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# Wildlife Use of Vernal Pools in an Urbanizing Landscape with a Focus on Population Vitality of Vernal Pool-Breeding Amphibians

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**WILDLIFE USE OF VERNAL POOLS IN AN URBANIZING LANDSCAPE**  
**WITH A FOCUS ON POPULATION VITALITY OF**  
**VERNAL POOL-BREEDING AMPHIBIANS**

By

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(in Wildlife Ecology)

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An Abstract of the Dissertation Presented  
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Vernal pools in the northeastern United States provide essential habitat for pool-breeding amphibians and provide resources for other forest-dwelling wildlife. These pools and pool-breeding amphibians in particular are threatened by land conversion associated with urbanization and urban-associated factors. The responses of these amphibians and of birds and mammals using vernal pools to intermediate levels of urban development are largely unknown. I used field observations and lab experiments to study the amphibians, birds, and mammals associated with vernal pools along an urban development gradient in greater Bangor, Maine.

In Chapter 1, I examined bird and mammal use and assemblage composition at 33 pools, with specific focus on the influence of impervious surface as an indicator of urbanization intensity. I detected 59 bird and mammal species using pools and the adjacent terrestrial areas. Within-pool vegetation and land cover types within 1,000 m of pools likely influenced assemblages with increases in impervious cover linked to shifts towards urban-affiliated species.

Chapters 2 focused on the associations between site characteristics in an urbanizing landscape and wood frog (*Lithobates sylvaticus*) larval morphology and survival. Differences in morphology were associated with urban land conversion, hydrology, within-pool vegetation, and conspecific density. Urbanization was positively associated with greater tadpole survival, development rate, and size.

In Chapter 3, I examined the carry-over effects of larval morphology and site characteristics, particularly urban-associated land conversion within 1,000 m, on newly emerged and post-breeding male wood frogs in 15 pools. Egg density had a salient influence with negative effects on larval and froglet responses, and the effects of urban-associated cover near pools at larval and adult stages suggest that the carry-over effects of urbanization from larval to froglet stages may not persist to adulthood.

Chapter 4 addresses the effects of urban-associated land conversion and road salt on breeding effort of wood frog, spotted salamander (*Ambystoma maculatum*), and the blue-spotted salamander (including the unisexual complex, *Ambystoma laterale-jeffersonianum*). All three taxa responded negatively to tree cover reduction, but had some positive responses that are indicative of the removal of breeding pools 300-1,000 m from a study pool resulting in displaced adults consolidating breeding in remaining pools.

## PREFACE

This dissertation reflects an effort to better understand responses of animals that use vernal pools across an urbanizing landscape with a focus on vernal pool-breeding amphibians. It is my hope that the information herein can assist those working to conserve vernal pools and the wildlife that use these important natural features. Because the research topic was relatively broad, I have studied several taxa and aspects of amphibian ecology. All chapters are “journal ready”, whereas appendices either provide supplemental information to chapters (Appendices A-C) or likely warrant publication (Appendices D-G and I) but for which substantial collaboration with other researchers is likely to increase the value of the resulting manuscript. The topics addressed in the chapters and appendices and the journals targeted for publication are listed, below.

- Chapter 1 addresses bird and mammal use and assemblage composition. This chapter was submitted to *Urban Ecosystems* and is in review.
- Chapter 2 focused on the relationship between site characteristics and wood frog larval morphology and survival. *Ecosphere* was the target journal for this chapter.
- Chapter 3 examined the effects of larval morphology and site characteristics that may persist to post-metamorphic stages. This chapter was submitted to *Urban Ecosystems* and is in review.
- Chapter 4 addresses the effects of urban-associated land conversion and road salt on breeding effort of wood frog, spotted salamander, and the blue-spotted salamander. This chapter was submitted to *Herpetological Conservation and Biology* and is in review.

- Appendices A-C provide supplemental information to chapters 1-3 and will be included as supporting information for submitted manuscripts if determined appropriate by journals.
- Appendix D describes aquatic insect assemblages and examines relationships between predatory insects and tadpole morphology. I will work with Dr. Greig to flesh out this appendix into a full-fledged manuscript and to determine the most appropriate journal for submission.
- Appendix E examined the changes in *Ranavirus* infection in wood frog throughout larval development and how these changes may relate seasonal shifts in site characteristics. I will continue to consult and coordinate with Drs. Matt Gray of the University of Tennessee, Emily Hall of Vanderbilt University, and Phillip deMaynadier of the Maine Department of Inland Fisheries and Wildlife to refine this manuscript and select a targeted journal for publication.
- Appendix F details detection of Ichthyophonus, a fungal pathogen, in wood frog larvae and aquatic insects. This report will be submitted to Dr. David Green at the USGS National Wildlife Health Center. I will consult with Dr. Green for his advice on how to best disseminate this information to an interested audience.
- Appendix G assesses the challenges of collecting blood from larval wood frogs for the purpose of examining white blood cell profiles. I also report preliminary white blood cell profile results for larval wood frog. I will continue to coordinate with Dr. Anne Lichtenwalner of the University of Maine and Lynda Leppert, blood analyst, to determine an appropriate outlet for dissemination.

- Appendix H reports all egg mass counts by site and species for vernal pool-breeding amphibians conducted throughout my graduate research. Coordinates of pools are provided. Additionally, summary statistics of clutch size (embryos per clutch) are reported for wood frog and spotted salamander by pool. This information is not intended for publication in another outlet, but is included as a point of reference for those who may study any of these pools in the future.
- Appendix I examined the relationship between road salt and food availability as observed during a controlled lab experiment. I plan to incorporate this appendix in a publication examining the influence of road salt contamination on wood frog larvae. This publication will address the comparative and/or interactive roles of road salt contamination with food availability, *Ranavirus* infection, insect predator pressure, and difference in source pool (i.e., population).



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My committee members have all expanded perspectives on ecological systems, provided crucial advice on study design and statistical analyses, and volunteered use of their lab and field supplies. Becky Holberton taught me to consider behavior and physiology as integral parts of wildlife ecology and introduced me to thinking of ecological issues in an evolutionary framework. Mike Kinnison helped me better understand the intersection of anthropogenic change, adaptation, and population genetics. Hamish Greig introduced me to the fascinating world of aquatic insects in vernal pools and illuminated layers of complexity within ecological communities.

This work would not have been possible without the support, encouragement, input, and thoughtful contributions of so many individuals at the University of Maine and beyond. Twenty-eight landowners and the University of Maine provided me access to vernal pools for three years of study. Matt Gray at the University of Tennessee provided tremendous assistance with study design, lab assistance, and sample analysis for ranavirus research. Edward “Davis” Carter and Jenny Asper at the University of Tennessee also helped with lab procedures, viral preparation, and sample processing. Emily Hall at the Washington State University led the study design and

conducted all sample processing for sampling eDNA of ranavirus in pool water. Joyce Longcore at the University of Maine helped with identification of a fungal infection in tadpoles. Anne Lichtenwalner at the University of Maine dedicated a great deal of time helping me improve blood collection and smear creation from small amphibian larvae. I am extremely grateful to Linda Leppert for analyzing hundreds of blood smears and for helping with troubleshooting smear creation techniques. I thank Brian McGill, Erik Blomberg, and Alessio Mortelleti at the University of Maine for their advice on the selection and implementation of statistical techniques. Their advice has been truly invaluable. I have had the pleasure of working with the “Of Pools and People” team. This interdisciplinary team, including Cyndy Loftin, Dana Bauer, Erik Nelson, Krista Capps, Jared Homola, Jessica Balukas, Lydia Kifner, and Kris Hoffmann, has provided advice on study design and analysis to produce results that can be integrated with social science research to further conservation outcomes. The Of Pools and People advisory board members have also contributed invaluable suggestions and have been a sounding-board for my research methods. Of this group, I am especially appreciative of the thoughtful advice of Ray Semlitsch, Phillip deMaynadier, and David Patrick.

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# **CHAPTER 1: BIRD AND MAMMAL USE OF VERNAL POOLS ALONG AN URBAN DEVELOPMENT GRADIENT**

## **1.1 Chapter Abstract**

Vernal pools are of conservation concern primarily because of their role as habitat for specialized pool-breeding amphibians, but their use by birds and mammals may also be of interest, especially from the perspective of the impact of urbanization. We describe camera-trapped wildlife (CTW) at 38 vernal pools along an urban development gradient in greater Bangor, Maine, USA. We detected 20 mammal and 39 bird taxa (29 contacted pool water; 39 detected at >1 site). Land cover type within 1,000 m (%), within-pool vegetation (%), and amphibian egg mass numbers explained a substantial portion of the variance (40.8%) in CTW assemblage composition. Submerged vegetation within pools and cover by water and impervious surfaces within 1,000 m of pools were key site characteristics defining assemblages. We scored the urban-affiliation of taxa and modeled the relationship between weighted assemblage scores for each site and impervious cover. Impervious cover within 1,000 m of pools was positively ( $p < 0.01$ ) related to site urban-affiliation scores. Use probability for red fox increased and snowshoe hare decreased with impervious cover at 1,000 m. These results indicate that within-pool vegetation and land cover types at 1,000 m influenced bird and mammal assemblages that used study pools and greater impervious cover at 100 and 1,000 m was correlated with a shift in assemblages from being dominated by urban-avoider to urban-adapted species. We encourage land use planners and managers to consider the influence of land use practices within 1,000 m of vernal pools on birds and mammals, especially near amphibian breeding pools.

## 1.2 Introduction

Vernal pools provide important seasonal sources of food or water for many species of birds and mammals (Silveira 1998; Colburn 2004; Mitchell et al. 2008), especially for those that prey on seasonally abundant pool-breeding amphibians or nutrient-rich aquatic vegetation early in spring (Shurin et al. 2006). For example, gray jays (*Perisoreus canadensis*), wild turkeys (scientific names not listed in the text are in Table 1.1), and raccoons are known to eat amphibians (Childs 1953; Murray et al. 2005; A. Calhoun, personal communication, 13 June 2017). Although the ecological roles of birds and mammals in vernal pool ecosystems are poorly known, there is evidence that they facilitate ecological processes such as nutrient transport, seed and egg dispersal, and regulation of amphibian populations (Childs 1953; Zedler 1987; Black and Zedler 1992), thereby making vernal pool ecological functions disproportionately large compared to their area (Calhoun et al. 2017).

Vernal pool conservation is challenging; pools are typically small and seasonally inundated, poorly regulated, and difficult to inventory (Calhoun et al. 2017). Conflicts between conservation and urbanization are most acute where economic growth converts forest into residential, commercial, and/or industrial developments (Windmiller and Calhoun 2008). These conflicts will escalate as urban areas expand >12.2 million ha throughout the US by 2051 (Lawler et al. 2014). As urban development replaces forest, habitat for forest-reliant wildlife is lost and fragmented (Fischer and Lindenmayer 2007); impervious surfaces alter hydrology and rapidly transport unfiltered anthropogenic chemicals into pools (Faulkner 2004); and novel threats to wildlife may increase, including human disturbance and predation from pets and subsidized predators (Hansen et al. 2005).

Shifts in bird and mammal assemblage composition in response to urban development have been well documented (Chace and Walsh 2006; McKinney 2008; Chupp et al. 2013). Urbanization typically involves a reduction in vegetation cover (McKinney 2006), a shift towards non-native plants (Aronson et al. 2014), and the addition of novel human structures and subsidies (e.g., food waste). Birds and mammals typically exhibit one of three responses to these changes (as coined by Blair 2001): avoidance, adaptation, or exploitation with these responses predominating in undeveloped/rural, suburban, and urban core areas, respectively (McKinney 2006). Examples of avoiders include area-sensitive birds (Friesen et al. 1995) and large predatory mammals that are persecuted by humans (Fischer et al. 2012). Examples of adapters include red squirrels, which thrive in areas where understory vegetation has been removed but trees remain (Racey and Euler 1982), and American robins, which benefit from increased forest edges (Minor and Urban 2010). Rock dove (*Columba livia*), house sparrows (*Passer domesticus*), and Norway rats (*Rattus norvegicus*) can exploit novel food and shelter in the urban core (Blair 1996, McKinney 2002).

Here we investigated bird and mammal assemblage composition and individual species use at vernal pools along an urban development gradient using motion-activated cameras. We used impervious cover to represent development intensity because it includes buildings and pavement and is thus linked to traffic and chemical, light, and noise pollution. Our primary objectives were to describe the composition of assemblages and examine how they corresponded to pool conditions and land cover types near pools at various spatial scales.

## **1.3 Methods**

### **1.3.1 Study area**

We conducted this study in the greater Bangor area in Maine, USA. i.e., within 18 km of downtown Bangor (44° 48' 8" N, 68° 46' 13" W) where there is 80-100% impervious cover (Maine Land Cover Dataset, MELCD; <http://www.maine.gov/megis/catalog/>). In general, impervious cover decreases and cover by mid-successional mixed forest (oak, *Quercus* spp.; Eastern hemlock, cover *Tsuga Canadensis*; white pine, *Pinus strobus*; American beech, *Fagus grandifolia*; poplar, *Populus* spp.; birch, *Betula* spp.; maple, *Acer* spp.; balsam fir, *Abies balsamea*) increases with distance from Bangor. Each site consisted of a vernal pool and the area within 1,000 m of its high-water mark. Sites were selected based on the presence of vernal pool-breeding amphibians and to represent the range of land cover types.

### **1.3.2 Camera trapping**

We placed infrared, motion-activated cameras (Bushnell Trophy Cam HD, Overland Park, Kansas; 18 m maximum detection) at 38 sites with 27, 35, and 11 sites surveyed in 2014, 2015, and 2016, respectively. At each pool one camera was placed within 2 m of the ground and within 3 m from the pool's high-water mark to capture as much of the pool as possible. Each site had a camera functioning 12-622 d (*median*=214, *IQR*=79-338). We identified species, behavior, and whether the animal(s) contacted pool water in photographs. All animals were detected between 15 May 2014 and 22 September 2016. We conducted this study in compliance with University of Maine Institutional Animal Care and Use Committee standards as no animals were handled.

For modeling, we used data from sites with >50 d of camera function between 14 May – 26 August 2014 – 2016 (33 sites; 63-293 d per site out of 315 total days, *median*=139, *IQR*=93-180). This “summer” season was selected to align with peak camera function among all sites (54.0% [4,711/8,725] of total camera days), to capture the most taxa (87.8% [52/58] of mammal and bird taxa), and to better meet assumptions of closure for occupancy modeling.

### **1.3.3 Site characteristics**

We quantified surrounding land cover types, pool vegetation, and amphibian egg masses. Using ArcView GIS 10.2 and MELCD (2004 all land use; 2011 impervious cover) we quantified tree, open water and non-forested wetland, and impervious cover within 100, 300, 600, and 1000 m from pool high-water marks. Distances matched spatial scales relevant to pool-breeding amphibians (Homan et al. 2004; Rittenhouse and Semlitsch 2007). We edited cover types to correct misclassifications and to reflect more recent aerial photographs (World Imagery; 10 July 2015).

We surveyed spring and summer vegetation at 27, 31, and 9 pools in 2014, 2015, and 2016, respectively. Spring surveys were conducted when vernal pool-breeding amphibian eggs were present in May and June. We conducted summer surveys after typical summer dry down in July and August. We visually estimated shrub, emergent, and submerged vegetation cover and measured woody vegetation canopy over pools using a spherical convex densiometer. We conducted vernal pool-breeding amphibian egg mass counts following Crouch and Paton (2000; April and May). Inter-annual means were used as covariates in further analyses including site characteristics.

### **1.3.4 Taxon sampling curves**

All statistical analyses were conducted using R version 3.3.1 (R Development Core Team 2016). We used the ‘vegan’ package (Oksanen et al. 2017) to create sample-based taxon sampling curves with camera-day as the sampling unit. We created species accumulation curves for sites with >30 camera days (35 sites) by adding species in order of detection. We created rarefaction curves across these 35 sites by randomly sampling (1,000 random permutations, sampling without replacement) all camera days and for the subset of days from the summer season (Gotelli and Colwell 2001).

### **1.3.5 Partial redundancy analysis (pRDA)**

We conducted a pRDA and variance partitioning using the ‘vegan’ package (Oksanen et al. 2017) to identify how site characteristics correlate with dominant gradients of variation in camera-trapped wildlife (CTW) assemblages among sites (Borcard et al. 2011). We conditioned the RDA on latitude and longitude to account for the portion of Curtis-Bray dissimilarity in assemblages attributed to spatial correlation (Spearman’s rank correlation=0.143,  $p<0.01$ ).

We represented CTW assemblage composition with a matrix of detection frequencies (proportion of camera-days a taxon was observed) of taxa x sites. We did not account for detection probabilities when examining assemblages because sparse detections of many taxa may produce occupancy estimates more misleading than ignoring non-detection altogether (Welsh et al. 2013) and because multi-species models may not be appropriate within groups of species that select habitat features at dissimilar scales (Dorazio et al. 2006; Royle and Dorazio 2008). We

used a square root ( $y + 1$ ) transformation on detection frequencies to dampen the influence of rare and super-abundant species.

We selected variables to represent vegetation, amphibian abundance, and land cover types. Within each category, we examined multivariate normality of variables and transformed across all variables as needed. We centered and column-standardized all explanatory variables to account for differences in units and assessed variables' categories for collinearity (pairwise Pearson product-moment bivariate correlations  $\geq 0.7$ ). When collinearity was detected, we used forward-step variable selection (Akaike Information Criterion [AIC]-based) to select three variables in each category with  $< 0.7$  correlation (Pearson correlation  $\leq 0.63$  among the nine selected variables).

We conducted Monte Carlo global permutation tests to determine the significance of the ordination, the pRDA axes, and each constraining variable. Because constraining variables are assessed sequentially for significance, we tested each variable as the first term in the model. Upon determining significance of the ordination ( $p = 0.001$ ), we compared the pRDA with an unconstrained, unconditioned principle component analysis to assess if extracted patterns in the pRDA likely represent actual dominant gradients (Legendre and Gallagher 2001). We similarly compared the "all species" pRDA ordination to pRDAs based on "limited species" data sets (i.e., removing species only observed at one site and/or the single most common species across all sites). Since all ordinations were similar, we assumed that the constraining variables in the pRDA are related to actual gradients of variation and that rare and abundant species were not highly influential in structuring the pRDA. We then calculated the proportion of variance



explained by each axis and overlaid generalized additive model (GAM) fitted surfaces on the ordination to examine the linearity of variation of each vector.

### **1.3.6 Modeling urban-affiliation**

We examined the relationship between urban development intensity, as indicated by impervious cover, and an index of assemblage urban-affiliation using linear modeling. We used AIC backward-step variable selection to select well-supported ( $\Delta AIC < 2$ ) models from a global model that included predictor terms of impervious cover within 100, 300, 600, and 1,000 m. To calculate an index of urban-affiliation, we scored each taxon on a scale of 1-4 with one for taxa that avoid and/or are greatly impaired by urbanization and four for taxa that benefit from urbanization. For each site, we multiplied the detection frequency of each taxon by its urban-affiliation score and averaged the products of all detected species, yielding an urban-affiliation index value. We did not incorporate a spatial variance structure into the model because of a lack of evidence of spatial autocorrelation among sites (Spearman's rank correlation=0.007,  $p=0.88$ ).

### **1.3.7 Single-species use probability modeling**

We fit single-season occupancy models (MacKenzie et al. 2006) using package 'unmarked' (Fiske et al. 2017) to examine the relationship between species-specific probability of use ( $\theta$ ) of a vernal pool and impervious cover while accounting for detection probability ( $p$ ; MacKenzie 2006a). We were interested in use, as opposed to occupancy, because species occupancy (i.e., home range) in an area containing a vernal pool does not necessitate their use of the pool. Following Trzcinski et al. (1999) we modeled species detected at  $>10\%$  of sites ( $\geq 4$  sites) during the summer season, using a 7-day camera function period as the sampling unit for

detections to increase detectability and precision of use probability estimates. We truncated the number of sampling periods to reduce excessive missingness in the dataset and maintain  $\geq 10$  sites with data for all sampling periods (24 periods, 24% missingness). In using single-season models we assume that use ( $\theta$ ; i.e., availability for detection) is constant across sampling units.

We identified three *a priori* detection covariates based on camera placement and the mean of interannual spring and summer within-pool vegetation and modeled four variables indicating intensity of urban development (impervious cover) within 1,000 m (Table 1.1). We expected detection to decrease with thicker vegetation (Emergent, Shrub) and to increase with the percent of a pool's basin captured in a camera's view (View; which varied from 10-80% [*median*=60, *IQR*=47-66]). All detection covariates were centered and column-standardized to account for differences in distributions.

Because our small sample size (33 pools) negated a complex model including terms for spatial autocorrelation, we fit one-covariate models to estimate detection and then ranked models by AICc (AIC, adjusted for small sample size). When  $>1$  model was  $\leq 2 \Delta AICc$  we tested additive models that included all combinations of covariates ranked above the null model. We retained the detection parameter from the top ranked model and repeated the process with use covariates to determine the best use model for each species. For best-fitting models we tested goodness-of-fit (1,000 bootstrap permutations; Mackenzie and Bailey 2004) and reassessed overdispersed models ( $\hat{c} > 1$ ) using quasi-AICc (QAICc) where  $\hat{c}$  is used as a variance inflation factor for comparing models for a more conservative model (Burnham and Anderson 2002). We tested fit of selected models that had  $\hat{c} \leq 4$  using Nagelkerke's (1991) R-squared index. Measures of fit (i.e.,  $\Delta QAICc$ , relative model weight,  $R^2_N$ ) were not assessed for models with  $\hat{c} > 4$  due to

probable inadequate model structure (Burnham and Anderson 2002). To avoid overstating the potential influence of impervious cover, when the model containing a null  $\theta$  covariate was  $\leq 2$   $\Delta\text{AICc}$  (or  $\Delta\text{QAICc}$ ) of the top model we considered the influence of  $\theta$  covariates to be no different from the null and only reported model structure and parameter estimates for the null model.

## **1.4 Results**

From 2014 to 2016 we detected 59 species (20 mammals, 39 birds) during 8,725 camera days at 35 of 38 sites (Table 1.2). We detected 11 predatory mammals, one omnivorous mammal, seven predatory birds, and 31 insectivorous and omnivorous birds (Table 1.2). Thirty-nine taxa were observed at  $>1$  site, and 29 taxa contacted pool water (Figure 1.1). We observed CTW bathing, drinking, feeding on vegetation, foraging for aquatic prey, preening, swimming, standing, and walking in pools. Fifty-two species were included in assemblage analyses and occupancy modeling.

Table 1.1 Predictor variables used to evaluate use and detection probability of species detected by camera traps at 33 vernal pools across an urban development gradient in 2014-2016 in greater Bangor, Maine, USA.

Parameter	Variable	Description
Detection	Emergent	Mean within-pool emergent vegetation cover (%)
	Shrub	Mean within-pool shrub cover (%)
	View	Mean pool basin* photographed (%)
Use	Imp100	Impervious cover within 100 m (%)
	Imp300	Impervious cover within 300 m (%)
	Imp600	Impervious cover within 600 m (%)
	Imp1000	Impervious cover within 1000 m (%)

\*Basin refers to inundated area at spring high-water

Table 1.2 Birds and mammals detected during a 2014-2016 camera trap survey at 38 vernal pools in Maine. Species detected during the summer season from 33 sites were used in an ordination and to quantify urban-affiliation of the bird and mammal assemblage at each site. Urban affiliation scores are: 1=avoids suburban and urban areas, 2=somewhat adaptable to suburban areas, 3=very adaptable to suburban areas, 4=adaptable to suburban and urban area; references for these are in Appendix Table A1.

Species	Observed swimming or wading in pool water (Y/N)	Sites detected (total, summer)	Only detected at sites with <10% or >20% impervious cover within 1,000 m	Mean summer season detection frequency	Urban-affiliation score (1-4)
<i>Birds</i>					
wood duck ( <i>Aix sponsa</i> )	Y	10, 9	-	0.0235	1
mallard ( <i>Anas platyrhynchos</i> )	Y	17, 15	-	0.0415	3
Canada goose ( <i>Branta canadensis</i> )	Y	1, 1	<10%	0.0003	3
hooded merganser ( <i>Lophodytes cucullatus</i> )	Y	1, 1	<10%	0.0004	1
common merganser ( <i>Mergus merganser</i> )	Y	1, 1	<10%	0.0002	3
ruffed grouse ( <i>Bonasa umbellus</i> )	Y	6, 2	-	0.0007	1
wild turkey ( <i>Meleagris gallopavo</i> )	Y	10, 4	-	0.0022	2
northern goshawk ( <i>Accipiter gentilis</i> )	N	2, 2	<10%	0.0004	1
sharp-shinned hawk ( <i>Accipiter striatus</i> )	Y	2, 1	<10%	0.0002	2
broad-winged hawk ( <i>Buteo platypterus</i> )	Y	1, 1	<10%	0.0002	2

Table 1.2, continued

Species	Observed swimming or wading in pool water (Y/N)	Sites detected (total, summer)	Only detected at sites with <10% or >20% impervious cover within 1,000 m	Mean summer season detection frequency	Urban-affiliation score (1-4)
American woodcock ( <i>Scolopax minor</i> )	Y	2, 1	-	0.0002	1
mourning dove ( <i>Zenaida macroura</i> )	N	2, 1	<10%	0.0001	3
great horned owl ( <i>Bubo virginianus</i> )	N	1, 1	<10%	0.0003	2
barred owl ( <i>Strix varia</i> )	Y	4, 3	<10%	0.0005	1
ruby-throated hummingbird ( <i>Archilochus colubris</i> )	N	1, 1	<10%	0.0003	3
northern flicker ( <i>Colaptes auratus</i> )	N	4, 2	<10%	0.0004	3
downy woodpecker ( <i>Dryobates pubescens</i> )	N	1, 1	>20%	0.0005	3
pileated woodpecker ( <i>Dryocopus pileatus</i> )	N	2, 2	-	0.0006	1
hairy woodpecker ( <i>Picoides villosus</i> )	N	4, 4	-	0.0015	3
eastern phoebe ( <i>Sayornis phoebe</i> )	N	2, 0	-	-	-
eastern kingbird ( <i>Tyrannus tyrannus</i> )	N	1, 1	<10%	0.0004	3
American crow ( <i>Corvus brachyrhynchos</i> )	Y	18, 14	-	0.0134	3
blue jay ( <i>Cyanocitta cristata</i> )	N	8, 4	-	0.0007	3

Table 1.2, continued

Species	Observed swimming or wading in pool water (Y/N)	Sites detected (total, summer)	Only detected at sites with <10% or >20% impervious cover within 1,000 m	Mean summer season detection frequency	Urban-affiliation score (1-4)
tufted titmouse ( <i>Baeolophus bicolor</i> )	N	1, 1	>20%	0.0004	3
black-capped chickadee ( <i>Poecile atricapillus</i> )	N	3, 1	>20%	0.0002	3
red-breasted nuthatch ( <i>Sitta canadensis</i> )	N	1, 0	-	-	-
hermit thrush ( <i>Catharus guttatus</i> )	N	5, 3	-	0.0013	1
Swainson's thrush ( <i>Catharus ustulatus</i> )	N	1, 0	-	-	-
American robin ( <i>Turdus migratorius</i> )	Y	11, 8	-	0.0034	3
gray catbird ( <i>Dumetella carolinensis</i> )	N	1, 1	-	0.0038	3
European starling ( <i>Sturnus vulgaris</i> )	N	2, 2	>20%	0.0004	4
common yellowthroat ( <i>Geothlypis trichas</i> )	N	1, 1	>20%	0.0002	3
black-and-white warbler ( <i>Mniotilta varia</i> )	N	1, 1	>20%	0.0005	1
yellow warbler ( <i>Setophaga petechia</i> )	N	1, 1	>20%	0.0002	3
northern cardinal ( <i>Cardinalis cardinalis</i> )	N	1, 0	-	-	-
rose-breasted grosbeak ( <i>Pheucticus ludovicianus</i> )	N	1, 0	-	-	-

Table 1.2, continued

Species	Observed swimming or wading in pool water (Y/N)	Sites detected (total, summer)	Only detected at sites with <10% or >20% impervious cover within 1,000 m	Mean summer season detection frequency	Urban-affiliation score (1-4)
common grackle ( <i>Quiscalus quiscula</i> )	Y	5, 2	>20%	0.0032	3
song sparrow ( <i>Melospiza melodia</i> )	N	1, 1	-	0.0024	3
American goldfinch ( <i>Spinus tristis</i> )	N	2, 2	-	0.0014	3
<i>Mammals</i>			-		
coyote ( <i>Canis latrans</i> )	Y	16, 8	-	0.0026	3
domestic dog ( <i>Canis familiaris</i> )	Y	11, 8	-	-	-
gray fox ( <i>Urocyon cinereoargenteus</i> )	N	1, 1	>20%	0.0001	2
red fox ( <i>Vulpes vulpes</i> )	Y	17, 8	-	0.0087	3
domestic cat ( <i>Felis catus</i> )	Y	8, 4	-	0.0066	4
bobcat ( <i>Lynx rufus</i> )	N	5, 3	-	0.0008	2
striped skunk ( <i>Mephitis mephitis</i> )	N	5, 2	-	0.0034	3
fisher ( <i>Martes pennanti</i> )	Y	10, 5	-	0.0034	1
weasel ( <i>Mustela</i> spp.)	Y	4, 0	-	-	-



Table 1.2, continued

Species	Observed swimming or wading in pool water (Y/N)	Sites detected (total, summer)	Only detected at sites with <10% or >20% impervious cover within 1,000 m	Mean summer season detection frequency	Urban-affiliation score (1-4)
raccoon ( <i>Procyon lotor</i> )	Y	30, 29	-	0.0805	4
black bear ( <i>Ursus americanus</i> )	Y	15, 14	-	0.0054	2
moose ( <i>Alces alces</i> )	Y	1, 1	<10%	0.0003	2
white-tailed deer ( <i>Odocoileus virginianus</i> )	Y	30, 24	-	0.0545	3
North American porcupine ( <i>Erethizon dorsatum</i> )	Y	13, 7	-	0.0028	2
muskrat ( <i>Ondatra zibethicus</i> )	Y	2, 2	-	0.0009	3
woodchuck ( <i>Marmota monax</i> )	N	3, 3	-	0.0045	3
eastern gray squirrel ( <i>Sciurus carolinensis</i> )	Y	29, 23	-	0.1161	4
red squirrel ( <i>Sciurus vulgaris</i> )	Y	12, 9	-	0.0246	3
eastern chipmunk ( <i>Tamias striatus</i> )	N	12, 10	-	0.0185	4
snowshoe hare ( <i>Lepus americanus</i> )	Y	10, 8	-	0.0080	2



Figure 1.1 Examples of camera-trapped wildlife (CTW) in contact with vernal in greater Bangor, Maine: fisher (A), black bear (B), barred owl (C), wood duck (D), white-tailed deer (E), and raccoon (F).

Among the 38 pools, 19 dried every year, 5 dried 1 or 2 years, and 14 never dried during 3 years (Table 1.3). Impervious cover was relatively low with <10% median cover across spatial scales and no site with >40% impervious cover at any spatial scale. Wood frog (*Lithobates sylvaticus*), spotted salamander (*Ambystoma maculatum*), and blue-spotted salamander (including the unisexual complex, *Ambystoma laterale - jeffersonianum*) egg masses were detected at 38, 31, and 23 sites, respectively. Among sites included in statistical analyses, the only site condition that clearly co-varied with impervious cover was tree cover (negatively, Figure 1.2).

Table 1.3 Site characteristics measured at 38 vernal pools during a 2014-2016 camera trap survey in Maine.

Characteristic	Range (median)
Hydroperiod (drying date)	June 6 – did not dry during study (24 pools [63%] dried $\geq$ 1 year)
Pool area at high-water mark (m <sup>2</sup> )	63-9,978 (420)
Impervious surface (%)	
<i>100 m radius</i>	0.0-34.5 (2.7)
<i>300 m radius</i>	0.0-36.8 (6.2)
<i>600 m radius</i>	0.0-38.4 (8.1)
<i>1,000 m radius</i>	0.3-37.9 (8.5)
Tree canopy density above pool (%)	
<i>Spring</i>	1.0-97.0 (40.8)
<i>Summer</i>	2.1-99.5 (51.6)
Shrub cover (%)	
<i>Spring</i>	0.0-77.5 (26.3)
<i>Summer</i>	0.0-80.0 (27.1)
Emergent vegetation cover (%)	
<i>Spring</i>	0.0-99.0 (11.3)
<i>Summer</i>	0.0-90.0 (37.1)
Submerged vegetation cover (%)	
<i>Spring</i>	0.0-60.0 (10.0)
<i>Summer</i>	0.0-90.0 (10.0)

Table 1.3, continued

Characteristic	Range (median)
Amphibian mean egg mass count	
<i>Wood frog</i> ( <i>Lithobates sylvaticus</i> )	1.3-300.7 (30.8)
<i>Spotted salamander</i> ( <i>Ambystoma maculatum</i> )	0.0-290.0 (16.2)
<i>Blue-spotted salamander</i> ( <i>Ambystoma laterale</i> ) <sup>1</sup>	0.0-2,065.0 (3.8)

<sup>1</sup> Includes the unisexual complex, *Ambystoma laterale* - *jeffersonianum*

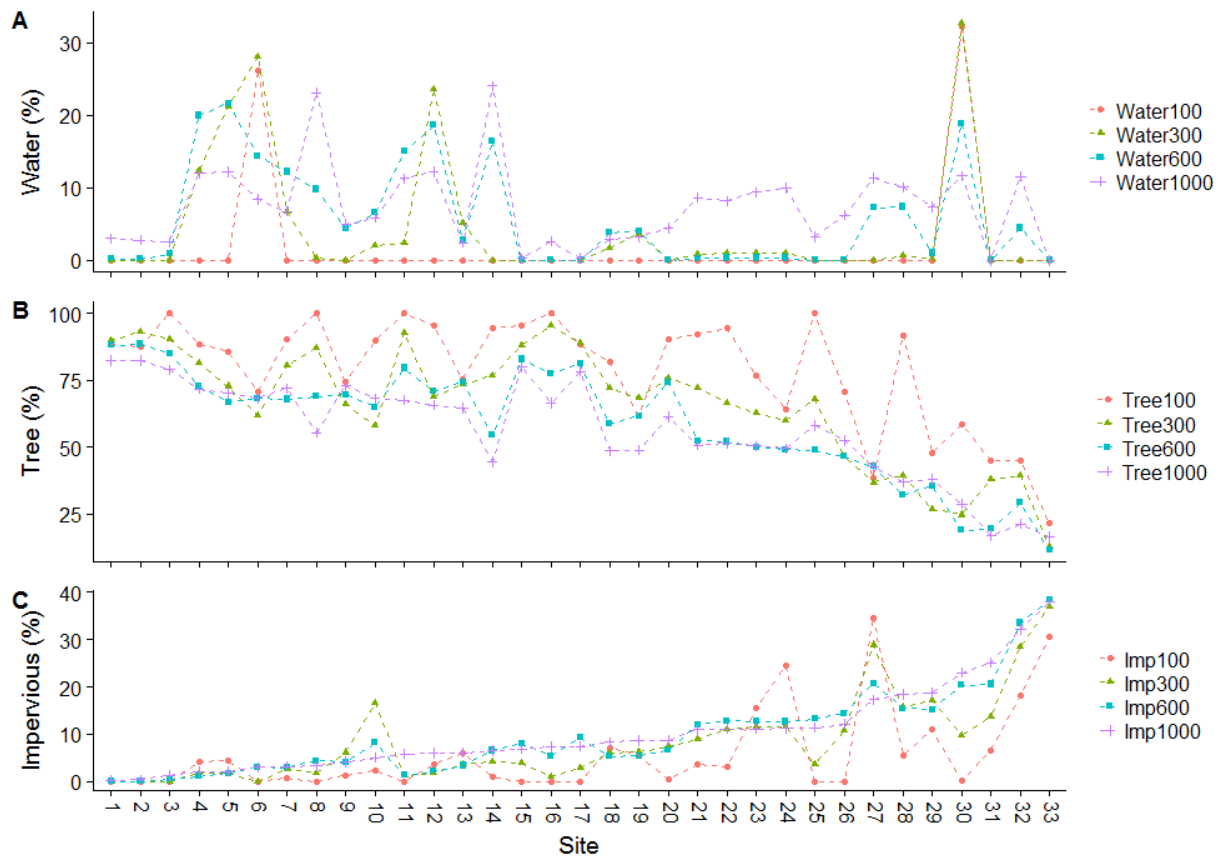


Figure 1.2 Water (A), tree (B), and impervious (C) cover within 100, 300, 600, and 1,000 m of 33 vernal pool sites with >50 days of camera function during a camera-trapped wildlife (CTW) survey during late spring and summer in greater Bangor, Maine in 2014-2016. Sites are arranged in ascending order based on impervious cover within 1,000 m and roughly indicate increased intensity of urbanization. Dashed lines connecting sites for each variable are for clarity in showing how all other variables change with urbanization intensity.

Species accumulation curves (SAC) indicated that we detected only a subset of the full bird and mammal assemblage because for most sites (73%) with >30 camera-days SAC did not approach an asymptote; and neither did rarefaction curves (Figure 1.3). However, rarefaction curves included an ‘elbow’, indicating that we captured the most rapid increase of species within the first 500 camera-days, followed by a slower increase (e.g., between camera-days 2,000 and 4,000 <10 species were added).

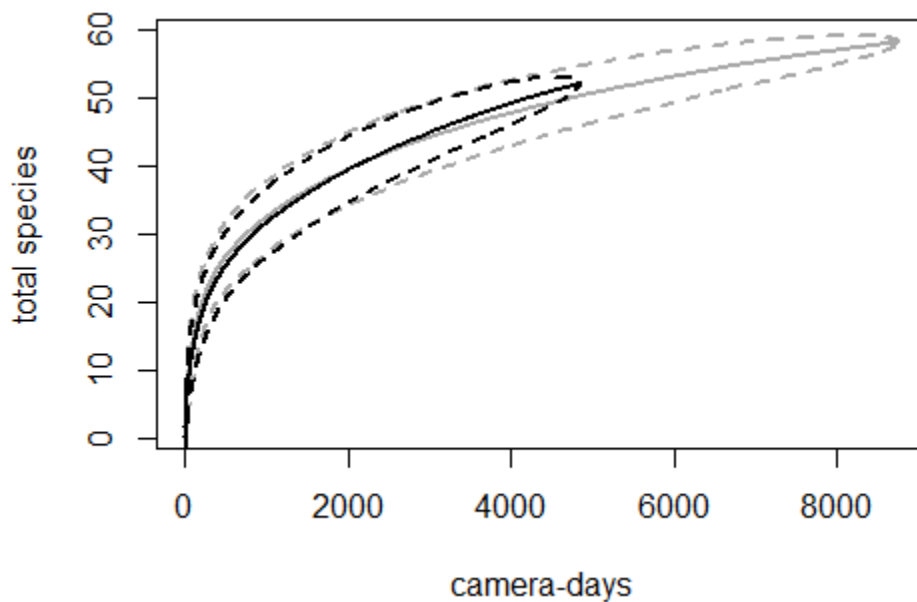


Figure 1.3 Rarefaction curves of camera-trapped bird and mammal taxa at 38 vernal pools with year-round (gray) and summer season (black) observations in Maine during 2014-2016. Curves (solid lines) are based on 1,000 random permutations, sampling without replacement. Dashed lines are standard deviations.

#### 1.4.1 Site characteristics corresponding to wildlife assemblages

Site characteristics and spatial distribution of sites affected CTW assemblages. All modeled land cover types, summer canopy cover, spring submerged vegetation cover, and *A. maculatum* egg mass counts were significant predictors of CTW assemblages ( $p < 0.05$ ). Summer

shrub cover and *L. sylvaticus* egg mass counts were marginally significant predictors ( $0.05 < p < 0.1$ ). Considerable variation in CTW assemblages among sites was constrained by site characteristics and Euclidean distance (40.8 and 26.4% of the variance, respectively). Land cover types, pool vegetation, and egg mass count variables respectively accounted for 19.4, 10.0, and 6.8% of variation in CTW assemblages (47.5, 24.4, and 16.8%, respectively, of the constrained, non-spatial variance).

The first canonical axis ( $p=0.003$ ), which explains 23.1% of the variation among assemblages (56.6% of constrained variance), is primarily described by Water1000 ( $r=0.51$ ) and Imp1000 ( $r=0.36$ ), summer canopy density above a pool ( $r=0.40$ ), and cover of spring submerged vegetation ( $r=-0.39$ ; Figure 1.4). The second pRDA axis ( $p=0.025$ ), which explains 9.2% of the variance among assemblages (22.6% of constrained variance), was positively correlated with spring submerged vegetation ( $r=0.72$ ) and to a lesser degree with summer shrub cover ( $r=0.29$ ) and amphibian egg mass counts ( $r=0.24-0.27$ ). All vectors varied roughly linearly in ordination space except Imp100 (Figure 1.4).

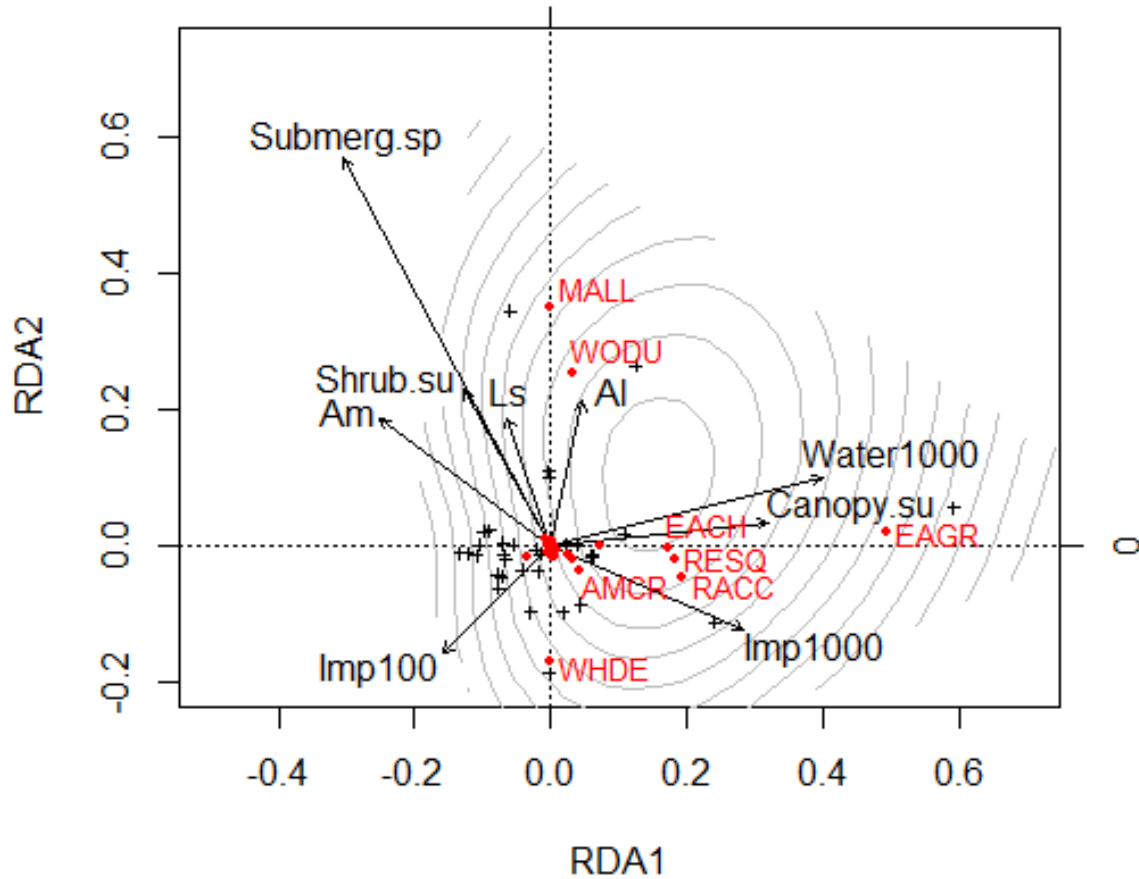


Figure 1.4 Partial redundancy analysis (pRDA) ordination for observation frequencies of 52 camera-trapped bird and mammal taxa across 33 vernal pools in Maine during 2014-2016. Sites are black crosses, red dots are observed taxa, and vectors represent site characteristics. Labeled taxa are: MALL=mallard (*Anas platyrhynchos*), WODU=wood duck (*Aix sponsa*), AMCR=American crow (*Corvus brachyrhynchos*), WHDE=white-tailed deer (*Odocoileus virginianus*), RACC=raccoon (*Procyon lotor*), EAGR=eastern gray squirrel (*Sciurus carolinensis*), RESQ=red squirrel (*Sciurus vulgaris*), and EACH=eastern chipmunk (*Tamias striatus*). Vector labels are: Canopy.su=mean density of summer tree canopy across years, Shrub.su=mean summer shrub cover, Submerg.sp=mean spring submerged vegetation cover; AI, Am, and Ls=mean egg mass counts for *A. laterale*, *A. maculatum*, and *L. sylvaticus*, respectively; and Imp100, Imp1000, and Water1000 are the percent impervious or open water cover within 100 or 1,000 m of pools. Variance explained: RDA1=56.6%; RDA2=22.6%. Contours (gray) represent change in impervious cover within 100 m across ordination space.



### **1.4.2 Assemblage composition along an urban development gradient**

The only significant covariate in both top models predicting urban-affiliation scores was Imp1000, which was positively related to urban-affiliation scores (Table 1.4, Figure 1.5).

Thirteen species were only detected at sites with <10% impervious cover within 1,000 m, and nine species were only detected at sites with >20% impervious cover within 1,000 m (Table 1.2). However, there was also considerable overlap of species across the development gradient: 16/52 taxa (30.8%) were detected at sites with <10% and at sites with >20% impervious cover within 1,000 m.

### **1.4.3 Detection and use models**

We modeled pool use for 19 species using single-season occupancy models (Table 1.5). Detection decreased with shrub and/or emergent cover for raccoon, white-tailed deer, eastern gray squirrel, red fox, North American porcupine, and fisher, but detection probabilities of waterfowl and domestic cat were highest in areas with greater shrub cover (Table 1.6, Figure 1.6, Appendix Figure A1). Models with View as a detection covariate (Table 1.6) indicated increased detection as more pool basin (%) was captured in photos (Appendix Figure A2). Red fox and snowshoe hare were respectively more and less likely to use pools with greater impervious cover within 1,000 m (Figure 1.7).

Table 1.4 Top ranked models predicting urban-affiliation scores of bird and mammal assemblages at vernal pools. The 52 modeled bird and mammal taxa were detected with camera-traps across 33 vernal pools in Maine during 2014-2016.

Model	Covariates	$\beta$ Estimate			AIC	$\Delta$ AIC
		(p-value)	95% CI lower	95% CI upper		
1	Imp100	-0.004 (0.347)	-0.012	0.004	-119.06	1.01
	Imp1000	0.012 (0.006)	0.004	0.020		
2	Imp1000	0.009 (0.005)	0.003	0.016	-120.07	NA

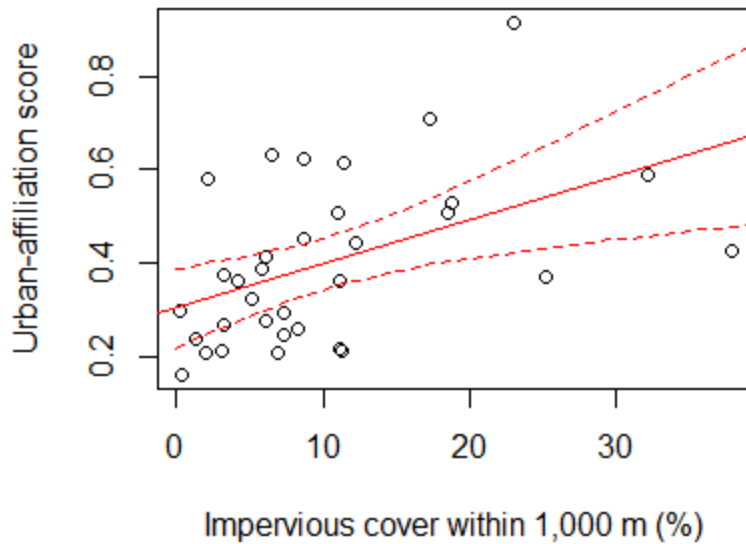


Figure 1.5 Predicted and observed urban affiliation-scores of bird and mammal assemblages at vernal pools in Maine during late spring and summer 2014-2016. Open circles are observed values, the solid line represents predicted values, and dashed lines represent the 95% confidence interval.

Table 1.5 Summary of the selected use models for 19 species at 33 vernal pools in Maine, USA, during summer 2014-2016. Model terms in parentheses represent detection (P) and use ( $\theta$ ). Model appropriateness and fit were assessed via an estimated overdispersion parameter ( $\hat{c}$ ) and the Chi-squared goodness-of-fit test p-value ( $p$ ), rank ( $\Delta\text{AICc}$  or  $\text{QAICc}$ ), relative model weight ( $w$ ), and Nagelkerke's R-square value ( $R^2_N$ ). Delta AICc or QAICc,  $w$ , and  $R^2_N$  were not assessed for models with  $\hat{c} > 4$  due to probable inadequate model structure (Burnham and Anderson 2002). Model covariate descriptions are in Table 1.1.

Species	Top model	$K$	$p$	$\hat{c}$	$\Delta\text{AICc}$ or $\text{QAICc}$ c	$w$	$R^2_N$
raccoon	$\theta(\cdot)\text{P}(\text{Shrub}+\text{Emergent})^{1,2}$	5	0.00	2.6	0.00	0.51	0.55
white-tailed deer	$\theta(\cdot)\text{P}(\text{Shrub})$	3	0.57	0.7	0.00	0.31	0.64
eastern gray squirrel	$\theta(\cdot)\text{P}(\text{Emergent})^1$	4	0.38	1.1	0.00	0.41	0.47
mallard	$\theta(\cdot)\text{P}(\text{Shrub})$	3	0.69	0.1	1.35	0.12	0.29
American crow	$\theta(\cdot)\text{P}(\cdot)$	3	0.00	4,299.0	-	-	-
black bear	$\theta(\cdot)\text{P}(\cdot)$	2	0.11	0.0	0.00	0.32	0.00
eastern chipmunk	$\theta(\cdot)\text{P}(\cdot)$	3	0.00	378.1	-	-	-
red squirrel	$\theta(\cdot)\text{P}(\text{View})$	3	0.96	0.0	1.83	0.22	0.63
wood duck	$\theta(\cdot)\text{P}(\text{Emergent})$	3	0.87	0.1	0.00	0.39	0.58
red fox	$\theta(\text{Imp1000})\text{P}(\text{Shrub})^2$	4	0.81	0.1	0.00	0.80	0.48
snowshoe hare	$\theta(\text{Imp1000})\text{P}(\cdot)$	3	0.18	0.5	0.00	0.23	0.21
American robin	$\theta(\cdot)\text{P}(\cdot)$	3	0.00	197.3	-	-	-
coyote	$\theta(\cdot)\text{P}(\cdot)$	2	0.08	0.8	0.06	0.35	0.00
North American porcupine	$\theta(\cdot)\text{P}(\text{Shrub})$	3	0.16	0.6	0.00	0.35	0.15
fisher	$\theta(\cdot)\text{P}(\text{View}+\text{Emergent})$	4	0.43	0.3	0.00	0.47	0.35

Table 1.5, continued

Species	Top model	$K$	$p$	$\hat{c}$	$\Delta\text{AICc}$ or $\Delta\text{QAICc}$		$R^2_N$
					$c$	$w$	
domestic cat	$\theta(.)P(\text{Shrub})$	3	0.48	0.1	1.84	0.09	0.54
wild turkey	$\theta(.)P(.)^2$	3	0.00	1,014.8	-	-	-
hairy woodpecker	$\theta(.)P(\text{View})$	3	0.55	0.0	1.71	0.09	0.32
blue jay	$\theta(.)P(.)$	2	0.18	0.7	0.74	0.23	0.00

<sup>1</sup>QAICc was used to assess model rank

<sup>2</sup>All other models were  $>2 \Delta\text{AICc}$  or  $\Delta\text{QAICc}$

Table 1.6 Estimated parameters ( $\beta$ ) and standard error (S.E.) for use ( $\theta$ ) and detection (P) parameters of the top ranked occupancy models predicting species use for species detected at  $\geq 4$  sites. Parameters were not estimated for grossly overdispersed models ( $\hat{c} > 4$ ).

Species	Parameter	Variable	$\beta$	S.E.
raccoon	$\theta$	Intercept	2.26	0.66
	P	Intercept	-0.73	0.10
	P	Shrub	-0.38	0.12
	P	Emergent	-0.36	0.11
white-tailed deer	$\theta$	Intercept	1.50	0.53
	P	Intercept	-1.28	0.13
	P	Shrub	-0.86	0.15
eastern gray squirrel	$\theta$	Intercept	0.86	0.39
	P	Intercept	-0.49	0.10
	P	Emergent	-0.48	0.12
mallard	$\theta$	Intercept	-0.06	0.37
	P	Intercept	-1.50	0.17
	P	Shrub	0.55	0.16
black bear	$\theta$	Intercept	0.66	0.76
	P	Intercept	-2.81	0.33
red squirrel	$\theta$	Intercept	-0.94	0.42
	P	Intercept	-1.32	0.24
	P	View	1.66	0.36
wood duck	$\theta$	Intercept	-0.54	0.44
	P	Intercept	-1.21	0.21
	P	Emergent	3.16	0.63
red fox	$\theta$	Intercept	-1.78	0.65
	$\theta$	Imp1000	2.01	0.77

Table 1.6, continued

Species	Parameter	Variable	$\beta$	S.E.
	P	Intercept	-1.32	0.24
	P	Shrub	-0.60	0.25
snowshoe hare	$\theta$	Intercept	-2.66	1.08
	$\theta$	Imp1000	-2.86	1.49
	P	Intercept	-1.84	0.29
coyote	$\theta$	Intercept	-0.80	0.70
	P	Intercept	-2.96	0.55
North American porcupine	$\theta$	Intercept	-0.88	0.55
	P	Intercept	-2.98	0.46
	P	Shrub	-1.02	0.46
fisher	$\theta$	Intercept	-0.78	0.74
	P	Intercept	-4.61	1.30
	P	View	1.07	0.49
	P	Emergent	-2.49	1.33
domestic cat	$\theta$	Intercept	-1.29	0.65
	P	Intercept	-2.83	0.60
	P	Shrub	1.51	0.35
hairy woodpecker	$\theta$	Intercept	0.46	1.11
	P	Intercept	-6.54	1.64
	P	View	3.31	1.34
blue jay	$\theta$	Intercept	5.96	45.5
	P	Intercept	-5.29	0.59

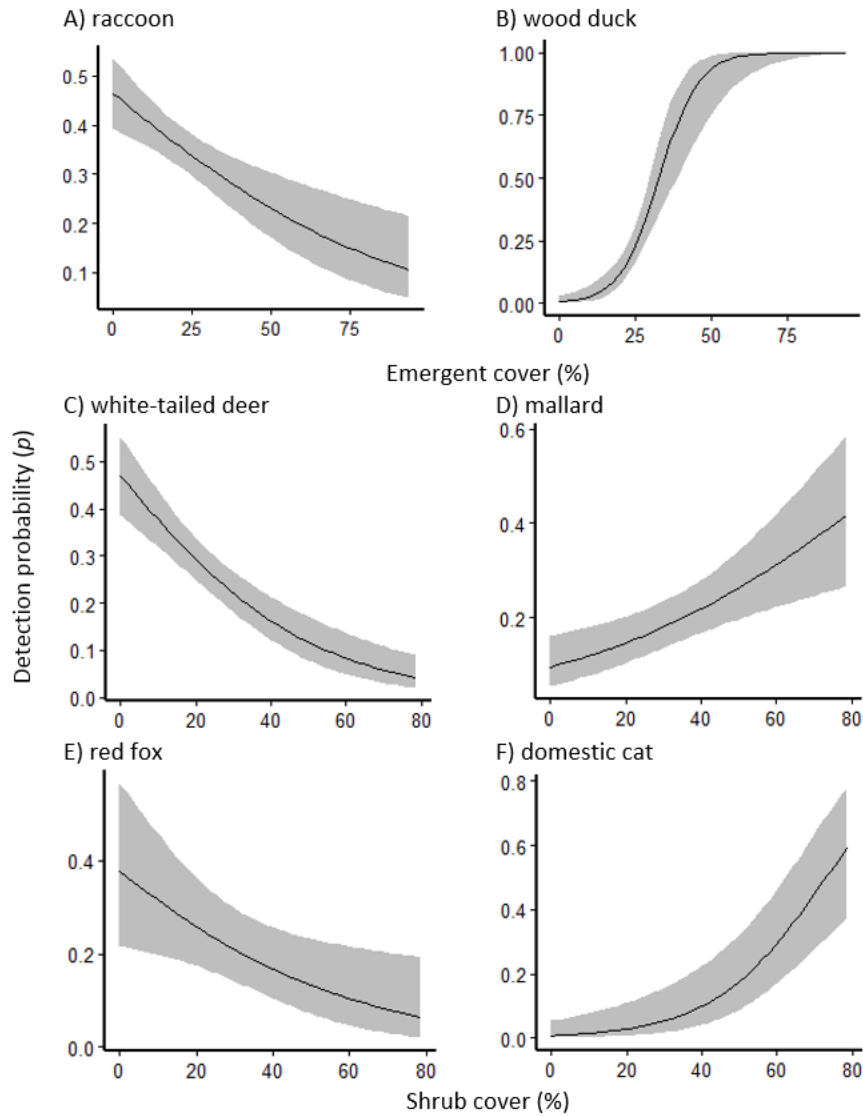


Figure 1.6 Relationships between detection probabilities and emergent vegetation and shrub cover modelled as covariate effects in single-season occupancy models for a subset of species. Shaded areas represent 95% confidence intervals. Curves for additional species' detection probabilities are in Appendix Figures A1 and A2.

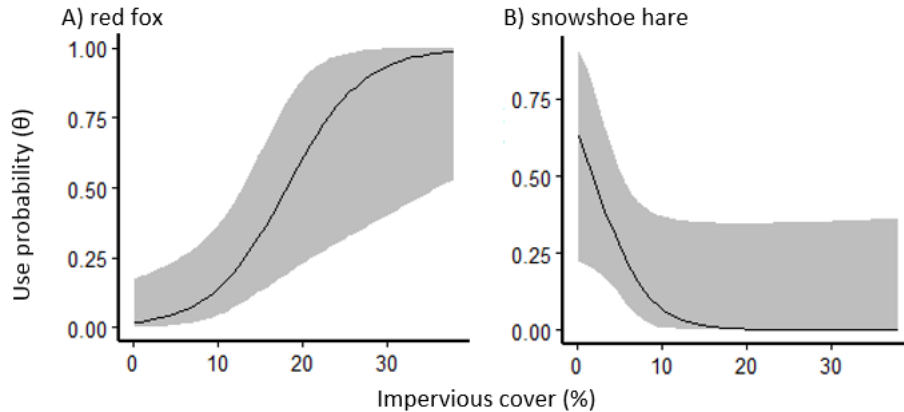


Figure 1.7 Relationships between use probabilities and impervious surface within 1,000 m of pools modelled as a covariate effect in single-season occupancy models. Shaded areas represent 95% confidence intervals.

## 1.5 Discussion

We observed 59 bird and mammal taxa during three years of camera-trapping, expanding insights into the composition of bird and mammal assemblages using vernal pools and how these assemblages may respond to site characteristics related to urbanization. Land cover types within 1,000 m and within-pool vegetation conditions strongly influence the composition of birds and mammals that use vernal pools (Figure 1.4). The abundance of pool-breeding amphibians may influence assemblages, but to a lesser degree.

Although our accumulation curves (Figure 1.3) indicate that we did not capture all species, the steep initial slope is characteristic of a community with a high proportion of common species (Thompson and Withers 2003). Additionally, with sufficient effort (1,000s of days) species undetected by camera traps are typically those considered rare (Tobler et al. 2008). Thus, we believe that we detected a high portion of the birds and mammals that regularly used studied pools (Table 1.2).



### 1.5.1 Land cover

Although we explored the less developed sector of the urban development gradient compared to similar studies (e.g., Blair 1996; Clergeau et al. 1998), we still detected wildlife responses to impervious cover within 1,000 m of pools. CTW trends corresponding with increased impervious cover may also signal wildlife responses to deforestation and, more generally, to urban development, especially since the pre-disturbance matrix in our study area was dominated by forest, and impervious and tree cover are negatively correlated (Figure 1.2). In our study, land cover types within 1,000 m of pools explained more variation in CTW assemblages than within-pool vegetation or amphibian egg numbers aligning with the idea that land cover types at broad spatial scales shape the set of species present at smaller scales (Johnson 1980) and/or limit resources that affect home range size (McLoughlin and Ferguson 2000). More specifically, our findings suggest little response of assemblages to small-scale (100 m) impervious cover when impervious cover is relatively high at large scales (1,000 m; Figure 1.4). However, assemblages at pools in relatively undisturbed larger landscapes (1,000 m) are predicted to respond to impervious cover at small scales (100 m). Consequently, the influence of development up to 1,000 m from pools may be dominant in determining the birds and mammals using pools even if areas directly adjacent to pools are undeveloped (Rodewald 2003, Hanowski et al. 2006).

The positive association we detected between impervious cover and urban-affiliation of CTW (Figure 1.5) further supports the idea that development within 1,000 m of pools may influence bird and mammal assemblages even in landscapes with relatively little urban development (e.g., 0.3-37.9% impervious cover within 1,000 m; Table 1.3, Figure 1.2). Although

we detected a significant association between impervious cover and use probabilities only for red fox (positive) and snowshoe hare (negative), these use probabilities varied predictably relative to species' urban affiliations (Table 1.2). These relationships align with previous research on community composition response to human disturbance (Beissinger and Osborne 1982; Nilon and VanDruff 1986; Croonquist and Brooks 1991).

The abundance of several urban-adaptable species (e.g., raccoon, eastern gray squirrel, American robin, and white-tailed deer) across study sites also may explain why use probability was not associated with impervious cover for 17 of the 19 modeled species (Table 1.5); i.e., the mean urban-affiliation score was 3 or 4 for 12 of the 17 species for which use was modeled but no response to impervious cover was detected (Tables 1.2 and 1.5). Because we only modeled data from frequently detected species (detected at >10% of sites), our analyses were biased towards urban adapters. Alternatively, we may not have detected use differences for species that respond to facets of urbanization other than impervious cover (e.g., vegetation composition, landscape fragmentation; Boren et al. 1999). Additionally, aiming cameras at pools may have biased detections towards urban adapters as small animals and especially canopy species, most of which are urban avoiders (e.g., Beissinger and Osborne 1982), were less likely to be detected (Tobler et al. 2008; Rowcliffe et al. 2011).

### **1.5.2 Within-pool vegetation**

Within-pool vegetation was the second-most important category of predictors (% variance explained). Our observations of white-tailed deer and moose foraging in pools suggest vegetation may be an attractant for some species. The negative effect of emergent and shrub cover on the detection probabilities for raccoon, eastern gray squirrel, red fox, white-tailed deer,

and North American porcupine (Table 1.6, Appendix Figure A.1) likely demonstrates that lower-strata vegetation within pools can provide cover for mammals that prefer dense vegetation (Fuller and Destefano 2003). Positive associations between vegetation and detection are difficult to interpret but may indicate that detection and use are confounded for species that respond to lower-strata vegetation via changes in abundance or frequency of use (Welsh et al. 2013).

### **1.5.3 Aquatic amphibians**

The associations between CTW and vernal pool-breeding amphibian egg numbers support the idea that amphibians provide food for birds and mammals (Figure 1.4). More specifically, spotted salamander and wood frog egg numbers were significant predictors of CTW and all three amphibian species had a similar direction of effect (Figure 1.4). Additionally, raccoon, mallard, and wood duck displayed behaviors commonly associated with foraging and may have preyed upon embryonic or larval amphibians and/or insects within-pools. Other studies have also documented birds and mammals consuming pool-breeding amphibians (Berven 1990) and aquatic invertebrates (Cox et al. 1998). Even if these suspected predator-prey interactions do not significantly shape bird and mammal assemblages they may be important for prey population dynamics. For example, Childs (1953) observed that in a single night a raccoon consumed all tadpoles in a pool. Additionally, diseases that can threaten amphibian populations, such as chytridiomycosis (Wake and Vredenburg 2008) and ranavirus, could be introduced by birds and mammals that move among pools (Gray et al. 2009).

## 1.6 Conclusions

Our results support commonly observed trends in bird and mammal assemblage composition along urban gradients, primarily an increase in species that can adapt to or even exploit human-altered landscapes. This occurs even with relatively low intensity development at spatial scales encompassing land quite distant from pools (e.g., 0.3-37.9% impervious cover within 1,000 m). Birds and mammals are likely important components of pool ecosystems and should be considered in management decisions even though they are not pool specialists. These animals can be thought of as surrogates that indicate natural system function (Hunter et al. 2016), and changes in their occupancy and assemblage composition with urbanization are likely to parallel degradation of pool-breeding amphibian populations and other vernal pool ecosystem functions. We encourage land use planners and managers to consider bird and mammal responses to zoning and land use practices within 1,000 m of pools and to select pools embedded in landscapes that are relatively undisturbed (i.e., fully functioning) within 1,000 m to implement vernal pool mitigation or conservation planning (Calhoun et al. 2017). We also encourage preferential conservation of pools that have sizable populations of breeding amphibians as they may provide important food for birds and mammals.

Because our primary aim was to document birds and mammals using vernal pools we did not compare use between vernal pool and non-vernal pool sites. Further study of such paired sites could help disentangle the effects of pool presence on bird and mammal assemblages from land cover type and local vegetation cover as well as identify possible bird and mammal community response to pool destruction. Comparing assemblages between pairs of pools with and without pool-breeding amphibian eggs and/or larvae could further elucidate the role of

amphibians in influencing use of pools by birds and mammals, and predation experiments could substantiate to what extent birds and mammals can prey on amphibian eggs and larvae.

## CHAPTER 2: INDICATORS OF WOOD FROG (*LITHOBATES SYLVATICUS*) CONDITION IN AN URBANIZING LANDSCAPE

### 2.1 Chapter Abstract

Wood frogs (*Lithobates sylvaticus*) are threatened by habitat degradation associated with urbanization. Urban development near pools may affect larvae with ramifications for population persistence and vitality. We studied larval development and documented key vernal pool and terrestrial characteristics within 1,000 m of 43 pools across the urban development gradient near Bangor, Maine, USA. Specifically, we examined how survival and morphological characteristics (e.g., developmental phenology, condition, body length, and tail length and shape) varied with characteristics at pool and landscape scales. Secondarily, we explored associations between morphology and survival. Differences in tadpole morphology were associated with urban land development, hydrology, within-pool vegetation indicative of light availability at the water's surface, and density of pool-breeding amphibian egg masses. Across all pools, tadpoles developed more slowly and were larger in pools with longer hydroperiods, while tadpoles in urban pools developed more quickly and were larger than those in rural pools with comparable hydroperiods. Tail length increased with canopy cover and was longer at urban pools than rural pools with comparable canopy cover. Morphology profiles also differed between rural and urban sites and among years, with 8% earlier development at urban sites. Survival in urban pools was predicted to be 15% greater than in rural pools, but across all pools (including those at intermediate intensities of urbanization), survival was not predicted to vary with either morphology or site characteristics. No strong relationship existed between developmental phenology and any condition or size metric. Because rural and urban tadpoles responded

similarly to within-pool conditions, our results support the need to maintain natural hydrology and vegetation conditions in pools even in developing areas. Although we detected benefits to tadpoles with increasing urbanization, urbanization is well-known to extirpate breeding populations; thus it is likely that wood frog population declines associated with urbanization are responding to stressors beyond the pool at terrestrial life stages.

## **2.2 Introduction**

Pool-breeding amphibians are often threatened by habitat loss and degradation as land development associated with urbanization encroaches near and even eliminates intermittently inundated pools (Zacharias et al. 2007; Windmiller and Calhoun 2008; Baldwin and deMaynadier 2009). More specifically, urban development has been associated with both extirpation and reduced occupancy of amphibians (Gibbs 1998a; Homan et al. 2004; Rubbo and Kiesecker 2005; Clark et al. 2008). Draining or impounding wetlands for development can eliminate breeding pools (Preisser et al. 2000; Beja and Alcazar 2003; Windmiller and Calhoun 2008), and adult road mortality and loss of critical post-breeding habitat via deforestation also likely contribute to population declines (Fahrig et al. 1995; Homan et al. 2004; Eigenbrod et al. 2008). Even when breeding populations persist in urbanizing areas (Le Viol et al. 2012; Scheffers and Paszkowski 2013), urban-associated factors (e.g., road runoff) can have sublethal influences (Bommarito et al. 2010; Brand et al. 2010) that may not be detectable in demographics until decades later (Griffis-Kyle 2007; Blaustein et al. 2011).

Accounting for sublethal effects of urbanization may be especially important in assessing population condition of species, such as wood frog (*Lithobates sylvaticus*), that are commonly surveyed by egg mass counts that can have high inter-annual variability (Berven 1990; Crouch

and Paton 2000; Capps et al. 2015). Sublethal responses to urbanization may also serve as early indicators of demographic declines that would not otherwise be detected for decades (e.g., Löfvenhaft et al. 2004; Gagné & Fahrig 2010). Measuring multiple responses (e.g., condition, survival) during post-embryonic life stages may provide better indicators of the impact of urbanization. For wood frogs, assessing larval responses may be appropriate as environmental conditions experienced during larval development may influence terrestrial stage morphology and performance (Berven 1990; Boes and Benard 2013). Larval wood frogs are sensitive to pool conditions that can be influenced by nearby vegetation and land use (Skelly et al. 2002; Watkins and Vraspir 2006; Karraker et al. 2008), and their survival may decline with road salt (Sanzo and Hecnar 2006; Karraker et al. 2008; Hall et al. 2017) and herbicide contamination (Relyea 2012) and light pollution (Perry et al. 2008).

Multiple site characteristics can vary with urbanization and may have unclear or conflicting influences on wood frog larvae. For example, road salt has been linked to larger tadpole size but also to reduced larval and froglet survival (Karraker et al. 2008; Dananay et al. 2015). Higher water temperatures may accelerate hatching (Herreid and Kinney 1967) and larval development, (Berven & Boltz, 2001; Skelly et al., 2002) thus allowing individuals to become free-swimming and able to move away from predation threats sooner in warmer urban waters. However, in warmer water wood frog larvae are slower and smaller (Herreid and Kinney 1967; Watkins and Vraspir 2006), potentially reducing their ability to evade predation and reducing survival and reproductive success during terrestrial stages. Impoundment of water in urban areas can increase the presence of predatory fish (Rubbo and Kiesecker 2005); however, if pools with longer hydroperiods lack fish and bullfrog predators they can support greater wood frog survival to metamorphosis (Rowe and Dunson 1995; Karraker and Gibbs 2009).



Shifts in within-pool vegetation with urbanization (Azous and Horner 2000) may influence wood frog larvae as they respond to vegetation structure (Skelly et al. 2002) and leaf litter composition (Rubbo and Kiesecker 2004). For example, fluctuations in canopy cover and leaf litter composition may influence larval development, growth, and survival (Werner and Glennemeier 1999; Skelly et al. 2002; Halverson et al. 2003; Rubbo and Kiesecker 2004). Changes in vegetation may also alter community interactions involving tadpoles. Submerged vegetation may reduce predation risk by providing cover for larvae (Formanowicz and Bobka 1989; Kopp et al. 2006), and reductions in canopy cover may increase the amphibian species richness (Skelly 2014), potentially changing competition and predation pressures.

Understanding the influence of urban development on wood frog persistence can be particularly challenging because a response to one condition can be mediated by another condition through synergistic or antagonistic effects (Relyea 2004; Marino et al. 2013). For example, tail morphology in wood frogs can respond to aquatic insect predators (Relyea 2012) as well as pool temperature during incubation (Watkins and Vraspir 2006). However, examining multiple metrics can provide a more comprehensive population status assessment than any single measure (Welsh et al. 2008) and can reduce potential error associated with extrapolating single measures, especially from early life stages (e.g., size at metamorphosis) to fitness (Earl and Whiteman 2015).

Previous research demonstrates that land cover type near pools influences wood frog breeding occupancy (Guerry and Hunter 2002; Porej et al. 2004; Gibbs et al. 2005; Gagné and Fahrig 2007) and terrestrial movements (Gibbs 1998b; Vasconcelos and Calhoun 2004), but few studies have examined the effect of gradations of natural and urban-associated land cover types

on amphibian larvae. Some studies have experimentally tested the effects of run-off associated with human development (e.g., pesticides, road salts) on larval growth and survival (e.g., Cothran et al. 2013; Hua et al. 2013; road salts, Sanzo & Hecnar 2006). Others have compared larval survival and size between forest and roadside pools (Karraker et al. 2008; Brady 2013; Hall et al. 2017) and examined the relationship between skeletal abnormalities and road proximity (Reeves et al. 2008). Although Shepack et al. (2017) experimentally introduced larvae into pools where breeding populations had been extirpated by urbanization and examined their survival and morphology, to our knowledge, no study has examined the relative influences of urban-associated land cover types at various spatial scales on the larval survival and development of a vernal pool-breeding amphibian.

In this paper, we examined larval wood frog responses to land cover type at the landscape scale (within 1,000 m), water quality, hydrology, within-pool vegetation, and indicators of amphibian community competition and predation across a gradient of low-intensity urbanization (0-38% impervious cover within 1,000 m). First, we examined relationships among developmental phenology, condition, body length, tail length, and tail shape within individuals and cohorts (i.e., site-year level) and determined if morphology varied among years and sites or between the least- and most-urbanized (i.e., rural and urban) pools. We then identified: 1) which morphological and/or site characteristics were important predictors of survival; 2) which site characteristics were important predictors of larval morphology; and 3) if the influence of these predictors varied with urbanization.

## **2.3 Methods**

### **2.3.1 Study area**

The greater Bangor, Maine area is located in the glaciated northeastern US, a region historically dominated by mixed coniferous-hardwood forest (Chapter 1). Bangor encompasses 90 km<sup>2</sup> with a population of approximately 33,000 (U.S. Census Bureau 2011). The 200 km<sup>2</sup> study area also included Orono, Hampden, and Old Town (populations of approximately 7,000-10,000; U.S. Census Bureau 2011). Developed land uses are primarily residential and commercial with development intensity extremes of nearly 100% impervious surface in downtown Bangor (44° 48' 8" N, 68° 46' 13" W) to <1% impervious surface (e.g., in the City Forest; Fry et al. 2011).

### **2.3.2 Site description**

Each site consisted of a wood frog breeding pool and the area within 1,000 m of the pool's high-water mark. We studied 36, 30, 13 sites (43 total) in 2014, 2015, and 2016, respectively. We selected sites to represent the range of development intensity at which wood frog breeding occurred using percent impervious cover within 1,000 m as an index of development. Selected sites had 0-34% impervious cover within 100 m and 0-38% within 1,000 m, 16-82% tree cover within 1,000 m, 0-100% above-pool tree canopy density, and 63-9,978 m<sup>2</sup> pool area at spring high water.

### 2.3.3 Measures of morphology

We conducted dip net surveys for wood frog larvae throughout larval development at 25, 30, and 12 pools (39 total) in 2014, 2015, and 2016, respectively. Surveys were conducted once every two weeks 10 June-19 August in 2014, and weekly 15 May-15 September in 2015 and 18 May-13 September in 2016. Up to 20 wood frog larvae were measured per survey. Within each pool, we sampled larvae from multiple locations, representing available vegetation and light conditions, to account for potential larval response to spatial variation within a pool. We recorded Gosner (1960) stage, mass, snout-vent length (SVL), tail length, and tail fin depth (following Relyea 2000).

To account for shifts in morphology with developmental stage and to satisfy assumptions of linearity in the relationship between morphological variables (Green 2001), we calculated individual morphological responses as the residuals of ANCOVA models where Gosner developmental stage was a factorial covariate (Packard and Boardman 1988; García-Berthou 2001). We calculated an index of relative developmental phenology (hereafter considered a morphological response and referred to as developmental phenology, Dev) as the residuals of the inverse of Julian day of measurement regressed on developmental stage (Table 2.1). This is appropriate because date of breeding does not influence date of metamorphosis (Berven 1990). Negative values represent a higher-than-expected Julian day given the developmental stage (i.e., relatively slow development), and conversely positive values represent relatively fast development. We calculated an index of relative condition (hereafter referred to as condition, Cond) as the residuals of square root-transformed mass (g) regressed on SVL and developmental stage. Condition is used to indicate amount of fats and other energetic reserves relative to body

size (reviewed by Green 2001). We calculated relative body length (Len) and tail length (TailL) as the residual of each of these raw responses regressed on SVL and developmental stage (Table 2.1). Because tail fin depth relative to body size (residuals of fin depth regressed against SVL and developmental stage) and fin depth relative to tail length (residuals of depth regressed against tail length and developmental stage; TailD) were highly correlated ( $r = 0.83$ , Pearson's correlation coefficient), we only used TailD as this provided a metric of tail shape and TailL already represented tail size.

For pool level analyses, we extracted aggregate morphology metrics: the 10, 50, and 90% quantiles and standard deviation (SD) from each site-year. Quantiles and SD better represent the variability within a population compared to the mean response (Cade and Noon 2003) and can be used to address the possibility that different segments within one population may be limited by different factors. We calculated aggregate measures for site-years with  $\geq 20$  tadpoles total collected during  $\geq 2$  site visits in a year.

#### **2.3.4 Egg mass surveys**

We conducted egg mass surveys at 43 pools to provide an index of the initial abundance of individuals in a pool as a baseline for estimating survival through metamorphosis. We also used the density of egg masses (number  $\times$   $m^{-2}$  pool area) to indicate conspecific competition (wood frog eggs) and predatory pressure from larval salamanders (spotted salamander [*Ambystoma maculatum*] and blue-spotted salamander [including the unisexual complex, *Ambystoma laterale* – *jeffersonianum*] eggs). We counted pool-breeding amphibian (wood frog, spotted salamander, and blue-spotted salamander) egg masses following the apparent peak of breeding using a dependent double-observer method to increase detection (Grant et al. 2005). If

eggs had been recently laid (within approximately two days) we revisited pools and counted new eggs.

Table 2.1 Larval wood frog responses from 2014-2016 in greater Bangor, Maine, USA. Missing data for some variables for some individuals and site-years account for differences in the number of site-year, site, and individual observations.

Response	Individual variables	Site-year variable(s) <sup>2</sup>	Site-years (sites, individuals)
Survival <sup>1</sup>	-	Sur	51 (33, -)
Developmental phenology (Julian day adjusted for developmental stage)	Dev	Dev10, Dev50, Dev90, DevSD	67 (39, 6,997)
Condition (mass adjusted for developmental stage and SVL)	Cond	Cond10, Cond50, Cond90, CondSD	65 (38, 6,692)
Body length (SVL adjusted for developmental stage)	Len	Len10, Len50, Len90, LenSD	65 (38, 6,919)
Tail shape (tail depth adjusted for tail length and developmental stage)	TailD	TailD10, TailD50, TailD90, TailDSD	66 (39, 5,788)
Tail length (adjusted for developmental stage and SVL)	TailL	TailL10, TailL50, TailL90, TailLSD	66 (39, 4,947)

<sup>1</sup> Responses only calculated for site-year

<sup>2</sup> 10, 50, 90, and SD indicate 10, 50, and 90% quantiles and standard deviation of responses within a site-year.

### 2.3.5 Estimation of survival

We used repeated counts of late stage (Gosner stage  $\geq 41$ ) tadpoles to calculate an index of survival (hereto referred to as survival). We conducted tadpole counts using dip net surveys at 42 sites (35, 26, and 12 pools in 2014, 2015, and 2016). The number of net sweeps was based on pool size at time of sampling (minimum of 2 sweeps, maximum of 50 sweeps; size measured at each sampling). This method of adjusting effort was to accommodate rapidly changing pool area with rain or rapid drying within a few days. In 2014, we calculated the number of sweeps per pool based on the estimated pool volume (one sweep per 25 m<sup>3</sup>). In 2015-16, we used pool surface area to determine the number of sweeps (one sweep per 25 m<sup>2</sup>). We measured length and width of a pool with a tape and maximum depth at pool center with a meter stick and used formulas for half of an ellipsoid or an ellipse to calculate volume or area. We recalculated the number of sweeps for the second and/or third day if the water surface area and/or depth had changed since the first day. Surveys began at a site when we detected a tadpole developed to Gosner stage  $\geq 41$  (approximate stage indicating pro-metamorphosis and thus a useful proxy of survival through metamorphosis) during morphology observations. We conducted 3 surveys within 3-4 days at each pool, where the net (0.3 m width x 0.22 m height) was gently dragged 1.5 m along the bottom and tadpoles were sampled without replacement. Sweep locations were selected to representatively sample the vegetation and light conditions in a pool.

We used package ‘unmarked’ (Fiske et al. 2017) to fit negative-binomial N-mixture models (MacKenzie et al. 2006) that estimated abundance ( $\lambda$ ) of tadpoles x m<sup>-2</sup> within sampled areas by site while accounting for imperfect detection ( $p$ ) (Royle 2004). We extrapolated the estimated tadpoles x m<sup>-2</sup> to the entire sampled pool area to estimate the abundance of tadpoles in



a pool. We then used the residuals of total abundance regressed against the number of egg masses to estimate survival by pool. We recognize that this method does not incorporate error in abundance estimates and egg mass counts. However, mark-recapture methods, which would theoretically provide more robust estimates of survival, are not feasible because embryos cannot be marked and marking techniques are unreliable for tadpoles (Grant 2008; Carlson and Langkilde 2013) or require a tissue sample at each capture for genetic analysis (Ringler et al. 2015), potentially affecting survival. We included pool depth, area, and number of dip net sweeps as visit-level detection covariates and number of wood frog egg masses and a vegetation density index as site-level detection covariates in models (Table 2.2). We created three global models which included all detection covariates using Poisson, negative binomial, and zero-inflated Poisson abundance distributions and selected the best global model using Akaike's Information Criterion adjusted for small sample size (AICc; models with  $\Delta\text{AICc} \leq 2$  were considered to be strongly supported). We tested the selected global model for goodness-of-fit (1,000 bootstrap permutations; Mackenzie and Bailey 2004). If the selected global model was overdispersed ( $\hat{c} > 1$ ) we inflated all subsequent estimated parameter standard errors by a factor of  $\sqrt{\hat{c}}$  and used a quasi-corrected AICc (QAICc) for further model selection (Burnham and Anderson 2002). We fit all combinations of covariates on detection and ranked models by QAICc and selected Beta ( $\beta$ ) parameters for the final model as those from models with  $\Delta\text{AICc} \leq 2$  and 95% confidence intervals (CI) excluding zero. We used the final model (results in Appendix B) to estimate the number of tadpoles  $\times \text{m}^{-2}$  of dipped area per site.

Table 2.2 Detection covariates used to estimate abundance of late stage wood frog tadpoles at 42 vernal pools across an urban development gradient in 2014-2016 in greater Bangor, Maine, USA.

Variable	Description
Veg	PC1 from a PCA of within-pool summer vegetation cover (shrub and emergent and submerged vegetation); positive relationship with cover
Depth	Maximum pool depth on day of sampling
Area	Estimated pool area on day of sampling
Dip	Number of dip net sweeps on day of sampling
Egg	Number of wood frog egg masses

### 2.3.6 Site characteristics

We used ArcView GIS10.2 and the Maine Land Cover Dataset (2004 all land use; 2011 impervious surface) to quantify the percent impervious and forest cover within 100, 300, 600, and 1,000 m from pool high water marks. We used impervious cover within 300 m (based on scales of land cover type identified as important during Random Forest Analyses, RFA) as an indicator of urbanization intensity. We assigned the 10 pools with the most impervious cover at 300 m (16.5-36.8%) to an “urban” group and the 10 pools with the least impervious cover at 300 m (0-1.9%) to a “rural” group.

We measured pool area at spring-high-water using a sub-decimeter accuracy GPS (Trimble Geo 7x, Westminster, Colorado) and maximum spring-high-water depth (April 17 - May 22) using a pole marked in centimeter increments (independent of rough pool measurements used to determine sample sizes for larval survival surveys). Hydroperiod was

determined by the Julian day that standing water was no longer present as observed during field visits or by trail cameras placed at pools for a separate study (Chapter 1). Because tadpoles can survive in small volumes of water (<1 L) for a short time, even small puddles were used to indicate standing water. To treat hydroperiod as a continuous variable in subsequent analyses, pools that did not dry in a given year were assigned Julian day 280 if they dried to within 10 cm or Julian day 300 if they had  $\geq 10$  cm of water at the deepest point (values only determine rank-order of continuous explanatory variables used in RFA; De'ath & Fabricius 2000). These values were selected because no pool dried during the study after Julian day equivalent 266 (23 September).

We measured vegetation in spring and summer within pool basins at 25, 36, and 11 sites in 2014, 2015, and 2016, respectively. Spring surveys were conducted 2-9 June 2014, 2-20 May 2015, and 19 May-7 June 2016 when vernal pool-breeding amphibian eggs were present. Summer surveys were conducted 24 July-21 August 2014, 22 July-17 August 2015, and 19 July-26 July 2016 at late summer dry down. We visually estimated percent cover of shrub, emergent vegetation, and submerged vegetation to the nearest 10%. We separately estimated duckweed (subfamily Lemnoideae) because we suspected it may be related to anthropogenic disturbance. Duckweed was estimated to the nearest 10%, using 1% to indicate any presence. Woody vegetation canopy density over pools was measured ~1 m above the ground near the pool center using a spherical convex densitometer.

We used water probes (Hach ©, Loveland, Colorado) at 43 pools to sample specific conductance, dissolved oxygen (DO), and water temperature; more specifically, 34 pools (31 pools once and 3 pools twice) 3 May-10 June 2014; 37 pools (2 pools once and 35 pools twice) 2

May-12 June 2015; and 12 pools (4 pools once, 6 pools twice, and 2 pools thrice) 6 May-16 June 2016. We did not adjust water temperature by Julian day because the correlation was small ( $r = 0.16$ , Pearson's correlation coefficient). On each date a pool was sampled, we collected and tested 1 L of surface water ~1 m from the water edge at each of three equidistant points around the perimeter. Only one sample was taken at pools that were almost dry and  $<2 \text{ m}^2$ . All testing was conducted at the pool edge within minutes of sample collection. Each metric was averaged by day and then year to calculate values used in analyses.

Some missing values occurred in site characteristic data:  $\leq 5\%$  in datasets used for analysis of tadpole responses. We used k-nearest neighbor ( $k\text{NN}$ ) imputation (package 'DMwR', Torgo 2010) to replace missing values with a weighted mean of the 10 nearest neighbors. Single imputation using  $k\text{NN}$  provides robust missing value estimates for datasets with  $\leq 20\%$  missing values (Troyanskaya et al. 2001).

Table 2.3 Explanatory variables used to predict larval wood frog responses in Random Forest Analyses. All variables except for impervious and tree cover were measured by site-year; impervious and tree cover were measured by site.

Abbreviation	Variable (unit)
ALDEN	<i>A. laterale</i> egg masses x m <sup>-2</sup>
AMDEN	<i>A. maculatum</i> egg masses x m <sup>-2</sup>
AREA	Pool area (m <sup>2</sup> )
DO	Dissolved oxygen (mg/L)
HYDRO <sup>1</sup>	Hydroperiod (Julian day of pool drying)
IMP100	Impervious cover within 100 m of pool (%)
IMP1000	Impervious cover within 1,000 m of pool (%)
IMP300	Impervious cover within 300 m of pool (%)
IMP600	Impervious cover within 600 m of pool (%)
LSDEN	<i>L. sylvaticus</i> egg masses x m <sup>-2</sup>
MAXDEPTH	Maximum pool depth at spring high water (m)
SPCAN	Spring tree canopy cover within pool (%)
SPCOND	Specific conductivity (mS/cm)
SPDUCK	Spring duckweed cover within pool (%)
SPEMERG	Spring emergent vegetation cover within pool (%)
SPSHRUB	Spring shrub cover within pool within pool (%)
SPSUBMG	Spring submerged vegetation cover within pool (%)
SUCAN	Summer tree canopy cover within pool (%)
SUDUCK	Summer duckweed cover within pool (%)
SUEMERG	Summer emergent vegetation cover within pool (%)

Table 2.3, continued

Abbreviation	Variable (unit)
SUSHRUB	Summer shrub cover within pool within pool (%)
SUSUBMG	Summer submerged vegetation cover within pool (%)
TEMP	Water temperature (C)
TREE100	Tree cover within 100 m of pool (%)
TREE1000	Tree cover within 1,000 m of pool (%)
TREE300	Tree cover within 300 m of pool (%)
TREE600	Tree cover within 600 m of pool (%)

<sup>1</sup> Julian day of drying adjusted for pools that did not dry (details in Methods).

### 2.3.7 Morphology profile analysis

We conducted principal components analyses (PCA) using the ‘vegan’ package (Oksanen et al. 2017) in R version 3.3.1 (R Development Core Team, 2016) to identify dominant gradients of variation in tadpole morphology and development within site-years and individuals (Borcard et al. 2011). To examine site-year profiles, we created a correlation matrix of 10, 50, and 90% quantiles and SD of developmental phenology, condition, body length, and tail length and depth for site-years with complete observations across these variables (64 site-years; 38 sites). To examine individual morphology profiles, we created a correlation matrix of developmental phenology, condition, body length, tail length, and tail shape variables for individuals with complete observations from site-years used in the site-year PCA. Variables were standardized to have a mean of zero and standard deviation of one to avoid unequal weighting among variables within the individual and site-year data sets, respectively. We used a Monte Carlo randomization test (1,000 permutations) to select significant axes ( $P < 0.01$ ) for further examination.

We compared the individual and site-year ordinations to examine the possibility that the tadpoles in a pool may display multiple, distinct profile ‘types’ (i.e., multiple morphology strategies within one pool). We assumed that the individual ordination was a fair representation of the relationships among morphology characteristics, even at the site-year level, as the relative eigenvector contributions of site-year variables to the correlation matrix were similar to those of the same variable type in the individual ordination (e.g., the relationships of Cond10, Cond50, and Cond90 relative to other site-year variables were similar to the relationship of Cond relative to other individual variables). Because individual and site-year profiles were similar, we used individual profiles to examine differences in morphological response (site and year effects and

between rural and urban groups). Because survival may not depend on all tadpoles in a pool (e.g., the smallest or slow-developing) and/or may be influenced by within-pool variance in responses, we used site-year profiles to examine the relative importance of aggregate responses (including within site-year variance) to predict survival.

To test for Site, Year, and Site x Year effects and urbanization level (Urban), Year, and Year x Urban effects in individual profiles, we conducted permutations of multiple analysis of variance (PERMANOVA, package 'vegan') for a site-year model and an urban-year model. The urban-year model was conducted for the subset of individuals included in rural and urban pools. We ran 5,000 permutations of PERMANOVA using a Bray-Curtis similarity matrix (Anderson and Walsh 2013) and nesting individuals by site-year for permutations (Anderson 2001). We used the F-value as a signal-to-noise ratio to indicate effect size (McCune et al. 1997; Short and Morris 2016). If there were significant differences in multivariate profiles identified in site-year or urban-year models, we conducted ANCOVAs to examine if there were differences among site, year, or urbanization level, and if these predictors changed the relationship between pairs of raw measurements used to calculate residuals (Table 2.1). We used eta-squared ( $\eta^2$ ) to indicate effect size (i.e., % change in the response accounted for by the predictor; Levine & Hullett 2002). If a model had significant main terms with medium to large effect sizes but a significant interaction term with a small effect size ( $\eta^2 < 0.05$ ) we dropped the interaction prior to interpreting the coefficients of the main effects. Mass, tail length, and tail depth regressed against SVL (both natural log-transformed) was examined in rural and urban pools for tadpoles with 6-22 mm SVL; these predictor ranges were limited to maintain comparable x-axis values between rural and urban sites.



For the site-year dataset, we used classification and regression trees (CART) analyses, specifically RFA to identify the relative importance of aggregate tadpole morphology variables in explaining survival. We conducted RFA using package ‘randomForest’ (Liaw and Wiener 2002) in R version 3.3.1 (R Core Team 2016). Classification and regression trees analyses are a non-parametric approach where data is recursively split into homogenous groups based on rank-order of continuous explanatory variables (De’ath and Fabricius 2000). These trees can be particularly useful in data exploration because they do not assume a specific statistical distribution for explanatory variables nor assume data independence and thus avoid potential concerns about pseudo-replication (Breiman 2001a). To avoid over-fitting and ensure robust classification by models, we conducted RFA, a method where many classification trees are constructed for each response variable and the dominant classification structure is selected (Breiman 2001b). We bootstrapped with replacement to build 10,000 regression trees (Random Forest error stabilized at approximately 1,000-2,000 trees for each response variable), using 2/3 of the data at each iteration. We calculated explanatory variable importance using the mean percent decrease in accuracy resulting from removal of each variable to rank the importance of explanatory variables. We used package ‘randomForestSRC’ (Ishwaran and Kogalur 2014) to create partial dependence plots (PDPs) that examine the marginal effects of predictor variables while holding all other predictors at average values (Friedman 2001; Cutler et al. 2007). Because PDPs display general trends all reported values are approximate.

### **2.3.8 Modeling tadpole response on site characteristics**

We conducted ANCOVAs to examine if urbanization level (rural, urban) or year changed the relationship between number of egg masses and estimated abundance of tadpoles that

survived to metamorphosis (i.e., survival) between rural and urban pools. We used eta-squared ( $\eta^2$ ) to indicate effect size (i.e., % change in the response accounted for by the predictor; Levine & Hullett 2002). Survival was examined for rural and urban pools with <70 egg masses; 4 urban site-years with 100-402 egg masses were dropped from analyses to maintain comparable x-axis values between rural and urban sites.

We used RFA following the same methods described above to identify the importance of site characteristics (Table 2.3) that may affect survival and aggregate measures of developmental phenology, condition, and tail length and shape (Table 2.1). We included the respective sample size (DEV.N, COND.N, LEN.N, TAILL.N, TAILS.N) as a predictor in each model because scatterplot trends suggested that variance decreased (condition, body length, and tail length) or increased (developmental phenology) with sample size. A decrease may be an artifact of larger sample size leading to increasingly precise estimates of group means, or a decrease or increase may be a result of site characteristics that affect the availability of tadpoles for sampling throughout the season (therefore the sample size) also affecting variance. Because we were interested in the influence of site conditions at the pool-scale, we modeled annual (site-year) responses.

For each survival or aggregate response with variance explained by RFA we conducted a separate ANCOVA for each of the best predictors identified during RFA (those for which we present partial dependence plots). Global model terms included urbanization level and an RFA predictor. We dropped non-significant interaction terms (F-test;  $P > 0.05$ ) and kept all main effects contained in significant interactions. We disregarded models with statistically significant

RFA predictors that did not have a similar range in values (x-axis distribution) across urbanization levels.

## 2.4 Results

For larval morphological responses, 20-327 tadpoles were sampled per site-year (median = 94), for a total of 6,997 tadpoles, with tadpoles sampled at Gosner stages 26-41.5 (one front leg emerged) and these ranged from 0.03-1.85 grams, 4-30 mm SVL, 3-40 mm tail length, and 2-20 mm tail depth. We estimated survival through metamorphosis for 51 site-years. Pool area and specific conductivity were greater in urban than rural pools (rural mean area = 429 m<sup>2</sup>, urban = 911 m<sup>2</sup>, Welch's  $t_{17} = -1.85$ ,  $P=0.08$ ; rural mean conductivity = 42.5 uS, urban = 378.3 uS,  $t_{17} = -3.50$ ,  $P<0.01$ ), as were scales of impervious cover not used to categorize urbanization levels. Tree cover was greater for rural pools at all scales (100 m:  $t_{17} = 5.97$ ,  $P<0.01$ ; 300 m:  $t_{25} = 8.14$ ,  $P<0.01$ ; 600 m:  $t_{23} = 8.94$ ,  $P<0.01$ ; 1,000 m:  $t_{19} = 8.10$ ,  $P<0.01$ ). There were no differences ( $P>0.1$ ) between urbanization levels for other site characteristics.

### 2.4.1 Morphological profile

Individual morphology profiles (Dev, Cond, Len, TailL, TailD) were best predicted by year in site-year models and by urbanization level in urban-year models. In both models, all predictors (main effects and interactions) were significant (PERMANOVA,  $P<0.001$ ), but main effects had larger effects than interaction terms (site-year:  $F_{\text{site} \times \text{year}} = 10.50$ ; urban-year:  $F_{\text{urban} \times \text{year}} = 17.25$ ). In the site-year model (including all sites), Year had a greater effect than Site and thus was relatively more important in distinguishing profiles ( $F_{\text{year}} = 86.93$ ;  $F_{\text{site}} = 36.41$ ). In the urban-year model (including only rural and urban sites), Urban had a

greater effect than Year ( $F_{\text{Urban } 1, 2240} = 102.27$ ;  $F_{\text{Year } 2, 2240} = 71.45$ ). Developmental phenology was not strongly associated with condition, body length, or tail morphology, but tail length and condition were positively associated, as were body length and tail depth (Figure 2.1). No distinct profile “types” (i.e., clusters) emerged in morphology profiles.

Developmental phenology was predicted to be 8% earlier in 10 urban sites than in 8 rural sites ( $F_{\text{Urban:1,3241}} = 1163.7$ ,  $P < 0.01$ ,  $\eta^2 = 0.13$ ; Figure 2.2). Across all 38 sites there were significant differences in developmental phenology ( $F_{\text{Site:37,6713}} = 145.96$ ,  $P < 0.01$ ,  $\eta^2 = 0.22$ ), with tadpoles at the earliest site developing 23% earlier than at the latest site. There were no other significant influences of Site, Year, or Urban with a medium to large effect size ( $\eta^2 \leq 0.07$ ) on pairs of morphology variables (Appendix B).

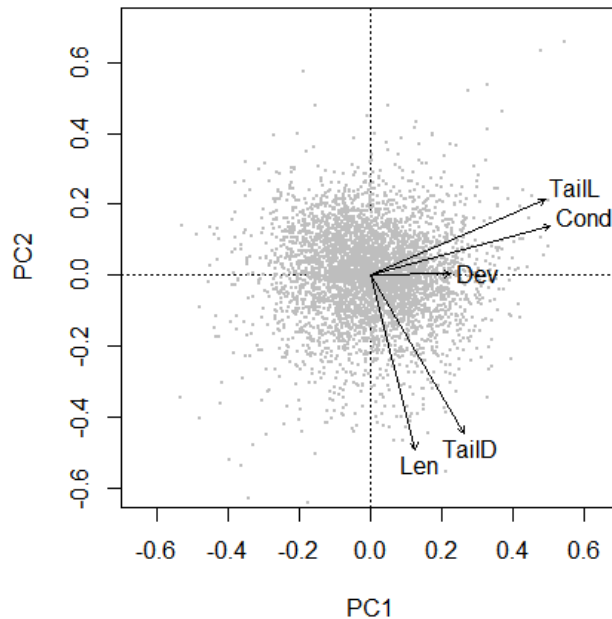


Figure 2.1 Principal components analysis (PCA) of individual wood frog tadpole morphology profiles. The first and second axes (PC1 and PC2) were significant ( $P < 0.001$ ) and account for 34.7 and 27.6% of the total variation, respectively. The first PC was positively described by relatively equal contributions from tadpole condition and tail length (0.64 and 0.62 respective eigenvalues). The second PC was negatively described by relatively equal contributions from body size and tail shape (-0.69 and -0.63 eigenvalues). Developmental phenology is poorly represented (communality value = 0.14 compared to 0.70-0.79 for other metrics).

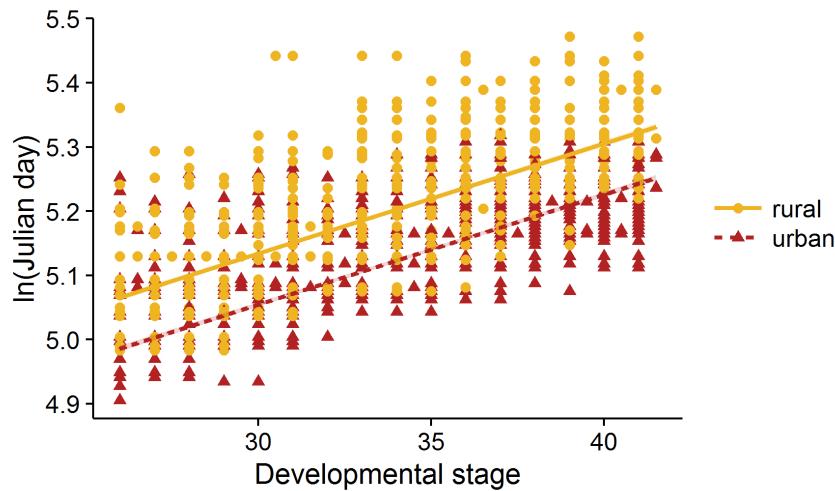


Figure 2.2 Individual wood frog tadpole developmental phenology values in 8 rural and 10 urban vernal pools (1,365 and 1,879 tadpoles, respectively) collected in Maine during 2014-2016. Each dot represents an individual. The represented equation is:  $\ln(\text{Julian day}) = 4.620 - 0.079\text{Urban} + 0.017\text{Stage}$ , adjusted  $R^2 = 0.64$ . This figure is for illustrative purposes. Population identity was accounted in the actual analysis.

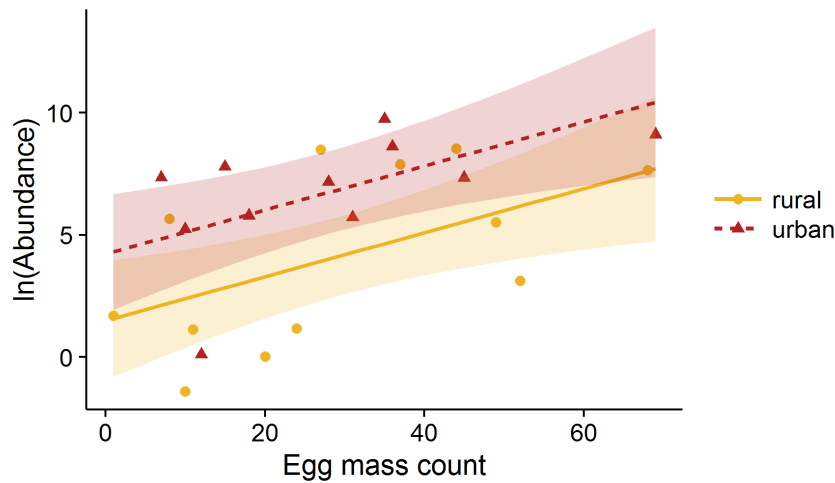


Figure 2.3 Survival of wood frog tadpoles through metamorphosis for rural and urban pools in terms of abundance of tadpoles to complete metamorphosis relative to the number of egg masses counted. Each dot represents a site-year. Shaded areas represent parameter 95% confidence intervals. The represented equation is:  $\ln(\text{Abundance}) = 1.487 + 2.728\text{Urban} + 0.090\text{Egg}$ , adjusted  $R^2 = 0.36$ .

## 2.4.2 Survival

Survival in urban pools was predicted to be 15% greater than in rural pools ( $F_{1,20} = 5.93$ ,  $P = 0.02$ ,  $\text{Adj-R}^2 = 0.36$ ; Figure 2.3). RFA survival models using morphology or site characteristic predictors did not explain any variation in the response (i.e., not different from random), thus predictor importance was not examined.

## 2.4.3 Developmental phenology

Tadpole phenology was predicted to be earlier in warmer pools with a shorter duration, lower conspecific density, and less tree cover. Later phenology was associated with longer hydroperiods, especially for pools drying after Julian day 200, 19 July (Figures 2.4a, c, and h; See Appendix B for ranking of predictors). Conversely, earlier developmental phenology was predicted by warmer water (Figures 2.4f and h) with the greatest increases in the lower third of the range of measured temperatures. Developmental phenology was also negatively associated with wood frog egg masses  $\times \text{m}^{-2}$ , an indicator of conspecific competition (Figures 2.4b and d). Finally, greater tree cover was associated with later phenology, with the greatest decrease at  $>60\%$  tree cover within 100 m of pools (Figures 2.4e and g) and a relatively linear association between tree cover within 1,000 m and developmental phenology (Figure 2.4j).

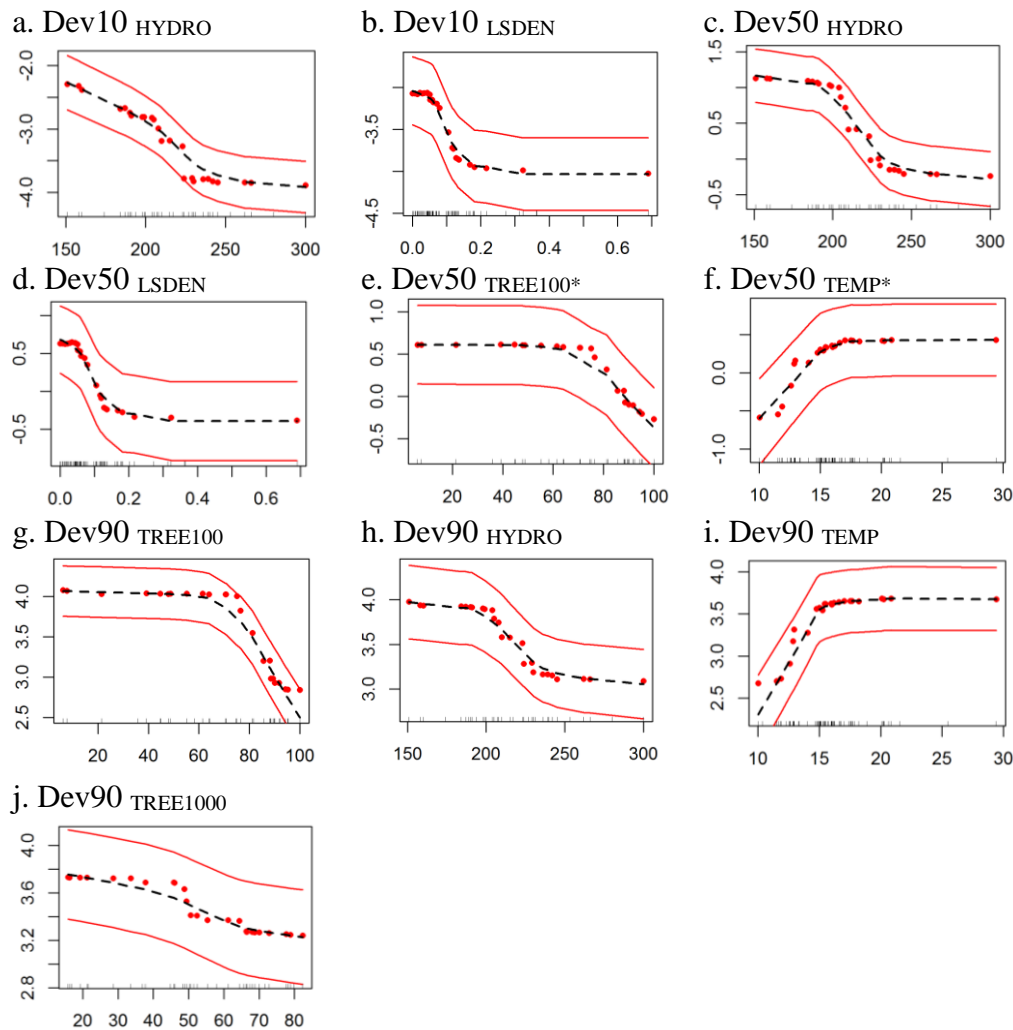


Figure 2.4. Partial dependence plots from Random Forest Analyses predictions of relative developmental phenology (adjusted for Julian day) for larval wood frog. Responses are 10, 50, and 90% quantiles of developmental phenology (Dev10, Dev50, and Dev90). See Table 2.3 for abbreviation legend for predictors (subscript). Dashed lines correspond to lowess smoothed lines representing the partial dependence between an explanatory variable and response. The solid lines indicate a smoothed error bar of  $\pm$  two standard errors. The dots indicate the partial values used to fit the lowess function. Plots marked with (\*) for the same response have roughly equivalent variable importance.

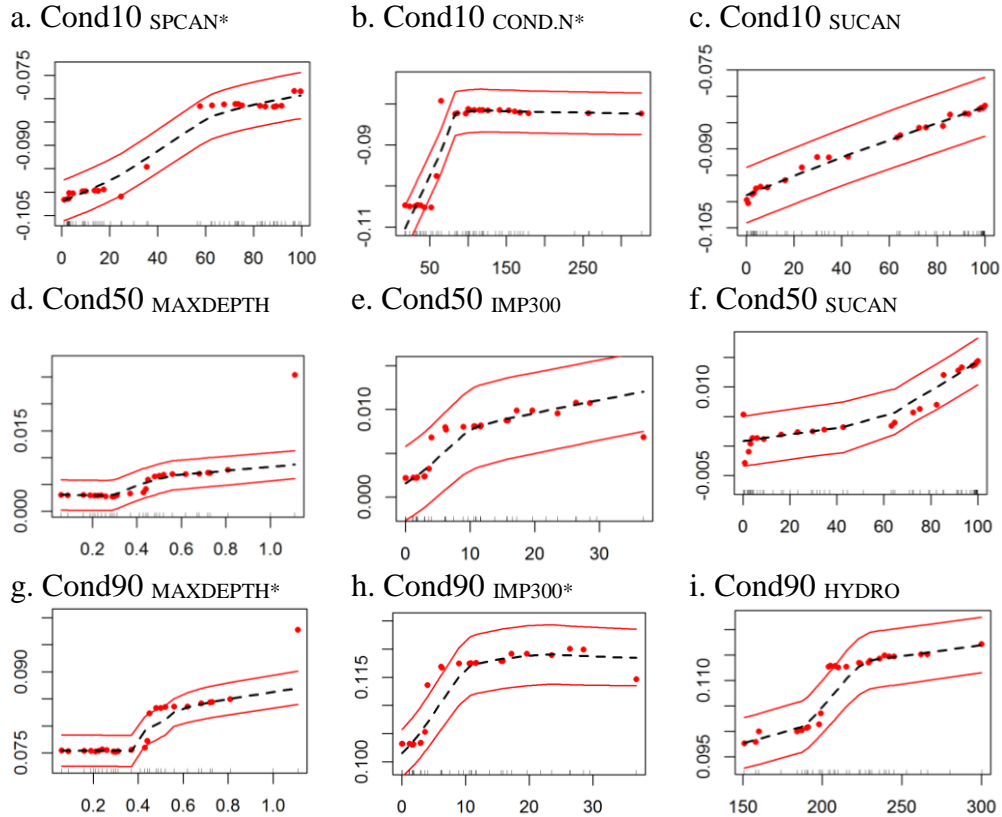


Figure 2.5 Partial dependence plots from Random Forest Analyses predictions of relative condition (mass regressed against SVL, adjusted for developmental stage) for larval wood frog. Responses are 10, 50, and 90% quantiles of condition (Cond10, Cond50, and Cond90). See Table 2.3 for abbreviation legend for predictors (subscript). Dashed lines correspond to lowess smoothed lines representing the partial dependence between an explanatory variable and response. The solid lines indicate a smoothed error bar of  $\pm$  two standard errors. The dots indicate the partial values used to fit the lowess function. Plots marked with (\*) for the same response have roughly equivalent variable importance.



#### **2.4.4 Condition**

Tadpoles in pools with a denser canopy cover and greater depth were predicted to be in better condition. Canopy cover was an important predictor of condition (roughly linearly associated with greater condition), as was greater depth and duration of water (positively associated with depths >0.4 m and longer hydroperiods; Figures 2.5a, c, d, f, g, and i). Impervious cover and sample size were also high-ranking predictors positively associated with condition (Figures 2.5b and h).

#### **2.4.5 Body length**

Site characteristics indicative of more water (depth, lower conspecific density, less emergent vegetation) were important predictors of greater body length. Pool depth and submerged vegetation were important predictors of body length, with deeper pools (especially increases in depth 0.3-0.6 m) and lesser emergent vegetation associated with longer bodies (Figures 2.6a, b, and f). Greater density of wood frog eggs (up to 0.2 egg masses x m<sup>-2</sup>) and sample size (up to 75 individuals) were associated with shorter body length (Figures 2.6e, g, and i). Although canopy was a top-ranking predictor, lowess smoothing of predicted trends suggests body length is relatively invariant to canopy cover (Figures 2.6c, d, and h).

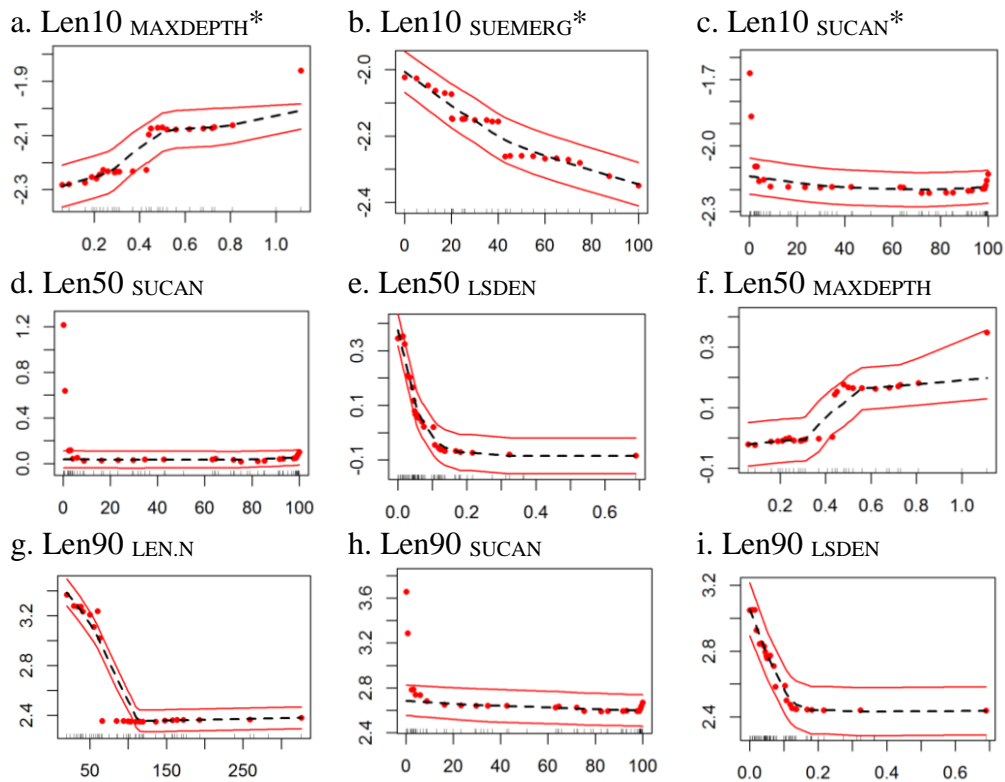


Figure 2.6 Partial dependence plots from Random Forest Analyses predictions of relative body length (adjusted for developmental stage) for larval wood frog. Responses are 10, 50, and 90% quantiles of body length (Len10, Len50, and Len90). See Table 2.3 for abbreviation legend for predictors (subscript). Dashed lines correspond to lowess smoothed lines representing the partial dependence between an explanatory variable and response. The solid lines indicate a smoothed error bar of  $\pm$  two standard errors. The dots indicate the partial values used to fit the lowess function. Plots marked with (\*) for the same response have roughly equivalent variable importance.

#### **2.4.6 Tail Length**

Site characteristics related to low-light conditions, greater urbanization, or lower conspecific competition were important predictors of greater tail length. The greatest increases in tail length were where spring canopy cover >30% or summer canopy cover >60% (Figures 2.7a-c, and h), sites with 0-10% impervious cover within 300 m (Figures 2.7d and j), and at sites with <40% spring emergent vegetation and/or <30% summer shrub cover (Figures 2.7f-g). Greater tree cover at 300 and 1,000 m was associated with shorter tails (Figures 2.7e and i). Wood frog egg density was also an important predictor of tail length, with lesser tail length associated with more wood frog egg masses  $\times \text{m}^{-2}$  within the lower third of the range of measured egg densities (Figures 2.7k and m).

#### **2.4.7 Tail shape**

Pool area was the most important predictor for tail shape (pools <50  $\text{m}^2$  predicted to have tadpoles with broader fins), followed by impervious surface with fin breadth expected to increase with up to 10% cover within 300 m (Figures 2.8a-d). However, as tail shape was predicted to be relatively invariant to pool area (Figures 2.8a and c), we hesitate to interpret the direction of association between area and tail shape.

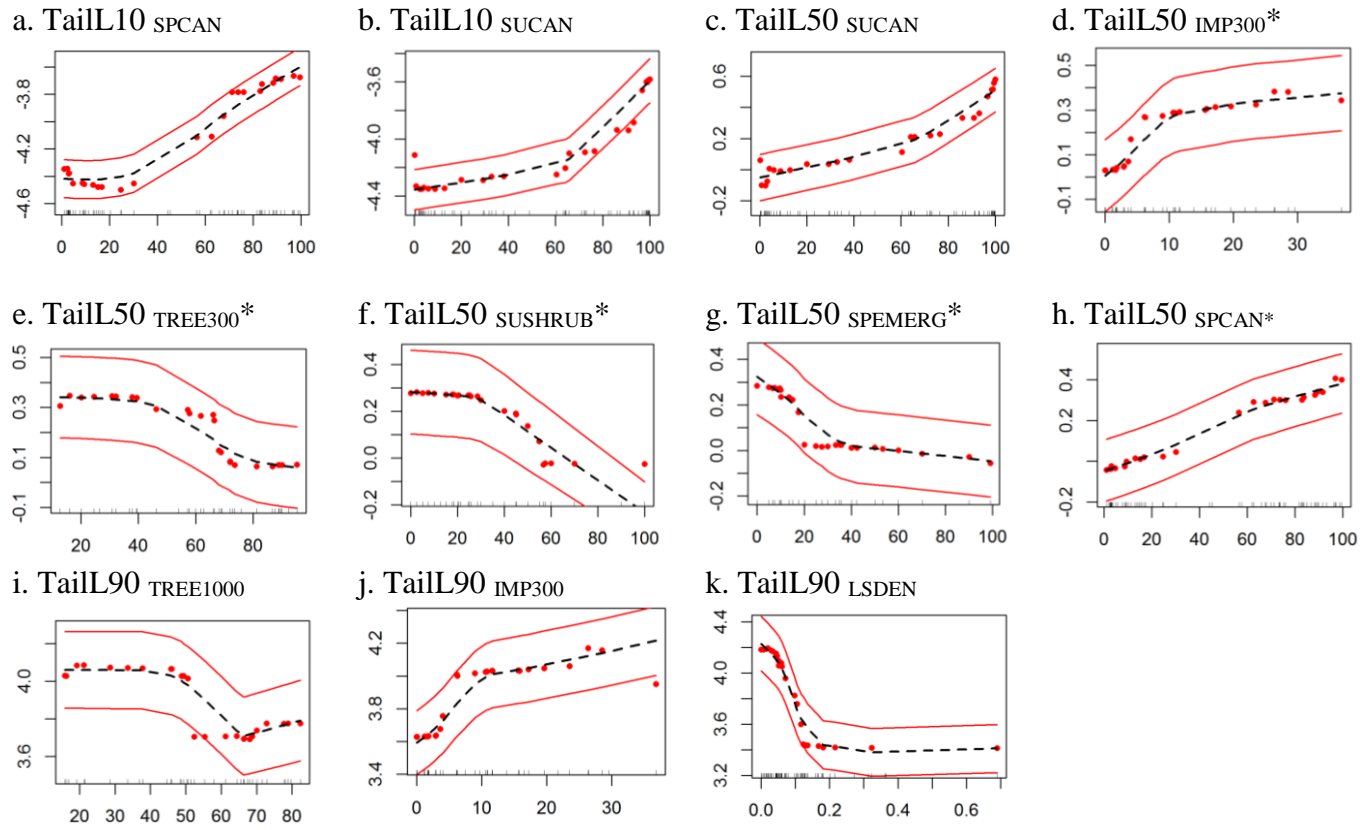


Figure 2.7 Partial dependence plots from Random Forest Analyses predictions of relative tail length (adjusted for developmental stage and body length) for larval wood frog. Responses are 10, 50, and 90% quantiles of tail length (TailL10, TailL 50, and TailL 90). See Table 2.3 for abbreviation legend for predictors (subscript). Dashed lines correspond to lowess smoothed lines representing the partial dependence between an explanatory variable and response. The solid lines indicate a smoothed error bar of  $\pm$  two standard errors. The dots indicate the partial values used to fit the lowess function. Plots marked with (\*) for the same response have roughly equivalent variable importance.

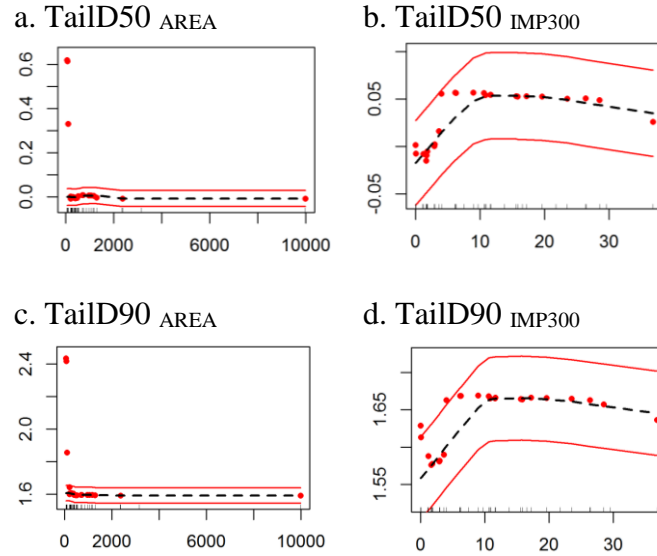


Figure 2.8 Partial dependence plots from Random Forest Analyses predictions of tail depth relative to tail length (adjusted for developmental stage) for larval wood frog. Responses are 50 and 90% quantiles of tail depth (TailD50, TailD90). See Table 2.3 for abbreviation legend for predictors (subscript). Dashed lines correspond to lowess smoothed lines representing the partial dependence between an explanatory variable and response. The solid lines indicate a smoothed error bar of +/- two standard errors. The dots indicate the partial values used to fit the lowess function.

### 2.4.8 Morphological variation within pools

Site characteristics indicative of more water (duration or depth) and higher light conditions (less canopy, greater submerged vegetation) were important predictors of variance in developmental phenology (positively associated at 0-20% spring submerged vegetation and >0.5 m maximum depth; Figures 2.9b and d), condition (positively associated with pool drying between Julian day 200-230 [19 July – 18 August] and negatively associated with canopy cover; Figures 2.9f-h), and body length (pool drying near Julian day 190 [9 July]; Figure 2.9j). Amphibian egg mass densities were also important predictors of variance; variance in developmental phenology was positively associated with 0-3 blue-spotted salamander eggs x m<sup>-2</sup>, and variance in tail length was negatively associated with wood frog egg density (Figures 2.9c

and l). Sample size - which was likely responsive to duration of larval presence, abundance, and within-pool detection probability - was also an important predictor of variation in developmental phenology (roughly linearly, positively correlated), condition (negatively associated with increased sample size up to 80 individuals), and body length (negatively associated with increased sample size up to 75 individuals; Figures 2.9a, e, and i). Impervious cover was an important predictor of variance in tail length (positively associated with increases in impervious cover within 300 m between 0-10%; Figure 2.9k).

#### **2.4.9 Differences in response to site characteristics between urbanization levels**

Developmental phenology, condition, body length, and tail length were greater in urban than rural pools when accounting for the influence of important site characteristics (Figure 2.10). The effect of these site characteristics was the same across urbanization levels ( $P > 0.05$  for interaction terms).

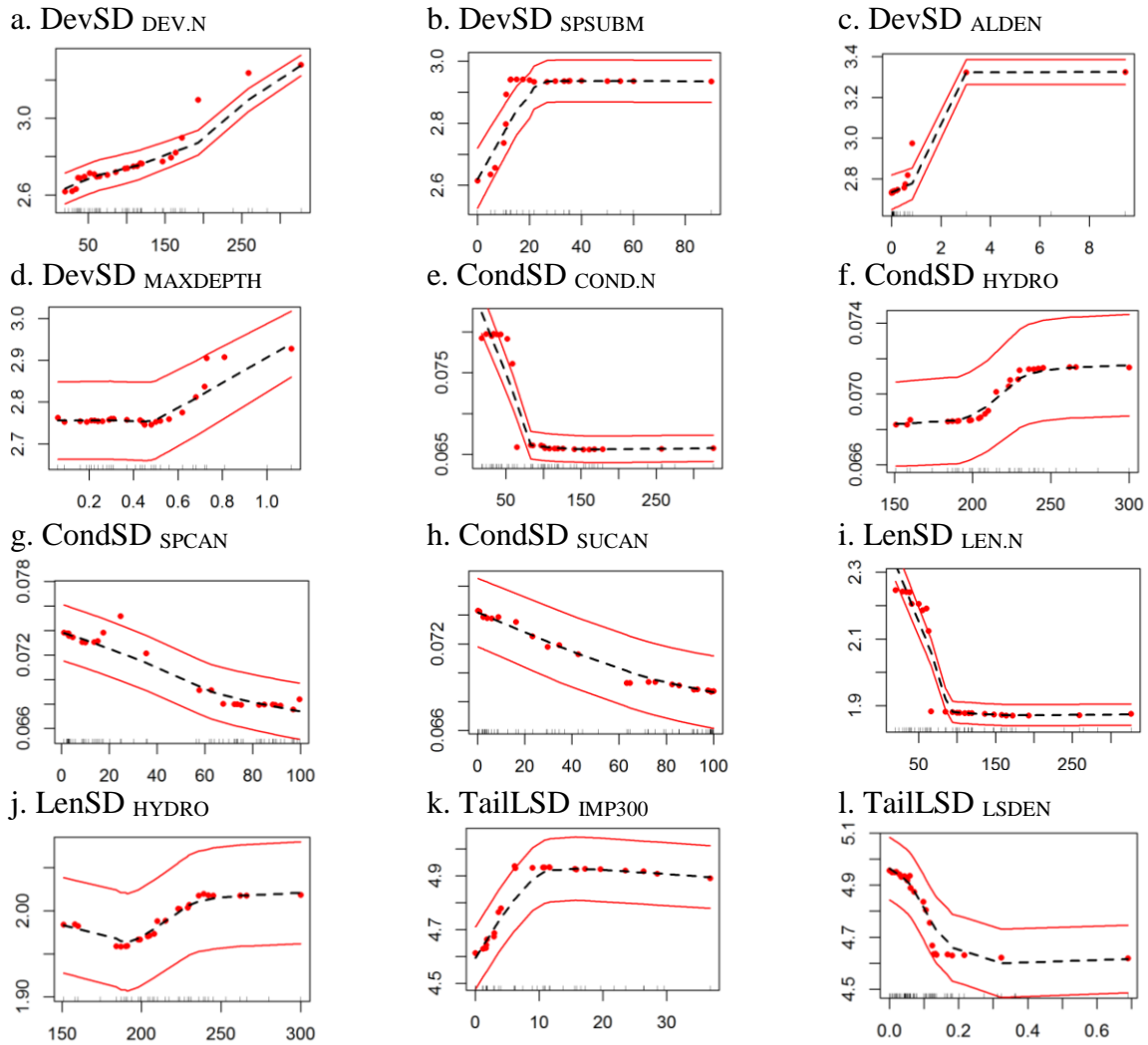


Figure 2.9 Partial dependence plots from Random Forest Analyses predictions of SD of relative developmental phenology (Dev), condition (Cond), body length (Len), and tail length (TailL) for larval wood frog. See Table 2.3 for abbreviation legend for predictors (subscript). Dashed lines correspond to lowess smoothed lines representing the partial dependence between an explanatory variable and response. The solid lines indicate a smoothed error bar of +/- two standard errors. The dots indicate the partial values used to fit the lowess function.

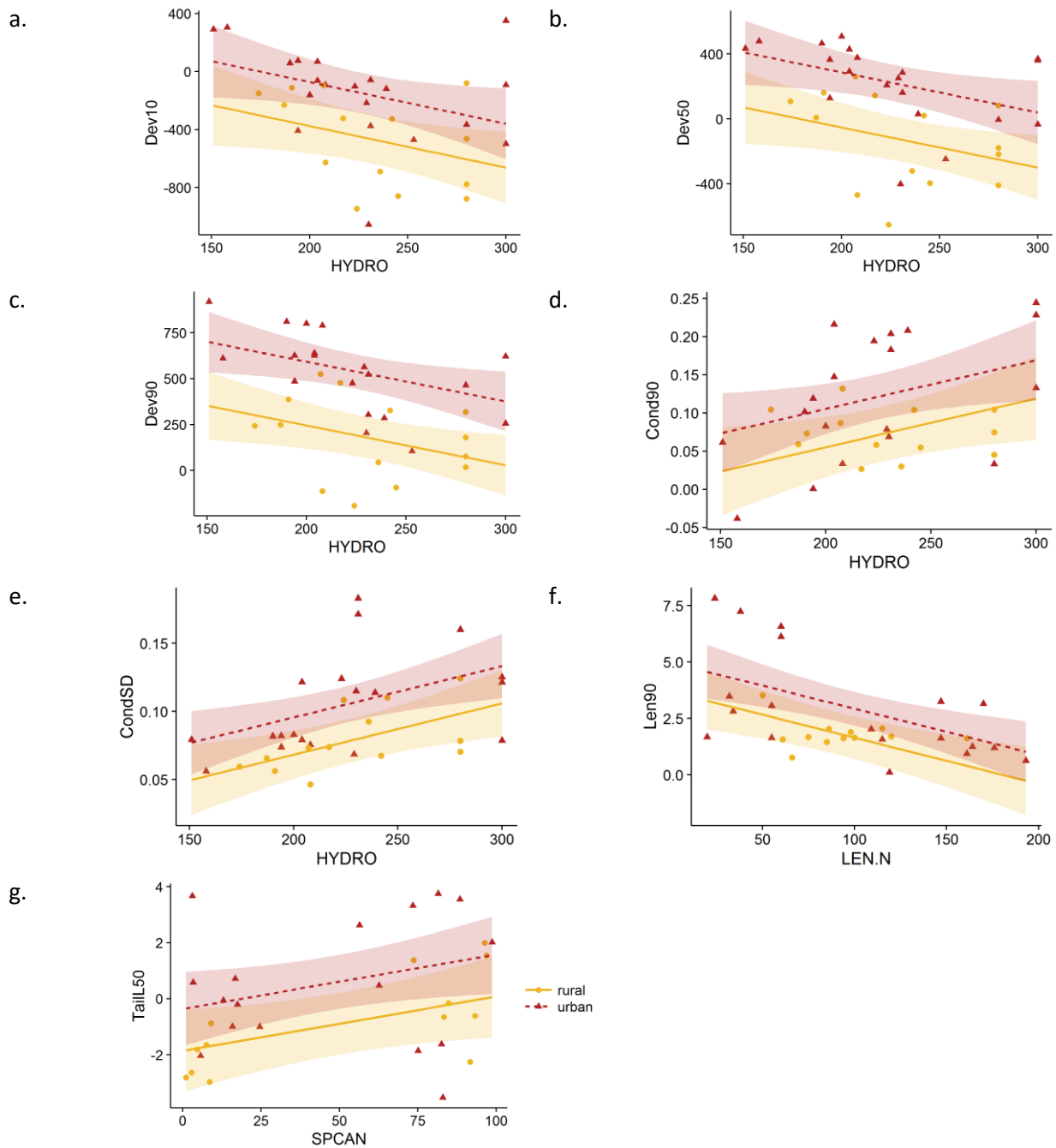


Figure 2.10. Associations between wood frog tadpole cohort responses and important site characteristics for which there were significant differences between rural and urban pools (Dev10:  $F_{\text{urban}:1,31} = 7.98$ ,  $P = 0.008$ ; Dev50:  $F_{\text{urban}:1,31} = 15.57$ ,  $P < 0.001$ ; Dev90:  $F_{\text{urban}:1,31} = 23.46$ ,  $P < 0.001$ ; Cond90:  $F_{\text{urban}:1,29} = 4.79$ ,  $P = 0.006$ ; CondSD:  $F_{\text{urban}:1,29} = 7.12$ ,  $P = 0.01$ ; Len90:  $F_{\text{urban}:1,28} = 4.81$ ,  $P = 0.004$ ; TailL50:  $F_{\text{SPCAN}:1,27} = 4.28$ ,  $P = 0.048$ ). See Tables 2.1 and 2.3 for abbreviation legend for responses and site characteristics, respectively. Points represent pool-year responses. Shaded areas represent parameter 95% confidence intervals.



## **2.5 Discussion**

This study provides evidence that human land disturbance surrounding pools (within 1,000 m) can influence tadpole survival and morphology, an issue that can influence subsequent terrestrial stages and potentially population vitality (Berven 1990; Boes and Benard 2013). Moreover, we compared the relative importance of land cover and site conditions within vernal pools and demonstrated that conditions both within and surrounding pools are likely to influence larval survival and morphology. The importance of site characteristics on multiple spatial scales emphasizes the complexity of wood frog larval response and the need to consider multiple variables when assessing population response to urbanization. Tadpole responses primarily varied with variables related to urbanization, pool hydrology, and within-pool vegetation, each covered in the next three sections.

### **2.5.1 Urbanization**

Urbanization was positively associated with earlier developmental phenology (both individuals and cohorts) and greater cohort survival. Additionally, changes in land cover type consistent with urbanization (lower forest cover and greater impervious cover) were associated with advanced developmental phenology and increased condition, tail length, tail depth, and greater variation in tail length. These responses seem counterintuitive given: 1) documented negative impacts of similar intensities of urbanization on adult wood frogs (Homan et al. 2004; Rubbo and Kiesecker 2005; Skidds et al. 2007; Clark et al. 2008); and 2) evidence that wood frog eggs and larvae are harmed by elevated road salt concentrations (Brady 2013). However, larval wood frogs have been observed to be larger at metamorphosis in stormwater wetlands (Scheffers and Paszkowski 2016) and have greater survival through metamorphosis after the

forest surrounding pools was cut (Semlitsch et al. 2009). Furthermore, some other amphibian species, including common green frog (*Pelophylax perezi*), southern leopard frog (*Rana utricularia*), pig frog (*R. grylio*), American bullfrog (*R. catesbeiana*), and eastern narrowmouth toad (*Gastrophryne carolinensis*), have also shown positive morphological and survival responses in urban areas (Scheffers and Paszkowski 2011; Iglesias-Carrasco et al. 2017) and in areas with less terrestrial habitat (Salice et al. 2011). Although survival through metamorphosis was positively correlated with greater urbanization in our study, further study of the daily survival rate may provide insight as to whether earlier metamorphosis in urban pools is an adaptive mechanism that allows tadpoles to “escape” them sooner.

We also demonstrate that within a landscape with relatively low-intensity urbanization (0-38% impervious surface within 1,000 m) larvae are likely influenced by land cover types indicative of urbanization at multiple spatial scales. For example, the relationship between tadpole developmental phenology and tree cover differed at different spatial scales. Tadpole developmental phenology was more sensitive to reductions in tree cover nearer to pools, with a dramatic change in predicted with initial reductions in tree cover (up to 30%) within 100 m of pools, whereas reductions in tree cover within 1,000 m were correlated with a steady change in developmental phenology (Figure 2.4). Additionally, even slight increases in impervious cover (0-10%) within 300 m were associated with morphological shifts. Other studies have found adult wood frogs to respond to land cover types at similar scales (Rubbo and Kiesecker 2005; Skidds et al. 2007) and with similar sensitivity (e.g., 88% habitat cover threshold within 30 m of pools, 44% cover threshold at 1,000 m; Homan et al. 2004); thus our findings suggest that larvae respond to land cover at scales similar to those affecting adults.

Because survival differed between urbanization levels but the RFA using site characteristics as predictors did not explain any variance in survival, some unmeasured difference between rural and urban pools may be responsible for differences in survival. Moreover, it is possible that positive tadpole responses to urbanization may reflect site conditions selecting out “weaker” tadpoles (i.e., select for a relatively narrow portion of the possible distribution of traits) while in natural systems tadpoles with a greater variability of traits may survive. Unmeasured factors may contribute to greater tadpole survival as well as size in urban areas. For example, we did not quantify invasive vegetation, but glossy buckthorn (*Rhamnus frangula*), an invasive plant in our study area, is associated with larger and faster developing wood frog tadpoles with higher survival (Stephens et al. 2013). Pathogens could also explain greater survival and larger morphology in urban pools given that *Batrachochytrium dendrobatidis* (Bd) and ranavirus (Rv) have higher rates of infection and occurrence in rural pools than urban pools (Crespi et al. 2015; Saenz et al. 2015). Additionally, advanced developmental phenology increase survival to emergence in the presence of Rv (Reeve et al. 2013), and this may explain greater survival even in infected urban populations. Urbanization may also alter aquatic insect communities and decrease predation pressure on wood frog larvae. For example, insecticides can reduce insect predator abundance, thereby increasing tadpole survival (Relyea 2005) and potentially increased feeding (Petranka and Hayes 2011; Cothran et al. 2013).

Greater larval survival and individual size may translate to advantages during terrestrial life stages in urban areas (Scheffers and Paszkowski 2016) as has been noted in rural areas (Berven 1990). However, because juvenile wood frog growth can respond to terrestrial conditions (Boone 2005; Dananay et al. 2015) larval benefits from urbanization may be

overshadowed by degradation of terrestrial habitat. Removal of tree cover, which protects from desiccation, can increase adult mortality (Rittenhouse et al. 2009). Other novel threats, such as roads, sewer grates, predatory pets, and lawn mowers may reduce abundance and survival of terrestrial wood frogs (Eigenbrod et al. 2008; Eigenbrod et al. 2009; Hastings et al. unpublished data). Alternatively, larval benefits from urbanization may persist beyond metamorphosis and lengthen the time breeding populations persist in urbanized areas and thus contribute to the multi-decadal time-lag noted for amphibians in urbanizing landscapes (Löfvenhaft et al. 2004; Blaustein et al. 2011; Gagné & Fahrig 2010; Metzger et al. 2009). Even if urbanization benefits larvae and adequate terrestrial habitat remains near pools, reduced habitat connectivity associated with urbanization can increase the risk of wood frog extirpation (Harper et al. 2008).

### **2.5.2 Hydrology**

Tadpole responses to hydroperiod and pool depth across an urbanization gradient were similar to those found by others in more natural landscapes. More specifically, our results of delayed developmental phenology and greater body length with longer hydroperiod align with other studies noting positive correlations between hydroperiod and wood frog larvae size (Karraker and Gibbs 2009) and larval period (Dimauro and Hunter 2002; Skelly 2004; Gervasi and Foufopoulos 2008). Although developmental phenology and condition in urban and rural pools had similar rates of change with increases in hydroperiod (Figures 2.4 and 2.5), urban pools were predicted to develop earlier (all quantiles), have greater condition (90% quantiles), and show more variance in condition than rural pools. This suggests that although hydroperiod is an important predictor of developmental phenology and condition, other factors related to impervious cover within 300 m (our metric for delineating rural and urban categories) influence

these responses. For example, predator pressure which leads to slower development (Relyea 2002) may decrease as urbanization levels increase.

Additionally, we found better condition and greater length associated with deeper pools perhaps because deeper pools have more volume and lower conspecific density, and thus competition, relative to pool area (Brooks and Hayashi 2002). Pool depth also may correlate with increased condition and body length because deeper pools are likely cooler at the bottom and cool water is linked to greater body size in wood frog tadpoles (Berven 1982). The influence of pool depth on condition and length may affect adult stages because larger tadpoles produce larger adults (Werner 1986; Berven 1990) and larger females produce more eggs (Berven 1988). This might explain the increase in reproductive effort with pool depth that has been observed by others (Dimauro and Hunter 2002; Calhoun et al. 2003).

### **2.5.3 Vegetation**

Across the urbanization gradient, within-pool vegetation conditions indicative of low-light conditions at a pool's surface were associated with greater condition, body length, and tail length (i.e, positive associations with canopy cover and negative associations with emergent vegetation or shrub cover). Our results align with Boes & Benard (2013), who observed larger wood frog metamorphs in closed canopy pools. However, our results run counter to Skelly et al. (2002), who observed slower mass gain in closed canopy cover. Low-light conditions could also facilitate increased condition and size by maintaining lower water temperatures which can increase size of wood frog larvae (Berven 1982).

#### **2.5.4 Morphology**

We did not detect strong relationships among larval developmental phenology, length, and condition (Figure 2.1) or between morphology and survival. Although we did not necessarily expect consistent (i.e., strong and directional) relationships among morphology metrics (Berven 1987; Berven and Chadra 1988), we did expect survival to increase with measures indicating size (Berven 1990). The lack of relationship between survival and morphology metrics suggests that unmeasured factors may determine survival or that a wide range of morphological responses may result in similar survival depending on the local context. Although larval survival was not well predicted by morphology, other size metrics may also be consequential for populations (i.e., breeding population size is limited by terrestrial density-dependent factors; Berven 1990). Given that larval body size is usually correlated with post-metamorphic body size (Boes & Benard 2013; Berven 1990, but see Earl & Semlitsch 2013), tadpole size may translate into fitness advantages associated with greater body size (Berven 1988).

#### **2.6 Conclusion**

Conserving amphibians in developing landscapes is challenging given the complexities of responses to urbanization, which can introduce a multitude of novel factors that may influence amphibians in both aquatic and terrestrial stages and habitats (Windmiller and Calhoun 2008). Our results indicate that a cohort of larvae respond to conditions within 1,000 m of pools as well as within-pool site conditions. This response may reflect the allele frequencies within the larval cohort and range of observed larval traits that are preprogrammed via epigenetics and maternal effects as well as the specific traits of surviving larvae (Relyea 2002; Donihue and Lambert 2015). Notably, within-pool conditions that benefit larval development (e.g., greater canopy

closure and pool depth) do not differ between rural and urban pools. Additionally, the site-characteristics most closely associated with urbanization (e.g., impervious cover, road salt contamination) were not necessarily the most important predictors of tadpole responses. This suggests that differences in response may be influenced by the confluence of changes across many site characteristics resulting from urbanization. Although we found responses in urban pools were more beneficial than in rural pools (e.g., greater survival), it is well-documented that ultimately urbanization extirpates breeding populations (Gibbs 1998a; Homan et al. 2004; Rubbo and Kiesecker 2005; Clark et al. 2008). Given the urban-associated benefits to larval stages, it is unlikely that wood frog population declines stem from larval responses to urbanization. We suspect that any urban-associated benefit to larval stages is outweighed by negative impacts to terrestrial life stages, and thus we suggest future study focus on this issue. We also recommend examining larval and terrestrial stage responses to greater intensities of urbanization than were represented in our study area because response strength may increase with urbanization.

**CHAPTER 3: THE INFLUENCE OF LAND COVER AND WITHIN-POOL  
CHARACTERISTICS ON LARVAL, FROGLET, AND ADULT  
WOOD FROGS ALONG AN URBANIZATION GRADIENT**

**3.1 Chapter Abstract**

Urbanization is known to extirpate wood frog (*Lithobates sylvaticus*) populations, but the mechanism is unknown. Although larvae may not respond directly to urbanization, within-pool conditions experienced by larvae may affect morphology and post-metamorphic survival (i.e., carry-over effects). We tested the carry-over effects of larval morphology and site characteristics, particularly land cover indicative of urbanization within 1,000 m, on newly emerged and post-breeding male wood frogs across an urbanization gradient in 15 pools in greater Bangor, Maine, USA. We raised field-captured larvae in microcosms and examined froglet morphology and locomotor performance at emergence and one month post-emergence. Larval mass was positively correlated with 50% of froglet responses, but was negatively associated with adult size. Among site characteristics, egg density had the most salient influence with negative effects on larval survival and morphology as well as on 11 of 14 froglet responses. Vegetation, hydrology, and urban-associated cover near pools also influenced froglet performance, and urban-associated cover and hydrology influenced adult morphology. Our findings support the idea that effects of conditions (e.g., hydrology) experienced by larvae can carry-over to terrestrial stages and have life-long consequences. However, conflicting directions of response to urban-associated cover suggest that the carry-over effects of urbanization from larval to froglet stages may not persist to adulthood and that terrestrial responses to urbanization experienced post-



emergence may override larval responses to urbanization. Thus it is likely that urbanization has the greatest impact on populations via direct effects on terrestrial stages.

### **3.2 Introduction**

It has been well established that urbanization, especially at higher intensities, can result in the extirpation of wood frog (*Lithobates sylvaticus*) populations (Gibbs 1998a; Homan et al. 2004; Rubbo and Kiesecker 2005; Clark et al. 2008; Windmiller et al. 2008). Wood frogs rely on fishless, vernal pools for breeding and larval development, and forest provides non-breeding adult habitat. This biphasic lifecycle means that disturbances in terrestrial areas primarily impact adults whereas within-pool disturbances primarily impact eggs and larvae. Terrestrial disturbances may also alter aquatic conditions and thus affect aquatic stage amphibians. For example, road salt (Sanzo and Hecnar 2006) and pesticides (Cothran et al. 2013) have been linked to reduced larval condition and survival in wood frogs. Although some research has addressed the effects of urban-associated changes in cover type on wood frogs at terrestrial stages (body size, Semlitsch et al. 2009; breeding population size, Veysey et al. 2011; Clark et al. 2008; Windmiller et al. 2008; movement ability, Cline and Hunter 2014; Cline and Hunter 2016; movement patterns, Hoffman and Hastings unpublished data), little work has focused on how urban development near pools may contribute to population declines via effects at the larval stage.

Although wood frog populations are harmed by urbanization, larval stages do not necessarily exhibit negative responses to terrestrial disturbances. For example, wood frog larvae have been observed to have equal if not greater survival, condition, and size in urban landscapes (Shepack et al. 2017; Chapter 2) and stormwater wetlands (Scheffers and Paszkowski 2016) and

had greater survival through metamorphosis after the forest surrounding pools was cut (Semlitsch et al. 2009). However, environmental conditions experienced during early life stages may have latent effects (i.e., “carry-over effects”) that influence later life stages (Pechenik 2004). Carry-over effects of larval conditions have been demonstrated for wood frog post-metamorphic morphology, locomotor performance, physiology, and survival (Relyea 2001a; Boes and Benard 2013; Crespi and Warne 2013). These effects may have life-long consequences, potentially influencing adult physiology and behavior (Denver 2009), fitness (Semlitsch et al. 1988; Berven 1990; Relyea and Hoverman 2003), and ecological processes (e.g., dispersal; Clobert et al. 2009). Additionally, because larval phenotype may not accurately indicate fitness, responses at later life stages (e.g., post-metamorphic) may be better indicators of fitness (Earl and Whiteman 2015).

Studies examining the carry-over effects of larval conditions in wood frogs have focused on the influence of *within-pool* conditions. These studies have demonstrated that differences in canopy cover (Boes and Benard 2013), accelerated drying (Gervasi and Foufopoulos 2008), water level and food availability (Crespi and Warne 2013), predator presence (Relyea 2001a; Barbasch and Benard 2011), larval density (Goater and Vandebos 1997), conductivity (indicative of road salt), and egg mass density (egg masses/m<sup>3</sup>; Green and Bailey 2015) during larval development influence post-metamorphic responses. However, because urban land development near pools can alter the larval environment in multiple ways, (e.g., introducing herbicides [Relyea 2012] and heavy metals [Peles 2013; Snodgrass et al. 2008], increasing water temperature [Watkins and Vraspir 2006], and shifting vegetation composition [Rubbo and Kiesecker 2004; Stephens et al. 2013] and predator community composition [Rubbo and Kiesecker 2005; Gibbs 1998; Urban et al. 2006]), examining the carry-over effects of land cover

type may integrate multiple influences of urbanization. Additionally, understanding the influence of urban-associated land conversion on carry-over effects to terrestrial stages is particularly relevant for vernal pool conservation which often involves conserving habitat some distance from a pool (Calhoun et al. 2005).

It is yet unknown how the effects of urban development on larvae contribute to conditions of terrestrial stage individuals and population declines. In this study we examined how urban-associated land cover types near pools influence conditions experienced during larval development and produce carry-over effects on post-metamorphic stages. We concurrently examined within-pool vegetation, hydrology, and conspecific density because these pool characteristics can result in carry-over effects in wood frogs (Goater and Vandebos 1997; Gervasi and Foufopoulos 2008; Boes and Benard 2013; Crespi and Warne 2013; Green and Bailey 2015). Additionally, because conditions experienced during terrestrial stages can override effects of larval conditions (Boone 2005; Dananay et al. 2015), we also examined the effects of conditions experienced by larvae on breeding adults. Specifically, our objectives were to examine the relative influences of landscape-scale and pool characteristics across an urbanization gradient on (1) larval morphology and survival to emergence, (2) newly metamorphosed froglet morphology and locomotor performance, and (3) adult male morphology.

### **3.3 Methods**

#### **3.3.1 Study Area**

The greater Bangor, Maine area is located in the glaciated northeastern US and covers 200 km<sup>2</sup> encompassing four towns: Bangor, Orono, Hampden, and Old Town (populations of approximately 7,000-33,000; U.S. Census Bureau 2011). Urban development is primarily

residential and commercial with urbanization intensity extremes of nearly 100% impervious surface in downtown areas to <1% impervious surface in conserved or lightly developed areas (e.g., Bangor City Forest; Fry et al. 2011). We have observed wood frogs breeding within the greater Bangor area in pools with 0-38% impervious cover within 1,000 m.

### **3.3.2 Site characteristics**

We selected site characteristics to measure in 15 pools that were likely to influence froglet responses based on a concurrent study of larval morphology and development in 30 pools (Chapter 2). We used ArcView GIS10.2 and the Maine Land Cover Dataset (2004 all land use; 2011 impervious surface) to quantify the percent impervious surface within 300 m and forest cover within 100 and 1,000 m from pool spring high-water marks. We selected 1,000 m based on previous evidence that wood frogs respond to conditions within 1,000 m (Homan et al. 2004; Rubbo and Kiesecker 2005; Skidds et al. 2007). We used impervious surface to represent urban development intensity because it includes buildings and pavement and is thus linked to traffic and chemical, light, and noise pollution. We measured spring-high-water depth using a pole marked in centimeter increments. Hydroperiod was determined by the Julian day that standing water was no longer present. Pools that dried to within 10 cm and  $\geq 10$  cm at the deepest point were assigned Julian day 280 and 300, respectively. We measured summer vegetation within pool basins 19 July-21 August 2014-2016 at late summer dry down by visually estimating percent cover of shrub, emergent vegetation, and submerged vegetation. Woody vegetation canopy density over pools was measured ~1 m above the ground using a spherical convex densitometer (lab-reared larvae pools: 6-100%, median=68%; adult pools: 31-67%, median=51%). We used water probes (Hach ©, Loveland, Colorado) to measure water

temperature 2 May-16 June 2014-2016. On each date a pool was sampled, we collected and tested 1 L of surface water ~1 m from the water edge at each of three equidistant points around the perimeter. Only one sample was taken at pools that were almost dry and  $<2 \text{ m}^2$ . All testing was conducted at the pool edge within minutes of sample collection. Pool temperature was averaged by day and then year for analyses.

We used wood frog egg mass density (the number of wood frog egg masses counted in a pool divided by pool area; egg masses/ $\text{m}^2$ ) to indicate conspecific competition at the larval stage. We counted egg masses after spring breeding (3-8 May 2015), following the apparent peak of breeding using a dependent double-observer method to increase detection (Grant et al. 2005). Observers walked through the pool and wore polarized sunglasses to increase egg mass detection. If eggs had been recently deposited (within approximately 2 days) we revisited pools and counted new masses. The maximum number of egg masses was used to calculate egg mass density.

Site characteristics from 2015 were used in analysis of froglet responses, and mean values from 2014-2016 were used in analysis of adult responses because adults were likely from multiple tadpole cohorts (years). We reduced the number of variables in both the 2015 and the 2014-2016 datasets using the 'Vegan' library (Oksanen et al. 2017). We conducted separate PCAs for all pool vegetation, land cover type, and hydrology variables and extracted axes values to represent these categories in the two datasets (6 PCAs, total). We also included wood frog egg density as a predictor of larval and froglet responses. Site characteristics variables were not highly correlated (Pearson's correlation coefficient  $<|0.36|$  for 2015 and 2014-2016 site characteristics).

### 3.3.3 Larval to post-metamorphic carry-over: morphology and performance measures

We conducted a microcosm experiment to assess the effects of environmental conditions experienced during early larval development on post-metamorphic morphology, survival, and locomotor performance in wood frogs. Post-metamorphic performance may reflect physiological condition and movement ability of a froglet, and thus can be useful to indicate aspects of individual condition not represented by morphology that are relevant to dispersal, migration, and resource selection ability. We captured 10 tadpoles per pool for 10 pools representing the available gradient of impervious surface cover within 1,000 m of the pools that had tadpoles surviving to Gosner (1960) stage 36-42 (median=40). We captured larvae (Gosner stage 36-42) from each pool between June 16-July 22 and transferred individuals from the field in 1 L plastic containers of pool water to a lab at the University of Maine. In the lab, we placed each larvae in 1 L of aged tap water in individual plastic containers that also had 200 cm<sup>2</sup> of terrestrial area. Small ramps allowed newly emerged froglets to leave the water at will. Each day, we checked microcosms for emerged froglets and for these individuals removed water containers to provide a larger (275 cm<sup>2</sup>) terrestrial area and to prevent drowning. We changed water every 72 hours to prevent fouling. Animals were kept under ambient light conditions. Each terrestrial microcosm contained leaf litter (primarily oak, *Quercus* spp.) approximately 2 cm deep and was misted with water daily. We fed larvae rabbit pellets and boiled romaine lettuce and fed froglets live flightless fruit flies, following Greenspan et al. (2012). Each microcosm was covered with a window-screen lid to prevent froglet escape.

We measured snout-vent length (SVL) and mass of each individual to assess individual morphology at time of capture. After emergence we conducted two “rounds” of post-

metamorphic locomotor performance trials and morphology measurements representing early and late froglet responses: first on days 1 and 2 after emergence and the second on days 29 and 30. We assessed performance for each froglet by conducting maximum jump trials on day 1 and 29, and endurance trials on day 2 and 30 following Boes and Benard (2013). We conducted maximum jump distance trials by placing a newly metamorphosed froglet in the center of a circular arena (1.5 m diameter plastic tub) under an opaque cup. The froglet rested under the cup during a 2-minute adjustment period before the cup was lifted and the distance of the first jump was recorded. Froglets that did not immediately jump were gently tapped on the urostyle. Three trials (separated by 6 minutes) were conducted for each froglet on the same day. The maximum jump distance (Jump) from the three trials in a day was used in analyses. To conduct endurance trials, we placed a froglet under an opaque cup on a circular track approximately 10 cm wide with walls 10 cm high for a 2-minute adjustment period. Upon lifting the cup, we recorded the total distance moved (Dist) and duration of movement (Duration) and calculated average speed (Speed). When a froglet did not immediately jump or came to a rest, it was gently tapped on the urostyle up to three times to encourage movement. Once a froglet did not jump after being tapped three times, the trial was concluded. On days 2 and 30, we measured SVL, mass, and hind leg length (following Boes and Benard 2013). We anesthetized froglets using MS-222 (Gentz 2007) prior to measurement to ensure their safety. After measurement, froglets were bathed in aged tap water with their head above the water until they regained locomotor ability.

### **3.3.4 Larval to adult carry-over: morphology measures**

During the 2 years prior to adult sampling, we measured tadpoles in the 9 pools where adults were sampled. We collected these data to predict breeding male size because breeding

adult males likely represent multiple cohorts and may have hatched within the previous 2 years. We conducted weekly tadpole surveys (see Chapter 2 for detailed methods) between 15 June -26 August 2014-2015, and measured SVL and mass of Gosner stages 40-41 tadpoles. Tadpoles at these developmental stages are typically at their largest size prior to completion of metamorphosis. We conducted ANCOVAs to determine if there were differences in SVL and relative mass (residuals of mass regressed against SVL) between years or developmental stages. Because there were no substantial differences (for those models where  $P < 0.1$ ,  $\eta^2 \leq 0.05$  indicated small effect size; Levine and Hullett 2002), we pooled observations across years and/or stages and calculated median SVL and relative mass.

We captured and measured adult male frogs to assess carry-over effects into adults. Due to logistic constraints, we could not capture enough females across pools to incorporate into analyses. Since wood frog survival to first reproduction is not different between sexes (Berven 1990), we used adult males as a proxy for adult wood frog responses. We used minnow traps to capture adult male wood frogs in 9 breeding pools (4 of the same pools from which tadpoles in the microcosm-rearing portion of the study were captured) from 2016 April 13-24. We weighed frogs, measured SVL, and toe-clipped new captures to prevent resampling. Given wood frog's high breeding fidelity to their natal pool (Berven and Grudzien 1990; Vasconcelos and Calhoun 2004), we assume that a high percent (>80%) of males were sampled at their natal pool.



Table 3.1 Larval, froglet, and adult frog response variables. Where a regressor variable is listed, pairs of response-regressor variables were examined for differences in relationship between rural and urban pools using ANCOVA. Relationships with regressors were examined for early and late froglet morphology and performance responses. Regressor variables with an (\*) were included as a covariate in linear mixed effect models.

Response variable	Regressor variable
ln(larval SVL)	Larval Gosner stage
ln(larval mass)	ln(larval SVL (mm))*
ln(froglet SVL)	-
ln(froglet mass)	ln(froglet SVL(mm))*
ln(froglet leg length)	ln(froglet SVL(mm))*
ln(maximum jump distance)	ln(froglet SVL(mm))
ln(duration)	ln(froglet SVL(mm))
ln(speed)	ln(froglet SVL(mm))
ln(distance moved)	ln(froglet SVL(mm))
ln(adult SVL)	-
ln(adult mass)	ln(adult SVL(mm))*

### 3.3.5 Statistical analysis

All statistical analyses were completed using program R (R Core Team 2016). Initially we conducted ANCOVAs for a subset of lab-reared individuals and a subset of adults from the four most rural and four most urban pools in each full dataset to examine if urbanization level influenced the relationships (i.e., rate of change) between pairs of likely size- or developmental stage-dependent responses and SVL or Gosner stage (Table 3.1). We used impervious cover within 300 m (lab-reared individuals: rural pools: 3-6%, urban: 22-27%; adults: 0.02-2% rural, 14-27% urban) to represent urbanization intensity because this was identified as an important site characteristic for predicting larval wood frog morphology (Chapter 2). Because there were no substantial differences in these relationships between urbanization levels (for those models where  $P < 0.1$ ,  $\eta^2 \leq 0.08$  indicated small effect size), we pooled all sites in each respective dataset for further analysis.

We tested for differences among sites using MANOVAs with response vectors of larval and early froglet responses ( $P < 0.05$ ) and logistic regression to test for differences in probability of survival to emergence ( $\chi^2 < 0.05$ ). Because of unexpectedly low survival to the second round of froglet measurements (1-6 observations per site; median = 2), we did not test for differences among sites for second measurements, but instead relied on differences among sites for early froglet measurements to indicate likely differences at the late froglet stage. We used ANOVA to test for among-site differences in adult responses ( $P < 0.05$ ) because of highly uneven sample sizes (8-53) and because we were examining only two responses. Prior to larval and early froglet MANOVAs, we conducted an ANOVA for each pair of variables used in ANCOVAs to identify which responses were size- or developmental stage-dependent and should be corrected for size or stage. For all response-regressor pairs except larval SVL-Gosner stage and duration-froglet SVL,

the regressor was significant ( $P < 0.1$ ) with at least a moderate effect size ( $\eta^2 > 0.2$ ). All regressions were interpreted using Type II sum of squares to reduce the influence of uneven sample sizes. We extracted residuals from significant relationships for use in MANOVAs. We conducted MANOVAs for early froglet responses (round 1) from sites with  $\geq 4$  complete cases to maintain similar sample sizes among sites (53 froglets from 8 sites, 4-8 individuals per site).

If differences among sites were detected for morphology, performance, and/or survival metrics, we used a two-step model selection process to identify which predictors within each predictor category (site characteristics, larval morphology) were likely influential and then compare the relative influence among those variables. Site characteristic variables were used to predict all responses (Figure 3.1a); larval morphology variables from lab-housed individuals were used to predict all froglet morphology and performance and survival metrics (Figure 3.1b); median larval morphology from late-stage field measured tadpoles was used to predict adult metrics (Figure 3.1c).

First we created a set of linear models for each morphology and performance response variable using R package 'lme4' (Bates et al. 2017) for continuous data and logistic regression models using package 'nlme' (Pinheiro et al. 2017) for binary survival data. For all responses except those from late stage froglets we nested by Site (random effect). We did not nest by Site for late stage froglets because of the small sample size and low per-site replicates (individuals). A single predictor was added to create competing models. Models of relative mass and leg length always included natural log-transformed SVL as a covariate because we were interested in the effect of these responses independent of body size. The influence of SVL was not interpreted in these models. We grouped models of each response by predictor category and selected the top models for a response within each category. We used library 'AICcmodavg' (Mazerolle 2017) to

rank models using Akaike’s information criterion adjusted for small sample size (AICc). We considered models  $\Delta AICc < 2$  that ranked above the null model to be plausible (Burnham and Anderson 2002). If  $> 1$  model met these criteria, we tested additive models that included all combinations of covariates in plausible models. Secondly, for each response, we compared all plausible models across predictor categories to determine the relative importance of predictors. Similar to the first step, if  $> 1$  model had  $\Delta AICc < 2$  we tested additive models that included all combinations of covariates these highly-ranked models. We examined the 85% confidence intervals (Arnold 2010) of each covariate in this final set of models (i.e., that ranked above the null model and had  $\Delta AICc < 2$  within its respective predictor category) to determine effect. An effect (predictor with an 85% CIs different from zero) of site characteristics but not of larval morphology suggests that site characteristics may influence later stages via an unmeasured morphology or physiologically related variable. In contrast, an effect of larval morphology but no effect of site characteristics suggests that an unmeasured difference among sites is responsible for the carry-over effect of larval morphology on terrestrial stages.

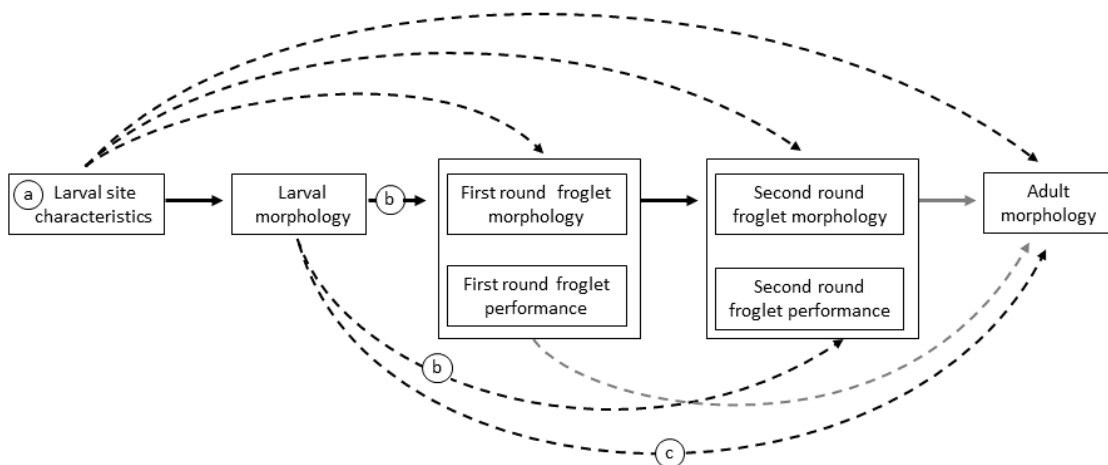


Figure 3.1 Direct and indirect effect pathways among site characteristics and stages of wood frog development. Solid lines represent direct effects and dashed lines represent indirect effects. Effects represented by black arrows were explored in our study; grey arrows were not addressed with our study design. Circled letters are for reference in the text.

## 3.4 Results

### 3.4.1 Summary statistics

Of 100 tadpoles captured, 57 froglets survived to emergence and were used in froglet model selection analyses. Of those 57, 21 survived until the second froglet performance trials (6 from urban pools, 13 from rural pools, and 2 from intermediate development intensities) and 14 completed second performance trials (5 from urban pools, 7 from rural pools). Larval morphology and early froglet morphology-performance profiles differed by site (MANOVA, Larval:  $F_{9,90}=8.88$ ,  $P<0.001$ ; Froglet: Pillai test  $F_{7,45}=2.06$ ,  $P<0.001$ ), as did survival to emergence ( $\chi^2_{9,100}=22.85$ ,  $P=0.007$ ). We measured 266 unique adult male frogs (8-53 per site), and adult SVL and mass adjusted for SVL differed by site (SVL:  $F_{8,257}=13.52$ ,  $P<0.001$ ,  $\eta^2=0.30$ ; Mass:  $F_{8,256}=13.51$ ,  $P<0.001$ ,  $\eta^2=0.17$ ).

Pool characteristic metrics for the set of 15 focal pools did not vary with urbanization (as indicated by impervious cover within 300 m;  $P>0.1$ ) with one exception. The hydrology metric (Hydro) decreased with urbanization ( $F_{1,8}=13.23$ ,  $P=0.01$ ) for the 10 tadpole source pools, but this trend was not universal across the set of 30 pools used to calculate principal component values ( $F_{1,28}=0.078$ ,  $P=0.78$ ).

At least one site characteristic had a statistical effect ( $<2\Delta AICc$  within each predictor category and with 85% CI excluding zero) on all larval, froglet, and adult responses except for late froglet leg length (Figure 3.2, Appendix C Table C2). Of those early froglet responses predicted by site characteristics, all except duration were also predicted by larval morphology. SVL was the only late stage response and mass was the only adult response predicted by site characteristics and larval morphology. (Figure 3.2, Appendix C Table C2). For those responses

for which both site characteristics and larval morphology had an effect, models with larval morphology predictors ranked above those with site characteristic predictors (Figure 3.2, Appendix C Tables C1-C2).

Table 3.2 Predictors of larval, froglet, and adult frog responses. Within a principal component, (–) indicates a negative and (+) a positive relationship. Pairs of numbers in parentheses after each variable in a PC refer to eigenvalue contributions within the 2015 and 2014-2016 datasets. Only one number is listed for each cover variable because cover was consistent among years.

Variable	Description
<i>Site characteristics</i>	
Veg	Vegetation PC1: canopy cover (+, 0.573, 0.552); shrub (-, -0.346, -0.291), emergent (-, -0.623, -0.574), and submerged vegetation cover (-, -0.404, -0.531)
Cover	Cover PC1: tree cover within 100 (+, 0.582) and 1,000 m (+, 0.573), impervious cover within 300 m (-, -0.576)
Hydro	Hydrology PC1: hydroperiod (+, 0.601, 0.629) and maximum depth (+, 0.571, 0.547), surface water temperature (-0.560, -0.552)
Egg	ln(Wood frog egg masses/m <sup>2</sup> )
<i>Larval morphology of tadpoles raised in the lab (individual measures)</i>	
L.Mass	Relative larval mass: ln(Larval mass (g)) <sup>1</sup>
L.SVL	Larval SVL: ln(SVL (mm))
<i>Larval morphology of tadpoles at pools where adults were measured (cohort measures)</i>	
L.Mass	Relative larval mass: Within-pool median of residuals of ln(Larval mass (g)) regressed against ln(Larval SVL (mm)) <sup>1</sup>
L.SVL	Larval SVL: Within-pool median ln(SVL (mm))

<sup>1</sup> Larval or froglet ln(SVL) is included as a covariate to account for variation in larval or froglet response, respectively, attributed to body size.

<sup>2</sup> Froglet morphology was assessed early (F1) and late (F2) in froglet development.

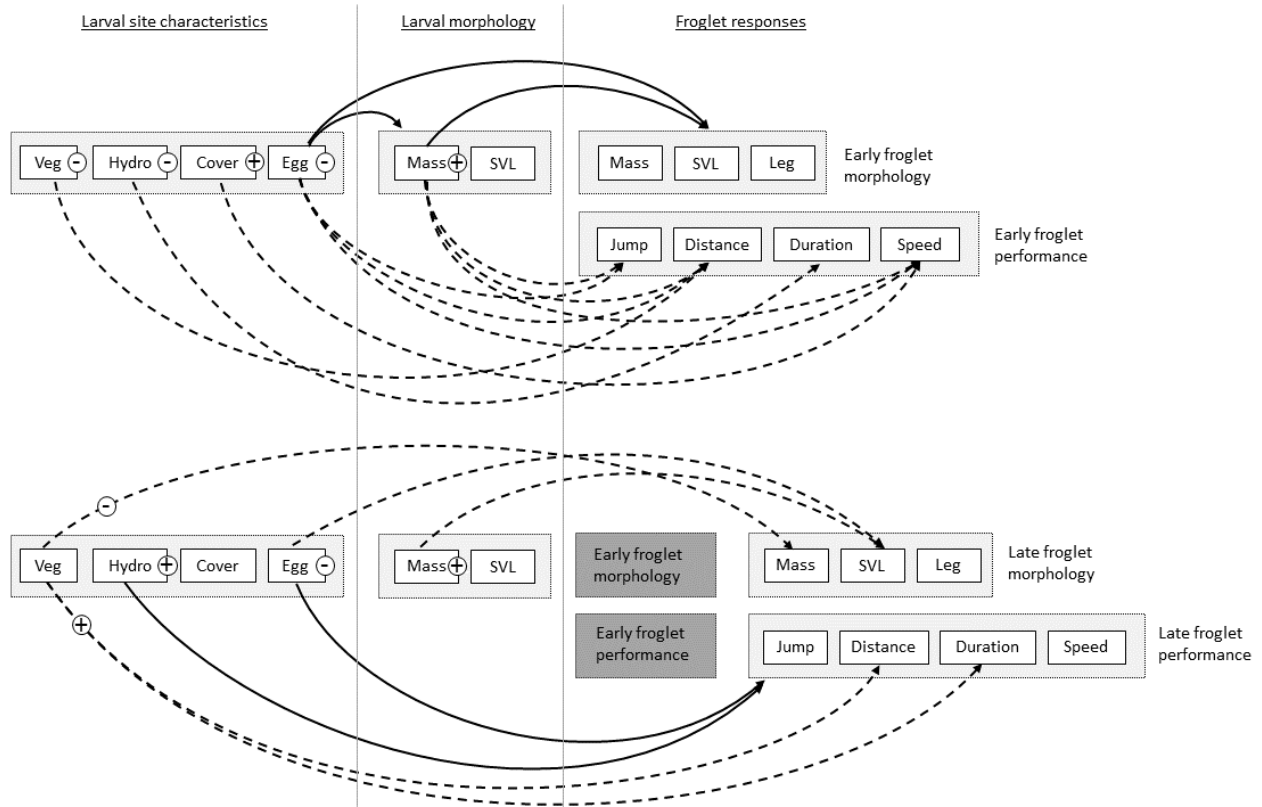


Figure 3.2 Carry-over effects of site characteristics and morphology on early froglet (top half) and late froglet (bottom half) morphology and performance. Arrows originate at the predictive parameter and point at the response. Arrows represent explanatory parameters from models with  $\Delta AIC_c < 2$  and that had 85% CIs excluding zero. Solid arrows that point at categories of variables (enclosed in gray boxes) indicate a statistical effect on all variables within a box. Dashed lines indicate an effect of one predictor on one response variable. Circled “+” and “-” indicate the direction of effect of a predictor on responses. Vegetation (Veg) has both positive and negative effects associated with different responses at the late froglet stage and thus the direction of effect is indicated on the appropriate arrow.



### 3.4.2 Site characteristic predictors

Tadpoles from pools with a higher egg density were predicted to have lower SVL and mass, and become froglets with shorter and slower jumps and lower endurance (but no effect on duration in early froglets; Figure 3.2, Appendix C Tables C1-C2). Vegetation (Veg), hydrology (Hydro), and land cover type (Cover) each had somewhat conflicting statistical effects across responses (Figure 3.2, Appendix C Tables C1-C2). Tadpoles from pools with vegetation characterized by less canopy and more herbaceous cover (negative Veg values, Table 3.2) were predicted to have lower mass and move shorter distances in endurance trials as early-stage froglets but farther distances in endurance trials and greater duration as late-stage froglets (Figure 3.2, Appendix C Tables C1-C2). Tadpoles from pools with higher hydrology values (primarily corresponding with longer hydroperiod and deeper water and secondarily with cooler water, Table 3.2) were predicted to have shorter jump duration as early-stage froglets, but be better jumpers (positive effect on all performance metrics) as late-stage froglets (Figure 3.2, Appendix C Tables C1-C2). Adults breeding in pools with greater hydrology values were predicted to have greater mass. Land cover type (characterized by more tree cover and less impervious cover, Table 3.2) was positively associated with early froglet jump speed, but negatively associated with adult SVL (Figure 3.3, Appendix C Tables C1-C2).

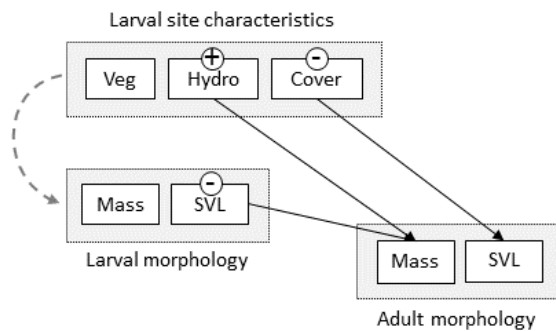


Figure 3.3 Relationships among site characteristics and larval and adult morphology. Solid arrows originate at the predictive parameter and point at the response and represent explanatory parameters from models with  $\Delta AICc < 2$  and that had 85% CIs excluding zero. The dashed arrow indicates the unmeasured but likely influence of site characteristics on larval morphology. Circled “+” and “-” indicate the direction of effect of a predictor on responses.

### 3.4.3 Larval morphology predictors

Froglet responses were positively associated with larval predictors; all larval predictors in top-ranked froglet response models that also had covariate estimates with 85% CI different from zero had a positive effect on responses (Figure 3.2, Appendix C Tables C2). Survival models with larval SVL and mass as predictors ranked above the null model, but only SVL had an 85% CI that did not include zero ( $\beta_{\text{Mass}}$ : -0.517, 2.02; Table 3.4); tadpoles with greater larval SVL were predicted to have a greater probability of survival to emergence (Tables C1-C2). Tadpoles with greater mass were predicted to have greater early froglet morphology and performance responses (for 6 of 7 metrics) and late froglet SVL. Larval SVL was the predictor in the top-ranked model of adult mass, with a negative effect on mass.

## 3.5 Discussion

Our findings demonstrate that both pool and landscape characteristics can influence larvae and have carry-over effects on post-metamorphic morphology and performance in an

urbanizing landscape. Although landscape characteristics had an effect on adult size, they had little statistical effect on froglets (only for early stage jump speed), which suggests that landscape characteristics primarily influence development during terrestrial stages.

Our observation that egg density negatively affects larval morphology and froglet locomotor performance aligns with well-studied relationships of increased conspecific density resulting in smaller (mass, volume, or body length) wood frogs at metamorphic climax (emergence of front legs; Wilbur 1977; Smith-Gill and Berven 1979; Berven and Chadra 1988; Berven 2009). Additionally, Goater and Vandenbos (1997) observed that the effects of experimentally controlled larval density on froglet morphology are detectable months after metamorphosis. Our results suggest that conspecific density in the field may have similarly long-lasting effects on juveniles, including consequences for post-emergence movement ability. Additionally, egg density may affect froglets indirectly via larval morphology: we observed that relative larval mass, which decreased with egg density, affected froglet morphology and performance and out-ranked competing models with egg density as a predictor. However, our findings that larval SVL was negatively associated with relative adult mass suggest a possible disconnect between egg density effects on larval stages and adult morphology. Terrestrial habitat quality may explain these conflicting effects between juvenile and adult stages: high-quality terrestrial habitat could be expected to support a relatively large population of fecund adults that would produce a greater egg density, which could have negative effects on larval size.

Our finding of a negative relationship between relative larval mass and adult size is unexpected based on published positive correlations between size of newly emerged froglets and one-year old size and survival (Berven 1990). Because this result is counterintuitive, we suspect that (at least for the breeding pools examined), aquatic and terrestrial habitat were not of similar

quality (i.e., high-quality aquatic conditions were set in a landscape with low-quality terrestrial conditions, or vice versa) and the effects of terrestrial factors may have masked the effect of larval size.

The positive relationships between larval and froglet mass and between larval SVL and survival in wood frog aligns with other studies (Goater and Vandembos 1997; Relyea 2001a; Berven 2009). Additionally, the positive effect of larval mass, froglet mass, and leg length across froglet performance measures is consistent with other research on wood frogs (Boes and Benard 2013) and newly emerged froglets of other species (Álvarez and Nicieza 2002; Orizaola and Laurila 2009). Our results indicate that larval mass, which corresponds with the amount of fats and other energetic reserves relative to body size (i.e., condition; reviewed by Green 2001), is a better predictor of froglet morphology (SVL, relative mass, and relative leg length) than is larval SVL and thus may be of greater importance to fitness. The effect of larval mass on froglet morphology suggests that greater late-stage larval metabolic reserves may help froglets move faster and farther immediately after emergence from a pool. This may be particularly important in fragmented urbanized landscapes where longer movements may be necessary to locate suitable overwintering areas and for juveniles to disperse to sustain genetic connectivity and colonize suitable breeding pools.

Although we did not detect an effect of hydrology on larval morphology, hydrology conditions that primarily corresponded with longer hydroperiod and deeper water, and secondarily with cooler water, indicated greater late stage froglet performance and larger relative mass of adults. The effect on late stage performance suggests that hydrology conditions experienced during larval development could benefit frogs at terrestrial stages and persist through adulthood. Other studies have noted wood frog larvae size and relative mass increased

with hydroperiod, pool depth, and cooler water (Karraker and Gibbs 2009; Herreid and Kinney 1967; Watkins and Vraspir 2006; however, see Rowe and Dunson 1995). In our study, we detected an effect of hydrology conditions experienced during larval development on *terrestrial* stages.

Vegetation indicative of high-light conditions (consistent with less-dense canopy cover) was correlated with greater mass in late stage froglets and aligned with other studies that have demonstrated that froglets from open-canopy pools emerge at greater size than those from closed-canopy pools (Werner and Glennemeier 1999; Skelly et al. 2002; Schiesari 2006). However, there is some inconsistency within the literature; both Halverson et al. (2003) and Boes and Benard (2013) observed larger wood frogs developing in closed-canopy pools. The inconsistent effect of vegetation that we observed between early and late performance (i.e. individuals from high-light pools jumped farther in early stage endurance trials (Distance), but had shorter jumps (Distance and Duration) as late stage froglets) may suggest that vegetation during the larval stage can influence terrestrial stages, but the direction of effect changes with time since emergence.

Cover type had no detectable effect on any larval or froglet response other than early stage froglet jump speed. The lack of effect of cover type on all other larval and froglet metrics is unexpected as it is well-demonstrated that anthropogenic contaminants associated with urban land cover can impact aquatic amphibians (Relyea 2005; Sanzo and Hecnar 2006; Karraker et al. 2008; Peles 2013). Given that urbanization likely impacts larvae but that we did not find that larval or froglet responses corresponded to the coarse measures of land cover conversion commonly used to assess compliance with regulations (i.e., the concentric circle zoning technique), we suspect that site-specific examinations that consider land cover and hydrology

within the watershed of each pool may more accurately assess water quality impairments from urban land conversion.

The positive association between adult size and urbanization might seem unexpected; however, degraded habitat and novel risks (e.g., road mortality, lawn mowers, pets) in urban areas may reduce survival and support fewer adults compared to natural areas. A lower density of adults in urbanized areas may reduce competition for food and produce larger adults (Harper and Semlitsch 2007; Berven 2009). This suggests that the negative effects of urbanization on wood frog populations may be more apparent in the demographics of breeding adults than in larval or froglet responses or in adult body condition and that measures of body condition may be counterintuitive in urban areas with degraded terrestrial habitat. Additionally, the effects of urbanization on breeding population size may be more apparent than on larval demographics or condition because adults interact directly with degraded terrestrial habitat and urban-associated risks, whereas larvae are buffered from contamination and novel risks by undeveloped areas near pools. Thus land cover experienced during terrestrial stages likely has a greater influence on population persistence.

### **3.6 Conclusion**

Our findings indicate that egg mass density, vegetation, and hydrology experienced during larval development can influence terrestrial stages even though their effects may not be expressed in larval and/or froglet morphology. Cover type near larval pools can also influence development at aquatic stages with carry-over effects to terrestrial stages; however, cover type experienced during terrestrial stages likely has a greater influence on population persistence. The effect of pool hydrology on adult mass supports the idea that site conditions experienced during

larval development have life-long consequences, but may not be adequately captured by larval or froglet morphology measurements alone. Although some researchers have examined the relative effects of pool and landscape-scale characteristics on morphology and survival throughout the wood frogs' life-cycle (Berven 2009; Green and Bailey 2015), understanding these relationships in urbanizing landscapes will help ensure that conservation actions are effective. Further study that examines the relative influence of larval and terrestrial conditions on adult morphology and performance, as well as survival to breeding in urbanizing landscapes, can enhance our understanding of which aspects of urbanization contribute to wood frog population declines.

## CHAPTER 4: EFFECTS OF URBANIZING LANDSCAPES ON VERNAL POOL- BREEDING AMPHIBIAN REPRODUCTIVE EFFORT

### 4.1 Chapter Abstract

Urban land conversion around vernal pools compromises terrestrial habitat quality, reduces pool water quality, and alters pool hydrology thereby potentially reducing amphibian reproductive effort and long-term population viability. We examined the effects of tree and impervious cover within 100 - 1,000 m of vernal pools and of road salt contamination (conductivity) on reproductive effort for wood frog (*Lithobates sylvaticus*), spotted salamander (*Ambystoma maculatum*) and blue-spotted salamander (including the unisexual complex, *Ambystoma laterale – jeffersonianum*) at 43 pools across an urbanization gradient near Bangor, Maine, USA. We studied the relationship between adult wood frog body condition and reproductive effort at six pools. Across all three species, reductions in tree cover across multiple scales (300-1,000 m) and increased pool conductivity were associated with reduced likelihoods of breeding and smaller breeding populations. Wood frog and spotted salamander populations were negatively associated with impervious cover near pools (100 m); however, these responses, along with blue-spotted salamander likelihood of breeding, were positively associated with impervious cover at larger scales (300-1,000 m). This increase may be explained if the removal of breeding pools consolidates breeding in remaining pools. Adult wood frog body size was positively associated with clutch size (embryos per clutch), but clutch size was negatively predicted by tree cover within 100 and 300 m and conductivity, suggesting lower competition among adults in urbanizing areas. Our results suggest that reproductive effort may be especially sensitive to impervious cover within 100 m and landscape change within 1,000 m. However,



positive responses to impervious cover  $\geq 300$  m from pools suggests that understanding the effects of urbanization may require an approach that treats amphibians from sets of pools as a single population.

## 4.2 Introduction

Pool-breeding amphibians in the northeastern United States, similar to most groups of amphibians worldwide, are threatened by urbanization and the resultant habitat loss, fragmentation, and degradation (Windmiller and Calhoun 2008; Baldwin and deMaynadier 2009). Because these amphibians usually require both aquatic and terrestrial habitats to complete their life cycles, they are sensitive to urban-associated perturbations in both environments (Gibbs 1998a; Homan et al. 2004; Rubbo and Kiesecker 2005).

Pool-breeding amphibians spend the vast majority of their lives in forested areas near breeding pools that provide post-breeding and overwintering habitat (Regosin and Windmiller 2003; Groff et al. 2016). The conversion of forest to urban-associated cover types (e.g., impervious surfaces) within 1,000 m of pools has been correlated with breeding population declines for wood frog (*Lithobates sylvaticus*) and spotted salamander (*Ambystoma maculatum*) – two species that use vernal pools as essential breeding habitat (Windmiller 1996; Homan et al. 2004; Skidds et al. 2007; Eigenbrod et al. 2008). Moreover, the effects of urbanization may have non-linear effects on these breeding populations, with different intensities of forest cover removal within 30-1,000 m corresponding to sharp declines in the likelihood of wood frogs and spotted salamanders breeding at a pool (Homan et al. 2004). These effects of urbanization on breeding population size have not been verified for these species in northern New England, where sensitivity to urbanization may differ from individuals in more southerly states with

greater intensities of urbanization, nor for breeding populations of blue-spotted salamander (including the unisexual complex, *Ambystoma laterale – jeffersonianum*) – another species for which vernal pools are essential breeding habitat. While blue-spotted salamander also select non-breeding habitat in forested areas (Ryan and Calhoun 2014), life history differences between spotted and blue-spotted salamanders (Homan et al. 2007; Hoffmann 2017) suggest that blue-spotted salamanders may have a distinct, species-specific response to urbanization.

Conservation of vernal pool-breeding species typically involves managing terrestrial habitat within some distance from a pool (Calhoun et al. 2005). Although management recommendations are based on documented distances that adult amphibians travel from pools during the non-breeding season, regulations typically permit some development well within the mean distances of terrestrial amphibian movements from pools (Calhoun et al. 2014), and it is not well understood how development within regulated “life zones” affects the vigor of amphibian populations. Some studies of blue-spotted salamander in urbanizing landscapes have indicated that breeding occupancy is related to forest cover near pools and a post-breeding preference for forests and wet meadows (Ryan and Calhoun 2014; Hoffmann 2017), but largely, the effects of urbanization near pools have not been well-studied for blue-spotted salamander.

Conversion of habitat in terrestrial areas to impervious cover, specifically, introduces a suite of pollutants, most notably road salt, which can travel via runoff or vehicle spray into pools and may be implicated in pool-breeding amphibian population declines (Sanzo and Hecnar 2006; Karraker et al. 2008; Collins and Russell 2009). Road salt contamination can not only harm larval stage wood frogs and spotted salamanders (Sanzo and Hecnar 2006; Karraker et al. 2008) but its impacts may persist to adulthood. For example, in wood frogs, larval exposure to road salt

contamination has been linked to reduced post-metamorphic survival (Dananay et al. 2015; Green and Bailey 2015) and metrics of physiological stress in adult male wood frogs increase with road salt contamination (Hall et al. 2017).

Terrestrial conditions in urbanizing landscapes may also alter the condition of breeding adults (see Patrick et al. 2008 for wood frogs and Homan et al. 2003 for spotted salamanders). Reduced body condition can lessen reproductive effort, as smaller bodied females typically laying clutches with fewer embryos (i.e., smaller clutch size) in both wood frog and spotted salamander (Wilbur 1977b; Kaplan and Salthe 1979; Woodward 1982; Berven 1988). However, there is some disagreement about the likely response of clutch size to urbanization. Clutch size of wood frogs breeding in roadside pools has been detected to be greater than or not different from that in forest pools, and spotted salamanders breeding in roadside pools had smaller clutch sizes (Karraker and Gibbs 2011; Brady 2013). Examining both breeding population size and clutch size may help elucidate the mechanisms of decline in urbanizing landscapes and begin to explain how female body condition may contribute to population declines in urban areas.

Here we examine the effects of both urban-associated land cover near pools and road salt contamination in pools to compare the relative effects of these common facets of urbanization on wood frog, spotted salamander, and blue-spotted salamander breeding populations. More specifically, we examine two facets of reproductive effort, the breeding population size as well as clutch size, to better understand how regulated “life zone” distances and road salt management strategies may impact populations in ways not captured by egg mass counts alone.

## **4.3 Methods**

### **4.3.1 Study Area**

We conducted this study in the greater Bangor area (44° 48' 8" N, 68° 46' 13" W) in Maine, USA. The 200 km<sup>2</sup> study area included Bangor, which encompasses 90 km<sup>2</sup> with a population of approximately 33,000, and Orono, Hampden, and Old Town (populations of approximately 7,000-10,000; U.S. Census Bureau 2011). Developed land uses are primarily residential and commercial with development intensity extremes of nearly 100% impervious surface in downtown Bangor (44° 48' 8" N, 68° 46' 13" W) to <1% impervious surface (e.g., in city conserved lands; Fry et al. 2011). Vernal pools included in the study are embedded in mixed coniferous-hardwood forest (see Chapter 1 for detail). Each site consisted of a vernal pool and the area within 1,000 m of its high-water mark. Sites were selected based on the presence of vernal pool-breeding amphibians.

### **4.3.2 Site Description**

Each site consisted of a wood frog and/or spotted salamander breeding pool and the area within 1,000 m of the pool's high-water mark. We studied 35, 41, and 36 sites (43 total) in 2014, 2015, and 2016, respectively. We selected sites to represent the range of development intensity at which vernal pool-breeding amphibian reproduction occurred in the greater Bangor area. Sites had 0-35 % impervious cover within 100 m, 0-38 % within 1,000 m, and 0-100 % tree canopy over pools. Pool area at spring high water ranged from 24 to 9978 m<sup>2</sup>.

### 4.3.3 Egg mass and embryo counts

We used counts of egg masses and embryos per clutch (clutch size) to indicate reproductive effort, with egg mass counts representing breeding population size or breeding presence. Following the apparent peak of breeding, we conducted egg mass counts for wood frog, spotted salamander, and blue-spotted salamander (including the unisexual complex, *Ambystoma laterale-jeffersonianum*) at 35, 37, and 36 pools (44 total) in 2014, 2015, and 2016, respectively. In 2014, double-observers counted egg masses for greater detection, but only one count was recorded per pool. In 2015 and 2016, dependent double-observer counts were used, following Grant et al. (2005) where the second observer was aware of the eggs detected by the first observer, but not vice versa. Thus the second observer detected at least as many eggs as the first observer. Blue-spotted salamander egg mass counts were only used to indicate detected breeding presence and were not used to indicate breeding population size because of the high variability in how many egg masses are laid by a single female (Wilbur 1971). In 2015 and 2016, we counted the embryos in a subset of wood frog egg masses in 22 and 27 pools (28 total) and of spotted salamander egg masses in 27 and 22 pools (29 total; 38 sites between both species). Even though each female spotted salamanders can produce multiple egg masses (typically a larger, primary mass and 1 to 2 secondary, smaller egg masses; Hunter et al. 1999), and thus more error is likely in analyses of spotted salamander egg masses than for wood frogs, these counts may still provide useful information. We counted embryos in a minimum of five egg masses for a species at a site using techniques described in Karraker (2007) and photographed egg masses. Embryos in images were counted using ImageJ (Schneider et al. 2012). During counting and embryo examination, egg masses were disturbed as little as possible and returned to their original location.

#### **4.3.4 Adult wood frog measurements**

We captured and measured adult male wood frogs to examine the correlation between adult size and resulting reproductive effort. Due to logistical constraints, we could not also capture enough females across pools to incorporate into analyses. Male size can be positively associated with increased breeding success (especially in male-skewed populations; Berven 1981) and may also be a suitable proxy for breeding female size since adult body size in both sexes can respond similarly to conditions such as juvenile population size (Berven 2009). We used minnow traps to capture adult male wood frogs in 9 breeding pools from 2016 April 13-24. We weighed frogs, measured SVL (snout-vent length), and toe-clipped new captures to prevent resampling. Given wood frog's high breeding fidelity to their natal pool (Berven and Grudzien 1990; Vasconcelos and Calhoun 2004), we assume that a high percent (>80%) of males were sampled at their natal pool.

#### **4.3.5 Site Characteristics**

We used ArcView GIS10.2 and the Maine Land Cover Dataset (2004 all land use; 2011 impervious surface) to quantify the percent impervious (IMP) and tree (TREE) cover within 100, 300, 600, and 1,000 m from pool high water marks. Tree cover was digitized from aerial photographs in disturbed and undisturbed areas, thus we cannot assume that tree cover represents forest. Although forest cover and tree cover in urbanizing areas do not necessarily provide the same understory characteristics, most tree cover in either area likely corresponds with ground shading, leaf litter, and increased soil moisture. We used water probes (Hach ©, Loveland, Colorado) to sample specific conductance (SPCOND), which is indicative of road salt contamination, at 43 pools between 2 May-16 June. On each date a pool was sampled, we

collected and tested 1 L of surface water ~1 m from the water edge at each of three equidistant points around the perimeter. All testing was conducted within minutes of sample collection. Specific conductance was averaged by day to represent salt contamination concentration throughout seasons when amphibians use pools. Measurements were collected >1 year at 31 sites; 27 sites had differences of  $\leq 40$   $\mu\text{S}$  between years and only four sites had a difference  $>150$   $\mu\text{S}$  between years (161-872  $\mu\text{S}$ ).

## **4.4 Analyses**

### **4.4.1 Egg mass counts**

In all analyses of breeding population size we only included counts from pools where the modeled species was detected at least one year. We attempted to account for detection probabilities of egg masses to estimate the number of wood frog and spotted salamander masses per site-year using n-mixture models in R software package ‘unmarked’ (Fiske and Chandler 2017) in R version 3.3.1 (R Core Team 2016). However, we chose not to interpret these results because the models performed poorly with improbable detection covariate values (e.g., greater pool sizes had greater detection probabilities) and unrealistically high estimates of egg masses (e.g., 150-1,000% the counted masses in approximately 1/3 of pools). For further analyses we modeled the maximum egg mass counts.

We ultimately examined the relationships between site characteristics (land cover and specific conductivity) and reproductive effort indicated by egg masses (detected reproductive population size of wood frog and spotted salamanders; likelihood of detected breeding of spotted and blue-spotted salamanders) by site-year using Random Forest analyses (RFA) of classification and regression trees (CART) in package ‘randomForest’ (Liaw and Wiener 2002).

We also examined differences in conductivity between pools with and without detected breeding using individual Mann–Whitney tests for spotted and blue-spotted salamander. To avoid overfitting and to ensure robust classification by models, we conducted RFA, a method where many classification trees are constructed for each response variable and the dominant classification structure is selected (Breiman 2001b). We bootstrapped with replacement to build 10,000 regression trees (Random Forest error stabilized at approximately 1,000-2,000 trees for each response variable), using 2/3 of the data at each iteration. We calculated explanatory variable importance using the mean percent decrease in accuracy resulting from removal of each variable to rank the importance of explanatory variables. We used package ‘randomForestSRC’ (Ishwaran and Kogalur 2014) to create partial dependence plots (PDPs) that examine the marginal effects of predictor variables while holding all other predictors at average values (Friedman 2001; Cutler et al. 2007). Because PDPs display general trends all reported values are approximate.

CARTs allow for high correlation of covariates and identify the relative importance of covariates while holding all other variables at their mean. By using multiple years of data from sites as separate observations we captured the among-year variation that is well-documented for the breeding population size of these amphibians (Berven 2009; Capps et al. 2015). We natural-log transformed abundance data so the model (CART) would have an unbiased treatment of high and low egg mass counts. RFAs were particularly appropriate because population size and likelihood of breeding likely does not respond linearly to land cover types and because there was high correlation ( $r > 0.59$ ) among all land cover type variables. Additionally, CART have been found to be more accurate than negative binomial regression models of count data, which can be



used to account for the overdispersion and non-normal distribution of count data (Wah et al. 2012).

#### **4.4.2 Clutch size**

Prior to modeling the effect of site characteristics on wood frog and spotted salamander clutch size (embryos per egg clutch), we examined the variability in embryo counts (i.e., detection probability) using embryos from 5 wood frog and 19 spotted salamander egg masses. These were counted 2-7 times (spotted salamander median = 2, wood frog median = 4 counts; 23 wood frog and 52 salamander counts for a total of 75 counts) with each egg mass being counted by  $\geq 2$  observers. We assumed that undercounting was more likely than over-counting due to embryos possibly being obscured by other embryos or glare in the photo. For each egg mass, we compared the maximum embryo count to all other counts (equal or fewer embryos than the maximum count) to determine the proportion of embryos detected in non-maximum counts. Because detection was relatively high (median non-maximum count detection = 98%; wood frog: 86-100%; spotted salamander: 81-100%), in further analyses of the entire embryo dataset we used the counted number of embryos per egg mass for clutches counted once and used the median count for clutches counted multiple times.

We examined the effect of Year on clutch size for wood frog and spotted salamander before examining the effects of site characteristics, breeding population size, or adult morphology. Using clutch sizes only from site-years where clutch size had been counted for  $\geq 5$  egg masses, we regressed clutch size on year. We initially compared model structure between models with and without Site as a random effect using Akaike's information criterion (AIC). We then examined the effects of Year in the model with the highest-ranking model ( $\Delta AIC=0$ ) for

both species. If Year had a significant effect ( $P < 0.05$ ), it was included as a fixed effect in all further models of both years of clutch size data for that species to compare years across all sites and estimating the effect size of year-to-year differences.

To examine the effects of land cover type at 100-1,000 m and specific conductivity on clutch size we fit linear models that included one predictor as well as Year if clutch size was different between years, as noted above, using package ‘nlme’ (Pinheiro et al. 2017). We then ranked models using AIC adjusted for small sample size (AICc) using package ‘AICcmodavg’ (Mazerolle 2017). We considered models  $\Delta AICc < 2$  that ranked above the null model to be plausible (Burnham and Anderson 2002). If  $> 1$  model had  $\Delta AICc < 2$  we tested additive models that included all combinations of covariates these highly-ranked models. We examined the 85% confidence intervals (Arnold 2010) of covariates in all plausible models to determine effect. Prior to fitting univariate models, we determined optimal model structure by comparing model fit among full models (including all land cover variables and specific conductivity as predictors) with no random effect or that had Site or Year as a random effect. We used the structure from the highest-ranking ( $\Delta AIC = 0$ ) model with the simplest structure; i.e., models without a random structure were selected over those with a random structure if both were  $\Delta AICc < 2$  from each other.

We examined the effect of breeding population size (egg mass count) on clutch size across all years and the effects of median adult SVL and median size-adjusted mass (residual of natural log-transformed mass plotted against natural log-transformed SVL) on 2016 clutch size by species for wood frog and spotted salamander using linear regression. We removed one site from analyses of the effects of adult morphology because it had clutch size for only two egg

masses in 2016. For both sets of models, we initially compared model structure between models without a random effect or with Site as a random effect. We then examined the effects of significant ( $P < 0.05$ ) predictors within the highest-ranking models ( $\Delta AIC = 0$ ).

## 4.5 Results

### 4.5.1 Detected breeding

We detected up to 426 and 391 egg masses of wood frogs and spotted salamanders, respectively, per site-year, and detected breeding at 108, 82, and 54 site-years for wood frogs, spotted salamanders, and blue-spotted salamanders, respectively (Table 4.1). Spotted salamander and blue-spotted salamander breeding was detected in pools with  $\leq 646 \mu S$ ,  $\leq 34\%$  impervious cover within 100 m,  $\leq 32\%$  and  $\leq 27\%$  (respectively) impervious cover within 1,000 m,  $\geq 36\%$  and  $\geq 8\%$  tree cover within 100 m, and  $\geq 17\%$  and  $\geq 29\%$  tree cover within 1,000 m.

Table 4.1 Reproductive responses to conditions in a developing landscape in greater Bangor, Maine, USA, modeled for three species. Observed range and medians are untransformed values. Egg mass counts are only provided for those pools where breeding presence was detected.

Response	Sample size: Pools / individuals	Range (median, mean)
Maximum egg mass count		
<i>Wood frog</i>	44 (-)	0-426 (22.5, 40.8)
<i>Spotted salamander</i>	34 (-)	0-391 (8.0, 36.2)
<i>Blue-spotted salamander</i>	27 (-)	-
Embryos per egg clutch		
<i>Wood frog</i>	27 (453)	120-1,469 (642.0, 650.8)
<i>Spotted salamander</i>	26 (744)	4-224 (97.0, 95.6)

Wood frog breeding population size, as indicated by egg mass counts, was predicted by cover types at multiple scales, with top-ranked predictors at 100-1,000 m (Figure 3.1a). Population size was predicted to negatively respond to urban-associated land cover at 100-600 m with small increases (0-5%) in impervious cover near pools (100 m) predicted to reduce population size and increases in tree cover >50% within 600 m associated with larger population sizes (Figure 3.2). However, 0-38% impervious cover at larger scales (600 and 1,000 m) was positively associated with population size at (Figure 3.2).

Spotted salamander breeding population size and the likelihood of detected breeding were generally negatively associated with urbanization. Both responses were predicted by cover types at multiple scales, with the most important predictor for each operating at relatively small scales (100 m for population size; 300 m for likelihood of breeding; Figures 3.1b-c). Spotted salamander population size was predicted to decrease with small increases (0-7%) in impervious cover near pools (100 m) but was positively associated with small increases (0-7%) in impervious cover at 600 m. Tree cover was positively associated with population size (1,000 m) and likelihood of detected breeding (300-600 m), with steep declines in population size predicted with tree cover losses at 1,000 m between 60-80% and in breeding likelihood predicted with losses of tree cover at 300 and 600 m below 70% and 50%, respectively (Figure 3.2). Increases in specific conductivity up to 650  $\mu\text{S}$  were associated with reductions in likelihood of breeding (Figure 3.2). Significant differences in conductivity between pools where breeding was and was not detected were detected with Mann-Whitney tests ( $U = 232$ ,  $P = 0.05$ ).

The most important predictor for the likelihood of detected breeding for blue-spotted salamander was tree cover within 1,000 m, which was positively associated with breeding likelihood up to at least 82% (Figures 3.1d and 3.2). However, impervious cover within 300 was

generally positively associated with breeding likelihood, with the strongest effect predicted between 0-7% impervious cover (Figure 3.2). No significant differences in conductivity between pools where breeding was and was not detected (Mann-Whitney,  $U = 206$ ,  $P = 0.72$ ).

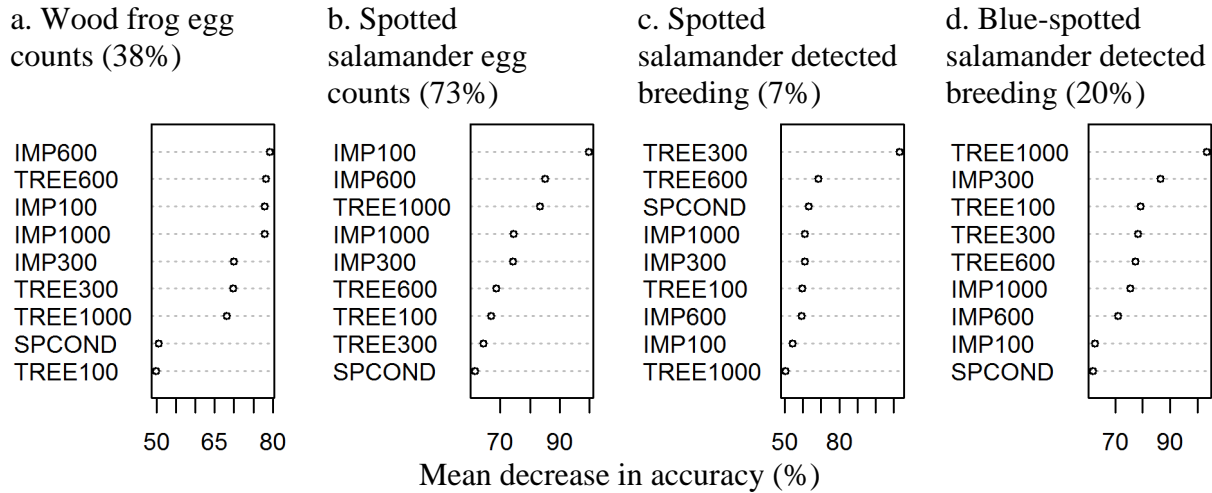
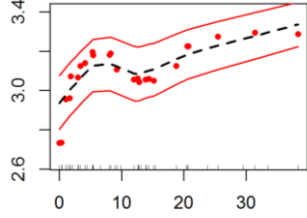


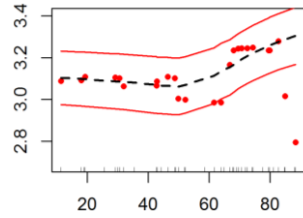
Figure 4.1. Variable importance plots from random forest models for wood frog (*Lithobates sylvaticus*) ln (mean egg mass count +1) regression trees (a and b) and classification trees (c and d). Plot shows the rank-order of explanatory variables along the y-axis and the percent average increase in mean square error when the values of the given variable are randomized while all others are held constant along the y-axis. Parenthetical percentages represent the variation explained for egg mass counts (wood frog and spotted salamander) and out of bag (OOB) estimate of error rate for detected breeding (spotted salamander and blue-spotted salamander).

**Wood frog egg mass count**

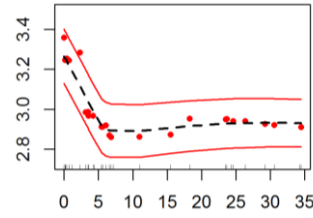
a. IMP600\*



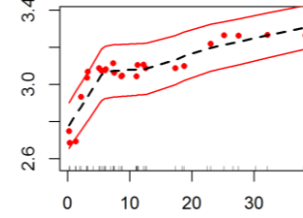
b. TREE600\*



c. IMP100\*

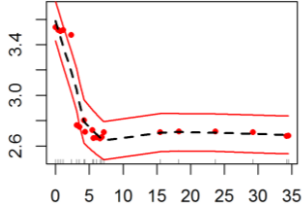


d. IMP1000

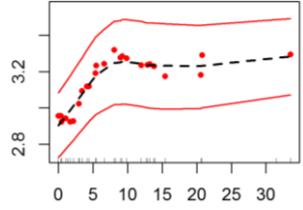


**Spotted salamander egg mass count**

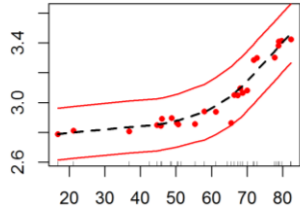
e. IMP100



f. IMP600\*

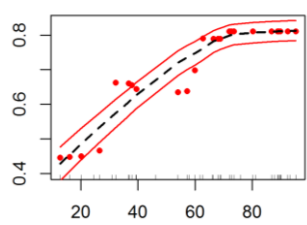


g. TREE1000\*

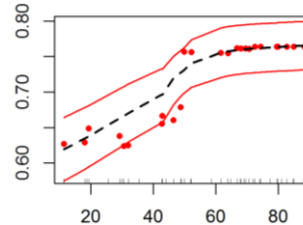


**Spotted salamander likelihood of detected breeding**

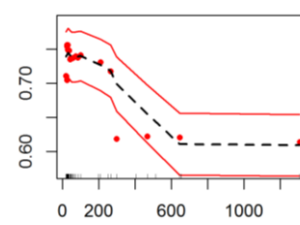
h. TREE300



i. TREE600

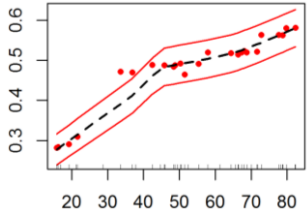


j. SPCOND



**Blue-spotted salamander likelihood of detected breeding**

k. TREE1000



l. IMP300

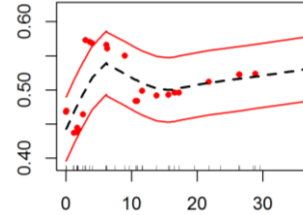


Figure 4.2. Partial dependence plots from random forest predictions of natural log-transformed wood frog egg mass counts (a-c), natural log-transformed spotted salamander egg mass counts (d-f) and likelihood of breeding presence (g-i), and likelihood of blue-spotted salamander breeding presence (j-k) plotted against impervious and tree cover within 100, 300, 600, and 1,000 m. In a partial dependence plot of marginal effects, only the relative values (and not the absolute values) of predicted responses can be interpreted. The black dashed line corresponds to a lowess smoothed line representing the partial dependence between an explanatory variable and response. The dashed red lines indicate a smoothed error bar of  $\pm$ two standard errors. The red dots indicate the partial values used to fit the lowess function.

#### 4.5.2 Clutch size

We counted embryos in 453 wood frog and 744 spotted salamander egg masses. Wood frog egg masses had 120-1469 (median = 642) and spotted salamanders had 4-224 (median = 97) embryos per egg mass (Table 4.1, Appendix H Tables H2-H3). There were differences between years for wood frog and spotted salamander clutch size (Wood frog: Site as a random effect;  $F_{1,425}=10.88$ ,  $P=0.001$ ; Spotted salamander: no random effect;  $F_{1,742}=10.02$ ,  $P=0.002$ ).

Wood frog clutch size decreased with tree cover within 100 and 300 m (Tables 4.2 and 4.4). Conductivity had a positive effect on wood frog clutch size, with increases of 100  $\mu\text{S}$  in conductivity predicted to increase wood frog clutches by 13 embryos (Table 4.3). There was no effect of site characteristics on spotted salamander clutch size nor of the effective breeding population size on clutch size (wood frog, Site as a random effect,  $F_{1,424}= 1.86$ ,  $P=0.17$ ; spotted salamander, no random effect,  $F_{1,741}=0.622$ ,  $P=0.43$ ). Median adult SVL had a positive effect on clutch size, and there was close to a statistically significant effect of size-adjusted mass (Site as a random effect;  $F_{\text{SVL}:1,72}=22.6$ ,  $P<0.001$ , Figure 4.3;  $F_{\text{Mass}:1,72}=3.70$ ,  $P=0.059$ ).

Table 4.2 Model ranking using only those wood frog models that ranked  $<2 \Delta\text{AICc}$  and ranked above the null model. Null models are included for reference. Observations are nested by Site (random effect).

	K	AICc	$\Delta\text{AICc}$	w	LL
TREE100 + Year + Site	5	5881.76	0	0.14	-2935.88
SPCOND + Year + Site	5	5882.02	0.26	0.12	-2936.01
TREE300 + Year + Site	5	5882.13	0.37	0.12	-2936.07
Year + Site (Null)	4	5882.26	0.5	0.11	-2937.13



Table 4.3 Estimates, standard errors, and 85% confidence intervals (CIs) of covariates of wood frog clutch size for models with  $<2\Delta AICc$  and that rank above null models. Covariates are listed in order of AICc of their respective model.

	$\beta$ estimate	SE	Lower CI	Upper CI
TREE100	-1.36	0.844	-2.61	-0.110
SPCOND	0.128	0.0845	0.00323	0.253
TREE300	-1.39	0.937	-2.78	-0.00317

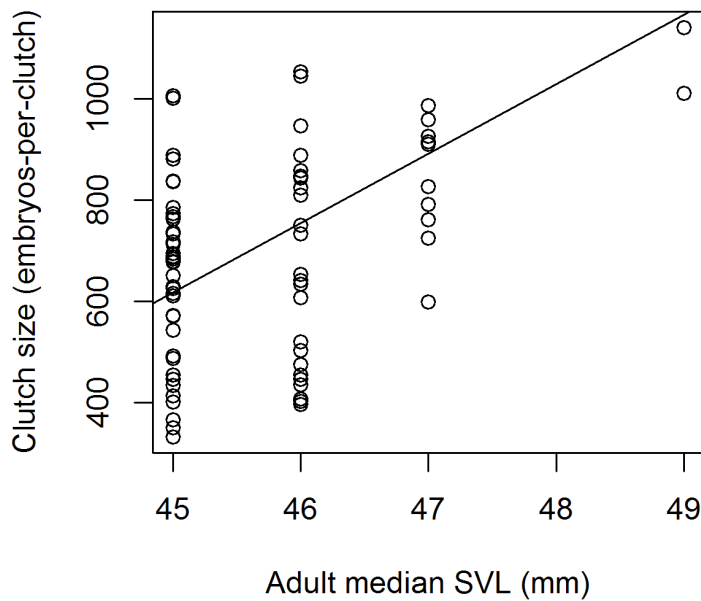


Figure 4.3 Wood frog clutch size plotted against adult median SVL in breeding pools in 2016. The regression line is based on all clutch sizes except those that SVL = 49 mm (clutch size counted for two egg masses).

## 4.6 Discussion

Our results suggest that decreases in tree cover (including canopy cover in urbanizing areas as well as forest) within 1,000 m, increases in impervious cover within 100 m, and increases in road salt contamination impact reproductive effort for wood frog, spotted salamander, and blue-spotted salamander. Increased specific conductivity and/or reductions in tree cover were associated with reduced breeding likelihoods and/or fewer egg masses for the three studied species. Similarly, egg mass counts for wood frog and spotted salamander greatly decreased with small increases (0-5%) in impervious cover within 100 m. Additionally, we detected declines in the likelihood of spotted salamander breeding corresponding to tree cover at <70% within 300 m and <50% within 600 m.

Smaller breeding populations or a reduced likelihood of breeding of our three study species correlating with less forest support similar findings in other studies. Windmiller et al. (2008) documented substantial declines in wood frog, spotted salamander, and blue-spotted salamander (complex) breeding emigration after 41% forest removal within 300 m of a pool; and Homan et al. (2004) detected thresholds of declines in spotted salamander breeding occupancy at 30% and 41% forest cover within 100 and 500 m, respectively. The substantial declines we detected may have corresponded with greater forest cover than those detected by Homan et al. (2004) because they selected study pools using remote sensing whereas we only studied pools with confirmed breeding presence of wood frog or spotted salamander.

Lower wood frog and spotted salamander breeding populations associated with relatively little impervious cover within 100 m of pools suggest that these species are especially sensitive to urban land conversion near pools. Additionally, these results align with studies that

demonstrate the importance of conserving forested areas near pools for pool-breeding amphibian adult habitat. For example, areas near pools provide important overwintering habitat for adult wood frogs and spotted salamander (40-60% of wood frog and spotted salamanders overwintering <100 m, Regosin et al. 1996; within 100 m, Regosin & Windmiller 2003; mean hibernacula distance of approximately 125 m and maximum distance of 317 m from breeding pools, Groff et al. 2016). Moreover, these are important areas for movements outside of the breeding season. Areas within 200 m encompass  $\geq 2/3$  of spotted salamander movements outside of the breeding season (Regosin et al. 1996), and areas within 152 m of pools support 95% of adult blue-spotted salamander (sexually breeding) movements (Ryan and Calhoun 2014) to be within 152 m of pools. Increases in impervious surfaces almost assuredly remove the burrows and uncompacted substrates on which these species rely for cover and overwintering (Madison 1997; Regosin et al. 2003). In areas where lightly compacted yet vegetated areas (e.g., lawns) are typically associated with impervious cover, as is the case in the Greater Bangor area, increases in impervious cover likely correspond to even greater increases in areas where burrows and uncompacted areas suitable for overwintering are removed.

The reduced likelihood of spotted salamander breeding in pools with higher specific conductivity supports other studies indicating that road salt contamination may eliminate breeding populations (Turtle 2000; Karraker et al. 2008; Collins and Russell 2009; Brady 2012). It is possible that our detected effect of specific conductivity may be confounded by road mortality which can contribute to extirpation of breeding populations of spotted salamanders (Gibbs and Shriver 2005) and may encompass the total effects of nearby roads rather than the effects conductivity alone.

Counterintuitively, impervious cover at larger scales (300-1,000 m) was positively associated with wood frog and spotted salamander breeding population size along with blue-spotted salamander likelihood of breeding. One hypothesis is that this relationship may be explained by the loss of breeding pools resulting in displaced adults and first year breeding recruits consolidating breeding in remaining pools. The idea that isolation of suitable breeding pools can increase breeding population size in those pools has been proposed by others (Calhoun et al. 2003; Baldwin et al. 2006; Veysey et al. 2011). Moreover, genetic analyses for wood frogs and spotted salamanders have indicated that populations become more isolated with increased road density within 1 km of pools (J. J. Homola, personal communication). This consolidation of breeding effort could lead to egg mass count-driven population assessments to inappropriately conclude that populations were stable or even benefiting from urbanization. Thus, we suggest that future studies examining the impacts of urbanization on an egg mass count-derived response consider amphibians from sets of pools as one population. This follows the recommendations of other studies to treat clusters of pools as single demographic units (Petranka et al. 2004; Zamudio and Wiczorek 2007; Veysey et al. 2011).

The clutch sizes we observed (wood frog: 120-1,469; spotted salamander: 4-224; Table 4.1) were similar to those in other locations throughout the northeastern US (wood frog: ~300-1,250 embryos per clutch in Maryland, Berven 1988; mean=664 embryos per clutch in Connecticut, Halverson et al. 2006; 514-1,012 in New York, Karraker & Gibbs 2011; ~800 in Connecticut, Brady 2013; spotted salamander: 7-228 in New York, Karraker & Gibbs 2011; ~100 in Connecticut, Brady 2012). However, the effects of tree cover near pools (100-300 m) and specific conductivity on wood frog clutch size suggest that clutch size may increase with urbanization intensity. Because female wood frog size has been positively correlated with clutch

size (Howard and Kluge 1985; Berven 1988), the effect of urbanization on clutch size may be linked to female body size. The positive correlation between adult male SVL and clutch size in a subset of study pools supports this relationship, if one assumes terrestrial habitat quality has a similar effect on the size of males and females. Female body size has also been positively correlated with clutch size for spotted salamander in temporary pools as well as for two other congeners (Kaplan and Salthe 1979; Woodward 1982). Thus, the lack of detected effect of land cover conversion or specific conductivity on spotted salamander clutch size in our study suggests no difference in female condition across the studied urbanization gradient.

Larger clutch sizes with increased urbanization aligns with Brady's (2013) observation that clutch size increased with female body size at a greater rate in high-salinity, roadside pools compared to low-salinity, woodland pools (but see Karraker and Gibbs 2011). However, greater embryo mortality was detected in roadside pools (Brady 2013) and sodium chloride-contaminated stormwater management pools (Snodgrass et al. 2008). Thus, the effect of larger clutch sizes with increasing urbanization intensity may not increase the breeding population size. Indeed, increased embryonic mortality with greater urbanization would favor those individuals with larger clutch sizes, and thus greater clutch size would be expected to evolve. It is also plausible that declines in breeding population size in urbanizing areas may reduce competition for resources among the remaining adults, allowing these adults to grow larger and potentially produce larger clutches (Harper and Semlitsch 2007; Patrick et al. 2008).

Our study supports the idea that urbanization, even at intermediate levels, limits pool-breeding amphibian populations by compromising reproductive effort and may affect the body condition of breeding wood frogs, specifically. Because urban-associated cover types near pools have the most consistent negative effects on egg mass numbers and these effects can be detected

at relatively low levels of cover, maintaining forest cover and other undisturbed areas within 100 m of breeding pools is likely especially important to avoid direct reductions in breeding population size and breeding occurrence. Furthermore, conservation of areas within 1,000 m may be best served to focus on maintaining a mosaic of intact breeding pools, each with sufficient, adjacent terrestrial habitat. Additionally, our results suggest that the effects of conductivity on clutch size are likely not responsible for these declines in breeding populations, but we note that other effects of road salt (e.g., increased larval mortality) may contribute to these declines.

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**APPENDIX A: SUPPLEMENTAL INFORMATION ABOUT BIRD AND MAMMAL  
USE OF VERNAL POOLS ALONG AN URBAN DEVELOPMENT GRADIENT**

Table A1 Bird and mammal species detected during a 2014-16 camera trap survey at 38 vernal pools in Maine. Species detections during the summer season (14 May – 26 August 2014 – 2016) from 33 sites were used in a partial redundancy analysis (pRDA) and to quantify urban-affiliation of the detected bird and mammal assemblage at each site. A numbered list of references follows the table.

Species	Reference supporting urban-affiliation score
<i>Birds</i>	
wood duck ( <i>Aix sponsa</i> )	Campbell, 2009
mallard ( <i>Anas platyrhynchos</i> )	Blair, 1996; Eakin et al., 2015
Canada goose ( <i>Branta canadensis</i> )	Eakin et al., 2015; Gosser & Conover, 1999
hooded merganser ( <i>Lophodytes cucullatus</i> )	Donaldson, Henein, & Runtz, 2007
common merganser ( <i>Mergus merganser</i> )	Donaldson, Henein, & Runtz, 2007
ruffed grouse ( <i>Bonasa umbellus</i> )	Campbell, 2009
wild turkey ( <i>Meleagris gallopavo</i> )	Fuller, Spohr, Harrison, & Servello, 2013; Gustafson, Parker, & Backs, 1994
northern goshawk ( <i>Accipiter gentilis</i> )	Bosakowski & Smith, 1997
sharp-shinned hawk ( <i>Accipiter striatus</i> )	Hager, 2009; Hansen & Urban, 1992
broad-winged hawk ( <i>Buteo platypterus</i> )	Bosakowski & Smith, 1997; Campbell, 2009; Hager, 2009
American woodcock ( <i>Scolopax minor</i> )	Horn, 1985
mourning dove ( <i>Zenaida macroura</i> )	Blair, 1996
great horned owl ( <i>Bubo virginianus</i> )	Bosakowski & Smith, 1997; Hager, 2009

Table A1, continued

Species	Reference supporting urban-affiliation score
barred owl ( <i>Strix varia</i> )	Bosakowski & Smith, 1997; Horn, 1985
ruby-throated hummingbird ( <i>Archilochus colubris</i> )	McCaffrey & Wethington, 2008
northern flicker ( <i>Colaptes auratus</i> )	Beissinger & Osborne, 1982; Horn, 1985; McIntyre, 1995
downy woodpecker ( <i>Dryobates pubescens</i> )	Beissinger & Osborne, 1982; Eakin et al., 2015; Horn, 1985
pileated woodpecker ( <i>Dryocopus pileatus</i> )	Beissinger & Osborne, 1982
hairy woodpecker ( <i>Picoides villosus</i> )	Latta, Musher, Latta, & Katzner, 2013
eastern kingbird ( <i>Tyrannus tyrannus</i> )	DeGraaf & Wentworth, 1986; Eakin et al., 2015
American crow ( <i>Corvus brachyrhynchos</i> )	McGowan, 2001
blue jay ( <i>Cyanocitta cristata</i> )	Aldrich & Coffin, 1979; Beissinger & Osborne, 1982; Horn, 1985
tufted titmouse ( <i>Baeolophus bicolor</i> )	Beissinger & Osborne, 1982; Dowd, 1992; Horn, 1985
black-capped chickadee ( <i>Poecile atricapillus</i> )	Beissinger & Osborne, 1982; Eakin et al., 2015
hermit thrush ( <i>Catharus guttatus</i> )	MacGregor-Fors, 2010; Manley et al., 2006
American robin ( <i>Turdus migratorius</i> )	Beissinger & Osborne, 1982; Blair, 1996; Minor & Urban, 2010

Table A1, continued

Species	Reference supporting urban-affiliation score
gray catbird ( <i>Dumetella carolinensis</i> )	Aldrich & Coffin, 1979; Beissinger & Osborne, 1982
European starling ( <i>Sturnus vulgaris</i> )	Aldrich & Coffin, 1979; Beissinger & Osborne, 1982; Blair, 1996
common yellowthroat ( <i>Geothlypis trichas</i> )	Eakin et al., 2015; Horn, 1985
black-and-white warbler ( <i>Mniotilta varia</i> )	Wilcove, 1985
yellow warbler ( <i>Setophaga petechia</i> )	Campbell, 2009; Tewksbury, Hejl, & Martin, 1998
common grackle ( <i>Quiscalus quiscula</i> )	Beissinger & Osborne, 1982
song sparrow ( <i>Melospiza melodia</i> )	Aldrich & Coffin, 1979; Beissinger & Osborne, 1982
American goldfinch ( <i>Spinus tristis</i> )	Blair, 1996; Eakin et al., 2015
<i>Mammals</i>	
coyote ( <i>Canis latrans</i> )	Ordenana et al., 2010
gray fox ( <i>Urocyon cinereoargenteus</i> )	Chupp, Roder, Battaglia, & Pagels, 2013; Ordenana et al., 2010
red fox ( <i>Vulpes vulpes</i> )	(Adkins and Stott 1998)
domestic cat ( <i>Felis catus</i> )	Chupp, Roder, Battaglia, & Pagels, 2013; Ordenana et al., 2010
bobcat ( <i>Lynx rufus</i> )	Ordenana et al. 2010; Joly and Myers 2001
striped skunk ( <i>Mephitis mephitis</i> )	Ordenana et al., 2010



Table A1, continued

Species	Reference supporting urban-affiliation score
fisher ( <i>Martes pennanti</i> )	Schwartz, Ruiz-Gonzalez, Masuda, & Pertoldi, 2012
raccoon ( <i>Procyon lotor</i> )	Chupp, Roder, Battaglia, & Pagels, 2013; Ordenana et al., 2010
black bear ( <i>Ursus americanus</i> )	Baruch-Mordo, Breck, Wilson, & Theobald, 2008; Joly & Myers, 2001
moose ( <i>Alces alces</i> )	Allen, Jordan, & Terrell, 1987; LaBonte, Kilpatrick, & Barclay, 2013
white-tailed deer ( <i>Odocoileus virginianus</i> )	Grund, McAninch, & Wiggers, 2002
North American porcupine ( <i>Erethizon dorsatum</i> )	Barthelmess, 2014; Odell & Knight, 2001
muskrat ( <i>Ondatra zibethicus</i> )	Cotner & Schooley, 2011
woodchuck ( <i>Marmota monax</i> )	Lehrer, Fredebaugh, Schooley, & Mateus-pinilla, 2010
eastern gray squirrel ( <i>Sciurus carolinensis</i> )	Bateman & Fleming, 2014; Williamson, 1983
red squirrel ( <i>Sciurus vulgaris</i> )	Racey & Euler, 1982
eastern chipmunk ( <i>Tamias striatus</i> )	Nilon & VanDruff, 1986
snowshoe hare ( <i>Lepus americanus</i> )	Joly & Myers, 2001

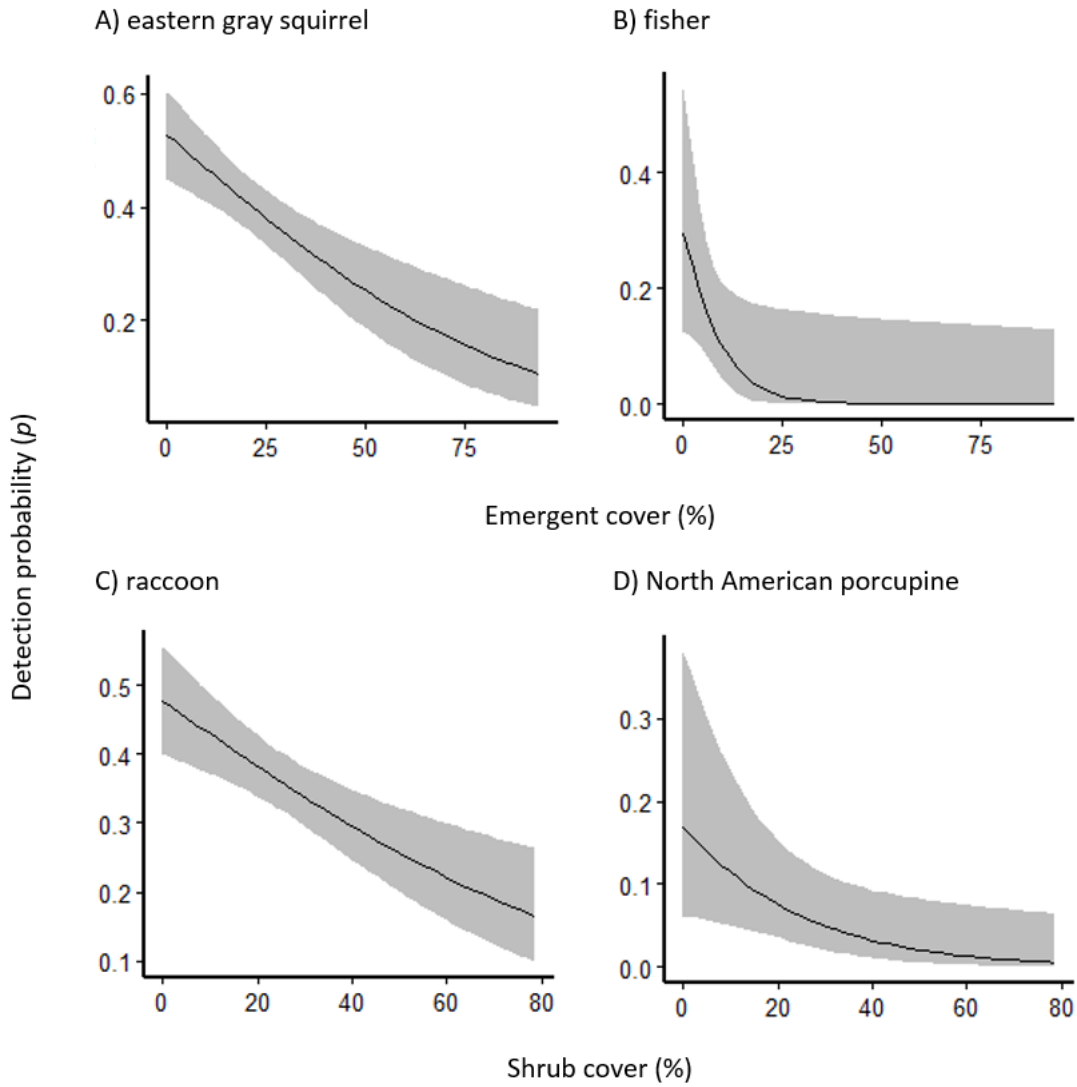


Figure A1 Relationship between detection probability and within-pool emergent vegetation and shrub cover modelled as covariate effects in single-season occupancy models for a subset of species. Shaded areas represent 95% confidence intervals.

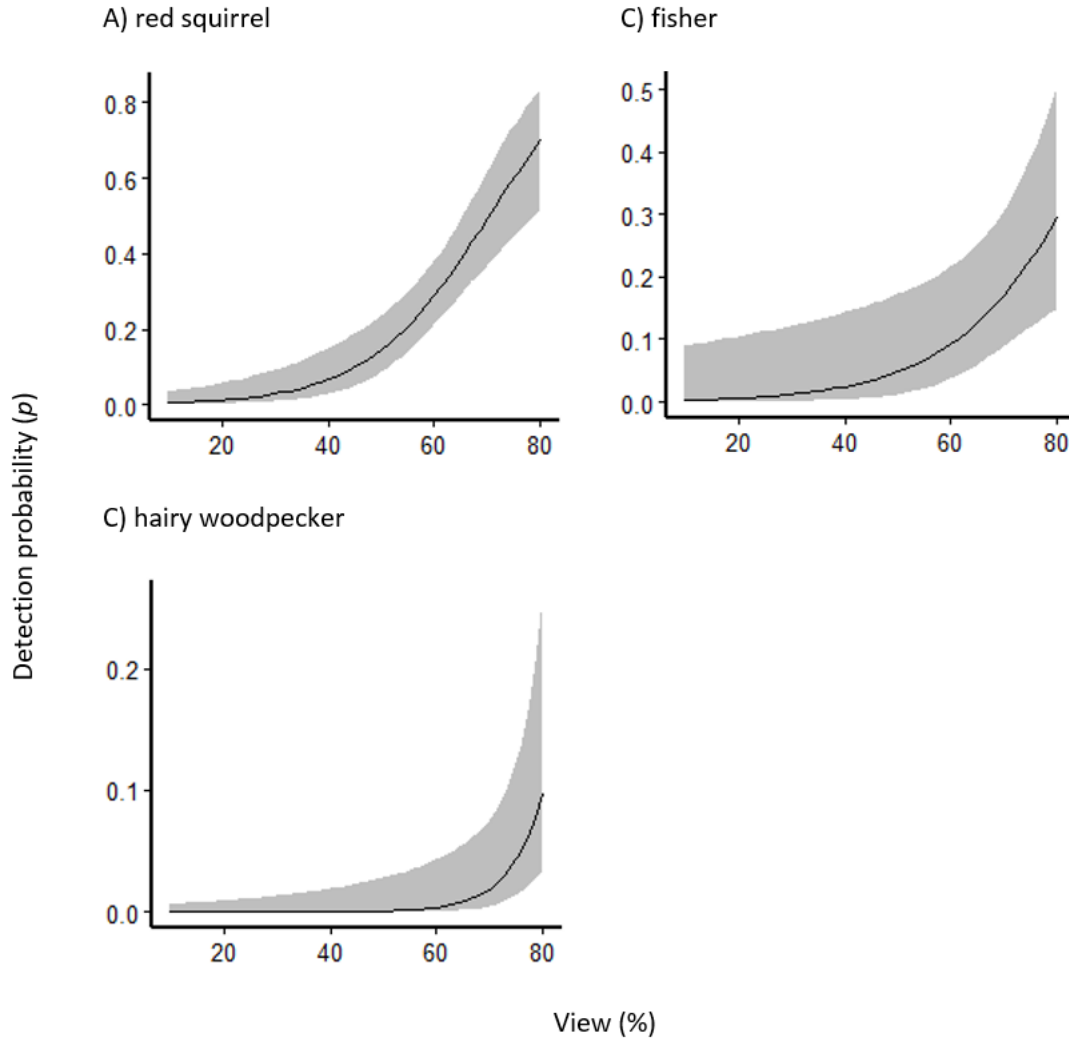


Figure A2 Relationship between detection probability and the percent of a pool basin captured in photos (View). View was modelled as a covariate effect in single-season occupancy models. Shaded areas represent 95% confidence intervals.

**APPENDIX B: SUPPLEMENTAL INFORMATION FOR CHAPTER 2 ABOUT WOOD  
FROG (*LITHOBATES SYLVATICUS*) LARVAE SURVIVAL AND MORPHOLOGY**

**B.1 Survival estimation of wood frog larvae**

Within the global models candidate set in Chapter 2, the model with the negative binomial distribution was the highest ranked and was slightly overdispersed ( $\hat{c} = 1.28$ ,  $\chi^2 = 16,379.7$ ,  $P = 0.163$ ). Two models were within  $\Delta\text{QAICc} \leq 2$  with all detection parameters in common except Dip (Table B1). Dip was not included in the final model because its 95% CI Beta estimate included zero. Beta estimates in the final model indicated that detection decreased with Veg, Depth, and Egg, but increased with Area (Table B2).

Table B1 Results of the top-ranked abundance models ( $\Delta\text{QAICc} \leq 2$ ). The null model is included for reference.

	K	QAICc	$\Delta\text{QAICc}$	$w$
$\lambda(.)p(\text{Veg}+\text{Depth}+\text{Area}+\text{Egg})$	8	1275.64	0.00	0.70
$\lambda(.)p(\text{Veg}+\text{Depth}+\text{Area}+\text{Dip}+\text{Egg})$	9	1277.32	1.68	0.30
$\lambda(.)p(0)$	4	1340.7	65.06	0.00

Table B2 Estimated detection parameters ( $\beta$ ), standard error (SE), and 95% CI of the selected model predicting wood frog tadpole abundance.

	$\beta$	SE	Lower CI	Upper CI
Veg	-0.18	0.03	-0.23	-0.13
Depth	-0.54	0.05	-0.62	-0.46
Area	0.73	0.15	0.47	0.98
Egg	-0.67	0.06	-0.78	-0.55

## B.2 Effect sizes of predictors of wood frog larvae morphology variable pairs

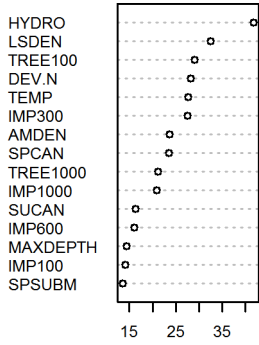
Table B3 Effect sizes (eta-squared,  $\eta^2$ ) of predictors for raw morphology variable pairs from univariate-ANOVAs examining site, year, and urbanization level effects associated with Chapter 2. The first variable listed in the pair-wise relationship is regressed against the second variable, and the main effect of Site, Year, or Urban and the respective interaction term were included as predictors. Symbols indicate statistical significance. Parenthetical numbers are degrees of freedom. Stage refers to developmental stage.

Pair-wise relationship	$\eta^2$ (Full data set)				$\eta^2$ (Subset of rural and urban site observations)	
	Site	Site- interaction	Year	Year- interaction	Urban	Urban- interaction
ln(Julian day)~stage	0.22*** (37, 6676)	0.03*** (37, 6676)	0.03*** (2, 6746)	0.02*** (2, 6746)	0.13*** (1, 3240)	0.02*** (1, 3240)
ln(mass)~ln(SVL)	0.03*** (37, 6676)	0.01*** (37, 6676)	0.00*** (2, 6746)	0.00*** (2, 6746)	0.00* (1, 3218)	0.01*** (1, 3218)
ln(SVL)~stage	0.05*** (37,6676)	0.03*** (37, 6676)	0.01*** (2, 6746)	0.01*** (2, 6746)	0.00*** (1, 3240)	0.00 (1, 3240)
ln(tail length)~ln(SVL)	0.07*** (37, 6676)	0.02*** (37, 6676)	0.01*** (2, 6746)	0.01*** (2, 6746)	0.01*** (1, 3218)	0.02*** (1, 3218)
ln(tail depth)~ln(tail length)	0.06*** (37, 6602)	0.01*** (37, 6602)	0.01*** (2, 6672)	0.00*** (2, 6672)	0.00*** (1, 3179)	0.00*** (1, 3179)

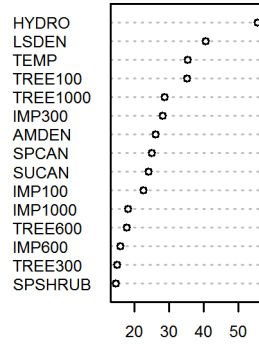
\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$

### B.3 Aggregate morphology responses of wood frog larvae to site characteristics

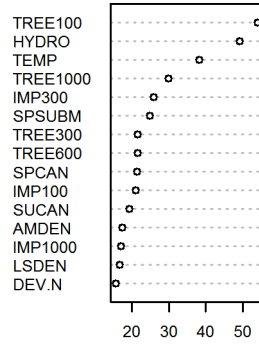
a. Dev10 (40%)



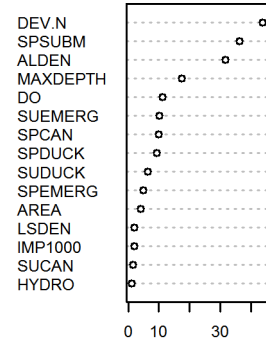
b. Dev50 (51%)



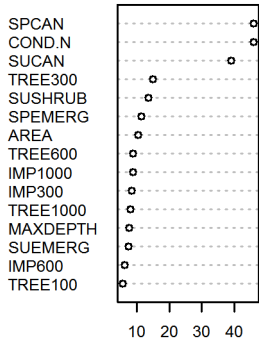
c. Dev90 (54%)



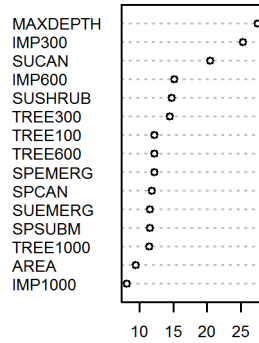
d. DevSD (15%)



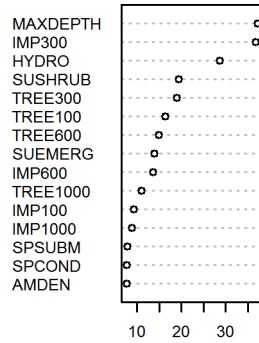
e. Cond10 (25%)



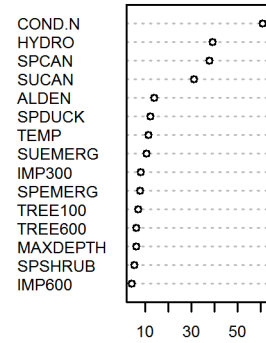
f. Cond50 (8%)



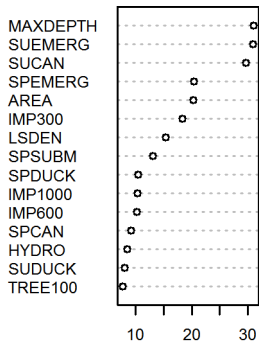
g. Cond90 (20%)



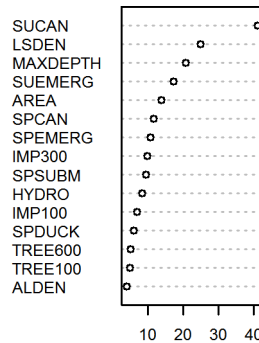
h. CondSD (33%)



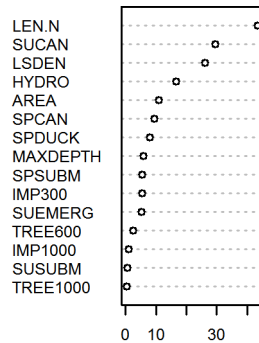
i. Len10 (15%)



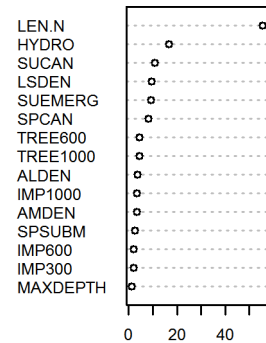
j. Len50 (17%)



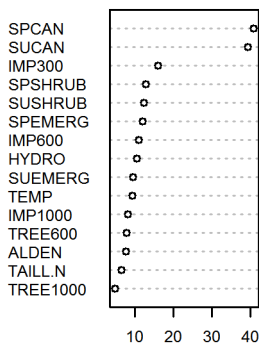
k. Len90 (19%)



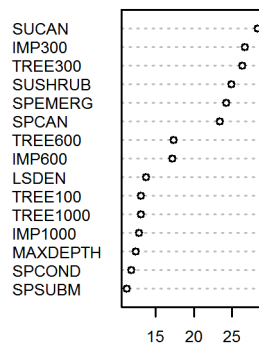
l. LenSD (10%)



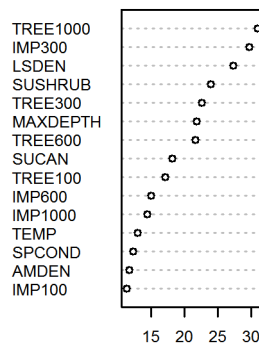
m. TailL10 (12%)



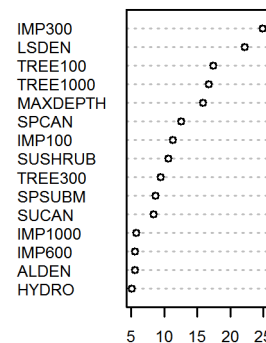
n. TailL50 (19%)



o. TailL90 (24%)



p. TailLSD (4%)



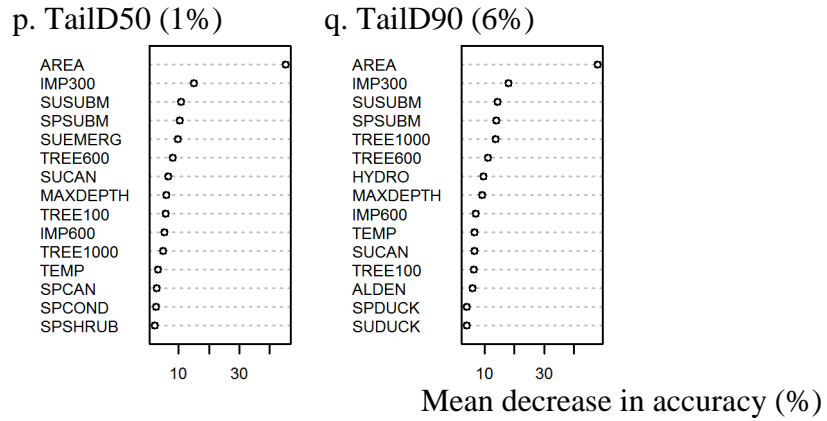


Figure B1 Variable importance plots from Random Forest Analyses models for larval wood frog examined in Chapter 2. Plots show the 15 top-ranked explanatory variables along the y-axis and the percent average increase in mean square error when the values of the given variable are randomized while all others are held constant along the x-axis. Parenthetical numbers note % variance explained by RFA. Models for TailD10 and TailDSD did not explain any variation in the response (i.e., not different from random), thus predictor importance was not examined for these responses.

**APPENDIX C: SUPPLEMENTAL INFORMATION ABOUT CARRY-OVER**

**EXPERIMENT MODEL RANKINGS AND COEFFICIENTS**

Table C1 Model ranking using only those models that ranked  $<2 \Delta AICc$  in their respective predictor category (SC = site characteristics, LM = larval morphology) and that ranked higher than the null model. Null models (NM) are included for reference. Observations are nested by Site (random effect) in all models except those for round 2 froglet responses.

	K	AICc	$\Delta AICc$	w	LL
<i>Larval morphology</i>					
Relative mass					
SC: L.SVL + Egg	5	-51.23	0	0.44	30.93
NM: L.SVL	4	-50.24	0.99	0.27	29.33
SVL					
SC: Egg	4	-131.27	0	0.82	69.85
NM: .	3	-126.65	4.62	0.08	66.45
<i>Survival to emergence</i>					
LM: L.SVL	3	129.33	0	0.64	-61.54
LM: L.Cond	4	130.79	1.46	0.31	-61.19
NM: .	2	134.32	4.98	0.05	-65.1
<i>Round 1 froglet morphology</i>					
Relative mass <sup>2</sup>					
LM: F1.SVL + L.Mass + L.SVL	6	-72.16	0	1	42.89
SC: F1.SVL + Egg	5	-50.9	21.27	0	31.01
NM: F1.SVL	4	-49.5	22.67	0	29.12



Table C1, continued

	K	AICc	$\Delta$ AICc	w	LL
<b>SVL</b>					
LM: L.Mass + L.SVL	5	-165.11	0	1	88.11
SC: Egg	4	-140.31	24.8	0	74.52
NM: .	3	-138.88	26.23	0	72.65
<b>Relative leg length</b>					
LM: F1.SVL + L.Mass + L.SVL	6	-128	0	0.94	70.81
SC: F1.SVL + Egg	5	-121.06	6.94	0.03	66.10
NM: F1.SVL	4	-119.87	8.13	0.02	64.30
<i>Round 1 froglet performance</i>					
<b>Maximum jump distance</b>					
LM: L.SVL + L.Mass	5	38.65	0	0.99	-13.83
SC: Egg	4	47.27	8.62	0.01	-19.31
NM: .	3	51.97	13.32	0	-22.79
<b>Distance moved</b>					
LM: L.SVL + L. Mass	5	88.69	0	0.59	-38.8
SC: Egg	4	99	10.3	0	-45.14
SC: Veg	4	99.03	10.33	0	-45.16
NM: .	3	99.16	10.47	0	-46.37
<b>Duration</b>					
SC: Hydro	4	60.87	0	0.77	-26.08
NM: .					

Table C1, continued

	K	AICc	$\Delta$ AICc	w	LL
<b>Speed</b>					
LM: L.Mass + L.SVL	5	62.19	0	0.95	-25.55
SC: Egg	4	69.19	7	0.03	-30.24
SC: Cover	4	70.79	8.6	0.01	-31.04
NM: .	3	71.51	9.32	0.01	-32.55
<i>Round 2 froglet morphology**</i>					
<b>Relative mass</b>					
SC: F2.SVL + Veg	4	-14.46	0	0.61	12.48
NM: F2.SVL	3	-13.59	0.87	0.39	10.5
<b>SVL</b>					
LM: L.SVL + L.Mass	4	-52.57	0	1	31.53
SC: Veg	3	-26.92	25.64	0	17.17
NM: .	2	-14.02	38.55	0	9.34
<b>Relative leg length</b>					
NM: .	3	-37.09	0	1	22.25
<i>Round 2 froglet performance**</i>					
<b>Maximum jump distance</b>					
SC: Hydro	3	13.19	0	0.66	-2.67
SC: Egg	3	14.56	1.36	0.33	-3.36
NM: .	2	21.94	8.75	0.01	-8.54
<b>Distance moved</b>					

Table C1, continued

	K	AICc	$\Delta$ AICc	w	LL
SC: Hydro	3	56.21	0	0.4	-24.1
SC: Egg	3	56.52	0.31	0.34	-24.26
SC: Veg	3	58.12	1.92	0.15	-25.06
NM: .	2	58.68	2.47	0.11	-26.88
<i>Duration</i>					
SC: Hydro	3	43.01	0	0.4	-17.5
SC: Egg	3	43.39	0.38	0.33	-17.7
SC: Veg	3	44.53	1.52	0.19	-18.26
NM: .	2	46.14	3.13	0.08	-20.61
<i>Speed</i>					
SC: Hydro	3	29.63	0	0.35	-10.81
SC: Egg	3	29.75	0.12	0.33	-10.88
NM: .	2	29.77	0.15	0.32	-12.43
<i>Adult morphology</i>					
<i>Relative mass</i>					
LM: SVL	5	-492.18	0	0.81	251.21
SC: Hydro	5	-488.02	4.16	0.1	249.13
NM: .	4	-487.83	4.35	0.09	248
<i>SVL</i>					
SC: Cover	4	-758.02	0	0.33	383.09
SC/LM: Cover + L.Mass	5	-757.66	0.36	0.27	383.95

Table C1, continued

	K	AICc	$\Delta$ AICc	w	LL
LM: Mass	4	-757.21	0.81	0.22	382.69
NM: .	3	-756.84	1.18	0.18	381.47

Table C2 Estimates, standard errors, and 85% confidence intervals (CIs) of covariates of larval survival; larval, froglet, and adult morphology; and froglet performance for models with  $<2\Delta\text{AICc}$  within their respective predictor category and that rank above null models. Covariates are listed in order of AICc of their respective model. Only those covariates with CIs that do not include zero are shown here. In two-covariate models,  $<2$  covariates had 85% CIs different from zero, thus single covariate models were more parsimonious; no two covariates shown are from the same model. The SVL terms that were paired with mass and leg length covariates to adjust for size-dependence are not shown here as they were not intended as predictors.

	$\beta$ estimate	SE	Lower CI	Upper CI
<i>Larval morphology</i>				
Relative mass				
Egg	-0.239	0.125	-0.436	-0.0426
SVL				
Egg	-0.0835	0.0271	-0.126	-0.0409
<i>Survival to emergence</i>				
L.SVL	4.93	1.92	2.25	7.84
<i>Round 1 froglet morphology</i>				
Relative mass				
L.Mass	0.524	0.0998	0.383	0.665
Egg	-0.0671	0.0348	-0.121	-0.0131
SVL				
L.Mass	0.270	0.0315	0.225	0.315
Egg	-0.0819	0.0391	-0.143	-0.0207
Relative leg length				
L.Mass	0.202	0.0601	0.117	0.287
Egg	-0.0329	0.0175	-0.0601	-0.00571

Table C2, continued

	$\beta$ estimate	SE	Lower CI	Upper CI
<i>Round 1 froglet performance</i>				
Maximum jump distance				
L.Mass	0.762	0.162	0.531	0.993
Egg	-0.354	0.114	-0.533	0.175
Distance moved				
L.Mass	1.10	0.232	0.770	1.43
Egg	-0.310	0.191	-0.609	-0.0111
Veg	-0.188	0.117	-0.371	-0.00530
Duration				
Hydro	-0.113	0.0344	-0.167	-0.0588
Speed				
L.Mass	0.751	0.190	0.480	1.02
Egg	-0.304	0.129	-0.506	-0.102
Cover	0.104	0.0572	0.014	0.194
<i>Round 2 froglet morphology</i>				
Relative mass				
Veg	-0.0499	0.0258	-0.0886	-0.0111
SVL				
L.Mass	0.357	0.0373	0.301	0.413
Egg	-0.151	0.0329	-0.200	-0.101
<i>Round 2 froglet performance #2</i>				

Table C2, continued

	$\beta$ estimate	SE	Lower CI	Upper CI
<b>Maximum jump distance</b>				
Hydro	0.180	0.0467	0.110	0.251
Egg	-0.276	0.0777	-0.394	-0.158
<b>Distance moved</b>				
Hydro	0.482	0.200	0.177	0.786
Egg	-0.775	0.333	-1.28	-0.268
Veg	0.561	0.297	0.109	1.01
<b>Duration</b>				
Hydro	0.341	0.132	0.139	0.542
Egg	-0.548	0.221	-0.884	-0.211
Veg	0.424	0.194	0.128	0.720
<b>Speed</b>				
Hydro	0.154	0.0871	0.0213	0.287
Egg	-0.250	0.144	-0.469	-0.0298
<b>Adult morphology</b>				
<b>Relative mass</b>				
L.SVL	-0.332	0.108	-0.509	-0.155
Hydro	0.0279	0.0166	0.000752	0.0551
<b>SVL</b>				
Cover	-0.00724	0.00383	-0.0135	-0.000940

## APPENDIX D: AQUATIC INSECT ASSEMBLAGES AND EFFECTS ON WOOD FROG TADPOLE MORPHOLOGY ACROSS AN URBANIZING LANDSCAPE

### D.1 Introduction

Urban development threatens vernal pools in the northeastern US and the amphibians for which these pools are essential breeding habitat (Homan et al. 2004; Windmiller and Calhoun 2008; Baldwin and deMaynadier 2009). Aquatic insects in these pools may be major predators of larval pool-breeding amphibians (Colburn et al. 2006). Changes in pool environments caused by urban development may shift the composition of insect assemblages (Williams 1996; Relyea 2002; Bischof et al. 2013) and resulting differences in amphibian and insect interactions may shift patterns of predation pressure (Eck et al. 2014). Predation pressure can influence wood frog (*Lithobates sylvaticus*) tadpole morphology, growth, and developmental rates (Relyea 2001b, 2004), thus adding complexity to understanding the total influence of urbanization on vernal pool amphibians.

Tadpole tail morphology may be influenced by differences in predator pressure (Calsbeek and Kuchta 2011). Predatory insect attacks can also cause sublethal damage to tadpole tails. Although individual larvae with tail damage may not have reduced survival and growth (Polich et al. 2013), tail damage among surviving tadpoles may indicate relative within-pool (i.e., population level) predator pressure on tadpoles.

Here we characterize the invertebrate assemblage in 29 vernal pools and examine their predatory pressures on wood frog tadpoles in an urbanizing landscape. Specifically, we studied how two urban-associated characteristics, impervious cover and road salt contamination,



influence assemblage composition and density of predatory insects. We also examined how the densities of predatory insects relate to larval wood frog tail damage, development, and morphology. We expected insect assemblages to shift with urban-associated characteristics, and that increased densities of predatory insects would correspond to a greater likelihood of tail damage, tadpoles with longer and broader tails, slower development, shorter bodies, and lower condition.

## **D.2 Methods**

### **D.2.1 Site characteristics**

We used ArcView GIS10.2 and the Maine Land Cover Dataset (2004 all land use; 2011 impervious surface) to quantify the percent impervious and tree cover within 100 m of the pools' high water marks. We quantified mean specific conductivity, hydroperiod, and summer canopy cover using 2014-2016 measurements from concurrent studies of a larger set of study pools that included the study pools here (Chapters 1-2). We used the multi-year average to reflect conditions over an extended time period to which insect assemblages likely respond. We used water probes (Hach ©, Loveland, Colorado) to sample specific conductance in pools during May and June 2014-2016. On each date a pool was sampled, we collected and tested 1 L of surface water ~1 m from the water edge at each of three equidistant points around the perimeter. Only one sample was taken at pools that were almost dry and  $<2 \text{ m}^2$ . All testing was conducted at the pool edge within minutes of sample collection. Each metric was averaged by day and then year to calculate values used in analyses. Hydroperiod was determined by the Julian day that standing water was no longer present as observed during field visits or by trail cameras placed at pools for a separate study (Chapter 1). We measured woody canopy cover over pools in summer. Canopy

density was measured ~1 m above the ground near the pool center using a spherical convex densitometer. See Chapter 2 for greater detail.

### **D.2.2 Aquatic insect surveys**

We used the density of predatory aquatic insects ( $m^{-2}$ ) during wood frog larval development to indicate the relative predatory pressure from predatory aquatic insects among pools. We conducted dip net surveys of aquatic insects once at 27 pools in 2015 from 16 June – 24 July, once at 10 pools in 2016 from 8 - 23 June, and a second time at 5 pools from 29 June – 22 July (29 different pools, total; 9 of the pools sampled in 2015 were also resampled in 2016). We followed the same dip netting methods described in Chapter 2 for tadpole abundance surveys, except that insect sampling was conducted within a 5-day window.

We followed Werner et al. (2007) to determine if an insect family was likely to prey upon tadpoles. Some families in orders Odonata (Aeshnidae, Cordulidae, Gomphidae, Libellulidae, and Corduliidae), Coleoptera (Dytiscidae, Hydrophilidae), and Hemiptera (Belostomatidae, Notonectidae) as well as all Megaloptera were categorized as predators (hereafter referred to as predatory insects). We combined Libellulidae and Corduliidae because of the difficulty in differentiating between these families and treated them as a single family throughout analyses. We counted and identified all insects to family except for some Coleopterans detected in 2015 in six pools that were not collected for lab identification. Because we could not determine whether these Coleopterans were likely tadpole predators, all 2015 observations from these pools were excluded from predator density analyses. To indicate daily relative density, we divided the number of individuals in each family detected during each visit by the total area of dip net

sweeps (number of sweeps x 0.39 m<sup>2</sup>) during that visit. Predatory insect density was summed by visit and averaged across visits within each year.

For a more complete representation of the aquatic insect assemblages, we also opportunistically identified aquatic insects during tadpole surveys (15 May – 8 June 2015 and 23 May – 6 September 2016). All opportunistic samples from 2015 were from pools where insect density surveys were conducted, and in 2016 they were conducted at pools surveyed to determine density as well as at an additional two pools which were sampled for insect density in 2015 (collected concurrently with tadpole sampling occurring at these pools in 2016, Chapter 2). Only 2015 observations (opportunistic and those from density surveys) were used in further analyses of insect composition.

### **D.2.3 Tadpole surveys**

We conducted wood frog tail damage surveys at 30 pools in 2015 and at 10 pools in 2016. During these surveys, we inspected tadpoles for the presence of tail damage (0,1) indicative of an escape from a predator. At least 34 tadpoles were sampled per site. We also collected data on tadpole morphology and development for the 23 sites (31 pool-years; 21 pools in 2015 and 10 pools in 2016) where tail damage and insect abundance was studied. These data were collected as part of a larger study of 39 vernal pools during 2014-2016, as described in Chapter 2. Residuals indicating relative tadpole developmental phenology, body condition, body length, tail length, and tail fin depth were calculated in this concurrent study. Tail measurements were excluded for tadpoles with tail damage.

## **D.2.4 Statistical analyses**

### **D.2.4.1 Insect assemblages**

We examined differences in assemblage composition among pools using 2015 observations from pools where all insect detections (density surveys and opportunistic detections) were identified to family. Although including opportunistic observations makes sampling effort inconsistent among pools, this increases the overall detection of families and thus may provide some useful information.

Initially, we examined similarities among assemblage composition using non-hierarchical cluster analyses in the ‘fpc’ package. We represented assemblage composition with a detection matrix (0,1) of family x site-year. We removed all families detected at <2 sites. If clusters were not stable, we did not categorize pools by assemblage “type” in further analyses but instead assumed that examination of gradients of variation among assemblages was likely more appropriate.

We then conducted a partial redundancy analysis (pRDA) using the ‘vegan’ package (Oksanen et al. 2017) to identify how hydroperiod and canopy cover (conditions which are associated with shifts in aquatic assemblages) as well as specific conductivity and impervious cover within 100 m (urban-associated characteristics) correlate with dominant gradients of variation in assemblages among sites (Borcard et al. 2011). Prior to conducting the pRDA, we assessed explanatory variables for collinearity (all pairwise Pearson product-moment bivariate correlations were  $\leq 0.56$ ). We conditioned the RDA on latitude and longitude to account for the

portion of Curtis-Bray dissimilarity in assemblages attributed to spatial correlation (Spearman's rank correlation=0.110, p=0.04).

We conducted Monte Carlo global permutation tests to determine the significance of the ordination, the pRDA axes, and each constraining variable. Because constraining variables are assessed sequentially for significance, we tested each variable as the first term in the model. Upon determining significance of the ordination (p=0.001), we compared the pRDA with an unconstrained, unconditioned principle component analysis to assess if extracted patterns in the pRDA likely represent actual dominant gradients (Legendre and Gallagher 2001). Since both ordinations were similar, we assumed that the constraining variables in the pRDA are related to actual gradients of variation. We then calculated the proportion of variance explained by each axis and each constraining variable and overlaid generalized additive model (GAM) fitted surfaces on the ordination to examine the linearity of variation of each vector.

We further examined how the natural log-transformed total density of predatory insects relates to hydroperiod, canopy cover, and urban-associated characteristics. We compared global models including Site or Year as nesting terms (random effects) with a model with no nesting term using Akaike's information criterion adjusted for small sample size (AICc) to determine the optimal fixed structure of the model. Because the global model without a random effect was highest ranked, we did not include a nesting term in subsequent models. We created a set of univariate linear models for predator density using R package 'lme4' (Bates et al. 2017) and then compared these models using AICc to select top ranking models using library 'AICcmodavg' (Mazerolle 2017). We considered models  $\Delta AICc < 2$  that ranked above the null model to be

plausible (Burnham and Anderson 2002) and examined the 85% confidence intervals (Arnold 2010) of each covariate in these models to determine effect.

#### **D.2.4.2 Tadpole responses**

We examined the statistical effects of insect predator density on tadpole tail damage, developmental phenology, and morphology. Initially, we examined if the likelihood of tadpole tail damage was cumulative throughout the season using logistic regression (package ‘nml’, Pinheiro et al. 2017). We used Site as a random term in these models to account for the lack of independence among tadpoles within the same pool. If tail damage was cumulative throughout the season ( $P < 0.05$ ), we included Julian day as a covariate in all further models of likelihood of tail damage.

For each tadpole response, we created a set of univariate linear models with the density of each predator family and the total predator density as covariates. We examined the response to multiple predatory families because predatory ability differs among families, i.e., the predation pressure of 10 individuals of family X might be different from that of 10 individuals of family Y (Roth and Jackson 1987). We created models of likelihood of tail damage using package ‘nml’ (Pinheiro et al. 2017) and linear models of tadpole development and morphology responses using ‘lme4’ (Bates et al. 2017). We then compared the set of models for each response using AICc to select top ranking models using library ‘AICcmodavg’ (Mazerolle 2017) and examined the 85% confidence intervals (Arnold 2010) of each covariate in plausible models to determine effect. If  $>1$  model met these criteria, we tested additive models that included all combinations of covariates in plausible models.

### **D.3 Results**

We detected aquatic insects in six orders and 24 families, with six orders and 12 families represented in abundance surveys. Although not targeted taxa, we also detected aquatic invertebrates in subclass Hirudinea (leech), order Isopoda (isopods), and phylum Mollusca (mollusks, primarily snails and clams). Dytiscidae and Libellulidae/Corduliidae, all of which are predators of wood frog tadpoles, were detected in 27 of 29 pools (Table D1).

Table D1 Detected presence of aquatic insects at 29 pools in greater Bangor, Maine. In 2015 and 2016, 27 and 10 pools were surveyed, respectively. Those taxa denoted with (\*) were categorized as predators in analyses.

<b>Order and Family</b>	<b>2015</b>	<b>2016</b>	<b>Total</b>
Coleoptera	26	10	28
Dytiscidae * <sup>1</sup>	25	10	27
Elmidae <sup>1</sup>	3	0	3
Gyrinidae	0	1	1
Haliplidae	0	1	1
Hydrophilidae * <sup>1</sup>	7	6	11
Noteridae	0	5	5
Scirtidae	1	0	1
Diptera <sup>2</sup>	7	2	9
Culicidae	0	2	2
Stratiomyidae	1	0	1
Tipulidae <sup>1</sup>	5	0	5
Hemiptera	16	4	17
Belostomatidae * <sup>1</sup>	2	1	3
Corixidae <sup>1</sup>	16	3	17
Gerridae <sup>1</sup>	6	3	9
Notonectidae *	1	2	3
Megaloptera	12	7	17
Corydalidae * <sup>1</sup>	12	7	17



Table D1, continued

<b>Order and Family</b>	<b>2015</b>	<b>2016</b>	<b>Total</b>
Sialidae *	0	1	1
Odonata	27	7	28
Aeshnidae * <sup>1</sup>	15	3	17
Gomphidae *	1	0	1
Lestidae <sup>1</sup>	13	2	13
Libellulidae/Corduliidae * <sup>1</sup>	26	6	27
Trichoptera <sup>3</sup>	17	3	17
Limnephilidae <sup>1</sup>	14	2	14
Hydropsychidae	1	0	1
Phryganeidae	1	0	1
Rhyacophilidae	0	1	1

<sup>1</sup> Families detected in 2015 and used in assemblage composition analyses.

<sup>2</sup> Diptera were detected in one pool in 2015 where no family was identified. This pool was not resampled in 2016.

<sup>3</sup> Trichoptera were detected in two pools in 2015 where no family was identified. In 2016, one pool was not resampled and no Trichoptera were detected in the pool that was resampled.

Site characteristics and the spatial distribution of sites affected aquatic insect assemblages detected in 2015. Summer canopy cover was a significant predictor of assemblages ( $p < 0.01$ ). Impervious cover within 100 m and specific conductivity were marginally significant predictors ( $0.05 < p < 0.1$ ). Considerable variation in assemblages among sites was constrained by site characteristics and relatively little variation was constrained by Euclidean distance (28.2 and 7.0% of the variance, respectively). Canopy cover, conductivity, and impervious cover respectively accounted for 13.1, 4.9, and 4.7% of variation in CTW assemblages (46.5, 17.4, and 16.5%, respectively, of the constrained, non-spatial variance).

The first canonical axis ( $p = 0.001$ ), which explains 15.2% of the variation among assemblages (50.0% of constrained variance), is primarily described by summer canopy cover ( $r = -0.93$ ), and to a lesser degree by specific conductivity (positively,  $r = 0.26$ ) and impervious cover within 100 m (negatively,  $r = -0.20$  Fig. 4). We note that impervious cover and conductivity were positively correlated ( $r = 0.52$ ) despite the differences in the constraints they place on RDA1. The second pRDA axis ( $p = 0.029$ ), which explains 6.8% of the variance among assemblages (22.5% of constrained variance), was positively correlated with hydroperiod ( $r = 0.37$ ) and negatively with impervious cover within 100 m ( $r = -0.36$ ). Most (10/12) modeled insect taxa were positively associated with RDA1, and negatively associated with RDA2 (8/12 taxa; Figure D1). All vectors varied roughly linearly in ordination space except specific conductivity (Figure D1). Insect assemblages detected in 2015 did not form distinctly different clusters (Jaccard similarity value 0.52 – 0.64), and thus pools were not categorized by assemblage type in further analyses.

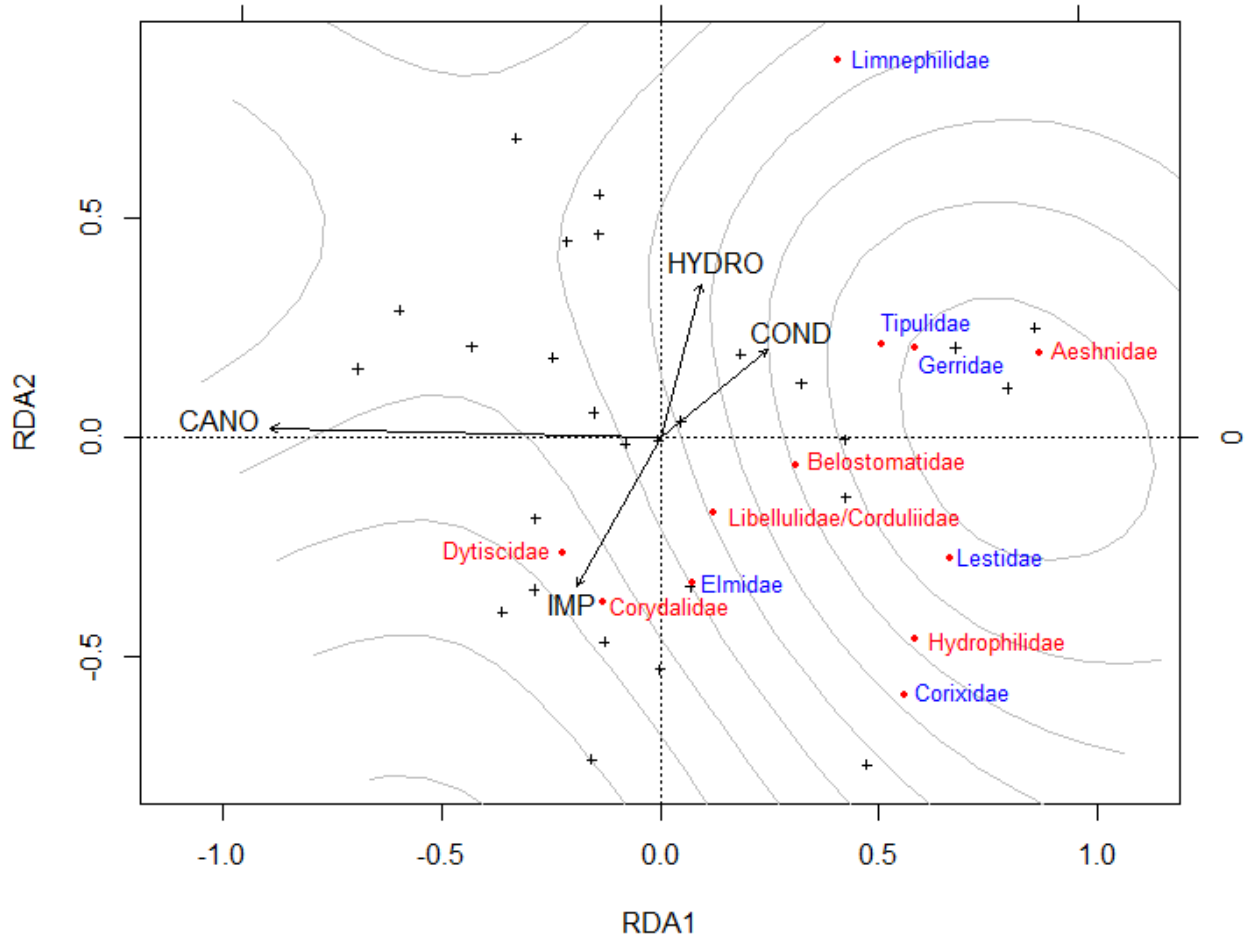


Figure D1 Partial redundancy analysis (pRDA) ordination for aquatic insect family detections at 27 vernal pools in Maine during 2014-2016. Sites are black crosses, red dots are observed taxa, and vectors represent site characteristics. Taxa labeled in red are categorized as likely predators of tadpoles and those labeled in blue are considered not likely to be predators. Vector labels are: CANO=mean density of summer tree canopy across years, HYDRO=mean hydroperiod, IMP=impervious cover within 100 m, and COND=mean specific conductivity. Constrained variance explained: RDA1=50.0%; RDA2=22.5%. Contours (gray) represent change in mean specific conductivity across ordination space.

Predatory taxa were detected throughout all pools and non-predatory taxa were detected in 24 of 27 pools included in the ordination. When examining all pools with predator density data in 2015 and 2016, predator density was predicted to decrease with longer duration of pool water (Figure D2, Table D2). Models of total predator density with other predictors (canopy, impervious cover, specific conductivity) did not rank higher than the null or have  $<\Delta 2AICc$ .

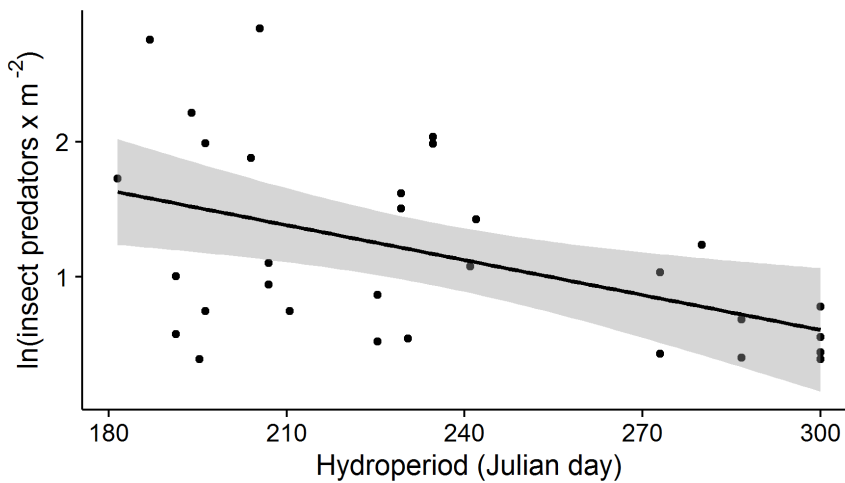


Figure D2 The density of insect predators ( $m^{-2}$ ) relative to the interannual mean pool hydroperiod. Each dot represents a site-year. The shaded area represents parameter 95% confidence intervals.

### D.3.1 Tail damage and depth and insect predators

Across 2015 and 2016, 46.7% of tadpoles surveyed for tail damage had a damaged tail (2,566 of 5,493 total individuals; 45.1% [2,020 / 4,479] in 2015; 53.8% [546 / 1,014] in 2016). Likelihood of tail damage increased with Julian day ( $\beta = 0.013$ ,  $SE = 0.0015$ , 90%  $CI = 0.012$ - $0.016$ ,  $P < 0.01$ ).

In pools where insect abundance was measured, 4,458 tadpoles were examined for tail damage and developmental phenology and morphology. Tadpoles were predicted to develop

faster in pools with greater densities of Hydrophilidae (Table D2). Tadpoles in pools with greater densities of Notonectidae were predicted to have greater body condition, shorter bodies, longer tails (if not damaged), and a greater likelihood of tail damage (Table D2). Greater densities of Dytiscid larvae were correlated with shorter tail fins (Table D2).

Table D2 Model ranking using only those models that ranked  $\leq 2 \Delta AICc$  and higher than the null model. Null models are included for reference. Observations are nested by Site (random effect) in all models except those total predator density. Direction of effect notes the covariate value for those covariates with a 85% CI that is different from zero.

	K	AICc	$\Delta AICc$	w	LL	Direction of effect
<i>Total predator density</i>						
Hydroperiod	3	64.32	0.00	0.87	-28.71	(-)
Null	2	69.97	5.66	0.05	-32.77	
<i>Likelihood of tail damage</i>						
Notonectidae density	4	5957.01	0.00	0.99	-2974.50	(+)
Null	3	5975.36	18.34	0.00	-2984.68	
<i>Developmental phenology</i>						
Hydrophilidae density	4	-58458.7	0.00	1.00	29233.35	(+)
Null	3	-58425.8	32.86	0.00	29215.91	
<i>Body condition</i>						
Notonectidae density	4	-9078.52	0.00	1.00	4543.27	(+)
Null	3	-9014.87	63.65	0.00	4510.44	
<i>Body length</i>						
Notonectidae density + Belostomatidae density	5	17837.43	0.00	0.62	-8913.71	(-), (-)
Notonectidae density	4	17839.43	2.00	0.23	-8915.71	(-)
Null	3	17875.49	38.05	0.00	-8934.74	
<i>Tail length</i>						
Notonectidae density + Sialidae density	5	15515.52	0.00	1.00	-7752.75	(+), (-)
Null	3	15599.96	84.44	0.00	-7796.98	
<i>Tail height</i>						
Dytiscid density	4	12261.93	0.00	1.00	-6126.96	(-)
Null	3	12290.87	28.94	0.00	-6142.43	

## **D.4 Discussion**

### **D.4.1 Insect assemblage composition and density**

Canopy cover strongly influenced the composition of aquatic insect assemblages in vernal pools across an urbanization gradient, with most families examined in the ordination (10 of 12) negatively correlated with canopy cover (Figure D1). The strong influence of canopy cover on insects in vernal pools provides further support for recent findings by Plenzler and Michaels (2015) that canopy cover may be a primary regulatory mechanism for invertebrate communities in ephemeral wetlands via food web pathways regulation. Our results also align with Relyea (2002), who observed the insect assemblage composition among closed canopy pools to be less variable than among open canopy pools and that Libellulidae, Aeshnidae, and Hydrophilidae occurred in greater densities in open canopy pools. Additionally, the family that we found to be most positively correlated with greater canopy cover, Dytiscidae, was noted to be more abundant in closed canopy pools by Relyea (2002). Impervious cover and road salt contamination may influence assemblages, but to a lesser degree than canopy cover. The relatively strong negative correlation of Limnephilidae with impervious cover, (the only Trichoptera family that was included in the assemblage ordination), suggests that water quality corresponding to impervious cover may exclude insects that are especially sensitive to water quality.

The lack of a strong influence of hydroperiod in structuring assemblages was unexpected given that water permanence is considered to limit the diversity of insects and drive the composition of these assemblages across temporary waters, including vernal pools (Williams 1996; Brooks 2000). However, another study of aquatic insect composition in temporary pools in

Ohio also did not detect an expected increase in family richness with longer hydroperiods (Plenzler and Michaels 2015).

Examination of families relative to the constraining environmental variable vectors in the assemblage ordination did not suggest an obvious pattern of response of predator and non-predator families (Figure D1). However, when grouping all insects in predatory families, we did detect a significant decrease of predatory insects in pools with a shorter hydroperiod (Figure D2). These results suggest that although hydroperiod may not predict the presence of any particular predatory family, in general, pools with shorter hydroperiods may have relatively high densities of individuals of whichever predatory families are present.

#### **D.4.2 Tadpole responses to insect density**

Contrary to previous findings that wood frog tadpoles have less tail damage later in the season (Blair and Wassersug 2000), in our pools tail damage within a pool was cumulative within a season, despite clear evidence of tail regeneration in tadpoles. This suggests that tadpoles in our study pools were under predatory pressure from insects throughout development and supports lab experiments demonstrating that larger insect predators can handle and some preferentially select larger tadpoles as prey (Brodie and Formanowicz 1983).

Our result that longer tadpole bodies were predicted at lower predator densities aligns with other studies demonstrating that insect predators can restrict tadpole body size. For example, the presence of Dyticidae, Belostomatidae, and Aeshnidae dragonflies can induce smaller body morphology and lower activity levels in wood frog tadpoles (Relyea 2003, 2004).



Advanced developmental phenology, greater body condition, and smaller tails with greater predator densities were unexpected patterns given past field and lab research on tadpole morphology responses to insect predators (e.g., Skelly and Werner 1990; Relyea 2003, 2004). Although wood frog tadpole morphology typically responds to the strongest predator in the presence of multiple predator families (Relyea 2003), it is possible that tadpole response to an entire assemblage of insect predators is different from the response to one to two predators. Additionally, because the number of tadpoles in our study was not controlled, removal of tadpoles by predators might have counterintuitive effects on the morphology of the surviving tadpoles (e.g., less competition for food in areas of the pool with relatively little predation pressure). The greater likelihood of tail damage with greater predator densities also supports the idea that greater predator densities may increase tadpole mortality and thus release remaining tadpoles from competition pressures. Tadpoles in our study could also access the entire pool, and thus some may have located areas within the pool where they could avoid predation pressure.

Our study provides evidence that canopy cover and hydrology may be driving forces for insect assemblage composition in vernal pools across an urbanizing landscape. We also provide a greater understanding of how insects in vernal pools may interact with larval amphibians, specifically wood frog. Our results suggest that increased predation pressure may, counterintuitively, result in increased size and condition of developing tadpoles. These findings suggest that additional studies of the relationship between predatory insects, larval survival, and the fitness of larvae that survive through metamorphosis will help to elucidate the effects of insect predation on wood frog populations.

## D.5 Supplemental Information

Table D3 Total individuals sampled during density sampling surveys by site and year. The number of site visits is “n”, and “Area” is mean pool area at spring high water (m<sup>2</sup>).

Site	Year	Coleoptera							Diptera			Hemiptera				Mega- loptera		Odonata			Trichoptera			n	Area
		Dytiscidae	Elmidae	Gyrinidae	Halplidae	Hydrophilidae	Noteridae	Scirtidae	Culicidae	Stratiomyidae	Tipulidae	Belostomatidae	Corixidae	Gerridae	Notonectidae	Corydalidae	Sialidae	Aeshnidae	Lestidae	Libellulidae/Cordulidae	Hydropsychidae	Limnephilidae	Rhyacophiliidae		
B06	2015	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	3	9	0	2	0	2	49	
B08	2015	5	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	9	0	1	0	3	146	
B10	2015	0	1	0	0	0	0	1	0	0	0	4	2	0	0	0	2	24	1	0	0	0	3	71	
B12	2015	2	0	0	0	0	0	0	0	6	0	0	0	0	2	0	5	2	13	0	0	0	3	839	
B13	2015	12	0	0	0	2	0	0	0	2	0	22	2	0	0	0	12	15	38	0	6	0	3	371	
B18	2015	10	3	0	0	3	0	0	0	5	0	13	0	0	0	0	0	3	27	0	3	0	3	285	
B18	2016	20	0	4	0	2	1	0	0	0	1	173	1	1	0	0	0	37	20	0	0	0	7	89	
B23	2015	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	3	39	
B25	2015	20	0	0	0	1	0	0	0	6	0	48	1	0	0	0	6	11	62	0	9	0	3	676	
H01	2015	11	0	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	7	1	0	0	3	184	
H01	2016	30	0	0	0	4	2	0	0	0	0	3	1	0	1	0	0	0	0	0	0	2	5	195	
H02	2015	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	20	0	0	0	3	225	

Table D3, continued

Site	Year	Coleoptera							Diptera			Hemiptera			Mega- loptera		Odonata			Trichoptera			n	Area	
		Dytiscidae	Elmidae	Gyrinidae	Halipidae	Hydrophilidae	Noteridae	Scirtidae	Culicidae	Stratiomyidae	Tipulidae	Belostomatidae	Corixidae	Gerridae	Notonectidae	Corydalidae	Sialidae	Aeshnidae	Lestidae	Libellulidae/Cordulidae	Hydropsychidae	Limnephilidae			Rhyacophiliidae
H02	2016	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	69
OR01	2015	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	5	0	2	152	
OR11	2015	7	0	0	0	0	0	0	0	0	0	3	0	0	0	0	1	4	4	0	12	0	3	326	
OR11	2016	39	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	14	11	0	1	0	6	305	
OR12	2015	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	14	0	0	0	2	10	
OR16	2015	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	3	0	3	164	
OR16	2016	7	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	3	0	0	0	3	223	
OR17	2015	6	0	0	0	2	0	0	0	0	5	0	1	0	0	5	0	4	0	17	0	18	0	3	316
OR23	2015	9	0	0	0	0	0	0	0	0	0	2	0	0	6	0	0	0	1	0	0	0	3	248	
OR25	2015	12	0	0	0	1	0	0	0	0	0	1	13	0	0	2	0	1	5	208	0	0	0	2	413
OR26	2015	10	0	0	0	0	0	0	0	0	0	0	0	0	6	0	4	0	2	0	21	0	3	237	
OR26	2016	9	0	0	0	2	0	0	0	0	0	0	3	0	1	0	2	0	0	0	6	0	6	264	
OR27	2015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	1	88	
OT05	2015	9	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	6	0	0	0	3	17	
OT05	2016	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	24	
OT06	2015	5	0	0	0	0	0	0	0	0	0	9	0	0	4	0	0	2	27	0	0	0	3	106	

Table D3, continued

Site	Year	Coleoptera							Diptera			Hemiptera				Mega- loptera		Odonata			Trichoptera			n	Area
		Dytiscidae	Elmidae	Gyrinidae	Halplidae	Hydrophilidae	Noteridae	Scirtidae	Culicidae	Stratiomyidae	Tipulidae	Belostomatidae	Corixidae	Gerridae	Notonectidae	Corydalidae	Sialidae	Aeshnidae	Lestidae	Libellulidae/Cordulidae	Hydropsychidae	Limnephilidae	Rhyacophiliidae		
OT06	2016	38	0	0	0	0	2	0	0	0	0	0	0	0	1	1	2	0	15	0	0	0	6	125	
OT10	2016	4	0	0	0	0	3	0	0	0	0	0	0	3	0	2	0	2	0	0	0	0	3	427	
OT12	2016	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	111	

## **APPENDIX E: EXAMINATION OF THE SEASONALITY OF *RANAVIRUS* (RV) INFECTION IN WOOD FROG LARVAE**

### **E.1 Introduction**

There is mounting evidence that ranavirus (Rv), an emerging viral pathogen, contributes to substantial amphibian population declines globally and can result in high mortality rates within populations (Lesbarreres et al. 2012; Gray and Chinchar 2015). Rv infection in amphibians has been detected on five continents and in every state within the continental US (Duffus et al., 2015; USGS NWHC, 2015). Even when mortality is <100%, the resultant population decline can exacerbate the risk of extinction from other stressors, such as habitat loss and degradation (Earl and Gray 2014; Brunner et al. 2015) or presence of other pathogens (Hoverman et al. 2012b; Landsberg et al. 2013). The consequences of this disease may be underrated because study of its ecosystem consequences and interactions have begun relatively recently (Chinchar et al. 2009). Although great strides have been made to understand the ecology, etiology, epidemiology, and other aspects of the disease, there continues to be a lack of information about how environmental factors contribute to mortality, incidence of illness, and timing of infection in populations (Lesbarreres et al. 2012).

Wood frog (*Lithobates sylvaticus*) tadpoles are especially susceptible to Rv infection (Hoverman et al. 2011) and observations of cohort die-offs in vernal pools are not uncommon throughout the Northeastern US (e.g., Brunner et al., 2011; Gahl & Calhoun, 2010; Wheelwright, Gray, Hill, & Miller, 2014). Loss of wood frogs in vernal pools due to unchecked Rv infections could degrade food web structure and resilience in these pools as tadpoles have important roles as consumers and prey (Duellman and Trueb 1994; Schiesari et al. 2009; Brodie and

Formanowicz 1983; Skelly 1997). Despite that wood frogs are prolific breeders and can thus absorb high mortality rates, they are also short-lived and have high breeding site fidelity (Berven 2009). This confluence of factors makes it so that consecutive years of die-offs within a pool greatly increase the probability of extinction of a breeding population. Moreover, the loss of wood frogs from vernal pools would likely alter these ecosystems in ways not yet understood (e.g., biogeochemically, food web contributions, the cascade of effects resulting from frogs transferring nutrients between aquatic and terrestrial systems; Gibbons et al. 2006; Capps et al. 2015). The potential consequences of Rv infection for wood frogs are especially concerning because we cannot know the risk to vernal pool ecosystems without information about which site conditions increase tadpole susceptibility and mortality. Surveillance studies that intensively monitor Rv infection in tadpole populations are needed to better understand the population consequences of Rv infection (Gray, Brunner, Earl, & Ariel, 2015).

During a preliminary investigation of Rv infection in 2014-15, we conducted wood frog tadpole surveys targeted at measuring growth and development and observed clinical signs consistent with Rv infection (hemorrhaging, lethargy, bloating) in 24 and 22 pools and die-offs in 1 and 4 pools in 2014 and 2015, respectively. We collected and sent a small subset of tadpole samples from 7 pools (years combined) to the USGS National Wildlife Health Center (NWHC) for lab analysis. Although virus culture rates were low (due to a logistical issue), samples from 3 pools tested positive for Rv, including 2 of the 5 pools with die-offs. The NWHC also suspected Rv infection in those samples that did not test positive but in which clinical signs of Rv were observed. If all tadpoles that had clinical signs of Rv were actually infected, 13 pools had tadpoles that were infected with Rv for at least two consecutive years, however, did not necessarily experience complete die-offs. Additionally, pools that had infected individuals are in

a variety of settings, ranging from suburban lawns to managed forests, and the timing of observed clinical signs and die-offs did not appear to be synchronous across pools. The disparate infection timing, prevalence (proportion of infection within a population), and mortality rates among pools supports the idea that site characteristics contribute to the response of a population to Rv infection. Because Rv has been observed to occur in pools for consecutive years (Hoverman et al. 2012a, personal observation), the known occurrence of Rv in these pools provided an opportunity to study its effects on wood frog tadpoles.

In 2016, we conducted a surveillance study to describe seasonal changes in (1) prevalence of Rv-infected tadpoles, (2) viral load in tadpoles, and (3) the waterborne Rv concentration in vernal pools. We also aimed to provide insights into how pool characteristics that change throughout the season may influence these responses. We also considered the effects of larval development. Additionally, we examined how prevalence and timing of infection was related to pool-wide tadpole die-offs and how tadpole Rv infection and development varied with concentrations of Rv DNA in pool water throughout the season.

## **E.2 Methods**

### **E.2.1 Tadpole sampling**

We surveyed wood frog tadpoles in 10 vernal pools in the Greater Bangor, Maine, area between 19 May and 13 September 2016 for Rv infection. We conducted surveys approximately weekly (4-17 days between visits, mean = 7.1 days, median = 7 days; 17 day gap only occurred once at the beginning of the season, and the next longest time between surveys was 11 days) surveys starting shortly after hatching and continued until metamorphosis or a die-off occurred.

During dip netting surveys, we captured up to 26 tadpoles from each pool and conducted larval examinations, noting developmental Gosner (1960) stage and measuring mass and body length. Although not necessarily indicative of Rv infection, we also noted any symptoms consistent with Rv infection. We calculated an index of relative condition (hereafter referred to as condition, Cond) as the residuals of square root-transformed mass (g) regressed on SVL and developmental stage. Condition is used to indicate amount of fats and other energetic reserves relative to body size (reviewed by Green 2001). Field equipment that came in contact with pool water or animals was disinfected at least 100 m from pools using 0.75-2% Nolvasan® S (chlorhexidine diacetate; (Bryan et al. 2009) after visiting each site.

Tadpoles in five of these pools were also monitored for Rv infection using quantitative polymerase chain reaction (qPCR). These pools (hereto referred to as “tadpole collection pools”) were selected for this more intensive surveillance because Rv had been detected or a die-off suggestive of Rv infection occurred in 2014 and/or 2015. When  $\geq 5$  tadpoles were captured, up to 15 tadpoles were euthanized by submersion in an aqueous 10g/L solution of MS-222 (tricaine methane sulfonate) for organ extractions. Our target sample size for genetic analyses was 15 tadpoles because in pools with 4,300-21,000 tadpoles (early spring estimated maximum population sizes in these 5 pools based on egg mass counts and clutch size estimates in 2014-15; See Chapters 2 and 4 for methods egg and embryo count methods) this sample size would likely detect high-prevalence Rv infection ( $\geq 20\%$  infection rate; sample size recommendations in Gray et al. 2015). We collected tadpoles for qPCR testing in one of the other five pools during a visit when symptoms of Rv were detected in tadpoles.



Tadpoles were placed on ice and immediately transferred to the lab where liver and kidneys were extracted and frozen (-80 C) until shipped to the University of Tennessee Rv Lab (under direction of M. Gray) for qPCR to detect and quantify Rv (viral load) in individuals. Each sample was run in duplicate, and samples without amplification were considered negative. We prioritized sample testing so that those collected at the last sampling date were tested first, followed by those collected from sequentially earlier dates of sampling until Rv was not detected in any tadpoles sampled from a visit. This method allowed us to archive samples from the beginning of the season and only test samples if ranavirus infection was detected in samples from later weeks, thus minimizing the number of samples that needed to be analyzed.

### **E.2.2 Pool water sampling (eDNA)**

Water sampling was conducted to determine the Rv titer in pool water. This non-invasively sampling technique has been validated to be effective for detecting Rv presence in wood frog tadpole populations, with concentrations of Rv in pool water positively correlated with viral load in tadpoles (Hall et al. 2016). Despite the benefits of this technique, we note that this technique is complementary to the Rv testing of individual tadpoles as it does not indicate the prevalence of infection within a population, does not specifically identify which host(s) contribute eDNA, and the diagnostic specificity (true negative rate) has not yet been estimated for this technique (Hall et al. 2016).

We collected water once every 1-2 weeks from 10 pools between 9 June and 9 August 2016. Three 250 ml samples were collected ~120 degrees apart on each pool shore; samples were taken from the edge of each pool prior to entering pools for tadpole capture. At each pool visit, water samples were either immediately filtered or put on ice, transferred to the lab, kept under

refrigeration, and filtered within 24 hours. We filtered 200 - 250 ml from each water sample using a vacuum pump onto one or two 0.45  $\mu\text{m}$  Cellulose nitrate filter funnels (Fisher scientific: CAT#1452045). Directly after filtering a round of pool water, a corresponding field- or lab-negative sample was processed using 250 ml of store-bought bottled water (Nestle). Filtration equipment was disinfected using 10% bleach solution after the processing each water sample. eDNA samples were placed in paper coin envelopes and then sealed into a snack bag with at least 10 ml of indicating silica, stored at room temperature, and shipped for DNA extraction and qPCR at Washington State University within 5 months of collection. During storage, we replaced silica beads if color change indicated the presence of excess moisture. Water sampling was conducted in cooperation with a separate study by E. Hall that focused on linking road-related disturbance with Rv die-offs in wood frog larvae using eDNA techniques.

### **E.2.3 Sampling site characteristics**

We measured water depth using a pole marked in centimeter increments and water probes (Hach ©, Loveland, Colorado) to sample specific conductance (indicating salinity), dissolved oxygen (DO), pH, and water temperature. On each date a pool was sampled, we collected and tested 1 L of surface water ~1 m from the water edge at each of three equidistant points around the perimeter. All testing was conducted at the pool edge within minutes of sample collection. All metrics except depth were averaged by day to calculate values used in analyses.

### **E.2.4 Statistical analysis**

All statistical analyses were completed using program R (R Core Team 2016). For analyses of relationships between eDNA and site and tadpole morphology characteristics we

assigned each measurement to a “Visit” based on the date of observation. Because eDNA sampling was often not conducted on the same day that site characteristics and tadpoles were measured, we associated measurements by “Visit”.

We examined the effect of site characteristics and tadpole morphology metrics on prevalence of tadpole infection in tadpole collection pools and on eDNA titer in all ten pools. We used a two-step model selection process to identify which predictors within each predictor category (site characteristics, larval morphology) were likely influential and then compare the relative influence among those variables. First we created a set of models for each response using R package ‘nlme’ (Pinheiro et al. 2017) for logistic regression for the probability of infection (prevalence) and using package ‘lme4’ (Bates et al. 2017) for eDNA titers. Because it is likely that prevalence and eDNA titer do not respond linearly to site or tadpole morphology characteristics, we considered both linear and quadratic models. Examination of correlation plots indicated possible quadratic relationships between eDNA titer and site and tadpole morphology characteristics. For prevalence, we created multivariate linear model with all single-order site characteristic terms and a separate model with all single-order tadpole morphology terms. Examination of the residuals did not indicate a quadratic relationship between prevalence and predictors. Thus, each set of models included all univariate linear models and model sets for eDNA titer also included a two-term model with the corresponding quadratic term added to a univariate model. We nested observations by site (Site as a Random effect) to account for the lack of independence among observations within a site. We grouped models of each response by predictor category and selected the top models for a response within each category. We used library ‘AICcmodavg’ (Mazerolle 2017) to rank models using Akaike’s information criterion adjusted for small sample size (AICc). We considered models  $\Delta AICc < 2$  that ranked above the

null model to be plausible (Burnham and Anderson 2002). Secondly, for each response, we compared all plausible models across predictor categories to determine the relative importance of predictors. We examined the 85% confidence intervals (Arnold 2010) of each covariate in this final set of models (i.e., that ranked above the null model and had  $\Delta AIC_c < 2$  within its respective predictor category) to determine effect.

### **E.3 Results**

We sampled 1,059 tadpoles in all 10 pools where pool water titers were sampled, with 546 in tadpole collection pools. We conducted qPCR analyses for 197 tadpoles at tadpole collection pools and an additional 15 tadpoles at one non-tadpole collection pool where tadpoles with symptoms consistent with Rv were detected, for a total of 212 tadpoles at 6 pools.

#### **E.3.1 Die-offs in pools with tadpole collection**

Apparent die-offs in two pools (H02, OT10) likely involved Rv. In these pools leading up to die-offs, the detected prevalence of Rv increased up to 75-100% and the viral titer in pool water was double that of the three other tadpole collection pools. Moreover, in H02, we never detected tadpoles in the late stages of metamorphosis (most advanced developmental Gosner stage detected = 34; hind limb developed to foot paddle stage, but no digit separation), suggesting that tadpoles did not complete metamorphosis. In OT10 we detected one tadpole as developmentally advanced as Gosner stage 40 one week prior to the last date of detection, thus it is possible that some tadpoles completed metamorphosis between these site visits. However, the maximum developmental stage detected during the last site visit when 15 tadpoles (our sampling target) were detected (29 June) was Gosner stage 37 (minimum = 31, median = 33), suggesting

that declines in tadpole detection at subsequent visits were not caused by emergence from pools. On the last date tadpoles were detected in OT10, we also detected four larval *Ambystoma* spp. which had symptoms consistent with Rv (hemorrhaging, mortality, swelling).

A die-off likely occurred in one other pool (OT12) as tadpoles were unexpectedly not detected despite tadpoles not yet nearing completion of metamorphosis. On the last two dates of detection at OT12 tadpoles were between Gosner stages 28 – 32 (Figure E1a). Although Rv was detected in pool water, it is unlikely that this die-off was primarily precipitated by Rv since Rv was not detected in any of the 16 tadpoles tested on the last two dates when tadpoles were detected in OT12. Although a tadpole in OT06 had our highest viral load of all tadpoles sampled, there is no evidence of a die-off at this pool as prevalence was low (1 of 15 tadpoles sampled) on the date tested tadpoles were collected, none of the 15 tadpoles sampled on the previous date were Rv-positive, and 15 tadpoles (our sampling target) were detected within a week of pool drying.

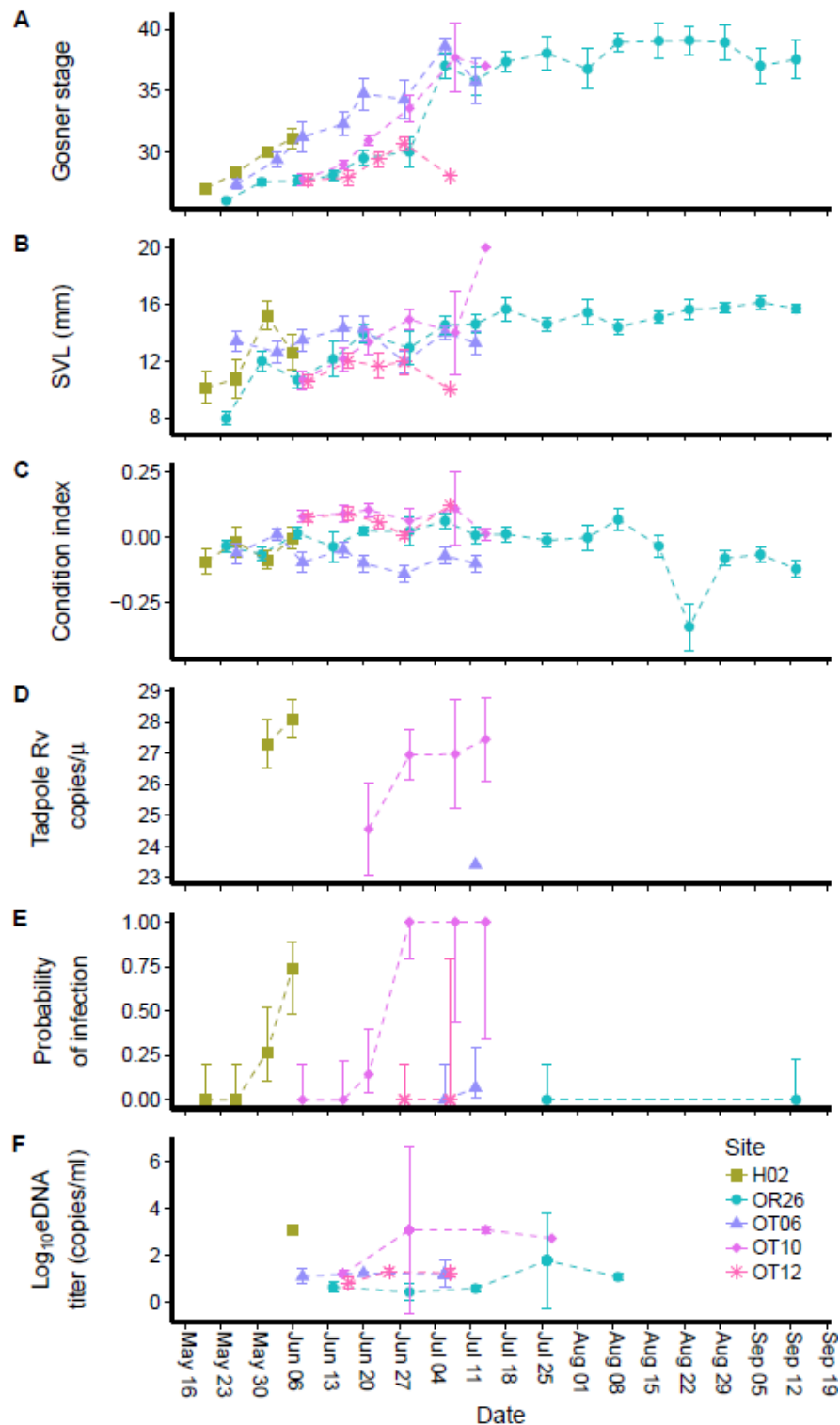


Figure E1 Seasonal trends in mean tadpole developmental stage (A), SVL (B), body condition (C), and Ranavirus (Rv; (D)) titer in tadpoles as well as the probability of infection (E) and the Rv titer in pool water (F) in 5 pools in 2016. Error bars represent 95% CIs. Tadpole titer was calculated for the subset of tadpoles that tested positive for Rv, only. All dates with larval Rv titer samples are reflected in (E).

### **E.3.2 Seasonal trends associated with Rv detection in tadpoles**

At both pools with suspected Rv-involved die-offs (H02 and OT10), tadpoles were Rv-positive at two and four sequential samplings, respectively, with increasing prevalence but decreasing viral titer in tissue (Figures E1d-e). In both pools, Rv-infected tadpoles were detected at approximately the same developmental stage (H02, mean Gosner stage = 30.0, 95% CI = 26.6-30.4; OT10, mean = 30.9, 95% CI = 30.5-31.3), and leading up to die-offs viral load in tadpoles decreased and prevalence increased (Figures E1a, d, and e). Both pools also had the highest eDNA concentration of the five pools where larvae were routinely sampled for Rv (Figure E1f). The progression of infection and prevalence throughout the season differed between the two pools with detection of Rv in tadpoles at H02 approximately three weeks earlier than at OT10 (Figures E1d-e). The die-off at H02 also appeared to progress more quickly, as we were not able to detect tadpoles at H02 within two weeks of the first Rv detection, but at OT10 tadpoles were detected until approximately one month after the first Rv detection. The number of tadpoles detected during and after the visit when Rv was detected declined slowly at OT10 (on 29 June, 15 of 15 tadpoles sampled; on 8 July, 3 of 3 tadpoles, 14 July, 2 of 2 sampled) compared to H02 where 15 tadpoles were detected during each of the two last visits. Tadpoles in H02 developed faster than in OT10 (progressed to a later Gosner stage at an earlier date; Figure E1a), and the die-off in H02 occurred earlier in the season than in OT10.

Of the five non-tadpole collection pools, the only one that had notably higher specific conductivity and a lower pH than the others (H02) also likely had a Rv-related die-off (Figures E2a and c). The one pool that had a consistently lower pH than the others (OT12) had a die-off that was not likely Rv-related (Figure E2c). No Rv-infected tadpoles were detected in the one pool that was notably deeper than the others (OR26), but Rv was detected in pool water in this

pool (Figures E2a and d). Tadpole morphology, DO, and water temperature did not vary appreciably among these five pools throughout the season (Figures E1a-c, E2b, and E3b).

Prevalence of infection was predicted to increase with pH and developmental stage, with pH as the predictor in the top-ranking model (Tables E1-E2). The probability of infection in a “typical” pool was predicted to increase notably after approximately pH 6.0 or Gosner stage 35, however, there was considerable among-pool variation (Figure E4).



Table E1 Model ranking using only those models that ranked  $<2 \Delta AICc$  in their respective predictor category (site characteristics and larval morphology) and that ranked higher than the null model. Null models (NM) are included for reference. Observations are nested by Site (random effect) in all models. Quadratic models included linear and squared covariate terms.

	K	AICc	$\Delta AICc$	w	LL
<i>Prevalence</i>					
pH	3	86.41	0	1	-40.11
Stage	3	133.25	46.84	0	-63.57
Null	2	178.58	92.17	0	-87.26
<i>eDNA</i>					
DO + DO <sup>2</sup>	5	557.5	0	1	-273.66
Stage	4	999.18	441.68	0	-495.54
Null	3	1013.62	456.12	0	-503.78

Table E2 Estimates, standard errors, and 85% confidence intervals (CIs) of covariates of eDNA and detected prevalence of Rv infection for models with  $<2\Delta AICc$  within their respective predictor category and that rank above null models. Covariates are listed in order of AICc of their respective model. Quadratic models included linear and squared covariate terms.

	$\beta$ estimate	SE	Lower CI	Upper CI
<i>Prevalence</i>				
pH	21.7	5.86	13.7	30.9
Stage	0.707	0.143	0.518	0.930
<i>eDNA</i>				
DO	-0.574	0.134	-0.767	-0.381
DO <sup>2</sup>	0.0578	0.0153	0.0358	0.0799
Stage	0.0527	0.0129	0.0342	0.0712

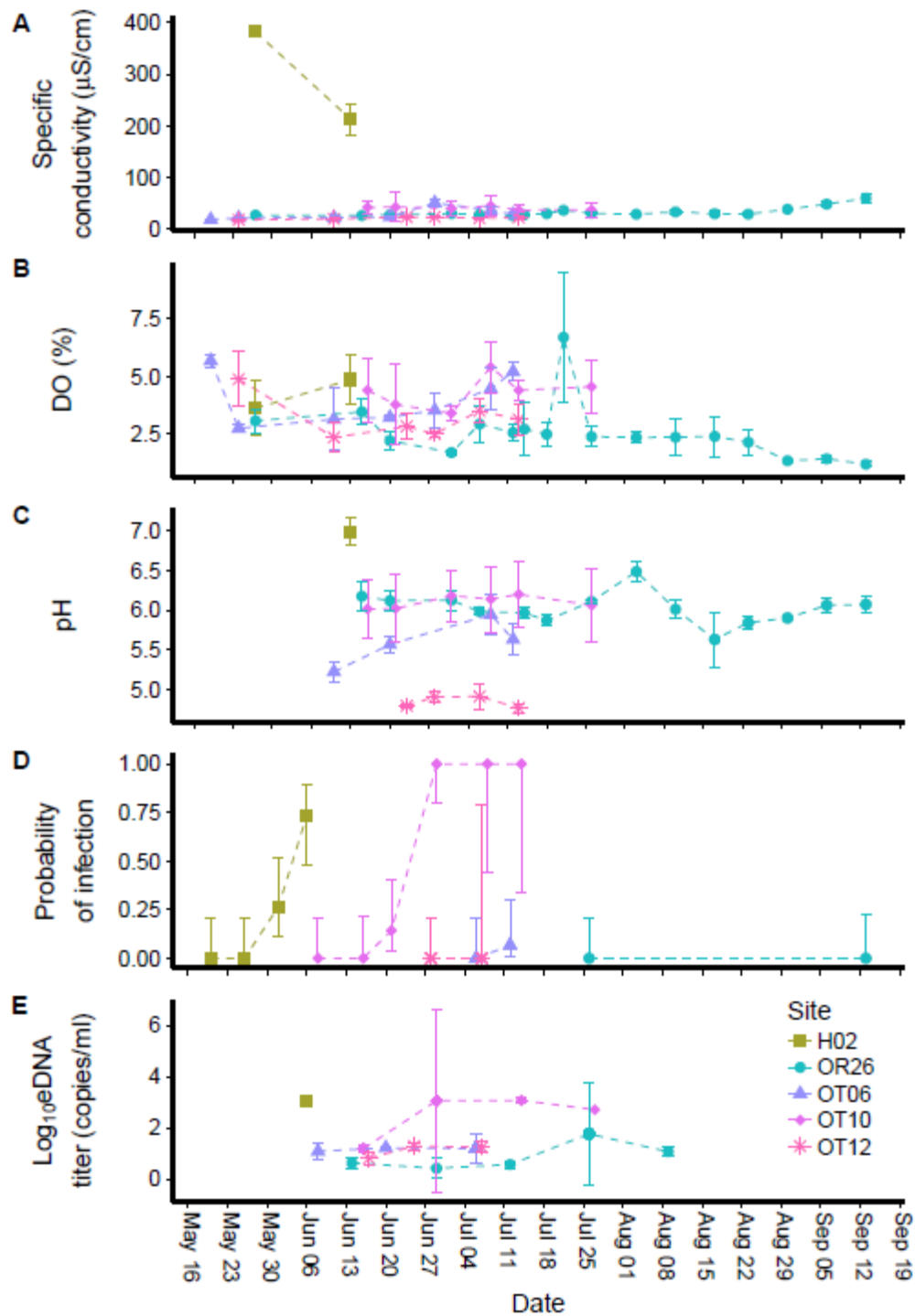


Figure E2 Seasonal trends in the mean specific conductivity (A), DO (B), and pH (C) in 5 pools in 2016. The probability of infection (D) and the Rv titer in pool water (E) are duplicated from Figure E1 for comparison. Error bars represent 95% CIs.

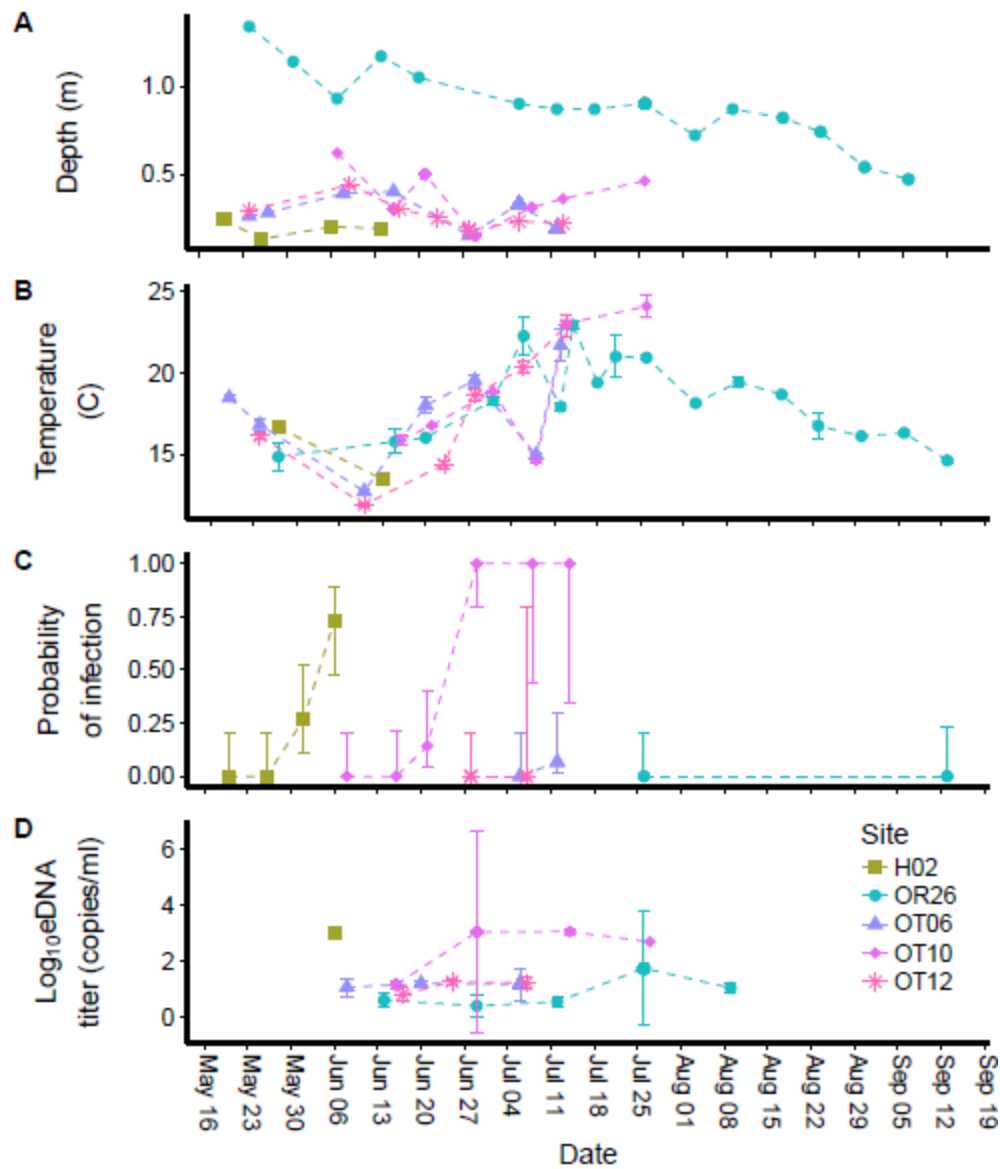


Figure E3 Seasonal trends in the mean pool depth (A) and temperature (B) in 5 vernal pools in 2016. The probability of infection (D) and the Rv titer in pool water (E) are duplicated from Figure E1 for comparison. Error bars represent 95% CIs.

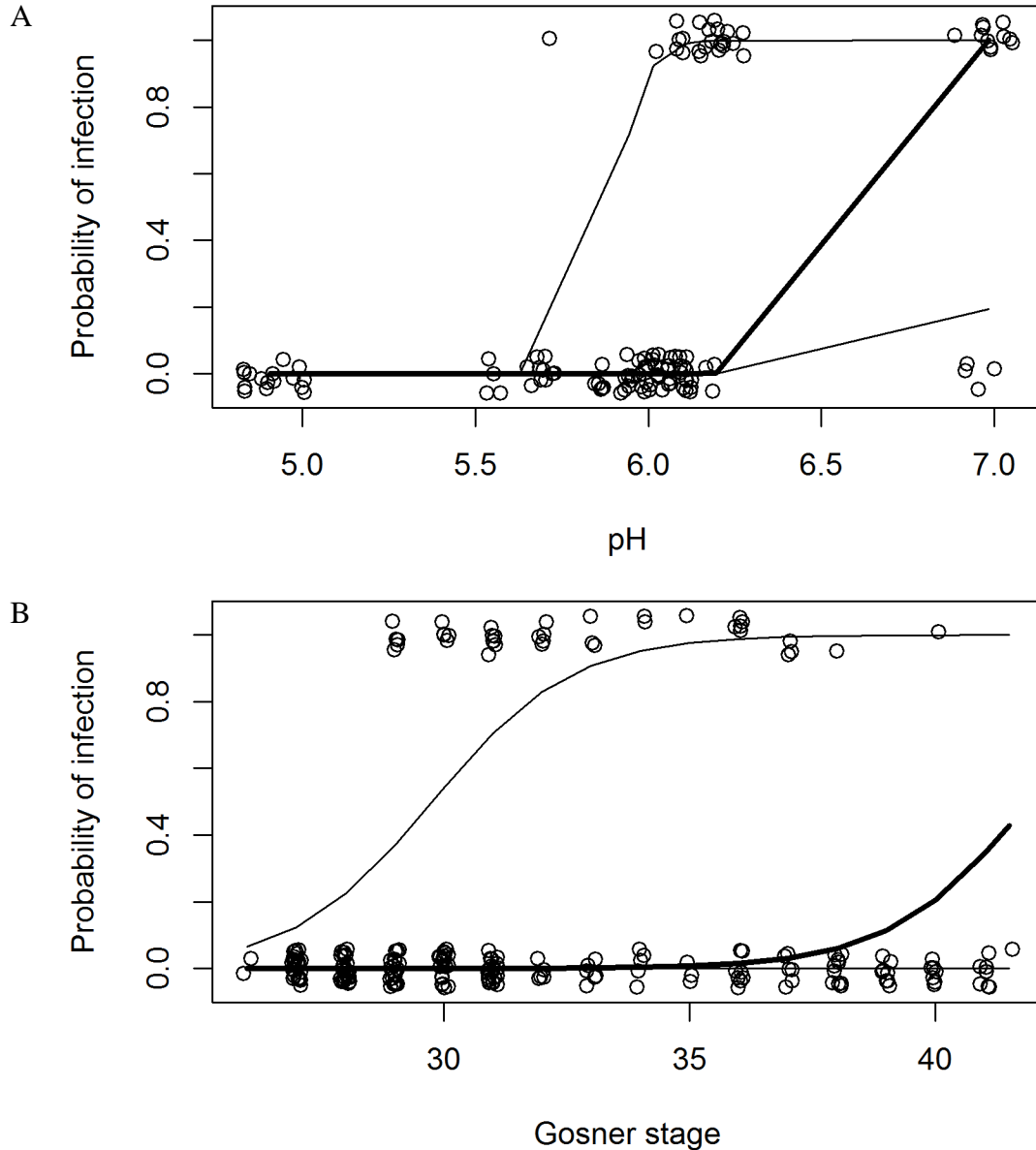


Figure E4 Predicted probabilities of Ranavirus detection in wood frog tadpoles corresponding to pool pH (A) and developmental (Gosner) stage (B) for five vernal pools. The thick line in the middle represents the predicted values across all pools, and the thin lines represent the extremes of the location of 85% of pools' logistic curves. Open circles represent individuals. Points are 'jittered' to show all individuals, but infection values for each individual is 0 or 1.

### **E.3.3 Rv detection and die-offs in pools with eDNA sampling**

We detected Rv in all pools during at least one visit, and the highest titers ( $>10^4$  copies  $\times$   $\text{ml}^{-1}$ ) were detected between mid-June and mid-July in two pools (Figure E5). Approximately a week prior to this time (8 June), we detected symptomatic tadpoles in OR16, the one of these two non-tadpole collection pools, and tested 15 individuals but Rv was not detected in any of these tadpoles. This pool and another pool without tadpole collection (B18 and OR16) had higher eDNA titers than H02 or OT10 where die-offs likely involved Rv (as confirmed by Rv detection in tadpoles; Figure E5). At both pools we detected tadpoles in the late stages of metamorphosis ( $\geq 41$  Gosner stage, front arms emerge within 24 hours), suggesting that some tadpoles in each pool completed metamorphosis. Although Rv was not detected in tadpoles collected on 8 June from OR16, we suspect a relatively slow die-off occurred in there as the number of detected tadpoles decreased from 15 (our sampling target) on 28 June to one tadpole on 20 July and the pool never dried. In contrast, there is no evidence that a die-off occurred at B18 as 14 tadpoles were detected on the last date of sampling (12 July) and the pool dried within the following week. There is no evidence of die-offs in the other three pools without tadpole collection (H01, OR11, OT05) as all three pools had tadpoles detected within the week prior to drying, no notable declines in the number of detectable tadpoles from week-to-week with the exception of samplings within a week of drying (5-15 tadpoles detected), and eDNA titers in these pools were  $<0.05$  copies  $\times$   $\text{ml}^{-1}$ .

In one pools with a likely Rv-involved die-off, OT10, eDNA titers remained relatively high during visits with high prevalence detection in tadpoles and approximately two weeks after the last tadpole detections. However, eDNA titers were substantially lower in another pool with a

likely Rv-involved die-off, OR16, near the time of die-off than had been measured two weeks earlier.

DO and developmental stage both had statistical effects ( $2\Delta AIC_c$  within each predictor category and with 85% CI excluding zero) on eDNA titer (Tables E1-E2). Pools with tadpoles at later developmental stages were predicted to have higher eDNA titers (Table E2 and Figure E6b). However, there was substantial variation in predicted values among pools (Figure E6).

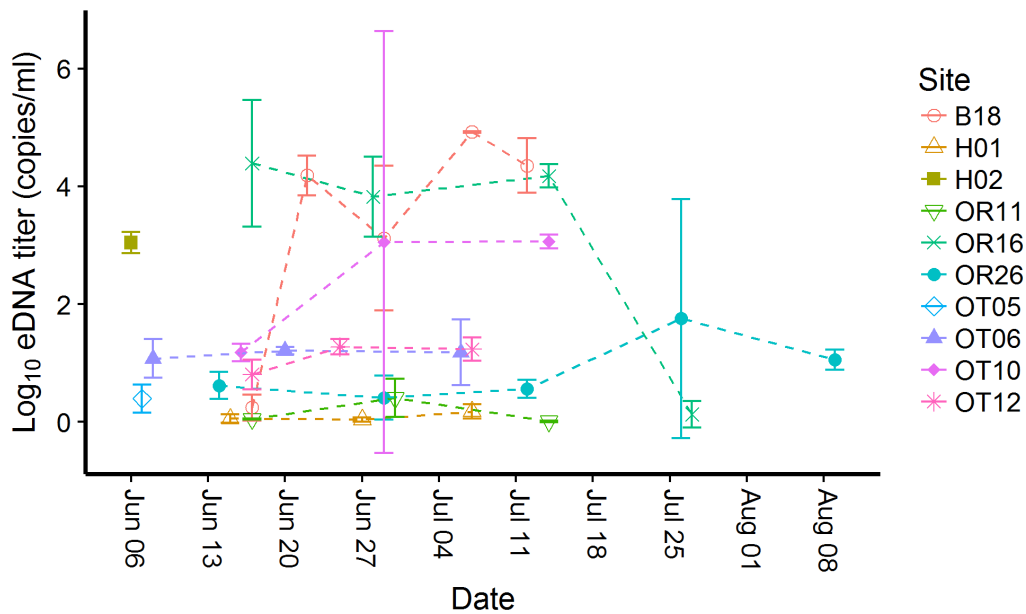


Figure E5 Seasonal trends in the mean Rv titer in pool water. Error bars represent 95% CIs.

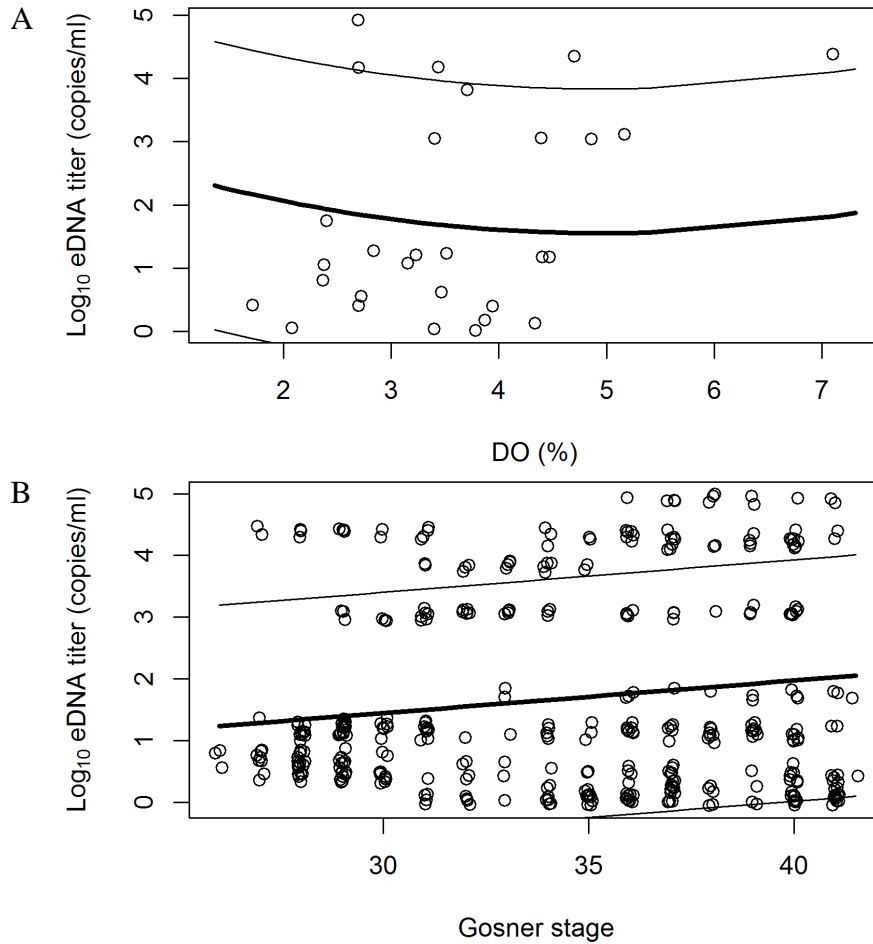


Figure E6 Predicted eDNA titer of Ranavirus corresponding to DO (A) and wood frog tadpole developmental (Gosner) stage (B) in ten vernal pools. The thick line in the middle represents the predicted values across all pools, and the thin lines represent the extremes of the location of 85% of pools' predicted curves. Open circles represent pool-visits in (A) and individuals in (B). Points in (B) are 'jittered' to show all individuals.

## E.4 Discussion

We detected the presence of Rv in pool water across all study pools but only detected tadpole declines and other evidence of Rv-related die-offs at three pools. Although PCR cannot determine if the detected virus is active (Miller et al. 2015), this suggests that Rv may be ubiquitous within our study area but that susceptibility may differ among pools. This contributes to a growing body of evidence that multiple factors, including physical and chemical site characteristics as well as anthropogenic stressors, may interact and effect amphibian susceptibility to Rv (Gray et al. 2009; Gahl and Calhoun 2010). Greater probability of infection in tadpoles in pools with higher pH (Table E2, Figure E4a) aligns with a lesser likelihood of infection of wood frog and spring peeper (*Pseudacris crucifer*) tadpoles in pools with lower pH (pH <4.5) observed by (Gahl and Calhoun 2010). In contrast to their finding that lower specific conductivity predicted a higher incidence of Rv die-offs and that Rv die-offs were not detected at pools with <60  $\mu$ S, one of our three pools that likely had a Rv die-off had the highest specific conductivity within the tadpole collection pools (approximately 200-400  $\mu$ S). However other studies have noted Rv infection of larval amphibians in pools with pH 7.35 (Bullfrog, *Lithobates catesbeianus*; Landsberg et al. 2013). DO predicted eDNA titer in our study pools, but interpreting the effect of this is difficult given the relatively small range of DO represented by our study pools and the quadratic shape of the relationship indicating the lowest predicted eDNA titer near 5% DO.

We detected a greater likelihood of infection and eDNA titer with developmental stages nearing metamorphosis (Figures E3b and E5b). These results support other studies providing evidence that susceptibility increases with tadpole developmental stage. Warne et al. (2011) observed wood frog tadpoles infected with Rv at developmental stages nearing metamorphosis to



have a greater likelihood of death, and die-offs typically observed at developmental stages near completion of metamorphosis in wood frogs and other amphibians (Green et al. 2002; Greer et al. 2005; Gahl and Calhoun 2010).

The apparent widespread occurrence throughout our study site in relatively undisturbed sites as well as at pools within 10 meters of roads, emphasizes the potential impact Rv may have on vernal pool-breeding amphibian populations. Additionally, as Rv titers in pool water and tadpoles were better predicted by pool chemistry than by tadpole developmental measures, it is possible that Rv infection and die-offs respond more strongly to pool physical characteristics than developmental stage.

## APPENDIX F: DETECTION OF *ICHTHYOPHONUS* IN WOOD FROG AND AQUATIC INSECTS

### F.1 Introduction

*Ichthyophonus* sp. infections leading to morbidity and mortality have been detected in multiple species of larval and adult frogs and salamanders from vernal pools and other wetland types throughout the eastern US (Mikaelian et al. 2000; Green et al. 2002; USGS National Wildlife Health Center (NWHC) 2009). *Ichthyophonus*-attributed wood frog (*Lithobates sylvaticus*) larvae die-offs in vernal pools have been reported many times including twice in Maine (Gahl and Calhoun 2008; Glenney et al. 2010). *Ichthyophonus* is currently thought to be endemic to amphibian populations, generally resulting in more cases of morbidity than mortality within a population (Green et al. 2002). However, whole cohort die-offs in multiple amphibians have also been attributed to *Ichthyophonus* and this is troubling as there has been little research on population effects (Green et al. 2002). Additionally, not much is known much about site conditions that may correlate with *Ichthyophonus* infections and/or die-offs in amphibians (David Green, personal communication, 20 November 2015). It is also unknown how this organism may be spread among amphibians, whether it persists in the environment, whether there are unidentified infective stages, and whether there are intermediate hosts (NWHC report, 30 November 2015).

*Ichthyophonus* can infect wood frog larvae in landscapes where Ranavirus – a lethal pathogen of amphibians – is also present (Gahl and Calhoun 2008; Glenney et al. 2010). *Ichthyophonus* and Ranavirus can co-occur in pools within an amphibian species (red-spotted newts, *Notophthalmus viridescens*; Glenney et al. 2010). Because symptoms of *Ichthyophonus*

overlap with those associated with Ranavirus infection in tadpoles (swelling in the thigh, rump, and tail; Densmore and Green 2007), it is possible that infections and die-offs attributed to Ranavirus may be facilitated or caused by Ichthyophonus. In 2015 and 2016, we surveyed wood frog tadpoles across an urbanizing landscape to examine which diseases might infect wood frog tadpoles and how this might vary with urbanization. This survey was not disease-specific and thus not designed to target Ichthyophonus. Because *Batrachochytrium dendrobatidis* (Bd) and tadpoles with symptoms consistent with Ranavirus had been detected within the study area prior to our study (Longcore et al. 2007; personal observation; Appendix E), we expected that some symptoms, such as swelling, lethargy, or emaciation, or die-offs to most likely be caused by these diseases.

## **F.2 Methods Field observations and USGS National Wildlife Health Center examination**

We conducted health surveillance surveys of larval wood frogs from 15 May – 15 September 2015 and 18 May – 13 September 2016 in 32 vernal pools in greater Bangor, Maine following methods in Chapter 2. During surveys we noted indications of disease, including small white structures embedded in the skin and/or visible within the body cavity in several larvae. In 2015, we collected and submitted 19 of these larvae from seven pools to the USGS National Wildlife Health Center (NWHC) for examination. We photographed a sample of these white structures from a wood frog skin sample through a light microscope. Morphologically similar white structures were detected in multiple aquatic insects examined in 2016. Additionally, we quantified within-pool and landscape scale site characteristics following methods in Chapter 2.

### F.3 Results

The NWHC confirmed muscle infections by *Ichthyophonus* sp., a fungal parasite, in 12 larvae from five pools via histological examination. Nearby pools there was little disturbance (0.0-5.6 % impervious cover within 100 m), and within 1,000 m of pools there was 2.0-27.5 % impervious cover. Furthermore, gross examination suggested muscle infection in five tadpoles for which histological examinations were not conducted, for a total of 17 of 18 wood frog samples with confirmed or suspected *Ichthyophonus* infections (NWHC report, 30 November 2015). The NWHC suggested that given the absence of other infectious skin diseases in the examined larvae, it is likely that all white structures were due to infection by *Ichthyophonus*. The NWHC noted 4 to >90 individual *Ichthyophonus* organisms in each tadpole, and suggested that *Ichthyophonus* infection may have been the cause of death for these animals, however, this was unclear as the intensity of *Ichthyophonus* infections was considered minimal to moderate.

In the field, we detected small white structures embedded in tadpoles in 11 pools, total, between 3 June – 28 July 2015 and 16 June – 20 July 2016 (Table F1, Figures F1 and F2). These structures were observed on the ventral sides and tails of tadpoles. Concurrently observed symptoms of infection included lethargy, hemorrhaging, emaciation, and bloating/swelling, and in 2015, 11 individuals with structures were found dead. Infected tadpoles were Gosner stage 25-40 (median = Stage 35 in 2016, Stage 32 in 2015) and SVL 6-20 mm (median = 13 mm in 2016, 14 mm in 2015). We also detected morphologically similar structures in five insect taxa in five pools in 2016, with two of these pools also having tadpoles with white structures (Table F2, Figure F3). During some surveys, the detected prevalence of these structures was relatively high (5-100% in 2015; 33-87% in 2016). Five pools where white structures were detected in 2015

were also sampled in 2016. Two pools had white structures detected on tadpoles both years, with 80-95% prevalence during at least one survey each year.

Table F1 Detected prevalence of white structures consistent with *Ichthyophonus* sp. in wood frog larvae in 32 pools in greater Bangor, Maine during 2015-2016.

Year	Overall prevalence (larvae with white structures detected; total larvae examined)	Prevalence within pools where structures were detected (larvae with white structures detected; total larvae examined)	Proportion of pools with detections (Pools with white structures detected; Total pools examined)
2015	3.6% (185; 5,071)	10.6% (185; 1,747)	32.2% (10; 31)
2016	5.9% (69; 1,160)	23.8% (69; 290)	25.0% (3; 12)

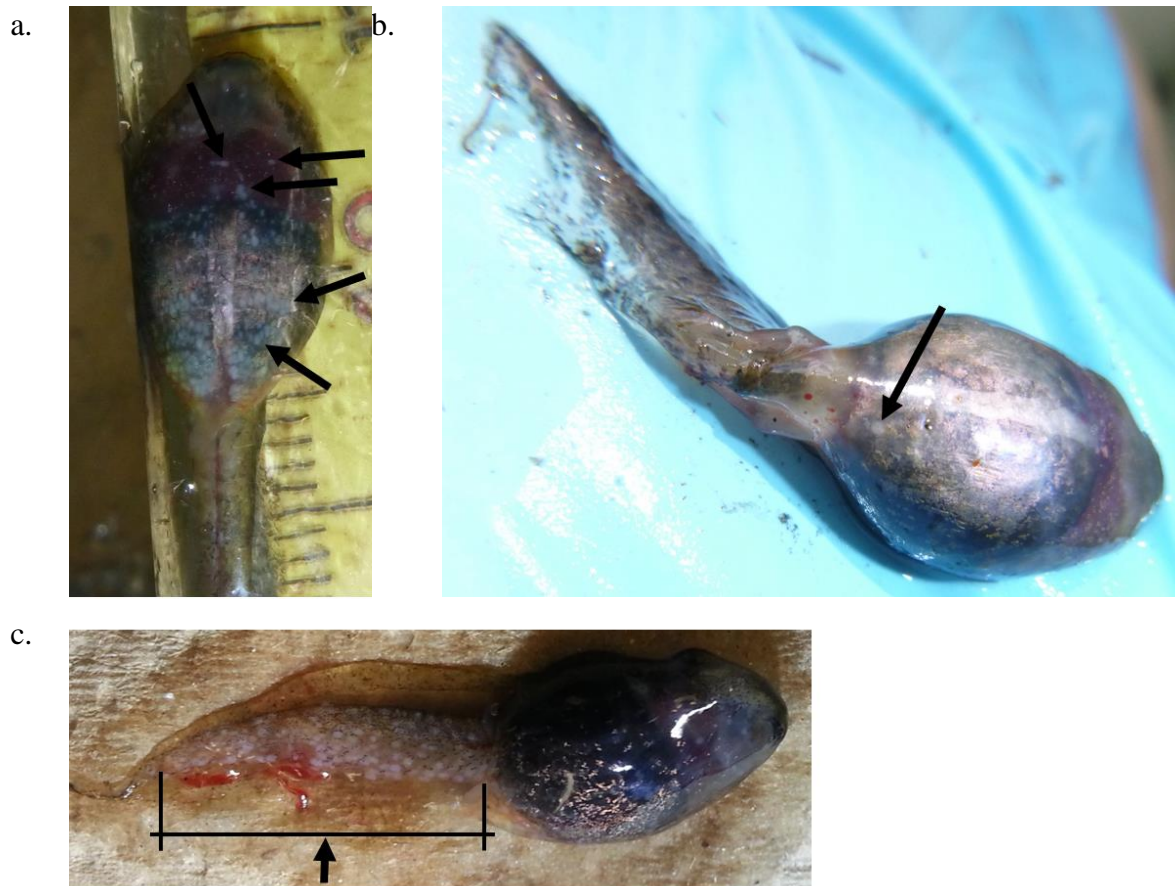


Figure F1 Tadpoles with suspected *Ichthyophonus* sp. infections observed on June 9 (a) and 22, 2015 (b) and June 28, 2016 (c). Arrows point to small white structures that are likely *Ichthyophonus* sp. spores in (a) and (b). The entire tail of (c) has multiple small white structures as indicated by the bracket. A single spore is indicated in (a), and multiple spores are present throughout the abdomen in (b) and especially visible against the red of the heart and lungs. Tadpoles in (a) and (c) also displays symptoms consistent with Ranavirus (hemorrhaging and tail fin degradation).

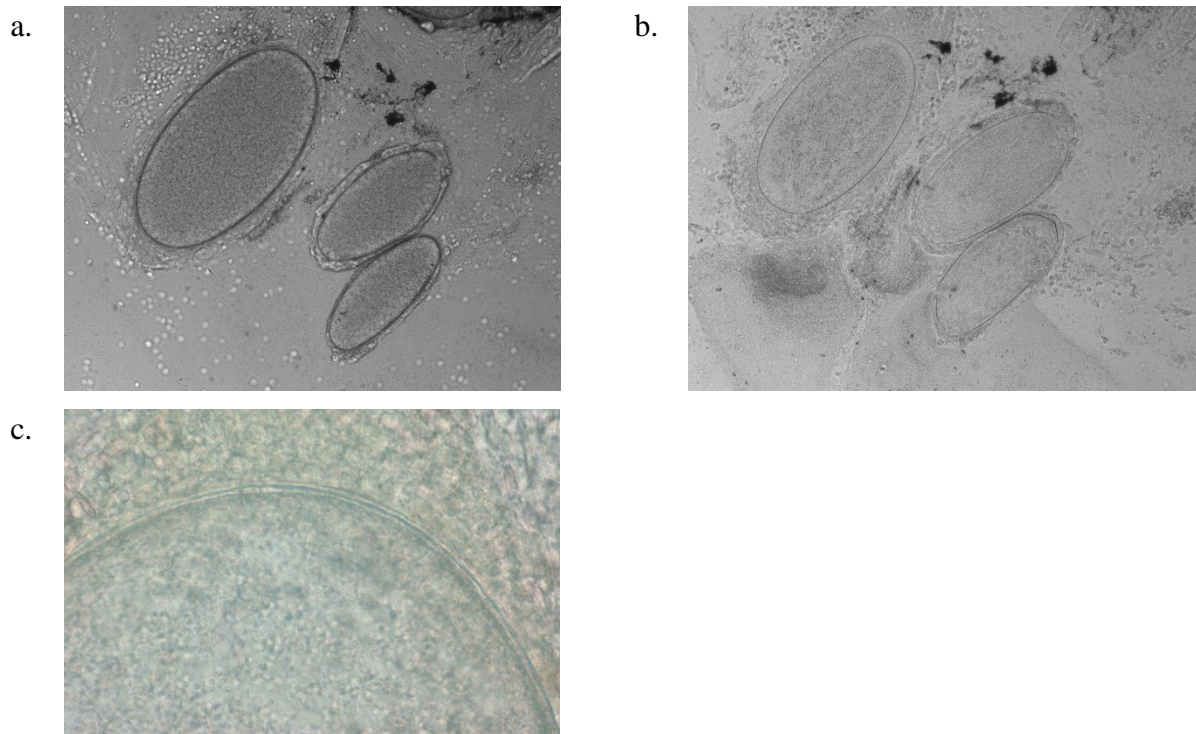


Figure F2 Photographs of foreign bodies removed from a wood frog tadpole on June 19, 2015. Images are of the same slide at 40X (a and b) and 100X magnification (c). Image b was taken after the cell wall was ruptured and intercellular fluid appears to be flowing out of the cell. Image (c) is of the upper left structure in images (a) and (b).

Table F2 Aquatic insects in which white structures consistent with *Ichthyophonus* sp. were detected in greater Bangor, Maine from June 3 to July 21, 2016.

Order/Suborder	Family	Genus
Megaloptera	Corydalidae	
Odonata/Anisoptera	Libellulidae	Sympetrum
Coleoptera	Noteridae	
Coleoptera	Dysticidae	
Coleoptera	Hydrophilidae	

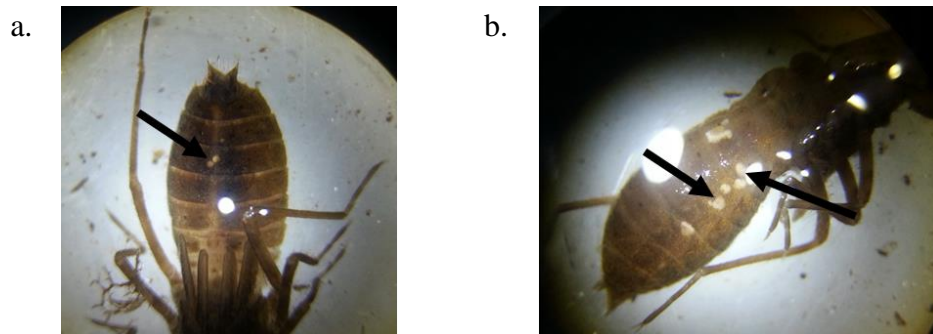


Figure F3 Photographs of a dragonfly larvae (Order Odonata, Suborder Anisoptera, Family Libellulidae) with small white structures consistent with the morphology of *Ichthyophonus* sp. (as noted by black arrows) from OT06 from 2016 – June 10, 2016.

In 6 of 11 pools where structures were detected some larvae exhibited concurrent hemorrhaging, a symptom consistent with Ranavirus infection (in pools with >20% prevalence during at least one survey during 2015 and with 87% prevalence in 2016). Of the pools where structures were detected in 2015 and 2016, two had larval amphibians (larval wood frog and/or *Ambystoma* sp.) with positive Ranavirus cultures (testing by USGS NWHC) in 2014, four had wood frog larvae with positive Ranavirus cultures in 2016 (testing in Matt Gray’s lab, University of Tennessee), four had larval wood frog die-offs in 2014, and one had a die-off in 2016 (Appendix E).

Pools where we detected structures in tadpoles had 0-31% impervious cover within 100 m and 0-38% impervious cover within 1,000 m. These pools dried as early as 6 June and others did not dry during the year sampled, and pool areas ranged from 103-3,147 m<sup>2</sup> at spring high water.

#### **F.4 Discussion and conclusions**

Because the NWHC suggested that all white structures detected in examined samples were caused by *Ichthyophonus*, we suspect that most, if not all abnormal white spots detected on



amphibian larvae in the field were also due to infection by *Ichthyophonus*. Our detections consistent with *Ichthyophonus* infection appeared to spike in June and July, with relatively high prevalence of infection apparent in some larval cohorts. Our results are similar to that of *Ichthyophonus*-associated mortality events reported for larval and adult frogs between April-August in the Midwest and glaciated northeastern North America (Mikaelian et al. 2000; Green et al. 2002).

Results from the NWCH indicated that *Ichthyophonus* infections were present in wood frog tadpoles in 3 of the 5 pools where we suspect there were Ranavirus-related die-offs in 2015. Our detection of Ranavirus and *Ichthyophonus* co-occurring in our study pools supports the idea that there may be a synergistic or facultative relationship between these pathogens. Additionally, given our detection of structures in aquatic insects that are morphologically similar to the *Ichthyophonus* spores detected on wood frog larvae, it is possible that aquatic insects in vernal pools may also be affected by and/or involved in the life cycle of *Ichthyophonus*.

Because there is evidence that *Ichthyophonus* may pose a substantial risk to pool-breeding amphibian larvae yet there is a lack of knowledge about the effects of this pathogen in amphibian larvae, we suggest future study of *Ichthyophonus* in vernal pool ecosystems. Possible interactions of this pathogen with Ranavirus increase the importance of understanding the effects of *Ichthyophonus* on pool-breeding amphibian larvae.

## **APPENDIX G: ASSESSMENT CHALLENGES AND PRELIMINARY EXAMINATIONS OF WHITE BLOOD CELL PROFILES IN WOOD FROG LARVAE**

### **G.1 Introduction**

White blood cell (WBC) profiles are good indicators of pre-capture physiological condition in amphibians because WBC composition and production responds to the long-term blood concentration of corticosterone (CORT), a glucocorticoid which is involved in energy regulation and immune responses (Davis et al. 2008). Types of WBCs include neutrophils, lymphocytes, basophils, eosinophils, and monocytes and are involved in immunological functions such as defense (e.g., lymphocytes and basophils) and response to infection (e.g., neutrophils; Davis et al. 2008). The ratio of neutrophils : lymphocytes (hereafter referred to as N : L ratios) have recently been used to indicate adult and larval amphibian physiological health responses to environmental conditions (Shutler and Marcogliese 2011; Burraco et al. 2013; Hota et al. 2013). N : L ratios better reflect chronic environmental conditions than do CORT levels as CORT in amphibians can change in response to acute (Glennemeier and Denver 2002) and chronic (Gendron, Bishop, Fortin, & Hontela, 1997) adverse environmental conditions. Although short-term activation of CORT is necessary for general life processes (e.g., mobilize energy to flee a predator), long-term activation can result in chronically elevated levels, leading to depleted energy reserves and/or the inability of an individual to mount an additional metabolic response to an acute stimuli (Glennemeier and Denver 2002).

Although WBC profiles have been used to assess the physiological health of amphibians, amphibian blood collection and smear creation methods, especially for relatively small tadpoles (i.e., <5 g), are poorly described in the literature. Several studies on amphibian larvae either do

not detail the number of WBCs counted to obtain N : L ratios (Rocha et al. 2010; Teixeira et al. 2012) or use WBC counts that are too low to be considered statistically robust for analysis of N : L ratios (Davis 2008; Davis and Milanovich 2010; Hota et al. 2013): statistically relevant sample sizes require  $\geq 100$  WBCs per slide (Houwen 2001). Although creating high quality blood smears for small amphibian larvae, can be logistically challenging, N : L ratios paired with other measurements (e.g., mass, growth rate) can be used to comprehensively assess the health of amphibian populations.

WBC profiles may be especially appropriate to assess the response of some larvae with highly variable morphology to environmental conditions (i.e., comparatively large larvae are not necessarily healthier). As part of an overarching study examining how urbanization contributes to wood frog (*Lithobates sylvaticus*) declines, we attempted to assess N : L ratios. The external morphology of wood frog tadpoles is highly variable as development patterns respond to pool size, hydrology, vegetation, and predator communities (Skelly 1997; Snodgrass et al. 2000; Veysey et al. 2011), thus N : L ratios are appropriate to assess how urbanization may contribute to wood frog declines at the larval stage. Additionally, N : L ratios may indicate imperiled populations before actual declines become noticeable. Despite the potential for WBC profiles to provide much needed information about larval amphibian health, there are several logistical challenges to successfully performing this technique in small larval amphibians. Here, we present our preferred suite of techniques for blood collection and smear creation for African clawed frog in the lab (i.e., preliminary technique selection) and wood frog larvae in the lab and field. We also examine the relationship between wood frog larval developmental metrics and WBC types in successful smears (smears with  $\geq 100$  WBCs).

## **G.2 Methods**

### **G.2.1 Blood collection and smear creation techniques**

In 2014, we attempted preliminary blood smears for 92 wood frog tadpoles from three (Gosner stages 27-40, median 36; approximately 0.1-1.0 g). Tadpoles were from six pools, of which three were relatively rural landscapes (0-7% impervious cover within 1,000 m) and three were in somewhat urbanized landscapes (27-38% impervious cover). We euthanized tadpoles by submersion in 10 mg/L MS-222 within 5 minutes of collection and tadpoles remained submerged for approximately 10 minutes prior to blood collection. We collected blood from euthanized tadpoles due to the small size of these animals relative to the volume of blood needed for N : L ratio analysis (5 - 10  $\mu$ l [Davis et al. 2008]; which exceeds the recommendation of no more than 10% of body mass for any amphibian <2.65g [Heatley and Johnson 2009]). Upon removal from MS-222, we dried tadpoles using an absorbent tissue, and made an incision on the ventral side at the heart with a No. 12 scalpel (hooked). We attempted to gently soak up any clear body fluid with an absorbent tissue prior to rupturing the exposed heart using a heparinized microcapillary tube (Davis and Maerz 2008). Immediately after blood collection, we created two blood smears on microscope slides using the coverglass technique (Houwen 2002). Within one month of collection a blood analyst stained the smears using a Wright-Giemsa stain (Volu-Sol, Salt Lake City, Utah; 2 minute stain, 2 minute buffer, and 10-dip rinse) and examined them for N : L ratios using a standardized count methodology (Houwen 2001).

Because all smears created from these preliminary attempts were unreadable for N : L ratios (< 30 WBCs per slide), we attempted to improve upon techniques. Blood cells were distorted and diluted, likely caused by contamination of blood with lymph and cerebrospinal

fluid (personal communication, L. L. Leppert). Thus, our technique, rather than a lack of WBCs in tadpole blood, was likely responsible for the low number of WBCs. We identified possible improvements at multiple steps between tadpole capture and completion of stained smears. Immobilizing tadpoles and using magnification during blood collection, collecting blood from anesthetized amphibians (to be euthanized immediately after collection), and pretreating the circulatory system (injecting the heart) with heparin sodium, an anticoagulant, prior to collection were suggested to potentially improve the quality of blood smears (personal communications, A. B. Lichtenwalner, J. A. Weber). Other researchers suggested that staining slides immediately after collection or using a different stain may reduce cell degradation (personal communications, A. K. Davis, L. L. Leppert). Another strategy identified to reduce lymph contamination was to deposit blood into a small area on a thin cytocentrifuge filter pad on top of a microscope slide to absorb lymph while blood cells would fall to the slide, below (personal communication, Jill Arnold).

To improve the technique used to make smears between the 2014 and 2015 wood frog larval seasons, we used 37 lab-reared (*Xenopus* 1, Inc., Dexter, Michigan) African clawed frog (*Xenopus laevis*) tadpoles which were approximately the same size as wood frog tadpoles. Tadpoles were reared in aged tap water maintained at approximately 20 C at a density of approximately 1.6 tadpoles per L and fed *Xenopus* tadpole food (Carolina Biological Supply Company, Burlington, NC) and cooked lettuce. We collected blood and created smears between 24 April and 29 May 2015. We attempted different techniques to (1) stabilize tadpoles (pinning, strapping with a rubber band, placing tadpoles into a tadpole-shaped divot cut in foam), (2) improve visibility of tadpoles (magnification, artificial lighting), (3) create an incision (No. 12 and No. 15 scalpel), (4) collect blood (heparinized microhematocrit tube, cardiac puncture using

a 27 gauge needle), (5) create smears (wedge-method using two microscope slides; making a smear by placing blood on a coverslip, dropping another coverslip onto the blood, and quickly pulling the coverslips apart at a slight angle), and (6) stain slides (Wright-Giemsa [same as used in 2014], Dip Quick [5 s in methanol, 5 s in eosin, 5 s in Thyozone stain, rinse with DI water], May-Grunwald (Sigma Aldrich Inc., St. Louis, MO; 5 m in stain, 3 m in buffer [pH 6.8], 15 m in 1:20 Giemsa solution, rinse with DI water). We also tested multiple placements and directions of the ventral incision, attempted to heparinize tadpole hearts (0.01 ml of 1000 iu/ml heparinized saline using a 27 gauge tuberculin syringe), and collected blood on euthanized as well as anesthetized tadpoles. Determination of success of these techniques was qualitative, with comparative targets of (1) reducing collection of non-blood body fluid and coagulated blood, (2) increasing the volume of undiluted blood collected, (3) a relatively even spread of readable blood cells across slides, (4) smears with undistorted WBCs, and (5) staining that contrasts WBC organelles for easier morphological distinction among WBC types. Two or more smears were created for each tadpole. We examined slides using an Olympus BX60 compound microscope.

Within a week of determining a preferred suite of techniques to create blood smears for clawed frog tadpoles, we were provided with several lab reared wood frog larvae from a separate research project. Between 1 – 3 June 2015, We conducted the preferred clawed-frog techniques on 33 wood frog larvae (Gosner stages 27-35, median 33; approximately 0.1-0.8 g) and made adjustments as needed to increase the success of smear creation for wood frog larvae.

Between 10 June – 14 July 2015, we created blood smears from wood frogs from 10 pools in the field. Five pools were relatively rural landscapes (4-11% impervious cover within 1,000 m) and five were in somewhat urbanized landscapes (27-38% impervious cover). We sampled 5-11 individuals per site (median = 10) and 1-4 slides were created per individual

(median = 2). We used the suite of techniques that were preferred to create blood smears from wood frog tadpoles in the lab. We also quantified developmental Gosner stage (Gosner 1960), mass, and SVL for tadpoles. Two or more smears were created for each tadpole in the lab and field. Field-collected smears that appeared to have an even, red spread indicative of a slide likely of having  $\geq 100$  WBCs were sent to a blood analyst to count types of WBCs using the standard methodology to determine N : L ratios (Houwen 2001). Neutrophils, lymphocytes, monocytes, eosinophils and basophils were counted for smears that had an abundance of WBCs nearing 100 (based on the approximation of the blood analyst). Relatively few smears neared 100 WBCs per slide, and thus interpreting the relationship between N : L ratios and conditions was not appropriate as some sites would be represented by a smear from one tadpole. However, these data are appropriate for a preliminary examination of how WBC counts and N : L ratios vary with wood frog tadpole development.

### **G.2.2 Statistical analysis**

Statistical analyses were conducted for wood frog WBC responses using program R (R Core Team 2016). We examined the relationship between the likelihood of an individual having at least one slide with  $\geq 100$  WBCs (i.e., a “successful” smear) and tadpole developmental responses (SVL, mass, stage) using logistic regression using R package ‘nlme’ (Pinheiro et al. 2017). For those individuals with at least 100 WBCs per slide, we examined the statistical effect of tadpole developmental responses on each WBC type (the number encountered during a standard white blood cell count used to determine N : L ratios) and on N : L ratios using linear models using package ‘lme4’ (Bates et al. 2017). We averaged the numbers of WBCs by type for individuals that had  $>1$  slide with 100 WBCs. To account for the lack of independence among

slides from the same individual, we included Site as a random term in all models. For each response, we created a set of univariate models, and then selected the top model(s) for each response using library ‘AICcmodavg’ (Mazerolle 2017) to rank models using Akaike’s information criterion adjusted for small sample size (AICc). We considered models  $\Delta AICc < 2$  that ranked above the null model to be plausible (Burnham and Anderson 2002). If  $> 1$  model met these criteria, we tested additive models that included all combinations of covariates in plausible models. We examined the 85% confidence intervals (Arnold 2010) of each covariate in these models to determine effect.

## **G.3 Results**

### **G.3.1 African clawed frog technique**

The suite of techniques that achieved the best smear for African clawed frogs used tadpoles included euthanasia, with blood collection beginning as soon as tadpoles did not respond to touch. Less blood was generally available for collection in tadpoles that continued to soak in MS-222 for a longer time, potentially because blood continued flowing immediately after death, but then may have quickly ceased to flow with additional time. We placed tadpoles in a supine position (ventral side-up) into a tadpole-shaped divot cut into a piece of soft foam and pinned tadpoles through the tail and nostrils to the foam. The tail was pinned at an angle through the tail to avoid the spinal cord and associated blood vessels to prevent bleeding. The tadpole was more stable when pinned into a divot than when pinned to a flat foam surface. Strapping tadpoles to the foam with a rubber band that was pinned at either end avoided rupturing blood vessels but did not adequately prevent tadpoles from shifting during incision.



We gently dabbed the skin dry with an absorbent lab tissue and then made a lateral (horizontal) 1 mm incision with a #15 scalpel to expose the heart. Creating a vertical incision (from vent towards nose) did not appear to increase the ease of blood collection, and the horizontal incision was easier to control. A small corner of the tissue was used to absorb interstitial fluid that flowed from the incision without disturbing any internal organs. However, attempts to absorb as much fluid as possible typically disturbed organs and resulted in uncontrolled bleeding into the abdominal cavity. With one hand, we positioned a heparinized microhematocrit tube in the incision and slightly agitated (ruptured) the heart while gently pinching either side of the base of the tail with thumb and forefinger of the other hand and then gently applying pressure to the lower abdomen. Applying pressure resulted in a greater volume of blood collected. The microhematocrit tube had a rubber bulb dispenser on the end which increased the accuracy and speed with which we could dispense the desired volume of blood on a slide. The bulb had the added benefit of greater dexterity in handling microhematocrit tubes during blood collection. We dispensed one drop of blood onto the end of the slide and created a smear using the wedge-method (Houwen 2002). The coverglass technique more often resulted in smears that did not have uniform coverage of red blood across the slide than the wedge-method, and the coverslip technique produced a relatively small smear. Although the coverglass and coverslip techniques did produce successful smears, we more reliably created successful smears using the wedge-method. Smears were stained immediately after drying using Wright-Giemsa stain. Other tested stains did not provide greater contrast in WBC organelles. Blood collection was aided by use of a tabletop, adjustable magnifying glass or a magnification visor (1.75-2.25 X) and a small lamp lighting tadpoles.

The #15 scalpel was easier to control compared to the #12 (hooked) scalpel, as the tip of the #12 could more easily rupture a non-target area with the slight tremor of a hand. Attempts to heparinize the heart and to collect blood using a heparinized syringe (through an incision as well as without an incision) ruptured the heart and resulted in blood quickly mixing with fluid in the body cavity. Additionally, less blood was collected with the syringe than the microhematocrit tube, and after the only a portion of this blood in the needle hub was successfully removed via microhematocrit tube and then transferred to a slide.

### **G.3.2 Wood frog technique**

The suite of techniques that achieved the best smear for wood frog tadpoles slightly differed from the best suite of techniques identified for African clawed frog tadpoles. We pinned tadpoles to the flat foam surface because they were typically less turgid than clawed frog tadpoles and “sunk” into the divot, making the angle of handling slightly more difficult. Additionally, the wood frog tadpoles did not shift as much as clawed frog tadpoles during incision and blood collection and thus did not require the extra stability of being positioned in a divot.

Gently applying pressure to the lower abdomen during blood collection forced intestines out of the incision over the heart and thus was discontinued. Incision with a # 12 (hooked) scalpel provided more accuracy in the position of the cut than a # 15 scalpel. We used the tip of the scalpel to puncture the skin at one end of the horizontal incision, and then, with the hooked end just under the surface of the skin and maintaining upwards pressure, we completed the horizontal incision. Additionally, after agitating the heart with a microhematocrit tube, we held

the microhematocrit tube at a downwards angle from the heart during collection to use gravity to increase the volume of blood collected.

### **G.3.3 Wood frog WBCs**

Twenty-one of the 91 individuals with smears examined for WBCs had successful smears (100 WBCs counted on at least one slide; 8 individuals had 2-3 successful smears), and seven pools had individuals with successful smears. Three of these pools were relatively urban (9 individuals) and four were relatively rural (12 individuals). On successful smears, we detected 16-83 lymphocytes (median = 53), 3-73 neutrophils (median = 22), 0-25 eosinophils (median = 4), 3-37 basophils (median = 13.5), and 0-20 monocytes (median = 3.5). Larvae with successful smears were 0.60-1.55 g (median = 0.97 g), had 14-21 mm bodies (median = 18 mm), and were stage 30-41 (median = 36.5).

In general, smears from later stage and larger tadpoles had fewer lymphocytes, more neutrophils and monocytes, and greater N : L ratios (Tables 1-2, Figure 1). Tadpole stage was the best predictor of lymphocytes, neutrophils, and (as would follow) N : L ratios (Table 1). Although mass was positively correlated with the likelihood of a successful smear (covariate estimate = 1.86) and was the covariate in the highest ranking model (Table 1), the 85% CI for mass included zero. No univariate model outranked the null model for eosinophils or basophils.

Table G1 Model ranking for the likelihood of a successful smear and numbers of white blood cell types using only those models that ranked  $<2 \Delta AICc$  and that ranked higher than the null model. Null models (NM) are included for reference. Observations are nested by Site (random effect) in all models.

	<b>K</b>	<b>AICc</b>	<b><math>\Delta AICc</math></b>	<b>w</b>	<b>LL</b>
<i>Likelihood of a successful smear</i>					
mass	3	97.39	0	0.32	-45.56
null	2	97.6	0.21	0.29	-46.73
<i>Neutrophils</i>					
stage	4	175.39	0	0.27	-82.45
mass	4	175.47	0.07	0.26	-82.48
null	3	175.53	0.14	0.25	-84.06
<i>Lymphocytes</i>					
stage	4	173.26	0	0.4	-81.38
null	3	173.31	0.05	0.39	-82.95
<i>Monocytes</i>					
SVL	4	126.22	0	0.51	-57.86
mass	4	127.9	1.67	0.22	-58.7
null	3	129.04	2.82	0.13	-60.82
<i>N : L ratio</i>					
stage	4	36.76	0	0.41	-13.13
null	3	37.41	0.65	0.3	-15

Table G2 Estimates, standard errors, and 85% confidence intervals (CIs) of covariates of white blood cell counts and N : L ratios for models with  $<2\Delta\text{AICc}$  and that rank above null models. Covariates are listed in order of AICc of their respective model. Only those covariates with CIs that do not include zero are shown here.

	$\beta$ estimate	SE	Lower CI	Upper CI
<i>Neutrophils</i>				
stage	3.05	1.51	0.852	5.25
mass	28.7	16.1	5.22	52.2
<i>Lymphocytes</i>				
Stage	-2.71	1.53	-4.94	-0.476
<i>Monocytes</i>				
SVL	1.35	0.419	0.739	1.96
mass	11.9	4.67	5.06	18.7
<i>N : L ratio</i>				
stage	0.116	0.0591	0.116	0.202

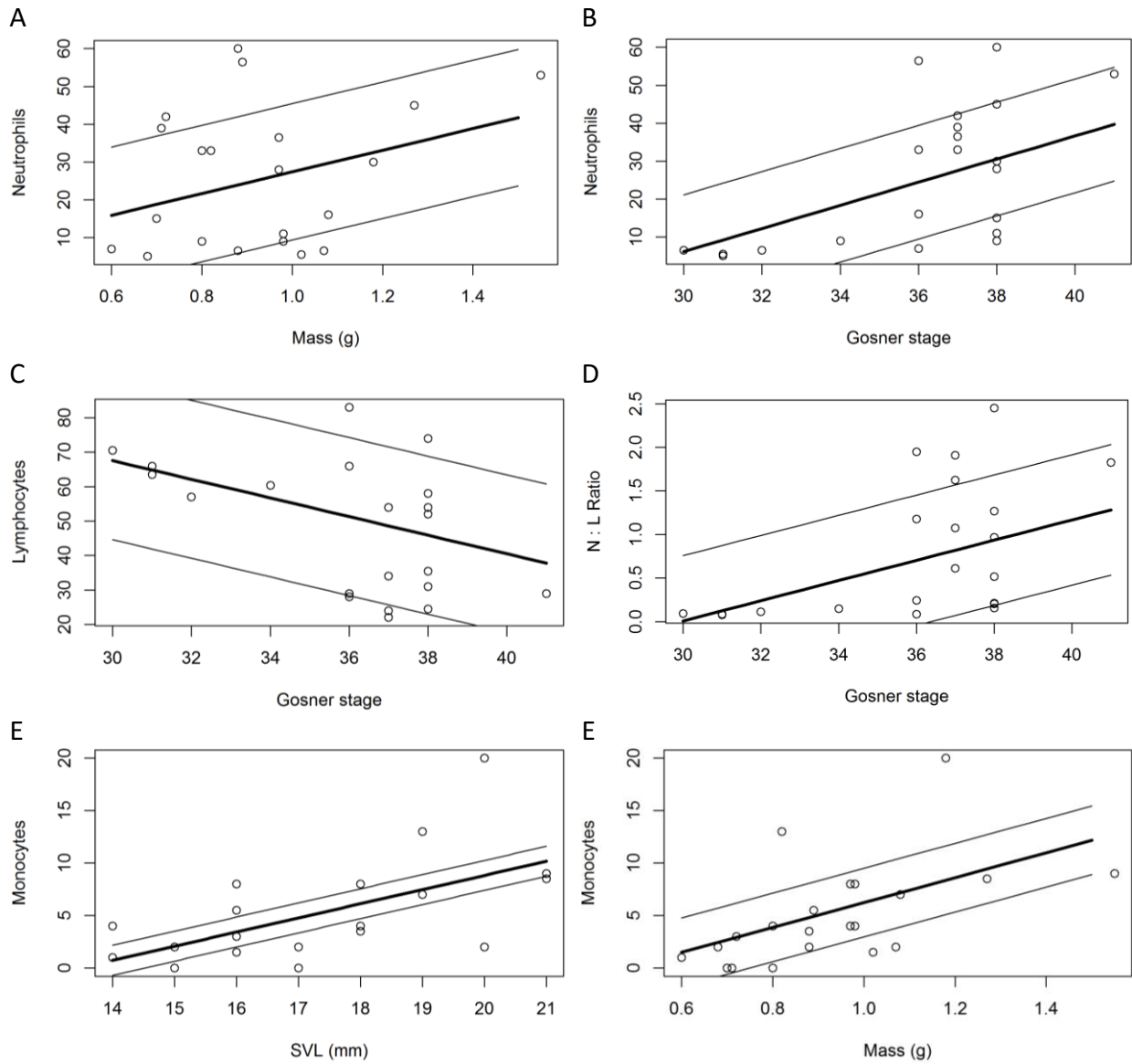


Figure G1 Predicted number of white blood cell types and N : L ratios corresponding to tadpole mass, stage, and SVL for 21 tadpoles with successful blood smears from six vernal pools. The thick line in the middle represents the predicted values across all pools, and the thin lines represent the extremes of the location of 85% of pools' predicted curves. Open circles represent individuals.

## G.4 Discussion

We successfully created blood smears from wood frog larvae as small as 0.6 grams, 14 mm SVL, and as early in development as stage 30. However, the majority (77%) of individuals sampled did not yield a successful smear. Although some of these tadpoles may have been too small (14 tadpoles were <0.6 g) for the techniques we used, 56 tadpoles with unsuccessful smears were an equivalent size and stage as those with successful smears. As tadpoles sampled at four pools did not yield any successful smears, it is possible that local conditions affect the success of the technique. For example, water temperature at time of sampling may influence viscosity and availability of blood for sampling, or within-pool conditions experienced by tadpoles during development may affect the availability or quality of blood. Moreover, any lymph contamination may dilute blood and make smears unsuitable for determining N : L ratios (Allender and Fry 2008; Teixeira et al. 2012).

We detected evidence of changes in WBC composition throughout wood frog larval development, with fewer lymphocytes and more neutrophils and monocytes nearer to the completion of metamorphosis and at larger sizes. Neutrophils and monocytes are phagocytic cells which are produced in response to infection and inflammation and lymphocytes defend against within-cell pathogens and mutations (Davis et al. 2008), thus these trends suggest a greater inflammatory response and suppressed immune function in wood frog larvae nearing metamorphic climax. These responses support the idea that during and shortly after metamorphosis, amphibians are particularly susceptible to adverse conditions, including disease, due to rapid metabolic and immune system restructuring (Duellman and Trueb 1994). The trends we detected in lymphocytes and neutrophils also align with expected increases of endogenous CORT production nearer to the end of metamorphosis (Rollins-smith 2001). Elevated CORT levels near metamorphosis may

reduce the ability of tadpoles to respond to environmental stimuli. For example, Chambers et al. (2011) observed that during metamorphosis (Gosner stages 41–45), wood frog larvae mounted a smaller CORT response to a metabolic challenge compared to earlier larval stages, thus indicating their relative inability to cope with additional “stressors” during this energetically demanding time in their lives.

WBC type fluctuations throughout development are similar to those detected for other larval anurans. Decreases in lymphocytes and increases in neutrophils and monocytes nearing metamorphic climax have also been observed for ornate frog (*Microhyla ornate*; Hota et al. 2013). Similar trends for lymphocytes and monocytes have been observed in bullfrog larvae (*Rana catesbeiana*) and Dubois’s tree frog (*Polypedates teraiensis*), but neutrophils increase with stage for these species (Davis 2008; Das and Mahapatra 2012).

Because WBC morphology changes throughout larval development in wood frog, it is essential to account for these fluctuations when studying deviations in WBC profiles due to additional challenges (e.g., anthropogenic disturbance, disease). Our work highlights some challenges in using WBC profiles to assess the immune and metabolic condition of wood frog larvae, and also provides information about the expected baseline WBC composition and abundance in wood frog larvae. Hematological parameters can be species-specific (Davis et al. 2008) and thus WBC profile reference values for wood frog larvae should be established prior to using WBC profiles to assess the metabolic and immune response of wood frog larvae to degraded environmental conditions.



**APPENDIX H: WOOD FROG, SPOTTED SALAMANDER, AND BLUE-SPOTTED SALAMANDER EGG MASS AND EMBRYO COUNTS**

Table H1 Maximum egg mass counts. We detected breeding at 133, 97, and 61 pool-years (66 pools; 44 pools included in statistical analyses) for wood frogs (*Lithobates sylvaticus*), spotted salamanders (*Ambystoma maculatum*), and blue-spotted salamanders (including the unisexual complex, *A. laterale* - *jeffersonianum*) in greater Bangor, Maine.

Site	<u>Wood frog</u>			<u>Spotted salamander</u>			<u>Blue-spotted salamander</u>			latitude, longitude
	2014	2015	2016	2014	2015	2016	2014	2015	2016	
B01	10	0	8	0	0	0	0	0	0	44.82467, -68.72979
B02*	8	-	-	0	-	-	0	-	-	roughly between B01 and B06
B03*	20	-	-	0	-	-	0	-	-	roughly between B01 and B06
B06	7	7	8	3	0	0	0	15	0	44.82302, -68.72926
B07*	35	-	-	7	-	-	0	-	-	44.833036, -68.731825
B08	33	48	45	1	6	3	0	0	0	44.794639, -68.800203
B09*	78	-	-	7	-	-	0	-	-	44.79015, -68.80201
B10	7	100	78	0	0	0	0	0	0	44.82342, -68.75153
B11*	8	-	-	0	-	-	0	-	-	44.824114, -68.749447
B12	18	44	39	0	0	0	0	0	0	44.81979, -68.74689
B13	7	38	41	0	0	0	0	0	0	44.81662, -68.74948
B14*	4	-	-	0	-	-	0	-	-	44.826716, -68.764152

Table H1, continued

Site	<u>Wood frog</u>			<u>Spotted salamander</u>			<u>Blue-spotted salamander</u>			latitude, longitude
	2014	2015	2016	2014	2015	2016	2014	2015	2016	
B15*	15	-	-	3	-	-	0	-	-	44.830622, -68.776919
B17	31	23	44	14	11	23	1	0	0	44.83455, -68.80389
B18	28	35	58	0	0	0	2	0	8	44.79787, -68.83723
B20	10	5	9	52	45	36	52	23	7	44.8632, -68.75721
B21	0	1	-	3	1	-	0	0	-	44.86317, -68.75737
B22	1	0	3	21	32	23	0	0	0	44.86382, -68.75237
B23	12	69	23	0	0	0	0	0	0	44.80212, -68.78862
B24*	0	-	-	0	-	-	42	-	-	44.833981, -68.804533
B25	6	0	66	53	78	18	206	43	0	44.833833, -68.803383
B26	49	8	33	49	77	86	1	2	0	44.86395, -68.75195
B27*	40	-	-	28	-	-	0	-	-	44.865022, -68.750297
H01	31	36	91	19	21	23	0	0	0	44.78024, -68.79009
H02	20	24	22	27	8	14	62	24	1	44.75848, -68.85593
H03*	167	-	-	12	-	-	4	-	-	44.72506, -68.83901
H04*	25	-	-	24	-	-	79	-	-	44.725981, -68.839553
H05*	62	-	-	220	-	-	88	-	-	44.726372, -68.839056
H06*	9	-	-	16	-	-	0	-	-	44.76884, -68.81378

Table H1, continued

Site	<u>Wood frog</u>			<u>Spotted salamander</u>			<u>Blue-spotted salamander</u>			latitude, longitude
	2014	2015	2016	2014	2015	2016	2014	2015	2016	
OR01	28	15	31	0	0	0	0	5	150	44.89837, -68.68651
OR02*	24	-	-	0	-	-	0	-	-	roughly between OR07 and 44.899278, -68.686958
OR03*	11	10	-	0	0	-	0	0	-	roughly between OR07 and 44.899278, -68.686958
OR04*	5	6	-	1	2	-	0	1	-	roughly between OR07 and 44.899278, -68.686958
OR05*	2	-	-	1	-	-	0	-	-	roughly between OR07 and 44.899278, -68.686958
OR06*	6	1	11	8	3	1	0	0	7	roughly between OR07 and 44.899278, -68.686958
OR07	11	2	1	8	10	14	51	16	12	44.89847, -68.68678
OR08	5	5	3	12	10	3	0	0	0	44.89807, -68.68738
OR09	2	9	-	7	3	-	0	44	-	44.89775, -68.68664
OR11	60	29	69	124	92	70	96	590	763	44.89423, -68.70352
OR12	426	301	175	65	96	76	0	0	0	44.88414, -68.68796
OR14*	8	-	-	0	-	-	22	-	-	roughly between OR07 and 44.899278, -68.686958
OR15	13	8	-	31	28	-	0	0	-	44.89336, -68.72401

Table H1, continued

Site	<u>Wood frog</u>			<u>Spotted salamander</u>			<u>Blue-spotted salamander</u>			latitude, longitude
	2014	2015	2016	2014	2015	2016	2014	2015	2016	
OR16	11	44	85	44	16	101	26	190	89	44.895389, -68.723372
OR17	402	157	73	131	86	120	20	3	0	44.87252, -68.70612
OR18	1	-	1	3	-	0	13	-	0	44.87265, -68.70502
OR19	6	20	25	0	0	0	189	51	34	44.90094, -68.67728
OR20*	11	-	-	55	-	-	0	-	-	44.89615, -68.72778
OR21	29	32	34	28	7	8	0	0	0	44.887556, -68.782631
OR22	-	11	-	-	23	-	-	5	-	44.892305, -68.656452
OR23	-	16	5	-	2	0	-	181	32	44.889115, -68.653327
OR24	-	15	21	-	36	7	-	44	1	44.889115, -68.653327
OR25	-	140	125	-	0	0	-	0	0	44.878547, -68.683408
OR26	-	34	119	-	82	56	-	1000	3130	44.879174, -68.682775
OR27	-	36	57	-	55	35	-	1296	654	44.885921, -68.686026
OR28	-	134	-	-	114	-	-	18	-	44.905639, -68.677889
OT01	52	-	-	0	-	-	0	-	-	44.93884, -68.68915
OT02*	2	-	-	0	-	-	0	-	-	roughly between OT07 and 44.935661, -68.689183
OT03*	6	-	-	0	-	-	0	-	-	roughly between OT07 and 44.935661, -68.689183

Table H1, continued

Site	<u>Wood frog</u>			<u>Spotted salamander</u>			<u>Blue-spotted salamander</u>			latitude, longitude
	2014	2015	2016	2014	2015	2016	2014	2015	2016	
OT04*	29	-	-	136	-	-	71	-	-	-, -
OT05	27	20	30	1	2	1	0	0	1	44.93947, -68.68961
OT06	37	24	62	80	76	53	0	0	0	44.934602, -68.687251
OT07	68	0	-	2	10	-	0	4	-	44.93426, -68.68809
OT08	124	49	103	391	233	244	13	6	2	44.93902, -68.67132
OT09	74	11	5	123	68	65	10	10	3	44.93902, -68.67132
OT10	156	24	95	355	86	382	72	25	21	44.93885, -68.6722
OT12	-	12	14	-	13	12	-	0	0	44.940437, -68.687158

\* Not included in statistical analyses

Table H2 Wood frog clutch size (number of embryos per egg mass) by site-year for 45 pool-years (27 pools) in greater Bangor, Maine. Summary statistics are based on clutch sizes of egg masses counted once and median clutch size for egg masses with >1 count.

Site	2015			2016		
	min	max	median	min	max	median
B01	-	-	-	686	1110	870
B08	537	959	744.5	333	1006	753
B10	483	659	559	395	715	516
B12	788	1035	900	599	987	868.5
B13	466	480	473	400	786	534
B17	-	-	-	468	619	511
B18	497	588	542.5	642	1110	776
B23	590	948	801	1011	1140	1075.5
H01	345	619	450	367	889	612
H02	403	1004	778.5	425	1469	872
OR08	394	664	590.5	247	247	247
OR09	455	774	599	-	-	-
OR11	366	366	366	351	837	626
OR12	308	600	511	534	828	708.5
OR16	437	1029	643	634	1053	824
OR19	-	-	-	180	828	643
OR21	395	945	667	397	844	455
OR24	-	-	-	534	891	549
OR25	398	879	641.5	543	903	693

Table H2, continued

Site	2015			2016		
	min	max	median	min	max	median
OR26	739	739	739	318	882	807
OR27	-	-	-	389	1130	826
OT09	120	554	258	355	503	393
OT10	378	766	571	272	703	472
OT12	499	972	801	537	899	761
OR15	524	963	687	-	-	-
OR22	323	606	421.5	-	-	-
OR28	211	911	542.5	-	-	-

Table H3 Spotted salamander clutch size (number of embryos per egg mass) by site-year for 43 pool-years (26 pools) in greater Bangor, Maine. Summary statistics are based on clutch sizes of egg masses counted once and median clutch size for egg masses with >1 count.

Site	2015			2016		
	min	max	median	min	max	median
B08	12	101	53.5	-	-	-
B17	46	202	125	35	161	74
B20	34	146	73	39	175	99
B22	4	150	75.5	7	170	82.5
B25	12	210	117.5	-	-	-
B26	5	176	117	20	210	111
H01	14	173	91	28	153.5	108
H02	39	134	74	13	185	76.5
OR07	14	147	85.5	-	-	-
OR08	9	221	99	-	-	-
OR11	21	156	109	13	153	108
OR12	23	178	81	9	199	124.5
OR16	10	173	89	11	169	95.5
OR17	10	206	88	33	210	109.5
OR21	47	122	96	30	124	93.5
OR24	-	-	-	10	168	130
OR26	14	145	70	10	187	106.5
OR27	31	150	93	23	212	122
OT06	8	117	51	-	-	-
OT08	30	205	98	40	164	101.25



Table H3, continued

Site	2015			2016		
	min	max	median	min	max	median
OT09	23	224	93	11	149	115.5
OT10	22	171	90	32	177	111.5
OT12	10	167	73	23	135	115
OR15	33	215	88	-	-	-
OR22	6	150	78.5	-	-	-
OT07	13	208	89	-	-	-

# APPENDIX I: INFLUENCES OF ROAD SALT AND FOOD AVAILABILITY ON WOOD FROG LARVAE

## I.1 Introduction

Habitat loss and degradation have been identified as major threats to amphibian persistence (Sodhi et al. 2008). Road salt is widely distributed on roadways and paved surfaces throughout areas with below-freezing temperatures in North America and can travel 100s of meters into wetlands (Trombulak and Frissell 2009). Thus, road salt contamination of amphibian breeding pools may contribute to risks to amphibians in these areas. Vernal pool-breeding amphibians, including wood frog (*Lithobates sylvaticus*), have been identified as particularly sensitive to road salt contamination (Collins and Russell 2009). Road salt contamination at environmentally relevant concentrations can reduce wood frog embryonic and larval survival, weight, activity, and time to metamorphosis and increase morphological abnormalities (Sanzo and Hecnar 2006; Karraker et al. 2008), and wood frog breeding is more likely to occur in pools without road salt contamination (Collins and Russell 2009).

Although high levels of sustained road salt contamination harm wood frogs at aquatic stages, larval responses to road salt in the field are likely complex. Road salt-induced reductions in individual survival may lessen competition for food and lead to detected increases in mass and survival among surviving larvae, as detected by Karraker et al. (2008) and others (Petranka and Doyle 2010; Dananay et al. 2015; Chapter 2). Additionally, effects on tadpoles in pools with low to moderate salt contamination (e.g., 500 – 2,000  $\mu\text{S}$ ) may not be clear as larval growth and survival in these pools may not be discernibly different than in uncontaminated pools. Increased variability in larval developmental phenology in salt-contaminated pools near roads (Hall et al.

2017) may further obscure the effects of road-related contamination on pool-breeding amphibian populations.

Here we studied the chronic exposure of wood frog larvae to a gradient of ecologically relevant road salt concentrations and examined growth and survival. We hypothesized that increases in tadpole size in pools with road salt contamination is linked to greater food availability (i.e., reduced competition). To test these hypotheses, we conducted an experiment where salt concentrations were crossed with high and low food availability.

## **I.2 Methods**

### **I.2.1 Salt x Food availability experiment**

We conducted an experiment to determine the influence of road salt contamination (as indicated by conductivity) and food availability on tadpole size and development by manipulating food and conductivity levels for individual tadpoles in small plastic containers in a lab. We manipulated food and road salt contamination (conductivity) levels, with seven conductivity levels crossed with two food availability levels. Within each food availability level, we used eight replicates for control conductivities and 13 replicates for each elevated conductivity, for 172 total experimental units (individual tadpoles). Control and elevated conductivity treatments had different numbers of replicates because we anticipated survival to differ between control and elevated conductivity levels (i.e., 64% in control and 41% in elevated conductivities; Sanzo and Hecnar 2006; Karraker et al. 2008), with a goal of  $\geq 5$  replicates per conductivity x food availability level surviving through the experiment (i.e., near metamorphic climax).

The control conductivity was similar to that noted for forest pond water (Karraker et al. 2008) and was within the range of specific conductivities we measured at pools in the greater Bangor area in 2014-15 (20-30  $\mu\text{S}$ ) in landscapes with a low level of human disturbance (unpublished data). The high conductivity level provides an ecologically relevant high endcap: we observed pools with conductivity up to 1,596  $\mu\text{S}$  in 2014-15 (unpublished data). Additionally, wood frog tadpoles raised in 2,000  $\mu\text{S}$  water have been documented to have significantly reduced larval survival compared to those raised at  $\sim 150$   $\mu\text{S}$  (Sanzo and Hecnar 2006). The concentrations of 500  $\mu\text{S}$  - 1,700  $\mu\text{S}$  were selected to represent the middle of the range of conductivity between which wood frog tadpole survival differences have been detected (differences between 500 and 3,000  $\mu\text{S}$ , Karraker et al. 2008; differences between 200 and 2,000  $\mu\text{S}$ , Sanzo and Hecnar 2006). The tadpoles in low and high food availability treatments were fed ground fish food (Tetramin  $\text{\textcircled{R}}$ ) at a rate of 4 and 8% of the mean body mass of the treatment per tadpole per day (to the nearest 0.01 g). We selected these rates of food availability because they have been observed to result in no difference in survival but in distinguishable differences in mass throughout larval development for wood frogs (Anholt et al. 2000, Berven and Boltz 2001). These food levels are also noted to not result in water fouling (Relyea 2002). Tadpoles were massed weekly to determine the mass of food to be given for the following week.

We collected five egg masses from a vernal pool (OT10) in the University of Maine University Forest, Old Town, ME without any known road salt contamination (42  $\mu\text{S}$  mean conductivity 16 June 2016) on 10 May 2016. Eggs were placed in 1 L of pool water and immediately transported to the University of Maine. Each egg mass was divided into seven sections and a section of each egg mass was placed in a rearing tub with 3 L of water of one of seven experimental conductivities: 25, 500, 800, 1,110, 1,400, 1,700, and 2,000  $\mu\text{S}$ . The 25  $\mu\text{S}$

control was created by mixing aged well water with deionized water. All other conductivities were created by mixing “control” water with road salt sourced from Maine Department of Transportation (Table 1) to achieve the target conductivity,  $\pm 6$  uS/cm. This method of achieving experimental conductivities was the same throughout all experiments. Slightly more embryos were allocated to the 25 and 2,000  $\mu$ S rearing tubs than intermediate conductivities to provide embryos for the salt x predator experiment, with <250 embryos/L (Karraker and Ruthig 2009) across all rearing tubs. Although others have documented differences in wood frog tadpole densities resulting in differences in individual mass after 10 days (Peacor and Pfister 2006) we did not anticipate early stage tadpoles that had been free swimming and feeding for only one day to have differences in mass resulting from differences in embryo densities.

Table II Road salt chemical composition results. Road salt was sourced from the Maine Department of Transportation in 2016 and represents a random sample from the MDOT stock of road salt applied to roads in the study area. Chemical composition analysis was performed by the University of Maine Analytical Lab.

<b>Element</b>	<b>Concentration (mg/kg)</b>
Al	< 16.4
Ca	479
Cu	< 3.3
Fe	< 16.4
K	3141
Mg	145
Mn	< 3.3
P	< 16.4
Zn	< 8.2

We checked egg masses daily for development. All 172 tadpoles needed for the experiment became free-swimming within 24 hours of each other and upon becoming free-swimming, were fed ground Tetramin ® *ad libitum* (following Berven and Boltz 2001). One day after tadpoles became free-swimming (16 May 2016), we distributed individuals among 2 L plastic containers containing 1 L of water of their corresponding experimental conductivities. To avoid maternal affects,  $\leq 25\%$  of the tadpoles in a treatment were from a single egg mass. We visually selected tadpoles to standardize initial size and mass among treatments and massed tadpoles prior to placement in the 2 L containers. We randomly allocated rearing containers to locations on a lab bench. Throughout rearing and the experiment, egg masses and tadpoles were maintained at room temperature (approximately 23 °C) in 12:12 light:dark conditions.

The experiment began as soon as tadpoles were placed in individual containers. We changed water in the experimental containers 3x per week and recorded mass, snout-to-vent length, tail fin height, and developmental stage for all tadpoles 1x per week. We measured survival from the time of placement into individual containers until the end of the experiment. Tadpoles euthanized because of signs of imminent death from abnormally high saline or unknown causes were considered to have survived until the day they were euthanized. The experiment ended after 28 days (2 days after the first tadpole developed to Gosner stage 42, just prior to completing metamorphosis) whereupon all tadpoles were euthanized and a final set of measurements was conducted. This experiment was conducted under approval from the University of Maine Animal Care and Use Committee (Protocol # A2016-03-10).

## I.2.2 Statistical Analyses

We calculated an index of final body mass (condition) and tail height (relative tail height) relative to body length using the residuals of mass and final tail height regressed against SVL. We then assessed all response variables (final stage, SVL, condition, change in mass, and relative tail height) for high correlation ( $|r| > 0.7$ ). We then examined the efficacy of the food availability manipulation by conducting a t-test ( $P=0.05$ ) and examining effect size using Cohen's  $d$  for final larval mass and SVL. We examined the effects of initial tadpole mass among conductivity levels using ANOVA and a Student's t-test between food availability levels ( $P=0.05$ ). If differences existed, we conducted Tukey's HSD pairwise comparisons to identify differences ( $P=0.05$ ). If initial tadpole morphology differed among treatment levels for a response, we examined the effects of food and conductivity treatments on the change between initial and final measurements as well as final measurements. For all further analyses we treated food level as a factor and conductivity as a continuous variable.

We examined interactions between food availability and conductivity treatments for the natural log-transformed final mass, SVL, condition, developmental stage, and survival to the end of the experiment using ANOVA ( $P=0.05$ ). If interactions were not significant, they were dropped and main effects were interpreted. If conductivity was a significant predictor of a response based on residuals (condition, relative tail height), we assessed the effects of conductivity on the relationship between morphology metrics to estimate predicted change in response values (as opposed to predicted change in residual values). Residual plots for all ANOVA models were checked for normality and response variables were natural log-

transformed to meet assumptions of normality if not normally distributed. All statistical analyses were performed using program R (R Core Team 2016).

### **I.3 Results**

We successfully manipulated food levels, with lower final mass and SVL in low compared to high food levels ( $P < 0.01$ , Cohen's  $d_{\text{Mass}} = -1.34$ , Cohen's  $d_{\text{SVL}} = -1.08$ ). Initial tadpole mass was different among conductivity levels ( $P < 0.01$ ) with 20 and 2,000 uS levels different from other conductivities ( $P < 0.01$ ). Responses were not highly correlated, with the highest correlation between mass and condition ( $|r| \leq 0.57$ ).

Survival was unexpectedly high (98.8%, 168 of 170 tadpoles) across all treatments, and thus we did not statistically examine effects of food or conductivity on survival. Tadpole condition was predicted to increase with increased conductivity ( $P = 0.03$ ,  $F_{\text{Cond } 1,159} = 4.54$ ) and food level, with food having a somewhat larger effect ( $P < 0.01$ ,  $F_{\text{Food } 1,159} = 7.49$ ; Figure 1). Mass was predicted to increase 5% for every increase in conductivity of 1 mS. The relationship between final mass and SVL differed with conductivity ( $P = 0.02$ ,  $F_{\text{Cond } 1,159} = 5.86$ ) with a 4% increase in mass predicted for every increase in conductivity of 1 mS at a given SVL. However, neither conductivity nor food was a significant predictor of natural log-transformed change in mass. Tadpoles were predicted to develop slower and have greater relative tail height at higher conductivities, but not significantly so (Development:  $P = 0.06$ ,  $F_{\text{Cond } 1,159} = 3.42$ ; Relative tail height:  $P = 0.052$ ,  $F_{\text{Cond } 1,159} = 3.83$ ). Food was not a significant predictor of final developmental stage or relative tail height. Conductivity was not a significant predictor of final SVL ( $P = 0.3$ ,  $F_{\text{Cond } 1,159} = 1.05$ ), but SVL was predicted to increase with more food ( $P < 0.01$ ,  $F_{\text{Food } 1,159} = 47.3$ ).



The interaction between food and conductivity levels was not significant for any modeled response.

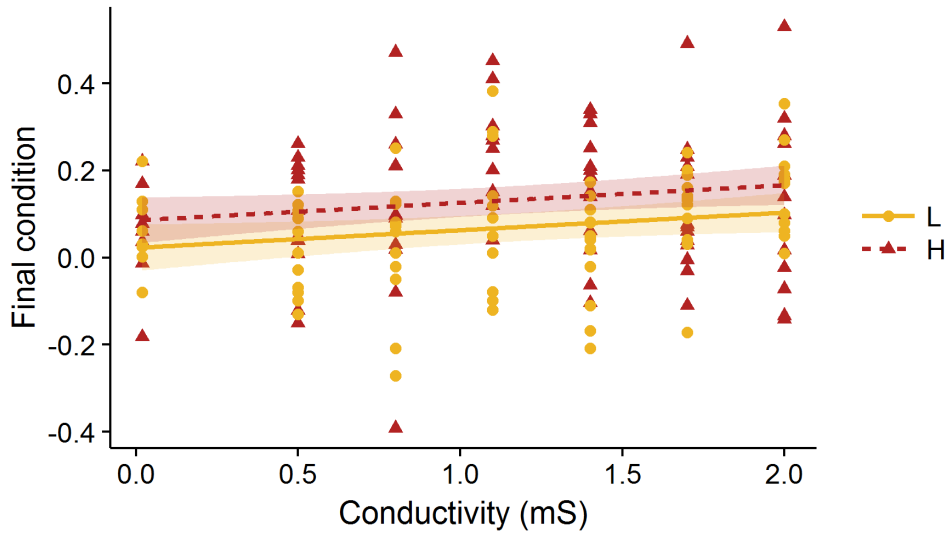


Figure 11 The relationship between wood frog tadpole final condition (mass adjusted for SVL) and conductivity from road salt contamination in a microcosm experiment. Conductivity and food availability levels had significant effects on final condition (L = low, H = high; Conductivity:  $P=0.03$ ,  $F_{1,159}= 4.54$ ; Food:  $F_{1,159}= 7.49$ ). Points represent individuals. Shaded areas represent parameter 95% confidence intervals.

#### I.4 Discussion

Our results indicate that in road salt contamination at ecologically relevant concentrations may not increase wood frog tadpole mortality rate aligns results from some studies (Dananay et al. 2015) but conflicts with lab and field observations of others (Sanzo and Hecnar 2006). Our detected effects of road salt on tadpole morphology are a little more ambiguous. Although tadpoles reared in higher conductivities had greater condition and greater relative tail height, there was no effect of conductivity on change in mass throughout larval development. A result of no change in body size with conductivity when food availability is controlled supports the hypothesis that increases in tadpole size observed in the field associated with elevated road salt

contamination are caused by reduced competition for food (Karraker et al. 2008) or changes in abundance of periphyton and zooplankton food resources (Dananay et al. 2015). Road salt may release algae from zooplankton grazing pressures, thus increasing food for tadpoles and promoting growth (Van Meter et al. 2011).

These results support the growing body of literature supporting the idea that road salt contamination primarily affects wood frog larvae through food web structure and food limitation. Although we did not detect negative effects to tadpoles associated with road salt, road salt exposure during larval development can reduce survival at embryo and froglet stages (Brady 2013; Dananay et al. 2015) and high salinity pools are not preferred for wood frog breeding (Collins and Russell 2009). Road side pools also have been documented to have breeding populations that produce embryos that are maladapted to high salinity conditions (Brady 2013). We encourage the reduction and avoidance of road salt use near vernal pools to help maintain ecosystem structure and functions which support healthy wood frog populations.

## **BIOGRAPHY OF THE AUTHOR**

Carly Jasmine Eakin was born in Lansing, Michigan and graduated from Laingsburg High School. She graduated from Michigan State University in 2006 with a Bachelor's in Landscape Architecture and in 2012 with a Masters in Fisheries and Wildlife. Carly has practiced as a professional landscape architect and currently works as the Technical Guidance Coordinator and a Wildlife Biologist at the Division of Fish and Wildlife for the Commonwealth of the Northern Mariana Islands. Carly's publications include "Enriching urban communities with green roofs for bird conservation" (Eakin, C, H Campa III, D Linden, DB Rowe, J Westphal, G Roloff. 2015. Proceedings from the 13<sup>th</sup> Annual Green Roof Conference. New York, NY. 05-08 October 2015.) and "Avian response to green roofs in urban landscapes in the Midwestern USA" (Eakin, C, H Campa III, D Linden, DB Rowe, J Westphal, G Roloff. 2015. Wildlife Society Bulletin. 39, 3:574-582.). She is a candidate for the Doctor of Philosophy degree in Wildlife Ecology from the University of Maine in 2018.