INFLUENCE OF LANDSCAPE ARRANGEMENT AND WETLAND CONDITION ON BREEDING DYNAMICS OF AMBYSTOMA MACULATUM (SPOTTED SALAMANDER) IN MAINE, USA

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INFLUENCE OF LANDSCAPE ARRANGEMENT AND WETLAND CONDITION ON BREEDING DYNAMICS OF AMBYSTOMA MACULATUM (SPOTTED SALAMANDER) IN MAINE, USA

By Amanda Frances Shearin

Thesis Advisors: Dr. Aram J.K. Calhoun and Dr. Cynthia S. Loftin


Ambystoma maculatum (spotted salamander) breeds in seasonal wetlands (vernal pools) as well as in wetlands with permanent hydroperiods. Ability to successfully recruit young in these latter breeding habitats may be particularly important in landscapes with low vernal pool density but may require alternative breeding strategies. We examined relationships among breeding habitat and landscape characteristics and A. maculatum occurrence, ovipositioning behavior, egg mass morphology, and embryo survival, and occurrences of other amphibian species during 2006-2010 in ten vernal pools and permanent waterbodies (seven fishless lakes and five stocked but naturally
fishless lakes) in Maine, USA, and in laboratory experiments. We also used automated audio recording devices to evaluate the effectiveness of generalized listener-based audio surveys for detecting relatively rare or audibly cryptic anurans in Maine.

Landscape-scale characteristics (number and area of ephemeral to semi-permanent wetlands within 500 m) were most important for predicting breeding occurrence by vernal pool amphibians (*Ambystoma maculatum, Lithobates sylvaticus* [wood frog]) in lakes, whereas lake-scale characteristics (e.g., vegetative cover, fish presence) were better predictors for species (e.g., *L. septentrionalis* [mink frog], *L. pipiens* [northern leopard frog]) associated with permanent waterbodies. *Ambystoma maculatum* breeding effort was greatest in lakes where more typical breeding habitats (e.g., vernal pools, beaver flowages) within 500 and 4000 m were less abundant. Egg masses and hatching larvae were approximately 13 and 33%, respectively, larger in vernal pools than in lakes. Survival of *Ambystoma maculatum* embryos to hatching while exposed to *in situ* predation was approximately 180% higher in vernal pools than both lake types. When compared with full-night surveys, generalized listener surveys may result in omissions and misclassifications of chorus sizes for certain species in Maine (e.g., *L. septentrionalis, L. palustris* [pickerel frogs]).

Vernal pool-centric conservation measures may fail to account for connectivity among pools and alternative permanent breeding habitats in maintaining population persistence in long lived species such as *Ambystoma maculatum*. Lakes potentially provide alternative breeding habitat for *Ambystoma maculatum* in landscapes with few or poor quality vernal pools or in drought years; however, vernal pools are the optimal breeding habitat for this species in our study landscape.
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Chapter 1

USE OF ALTERNATIVE BREEDING HABITATS BY AMBLYSTOMA MACULATUM (SPOTTED SALAMANDER)

Introduction

Conservation strategies for vernal pool breeding amphibians commonly focus on fixed terrestrial buffers around classic breeding pools (Calhoun 2003) without consideration of alternative, non-ephemeral breeding sites. Although this strategy offers protection to individuals in seasonal breeding pools and their immediate terrestrial habitat, it does not consider the role of alternative breeding habitats in maintaining amphibian populations, particularly in landscapes where traditional vernal pools may be degraded or lost owing to development or in landscapes with limited wetland area. The importance of alternative breeding sites to pool-breeding amphibian populations may depend on characteristics of individual wetlands as well as the configuration of wetland habitat in the landscape (Baldwin et al. 2006; Cushman 2006; Freeman 2010; Jacobs and Houlanhan 2011; Veysey et al. 2011).

*Ambystoma maculatum* (spotted salamander) breeds in vernal pools (Hunter et al. 1999) and also deposits eggs in beaver (*Castor canadensis*) impounded flowages (Cunningham et al. 2007; Karraker and Gibbs 2009) and in permanent waterbodies with and without fish (Kolozsvary 2003; Egan and Paton 2004). *Ambystoma maculatum* is secure throughout its range, although urbanization (Windmiller 1996), forestry practices (deMaynadier and Hunter 1998), genetic isolation of subpopulations through habitat fragmentation (Gibbs and Reed 2007; Zamudio and Wieczorek 2007), road mortality
(deMaynadier and Hunter 2000; Karraker and Gibbs 2011), and loss of vernal pools
(Smith 1999) potentially affect this species’ abundance. *Ambystoma maculatums*’s use of
multiple breeding habitats may allow it to persist despite habitat modifications
throughout much of its range (Karraker and Gibbs 2009). This species may successfully
produce offspring in non-vernal pool habitats by responding behaviorally and
morphologically to avoid or minimize stresses (Petranka et al. 1998; Yurewicz 2004;
Urban 2007). While both landscape- and wetland-scale characteristics influence *A.
maculatum* occurrence and reproductive output in vernal pools (Skidds et al. 2007;
Veysey et al. 2011), these relationships and resulting conservation recommendations may
not be transferable to other breeding habitats (Cushman 2006).

**Fishless Lakes as Potential Breeding Habitat**

In Maine, USA, wetlands range from seasonally inundated, such as vernal pools,
to permanent lakes with and without fish. Vernal pools provide optimal breeding habitat
for *A. maculatum* and other pool-breeding amphibians that lack chemical or physical
defenses against fish predators (Hunter et al. 1999; Calhoun and Klemens 2002).
Fishless lakes may provide potential amphibian breeding habitat at the opposite end of
the hydroperiod continuum. While fishless lakes co-occur with vernal pools in the
eastern coastal region of Maine, it is unknown if these lakes provide alternative breeding
habitat for amphibian species palatable to fish, particularly in landscapes with low
functional connectivity among breeding sites (Compton et al. 2007; Leibowitz and
Brooks 2008) or inter-pool distances above maximum migration thresholds (Gibbs 2000).
Stocking programs and illegal fish introductions to fishless lakes during the 20th century
have reduced the density of and altered invertebrate communities in fishless lakes in Maine (Schilling et al. 2008). However, it is unknown if fish introductions to fishless lakes have similarly altered amphibian assemblages or the availability of this habitat for breeding *A. maculatum*. Furthermore, there are no spatially explicit, multi-scale studies (Cushman 2006) examining landscape and wetland relationships to *A. maculatum*’s breeding in lakes and their role in long-term and large-scale amphibian conservation.

**Local and Landscape Determinants of Breeding Habitat Selection**

The goal of Chapter 2 was to investigate characteristics of alternative breeding habitats (fishless and recently stocked fishless lakes) used by *A. maculatum* to determine their potential role in landscape-scale conservation of vernal-pool breeding species. Specifically, we investigated the following questions: (1) do breeding site characteristics such as hydroperiod (i.e., permanent or seasonal) fish presence, vegetation cover, and amphibian assemblages affect reproductive effort, size, and age distribution of *A. maculatum*, and (2) does type, density, and number of potential wetland breeding sites (including classic vernal pools and alternative sites) affect *A. maculatum* reproductive effort in each of these breeding habitats? Furthermore, we characterized amphibian assemblages among fishless and recently stocked lakes and examine lake- and landscape-scale characteristics related to presence of each species.

**Ambystoma maculatum Responses to Breeding Habitat**

The *A. maculatum* embryonic stage may incur the greatest cumulative predation of all life stages owing to the duration of embryo development (up to eight weeks in
Maine; Smith 1999) and phenology of co-occurring predators also undergoing metamorphosis. Variable ovipositioning strategies by *A. maculatum* females among breeding habitats may confer different survival rates to offspring (Ireland 1989; Brodman 1995; Windmiller 1996). For example, ovipositing in or adjacent to abundant littoral vegetation also provides refugia that may mediate effects of predators (Formanowicz and Boba 1989; Egan and Paton 2004; Kopp et al. 2006; Hartel et al. 2007). It is unknown, however, if *A. maculatum* alters ovipositioning strategy in response to different predator assemblages and whether these strategies are effective in reducing predation of embryos. *Ambystoma maculatum* egg mass morphology (e.g., color [Rowe et al. 1994; Petranka 1998]; clutch size [Hecnar and M’Closkey 1997]) also may enhance embryo survival to hatching. While these morphological variations have been reported separately for vernal pools and small ponds, there are no explicit and simultaneous comparisons of variations among breeding habitats within a region, nor is there a comprehensive understanding of how morphological plasticity is influenced by predator assemblages. In Chapter 3, we examined *A. maculatum* ovipositioning strategies, egg mass morphology, and embryo survival among ‘traditional’ (vernal pools) and ‘alternative’ (fishless and stocked lakes) breeding habitats to reveal potential mechanisms for successful breeding among multiple habitats. Our objectives were to (1) identify differences in oviposition site selection among breeding habitats for *A. maculatum*, (2) identify differences in egg mass morphological characteristics among breeding habitats, and, (3) determine *in situ* embryo survival rates among lake and vernal pool breeding habitats when predators are allowed and excluded from developing egg masses.
Using Automated Audio Recording Devices to Inform Anuran Call Surveys

Accurate detection and abundance measures are essential for documenting trends in amphibian populations. Audio surveys are used extensively for long-term and rapid population monitoring of vocalizing anurans (Dorcas et al. 2009). Large-scale, volunteer-based audio survey programs, such as the North American Amphibian Monitoring Program (NAAMP; http://www.pwrc.usgs.gov/naamp), are designed to monitor anurans over diverse spatial and temporal scales (Weir and Mossman 2005). The unified NAAMP protocol standardizes data collection across multiple regions and years; however, the generality of the protocol may lead to poor detection of species that require different survey parameters. Automatic recording systems (ARS) may be useful for identifying sampling times and environmental variables that can improve detection of target anuran species. ARS are digital or cassette-tape based automated audio recording systems that can be programmed to record a specified interval over a given time period (Peterson and Dorcas 1992). In Chapter 2, ARS were one of four detection methods used to characterize amphibian assemblages at fishless and stocked lakes and vernal pools. In Chapter 4, we used ARS recordings generated from these surveys to determine if the standardized NAAMP protocol detects and accurately describes calling choruses for all anuran species in Maine. The specific objectives of this study were to (1) determine if surveys conducted during the NAAMP-specified time period (0.5 h past sunset to 0100 h) identified all species known to be present and captured the maximum chorus size for that night; (2) describe temporal calling patterns for anurans in central Maine; and, (3) describe environmental variables that predict calling occurrence.
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Chapter 2

INFLUENCE OF WETLAND AVAILABILITY AND CONDITION ON USE BY A VERNAL POOL AMPHIBIAN, AMBystoma MACulatum (SPOTTED SALAMANDER) IN MAINE, USA

Chapter Abstract

Conservation strategies for pool-breeding amphibians usually focus on fixed terrestrial buffers around seasonal wetlands or vernal pools. Current management strategies overlook alternative breeding sites, including permanent wetlands that may support the regional viability of pool-breeding amphibian assemblages. For example, the value of a vernal pool to Ambystoma maculatum (spotted salamander) local persistence may depend on characteristics of the vernal pool as well as landscape setting, including condition and availability of alternative breeding habitats. Our investigation focused on A. maculatum presence and breeding effort in permanent fish-containing (n=5) and fishless (n=7) lakes during 2006-2009 in a relatively undeveloped, forested landscape in eastern Maine, USA to determine effects of breeding site (e.g., hydroperiod, fish presence) and landscape (e.g., wetland area and density) characteristics on breeding dynamics. We used presence and egg mass abundance of A. maculatum as indicators of relative reproductive effort between lake types, and we documented presence of other amphibian species in lakes. We examined growth rates and age of breeding A. maculatum adults among fishless and fish-containing lakes and nearby (within 5 km) vernal pools (n=11) to relate breeding habitat conditions with potential recruitment and fitness characteristics.
Landscape-scale characteristics (e.g., number and area of ephemeral to semi-permanent wetlands within 500 m) were most important for predicting breeding occurrence by vernal pool amphibians (*A. maculatum, Lithobates sylvaticus* [wood frog]) in lakes, whereas lake-scale characteristics (e.g., vegetative cover, fish presence) were better predictors for species (e.g., *L. septentrionalis* [mink frog], *L. pipiens* [northern leopard frog]) associated with permanent waterbodies. *Ambystoma maculatum* breeding effort was greatest in lakes where more typical breeding habitats (e.g., vernal pools, beaver [*Castor canadensis*] impounded flowages) within 500 and 4000 m were less abundant. *Ambystoma maculatum* males (4 – 7 years old) grew faster in fishless than in fish-containing lakes and vernal pools, however, growth rates of females did not differ among habitats. Landscape context affects the use of non-vernial pool breeding habitat by *A. maculatum*. Vernal pool-centric conservation measures may fail to account for connectivity among pools and alternative permanent breeding habitats in maintaining population persistence in long lived species such as *A. maculatum*, in particular where ephemeral wetlands are not abundant.

**Introduction**

Efforts to conserve *Ambystoma maculatum* (spotted salamander) in the glaciated northeastern USA may be improved by considering atypical breeding habitats, including permanent water bodies. Conservation strategies for vernal pool breeding amphibians commonly focus on fixed terrestrial buffers around classic breeding pools (Calhoun 2003) without consideration of alternative, non-ephemeral breeding sites. Although this strategy offers protection to individuals in seasonal breeding pools and their immediate
terrestrial habitat, it does not consider the role of alternative breeding habitats in maintaining amphibian populations, particularly in landscapes where traditional vernal pools may be degraded or lost owing to development or in landscapes with limited wetland area. The importance of alternative breeding sites to pool-breeding amphibian populations may depend on characteristics of individual wetlands as well as the configuration of wetland habitat in the landscape (Baldwin et al. 2006; Cushman 2006; Freeman 2010; Jacobs and Houlanah 2011; Veysey et al. 2011).

*Ambystoma maculatum* breeds in vernal pools (Hunter et al. 1999) and also deposits eggs in beaver (*Castor canadensis*) impounded flowages (Cunningham et al. 2007; Karraker and Gibbs 2009) and in permanent waterbodies with and without fish (Kolozsvary 2003; Egan and Paton 2004). *Ambystoma maculatum* is secure throughout its range, although urbanization (Windmiller 1996), forestry practices (deMaynadier and Hunter 1998), genetic isolation of subpopulations through habitat fragmentation (Gibbs and Reed 2007; Zamudio and Wieczorek 2007), road mortality (deMaynadier and Hunter 2000; Karraker and Gibbs 2011), and loss of vernal pools (Smith 1999) potentially affect this species’ abundance. *Ambystoma maculatum*s’s use of multiple breeding habitats may allow it to persist despite habitat modifications throughout much of its range (Karraker and Gibbs 2009). This species may successfully produce offspring in non-vernal pool habitats by responding behaviorally and morphologically to avoid or minimize stresses (Petranka et al. 1998; Yurewicz 2004; Urban 2007; Shearin 2012 [Chapter 3]). While both landscape- and wetland-scale characteristics influence *A. maculatum* occurrence and reproductive output in vernal pools (Skidds et al. 2007; Veysey et al. 2011), these
relationships and resulting conservation recommendations may not be transferable to other breeding habitats (Cushman 2006).

Landscape characteristics affect *A. maculatum*’s breeding occurrence and reproductive output in ephemeral to semi-permanent wetlands. Pools relatively isolated from other pools or wetlands may contain greater egg mass densities than are found in individual pools within clustered wetlands (Calhoun et al. 2003; Baldwin et al. 2006; Veysey et al. 2011). Greater forest extent, density, adjacency, and age in terrestrial landscapes surrounding wetlands may increase *A. maculatum*’s reproductive output in ephemeral wetlands (Windmiller 1996; Guerry and Hunter 2002; Baldwin et al. 2006; Skidds et al. 2007; Homan et al. 2008). Terrestrial habitat characteristics also may influence body condition and population age structure of breeding *A. maculatum* (Blomquist 2008; Homan et al. 2008). While increasingly more studies examine the role of landscape characteristics on *A. maculatum* population dynamics in classic breeding habitats (e.g., Zamudio and Wieczorek 2007; Veysey et al. 2011), our knowledge of breeding in atypical habitats is largely restricted to within-wetland characteristics (Hecnar and M’Closkey 1997; Egan and Paton 2004; Karraker and Gibbs 2009). Integration of landscape and local-scale characteristics (Cushman 2006) is essential for understanding whether non-vernal pool habitats contribute to *A. maculatum* conservation or act as ecological sinks by drawing breeding individuals away from more productive wetlands (Dimauro and Hunter 2002).

Within-wetland characteristics also affect *A. maculatum* breeding dynamics. Hydroperiod influences breeding habitat selection and reproductive output (Babbitt et al. 2003; Kolozsvary 2003; Babbitt 2005; Baldwin et al. 2006; Vsey et al. 2011) in part by
structuring predator and competitor communities (Colburn 2004; Werner et al. 2007). For example, long hydroperiod wetlands support more drought-intolerant invertebrate (e.g., certain Belostomatids and Dysticids) and vertebrate predators (e.g., Notophthalmus viridescens [eastern newt] [Petranka 1998; Wells 2007]) than do ephemeral wetlands. Fish (e.g., centrarchids [Semlitsch 1988], Lepomis macrochirus and L. cyanellus [sunfish] [Dwyer 2009]; Notemgonus crysoleucas [golden shiner] [Shearin et al., unpublished data]) may be present in permanent wetlands used for breeding, and their presence is associated with lower A. maculatum egg mass densities (Egan and Paton 2004).

Within-wetland vegetation and substrate characteristics also influence breeding effort by A. maculatum and other vernal pool species by providing egg mass attachment sites (Seale 1982; Egan and Paton 2004) and refugia (Formanowicz and Bobka 1989). Abundant littoral vegetation likely provides refugia from predators (Kopp et al. 2006; Hartel et al. 2007; Purrenhage and Boone 2009; although see Porej and Hetherington 2005), and the abundance of woody vegetation and percent vegetative cover is positively associated with increased reproductive output (Egan and Paton 2004). Amphibian assemblages within wetlands also may affect A. maculatum occurrence and abundance, as predation by amphibian larvae and invertebrates in some temporary wetlands may exceed that of permanent wetlands (Petranka and Kennedy 1999; Gunzburger 2004). For example, A. maculatum survival is lower in pools with A. opacum (marbled salamander) and A. jeffersonianum (Jefferson salamander) (Skidds et al. 2007), golf course ponds with L. catesbeianus (Boone et al. 2008), and created wetlands with L. clamitans (Vasconcelos
and Calhoun 2004). However, amphibian assemblages are rarely included as wetland-scale variables in models predicting *A. maculatum* breeding habitat selection.

In Maine, USA, wetlands range from seasonally inundated such as vernal pools to permanent lakes with and without fish. Vernal pools provide optimal breeding habitat for *A. maculatum* and other pool-breeding amphibians that lack chemical or physical defenses against fish predators (Hunter et al. 1999; Calhoun and Klemens 2002). Fishless lakes may provide potential amphibian breeding habitat at the opposite end of the hydroperiod continuum. While fishless lakes co-occur with vernal pools in the eastern coastal region of Maine, it is unknown if these lakes provide alternative breeding habitat for amphibian species palatable to fish, particularly in landscapes with low functional connectivity among breeding sites (Compton et al. 2007; Leibowitz and Brooks 2008) or inter-pool distances above maximum migration thresholds (Gibbs 2000). Furthermore, there are no spatially explicit, multi-scale studies (Cushman 2006) examining landscape and wetland relationships to *A. maculatum’s* breeding in lakes. This study investigates characteristics of breeding habitats used by *A. maculatum* to determine their potential role in landscape-scale conservation of vernal-pool breeding species. Specifically, we investigated the following questions: (1) do breeding site characteristics such as hydroperiod (i.e., permanent or seasonal) fish presence, vegetation cover, and amphibian assemblages affect reproductive effort, size, and age distribution of *A. maculatum*, and (2) does type, density, and number of potential wetland breeding sites (including classic vernal pools and alternative sites) affect *A. maculatum* reproductive effort in each of these breeding habitats?
Materials and Methods

Study Area

We surveyed seven fishless lakes (‘fishless’) and five historically fishless lakes containing introduced fish (‘stocked’) (see Schilling et al. 2008 for detailed study site and faunal descriptions) in the Eastern Coastal Plain and Foothills biophysical regions of Maine (Krohn et al. 1999) during the 2006 to 2009 amphibian breeding seasons (Figure 2.1). Lakes were characterized by permanent hydroperiods and surface areas ranging 1.4 to 10.1 ha. We also selected 11 vernal pools within 5 km of 10 lakes to compare A. maculatum adult reproductive effort, physical characteristics, and age in lakes to seasonal wetlands. Pools were 0.02 to 0.7 ha at the high water mark and dried by the end of August each year. Lakes and vernal pools were surrounded by commercially managed forests of mixed hardwoods (e.g., Acer rubrum [red maple], Fagus grandifolia [American beech]) and softwoods (e.g., Abies balsamea [balsam fir], Picea spp. [spruce], Pinus strobus [white pine]).

Field Methods

Characterization of Potential A. maculatum Breeding Habitats and Surrounding Landscapes.

Landscapes. We mapped lake and vernal pool perimeters on-foot during July 2009 with a backpack-mounted Global Positioning System (Trimble GeoExplorer®, Trimble, Sunnyvale, CA) attached to an external antennae (Trimble® Tempest™, Trimble, Sunnyvale, CA) and lake areas <2 m deep (‘ovipositioning zone’) from canoes with GPS and a handheld sonar (30.5 m range, Fish-Ray™, Manta Company, Portland,
we chose 2 m as our maximum mapping depth, as it captured the maximum ovipositioning depth for most amphibian species in our study area (Gilbert et al. 1994; Hunter et al. 1999; Kolozsvary 2003). We digitized GPS data from the perimeter and ovipositioning zone into nested polygons in ArcMap version 9.3 (Esri®, Redlands, CA) to generate total vernal pool, lake, and ovipositioning zone area.

We characterized aquatic vegetative cover in the lake ovipositioning zone during 13 July-21 August 2009. Vegetation present at this time represents available attachment sites in early spring as well as potential refugia during amphibian larval development. We placed 30-40 (number relative to lake size) quadrats (1 m²) in the ovipositioning zone, visually estimated percent vegetative (submerged, aquatic, emergent) cover within each, and calculated mean percent cover for each site. We also characterized in situ pH
during 11-26 May 2010 with a handheld meter (Orion 230A plus portable pH meter: Thermo Orion, Beverly, MA), as certain amphibian species are more sensitive (e.g., *L. pipiens*) to this characteristic than others (e.g., *A. maculatum*) (Wells 2007). We verified handheld meter pH estimates with closed cell pH estimate for a subset of samples. We calculated mean pH for each lake from samples taken at three random locations 10 cm below the water surface and within one hour of sunrise (Gahl and Calhoun 2010).

We characterized wetland number, type, and area surrounding each lake within 500 m to accommodate maximum dispersal distances for *A. maculatum* (427.6 m [Veysey et al. 2009]; 476 m [Montieth and Paton 2006]) and 4000 m, a conservative distance estimate for genetic connectivity of *A. maculatum* (Zamudio and Wieczorek 2007). We created 500 m and 4000 m buffers around lakes and quantified characteristics from publically available geospatial data (stream length, STRMLENGTH; number of wetlands [‘wetland density’], WLNO; total wetland area, WLAREA; [http://www.maine.gov/megis/](http://www.maine.gov/megis/) within each buffer. We included wetland types extracted from the National Wetlands Inventory (NWI) (Cowardin et al. 1979) that are considered potential breeding habitat for vernal pool species (emergent, PEM; scrub-shrub, PSS; forested, PFO; unconsolidated bottom, PUB) and those with NWI water regime and special modifiers indicating seasonally flooded/saturated (E), semipermanently flooded (F), permanently flooded (H), beaver modified (b), partly drained/ditched (d), diked/impounded (h), and excavated (x) (Cowardin et al. 1979; [http://www.maine.gov/megis/](http://www.maine.gov/megis/)). All spatial analyses were conducted with ArcMap, version 9.3 (Esri®, Redlands, CA).
Characterization of Amphibian Assemblages at Lakes with Breeding *A. maculatum*.

We used automated and listener-based audio surveys, visual encounter surveys (VES), baited trapping, and littoral sweeps with a dipnet two to three times each year during 2006 and 2007 to target Maine’s three amphibian breeding periods (March – April; May; June – July; Maine Amphibian Monitoring Program, www.maineaudubon.org). We noted additional detections from audio, VES, and trap surveys conducted during concurrent studies (Shearin et al., *in press*) at sites during 2008-2009. Survey methods were pooled across years and methods to generate a single presence (1) or absence (0) value for each species and life stage. Observed life stages were divided into two groups: breeding (eggs, larvae, calling adults during breeding season) or other (non-calling adults and juveniles) to determine how amphibians were using the lakes. We also calculated the number of species detected at each site pooled over year, method, and life stage.

Detection methods are described below. The *A. laterale-jeffersonianum* complex was not detected at any lake, and thus was omitted from further analyses.

Automated and Listener-based Audio Surveys. We used a combination of five, tape-based automated audio recording systems (ARS, designed after Peterson and Dorcas 1992) and five digital-based ARS developed at the University of Maine, Orono (Shearin et al., *in press*) to detect calling anurans at each lake. Digital and tape-based ARS were deployed haphazardly among lakes during the duration of our study and programmed to record a two- to three- minute audio clip every hour from 0.5 h past sunset until sunrise (methods described in Shearin et al., *in press*). We supplemented the ARS with listener-based surveys to record calling amphibians for five minutes during a random time between 0.5 h past sunset to 0100 h (NAAMP; http://www.pwrc.usgs.gov/naamp).
**Visual Encounter Surveys.** We detected amphibian species during each of three breeding periods with visual encounter surveys (VES, Crump and Scott 1994) conducted by a single surveyor within lake ovipositioning zones from canoes and via snorkeling and around lake perimeters on-foot.

**Trapping.** Minnow (vinyl-coated, steel mesh, 42 cm long cage with 2.5 cm openings at each end) and crayfish (1 mm plastic mesh, 50 cm long cages with 2.5 cm openings at each end) traps were baited with a dog biscuit, secured near shore to emergent vegetation with floating line, and deployed overnight (12 hours) at each lake to capture amphibian adults and larvae. We deployed two traps per 25 m² of suitable wetland habitat (e.g., littoral zones < 2 m), and an additional trap with each doubling of habitat area (Adams et al. 1997).

**Littoral Sweeps.** We conducted five littoral sweeps with dipnets at up to 10 random locations within the ovipositioning zone at each lake. Two surveyors positioned at the bow and stern of a canoe swept 1.5 m² along the substrate, meeting at the canoe mid-point.

*Ambystoma maculatum* **Physical Characteristics and Age among Breeding Habitats.** We examined effects of breeding habitat on *A. maculatum* body condition and age (Homan et al. 2003) with morphometrics and skeletochronology. We captured adult *A. maculatum* in 2008 and 2009 at fishless lakes (n<sub>2008</sub> = 2, n<sub>2009</sub> = 3), stocked lakes (n<sub>2008</sub> = 4, n<sub>2009</sub> = 4), and vernal pools (n<sub>2008</sub> = 6, n<sub>2009</sub> = 8) during 16 April–7 May 2008 and 17 April–7 May 2009. Adults were captured in minnow traps deployed overnight in areas with historically dense egg mass concentrations, because breeding adults are more
numerous near these regions (Windmiller 1996). We determined sex, snout-to-vent (SVL) length, and mass of each individual.

In 2009, we excised the distal and second phalange from the fourth toe of each captured adult’s right rear foot to determine age and growth rates with skeletochronology, which examines lines of arrested growth (LAG) on cross sections of bone and is a common aging technique for *A. maculatum* (Flageole and LeClair 1992; Homan et al. 2003; Eden et al. 2007) and other amphibian species (Russell et al. 1996; Blomquist 2008). LAGs are formed in bone tissue during periods of slow growth (e.g., winter), with each LAG representing one year of growth. The width of the spaces between these lines (inter-LAG) can be used to compare relative growth rates (Homan et al. 2003). Excised toe specimens were prepared at the University of Maine Veterinary Diagnostic Laboratory (Orono, ME) as per Homan et al. (2003). Up to three sections were mounted per slide, and measurements were made on the clearest section. We captured images of slides at 20x and 40x magnification with SPOT™ Basic Imaging Software (Diagnostic Instruments, Inc.) and a camera-mounted compound microscope. Images were post-processed in ImageJ version 1.43u (W. Rasband, National Institutes of Health, USA) with the enhance contrast and sharpen tools.

We determined age by counting LAGs in digital photos in ImageJ (Figure 2.2). Counts were made independently by two researchers and compared for consistency. The mean number of counts was taken as the estimated age if individual counts were unequivocal. Only lines that appeared continuous for at least half of the bone perimeter cross section were counted. Salamander growth rates may be affected by age, with the fastest growth observed prior to reaching sexual maturity (Eden et al. 2007). To account
for these possible differences, we first separated individuals by sex and then into one of three age categories based on LAG counts (juvenile, 1-3 years; maturing, 4-7 years; adult, ≥ 8 years). Growth rate was assessed for the three outermost inter-LAG areas and represented approximately the previous three years of growth. We measured inter-LAG width at three random locations for each of the three inter-LAGs with the ruler tool in ImageJ and calculated the mean width for each inter-LAG.

*Ambystoma maculatum Reproductive Output in Lakes and Vernal Pools.* We surveyed 12 lakes originally surveyed in 2006-2007 and seven (six stocked, 1 fishless; 0.8 to 5.2 ha) additional lakes within 15 km during 2009. We also surveyed 11 vernal pools within 5 km of the original 12 lakes to compare reproductive effort in classical breeding habitats with lakes. We defined breeding effort as the number of egg masses relative to ovipositioning area in lakes or total area of vernal pools (all vernal pool depths were < 2 m). We quantified egg masses with canoe and on-foot VES conducted twice at all lakes (12 original, seven new) and vernal pools during 21 April – 10 June (peak breeding period for this species). We assigned lakes to one of six geographic regions (REGION). Lakes within a region were within 5 km of one another to encompass genetic relatedness (Zamudio and Wieczorek 2007) and not separated by major rivers or roads.
Figure 2.2. Example of phalange specimen used for skeletochronology. Numbers represent individual lines of arrested growth (LAG) and are equivalent to one year of growth. Arrow shows an example inter-LAG area used to estimate growth rates.

Statistical Analyses

All hypothesis tests were evaluated at $\alpha = 0.05$. All analyses were conducted in R version 2.11.1 (The R Foundation for Statistical Computing) unless otherwise noted.

Amphibian Community Characteristics at *A. maculatum* Breeding Lakes. We predicted within-lake amphibian species richness with Poisson regression models of within lake variables (TYPE, PH, VEGCOVER, MAXDEPTH, LAKEAREA) and
landscape characteristics (WLNO 500, WLAREA 500, STRMLENGTH 500) within 500 m of the lake perimeter (Table 2.1). We did not use 4000 m characteristics in these models to avoid overspecifying our models with too many predictive variables given our small sample size. Relationships among habitat and landscape variables often are species-specific (Cushman 2006), so we created separate logistic regression models for predicting presence of each detected amphibian species based on the same lake and landscape characteristics described for species richness. We described lake size with total lake area rather than ovipositioning area to avoid assumptions regarding species’ use of the lake. We used an information theoretic approach to identify the best model describing species richness and species presence/absence (Burnham and Anderson 2002). We converted model AIC values to QAIC\(_c\) to correct for small sample sizes. Model selection uncertainty was evaluated with \(\omega_i\), and we present only the models for each analysis belonging to the 95% confidence set (Burnham and Anderson 2002). As in similar studies (e.g., Cunningham et al. 2007; Jacobs and Houlanah 2011), we did not apply models described in MacKenzie et al. (2006) to address imperfect detection, because we did not assume equal detection probabilities among sites and therefore may have underestimated site occupancy for certain species.

**Ambystoma maculatum Physical Characteristics and Age among Breeding Habitats.**

Mass and SVL were analyzed separately for each sex with linear mixed models, with random effects specified as trapping site nested within year and ecosystem type (stocked lake, fishless lake, vernal pool) as the fixed effect. Female SVL was square root transformed to meet assumptions of normality. Male SVL and female mass could not be normalized; therefore, we used separate Kruskal-Wallis tests to determine
Table 2.1. Lake- and landscape-scale characteristics used in model building.

<table>
<thead>
<tr>
<th>Variable Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lake characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>TYPE</td>
<td>Stocked (TYPEFS) or fishless (TYPEFL) status as reported by Schilling et al. 2008</td>
</tr>
<tr>
<td>PH</td>
<td>pH of lake determined from 2009 water sampling</td>
</tr>
<tr>
<td>VEGCOV</td>
<td>Mean percent vegetative cover determined during 2009 quadrat surveys in the ovipositioning zone at each lake</td>
</tr>
<tr>
<td>MAXDEPTH</td>
<td>Maximum lake depth obtained from public data (<a href="http://pearl.maine.edu/">http://pearl.maine.edu/</a>)</td>
</tr>
<tr>
<td>LKAREA</td>
<td>Total area of lake determined from 2009 perimeter surveys</td>
</tr>
<tr>
<td><strong>Landscape characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>STRM 500, 4000</td>
<td>Total length (m) of streams within 500 and 4000 m of lake perimeters</td>
</tr>
<tr>
<td>WLNO 500, 4000</td>
<td>Number of potential breeding wetlands (‘wetland density’) within 500 and 4000 m of lake perimeters</td>
</tr>
<tr>
<td>WLAREA 500, 4000</td>
<td>Total area (ha) of potential breeding wetlands within 500 and 4000 m of lake perimeters</td>
</tr>
</tbody>
</table>

differences in these variables among ecosystems. We examined age and growth rate differences among ecosystem types and between hydroperiods (pooled lakes versus vernal pools) with linear mixed models and Kruskal-Wallis tests.

**Landscape Characteristics and Amphibian Assemblage Effects on A. maculatum**

**Reproductive Output in Lakes and Vernal Pools.** We predicted the density of A. *maculatum* egg masses based on lake (TYPE) and landscape variables (STRM500, STRM4000, NOWL500, NOWL4000, WLAREA500, and WLAREA4000) at 500 m and 4000 m with generalized linear mixed models, with lake REGION as the random effect and lake area as the offset. We present the best models remaining in the 95% confidence set. Models were fit and parameters were estimated with restricted maximum likelihood estimation with the REML function in R (Zuur et al. 2009). We examined the effects of random variables and nesting structure with variance estimates and eliminated variables
with small (<0.01) variance estimates. We compared relative egg mass densities (egg masses per m\(^2\) ovipositioning habitat) among lakes and vernal pools with Bonferroni-corrected (\(\alpha = 0.03\)) Kruskal-Wallis tests, as data could not be normalized.

We used K-means cluster analysis (SYSTAT Version 12, SYSTAT Software, Inc.) to separate the original 12 lakes into two groups (A, B) based on amphibian species presence/absence (combined breeding and other occurrences) with Euclidean distances and 100 iterations. *Ambystoma laterale-jeffersonianum* was not present at any lake and was excluded from this analysis. We examined the relationships between amphibian assemblage group and log transformed egg mass density (egg masses per m\(^2\) ovipositioning habitat) with ANOVA.

### Results

#### Amphibian Assemblages in Lakes

*Lithobates catesbeianus* and *L. clamitans* breeding and other life stages were detected at all sites (Table 2.2). We did not create predictive models for breeding *A. maculatum* and *Pseudacris crucifer* and non-breeding life stages of *Hyla versicolor* given their presence at all lakes. Presence of breeding and non-breeding life stages varied for all other amphibian species (Table 2.2). Lake type was the best predictor of species richness, with fewer species detected in fishless (mean ± 1 SE = 7.86 ± 0.51) than in stocked lakes (mean ± 1 SE = 10.00 ± 0.32) (Table 2.3). Lake and landscape
Table 2.2. Species presence (1) and absence (0) by site categorized by life stage (B = breeding [presence of calling adults, eggs, or larvae] and O = Other [presence of non-vocalizing adults and juveniles]).

<table>
<thead>
<tr>
<th>SITE</th>
<th>AMLA</th>
<th>AMMA</th>
<th>ANAM</th>
<th>HYVE</th>
<th>LICA</th>
<th>LICL</th>
<th>LIPA</th>
<th>LIPI</th>
<th>LISE</th>
<th>LSY</th>
<th>NOVI</th>
<th>PSCR</th>
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</table>

*Species names are abbreviated as follows: AMLA = A. laterale-jeffersonianum complex, AMMA = A. maculatum, ANAM = A. americanus, HYVE = H. versicolor, LICA = L. catesbeianus, LICL = L. clamitans; LIPA = L. palustris; LIPI = L. pipiens; LISE = L. septentrionalis; LISY = L. sylvaticus; NOVI = N. viridescens, and, PSCR = P. crucifer.
characteristics affecting breeding presence differed by species (Table 2.3). Lake characteristics were the strongest predictors of breeding and non-breeding life stages for most species, whereas, landscape characteristics were the best predictors for species with terrestrial adult stages (L. palustris, and L. sylvaticus and non-breeding A. maculatum [Table 2.3]).

Ambystoma maculatum Physical Characteristics and Age among Breeding Habitats

We captured more males (N_{Total}=156, N_{Fishless}=32, N_{Stocked}=66, N_{Vernalpool}=58) than females (N_{Total}=81, N_{Fishless}=20, N_{Stocked}=27, N_{Vernalpool}=34) in all ecosystems during both 2008 and 2009. There were no mass differences among ecosystems for males (model –LL=-419.06, P_{fishless-stocked}=0.30, P_{fishless-vernepool}=0.32) or females (Kruskal-Wallis $X^2$=3.87, df=2, P=0.14). SVL also did not differ among ecosystems for males (Kruskal-Wallis $X^2$=2.42, df=2, P=0.30) or females (LL=-34.63, P_{fishless-stocked}=0.74, P_{fishless-vernepool}=0.95). Female age did not differ among ecosystems (Kruskal-Wallis $X^2$=0.58, P = 0.75) or hydropools (Kruskal-Wallis $X^2$=0.12, P = 0.73). Male age also did not differ among ecosystems (Kruskal-Wallis $X^2$=0.36, P = 0.84) or hydropools (Kruskal-Wallis $X^2$=0.21, P = 0.65). Inter-LAG widths were correlated for both sexes (P < 0.001 for all Pearson-product moment correlations). We analyzed only the middle inter-LAG area as this was the growth area least likely affected by endosteal resorption (Eden et al. 2007) while also representing a full year of growth. Female growth rate was not affected by ecosystem type (linear mixed effect $t_{stocked}=-0.74$, $P_{stocked}=0.48$; linear mixed effect $t_{vernepool}=-0.74$, $P_{vernepool}=0.10$), however, growth rates were 30 and 52% slower, respectively, for females in the sexually maturing (linear mixed effect $t=-2.65$, P
Table 2.3. Results of stepwise Poisson and logistic regression models for species richness and presence/absence of breeding and non-breeding (other) life stages by species. The 95% confidence set is shown for each set of models. For *N. viridescens*, only one model is shown owing to overspecification in models containing additional variables. Not all species and life stages could be modeled owing to few detections or occurrences at all sites.

<table>
<thead>
<tr>
<th>Variables by species</th>
<th>$B$</th>
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= 0.01) and adult (linear mixed effect $t = -3.58, P = 0.001$) categories than the juvenile category. Maturing male growth rates were 27 and 30% slower, respectively, in vernal pools (linear mixed model $t = -2.48, P = 0.03$) and stocked lakes (linear mixed model $t = -2.55, P = 0.03$) than in fishless lakes, but did not differ among age categories (linear mixed model $t = 1.78, P = 0.08$) (Figure 2.3).

**Landscape Characteristics and Amphibian Assemblages Effects on A. maculatum**

**Reproductive Output in Lakes**

Although several landscape characteristics (wetland area within 500 and 4000 m, number of wetlands within 500 and 4000 m, and stream length within 4000 m) were correlated (Table A.1), we included all variables in model building because no one variable was strongly correlated with all others. Total *A. maculatum* egg mass density in lakes was negatively affected by number of potential breeding wetlands at 500 and 4000 m ($\omega_t = 0.77, LL = -33.64, K = 4, QAIC_c = 78.14, \Delta_i = 0.00$). A competing model also indicated a positive effect of stream length at 500 m on egg mass density ($\omega_t = 0.20, LL = -33.11, K = 5, QAIC_c = 80.84, \Delta_i = 2.70$). Egg mass density was eight times greater in vernal pools (0.09 ± 0.03 mean egg masses per m$^2$ ovipositioning area) (Kruskal-Wallis $X^2 = 8.6, df = 2, P = 0.01$) than lakes. Egg mass density was similar between stocked (0.01 ± 0.003 mean egg masses per m$^2$ ovipositioning area) and fishless (0.01 ± 0.005 mean egg masses per m$^2$ ovipositioning area) lakes (Kruskal-Wallis $X^2 = 0.53, df = 1, P = 0.46$).
Figure 2.3. Differences in inter-LAG width for males by ecosystem type and life stage (juvenile (< 4 years); maturing [4 – 7 years]; and, adult (> 7 years old)). Significant differences among ecosystem types at P < 0.05 are marked with asterisks. Sample sizes are indicated above each bar.

Amphibian assemblage Group A contained five lakes (all fishless) and was characterized by no occurrences of *A. americanus* and *N. viridescens* and only one occurrence of *L. pipiens*. Group B contained the remaining seven lakes (all stocked lakes; two fishless lakes) and was characterized by *N. viridescens* at all lakes, two occurrences of *A. americanus*, and six occurrences of *L. pipiens*. There was no effect of assemblage group on the log density of *A. maculatum* egg masses ($F_{(1, 10)} = 2.80, \ P = 0.13$).

**Discussion**

Our study is the first to relate breeding habitat and landscape scale characteristics to breeding effort, relative fitness, and amphibian community assemblages in larger,
permanent lakes occurring within landscapes with ephemeral breeding habitat; other
studies have reported breeding habitat and landscape scale predictors of *A. maculatum*
abundance in ephemeral pools (Regosin et al. 2005; Harper et al. 2008; Windmiller et al.
2008; Veysey et al. 2011). *Ambystoma maculatum* breeding effort in non-vernal pool
habitat in urbanizing (Veysey et al. 2011) and agricultural (Guerry and Hunter 2002)
landscapes is determined by characteristics of the landscape rather than within-wetland
features. We found a similar relationship between landscape-scale variables (wetland
number and area) and *A. maculatum* breeding effort in the fishless lakes, stocked
historically fishless lakes, and vernal pools in our study landscape. Although breeding
habitat characteristics (e.g., fish presence) also potentially affect population fitness and
productivity (Cushman 2006; Homan et al. 2008), our study supports the need for
landscape-scale and spatially explicit conservation approaches (Cushman 2006),
particularly for regions facing forest conversion to residential and commercial
development (Stein et al. 2005; White and Mazza 2008).

**Effects of Within-lake Characteristics on Amphibian Assemblages**

*Ambystoma maculatum* presence and breeding effort were not affected by within-
lake characteristics such as fish presence, lake area, depth, or amphibian assemblage.
Assemblages of amphibian species that traditionally breed in non-ephemeral habitat were
relatively similar in fishless and stocked lakes, although two species (non-breeding *N.
viridescens* and breeding *L. pipiens*) were more likely to occur with fish. *Notophthalmus
viridescens* adults are unpalatable to fish (Gill 1978; Rohr et al. 2002), although certain
fish species may exclude this species from ponds owing to competition for prey resources
(Bristow 1991; Smith et al. 1999). *Notophthalmus viridescens* breeding was associated with larger lakes in our study, which may optimize density-dependent growth and survival (Petranka 1989) and reduce larval cannibalism (Harris 1987). *Lithobates pipiens* breeding also was positively associated with fish presence. Egg masses are relatively resistant to fish predation (Salthe 1963; Grubb 1972), and fish presence may reduce the abundance of superior competitors such as *L. sylvaticus* (Werner and Glennemeier 1999). Fishless lakes in our study contained greater densities of several odonate families (e.g., Aeshnidae) (Schilling et al. 2009a) that induce defensive morphological responses in *L. pipiens* larvae (Relyea and Werner 2000). Fish presence was not linked to presence of other short-lived anurans (e.g., *P. crucifer* [Babbitt et al. 2003, Wells 2007]) in our study lakes.

Greater maximum lake depth was positively associated with breeding and non-breeding *L. septentrionalis* presence. Males call while floating in deep water (Bevier et al. 2004) and are positively associated with lakes >1.5 ha (Popescu and Gibbs 2009). Lake area in our study was not correlated with maximum lakes depth, so availability of deepwater habitat rather than lake area may drive site selection by *L. septentrionalis* in our region.

*Lithobates catesbeianus* and *L. clamitans*, two potential *A. maculatum* predators (Boone et al. 2008), were present in all lakes, with *L. catesbeianus* more abundant in stocked lakes, and *L. clamitans* more abundant in fishless lakes. *Lithobates catesbeianus* larvae are less susceptible to fish predation and are superior competitors compared to *L. clamitans* when fish are present (Wells 2007). While amphibian assemblage presence did not affect *A. maculatum* breeding effort in this study, co-occurring species abundance
may affect this metric. For example, increasing *L. sylvaticus* egg mass density is associated with an increased proportion of white to clear *A. maculatum* egg masses (Petranka et al. 1998), and greater densities of *A. opacum* reduce *A. maculatum* larval survival (Stenhouse et al. 1983). Additional studies are needed to determine how abundance of co-occurring amphibian predators and competitors affects *A. maculatum* breeding effort in lakes.

**Landscape-scale Predictors of *A. maculatum* Breeding Effort and Amphibian Assemblages**

Wetland number and area in the focal landscape was related to *A. maculatum* breeding effort in our study, suggesting that the role of lakes in maintaining amphibian diversity and population persistence in a landscape may be affected by availability of wetlands that potentially are used for breeding. *Ambystoma maculatum* egg mass density was negatively associated with wetland area within 500 and 4000 m of lakes, suggesting that lakes distant from ephemeral wetlands support larger breeding populations. This trend may be driven by limited availability of alternative breeding habitats in landscapes with few vernal pools (Calhoun et al. 2003), a relationship also reported for isolated vernal pools in Maine (Baldwin et al. 2006) and New Hampshire (Veysey et al. 2011). In our study landscape, the probability that a lake contains ≥ 20 egg masses, the criteria for determining regulatory jurisdiction for vernal pools in Maine (Natural Resources Protection Act, Title 38 Maine Revised Statutes Annotated §§ 480-A, Chapter 335, Section 9) was positively associated with wetland area in 4000 m and negatively associated with wetland density at this same distance. Larger breeding populations of *A.
*maculatum* among all potential breeding habitats would be expected in landscapes with abundant ephemeral wetland area, whereas alternative breeding habitats (e.g., lakes) would receive greater use in landscapes where vernal pools are more scarce.

Breeding probability of *L. sylvaticus*, another species known to breed in short-hydroperiod habitat, was negatively associated with increasing wetland area within 500 m. Although this species was less likely than *A. maculatum* to breed in lakes in our study landscape, they do breed in natural waterbodies containing fish (Hecnar and M’Closkey 1997; Cunningham et al. 2007; Karraker and Gibbs 2009). *Lithobates sylvaticus* colonize new breeding sites in response to changes in habitat quality in natal ponds (Hopey and Petranka 1994; DiMauro and Hunter 2002; Petranka and Holbrook 2006; Patrick et al. 2008); occurrence of breeding *L. sylvaticus* in lakes may indicate fewer available alternative breeding habitats or reduced habitat quality in natal pools.

Landscape-scale characteristics were better predictors than within-lake conditions of *L. palustris* and *H. versicolor* presence; these two species typically breed in permanent wetlands. Probability of *L. palustris* breeding was greater in lakes with less wetland area within 500 m, perhaps reflecting fewer breeding (e.g., well-connected beaver-modified wetlands [Cunningham et al. 2007]) or foraging (e.g., wetland edges, streams, and beaver meadows [Gibbs 1998; Hunter et al. 1999]) habitats in these landscapes. Non-breeding *L. palustris* were associated with less vegetated cover within ponds, consistent with foraging habitat preferences in a similar species (*L. pipiens*) we encountered in both lake types (Pope et al. 2000). *Hyla versicolor* was ubiquitous among our study lakes, and breeding probability was associated with greater density of wetlands within 500 m of lakes (Price et al. 2005). Since lakes in our study are surrounded by mostly
unfragmented landscapes, greater wetland density likely supports relatively large *H. versicolor* metapopulations (Brodman 2008).

**Linkages among Breeding Habitats and Landscape Characteristics**

*Ambystoma maculatum* populations are regulated by interactions in aquatic and terrestrial habitats (Windmiller 1996), and dissimilar growth rates for individuals among lakes and vernal pools may indicate differences in breeding or terrestrial habitat quality. In stocked lakes, fish introductions may affect both wetland and terrestrial habitat quality by reducing transfer of invertebrate biomass to terrestrial ecosystems, which limits prey resources available to terrestrial vertebrate predators (Finlay and Vrendenberg 2008). Fewer terrestrial prey resources contribute to longer travel distances for successful foraging (Anholt et al. 2000), which may result in slower growth. Slower growth rates in vernal pool-breeding adults may be attributed to density-dependent intra-specific interactions in the aquatic (Walls 1998; Brodman 1999) and terrestrial environments (Regosin et al. 2003, 2004; Harper and Semlitsch 2007) owing to larger breeding populations (using egg mass counts as a proxy for population size [Crouch and Paton 2000; Egan and Paton 2004]) in these systems.

The maximum initial stocking time for lakes in our study was ~50 years before our study (DeGoosh 2008). The *A. maculatum* lifespan exceeds 20 years (Flageole and LeClaire 1992), but the median ages for males and females in our study were five and four, respectively. We did not observe differences in median age *A. maculatum* among lakes and vernal pools. Greater energy allocation to reproduction may explain the slower growth rates observed in sexually maturing and adult female salamanders (Eden et al.
2007) across our study systems, and this allocation also may obfuscate any potential differences in growth rates among ecosystems. Our sample size for *A. maculatum* in fishless lakes was small (*n* _juveniles_ = 0; *n* _maturing_ = 9; *n* _adults_ = 1), and thus we may not have fully captured the age distribution for this ecosystem type. Only 3% of our samples were ≥ 10 years old, so it is unlikely that endosteal resorption led to age underestimates in the majority of our samples.

**Conservation Implications**

Conservation efforts for *A. maculatum* typically focus on vernal pools within terrestrial buffers of a fixed width (e.g., Calhoun et al. 2003; Oscarson and Calhoun 2008; Windmiller et al. 2008). Our results highlight the need for a management approach to amphibian conservation from a landscape perspective that simultaneously considers the role of a variety of wetland types, landscape setting, and within-wetland characteristics. Alternative wetland types, such as beaver-modified wetlands (Cunningham et al. 2007; Karraker and Gibbs 2009), may provide potential breeding habitat for amphibians typically associated with ephemeral wetlands. Longer hydroperiod wetlands may provide a bet-hedging strategy for maintaining local amphibian populations in years of drought (Kolozsvary 2003). Production from permanent wetlands proximal to vernal pools may provide sources of colonizers to avoid local extinctions (Gibbs 2000; Karraker and Gibbs 2009). A multi-scale conservation approach potentially maintains connectivity among these breeding habitats (Semlitsch and Bodie 1998; Rothermel 2004; Cushman 2006; Knapp et al. 2007).
Habitat destruction is a leading cause of amphibian declines worldwide (Beebee and Griffiths 2005), and understanding how anthropogenic-modified wetlands affect and are affected by system-wide characteristics is key to conserving critical habitat. Fish introductions to historically fishless lakes in Maine may affect habitat quality for native amphibians. Approximately two generations of *A. maculatum* have passed since fish stocking began, and while within-lake responses to fish stocking may be observed within several years, landscape-scale and metapopulation changes may occur over many decades (Knapp and Matthews 2000; Pilliod and Peterson 2001) and large spatial scales (Petranka and Holbrook 2006). High natal site fidelity (Vasconcelos and Calhoun 2004) coupled with presence of juvenile *A. maculatum* in all three systems suggests that juvenile recruitment is occurring in these lakes. *Ambystoma maculatum* recruits from permanent wetlands may contribute to landscape-scale genetic diversity, particularly given its ability to disperse over large areas (Zamudio and Wieczorek 2007). Additional studies are needed to determine if larval recruitment rates sufficiently compensate for lower embryonic and larval survival in stocked lakes and to describe effects of fish introductions on aquatic-terrestrial energy dynamics that could affect juvenile and adult terrestrial habitat quality.

**Chapter Acknowledgements**

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Chapter 3

BREEDING HABITAT AFFECTS SPOTTED SALAMANDER (AMBYSTOMA MACULATUM) OVIPOSITIONING CHOICE, EGG MASS MORPHOLOGY, AND EMBRYO SURVIVAL

Chapter Abstract

Ambystoma maculatum Shaw (spotted salamander) breeds in seasonal wetlands (i.e., vernal pools) as well as in wetlands with semi-permanent to permanent hydroperiods including beaver flowages, slow-moving streams, agricultural ponds, and lakes. *Ambystoma maculatum* commonly are reported breeding in semi-permanent to permanent waters, however, strategies for successful reproduction and recruitment under increased pressure of predation are not well known. Maintaining viable local populations of *A. maculatum* in breeding habitats with permanent hydroperiods may require alternative breeding strategies. Ability to successfully recruit young in these breeding habitats may be particularly important in landscapes with low vernal pool density.

We examined female *A. maculatum* ovipositioning behavior, egg mass morphology, and embryo survival during 2006-2010 in ephemeral (ten vernal pools) and permanent waterbodies (seven fishless lakes and five stocked but naturally fishless lakes) in Maine, USA, and in laboratory experiments. Egg masses in permanent hydroperiod habitats (lakes) were oviposited at greater depths within the available water column than in vernal pools and were deposited closer to shore in stocked lakes than in fishless lakes or vernal pools. Egg masses and hatching larvae were approximately 13 and 33%, respectively, larger in vernal pools than in lakes. Survival of *A. maculatum* embryos to
hatching while exposed to in situ predation was approximately 180% higher in vernal pools than both lake types. Lakes potentially provide alternative breeding habitat for A. maculatum in landscapes with few or poor quality vernal pools; however, vernal pools are the optimal breeding habitat for this species in our study landscape.

**Introduction**

In northeastern North America, *Lithobates sylvaticus* (wood frogs), the *Ambystoma laterale-jeffersonianum* complex (blue-spotted salamander complex), and A. maculatum (spotted salamanders) typically breed in vernal pools, and these seasonal wetlands often are cited in management plans or regulations as optimal breeding habitat for these species (Calhoun et al. 2003; 2005). Vernal pools may be limiting in some landscapes, and vernal pool-associated amphibians may breed in alternative habitats including created pools (Vasconcelos and Calhoun 2006; Petranka et al. 2007; Korfel et al. 2010), beaver flowages (Cunningham et al. 2007; Karraker and Gibbs 2009), roadside ditches or skidder ruts (DiMauro and Hunter 2002), and permanent ponds (Hecnar and M’Closkey 1997; Egan and Paton 2004). Typically, indicator species enjoy high recruitment success in vernal pools owing to the reduced mortality of eggs and larvae from fish and invertebrates more commonly associated with permanent waters and absent from pools (Petranka 1998; Colburn 2004; Vasconcelos and Calhoun 2006). Yet production from “alternative” (i.e., non vernal-pools) breeding habitats may contribute significantly to local genetic diversity and juvenile recruitment (Karraker and Gibbs 2009); however, strategies for success in permanent waters are not well understood.
Hydroperiod, or the duration of inundation, is a key factor shaping amphibian and invertebrate communities across wetland types (Stokes and McPeek 2003; Colburn 2004; Urban 2004; Babbitt 2005). Hydroperiod shapes amphibian (Babbitt 2005) and invertebrate (Colburn 2004) communities in fishless ephemeral wetlands, whereas, top predators such as fish affect amphibian (Wilbur 1987; Skelly 1997) and macroinvertebrate (Stokes and McPeek 2003) population structure in permanently inundated habitats. Species that successfully breed in wetlands along a hydroperiod gradient may require multiple strategies for avoiding or resisting predation by diverse predator assemblages.

Although characterized by permanent hydroperiods, fishless lakes provide potential breeding habitat for amphibians that lack defenses against fish predators. Larvae of *L. sylvaticus* (wood frogs) and *L. pipiens* (leopard frogs), for example, occur in permanent lakes lacking predatory fish in Ontario, Canada (Hecnar and M’Closkey 1997). It is unknown, however, how predation of fish-vulnerable amphibian species differs among fishless vernal pools and fishless lakes. Vernal pool invertebrate species typically shift to different but closely related species along the hydroperiod gradient from ephemeral to semi-permanent pools (Wiggins et al. 1980; Williams 1997; Stoks and McPeek 2003; Colburn 2004). Consequently, similar invertebrate communities may inhabit semi-permanent vernal pools and fishless lakes; however, fishless lakes support drought-intolerant predators (e.g., certain Belostomatids and Dysticids) not typically found in periodically drying pools (Colburn et al., 2008). On the other hand, predation by invertebrates and amphibian larvae in seasonal wetlands may exceed that of permanent wetlands for some species (Petranka and Kennedy 1999; Gunzburger 2004).
Therefore, it is unclear if fishless lakes provide suitable breeding habitat for species palatable to fish that require a relatively long hydroperiod for larval development.

The *A. maculatum* embryonic stage may incur the greatest cumulative predation of all life stages owing to the duration of embryo development (up to eight weeks in Maine; Smith 1999) and phenology of co-occurring predators also undergoing metamorphosis. In both ephemeral and permanent breeding habitats, trichopteran larvae (e.g., *Ptilostomis* spp.) are active in early spring (Wiggins 2005) and may completely consume *A. maculatum* embryos within 24 hours of egg mass deposition (Kolozsvary 2003; A.F. Shearin, personal observation) before other egg mass predators become active (Rowe et al. 1994). In prey-limited ephemeral wetlands, early-hatching *L. sylvaticus* larvae prey on *A. maculatum* embryos (Petranka et al.1998). *Lithobates clamitans* and *L. catesbeianus* overwinter as larvae and may be abundant in both permanent waterbodies (Cunningham et al. 2007) and semi-permanent ephemeral wetlands (Vasconcelos and Calhoun 2006) in early spring. *Lithobates clamitans* consumes up to 100% *L. sylvaticus* embryo in long hydroperiod created wetlands in Maine (Vasconcelos and Calhoun 2003), and may similarly consume *A. maculatum* embryos. Fish also are present in early spring in lakes containing persistent fish populations. Wetlands containing abundant embryo predators may become biological sinks for *A. maculatum* populations.

Unlike other vernal pool breeding amphibians in the northeastern United States, *A. maculatum* is largely secure throughout its range (excluding Delaware, New Jersey, and Oklahoma, Hammerson 2004). While other pool-breeding species actively avoid ovipositing in water bodies with fish (e.g., *A. barbouri* [Kats and Sih 1993] and *L.
sylvaticus [Hopey and Petranka 1994]), A. maculatum breeds in wetlands with fish (Ireland 1989; Sexton et al. 1994; Egan and Paton 2004), beaver flowages (Cunningham et al. 2007), and permanent lakes (Shearin et al., unpublished data). However, reproductive output usually is greatest in fishless, long-hydroperiod seasonal pools (Kolozsvary 2003; Baldwin et al. 2006; Skitts et al. 2007). Ambystoma maculatum’s use of multiple breeding habitats may enable its persistence, and variable breeding strategies may be necessary for successful reproduction in wetlands along the hydroperiod gradient.

Variable ovipositioning strategies among breeding wetlands may confer different survival rates to offspring. For example, A. maculatum eggs laid in groups at shallow depths have greater survival than those laid singly at greater depths (Brodman 1995); however, eggs laid at shallow depths risk greater mortality owing to freezing (Ireland 1989). Breeding females may concentrate egg masses in northern portions of breeding habitats to maximize solar heating (Windmiller 1996) thereby reducing the duration of embryonic development and exposure to predators. Ovipositing in or adjacent to abundant littoral vegetation also provides refugia that may mediate effects of predators (Formanowicz and Boba 1989; Egan and Paton 2004; Kopp et al. 2006; Hartel et al. 2007). It is unknown, however, if A. maculatum alters ovipositioning strategy in response to different predator assemblages and whether these strategies are effective in reducing predation of embryos. For example, in breeding habitats with abundant visual predators, we might expect A. maculatum to cluster egg masses at the water surface, similar to communal L. sylvaticus egg mass clusters (Seal 1983).

Ambystoma maculatum egg mass morphology also may enhance embryo survival to hatching. Egg masses are surrounded by a protective mucoid envelope that reduces
predation by fish (Ward and Sexton 1988; Semlitsch 1988; Ireland 1989) and risk of
desiccation (Nyman 1987). Egg masses may be white, clear, or intermediate owing to
inherited protein composition in the outer envelope (Hardy and Lucas 1991; Ruth et al.
1993; Petranka 1998), and the effects of egg mass color on predation may be taxa-
dependent (Rowe et al. 1994; Petranka et al. 1998). Clutch size (number of eggs per
mass) is greater in ponds with predatory fish than fishless ponds and ponds with non-
predatory (small) fish (Hecnar and M’Closkey 1997), perhaps as a predator-swamping
response. While these morphological variations have been reported separately for vernal
pools and small ponds, there are no explicit and simultaneous comparisons of variations
among breeding habitats within a region, nor is there a comprehensive understanding of
how morphological plasticity is influenced by predator assemblages.

*A. maculatum* breeds in lakes and other non-vernal pool wetlands, however, it is
unclear if this species’ ovipositioning choices or morphological adaptations in egg
masses maximize its breeding success among habitats with different hydroperiods and
predator assemblages. Alteration of egg mass morphologies and ovipositioning strategies
may increase survival from predation in long hydroperiod habitats, or, conversely, these
habitats may become biological sinks for local populations that lose annual reproductive
effort to predators. We examined *A. maculatum* ovipositioning strategies, egg mass
morphology, and embryo survival among wetlands differing in hydroperiod and predator
assemblages in Maine to reveal potential mechanisms for successful breeding among
multiple habitats. Our objectives were to (1) identify differences in oviposition site
selection among three potential breeding habitats (vernals pools, fishless lakes, and
stocked lakes) for *A. maculatum*, (2) identify differences in egg mass morphological
characteristics among breeding habitats, and, (3) determine in situ embryo survival rates when predators are allowed and excluded from developing egg masses.

**Materials and Methods**

**Study Area**

All field studies were conducted at seven naturally fishless lakes, five stocked lakes, and ten vernal pools found within 5 km of naturally fishless and naturally fishless but stocked lakes in the Eastern Coastal Plain and Foothills biophysical region of Maine, USA, (Krohn et al. 1999) (Figure 3.1) during April-May 2006-2009. We selected a subset of lakes previously surveyed for invertebrate and fish community composition (see Schilling et al. 2008, 2009 for descriptions of fish and invertebrate assemblages). Lakes were characterized by permanent hydroperiods and surface areas ranging 1.4-10.1 ha. Vernal pools ranged 0.02-0.7 ha at the high water mark and typically dried by the end of August. Lakes and vernal pools were located in largely unfragmented commercial forests dominated by *Abies balsamea* (balsam fir), *Acer rubrum* (red maple), *Picea rubens* (red spruce), and *Pinus strobus* (Eastern white pine).

**Field and Laboratory Methods**

**Site Characteristics.** We characterized vegetation and substrate characteristics within the ‘ovipositioning zone’ (defined here as all portions of lakes < 2 m in depth and the entire extent of vernal pools) for lakes and vernal pools during 13 July-21 August 2009. While *A. maculatum* typically oviposit egg masses before full leaf-out, distribution
Figure 3.1. Locations of fishless lakes (n=7), stocked lakes (n=5), and vernal pools (n=10) in Hancock and Washington Counties, Maine (USA). Sites are denoted with circles. Not all sites appear as individual circles owing to the close proximity of some sites.

of woody and emergent patches remained consistent at lakes and vernal pools during our study, so vegetation present in summer was indicative of vegetation available for ovipositioning in early spring. Vegetation sampling during summer allowed us to characterize both available attachment sites in early spring as well as potential refugia during larval development. Quadrats (1 m$^2$) were placed haphazardly in vernal pools (5-15 quadrats per pool) and lakes (30-40 quadrats per lake), in the ovipositioning zone. We identified and estimated percent cover for each live and dead vegetative species within quadrats and categorized each species as submerged/aquatic, emergent, woody, or sphagnum. We estimated the portion of quadrat substrates covered by boulders, gravel, leaf litter, organic matter, sand, submerged logs, or submerged sticks, and grouped these
as potential sites for amphibian ovipositioning (submerged logs, submerged sticks) or non-ovipositioning habitat (boulders, gravel, leaf litter, organic matter, sand). We quantified the percent cover of floating sticks and logs within each quadrat, as these also provide ovipositioning sites. A single surveyor estimated percent cover using a training set of known coverages to maintain consistency among quadrats and sites.

We collected lake and vernal pool chemical characteristics associated with *A. maculatum* reproductive output and embryo survival (pH [Portnoy 1990; Rowe and Dunson 1993], water temperature [Karraker et al. 2008], dissolved oxygen [Karraker et al. 2008], and apparent color) during 11-26 May 2010 from 0600 to 0800 h. We took *in situ* measurements with handheld meters (pH and water temperature: Orion 230A plus portable pH meter: Thermo Orion, Beverly, MA; dissolved oxygen: Hanna Instruments HI9143) 10 cm below the water surface, at least 1 m from vegetation, and within 2 m of shore at three random locations per lake or vernal pool. We determined apparent color on three collected water samples per site with an Orbeco-Hellige Aqua Tester (Orbeco Analysis Systems, Farmingdale, NY). We chilled water samples while particles settled and determined apparent color within 24 hours of collection.

**Egg Mass Oviposition Characteristics.** We characterized ovipositioning sites for *A. maculatum* egg masses in each of the fishless lakes, stocked lakes, and vernal pools, during spring 2006 – 2009 visual encounter surveys (Shearin 2012 [Chapter 2]). For each egg mass found, we measured egg mass depth, total depth of the water column at the ovipositioning site, and distance to shore and counted egg masses (‘group size’) oviposited within 1 m² around each egg mass. We visually estimated percent terrestrial and aquatic vegetative cover above egg masses by category: none; 1-25%; 26-50%; 51-
Observers calibrated their cover estimates with a training set of known cover percentages before each survey. We assigned a degree location referenced to the lake (2007, 2008) or vernal pool (2008) center for each egg mass. We also noted the egg mass attachment site and dominant (>30% surface area) vegetation (submerged/aquatic, emergent, woody, or sphagnum) and substrate (boulders, gravel, leaf litter, organic matter, sand, submerged logs, or submerged sticks) category for 1m² around each egg mass.

**Egg Mass Morphology.** We documented *A. maculatum* egg mass morphology *in situ* for egg masses at all sites in 2008 and 2009 either from canoes or by snorkeling. We recorded the longest radius of each egg mass and a second radius perpendicular to the first, and we used ocular counts to estimate number of embryos. Ocular embryo estimates were correlated with ovagram counts (see Karraker 2007) of 24 egg masses (Pearson’s product moment correlation: \( t = 8.82, \text{df} = 22, p < 0.001 \)), thus ocular estimates were used for all subsequent embryo counts. We inserted a pin into the outer envelope at three positions on the egg mass, and we measured the distance between the outer envelope wall and the outermost egg to the nearest 0.25 cm to determine if envelope thickness differed by breeding habitat. Mean egg mass thickness was categorized by 0.25 cm increments (e.g., category 1=0 to 0.24 cm, 2=0.25 to 0.49 cm, etc.). Egg masses were classified as either cloudy or clear.

We deployed minnow traps at all stocked lakes and vernal pools and five of seven fishless lakes during early April to early May in 2008 and 2009 to capture breeding female *A. maculatum*, as egg mass size may be confounded by female body size in the breeding population (Cunnington and Brooks 2000). Traps were not deployed at two
fishless lakes owing to landowner-restricted road access during April. We checked minnow traps daily for three to five nights at each site once we observed the first egg masses.

**In situ Egg Mass Predator Exclusion Experiments.** We randomly selected three to six freshly deposited (within 72 hours), clear *A. maculatum* egg masses per site with no evidence of predation from each of five fishless and five stocked lakes and seven vernal pools in April 2009. We recorded morphological measurements and ovipositioning site characteristics for each egg mass and randomly assigned each to control, closed, or open treatments. Control treatment egg masses were returned to their original attachment site following morphological measurements, whereas egg masses in the open and closed treatments were placed inside a mesh box (two 0.5 liter plastic berry baskets inverted and held together with cable ties) and enclosed with mosquito netting (1 mm gauge, 200 holes per 5.5 cm$^2$). Closed treatment mosquito netting excluded predators >1 mm diameter access to egg masses. Two, 10 cm holes were cut in the mosquito netting in the open treatments to allow access by larger predators. The open treatment was designed to test for potential effects of the basket on embryo survival while allowing predation. Baskets were affixed with string to the original attachment site.

We monitored masses weekly for embryo development, and we enclosed the egg masses with intact netting just prior to hatching to prevent larvae from leaving the mass. The number of hatched larvae, unhatched live embryos, and unhatched dead (embryos with discolored egg sacs or lacking movement) embryos were quantified at the end of the experiment during 15-19 June when 75% of embryos in each mass had hatched or were missing from the mass and assumed predated. We recorded the number of predators
present in each egg mass and enclosure at the end of the experiment. Three hatched larvae were collected from each mass, transported on dry ice, and stored them in a -20°C Celsius freezer until they were freeze-dried (Labconolymph-Lock 4.5 Freeze Dry System, Labconco Corporation, Kansas City, MO) for 24 hours and weighed. We used freeze drying to determine larval mass upon hatching (Cunnington and Brooks 2000), as this method allowed us to transfer specimens from the field to the laboratory without altering mass through desiccation or absorption of preservatives.

**Statistical Analyses**

All statistical analyses were conducted in R version 2.11.1 and Systat version 12 and evaluated at alpha = 0.05 unless otherwise noted.

**Site Characteristics.** We tested for vegetation and substrate differences among lake and vernal pool ovipositioning habitats with multi-response permutation procedures (MRPP) (McCune and Grace 2002) at two levels: among breeding habitats (stocked lakes, fishless lakes, vernal pools) and between lake types. We tested for differences among ecosystems with ANOVA (α = 0.1; Schilling et al. 2009) and the n/sum(n) weighting factor and Sorensen (Bray-Curtis) distance measure.

We determined relationships among vegetation and substrate categories among the three breeding habitats and between lake types with non-metric multidimensional scaling (NMDS) (McCune and Grace 2002), which does not assume normality or linearity among variables (McCune and Grace 2002). We used autopilot mode (‘slow and steady’) with 250 runs on actual data and 250 runs on randomized data, and we determined the number of axes retained with skree plots. We tested for differences
among NMDS scores on retained axes for ecosystem and lake types with ANOVA (α = 0.1). All analyses were performed in PCORD version 5.0 (MjM Software, Gleneden Beach, OR).

We determined multicollinearity among water quality characteristics with Pearson’s product moment correlations and tested for ecosystem effects on each characteristic with individual linear models. Dissolved oxygen and apparent color were rank and log transformed, respectively, to meet assumptions of normality.

**Egg Mass Oviposition Characteristics.** We compared estimated percent vegetative cover in the water above egg masses, egg mass distance from shore, egg mass depth to total water depth ratio, and group size (as a measure of communal clustering) among breeding habitats with Bonferroni-corrected Kruskal-Wallis tests. We repeated each Kruskal-Wallis test to reach the smallest probability value for differences among treatments. We determined differences in egg mass cardinal distribution among ecosystems with circular statistics (Zar 1999) and determined if egg mass degree locations adhered to a von Mises (circular normal) distribution for each breeding habitat with a Kuipers V test. We compared the median vector of ovipositioning location among ecosystems with the non-parametric Mardia-Watson Wheeler test (Batschelet 1981), given that egg masses only in stocked lakes adhered to the von Mises distribution (V = 1.31, P > 0.15).

We calculated the expected number of egg masses for each vegetation and substrate category per site as:

\[
\text{No. quadrats dominated by each category} \quad \text{Total no. quadrats} * \text{Total no. egg masses}
\]
We compared the number of observed and expected egg masses for each vegetation and substrate category with Pearson’s Chi Square tests, and evaluated comparisons with Bonferroni-adjusted significance values to control family-wise error rates. We also determined relationships among the following quadrat characteristics with Pearson’s product moment correlations: distance to shore, water depth, total vegetative cover (cover values for all species combined), and persistent vegetative cover (woody and emergent vegetation providing attachment sites).

**Egg Mass Morphology.** We compared egg mass area (square root transformed; ANOVA), mean envelope thickness (Kruskal-Wallis tests), and the number of clear relative to cloudy egg masses (Poisson regression; offset is number of cloudy egg masses) among ecosystems. Data were pooled over sites and years to account for small egg mass counts in individual sites. Mass and SVL of captured females were compared among fishless lakes, stocked lakes, and vernal pools with ANOVA.

**In situ Egg Mass Predator Exclusion Experiments.** We used an information theoretic approach to identify the best generalized mixed model predicting the effects of breeding habitat and treatment on the proportion of surviving embryos and larvae (Burnham and Anderson 2002). Fixed effects included breeding habitat (‘TYPE’) nested within treatment (‘TRT’), presence/absence of chironomid larvae (‘CHIRO’) (these could not be completely excluded from closed and open treatments owing to the gauge of the netting and thus were included in all models), the total number of predators found in exclosures at the end of the experiment (‘NO.PREDS’), and the mean envelope width (‘ENV’). Site (‘1|SITE’) was the random effect. The initial model was fit with maximum likelihood estimation. Individual terms were dropped based on smallest coefficient values, and the
model was refit until the smallest AIC value was achieved. As there are currently no statistical tests for determining fit, model parameters for the best model were estimated with restricted maximum likelihood estimation with the REML function in R (Zuur et al. 2009). The effect of random variables was assessed by examining variance estimates. We used linear models to determine the effects of treatment, breeding habitat, and the presence of chironomid larvae on dry mass (rank transformed) of hatched larvae. Linear mixed models were not used for this analysis, because the variance estimate for site was small (<0.001) and thus not properly fitted in the model. Chironomid larvae were unexpected but ubiquitous among all treatments and ecosystems, therefore, we also created a final linear mixed model to determine effects of breeding habitat, exclosure treatment, pH, and water color on the probability of chironomid larvae being present within egg masses.

**Results**

**Site Characteristics**

Vegetation and substrates differed significantly among ecosystems (T = -2.66, P = 0.02) and were more similar within lakes than within vernal pools, as indicated by within group distances (µ_fishless = 0.44, µ_stocked = 0.44, and µ_vernalpool = 0.66). Vegetation and substrate homogeneity within lake types was significantly greater than expected (A=0.08), however, differences between lake types were significant (T = -1.51, P = 0.08).

Vegetation and substrate variability (89.3%) was captured by two axes (axis 1: emergent vegetation, [+]; axis 2: woody vegetation [-]) identified in NMDS (r² axis 1 = 0.48, r² axis 2 = 0.41) among breeding habitats (Figure 3.2a). The final stress was 11.74,
which was within an acceptable range for ecological data and indicated that this configuration was appropriate for our data (McCune and Grace 2002). Axis 1 scores were most variable for vernal pools, whereas, all ecosystems were similarly variable for axis 2 (Figure 3.2a).

Emergent and woody vegetation (axes 1 and 2, respectively) captured 94.1% of the variability ($r^2$ axis 1 = 0.68, $r^2$ axis 2 = 0.26; final stress = 5.56) between lake types (Figure 3.2b). NMDS scores were significantly different between lake types for axis 1($F_{1,10} = 5.91, P = 0.04$) and not for axis 2 ($F_{1,10} = 2.45, P = 0.15$). Stocked lakes were positively associated with axis 1 ($t_{axis1} = 2.43, P = 0.04$) and negatively associated with axis 2 ($t_{axis2} = -1.57, P = 0.15$). Emergent vegetation was more variable in fishless lakes, whereas, woody vegetation was more variable in stocked lakes, as evidenced by more variable axes 1 and 2 scores for each respective breeding habitat (Figure 3.2b).

Several water chemistry characteristics were correlated (Table 3.1). pH was 14 and 6% greater in stocked lakes than fishless lakes or vernal pools, respectively ($F_{2,30} = 4.67, P = 0.02$). Mean water temperature ($F_{2,30} = 8.40, P = 0.001$) and dissolved oxygen ($F_{2,30} = 4.91, P = 0.01$) were 35 and 26% lower, respectively, in vernal pools, and apparent color ($F_{2,30} = 9.48, P < 0.001$) was darker in vernal pools.
Figure 3.2. Results of NMDS relating vegetation and substrate characteristics among lake and vernal pool types; (a) comparison among fishless lakes (‘FL’), stocked lakes (‘ST’), and vernal pools (‘VP’); and (b) comparison between stocked and fishless lakes. Lake variables are abbreviated as follows: COV.EM = percent emergent vegetation cover, COV.SS = percent woody vegetation cover; COV.SUBA = percent aquatic vegetation cover; COV.SO = percent sphagnum cover; PERC.OVI = percent cover by substrates suitable for ovipositioning (submerged logs and sticks); and, PERC.FLCO = percent cover by floating sticks and logs suitable for ovipositioning.
Table 3.1. Pearson’s product moment correlation table for water quality characteristics among lakes and vernal pools (df = 31 for all comparisons). Abbreviations are as follows: DO = dissolved oxygen, and, Temp = water temperature. Significant correlations at P < 0.05 are noted in bold.

<table>
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<tr>
<th>Variable</th>
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<th>DO</th>
<th>Temp</th>
<th>Color</th>
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</table>

Egg Mass Oviposition Characteristics

All ovipositioning variables differed among breeding habitats (Figure 3.3), including egg masses deposited deeper in vernal pools and closer to shore in stocked lakes. Egg masses were distributed non-randomly in all ecosystems, and the median ovipositioning angle differed by ecosystem (Mardia-Watson Wheeler W_{allcomparisons} > 11.35, P_{allcomparisons} < 0.003, Figure 3.4). In all ecosystems, egg masses were attached to dead woody vegetation more often than expected based on oviposition site availability, however, egg masses in fishless and stocked lakes were attached to emergent vegetation less than expected (Table 3.2). The proportion of observed substrates relative to expected substrates below egg masses also differed by breeding habitat; egg masses were oviposited more often than expected above organic matter and less than expected above woody debris and leaves in fishless lakes (Table 3.2). Egg masses were deposited as expected above all four categories of substrate in vernal pools and stocked lakes. Correlations among ovipositioning characteristics varied with distance from shore and breeding habitat (Table 3.3), with percent vegetative cover increasing with distance from shore for all habitats.
Figure 3.3. Differences among breeding habitats for the following ovipositing characteristics: (a) distance to shore, (b) group size (the number of egg masses within 1 m$^2$ of each surveyed egg mass), (c) ratio of egg mass depth to the total depth of the water column, and, (d) percent vegetative cover above egg masses from ocular estimates. Significant differences are denoted as * P < 0.05, **P < 0.01, and ***P > 0.001; different letters indicate significant differences among breeding habitats.
Figure 3.4. Median angles of egg mass deposition based on breeding habitat. The line radiating from the center indicates the median angle and the arc represents the 95% confidence interval around the median. Analyses are presented separately as follows: (a) stocked lakes pooled over 2007 and 2008, (b) fishless lakes pooled over 2007 and 2008, and, (c) vernal pools, surveyed in 2008 only.
Table 3.2. Pearson’s Chi Square tests for observed versus expected use of attachment sites and substrates by breeding habitat. The Bonferroni-corrected significance values for tests within each breeding habitat were 0.01 and 0.013 for attachment sites and substrates, respectively (denoted by bold text). Attachment site codes are as follows for a) non-vegetated/dead vegetation: GRD = ground (egg mass unattached on the substrate); WDY.D = woody debris (submerged logs or sticks; floating sticks or logs); and, b) live vegetation: AQ/SUB = aquatic and submerged vegetation; EM = emergent vegetation; and, WDY = woody vegetation. Substrate codes are as follows: OM = organic matter; HRD = hard substrates (boulders, gravel, or sand); WDY= woody debris (submerged logs or sticks); and, LVS = leaves. Observed and expected values are abbreviated as Obs. and Exp.

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<th>Breeding Habitat</th>
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<th>Live Vegetation Attachment Sites</th>
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<tr>
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<tr>
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<td>Vernal Pools</td>
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<td>0.77</td>
<td>0/0</td>
<td>--</td>
<td>--</td>
<td>4/4</td>
<td>0</td>
<td>1.00</td>
<td>47/51</td>
<td>0.16</td>
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</table>
Table 3.3. Pearson’s product moment correlations among available ovipositioning characteristics by breeding habitat. Ovipositioning characteristics are abbreviated as follows: PersVEG (total persistent vegetative cover), TotVEG (total vegetative cover), Depth (water depth), and, Dist (distance from shore). Significant correlations at P < 0.05 are noted in bold.

<table>
<thead>
<tr>
<th>Variable</th>
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<th>TotVEG</th>
<th>Depth</th>
<th>Dist</th>
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<td>R</td>
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</tr>
<tr>
<td>Fishless lakes</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PersVEG</td>
<td>1.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotVEG</td>
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<td>0.00</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Depth</td>
<td>0.14</td>
<td>0.22</td>
<td>0.18</td>
<td>0.05</td>
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<td>Dist</td>
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<td>&lt;<strong>0.001</strong></td>
<td><strong>0.35</strong></td>
<td>&lt;<strong>0.001</strong></td>
</tr>
<tr>
<td>Stocked lakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PersVEG</td>
<td>1.0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotVEG</td>
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<td>0.82</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Depth</td>
<td>0.05</td>
<td>1.00</td>
<td>0.17</td>
<td>0.21</td>
</tr>
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<td><strong>0.67</strong></td>
<td>&lt;<strong>0.001</strong></td>
<td><strong>-0.31</strong></td>
<td>&lt;<strong>0.001</strong></td>
</tr>
<tr>
<td>Vernal pools</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PersVEG</td>
<td>1.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotVEG</td>
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<td>0.32</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Depth</td>
<td>-0.10</td>
<td>1.00</td>
<td><strong>0.42</strong></td>
<td>&lt;<strong>0.001</strong></td>
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<tr>
<td>Dist</td>
<td><strong>0.50</strong></td>
<td>&lt;<strong>0.001</strong></td>
<td><strong>0.26</strong></td>
<td><strong>0.01</strong></td>
</tr>
</tbody>
</table>

**Egg Mass Morphology**

The number of embryos per mass was correlated with egg mass area (Pearson’s product moment correlation $t_{93} = 4.75$, $P < 0.001$) and mean envelope width (Pearson’s product moment correlation $t_{100} = 3.00$, $P = 0.003$). The largest egg masses were found in vernal pools ($F_{2, 92} = 4.78$, $P = 0.01$), however, the number of embryos per mass did not differ by breeding habitat ($F_{2, 108} = 1.27$, $P = 0.29$). Breeding habitat did not have an effect on adult female mass ($F_{2, 78} = 1.24$, $P = 0.29$) or SVL ($F_{2, 78} = 1.24$, $P = 0.30$). Egg mass outer envelope width was marginally thicker in stocked lakes (Kruskal-Wallis $X^2 = 6.03$, df = 2, $P = 0.05$; median envelope category = 5 [1.00-1.25 cm]), although this difference may not be biologically relevant (mean difference stocked vs. fishless lakes =
0.14 mm; stocked lakes vs. vernal pools = 0.11 mm; median envelop category for both fishless lakes and vernal pools = 4 [0.75-0.99 cm]; median envelope category for stocked lakes = 5 [1.00-1.24 cm]). Breeding habitat did not have an effect on the ratio of cloudy to clear masses \((|Z|_{\text{allecosystems}} < 1.31, P > 0.19)\). We did not observe egg masses that were intermediate between clear and cloudy (Ruth et al. 1993).

**In situ Predator Exclusion Experiment**

Embryo survival was greatest in vernal pools in treatments allowing predators access to egg masses (control and open treatments; Tables 3.4; B.1), although survival was similar among breeding habitats when large predators were excluded (closed treatment). One predator (chironomid larvae) reduced the proportion of surviving larvae across all treatments and ecosystems. Chironomid presence also significantly reduced larval dry mass, although there was no interaction between chironomid larval presence, treatment, and breeding habitat (Tables 3.4; B.1). We did not find a strong relationship among treatment, breeding habitat, and pH and the probability of chironomids being present in egg mass exclosures; however, chironomids had a slightly greater probability of being present in vernal pools (Tables 3.4; B.1). Larval mass at hatching was greatest in vernal pools across exclosure treatments.

**Discussion**

Through its widespread distribution on the landscape and biphasic life history, *A. maculatum* serves as an energetic link among uplands, vernal pools, and other wetland habitats (Gibbons et al. 2006; Regester et al. 2006). Successful breeding habitats for *A.
Table 3.4. Variables remaining in the best generalized mixed models predicting larval dry mass, proportion of surviving offspring, and the presence of chironomid larvae at the end of the *in situ* predation experiment. AIC values for each model step are included in Appendix B.

<table>
<thead>
<tr>
<th>Model</th>
<th>Variables Remaining in Best Model</th>
<th>Coefficient ±SE</th>
<th>P-value</th>
<th>AICw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of surviving</td>
<td>TRTCONTROL</td>
<td>-3.35 ± 0.36</td>
<td>&lt;0.001</td>
<td>0.66</td>
</tr>
<tr>
<td>offspring</td>
<td>TRTOPEN</td>
<td>-3.08 ± 0.28</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHIRO1</td>
<td>-1.20 ± 0.21</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ENV</td>
<td>0.28 ± 0.25</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRTCLOSED:TYPESTOCKED</td>
<td>0.28 ± 1.22</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRTCONTROL:TYPESTOCKED</td>
<td>1.55 ± 1.31</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRTOPEN:TYPESTOCKED</td>
<td>1.52 ± 1.27</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRTCLOSED:TYPEVERNALPOOL</td>
<td>2.09 ± 1.21</td>
<td>0.09</td>
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</tr>
<tr>
<td></td>
<td>TRTCONTROL:TYPEVERNALPOOL</td>
<td>3.89 ± 1.27</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRTOPEN:TYPEVERNALPOOL</td>
<td>3.54 ± 1.24</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Larval dry mass</td>
<td>TRTCONTROL</td>
<td>13.77 ± 7.22</td>
<td>0.06</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>TRTOPEN</td>
<td>13.05 ± 7.21</td>
<td>0.07</td>
<td></td>
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<tr>
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<td>TYPEFS</td>
<td>16.37 ± 9.05</td>
<td>0.07</td>
<td></td>
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<tr>
<td></td>
<td>TYPEVP</td>
<td>19.80 ± 7.81</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHIRO1</td>
<td>-19.56 ± 6.29</td>
<td>0.002</td>
<td></td>
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<tr>
<td>Chironomids present</td>
<td>TYPESTOCKED</td>
<td>3.72 ± 2.30</td>
<td>0.11</td>
<td>0.56</td>
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<tr>
<td></td>
<td>TYPEVERNALPOOL</td>
<td>3.35 ± 1.92</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WATERCOLOR</td>
<td>0.03 ± 0.02</td>
<td>0.10</td>
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</table>

*maculatum* must have suitable ovipositioning habitat and sufficiently long hydroperiods for embryos and larvae to complete metamorphosis while also limiting predators. Hydroperiod may fluctuate annually in ephemeral wetlands (Kolozsvary 2003; Skidds et al. 2007), affecting inter-annual breeding success. Breeding in fishless lakes may be one strategy to avoid embryo death due to early pool drying. We detected differences among breeding habitat, morphological, and behavioral characteristics that may maximize embryo survival depending on the breeding habitat selected for ovipositioning. These flexible breeding strategies, if effective, may be one explanation for *A. maculatum*’s population persistence despite continued losses of its primary breeding habitat.
Ovipositioning site selection is a function of both site availability (Egan and Paton 2004) and environmental cues perceived by breeding females (Kats and Sih 1993; Hopey and Petranka 1994). Vernal pool vegetation and substrate characteristics were more variable than those of lakes, reflecting hydroperiod and size effects on vernal pool vegetation diversity (Babbitt et al. 2003; Kolozsvary 2003; Babbitt 2005). However, females in vernal pools oviposited equally above all substrates suggesting that within pool conditions (e.g., predator distributions) may be more spatially uniform than lakes. Egg masses in stocked lakes were concentrated closer to shore in areas with fewer preferred attachment sites (e.g., woody vegetation [Egan and Paton 2004]) but greater vegetative cover, perhaps to avoid fish predation. *Notemgonus crysoleucas* was present in all five stocked lakes and readily consumes prey from both the littoral and pelagic zones of lakes (Reebs 2002; Christensen and Moore 2008). A second fish species, *S. fontinalis*, is stocked annually at four of the five stocked lakes, and forages within 2 - 4 m of shorelines during May – June (Biro 1998, Bonney 2009), a period and shore area that overlaps with *A. maculatum* embryonic development in stocked lakes.

Egg mass ovipositioning depth may be a function of the ephemeral hydroperiod of vernal pools, whereas, egg mass depth in longer hydroperiod systems (e.g., lakes) may optimize protection from predators. Shallow egg masses (< 0.5 m) were reported for small (< 0.1 ha), shallow (< 1.2 m), mostly semi-permanent ponds in Rhode Island (Egan and Paton 2004), however, egg masses were even shallower (0.1 ± 0.01 m) for vernal pools in our study, suggesting that ovipositioning depth is highly variable among regions. Vernal pools were significantly colder than lakes during May surveys, thus shallow ovipositioning may maximize solar heating to encourage rapid embryo development and
hatching before pool dry down. Alternatively, slower embryonic development at lower temperatures may produce larger larvae that escape gape-limited predators (Urban 2007). Given that *in situ* embryo survival with predators was nearly 180 percent greater in vernal pools than lakes, predation pressure may be less important than pool dry-down for regulating survival in vernal pools. In contrast, lakes do not dry, and thus egg masses may be oviposited at depths that maximize protection from predators or facilitate embryonic development.

Additional breeding habitat characteristics and ovipositioning strategies may affect embryo survival. Darker apparent water color in vernal pools may better camouflage egg masses from visual predators, as egg masses were more visible to surveyors in lakes than vernal pools (A.F. Shearin, personal observation). Females did not consistently oviposit egg masses in the northern portions of lakes and vernal pools as in Massachusetts (Windmiller 1996) suggesting that other factors (e.g., ovipositioning sites available) besides maximizing solar heating affect choice of ovipositioning sites in the ecosystems we studied. Although we expected greater communal clustering in lakes to avoid predators, egg mass group size in vernal pools was nearly double that of stocked lakes, perhaps owing to more satellite masses in vernal pools (identified as one to two small egg masses within 5 cm of a larger, primary mass; Hunter et al. 1999) rather than predator avoidance. While *A. maculatum* survival is associated with increasing pH (Brodman 2002), embryo survival was less in stocked lakes (mean pH = 5.55 ± 0.15) than vernal pools (mean pH = 5.19 ± 0.12), suggesting that biotic interactions were more important than small differences in pH for predicting survival.
Egg mass morphological characteristics differed among breeding habitats, and when coupled with ovipositioning strategies, these differences may affect embryo survival. Egg masses were approximately 13% larger in vernal pools but were similarly sized in fishless and stocked lakes, suggesting that hydroperiod (Woodward 1982) rather than fish presence influences this characteristic. Woodward (1982) also found significantly larger egg masses in temporary than in permanent small (<0.13 ha), shallow (<0.1 m) ponds in Connecticut, but the magnitude of this difference (3%) was smaller than our observations. Egg mass size also may be related to breeding habitat quality, with larger egg masses in forested vernal pools than roadside ditches in New York (USA) (Karraker and Gibbs 2011). Since there were no differences in female body size among ecosystems, females in large permanent lakes and other alternative breeding habitats (Karraker and Gibbs 2011) may invest fewer energetic resources in reproductive output (Kaplan 1987; Cunnington and Brooks 2000) compared with their vernal pool counterparts. In situ larval dry mass at hatching also was greatest in vernal pools regardless of exclosure treatment and may be attributed to egg size, with embryos in larger eggs hatching at larger sizes than those from smaller eggs (Dushane and Hutchinson 1994; Petranka 1998) or longer development times.

While Cunnington and Brooks (2000) found no effects of fish on A. maculatum egg size, predation resistance likely occurs at the egg mass rather than the egg level. Fish readily consume embryos removed from the protective mucoid capsule (Semlitsch 1988), and it is this outer envelope (Semlitsch 1988; Ireland 1989), not the individual egg that is the first mechanical defense against predation. In situ embryo survival was greatest in vernal pool control and open in situ enclosure treatments; however, inherent survival (i.e.
closed treatment that excluded predators) was not statistically different among ecosystems, suggesting that the effectiveness of outer envelopes varied by predators present. We selected egg masses for the in situ experiment with similar mucoid envelope widths to avoid confounding embryo survival with egg mass morphology; however, we observed outer envelopes in stocked lakes that were 17 and 14% thicker, respectively, than in fishless lakes and vernal pools.

Thicker envelopes may provide greater predator resistance to fish and other predators over multiple A. maculatum generations. Fish have been stocked in four of the five stocked lakes for at least 30 years (DeGoosh 2007), and fish have been present in the fifth lake for at least 10 years. Ambystoma maculatum has a lifespan near 25 years (Flageole and LeClair 1992), with females reproducing around ages four (Wilbur 1977) to seven (Flageole and LeClair 1992). Using seven as a conservative estimate for age of first female reproduction, lakes contained at least four generations. While genetic correlations among A. maculatum breeding habitats may extend as far as 4.8 km, (Zamudio and Wieczorek 2007), females routinely avoid outbreeding by mating with related males (Chandler and Zamudio 2008). It is possible that selection is occurring at a microgeographic scale, particularly if the selection pressure by fish remains static (e.g., fish populations are always present in lakes due to repeated stocked and natural reproduction) (Urban 2010). Long-term surveys (Sexton et al. 1998; Brodman 2002) are needed to determine if differences in egg mass envelope thickness become more pronounced among breeding habitats over time and whether this characteristic, when coupled with ovipositioning strategies, offers greater predation resistance.
The presence of chironomids reduced *in situ* embryo survival and dry mass more than exclosure treatments or ecosystem. Chironomid larvae are ubiquitous components of freshwater ecosystems (Pinder 1986) and are a prey item for amphibian larvae including *A. maculatum* (Petranka 1998). Greater rates of chironomid infestations of *A. maculatum* egg masses have been reported in vernal pools with lighter apparent water color (LeClair and Bourassa 1981), although this variable did not appear to have a strong effect on predicting chironomid presence in our study. Chironomid community composition is controlled by water chemistry and macrophytic habitat in Maine (Woodcock et al. 2005) and hydroperiod in general (Pinder 1986). Several water chemistry characteristics (pH, water temperature, apparent water color, and dissolved oxygen) differed among breeding habitats in our study and thus our sites likely contained different chironomid taxa with similar ecological roles as *A. maculatum* embryo predators.

Our study identified few morphological (e.g., egg mass envelope thickness and size) and behavioral breeding characteristics (e.g., ovipositing near abundant vegetation in stocked lakes) in *A. maculatum* that may maximize embryo predation resistance among variable breeding habitats. The lack of widespread egg mass morphological differences among lakes may reflect the relatively short stocking time or similar predation rates in these systems. Embryo survival in stocked lakes may be too low to maintain local populations (Sternhouse 1997) in this ecosystem. However, recruitment of offspring from fishless and stocked lakes may contribute to maintaining genetic diversity and metapopulations over time, particularly in periods of drought when vernal pool amphibian production is low (Karraker and Gibbs 2009). Conservation of breeding
habitats along the hydroperiod gradient from ephemeral to permanent may enhance long-term pool-breeding amphibian conservation in landscapes where ephemeral wetlands are threatened or scarce and in vernal pool rich landscapes where juvenile recruitment may be low during periods of drought.

Chapter Acknowledgements

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Chapter 4

EVALUATION OF LISTENER-BASED ANURAN SURVEYS WITH AUTOMATED AUDIO RECORDING DEVICES

Chapter Abstract

Volunteer-based audio surveys are used to document long-term trends in anuran community composition and abundance. Current sampling protocols, however, are not region- or species-specific and may not detect relatively rare or audibly cryptic species. We used automated audio recording devices to record calling anurans during 2006-2009 at wetlands in Maine, USA. We identified species calling, chorus intensity, time of day, and environmental variables when each species was calling and developed logistic and generalized mixed models to determine the time interval and environmental variables that optimize detection of each species during peak calling periods.

We detected eight of nine anurans documented in Maine. Individual recordings selected from the sampling period (0.5 h past sunset to 0100 h) described in the North American Amphibian Monitoring Program (NAAMP) detected fewer species than were detected in recordings from 30 minutes past sunset until sunrise. Time of maximum detection of presence and full chorusing for three species (green frogs, mink frogs, pickerel frogs) occurred after the NAAMP sampling end time (0100 h). The NAAMP protocol’s sampling period may result in omissions and misclassifications of chorus sizes for certain species. These potential errors should be considered when interpreting trends generated from standardized anuran audio surveys.
Introduction

Accurate detection and abundance measures are essential for documenting trends in amphibian populations. Audio surveys are used extensively for long-term and rapid population monitoring of vocalizing anurans (Dorcas et al. 2009). Large-scale, volunteer-based audio survey programs, such as the North American Amphibian Monitoring Program (NAAMP; http://www.pwrc.usgs.gov/naamp/), are designed to monitor anurans over diverse spatial and temporal scales (Weir and Mossman 2005). NAAMP is a North American program instituted in 1997; in the US, it is a collaborative effort among state wildlife agencies, non-profit organizations, and the U.S. Geological Survey to document trends in anuran populations with volunteer-based call surveys (Weir et al. 2005). The unified sampling protocol adopted by participating states in 2001 (Weir and Mossman 2005) directs volunteers to identify calling anurans during a five-minute listening period at up to ten stops along a designated route. All surveys must begin 0.5 hours after sunset or later and terminate by 0100 hours. Each species identified during a listening period is assigned a Calling Index (CI) code from 1 to 3, with CI 1 representing non-overlapping individuals of that species calling and CI 3 representing a full chorus (Mossman et al. 1998; Weir and Mossman 2005). CI is correlated with the number of calling males for certain species, such as green frogs (Lithobates clamitans Latreille) (Nelson and Graves 2004), North American bullfrogs (L. catesbeianus Shaw) (Shirose et al. 1997), and Fowler’s toads (Anaxyrus fowleri Hinckley) (Shirose et al. 1997), and it can be used to estimate relative abundance. Declining CI over multiple years often is used as an indicator of declining populations (Gibbs et al. 2005). Environmental variables, such as wind speed and cloud cover, also are noted at each NAAMP listening
stop or at the start and end of the survey, as these conditions may affect calling initiation and detection (Weir et al. 2005). Standardized volunteer listener surveys are inexpensive, repeatable, applicable across multiple regions, and foster public involvement in amphibian research. Calling surveys alone, however, may be insufficient for detecting species that call infrequently, vocalize outside the protocol’s monitoring windows, or are rare and may require pairing with more intensive sampling methods such as pitfall traps or automated audio recording devices (Crouch and Paton 2002). Commission errors (false inclusions) also may obfuscate accurate assessment of population trends in long-term amphibian datasets (Lotz and Allen 2007; McClintock et al. 2010). Furthermore, species omissions and misclassification of CI can affect interpretations of long-term trends based on audio data (Lotz and Allen 2007), although models may correct for imperfect detections in some scenarios (e.g., Royle 2004; Nichols et al. 2007; Mackenzie et al. 2009; Miller et al. 2011).

The unified NAAMP protocol standardizes data collection across multiple regions and years; however, the generality of the protocol may lead to poor detection of species that require different survey parameters. The NAAMP sampling period is appropriate for detecting many species, such as Fowler’s toads, which call most frequently within two hours after sunset (Tupper et al. 2007). Species that call before or after this time period may not be detected. Southern leopard frog (L. sphencephalus Harlan) for example, calls primarily from midnight until dawn in North Carolina and could be missed by surveys conducted during evening hours (Bridges and Dorcas 2000). In the northeastern United States, mink frogs (L. septentrionalis Baird) routinely call later than the 0100h end time for NAAMP surveys. Peak mink frog detection usually occurs during 0300-
0400 h following peak calling activity (Bevier et al. 2006). Surveys conducted before this time may fail to detect full choruses given that estimation of local population sizes would warrant such a distinction (Popescu and Gibbs 2009). While these studies indicate that audio surveys conducted before 0100 h may fail to detect certain species, there currently are no paired studies explicitly comparing species detections and CI classifications before and after the 0100 h NAAMP end time, although detection probabilities are known to vary throughout the NAAMP sampling window (Royle 2004; Weir et al. 2005).

Each state participating in NAAMP offers sampling guidance for dates with the best probabilities of detecting target species. In Maine, there are three sampling periods that target early spring (wood frog [*L. sylvaticus* Le Conte], spring peeper [*Pseudacris crucifer* Wied-Neuwied]), late spring (American toad [*Anaxyrus americanus* Holbrook], gray treefrog [*Hyla versicolor* Le Conte], pickerel frog [*L. palustris* Le Conte], northern leopard frog [*L. pipiens* Schreber]), and early summer (green frog, bullfrog, mink frog) breeders (Maine Amphibian Monitoring Program, www.mainaudubon.org). Guidance for when to sample within these periods is limited to temperature thresholds and the occurrence of light precipitation. Recommended sampling dates differ among the three Maine sampling regions (coastal, interior, northern Maine) and are based on anuran species present in each; however, the unified protocol requires that surveys be conducted during 0.5 h after sunset to 0100 h.

While general guidance simplifies the sampling protocol, environmental variables may significantly influence the probability of detecting certain species. Wood frogs and spring peepers may vary calling initiation by as much as three weeks based on
environmental variables (Mossmen et al. 1998). Other studies have documented the importance of environmental variables for predicting calling occurrence (e.g., Oseen and Wassersug 2002; Weir et al. 2005; Saenz et al. 2006; Steelman and Dorcas 2010). The number and effect of environmental variables on calling may differ among breeding strategies, with explosive breeders (e.g., wood frogs) influenced by fewer variables than their prolonged breeding counterparts (Wells 1977) that call over several weeks or months (e.g., green frogs) (Oseen and Wassersug 2002). Prolonged breeders must regulate calling effort over a longer period of time and thus are exposed to a greater range of environmental conditions (Wells 1977).

Automatic recording systems (ARS) may be useful for identifying sampling times and environmental variables that can improve detection of target anuran species. ARS are digital or cassette-tape based automated audio recording systems that can be programmed to record a specified interval over a given time period (Peterson and Dorcas 1992). They can be used to monitor sites over multiple nights, extended time periods, and under various environmental conditions. ARS also remove any potentially confounding effects of human disturbance during a survey. Recordings can be permanently archived and reviewed repeatedly by multiple listeners for accurate species identification and CI designation. Digital ARS data also can be manipulated with widely available software to improve recording quality, remove interference, and isolate audibly cryptic species that may be masked by louder species (Dorcas et al. 2009). ARS have been used extensively for detecting calling anurans (e.g., Steelman and Dorcas 2010), birds (e.g., Brandes 2008), and other vocalizing species.
We used ARS to monitor calling anurans at lakes and vernal pools in Maine to determine if the standardized NAAMP protocol detects and accurately describes calling choruses for all anuran species in our region. The specific objectives of our study were to (1) determine if surveys conducted during the NAAMP-specified time period (0.5 h past sunset to 0100 h) identified all species known to be present and captured the maximum CI for that night; (2) describe temporal calling patterns for anurans in central Maine; and, (3) describe environmental variables that predict calling occurrence.

**Methods**

**Study Area**

ARS were deployed during 2006-2009 at lakes and vernal pools in the Western and Interior Mountains and Foothills (12 lakes) and the Eastern Coastal Plain and Foothills (12 lakes and four vernal pools) biophysical regions of Maine (Krohn et al. 1999) (Figure C.1). Lakes were characterized by permanent hydroperiods and surface areas from 1.4 to 10.1 ha. Vernal pools typically dried by the end of August, with surface areas ranging 0.02-0.7 ha at high water. Sites chosen were a subset of those surveyed for invertebrate, fish, and amphibian community composition (Schilling et al. 2008; Shilling et al. 2009; Shearin unpublished data) and are located within Maine’s ‘central’ NAAMP region (Zone 2) in Franklin, Hancock, Oxford, Piscataquis, and Washington Counties (Figure C.1). Nine anuran species (American toad, gray treefrog, North American bullfrog, green frog, pickerel frog, northern leopard frog, mink frog, wood frog, spring peeper) occur in this region (Hunter et al. 1999).
ARS Deployment

We used a combination of five, tape-based ARS (designed after Peterson and Dorcas [1992]) and five digital-based ARS developed at the University of Maine, Orono, to record calling anurans. Each digital ARS consisted of a USB recorder (iKEY® Plus, GCI Technologies Corporation, Edison, NJ), two-channel, eight event timer (DT-04, SuperFeeder®, Hermitage, TN), microcontroller-operated electronic trigger circuit, voltage regulator (12 to 5 volt DC), 4 GB capacity USB drive, stereo microphone (iKEY® Plus, GCI Technologies Corporation, Edison, NJ), aluminum, 1 m tripod (DT-120D, Fox®), waterproof case (Pelican™ Products, Inc., Torrance, CA, 24 X 18 X 11 cm), and two 6-volt DC batteries. Microphones were housed in a 20 cm-long section of 5 cm diameter polyvinylchloride (PVC) piping to prevent wind and rain interference (see Peterson and Dorcas 1992). Digital and tape-based ARS were deployed haphazardly among 24 lakes and vernal pools during the duration of our study. ARS were often removed from a site once a species was detected, and the number of deployment days and sampling occasions varied by site and year. We placed a single ARS within 0.5 m of shoreline under vegetation to provide protection from sunlight and to reduce interference from wind and precipitation. ARS were programmed to record an approximate two minute audio clip every hour from 0.5 h past sunset until sunrise. This time interval constituted a single recording night and maximized the number of nights and recordings per night captured by each ARS deployment.
Recording Analyses

Only nights containing recordings from all hours between 0.5 h past sunset and sunrise were used for analysis. Time (minutes) past sunset was noted for each recording. Recordings were assigned a Julian date (DAY) based on the start date of each recording night. Audio recordings were reviewed by one of five listeners. A single listener also reviewed random recordings from each night to ensure that all listeners correctly and consistently assigned CI values. CI code (1-3) for each species per recording was assigned following NAAMP detection protocols: CI 1 for individuals with no overlap between calls, CI 2 for overlapping individuals, and CI 3 for a constant, overlapping chorus (Weir and Mossman 2005). We also created an additional category (CI 0) for no calls.

We used environmental data collected nightly from the nearest weather station within 30 km (National Oceanic Atmospheric Administration weather stations, http://lwf.ncdc.noaa.gov/oa/climate/stationlocator.html). We determined maximum (MAXT) and minimum (LOWT) temperature, relative humidity (HUMID) at 2400 h, precipitation during the recording night (PRECIP), and presence (1) or absence (0) of precipitation within 24 h prior to the recording. The fraction of the moon illuminated for each night (MOON) (Weir et al. 2005) was obtained from the U.S. Naval Observatory (http://www.usno.navy.mil/USNO/astronomical-applications/data-services/frac-moon-ill). Cloud cover (CLD) and wind speed (WIND) at midnight during each recording session were obtained from Weather Underground (www.wunderground.com). Cloud cover was converted to a sky condition code based on NAAMP categories from 0 (clear or few clouds) to 8 (showers). Wind speeds were categorized as Beaufort wind speed
codes from 0 (wind speed < 1.6 km per hour) to 5 (wind speeds 30.6 to 38.6 km per hour), which is consistent with NAAMP protocols.

Individual audio recordings were classified as falling within the NAAMP sampling time period (0.5 h past sunset to 0100 h; NAAMP) or within a COMPLETE recording session (0.5 h past sunset to sunrise; COMPLETE). Recordings occurring from 0.5 h past sunset until 0100 h occurred during both the NAAMP and full night sampling intervals, and therefore were classified as both NAAMP and COMPLETE. Recordings occurring from 0100 h to sunrise were outside the NAAMP sampling interval, and therefore were classified only as COMPLETE. A single recording (INDIVIDUAL) was chosen randomly from each recording night within the NAAMP sampling time to represent a typical NAAMP survey that identifies species at a site and assigns a CI during a single five-minute listening period. We determined the number of species identified for the randomly chosen INDIVIDUAL sessions as well as the median number of species for all the NAAMP (0.5 h past sunset until 0100h) and COMPLETE (any time between 0.5 h past sunset and sunrise) sessions for each recording night. Paired median numbers of species from INDIVIDUAL, NAAMP, and COMPLETE sessions were compared across nights with Wilcoxon Signed Rank Tests, because data were not normally distributed (Sprent and Smeeton 2001). We determined the number of times each species was detected during the INDIVIDUAL, NAAMP, and COMPLETE sessions and calculated the percentage of nights the INDIVIDUAL and NAAMP sessions omitted species compared with the COMPLETE session. We also determined the median CI for each species for the NAAMP and COMPLETE recording sessions and compared these with the INDIVIDUAL CI for each recording night with Wilcoxon Signed Rank Tests. We
calculated the percentage of sessions that the INDIVIDUAL session CI (as a proxy for a typical NAAMP sampling night) over- or underestimated the median CI.

We also wanted to determine if NAAMP volunteers in Maine were surveying during the entire NAAMP sampling time period. We accessed data for 2006-2009 from the Maine NAAMP website (http://www.pwrc.usgs.gov/naamp; accessed 15 March 2011) and identified survey end times for each route per sampling time period and year. We identified the time of night for which 80% of surveys were completed (NAAMP volunteer end time). We randomly selected individual recordings from within the NAAMP protocol time period truncated by the NAAMP volunteer end time and determined species detected and their respective CI. We calculated the median CI for each species and calculated the percentage of nights these matched the median CI from the COMPLETE session. We also determined the total number of detections by species pooled over route, sampling period, and year during 2006-2009.

**Time as a Predictor of Species Presence and Maximum Calling Index**

We used logistic regression to model the relationship between time (minutes after sunset) of INDIVIDUAL sessions and the probability of detecting a calling species. We did not include environmental or date variables in this analysis, because the NAAMP sampling time period remains static throughout the sampling season. Thus, we wanted to determine if species could be detected during the NAAMP sampling time period for all nights and conditions when a species is present, as would be the realistic case for volunteers conducting NAAMP surveys. Only recordings from COMPLETE sessions where a species was detected at least once were used for this analysis. Each recording
was classified as a 1 (CI 1, 2, 3), or 0 (CI 0). Time was included in the model as both a first and second order term to produce a quadratic relationship between time and calling probability. Similar models were created to describe the relationship between time and the probability of detecting a CI 3, including only audio recordings with CI values 1, 2, or 3 and classified as 1 (CI 3) or 0 (CI 1, 2). We did not detect CI 3 for pickerel frogs; therefore, we used CI 2 as the maximum chorus value for this species (Crouch and Paton 2002). We included only COMPLETE nights during which a species was present, which limited number of recording sessions for a given site or year and required pooling our analysis over these variables. Model fit for each species was evaluated with likelihood ratio tests comparing models with and without time (null model) as a predictor (Agresti 2007). We added the time with the greatest detection probability to the earliest and latest observed sunset time during which a species was detected to determine if predicted peak times of detection and CI 3 were within the NAAMP specified sampling period.

**Environmental Variables as Predictors of Species Detection**

We used an information theoretic approach to determine the best generalized mixed model describing the probability of detecting each species during the full survey season (April to August) based on environmental variables (Burnham and Anderson 2002). Mixed models are useful for analyzing unbalanced data, separating between-group and between-individual effects, and for datasets with nested fixed effects (Veysey et al. 2009; Zuur et al. 2009). We pooled hourly environmental variables and species detections over the COMPLETE session to generate a single summary value for each COMPLETE night, because hourly environmental data recorded at weather stations may
not represent hourly changes in environmental conditions at the recording sites. The
response term was detection or nondetection of a given species on each night. Among
explanatory variables we considered, the fixed effects in our models including nine
environmental variables typically collected as part of the NAAMP sampling protocol
(DAY, the quadratic day term DAYSQ, MOON, WIND, CLD, LOWT, the quadratic
temperature term LOWTSQ, PRECIP, PRECIP24) and one interaction term
(MOON*CLD) (Weir et al. 2005). HIGHT (|r| > 0.20, P < 0.01 for all variables except
MOON) and HUMID (|r| > 0.30, P < 0.001 for all variables except WIND and CLD)
were correlated with other variables and dropped from the analysis. LOWT, LOWTSQ,
DAY, and DAYSQ were centered to reduce autocorrelation between the main and
quadratic term. Random variables included YEAR and SITE and were initially fit as
SITE nested within YEAR with the notation (1|YEAR/SITE). The initial model was fit
with maximum likelihood estimation. We dropped individual terms based on smallest
coefficient values and refit the model to achieve the smallest AIC value. Model
parameter fit for the best model was estimated with restricted maximum likelihood
estimation with the REML function in R (Zuur et al. 2009). We examined the effect of
random variables with variance estimates. SITE and YEAR were first unnested and fit as
individual random variables with the command (1|SITE) + (1|YEAR) for small (<0.001)
or large (>20) variance estimates. If variance for either random effect remained too large
or small, we dropped the term as a random effect, and the model was refit with the
variable as a fixed effect. If this failed to correct the variance estimate of the remaining
random term, we refit the model as a logistic model pooled over site and year.
We created additional models for four species targeted during early (wood frogs, spring peepers) and late (gray treefrogs, northern leopard frogs) spring NAAMP sampling periods in Maine. NAAMP monitoring for these species is limited to a three to four week window in April and a minimum temperature threshold (5.6 and 10° C for the early and late spring breeders, respectively [Maine Amphibian Monitoring Protocol, www.maineaudubon.org]). Of these species, wood frogs and northern leopard frogs are considered explosive breeders and may be less affected by temporal changes in environmental variables than spring peepers and gray tree frogs, which have longer breeding periods (Oseen and Wassersug 2002). Limiting this analysis to April allowed us to examine small changes in environmental variables that could affect calling behavior and thus detection probabilities. We used logistic regression to determine which environmental variables examined in the full season (April to August) model were most important for predicting species calling during April. We pooled over site and year as there were too few replications in the April dataset for each variable to create random effects in generalized mixed models. The quadratic terms DAYSQ and LOWTSQ were excluded from the models, because our target species call beyond April and thus were not expected to follow a quadratic pattern for April alone. An information theoretic approach was used to determine the best model. All statistical analyses were performed in R Statistical Software version 2.11.1 (R Development Core R Team 2010).
Results

Comparison of Recording Sessions

We collected recordings for 137 COMPLETE (0.5 h past sunset to sunrise) sessions, 75% of all nights that ARS were deployed. Each session contained 7 to 12, two- to three- minute recordings made hourly after sunset. Eight species were recorded during the study: gray treefrog, bullfrog, green frog, pickerel frog, leopard frog, mink frog, wood frog, and spring peeper. One additional species (American toad) found in Maine was not recorded with ARS; however, it was detected at our survey sites with visual encounter surveys (Shearin, unpublished data). The number of nights and dates each species was recorded varied by year and species (Table 4.1).

The number of species detected and their CI differed by recording session. Fewer species were detected during INDIVIDUAL sessions (median no. species ± 1 SE: 1 ± 0.09) than during the NAAMP (2 ± 0.10 species; Wilcoxon Z statistic = -6.86, P < 0.001) and COMPLETE (2 ± 0.10; Wilcoxon Z statistic = -7.31, P < 0.001) recording sessions. The number of times a species was omitted during the INDIVIDUAL and NAAMP sessions compared with COMPLETE sessions differed by species (Table 4.2). Species were omitted more frequently during INDIVIDUAL sessions than during NAAMP. Approximately 80% of Maine NAAMP surveys during 2006-2009 were completed by 2300 h (Figure C.2). Number of species detections by NAAMP volunteers varied by species (Table 4.1). The median CI differed significantly by recording session for some species (Table 4.2). INDIVIDUAL session CIs were congruent with COMPLETE session median CIs for greater than 50% of recording nights for all species (Table 4.2).
Table 4.1. Species detections by year, species omissions by recording session, and species detections reported by NAAMP volunteers during 2006-2009. INDIVIDUAL session is abbreviated as ‘INDIV’.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dates Detected</th>
<th>Total No. Nights Detected</th>
<th>No. Detections by Year (No. Nights, No. Sites)</th>
<th>No. Omissions by Session Type</th>
<th>No. Detections by NAAMP Volunteers (% of All NAAMP Detections)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2006 (29, 24) 2007 (40, 11) 2008 (65, 12) 2009 (13, 3)</td>
<td>INDIV</td>
<td>NAAMP</td>
</tr>
<tr>
<td>Gray treefrog</td>
<td>16 Apr-18 Jul</td>
<td>66</td>
<td>9 10 43 4</td>
<td>14 0</td>
<td>624 (14)</td>
</tr>
<tr>
<td>Bullfrog</td>
<td>1 Jun-21 Jul</td>
<td>35</td>
<td>13 17 2 3</td>
<td>10 3</td>
<td>200 (4)</td>
</tr>
<tr>
<td>Green frog</td>
<td>3 May-11 Aug</td>
<td>62</td>
<td>21 25 8 8</td>
<td>18 4</td>
<td>605 (13)</td>
</tr>
<tr>
<td>Pickerel frog</td>
<td>18 Apr-7 Jun</td>
<td>6</td>
<td>0 2 4 6</td>
<td>5 2</td>
<td>81 (2)</td>
</tr>
<tr>
<td>No. leopard frog</td>
<td>17-21 Apr</td>
<td>7</td>
<td>0 0 7 0</td>
<td>2 1</td>
<td>18 (&lt;1)</td>
</tr>
<tr>
<td>Mink frog</td>
<td>23 Jun-4 Aug</td>
<td>12</td>
<td>9 3 0 0</td>
<td>8 3</td>
<td>30 (&lt;1)</td>
</tr>
<tr>
<td>Wood frog</td>
<td>16 Apr-5 Jun</td>
<td>20</td>
<td>0 3 15 2</td>
<td>5 1</td>
<td>680 (&lt;1)</td>
</tr>
<tr>
<td>Spring peeper</td>
<td>16 Apr-3 Jul</td>
<td>60</td>
<td>3 9 42 6</td>
<td>9 0</td>
<td>1886 (42)</td>
</tr>
</tbody>
</table>
Table 4.2. Percentage of INDIVIDUAL session Calling Index (CI) randomly selected from NAAMP (0.5 h after sunset to 0100 h) and truncated NAAMP (0.5 h after sunset to 2300 h) sessions that match, overestimate, or underestimate the median CI for the COMPLETE session, and paired Wilcoxon Signed Rank tests for median CI by species and recording session. Significant differences at $\alpha = 0.05$ are noted in bold type.

<table>
<thead>
<tr>
<th>Species</th>
<th>INDIVIDUAL CI Comparisons with COMPLETE Session</th>
<th>Wilcoxon Signed Rank Results for CI Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAAMP Session</td>
<td>Truncated NAAMP Session</td>
</tr>
<tr>
<td></td>
<td>% of Total Nights Detected*</td>
<td>% of Total Nights Detected*</td>
</tr>
<tr>
<td></td>
<td>Match</td>
<td>Under</td>
</tr>
<tr>
<td>Gray treefrog</td>
<td>62</td>
<td>6</td>
</tr>
<tr>
<td>Bullfrog</td>
<td>77</td>
<td>17</td>
</tr>
<tr>
<td>Green frog</td>
<td>62</td>
<td>28</td>
</tr>
<tr>
<td>Pickerel frog</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>No. leopard frog</td>
<td>86</td>
<td>0</td>
</tr>
<tr>
<td>Mink frog</td>
<td>58</td>
<td>33</td>
</tr>
<tr>
<td>Wood frog</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Spring peeper</td>
<td>62</td>
<td>10</td>
</tr>
</tbody>
</table>

* Percentages for each species may not add up to exactly 100% due to rounding. See Table 4.1 for total number of nights detected by species.

b The comparison between the COMPLETE and NAAMP sessions for northern leopard frogs was not performed, because values were identical for these two session types.
INDIVIDUAL calling codes from the NAAMP volunteer end time truncated session were congruent with COMPLETE median calling codes for > 50% of recording sessions for all species except mink frogs (Table 4.2).

**Time as a Predictor of Species Presence and Maximum Calling Index**

CI varied temporally and among species (Figure C.3). Mean CI for six species did not exceed CI 2 for the COMPLETE session, whereas, the mean CI for gray treefrogs and spring peepers exceeded CI 2 within two and four hours of sunset, respectively, and declined thereafter. The predicted time of maximum detection probability varied among species (Figure 4.1). Maximum predicted detection probability also varied by species and exceeded 80% for all species except pickerel frogs. The time of maximum probability for detecting a CI 3 (or CI 2 for pickerel frogs) also varied among species (Figure 4.2). We were least likely to detect a CI 3 for bullfrogs and leopard frogs; the probability of detecting this value was <50% throughout the sampling night. For each species, the maximum detection probability and detection of CI 3 occurred at different times, except for gray treefrogs (Table 4.3). For this species, both maximum detection probability and detection of CI 3 was greatest 32 minutes after sunset and declined throughout the night.

**Environmental Variables as Predictors of Species Detection**

Multiple environmental variables were predictive of presence during the full sampling period for seven of eight species (Table C.1). Bullfrog presence was explained only by date (DAY and DAYSQ). Multiple environmental variables also were needed to
Figure 4.1. Logistic regression models relating time (minutes past sunset) to the probability of detecting eight Maine amphibian species: a) gray treefrog, b) bullfrog, c) green frog, d) pickerel frog, e) northern leopard frog, f) mink frogs, g) wood frog, and, h) spring peeper.
Figure 4.2. Logistic regression models relating time (minutes past sunset) to the probability of detecting a maximum Calling Index (CI) for eight Maine amphibian species: a) gray treefrog, b) bullfrog, c) green frog, d) pickerel frog, e) northern leopard frog, f) mink frog, g) wood frog, and h) spring peeper. CI 3 was used as the maximum CI value for species all but pickerel frogs; CI 2 was used as the maximum CI for this species.
Table 4.3. Predicted times maximizing detection probability and maximum Calling Index (CI) detection by species. Time of night intervals were created by adding the predicted minutes after sunset to the earliest and latest sunset times observed during sampling periods for each species. Species are arranged in chronological order of detection times.

<table>
<thead>
<tr>
<th>Species</th>
<th>Predicted Min. after Sunset Maximizing Detection (Time of Night)</th>
<th>Predicted Min. after Sunset Maximizing CI 3 Detection Probability (Time of Night)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gray treefrog</td>
<td>32 (1953 – 2102 h)</td>
<td>32 (1953 – 2102 h)</td>
</tr>
<tr>
<td>No. leopard frog</td>
<td>32 (1955 – 1958 h)</td>
<td>216 (2259 – 2302 h)</td>
</tr>
<tr>
<td>Wood frog</td>
<td>100 (2100 – 2123 h)</td>
<td>154 (2154 – 2217 h)</td>
</tr>
<tr>
<td>Spring peeper</td>
<td>159 (2200 – 2308 h)</td>
<td>32 (1953 – 2101 h)</td>
</tr>
<tr>
<td>Bullfrog</td>
<td>275 (2403 – 0100 h)</td>
<td>319 (2447 – 0144 h)</td>
</tr>
<tr>
<td>Mink frog</td>
<td>298 (0101 – 0128 h)</td>
<td>502 (0425 – 0452 h)</td>
</tr>
<tr>
<td>Green frog</td>
<td>335 (0103 – 0205 h)</td>
<td>325 (2453 – 0155h)</td>
</tr>
<tr>
<td>Pickerel frog</td>
<td>422 (0225 – 0319 h)</td>
<td>402b (0205 – 0259 h)</td>
</tr>
</tbody>
</table>

aCI 3 not detected; CI 2 used as maximum value

predict calling during April by early spring breeders, and these models differed from models for the full sampling time period (Table C.1). Leopard frogs were detected only in April, and there were no differences among variables included in final models among sampling seasons.

**Discussion**

**Species Omissions and Calling Index Underestimates**

We compared anuran species and their respective CI detected during the NAAMP-specified time period with those detected during the COMPLETE recording session. INDIVIDUAL sessions consistently detected one fewer species than
COMPLETE sessions. Species omitted during an INDIVIDUAL sampling session may be detected during subsequent surveys. For example, gray treefrogs were omitted during 21% of INDIVIDUAL sessions compared with detections for COMPLETE sessions; however, no omissions occurred for the entire NAAMP session. This indicates that multiple surveys conducted during random sampling times within the NAAMP time period should eventually detect this species. For other species, however, omissions spanned the duration of the NAAMP sampling session. INDIVIDUAL session omission rates for pickerel frogs and mink frogs were 83 and 67%, respectively, and omission rates for the NAAMP session were 33 and 25%, respectively. The predicted time of maximum detection of both species occurred after 0100 h. Surveys conducted during the NAAMP time period (i.e., ending by 0100 h) are likely to omit these species.

CI underestimates may be particularly problematic if used as an indicator of population size. For example, bullfrogs are considered invasive outside their native range (Ficetola et al. 2007), and CI is sometimes used as a surrogate for its population size (Shirose et al. 1997). While bullfrogs called throughout the sampling period in our study area, the time of predicted peak detection occurred just before or after 0100 h, which is two hours after most NAAMP volunteer surveys in the area ceased. Bridges and Dorcas (2000) also reported peak calling times after 0100 h for this species. While naive occupancy estimates can be corrected using detection probabilities (e.g., Weir et al. 2005), surveys that consistently omit or underestimate abundance due to improper sampling times may fail to adequately describe population trends.

In our study area, two additional species, northern leopard frogs and mink frogs, are of particular conservation concern. Northern leopard frogs are declining throughout
their range (Hinshaw 1999) and are listed as a Species of Special Concern in Maine (www.maine.gov.ifw/wildlife/species/endangered_species/specialconcern). We detected this species on only seven COMPLETE nights (~5%) during our study, an observation consistent with reports by NAAMP volunteers during the same period (14 detections, < 1%). Infrequent detections suggest that this species either is rare or is missed by audio surveys. Maine represents the southern range for mink frogs (Stockwell 1999), and their distribution may be particularly sensitive to climate change (Popescu and Gibbs 2009). We predicted that the best full chorus detection time for this species is around 0400 h, which is consistent with observations by Bevier et al. (2006). The mink frog CI for INDIVIDUAL sessions selected from both the full NAAMP and volunteer-truncated NAAMP sessions underestimated the median CI for 33 and 42% of COMPLETE sessions, respectively. It is likely that surveys conducted during the NAAMP time period consistently underestimate CI and thus may fail to detect small changes in CI (such as from CI 3 to 2) that may reflect changes in population sizes.

Species detection and detection of a full chorus may not be represented equally by volunteer-based listener surveys. Time of survey with the greatest probability of detection for four species did not overlap with the time with the greatest probability of detecting their full chorus. For example, the best time to detect a full northern leopard frog chorus occurred three hours after the best time to detect presence, although both times occurred within the NAAMP protocol sampling time. This may have implications for chorus size estimates within individual routes, where there is a greater probability of detecting full choruses at stops sampled later in the run. This discrepancy may not affect occupancy models, however, relative abundance estimates may be underestimated with
this truncated sampling time without correcting for the latent CI (the maximum CI achievable at a site) (Royle 2004; Royle and Link 2005; MacKenzie et al. 2009). For example, mink frog CI underestimates in our study increased from 33 to 42% of nights for NAAMP sessions truncated at 2300 h.

**Environmental Variables as Predictors of Peak Calling**

Time is often a predictor of anuran calling, however, other variables may influence temporal calling patterns. In our study, time was modeled separately and then removed from analyses to examine environmental variables alone. This method allowed us to predict which date or environmental variable most affected calling so that we could determine which conditions within these breeding times optimized species or full chorus detection. Environmental variables affecting calling differed among species. For example, precipitation generally is thought to positively affect anuran calling, and volunteers may be instructed to conduct surveys on nights with light rain above a certain temperature threshold. Our results are consistent with other studies (Oseen and Wassersug 2002; Saenz et al. 2006; Steelman and Dorcas 2010) that found no effect of precipitation on calling occurrence by bullfrogs, pickerel frogs, and northern leopard frogs. Rain also was not associated with calling detection for bullfrogs and pickerel frogs in Maryland (Weir et al. 2005). As reported by Bevier et al. (2004), increased precipitation negatively affected calling detection for mink frogs; the occurrence of precipitation in the 24 hours preceding the recording, however, positively affected calling detection for this species. In contrast to Saenez et al. (2006), we found a negative relationship between precipitation and calling by spring peepers. Precipitation was
included in the final model for April-only spring peeper detection, although this variable appeared to have little effect. The contrast between our study and Saenz et al. (2006) highlights the need to consider survey location when determining effects of environmental variables on calling patterns. Saenz et al. (2006) surveyed amphibians in eastern Texas, where prolonged periods with no precipitation are common. In contrast, Maine is characterized by cool springs and summers with frequent precipitation. Precipitation likely is not a limiting factor in our region, and thus heavy rains may affect calling through other mechanisms.

Wind speed negatively affected calling detection in green frogs, northern leopard frogs, and mink frogs in our study. Wind has also been reported to have a negative effect on detection of green frogs, and other ranids, in Maryland (Weir et al. 2005). However, Oseen and Wassersug (2002) found no effect of wind on green frogs, wood frogs, or spring peepers in New Brunswick, Canada, although they did see an effect on bullfrogs. As recommended in the NAAMP protocol, in our study we did not examine recordings from nights with wind codes greater than three, thus, it is unlikely that the negative effect of wind speed on calling detection in our study was the result of direct interference from wind.

We found that different combinations of variables predicted calling probability among species. This finding was similar for anurans in Maryland (Weir et al. 2005), thus suggesting the need for volunteers to record multiple environmental variables during surveys. Ideally, environmental data should be collected concurrently with call data (Dorcas et al. 2009). Additional environmental variables not routinely collected by volunteers also may be important. For example, water temperature was a strong predictor
of calling by bullfrogs and green frogs, although this variable became less important later in the breeding season (Oseen and Wassersug 2002).

We also found differences in predictive environmental variables between April-only and full-season surveys for early spring breeders. For example, northern leopard frogs were detected only in April, and wood frogs were the only explosive breeder that called beyond April. In contrast to Oseen and Wassersug (2002), we found that additional variables were needed to predict calling during April compared with the full season. Date was the strongest predictor for the full season and had a negative effect on the probability of detecting wood frogs. This species called primarily during April and early May, therefore, it is likely that other environmental variables had less effect as breeding declined. Fewer variables were needed to predict calling by gray treefrogs and spring peepers during the full season compared with April alone. Since calling grows more energetically expensive with warmer temperatures as a function of increased calling rates toward the end of the breeding season (Taigen et al. 1985; Wells et al. 1996), it is possible that individuals calling later in the season are responding to cues that minimize energy expenditure. Spring peepers, for example, were not strongly affected by cloud cover and low temperatures in April. Over the full sampling season, however, calling was positively affected by temperature and negatively affected by precipitation and the cloud cover-moon interaction. Individuals reduced calling on nights where precipitation may have interfered with call transmission and on moonlit cloudless nights, when individuals potentially were more visible to predators (Weir et al. 2005).

One weakness in our dataset was the inclusion of multiple sites with single surveys (COMPLETE nights). Models described in MacKenzie et al. (2006) can model
detection probabilities for datasets with missing observations, although these models assume that detection probabilities for a given species are equal among sites. Since we did not make this assumption with our dataset, our study may therefore underestimate site occupancy given imperfect detection. Thus, our study should not be used as a proxy to other NAAMP-related modeling studies (e.g., Royle 2004; Royle and Link 2005; Weir et al. 2005; Weir et al. 2009; Miller et al. 2011). Instead, our study describes entire night calling patterns throughout the night, beyond typical monitoring periods, that typically are not included in analysis of audio data and can be used to inform preexisting NAAMP detection and occupancy modeling. Furthermore, our study illustrates that additional survey effort may not be required to precisely model calling patterns for certain species in Maine (those that call primarily during the NAAMP time period). Using our naïve detection probabilities, gray treefrogs for example were always detected during the NAAMP interval and not detected during only 21% of INDIVIDUAL sessions.

Our study also contributes to modeling the maximum attainable CI (‘latent CI’) (Royle 2004; Royle and Link 2005), which is derived from the observed CI, with greater observed CI values indicating a greater potential latent CI for a given site and species (Royle 2004; Royle and Link 2005). These models are built on values derived from a limited (e.g., NAAMP volunteer-truncated) sampling time, which may underestimate the true latent CI for species that routinely call in greater abundance or activity (depending on the definition of CI as an indicator of population size or activity level) during the post-NAAMP period. For example, at one site with multiple COMPLETE night surveys, we recorded mink frog CI values of (0, 1, 1) and (1, 2, 2) during NAAMP and COMPLETE sessions, respectively, resulting in a predicted lower latent CI for the former due to
sampling bias rather than actual differences in abundance. Full night audio surveys, such as those presented here, can thus be used to evaluate whether modeled latent CIs are accurate or a reflection of the truncated NAAMP sampling interval. Current detection and occupancy modeling corrects for imperfect detection during the NAAMP sampling period; however, pairing NAAMP surveys with a subset of ARS-based full night surveys will allow researchers to determine the precision and effectiveness of these corrections across multiple species.

The longer NAAMP listening period at each stop accounts for resumption of normal calling activity following potential surveyor-induced disturbances (e.g., arrival at the site or passing cars) at the beginning of the survey period, although few studies (e.g., Granda et al. 2008) have examined surveyor impacts on calling patterns. Because ARS are remote and automated, human disturbance during the recording period is not an issue. While the two minute time intervals used in our study allowed us to collect recordings hourly over several nights, this abbreviated time interval may have affected detection of certain species. There is no consensus on an ideal time interval for anuran call surveys. However, this abbreviated time interval may affect detection of certain species. Shirose et al. (1997) found no difference in detecting North American bullfrogs between three and five minute surveys, and Gibbs et al. (2005) proposed that one minute surveys detected most species in New York State (USA). In contrast, other studies (Crouch and Paton 2002; Pierce and Gutzwiller 2004) found that 10-15 minute surveys are needed for detecting at least 90% of species. It is possible that our abbreviated recordings failed to detect all calling species. Additional studies are needed to determine the optimum
recording interval to detect all calling anurans using ARS while accounting for nocturnal calling patterns.

Standardized volunteer-based audio surveys track long-term and regional changes in anuran populations. A unified sampling time period (0.5 h past sunset to 0100 h) allows for standardized data comparisons across sites and years. Most species are captured during this time period; however, our study suggests that it may not be sufficient for detecting certain species, such as pickerel frogs and mink frogs, and their full choruses in Maine. Further, certain species, such as northern leopard frog, were rarely detected in our study and were likewise rarely detected in NAAMP data for the same period. Whereas other studies have considered only a single site (e.g., Steelman and Dorcas 2010) or season (e.g., Oseen and Wassersug 2002), we suggest expanding survey efforts across multiple sites and seasons for pickerel frogs and mink frogs to better describe temporal calling patterns and relationships with environmental variables. Although detectability estimates can correct for false absences, consistently missing a species due to improper sampling times may underestimate occupancy even when detectability corrections are applied. Studies with longer survey times and additional sampling periods targeting rarely detected species may improve accuracy of the current NAAMP sampling protocol.

Chapter Acknowledgements

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Scientists. We thank B. Agius, D. Bavaro, G. Dixon, L. Keener-Eck, D. Morgan, J. Noll, J. Rowe, J. Shrader, J. Torzewski, and J. White for assistance in the field and laboratory. We thank landowners in western and Downeast Maine for access to lakes and wetlands on their properties. Automated recording systems were generously constructed by K. Lesniewicz and G. Dixon (University of Maine) or loaned by L. Bailey (USGS-Patuxent Wildlife Research Center). The research was improved by guidance and recommendations from W. Halteman, K. Simon, and W. Glanz. Review comments provided by L. Bailey, L. Weir, and two anonymous reviewers improved the manuscript. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Government. This is University of Maine Agricultural and Forest Experiment Station Publication Number 3257.

**Chapter References**


Chapter 5
SUMMARY OF FINDINGS

Study Overview

This study was undertaken to gain a better understanding of breeding habitats used by *Ambystoma maculatum* (spotted salamanders) in eastern Maine (USA), particularly this species’ use of currently fishless (‘fishless’) and historically (but now containing introduced fish; ‘stocked’) lakes. We conducted this study during 2006-2010 at eight fishless lakes, 11 stocked lakes, and 14 reference vernal pools in Hancock and Washington Counties, Maine. Specifically, we investigated embryo survival, adult fitness characteristics and behavior, and offspring morphology in these ‘alternative’ (non-vernal pool) breeding habitats compared with traditional (vernal pool) habitats. We also determined lake and landscape predictors of *A. maculatum* reproductive output (egg mass counts) in lakes. Because fishless lakes in Maine contain unique invertebrate assemblages (Schilling et al. 2009) and studies elsewhere (e.g. Knapp and Matthews 2000; Vrendenburg et al. 2007) have documented amphibian community shifts in response to fish introductions, we also determined amphibian assemblages among fishless and stocked lakes and related these assemblages to *A. maculatum* reproductive output. Finally, we investigated how data obtained from Automated Audio Recording Devices (ARS) can be used to inform listener- and volunteer-based anuran surveys, such as the North American Amphibian Monitoring Program (NAAMP).
Key Findings

Use of Fishless and Stocked Lakes by Vernal Pool Amphibians

Two of the three vernal pool indicator amphibian species were found breeding in fishless and stocked lakes. *Lithobates sylvaticus* (wood frog) bred in six of seven fishless lakes, four of five stocked lakes, and all reference vernal pools (n=14). *Ambystoma maculatum* also bred in all reference vernal pools (n=14), all stocked lakes (n=11), and seven of eight fishless lakes. Salamanders in the *A. laterale-jeffersonianum* complex (blue spotted salamander complex) were not detected breeding at any lakes and in four of 14 vernal pools.

Lake and Landscape Predictors of *Ambystoma maculatum* Reproductive Output in Lakes

While *A. maculatum* was detected breeding at all but one lake, vernal pools on average contained eight times more egg masses than fishless or stocked lakes. Increased reproductive output at lakes was associated with less potential breeding wetland area within 500 (the maximum dispersal distance for *A. maculatum*; 427.6 m [Veysey et al. 2009]; 476 m [Montieth and Paton 2006]) and 4000 m (a conservative distance estimate for *A. maculatum* genetic connectivity [Zamudio and Wieczorek 2007]) of lakes. This suggests that lakes distant from ephemeral wetlands support larger breeding populations. This trend may be driven by limited availability of alternative breeding habitats in landscapes with few vernal pools (Calhoun et al. 2003), a relationship also reported for isolated vernal pools in Maine (Baldwin et al. 2006) and New Hampshire (Veysey et al. 2009).
2011). *Ambystoma maculatum* presence and breeding effort were not affected by within-lake characteristics such as fish presence, lake area, depth, or amphibian assemblage.

**Ambystoma maculatum Embryo Survival among Lakes and Vernal Pools**

*Ambystoma maculatum* embryo survival to hatching was approximately 180% higher in vernal pools than in lakes, and hatching larvae were 33% larger in vernal pools than in lakes. This suggests that, while some embryos survive to hatching in lakes, vernal pools produce significantly more larvae than may be more able to escape predation (Urban 2007) than their smaller counterparts.

**Ambystoma maculatum Morphological, Fitness, and Demographic Responses to Breeding Habitat**

Fitness characteristics (mass and snout-to-vent length) did not differ among breeding habitats, although male *A. maculatum* (4-7 years old) grew faster in fishless lakes than in stocked lakes or vernal pools. Individuals of all age classes (juvenile [1-3 years old], sexually maturing [4-7 years old]), and adults [≥8 years old]) were present in all breeding habitats. High natal site fidelity (Vasconcelos and Calhoun 2004) coupled with presence of juvenile *A. maculatum* in all three systems suggests that juvenile recruitment is occurring in these lakes. Egg mass outer membranes were thicker in stocked lakes than fishless lakes and vernal pools, although it is unclear if this response reduces predation.
Amphibians Assemblages in Fishless and Stocked Lakes

Assemblages of amphibian species that traditionally breed in non-ephemeral habitats were relatively similar in fishless and stocked lakes, although two species (*Notophthalmus viridescens* [eastern newt] and *L. pipiens* [pickerel frog]) were more likely to occur with fish. Both species are unpalatable to fish (Salthe 1963; Grubb 1972; Gill 1978; Rohr et al. 2002). Larvae of two potential *A. maculatum* predators (Boone et al. 2008), *L. catesbeianus* [bullfrog] and *L. clamitans* [green frog], were present in all lakes, with *L. catesbeianus* more abundant in stocked lakes, and *L. clamitans* more abundant in fishless lakes.

Effectiveness of Listener-based Anuran Surveys

We detected eight of nine anurans documented in Maine. Individual recordings selected from the sampling period (0.5 h past sunset to 0100 h) described in NAAMP detected fewer species than were detected in recordings from 30 minutes past sunset until sunrise. Time of maximum detection of presence and full chorusing for three species (*L. clamitans*, *L. palustris*, *L. septentrionalis*) occurred after the NAAMP sampling end time (0100 h). The NAAMP protocol’s sampling period may result in omissions and misclassifications of chorus sizes for certain species. These potential errors should be considered when interpreting trends generated from standardized anuran audio surveys.

Conclusions

Conservation efforts for *A. maculatum* typically focus on vernal pools within terrestrial buffers of a fixed width (e.g., Calhoun et al. 2003; Oscarson and Calhoun 2008;
Windmiller et al. 2008). Our results highlight the need for a management approach to amphibian conservation from a landscape perspective that simultaneously considers the role of a variety of wetland types, landscape setting, and within-wetland characteristics. Longer hydroperiod wetlands may provide a bet-hedging strategy for maintaining local amphibian populations in years of drought (Kolozsvary 2003). Production from permanent wetlands proximal to vernal pools may provide sources of colonizers to avoid local extinctions (Gibbs 2000; Karraker and Gibbs 2009). However, our results indicate that vernal pools are the more productive breeding habitat for A. maculatum. Conservation efforts should continue to consider vernal pools as primary breeding habitats while also incorporating a multi-scale approach to maintain connectivity among multiple breeding habitat types (Semlitsch and Bodie 1998; Rothermel 2004; Cushman 2006; Knapp et al. 2007).

**Chapter References**


Kolozsvary, M.B. 2003. Hydroperiod of wetlands and reproduction in wood frogs (Rana sylvatica) and spotted salamanders (Ambystoma maculatum). PhD Dissertation, University of Maine, Orono, USA.


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Gunzburger, M.S. 2004. The role of tadpole predation in the habitat of the green treefrog (Hyla cinerea). Ph.D. Dissertation. Florida State University, Tallahassee, FL.


APPENDIX A

ADDITIONAL DATA FOR CHAPTER 2

Table A.1. Pearson’s product moment correlations for landscape characteristics surrounding lakes (df=17 for all comparisons). Significant correlations at P < 0.10 are noted in bold.

<table>
<thead>
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<th>Variable</th>
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<th>STRM4000 R P</th>
<th>NOWL500 R P</th>
<th>NOWL4000 R P</th>
<th>WLAREA500 R P</th>
<th>WLAREA4000 R P</th>
</tr>
</thead>
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<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STRM4000</td>
<td>-0.02 0.94</td>
<td>1.00</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOWL500</td>
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<td>-0.15 0.53</td>
<td>1.00</td>
<td>-</td>
<td></td>
<td></td>
</tr>
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<td>-0.13 0.59</td>
<td>1.00</td>
<td>-</td>
<td></td>
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<td>WLAREA500</td>
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<td>0.10 0.67</td>
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<td>-0.07 0.79</td>
<td>1.00</td>
<td>-</td>
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<td>WLAREA4000</td>
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<td>0.52 0.02</td>
<td>-0.25 0.30</td>
<td>0.90 &lt;0.01</td>
<td>-0.25 0.31</td>
<td>1.00</td>
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APPENDIX B
ADDITIONAL DATA FOR CHAPTER 3

Table B.1. AIC values for *in situ* *A. maculatum* embryo enclosure experiment

1. Proportion of surviving offspring

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<th>AICc</th>
<th>ΔAICc</th>
<th>w</th>
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</thead>
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<td>2</td>
<td>TRT/TYPE+CHIRO+ALLPRED+ENVWIDTH+(1</td>
<td>SITE)</td>
<td>-200.1</td>
<td>13</td>
<td>430.99</td>
<td>1.14</td>
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<tr>
<td>3</td>
<td>TRT/TYPE+CHIRO+ENVWIDTH+(1</td>
<td>SITE)</td>
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<td>12</td>
<td>429.85</td>
<td>0.00</td>
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<tr>
<td>4</td>
<td>TRT/TYPE+CHIRO+(1</td>
<td>SITE)</td>
<td>-259.9</td>
<td>11</td>
<td>531.18</td>
<td>101.33</td>
</tr>
<tr>
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<td>1+(1</td>
<td>SITE)</td>
<td>-759.10</td>
<td>2</td>
<td>1522.34</td>
<td>1092.49</td>
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2. Larval dry mass

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<th>K</th>
<th>AICc</th>
<th>ΔAICc</th>
<th>w</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TRT/TYPE*CHIRO</td>
<td>-542.76</td>
<td>16</td>
<td>1124.97</td>
<td>9.74</td>
<td>0.004</td>
</tr>
<tr>
<td>2</td>
<td>TRT/TYPE*CHIRO</td>
<td>-549.19</td>
<td>11</td>
<td>1123.76</td>
<td>8.53</td>
<td>0.008</td>
</tr>
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<td>3</td>
<td>TRT+TYPE*CHIRO</td>
<td>-549.93</td>
<td>7</td>
<td>1115.23</td>
<td>0.00</td>
<td>0.566</td>
</tr>
<tr>
<td>4</td>
<td>TYPE*CHIRO</td>
<td>-552.57</td>
<td>5</td>
<td>1115.85</td>
<td>0.62</td>
<td>0.414</td>
</tr>
<tr>
<td>Null</td>
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<td>-559.84</td>
<td>2</td>
<td>1123.82</td>
<td>8.59</td>
<td>0.008</td>
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3. Chironomids present

<table>
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<th>K</th>
<th>AICc</th>
<th>ΔAICc</th>
<th>w</th>
</tr>
</thead>
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<td>92.73</td>
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<td>TYPE+color+(1</td>
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<td>5</td>
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<td>92.36</td>
<td>1.81</td>
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APPENDIX C

ADDITIONAL DATA FOR CHAPTER 4

Figure C.1. Locations of lakes and vernal pools where we deployed ARS during 2006-2009. Lakes (n=24) and vernal pools (n=4) are denoted by circles. Sites in close proximity to one another may not appear as independent circles due to overlapping.
Figure C.2. Cumulative density of survey end times for NAAMP volunteers during 2006-2009.
Figure C.3. Mean Calling Index (CI) ± 1 standard error by hour after sunset for eight species: a) gray treefrog, b) bullfrog, c) green frog, d) pickerel frog, e) northern leopard frog, f) mink frog, g) wood frog, and, h) spring peeper. Individual recordings originally measured in minutes after sunset were grouped into hours after sunset to produce mean CI.
Table C1. Parameter estimates for variables retained in best models fit by generalized mixed models and stepwise logistic regression for predicting detection of calling amphibians by environmental variables for all species during the full sampling season (April – August) and spring breeders (April only). Significant parameters at α = 0.05 are in bold text.

<table>
<thead>
<tr>
<th>Species</th>
<th>Full Year Models</th>
<th>April Only Models</th>
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<tbody>
<tr>
<td></td>
<td><strong>Variables</strong></td>
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<td>Gray treefrog</td>
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<tr>
<td></td>
<td>DAY</td>
<td>-0.01 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DAYSQ</td>
<td>-0.002 ± 0.0005</td>
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<tr>
<td></td>
<td>MOON</td>
<td>1.78 ± 1.31</td>
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<tr>
<td></td>
<td>CLD</td>
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<tr>
<td></td>
<td>PRECIP</td>
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</tr>
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<td></td>
<td>MOON*CLD</td>
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<tr>
<td>Bullfrog</td>
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<td></td>
<td>DAY</td>
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<td>Green frog</td>
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<tr>
<td></td>
<td>DAY</td>
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<td>DAYSQ</td>
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<tr>
<td></td>
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<td></td>
<td>LOWTSQ</td>
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<td></td>
<td>PRECIP</td>
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<td>Pickerel frog</td>
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<td>LOWT</td>
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<td></td>
<td>LOWTSQ</td>
<td>-0.08 ± 0.05</td>
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<tr>
<td>Northern leopard frog</td>
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<td></td>
<td>DAY</td>
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<td></td>
<td>DAYSQ</td>
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<tr>
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Table C.1 Continued

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APPENDIX D

EFFECTS OF VERTEBRATE AND INVERTEBRATE PREDATORS FROM THREE BREEDING HABITATS ON AMBLYSTOMA MACULATUM (SPOTTED SALAMANDER) LARVAL GROWTH AND SURVIVAL IN TANK EXPERIMENTS

Abstract

Ambystoma maculatum Shaw (spotted salamander) in Maine commonly breeds in ephemeral wetlands (vernal pools) but also breeds in wetlands ranging in hydroperiod from semi-permanent to permanent. These breeding sites may be particularly important in landscapes with low vernal pool densities reflecting topographic features or anthropogenic modifications. Hydroperiod characteristics structure predator communities, and thus developing A. maculatum larvae encounter different predation risks among dissimilar wetland types. It is unknown if plasticity in larval behavioral and morphological responses to predators allow A. maculatum to persist in a variety of wetland types. We designed a series of related experiments to test the effects of abundant vertebrate and invertebrate predators from a range of hydroperiods on the survival, morphology, and behavior of A. maculatum larvae. One vertebrate predator combination characteristic of lakes with introduced fish (green frog [Lithobatus clamitans] larvae and golden shiners [Notemgonus crysoleucas]) caused up to 100% A. maculatum larval mortality. Salamander larvae also responded morphologically and behaviorally to visual and olfactory cues from a ‘non-predatory’ fish (N. crysoleucas) by reducing activity and growing smaller dorsal fins. This study highlights morphological and behavioral
responses that may allow immature *A. maculatum* to survive to metamorphosis under different predator conditions while demonstrating lethal and sub-lethal effects of a non-predatory fish.

**Introduction**

Vernal pool breeding amphibians in the northeastern U.S. use multiple wetland and terrestrial habitats throughout their complex life cycles. Throughout its range, one vernal pool-breeding species, *Ambystoma maculatum* (spotted salamander), breeds in wetlands spanning a range of hydroperiods (Hecnar and M’Closkey 1997; Egan and Paton 2004; Karraker and Gibbs 2009) although the greatest juvenile recruitment occurs in seasonally-drying vernal pools (Petranka 1998). In Maine (USA), *A. maculatum*, breeds in ephemeral wetlands (Calhoun et al. 2003; Calhoun et al. 2005; Baldwin et al. 2006), beaver flowages (Cunningham et al. 2007), anthropogenic pools (DiMauro and Hunter 2002; Vasconcelos and Calhoun 2004; Baldwin et al. 2006), and permanent fishless and fish-containing lakes (Shearin 2012 [Chapter 2]). Unlike other vernal pool breeding amphibians (e.g., *L. sylvaticus* and *A. laterale-jeffersonianum* [blue-spotted salamander complex]) in the northeastern U.S., *A. maculatum* is largely secure throughout its range (excluding Delaware, New Jersey, and Oklahoma) (Hammerson 2004). It is unknown if *A. maculatum*’s use of multiple breeding habitats enables its persistence despite losses in ephemeral breeding habitats due to anthropogenic habitat modifications (deMaynadier and Hunter 1998; Pillsbury and Miller 2008; Windmiller and Calhoun 2008; Windmiller et al. 2008).
As a species, *A. maculatum* likely encounters different predator communities among its breeding habitats, because hydroperiod structures amphibian (Babbitt 2005) and invertebrate (Colburn 2004) communities in fishless ephemeral wetlands, whereas, in permanently inundated habitats, fish predation structures amphibian (Wilbur 1987; Skelly 1997) and macroinvertebrate (Stoks and McPeek 2003) populations. For any species to coexist across its range with diverse predator groups, individuals must respond behaviorally or morphologically to avoid or resist predation. Amphibian responses to predators vary by species and ontogeny. Adults, such as *Lithobates sylvaticus* (wood frogs) (Hopey and Petranka 1994) and *A. barbouri* (streamside salamanders) (Kats and Sih 1992), reduce offspring exposure to predators by avoiding ovipositing in waterbodies containing fish. *Amphystoma maculatum* adults do not avoid ovipositing with fish (Ireland 1989; Sexton et al. 1994) but do avoid wetlands with *A. talpoideum* (mole salamander) larvae (Semlitsch and Walls 1993) and low pH waterbodies with predatory trichopteran larvae (Rowe et al. 1994). Adult *A. maculatum* also alter ovipositioning site selection within breeding habitats, perhaps to maximize embryo survival (Shearin 2012 [Chapter 3]). However, for all pool-breeding amphibians, once breeding concludes and adults return to terrestrial habitats, predation resistance is dependent upon embryonic and larval responses to predators.

Juvenile recruitment to local amphibian populations is related in part to larval avoidance of predators prior to metamorphosis. For example, trout predation of *L. muscosa* (mountain yellow-legged frog) larvae has resulted in extirpation of this species from >90% of its historic range by reducing and eliminating juvenile recruitment (Knapp and Matthews 2000; Vrendenburg et al. 2007). Knapp (2005) suggests that spatial and
temporal overlap in habitat use between *L. muscosa* larvae and predatory fish enhance predation opportunities since larvae possess few anti-predator responses. Larval anti-predator defenses are well documented across amphibian taxa and are often induced by chemical cues produced during predation events (Petranka et al. 1987; Chivers and Smith 1998; Schoepchner and Relyea 2005; 2009). Morphological responses include enhanced musculature for faster swimming, changes in pigmentation, and reduced anatomical features that are otherwise attractive to predators (e.g., Relyea 2003; Relyea 2004; Urban 2007a). Behavioral responses include increased time in refugia, reduced activity and feeding time, and shifts in microhabitat use (Petranka and Hayes 1998; Yuriweiz 2004; Schoepchner and Relyea 2009).

Predators themselves may behave differently or display morphological modifications in response to interspecific competition and predation, which in turn affects rates of amphibian predation. Odonate species in fishless lakes, for example, exhibit adaptive phenotypic (Petrin et al. 2010) and behavioral (Strobbe et al. 2011) plasticity in response to fish. Zygopteran larvae in fishless lakes forage less and reduce movement in the presence of predaceous odonates but do not recognize fish as predators (Stoks et al. 2003). The range of responses in both predators and prey, however, is effectively constrained by the physiological limits of the organism (Smith 1987; Alford and Harris 1988) and is often mediated by abiotic wetland characteristics such as vegetative cover (Kopp et al. 2006; Hartel et al. 2007).

It is widely believed that *A. maculatum* breeds primarily in vernal pools to avoid predation of offspring by fish (Clark 1986). Several fish species (e.g., *Lepomis macrochirus* and *L. cyanellus* [sunfish] [Dwyer 2009]) are documented predators of *A.*
Ambystoma maculatum larvae, and other species, such as Notemogonys crysoleucas (golden shiner) are likely predators due to opportunistic feeding in both the littoral and pelagic zones (Christensen and Moore 2008), although this had not been documented. Many historically fishless lakes used for breeding by A. maculatum now contain introduced fish (Schilling et al. 2008). Fish introductions to historically fishless lakes have been linked to the decline and extirpation of several amphibian species (e.g., Hyla regilla [Pacific treefrog] and L. muscosa) (Knapp 2005), but it is unclear how fish predation of A. maculatum larvae compares to other vertebrate and invertebrate predators encountered across A. maculatum’s potential breeding habitats. Several studies suggest that predation pressure due to invertebrates and amphibian larvae in temporary wetlands may exceed that of permanent wetlands for certain amphibian species (Petranka and Kennedy 1999; Gunzburger 2004). While studies have examined predation of A. maculatum larvae among ephemeral wetlands (Kolozsvary 2003; Urban 2007; 2008), there are no explicit comparisons of larval survival among this species’ primary (e.g., vernal pools) and secondary (e.g., lakes) breeding habitats.

Ambystoma maculatum larval anti-predator morphological and behavioral responses have been documented for fish (L. macrochirus and L. cyanellus [sunfish], Dwyer 2009), invertebrate (Dystiscus spp., Urban 2010), and amphibian (A. opacum [marbled salamander], Notophthalmus viridescens [red-spotted newt], Urban 2007a, 2010) predators; however, the effectiveness and extent of these response is rarely compared among multiple predator assemblages characteristic of A. maculatum’s varied breeding habitats (but see Urban 2008 for a comparison of ephemeral wetlands). Anti-predator responses may initially enhance larval survival but may be offset by long-term
costs, such as smaller size at metamorphosis due to reduced foraging time (Dwyer 2009). Furthermore, certain anti-predator responses may not be effective against all predators encountered by a species among multiple breeding habitats. By understanding *A. maculatum* larval responses to predators, and how these responses are affected by other factors (such as timing of interactions and availability of refugia), we can better understand relative threats to larval survival posed by predators across multiple breeding habitats.

Managers often have limited financial resources to conduct long-term juvenile recruitment studies in individual wetlands to determine their importance in maintaining local amphibian populations. For example, certain long hydroperiod wetlands may be essential for long-term population persistence of *A. maculatum* (Karraker and Gibbs 2009) in landscapes with low vernal pool density or in years of drought when recruitment from ephemeral pools is low. Conversely, permanent hydroperiod breeding sites may be ecological sinks for *A. maculatum* due to reduced offspring survival (DiMauro and Hunter 2002; Vasconcelos and Calhoun 2006; Korfel et al. 2010). While embryo survival is lower in permanent lakes in Maine with and without introduced fish than in vernal pools (Shearin 2012 [Chapter 3]), larval survival has not been explicitly compared among these systems. Consequently, the relative contributions of lakes to local recruitment are relatively unknown, although it is likely that some juvenile recruitment is occurring in lakes based on age estimates of lake-breeding individuals (Shearin 2012 [Chapter 2]).

Knowledge of relative predation rates encountered by *A. maculatum* larvae among multiple breeding habitats and the range of behavioral and morphological responses to
predators will elucidate whether non-velar pool breeding habitats are potential ecological sinks. Therefore, the specific objectives of this study were to (1) compare predation rates and direct *A. maculatum* larval responses to fish and top vertebrate and invertebrate predators from permanent and ephemeral breeding habitats in Maine and to (2) quantify indirect responses of *A. maculatum* larvae to visual and olfactory cues from a fish predator, as direct interactions with fish may be limited by littoral vegetation and refugia in field settings (Kopp et al. 2006; Hartel et al. 2007). Addressing the first objective, we designed laboratory experiments to test relative predation rates of a fish (*N. crysoleucas* [golden shiner]) in comparison to potential top vertebrate (*L. clamitans* [green frog] and *L. sylvaticus* [wood frog]) and invertebrate predators (odonate larvae) characteristic of lakes and vernal pools in Maine and investigated how cover, interspecific predator interactions, and timing of predator-prey interactions affect *A. maculatum* larvae’s morphological and behavioral responses. We hypothesized that larval survival would be lowest and morphological and behavioral responses most pronounced with fish because fish avoidance is thought to be the primary reason *A. maculatum* typically breed in vernal pools (Clark 1986), while cover would reduce effects of all predators. Addressing the second objective, we hypothesized that *A. maculatum* larvae would increase use of refugia in response to fish cues resulting in smaller larvae owing to reduced foraging time or events. We also hypothesized that olfactory cues would induce stronger responses in larvae than visual cues owing to the ability of other vernal pool breeding amphibians to recognize novel predators based on olfactory cues (Ferrari et al 2009).
**Materials and Methods**

**Location Description and Collection of A. maculatum Larvae**

We conducted *A. maculatum* larval predation and behavior trials in a heavily forested (> 50% canopy closure) section of the University of Maine’s Demeritt Experimental Forest in Orono, ME, USA, during two field seasons 2009-2010. Larvae used in the experiments were reared from clear and cloudy *A. maculatum* egg masses collected during 15-25 April 2009 and 5-8 April 2010 within 24 hours of ovipositioning in fishless roadside ditches in Penobscot County, ME, and egg masses were housed in separate 60 L plastic ‘stock’ pools filled with well water until hatching. These egg masses served as the baseline condition for assessing effects of predators, as these individuals had not been previously exposed to vertebrate or invertebrate predation. Upon hatching, larvae (n=5 and 20 for aquaria and pools, respectively) were moved to individual ‘stock’ aquaria (38 L) and pools (132 L) and fed a 5 g mixture of live chironomid and dipteran larvae every two to three days. Experiments are summarized in Table D.1.

**Laboratory Methods**

**Predation by Fish and Odonates from Three Aquatic Ecosystems on A. maculatum**

**Larvae and the Effectiveness of Cover in Reducing Predation.** *Ambystoma maculatum* larvae (n=5; mean mass = 30.0 ± 20.0 mg, mean total length = 1.6 ± 0.2 cm) were collected from stock pools and randomly assigned to 40 glass aquaria (38 L) filled
<table>
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<th>Treatments</th>
<th>Response Variables</th>
<th>Duration</th>
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| Predation by fish and odonates from three aquatic ecosystems on *A. maculatum* larvae and the effectiveness of cover in reducing predation | Predators 1. Vernal pool odonates 2. Fishless lake odonates 3. Stocked lake odonates 4. Fish  
Cover 1. Cover 2. No cover | Location and number of predators  
Location and number of larvae  
Final larval length and mass | 3 days |
| Effects of interactions among odonates and fish on predation rates of *A. maculatum* larvae | 1. Control – no predators added 2. *N. crysoleucas* and odonate larvae (*Cordulia* spp. and *Leucorrhinia* spp.) from fishless lakes 3. *N. crysoleucas* and odonate larvae (*Cordulia* spp., *Ladona* spp., and *Leucorrhinia* spp.) from stocked lakes | Locations and number of predators  
Locations and numbers of larvae | 18.5 h |
| Predation and development of *A. maculatum* embryos and larvae with vertebrate lake and vernal pool predators | 1. Control (no predators) with clear or cloudy egg masses and larvae 2. *L. sylvaticus* larvae with clear or cloudy egg masses and larvae 3. *L. clamitans* larvae with clear egg masses and larvae 4. *N. crysoleucas* with clear egg masses and larvae 5. *L. clamitans* larvae and *N. crysoleucus* with clear egg masses and larvae | Egg mass area  
Outer egg mass envelope width  
Embryo survival  
Hatching larval mass and length  
Larval survival  
Larval mass and length  
Emergence date  
Metamorph total length, head width, tail length, right fore- and hindlimb length, and mass | 4 weeks 8 weeks |
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<td>2009 Larval locations</td>
<td>2009</td>
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<td>2. Olfactory</td>
<td>Larval total length and mass</td>
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<td>Metamorph emergence date</td>
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<td>2. Olfactory</td>
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with well water on 28 June 2009. Four predator (vernal pool odonates fishless lake odonates, stocked lake odonates, and fish) and two cover (no cover, cover [35 g of dried leaves and sticks per aquarium]) treatments with four replicates each were randomly assigned to each aquarium. *Notemgonus crysoleucas* were collected during 25-28 June 2009 from two stocked lakes in Hancock County, ME. We collected odonate larvae during 25-28 June 2009 with dipnets swept through the littoral zone at two fishless (Leucorrhinia spp.) and one stocked (Ladona julia Uhler 1857) lake in Hancock County, ME (Odonate identification by Paul M. Brunelle, Atlantic Dragonfly Inventory Project, New Brunswick, Canada). *Leucorrhinia* spp. are presumably top predators in Northeastern U.S. fishless lakes (Bendell and McNicol 1995; Strong and Robinson 2004; Schilling et al. 2009FB). *Ladona* spp. are common in fishless and stocked lakes in Maine (Schilling et al. 2009FB) and may successfully coexist with fish through avoidance of exposed microhabitats (Pierce 1988). Odonates (*Aeshna tuberculifera* Walker 1908) were collected from a vernal pool in Penobscot County, ME on 25 June 2009. This species is abundant throughout vernal pools in the Northeast (A. Remsburg, personal communication). We added two odonate larvae to the stocked lake odonate treatment while we added five larvae to the vernal pool and fishless lakes odonate treatments because we captured approximately half the number of odonates using dipnets in the stocked system than the others. Predators were added to aquaria on 29 June 2009 after allowing *A. maculatum* at least 24 hours to acclimate. Aquaria were covered with black greenhouse shade cloth to prevent disturbance by animals and reduce evaporation. We noted the location and numbers of predators and salamander larvae once daily (~1200 h)
with binoculars from 5 m away during 30 June to 2 July. The experiment was terminated on 2 July, at which point we recorded the mass and total length of remaining larvae.

**Effects of Interactions among Odonates and Fish on Predation Rates of *A. maculatum* Larvae.** In July 2009, we examined how interactions among fish and odonate larvae affected predation rates of *A. maculatum* larvae. We also determined if odonate larvae from fishless lakes behave differently when exposed to fish than odonate larvae from stocked lakes that coexist with fish. Twelve, 38 L glass aquaria were filled with well water. We placed dried leaves on the bottom of each aquarium to provide refugia for predators and salamander larvae. Aquaria were assigned one of three following predator treatments: (1) control – no predators added; (2) *N. crysoleucas* (n = 5 aquaria⁻¹) and odonate larvae (*Cordulia* spp. and *Leucorrhinia* spp.) from fishless lakes (n = 5 aquaria⁻¹); and, (3) fish *N. crysoleucas* (n = 5 aquaria⁻¹) and odonate larvae (*Cordulia* spp., *Ladona* spp., and *Leucorrhinia* spp.) from stocked lakes (n = 5 aquaria⁻¹). Each treatment was replicated four times. Odonates and fish were collected at the same time as specimens used in the previous experiment and were housed in separate 132 L plastic pools until the start of the experiment. We placed ten salamander larvae (1.5 – 2.0 cm total length) into each aquarium on 7 July 2009. Larvae were allowed to acclimate for four hours before predators were added. Once predators were added, we noted locations and number of predators and prey at 0.5, 1.0, and 18.5 h thereafter. Predator and prey locations were classified as either ‘hiding’ (under cover) or ‘not hiding’ (not under cover or in the water column). The experiment was terminated after the final observation (18.5 h). Fish were euthanized and gut contents extracted to confirm that fish were consuming
salamander larvae (identified by the presence of mottled epidermis or discernible body parts.

**Predation and Development of *A. maculatum* Embryos and Larvae with Vertebrate Lake and Vernal Pool Predators.** We undertook laboratory feeding trials in 2010 to determine effects of dominant vertebrate predators common to fishless lakes, stocked lakes, and vernal pools in our study region on *A. maculatum* embryo and larval survival. Five clear or three cloudy (number of replicates represent relative abundance of each color morphotype in the study area) *A. maculatum* egg masses were placed in each of 35 60-L plastic pools filled with well water. We marked egg masses with unique colors of visible implant elastomer injected in three locations in the outer envelope of each egg mass (Regester and Woosley 2005) and photographed each with a ruler for scale. We measured egg mass width, length, width of outer envelope in three locations, and number of embryos from photographs in ImageJ version 1.43u (National Institutes of Health, USA).

*Lithobates clamitans* larvae (Gosner stages 26-30 [Gosner 1960]), *L. sylvaticus* egg masses, and *Notemgonus crysoleucas* (golden shiner) (total length 5.5-9.7 cm) were collected from vernal pools (*L. sylvaticus*) and lakes (*L. clamitans* and *N. crysoleucas*) in Hancock County on 5-12 April and housed in plastic pools filled with well water. These species were chosen as representative fish and amphibian predators because they were present in all stocked lakes (*L. clamitans* and *N. crysoleucas*), fishless lakes (*L. clamitans*), and vernal pools (*L. sylvaticus*) in related studies (Schilling et al. 2008; Shearin 2012 [Chapter 2]). *Lithobates sylvaticus* embryos were allowed to develop until hatching. We added fish and amphibian predator treatments (5 replicates each) to pools
containing *A. maculatum* egg masses on 29 April as follows: (1) control (no predators) with clear or cloudy egg masses; (2) vernal pool predator (*L. sylvaticus* larvae \[n = 40 pool\(^{-1}\)]\) with clear or cloudy egg masses to test effects of egg mass color polymorphism on predation resistance reported elsewhere (Petranka et al. 1998); (3) lake amphibian predator (*L. clamitans* larvae \[n = 40 pool\(^{-1}\)]\) with clear egg masses; (4) lake fish predator (*N. crysoleucas* \[n=10\]) with clear masses; and, (5) lake amphibian (*L. clamitans* larvae \[n = 40 pool\(^{-1}\)]\) and fish (*N. crysoleucas* \[n=5 pool\(^{-1}\)]\) predators with clear egg masses. An equal 15 g mixture of commercial fish flakes, rabbit chow, and dipteran larvae was added to each tank every two to three days as a predator feeding supplement. Pools were covered with white canvas cloth to prevent unintended predation and reduce evaporation and were cleaned weekly with partial water changes. Deceased predators were replaced throughout the experiment with individuals housed in separate stock tanks (but collected at the same time as the original set of predators) to maintain equal predator densities among replicates. Egg masses were photographed on 28 April and 6 and 23 May, and we determined egg mass area and envelope width (as measures of egg mass dissolution from predation or perturbation by predators) and the number of dead (identified by a cloudy or discolored appearance) and live embryos on each date. As larvae began to hatch, egg masses were surrounded with mesh mosquito netting (1 mm gauge, 200 holes per 5.5 cm\(^2\)) to prevent escape. On the final photograph date (23 May), we removed egg masses from the mosquito netting and quantified the number of live and dead hatched larvae and live and dead unhatched embryos. We also searched each pool for any live or dead escaped larvae. The number of surviving embryos/larvae for any time period was calculated as the sum of live and dead hatched larvae plus unhatched live embryos. Dead
hatched larvae were included in this calculation because they survived through embryonic development. We determined body mass with a digital balance and larval length with a ruler and digital photos (ImageJ) for up to five randomly selected larvae from each pool.

We also examined effects of the aforementioned predators on *A. maculatum* larval survival and growth. On 27 May, we added 20 recently hatched *A. maculatum* larvae from stock pools to 139 L pools assigned one of the following treatments (each replicated five times): (1) control (no predators) with larvae from clear egg masses; (2) control (no predators) with larvae from cloudy egg masses; (3) vernal pool predator (*L. sylvaticus* larvae [n = 40 pool⁻¹]) with larvae from clear egg masses; (4) vernal pool predator (*L. sylvaticus* larvae [n = 40 pool⁻¹]) with larvae from cloudy egg masses; (5) lake amphibian predator (*L. clamitans* larvae [n = 40 pool⁻¹]) with larvae from clear egg masses; (6) lake fish predator (*N. crysoleucas* [n=10 pool⁻¹]) with larvae from clear masses; and, (7) lake amphibian (*L. clamitans* larvae [n = 40 pool⁻¹]) and fish (*N. crysoleucas* [n=5 pool⁻¹]) predators with larvae from clear egg masses. Predators were selected from stock pools as described above for the embryo predation portion of this experiment.

Larvae and predators were fed the same 15 g mixture of commercial fish flakes, rabbit chow, and dipteran larvae every two to three days and as needed when food supplies were diminished. Pools were cleaned weekly using partial water changes and covered with white canvas cloth. Dead predators were replaced to maintain uniform predator densities among replicates per treatment. The number of remaining *A. maculatum* larvae was quantified for each pool during 1-2 August. One pool (cloudy egg mass control treatment) dried unexpectedly during the experiment and was eliminated.
from analyses. We randomly selected five larvae per pool and weighed them by first blotting them on a paper towel to remove excess moisture and then placing individuals on a balance. Each larva was photographed and total length and head width were calculated from digital photographs in ImageJ. We tracked emergence date for metamorphosing juveniles and took a photo of and weighed each until 30 August. Metamorph physical characteristics (total length, head width, tail length, length of right forelimb, and length of right hindlimb) were calculated from digital photographs in ImageJ.

**Sublethal Morphological and Behavioral Responses of *A. maculatum* larvae to Cues from a ‘Non-predatory’ Fish (*N. crysoleucas*)**. We performed two tank experiments during 2009-2010 to describe nonlethal effects of a ‘non-predatory’ fish (*N. crysoleucas*) on the behavior and development of *A. maculatum* larvae. We also determined if *A. maculatum* larvae, which are mostly nocturnal, behave differently in response to fish (which are mostly diurnal) cues based on time of first exposure. Asynchronous activity periods may in part explain *A. maculatum*’s ability to avoid fish predation. In July 2009, we filled glass aquaria (38 L, n = 24) with 30 L of well water. Aquaria were randomly assigned treatments using a 3 (predator cue) x 2 (time of day) factorial treatment design with each treatment combination replicated four times. Predator cue treatments were as follows: 1) control – no predator cues; 2) olfactory cues; and, 3) visual cues. The olfactory cue treatment consisted of adding 2 L of water collected from 60 L plastic pools in which 50 *N. crysoleucas* individuals had been housed for approximately 30 days. Water containing fish cues was added every two to three days. To keep aquaria water levels consistent among treatments, 2 L of well water was removed from each aquarium in the olfactory cue treatment prior to adding the water containing fish cues. For the
visual cue treatment, we placed five *N. crysoleucas* each in clear plastic containers (6 L, 35 x 20 x 13 cm) filled with well water. Containers were placed in the aquaria and weighted with rocks to prevent the container from floating. Temporal treatments were as follows: 1) day - predator cues added at 12:00 h, and 2) night – predator cues added at 00:00 h to correspond with peak *A. maculatum* feeding activity (Branch and Altig 1981). *Ambystoma maculatum* larvae (n = 5; total length = 1.67 ± 0.27 cm; mass = 0.04 ± 0.02 g) were added to each aquarium on 10 July 2009. We allowed larvae to acclimate for four hours. A single larval refuge was constructed in each aquarium out of a 15 x 15 cm slate tile held up by four small rocks. Predator cues were added to aquaria in the day treatment at 12:00 h on 10 July 2009. We added night treatment predator cues at 00:00 h on 11 July 2009. Salamander locations and numbers were initially documented at 0, 0.5, 1.0, 1.5, 2.0, 24, and 48 h after predator cues were added, and then weekly thereafter until 2 September 2009. Water volume was kept consistent among treatments throughout the duration of the experiment. *Ambystoma. maculatum* larvae were fed a 5 g mixture of live chironomid and dipteran larvae every three days. Fish were fed commercial fish flakes on the same schedule. We added fish flakes to the larval region of the control and visual cue treatments to ensure that differences among larval behavior and development were due to predator cues not predator food. On 3 September 2009, we measured larval mass after first blotting individuals with a paper towel to remove excess water. Larval total length was measured using a ruler. Larvae were returned to aquaria and we continued to apply the predator cue treatments during larval metamorphosis. The experiment was terminated on 6 October 2009, and we recorded *A. maculatum* metamorph mass.

Physical characteristics (total length, snout-to-vent length [SVL], width of dorsal and
ventral regions surrounding the tail musculature, tail length, right and left forelimb length, and right and left hindlimb length) were determined from digital photographs in ImageJ.

We repeated this experiment in 2010 without the temporal cue addition treatments and with a modified design to maximize water exchange and cue exposure between fish and larval chambers. We filled 15 clear plastic storage boxes (39 L, 88 x 42 x 15 cm, Sterilite®) with approximately 35 liter of well water on 6 June 2010. Storage boxes were assigned one of the following three treatments, each replicated five times: (1) control - no fish cues; 2) visual – visual fish cues only; and, 3) olfactory – olfactory fish cues only. For the control treatment, we placed a smaller clear plastic storage box (11 L, 42 x 29 x 16 cm, Sterilite®) within the larger box, filled it with water from the larger box to equalize the height of the water, and weighted it down with rocks. For the visual treatment, we used the same container set-up as for the control treatment but placed five N. crysoleucas (mean total length = 6.5 cm) in the smaller container. For the olfactory treatment, we drilled 500, 0.6 cm holes in four sides and the bottom of 11 L white plastic dishpans (34 x 32 x 14 cm, Rubbermaid®) and placed a single dishpan in each of the remaining five 39 L storage boxes. We placed five N. crysoleucas (mean total length = 6.5 cm) in each dishpan. Five A. maculatum larvae (mean mass = 17.9 ± 7.1 mg; mean total length = 1.3 ± 0.07 cm) were placed in each of the 39 L storage boxes in the space outside of the smaller fish cue containers (hereafter referred to as the ‘larval region’ and ‘predator cue containers’). A single larval refuge was constructed out of a 15 x 15 cm slate tile held up by four small rocks. Containers were covered with fitted plastic lids each with 50, 1 cm wide drilled plastic holes to allow water exchange. Larvae were fed a
5 g mixture of live chironomid and dipteran larvae every three days. Fish were fed commercial fish flakes on the same schedule, and fish flakes were added to the larval region of the control and visual cue treatments. Every two days, water in the predator cue container for the olfactory treatment was drained into the larval region to ensure adequate water exchange between larvae and predators. Predator cue containers in the visual and control treatments also were picked up and replaced to mimic disturbance in the olfactory cue treatment. Tanks were checked weekly until 17 August. During each check, we classified larval locations as either hiding or not hiding. Larvae were classified as hiding if they were found under refuges or predator cue containers and considered not hiding if they were found in open water. Dead larvae were replaced to maintain equal densities among replicates and treatments until 29 June, at which point we ceased replacement to monitor the effects of cues on larval survival. Dead fish also were replaced as needed.

On 17 August, larvae were photographed and weighed after first blotting each larva on a paper towel to remove excess water. Larval physical characteristics (total length, length of right hind limb, length of right forelimb, and tail length) were determined from digital photographs in ImageJ. We also measured the same characteristics at metamorphosis and noted emergence date (day number).

**Statistical Analyses**

All statistical analysis were performed in Systat version 12 (Systat Software, Inc.) and R version 2.11.1 (The R Foundation for Statistical Computing) and evaluated at $\alpha = 0.05$. 
**Predation by Fish and Odonates from Three Aquatic Ecosystems on *A. maculatum***

**Larvae and the Effectiveness of Cover in Reducing Predation.** We determined effects of cover and predator treatment on the number of salamander larvae over time and the proportion of hiding salamander larvae, anisopteran larvae, and fish with generalized linear mixed models using REML estimation (sample date and tank replication as random effects). Since the presence of refugia could allow more individuals to hide, we analyzed the effect of predator treatment on prey and predator behavior for cover treatments separately. For the no cover added treatment, we considered individuals found in corners to be hiding. The effects of predator and cover treatment on salamander larval length and mass were analyzed with linear mixed models and Kruskal-Wallis tests, respectively.

**Effects of Interactions among Odonates and Fish on Predation Rates of *A. maculatum* Larvae.** We determined effects of predator treatment on the number of *A. maculatum* larvae remaining at 0.5, 1.0, and 18.5 h after predators with generalized linear mixed effects models. We fit models using REML estimation and used time period as a random effect. We examined the effects of predator treatment and anisopteran origin (fishless or stocked lakes) on the proportion of salamander and anisopteran larvae hiding over time using generalized linear models.

**Predation and Development of *A. maculatum* Embryos and Larvae with Vertebrate Lake and Vernal Pool Predators.** We used linear mixed models to determine the effects of predator treatment on egg mass area and envelope width over time. Time was treated as a random effect, with treatment and egg mass replication nested respectively within time. We used generalized linear mixed models to determine effects of predator treatment on the proportion of surviving embryos/larvae over time using REML.
Random effects were nested as described above. Larval mass and length at hatching could not be normalized, and thus we used Kruskal-Wallis tests to determine the effect of predator treatment on each characteristic. We used a Bonferroni corrected $\alpha = 0.006$ to account for multiple comparisons among treatments.

We used generalized linear mixed models to determine the effect of predator treatment on the number of *A. maculatum* larvae surviving at the end of the experiment using REML estimation and tank replication as a random effect. We used separate linear mixed models to examine the effects of predator treatment on larval mass, length, and head width. Because body size is often negatively correlated with salamander larval densities in mesocosm studies, we accounted for larval density by creating a variable with four categories corresponding to the number of remaining larvae per tank (0-5, 6-10, 11-15, and 16-20 larvae). This variable was added to the model as a random effect; tank replicate was nested within the larval density effect.

**Sublethal Morphological and Behavioral Responses of *A. maculatum* larvae to Cues from a ‘Non-predatory’ Fish (*N. crysoleucas*).** We tested the effects of predator treatment and cue addition time in 2009 on the proportion of larvae hiding at two time scales (short term $[\leq 48$ hours of predator additions$]$ and long term [entire duration of the experiment]) with generalized mixed models using REML approximation and tank replication as a random effect. Minutes from the beginning of the experiment was treated as a fixed effect in order to examine how behavior changed over time in response to treatments. The effect of predator treatment and addition time on the number of larvae remaining over time was evaluated with generalized linear mixed models using REML estimation and sampling and tank replication as random effects. We performed a
principle components analysis (PCA) on morphological characteristics of remaining larvae at the end of the experiment. From the PCA, we determined which characteristics were grouped, and selected the characteristic with the highest component loading in each group for further analysis. We used separate linear models to determine the effects of predator treatment, time of predator addition, and number of larvae remaining at the end of the experiment on this subset of larval characteristics.

We used generalized linear mixed models to determine the effect of predator cues on the proportion of larvae hiding over time in 2010. We examined correlations among larval and metamorph morphological characteristics using a Pearson’s product moment correlation matrix. Only characteristics that were uncorrelated with others were retained for further analysis. We used linear mixed effects models, generalized linear models, and Kruskal-Wallis tests to determine effects of predator cues on remaining larval and metamorph characteristics and emergence date.

**Results**

**Predation by Fish and Odonates from Three Aquatic Ecosystems on A. maculatum Larvae and the Effectiveness of Cover in Reducing Predation**

All predator treatments significantly reduced the number of larvae ($|Z|_{alltreatments} > 3.32, P_{alltreatments} < 0.001$) (Figure D.1). Cover also decreased the number of remaining larvae across treatments ($Z = 2.84, P < 0.01$), but there was no interaction among predator and cover treatment ($|Z|_{allinteractions} = 0.81, P_{allinteractions} > 0.42$). For tanks with cover, there was no effect of predator treatment on the proportion of salamander larvae hiding ($|Z|_{alltreatments} < 1.33; P_{alltreatments} > 0.18$); however, the effect of fish on larval behavior
could not be assessed as all *A. maculatum* larvae were consumed before the first behavioral assessment in this treatment. The proportion of predators hiding also did not differ among treatments with cover added (*|Z|* _alltreatments_ < 0.79; *P* _alltreatments_ > 0.43). For tanks without cover, anisopteran larvae from stocked lakes caused a greater proportion of salamander larvae to hide (*Z* = 2.42, *P* = 0.02); however, we could not assess the effects of vernal pool anisopterans on *A. maculatum* behavior as all salamander larvae were consumed in this treatment before the first behavioral assessment. Fewer vernal pool anisopterans hid in tanks without cover (*Z* -2.31, *P* = 0.02) than for the other predator treatments. Larval length did not differ by predator treatment (*|T|* _alltreatments_ < 1.89, *P* _alltreatments_ > 0.06), cover (*T* = -1.98, *P* = 0.05) or the total number of larvae (*T* = 1.69, *P* = 0.09).

Larval mass also was not affected by predator treatment (*X^2_2.56, df=3, p=0.47) or cover treatment (*X^2_2.47, df=1, p=0.12) in individual Kruskal-Wallis tests. The number of remaining larvae per tank was not correlated with larval mass (Pearson’s product moment correlation *t*=0.32, *df*=137, *p*=0.75). Mass and total length were significantly correlated (Pearson’s product moment correlation *t*=23.29, *df*=137, *p*<0.001).
Figure D.1. Mean number of *A. maculatum* larvae (± 1 SE) remaining over time by fish and anisopteran predator treatment. Data are separated by cover treatment with A) cover added and B) cover not added.
Effects of Interactions among Odonates and Fish on Predation Rates of *A. maculatum* Larvae

Both predator treatments significantly reduced the number of larvae compared with the control (\(|Z|_{\text{all treatments}} > 3.72, P_{\text{all treatments}} < 0.001\)) (Figure D.2), although there was no difference between predator treatments (\(Z = 0.27, P = 0.79\)). Salamander tissue was identified in the gut contents of at least one fish in every tank. Anisopteran larvae were not consumed by fish. The proportion of hiding salamander (\(|Z|_{\text{all treatments}} < 1.59, P_{\text{all treatments}} > 0.11\)) and anisopteran (\(|Z| = 0.85, P = 0.40\)) larvae did not differ by predator treatment over time. No fish were observed hiding during the experiment.

Predation and Development of *A. maculatum* Embryos and Larvae with Vertebrate Lake and Vernal Pool Predators

The number of surviving embryos was reduced by all predator treatments compared with the clear egg mass control (\(|Z|_{\text{all treatments}} > 4.77, P_{\text{all treatments}} < 0.001\)), except for clear egg masses developing with *L. sylvaticus* larvae (\(Z = 0.009, P = 0.99\)) (Figure D.3). Egg mass area (as a measure of egg mass dissolution) was not significantly affected by predator treatment (\(|T|_{\text{all treatments}} < 1.71, P_{\text{all treatments}} > 0.09\)) or correlated with the proportion of surviving embryos/hatching larvae (Pearson’s product moment correlation, \(T = -1.78, df = 458, P = 0.08\)). Mean envelope width also was not significantly affected by predator treatment (\(|T|_{\text{all treatments}} < 1.99, P_{\text{all treatments}} > 0.05\), although envelope width
Figure D.2. Mean number of *A. maculatum* larvae (± 1 SE) remaining over time by fish-anisopteran origin predator treatment.

Figure D.3. Boxplot of final proportion of *A. maculatum* embryos remaining by egg mass color and predator treatment (± 1 SE). * indicates significant difference compared with clear-control at P < 0.001.
was marginally thinner in the *L. clamitans* treatment (T = -1.99, P = 0.05). Mean envelope width was not correlated with the proportion of surviving embryos/larvae (Pearson’s product moment correlation, T = -0.42, df = 167, P = 0.67). Larval mass and total length at hatching were not correlated (Pearson’s product-moment correlation, T = 0.83, df = 143, P = 0.41). Predator treatment had a significant effect on newly hatched larval total length (Kruskal Wallis $X^2 = 48.94$, df = 6, $P < 0.001$, Figure D.4) but not on larval mass (Kruskal Wallis $X^2 = 4.55$, df = 6, P = 0.60).

All predator treatments except for ‘clear’ *L. sylvaticus* larvae reduced the number of larvae at the end of the experiment ($|Z|_{\text{alltreatments}} > 3.18$, $P_{\text{alltreatments}} < 0.01$) (Figure D.5). Larval mass, total length, and head width were highly intercorrelated ($R_{\text{allcomparisons}} > 0.89$, $P_{\text{allcomparisons}} < 0.001$). Predator treatment also affected salamander length, mass, and head width (Table D.2).

**Sublethal Morphological and Behavioral Responses of *A. maculatum* larvae to Cues from a ‘Non-predatory’ Fish (*N. crysoleucas*)**

In 2009, the proportion of larvae hiding decreased for all treatments over time within the first 48 hours of the experiment (Figure D.6); however, time had no effect on the proportion of larvae hiding for the duration of the experiment, indicating that the effects of predator cues decreased over time (Figure D.7). The number of larvae declined over time in all treatments, but the visual cue treatment significantly reduced the number of remaining salamander larvae ($Z = -2.11$, $P = 0.03$) compared to the olfactory and control treatments (Figure D.8).
Figure D.4. Boxplot of *A. maculatum* larval length (± 1 SE) at hatching by predator and egg mass color treatment. **P < 0.01** indicates significant differences for the *L. clamitans* treatment compared with control-clear and control-cloudy treatments and the cloudy-*L. sylvaticus* treatment compared with the cloudy-control treatment.

Figure D.5. Boxplot depicting effects of predator treatment on the number of remaining *A. maculatum* larvae at the end of the experiment. Symbols above bars indicate significant differences (**P < 0.01, ***P < 0.001).
Table D.2. Results of linear mixed model predicting the effects of predator treatment on *A. maculatum* larval characteristics after eight weeks. The *N. crysoleucas* - *L. clamitans* treatment was eliminated because all larvae were consumed. Text in bold indicates significance at $P < 0.05$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Larval length</th>
<th></th>
<th></th>
<th>Larval mass</th>
<th></th>
<th></th>
<th>Head width</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate ± 1 SE</td>
<td>T</td>
<td>P</td>
<td>Estimate ± 1 SE</td>
<td>T</td>
<td>P</td>
<td>Estimate ± 1 SE</td>
<td>T</td>
<td>P</td>
</tr>
<tr>
<td>Control-cloudy</td>
<td>-0.51 ± 0.25</td>
<td>-2.09</td>
<td><strong>0.04</strong></td>
<td>-0.12 ± 0.06</td>
<td>-2.01</td>
<td><strong>0.05</strong></td>
<td>-0.09 ± 0.06</td>
<td>-1.56</td>
<td>0.12</td>
</tr>
<tr>
<td><em>N. crysoleucas</em></td>
<td>0.57 ± 0.32</td>
<td>1.80</td>
<td>0.08</td>
<td>0.13 ± 0.07</td>
<td>1.80</td>
<td>0.08</td>
<td>0.10 ± 0.08</td>
<td>1.22</td>
<td>0.23</td>
</tr>
<tr>
<td><em>L. clamitans</em></td>
<td>0.31 ± 0.28</td>
<td>1.11</td>
<td>0.27</td>
<td>0.10 ± 0.07</td>
<td>1.49</td>
<td>0.14</td>
<td>0.16 ± 0.09</td>
<td>1.81</td>
<td>0.07</td>
</tr>
<tr>
<td><em>L. sylvaticus</em>-clear masses</td>
<td>0.23 ± 0.19</td>
<td>1.22</td>
<td>0.23</td>
<td>0.08 ± 0.04</td>
<td>1.87</td>
<td>0.07</td>
<td>0.09 ± 0.04</td>
<td>2.15</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td><em>L. sylvaticus</em>-cloudy masses</td>
<td>0.32 ± 0.21</td>
<td>1.54</td>
<td>0.13</td>
<td>0.08 ± 0.05</td>
<td>1.55</td>
<td>0.13</td>
<td>0.06 ± 0.05</td>
<td>1.12</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Figure D.6. Mean proportion of *A. maculatum* larvae (± 1 SE) hiding by treatment within 48 hours of initial predator cue addition. Cues added during A) day (1200 h) and B) night (0 h)
Figure D.7. Mean proportion of *A. maculatum* larvae (± 1 SE) hiding by treatment over the duration of the experiment. Data are pooled over cue addition time treatment (day and night) as there was no effect of this variable.

Figure D.8. Mean number of *A. maculatum* larvae (± 1 SE) remaining over time by predator cue treatment.
There was no effect of predator cue addition time on the number of larvae remaining ($Z = 0.16; P = 0.87$).

PCA analysis indicated associations among several larval morphological characteristics (Figure D.9). SVL had the highest loading on principal component 1 and thus was chosen as the representative variable from this set for further analysis. Ventral and dorsal tail widths were associated with principle components 2 and 3, respectively. SVL was rank transformed to meet assumptions of normality, and was negatively affected by the visual predator treatment ($T = -2.34, P = 0.02$) and the number of larvae remaining ($T = -4.55, P < 0.001$). There was no effect of predator treatment ($|T|_{alltreatments} < 1.12, P > 0.27$), cue addition time ($T = 0.57, P = 0.57$), or number of remaining larvae ($T = -1.04, P = 0.30$) on ventral tail width. Dorsal tail width was significantly lower in the olfactory-nocturnal cue addition treatment ($T = -3.04; P = 0.004$), although there was no effect of the number of larvae remaining on this variable ($T = -0.52, P = 0.61$). In 2010, tank replication and time period did not account for any variability in the generalized linear mixed model and thus a generalized linear model with no random effects was fitted to predict the effect of predator cue treatment on the proportion of larvae hiding. The olfactory cue treatment caused a greater proportion ($Z = 3.49, P < 0.001$) of larvae to hide than the visual treatment ($Z = -0.59, P = 0.56$) compared with the control (Figure D.10). Larval morphological characteristics were highly intercorrelated (Table D.3), so only total and forelimb length were retained for analysis.

There was no effect of predator treatment on larval length according to a linear mixed effects model with tank replication as a random effect ($|T|_{alltreatments} < 0.43$,
Figure D.9. Results of PCA relating larval morphological characteristics for the 2009 experiment examining effects of olfactory and visual cues from *N. crysoleucas* on *A. maculatum* larvae. Characteristics are abbreviated as follows: hindlimb length (hind.length.cm), forelimb length (fore.length.cm), mass (mass.g), total length and SVL (shown as one variable tot.svl.length.cm), dorsal tailfin width (dors.tail.cm), and, ventral tailfin width (vent.tail.cm). Numbers represent individual larvae.
Figure D.10. Mean proportion of *A. maculatum* larvae hiding (± 1 SE) by predator cue treatment over time.

Table D.3. Pearson’s product-moment correlation table for experiment examining effects of visual and olfactory cues on *A. maculatum* larval characteristics (2010). Text in bold indicates significance at \( P < 0.05 \).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mass R</th>
<th>Mass P</th>
<th>Total length R</th>
<th>Total length P</th>
<th>Head width R</th>
<th>Head width P</th>
<th>Hindlimb length R</th>
<th>Hindlimb length P</th>
<th>Forelimb length R</th>
<th>Forelimb length P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total length</td>
<td>0.95</td>
<td>&lt;0.001</td>
<td>0.90</td>
<td>&lt;0.001</td>
<td>0.90</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head width</td>
<td>0.88</td>
<td>&lt;0.001</td>
<td>0.91</td>
<td>&lt;0.001</td>
<td>1.00</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forelimb length</td>
<td>0.88</td>
<td>&lt;0.001</td>
<td>0.90</td>
<td>&lt;0.001</td>
<td>0.90</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Hindlimb length</td>
<td>-0.13</td>
<td>0.54</td>
<td>-0.01</td>
<td>0.97</td>
<td>-0.01</td>
<td>0.98</td>
<td>-0.09</td>
<td>0.68</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Tail length</td>
<td>0.85</td>
<td>&lt;0.001</td>
<td>0.94</td>
<td>&lt;0.001</td>
<td>0.88</td>
<td>&lt;0.001</td>
<td>0.85</td>
<td>&lt;0.001</td>
<td>0.02</td>
<td>0.93</td>
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</table>
Forelimb length could not be normalized, and thus a Kruskal-Wallis test found no effect of predator cue treatment on this characteristic (Kruskal-Wallis $X^2 = 0.35$, df = 2, $P = 0.84$). Metamorph morphological characteristics also were highly intercorrelated (Table D.4), and thus only total length and hind limb length were retained in the analysis. According to linear models, predator cue treatment did not have an effect on metamorph total ($F_{2,15} = 0.50$, $P = 0.62$) and hind limb ($F_{2,15} = 0.58$, $P = 0.57$) length. Emergence date was not affected by predator cue treatment (Kruskal Wallis $X^2 = 3.73$, df=2, $p=0.16$); however, few larvae survived to metamorphosis in any treatment (n = 4, 8, and 6 for control, olfactory, and visual cue treatments, respectively).

**Discussion**

**Vertebrate and Invertebrate Predator Effects on Larval Survival**

*Notemgonus crysoleucas* is typically considered a non-predatory fish, however, our results show that even this small fish can cause significant salamander larval mortality. The term ‘predatory fish’ typically applies to larger species (e.g., sunfish and trout) while ‘non-predatory’ refers to smaller albeit predatory species (e.g., minnows), herbivores, and scavengers (Hecnar and M’Closkey 1997). Regulated vernal pools in Maine are partially defined under the Maine Natural Resources Protection Act (NRPA) Chapter 335 as lacking ‘viable populations of predatory fish’. This specification applies primarily to vernal pools in forested floodplains that are occasionally inundated with fish.
Table D.4. Pearson’s product-moment correlation table for experiment examining effects of visual and olfactory cues on *A. maculatum* metamorph characteristics. Text in bold indicates significance at *P* < 0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mass</th>
<th>Total length</th>
<th>Head width</th>
<th>Hindlimb length</th>
<th>Forelimb length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>P</td>
<td>R</td>
<td>P</td>
<td>R</td>
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<td>-</td>
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<tr>
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<td>Head width</td>
<td>0.83</td>
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<td>0.91</td>
<td><em>&lt;0.001</em></td>
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<tr>
<td>Forelimb length</td>
<td>0.82</td>
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<td>0.87</td>
<td><em>&lt;0.001</em></td>
<td>1.00</td>
</tr>
<tr>
<td>Hindlimb length</td>
<td>0.50</td>
<td>0.03</td>
<td>0.53</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Tail length</td>
<td>0.93</td>
<td><em>&lt;0.001</em></td>
<td>0.97</td>
<td><em>&lt;0.001</em></td>
<td>0.83</td>
</tr>
</tbody>
</table>

during spring flooding of nearby rivers and streams. The term ‘predatory’ is not explicitly defined but is largely interpreted in Maine to include salmonids (e.g., *Salmo salar* [landlocked salmon] and *Salvelinus fontinalis* [eastern brook trout]) and centrarchids (e.g., *Lepomis gibbosus* [pumpkinseed sunfish]). Species typically used as bait (e.g., *N. crysoleucas*) are not explicitly included in this definition.

Non-predatory fish predation has been previously thought to have a minimal impact on amphibians (Hecnar and M’Closkey 1997), but recent studies have demonstrated significant impacts of non-predatory fish on ambystomatids. *Pimephales promelas* (fathead minnows) reduced larval survival and growth in *A. macrodactylum* (long-toed salamanders) largely due to interspecific competition for zooplankton (Pearson and Goater 2009). Pagnucco et al. (2011) documented consumption of *A. macrodactylum* by small-bodied native (*Couesius plumbeus*) lake chub as small as 70 mm. Fish in our study were of a similar small size (60 to 90 mm).

While our experiments greatly simplify fish-salamander interactions and habitat structure, *N. crysoleucas* readily consumes prey from both the littoral and pelagic zones of lakes (Christensen and Moore 2008) and tailor feeding times based on prey activity (Reebs 2002), and thus it is possible that refuges provide only minimal protection from
fish predation. The mucoid capsule surrounding *A. maculatum* egg masses prevents centrarchid fish from penetrating the egg mass (Semlitsch 1988; Ireland 1989); however, centrarchids readily feed on *A. maculatum* eggs extracted from the outer envelope (Semlitsch 1988). Fish predation of *A. maculatum* eggs thus may be rare unless the mucoid capsule is compromised (Ward and Sexton 1981). Perturbation of egg masses by swimming fish may have caused mucoid capsules to become dissolute. We observed fish routinely swimming through egg masses, although we do not know if fish actively did so to dislodge embryos or simply to use egg masses as cover.

*Lithobates clamitans* also significantly reduced larval survival, which is consistent with observations of this species consuming *L. sylvaticus* egg masses (Petranka and Kennedy 1999; Vasconcelos and Calhoun 2006) and reducing *A. maculatum* reproductive success (Vasconcelos and Calhoun 2006). *Lithobates clamitans* larvae are common in historically fishless lakes and those containing introduced fish in Maine (Shearin 2012 Chapter 2), and *A. maculatum* larvae co-occur with this predator in natural settings. The combination of *L. clamitans* and *N. crysoleucas* resulted in 100% larval mortality, and these species routinely co-occur in permanent lakes in our region (Shearin 2012 [Chapter 2]). *Lithobates sylvaticus* larvae did not reduce survival of ‘clear’ *A. maculatum* larvae, although they did reduce survival of ‘cloudy’ larvae. The reasons for this are not clear, although *L. sylvaticus* larvae also significantly reduced embryo survival in cloudy egg masses. In other regions (e.g., southern Appalachia), clear egg masses are more vulnerable to predation by *L. sylvaticus* larvae (Petranka et al. 1998).

Odonates are top predators in many systems, particularly those lacking predatory fish (Bendell and McNicol 1995; Strong and Robinson 2004; Schilling et al. 2009FB). In
our study, vernal pool anisoptera (*A. tuberculifera*) consumed similar amounts of *A. maculatum* larvae regardless of cover treatment, thus supporting earlier findings that invertebrate predation may be significant in temporary wetlands (Petranka and Kennedy 1999; Gunzburger 2004).

**Larval Morphological Responses to Predators**

Invertebrate and vertebrate predators induced morphological responses in *A. maculatum* larvae with potential implications for predator avoidance or resistance. Larval head width was greatest in the *L. sylvaticus* (larvae from clear egg masses) treatment (although this difference was only 0.3 ± 0.2 mm) while larval mass was greater for *N. crysoleucas* and *L. sylvaticus* (larvae from clear egg masses) treatments. Larger body size in response to fish presence is not surprising given that rapidly achieving a larger body size may allow larvae to escape gape-limited predators (Urban 2007b), although even the smallest fish in our study had sufficient gape widths to consume even large larvae. Larger larvae may be able to escape predation through stronger tail musculature, (Relyea 2001; 2003; 2004; Teplitsky et al. 2005, but see Anderson and Petranka 2003), although larger tailfins and faster swimming speeds are not always associated with greater survival, particularly in response to odonate predators (van Buskirk 2000; Johnson et al. 2008). While larger tail fins may lure predator strikes away from the core body (Van Buskirk et al. 2003), we observed smaller tail fins in larvae exposed to olfactory fish cues although it is unclear if this response is due to reduced feeding activity (Wilson et al. 2005; Dwyer 2009).
Greater head and body sizes in the *L. sylvaticus* and *L. clamitans* treatments are more surprising. *Lithobates sylvaticus* did not cause significant larval mortality, and thus wider head width may not be an anti-predation response. In an ecological role reversal, *A. maculatum* larvae become predators of *L. sylvaticus* larvae as the former grows larger (Petranka et al. 1998). A wider head may allow salamander larvae to consume larger *L. sylvaticus* prey.

We detected a negative relationship between SVL and the number of larvae remaining in the predator cue experiment. Increased intraspecific competition can lead to slower growth rates among conspecifics, although this in turn induces longer bodies, higher activity rates, and longer tails (Relyea 2004). We do not attribute differences in SVL to competition for several reasons. First, initial *A. maculatum* larval densities were low (n=5 tank⁻¹), and we supplied abundant food resources such that prey was never exhausted. Second, SVL was shortest in the visual cue treatment, and this treatment also had significantly fewer larvae.

**Larval Behavioral Responses to Predators**

*Ambystoma maculatum* larvae responded to olfactory cues by hiding but did not respond to visual cues. Reduced activity is a common response to predation risk in many taxa (e.g., Sih et al. 1992; Relyea 2001; Schoepper and Relyea 2005; Schoepper and Relyea 2009; but see Walls 1995; Dwyer 2009). For example, *L. sylvaticus* and A. *americanus* reduce foraging rates and activity and move away from invertebrate predator stimuli (Petranka and Hayes 1998). Limited activity reduces the risk that prey will be detected. Larvae used in the cue experiment hatched from egg masses oviposited in fish-
free habitats (e.g., ditches and ephemeral wetlands) and thus fish cues were a novel stimulus. A generalized recognition of novel predators has been demonstrated for *L. sylvaticus* based on the intensity of the perceived threat (Ferrari et al 2009). Since fish could not access prey in this experiment, salamander larvae were not responding to alarm cues from crushing or digestion of captured prey (Schoepner and Relyea 2005; Schoepner and Relyea 2009). *Lithobates sylvaticus* also exhibit strong responses to injured conspecifics by reducing movement (Ferrari and Chivers 2010). In our predation trials, predators had direct access to prey and fish consumed all salamander larvae, so we were unable to assess prey responses to alarm cues due to fish consumption.

Anisopterans from fishless lakes, however, did cause more salamander larvae to hide than did anisopterans from stocked lakes, although we could not assess the effects of vernal pool anisopterans on salamander behavior due to heavy predation. Yuriweiz (2004) found reduced activity by *A. maculatum* larvae in the presence of non-predatory Anax larvae, so it is likely that hiding may be more pronounced in response to predatory species.

While more larvae consistently hid in the olfactory fish cue treatment, the proportion of larvae hiding declined over time. This may be due to two reasons. First, escape performance in larval *A. maculatum* is positively correlated with early larval development and negatively correlated with body size and tail resorption (Landberg and Azizi 2009). There also is evidence that morphological changes induced by predator cues early in ontogeny can be reversed later when cues are removed (Relyea 2003). It is possible that behavioral responses may similarly reverse, particularly if predator cues are unaccompanied by more powerful alarm cues from injured conspecifics.
Time of first predator cue addition also affected larval behavior, with more salamander larvae hiding in the nocturnally-added olfactory cue treatment. This suggests that predator cues encountered early in larval development may persist throughout various ontogenetic stages. *Lithobates sylvaticus* also exhibits a time-sensitive response to predator cues (Ferrari and Chivers 2010). Barbasch and Bernard (2011) demonstrated that *L. sylvaticus* larvae exposed to predaceous odonates as larvae were more terrestrial as metamorphs. In natural systems, *A. maculatum* larvae may benefit from temporal segregation from fish during time of maximum activity. Many fish are diurnal feeders, while *A. maculatum* actively feeds at 00:00 h (Branch and Altig 1981). *Notemgonus crysoleucas* migrate from their littoral feeding zones during the day to pelagic surface waters at night presumably to avoid predators (Carpenter and Kitchell 1993; Christensen and Moore 2008), although temporal activity patterns vary widely in relation to prey availability (Reebs 2002), and this species can be active at any time in the littoral zone. We observed diurnal fish feeding likely due to abundant food resources and lack of predators. Nocturnal predator cues may be perceived as stronger threats than diurnal cues because *A. maculatum* actively feeds during this time and is more vulnerable to predation.

We also observed fewer anisopterans from vernal pools hiding in the presence of fish than those from fishless or stocked lakes. It is possible that vernal pool anisopterans do not recognize fish as potential predators, as do *Enallagma species in fishless lakes* (Stoks et al. 2003), although we did not find differences in the proportion of hiding odonates from stocked and fishless lakes in the presence of fish. In contrast, Hopper
(2001) reported differential responses to fish cues by odonates from fishless and fish-containing lakes.

**Summary and Conservation Implications**

Morphological adaptations to predators may be more efficient than behavioral adaptations for certain species, particularly species with limited time for development (Steiner and Pfeiffer 2007). *Lithobates sylvaticus* larvae reduce activity in response to odonate predators (Fraker 2010), and morphological defenses allow this species to feed without increasing predation risk (Relyea and Werner 1999; Relyea 2001). Because *A. maculatum* typically oviposit in habitats with longer hydroperiods and gape-unconstrained predators, a slower growth strategy that accumulates low but constant predation risk may be advantageous (Urban 2007b). This may in part explain why we saw few morphological differences among direct and indirect predator treatments. This slow development strategy is observed in natural systems, with higher densities and longer development times in breeding wetlands with more diverse invertebrate communities (Yuriweiz 2004).

Fish and *A. maculatum* may coexist through behavioral and morphological adaptations of larvae, and through periodic salamander re-colonization of fish-containing habitats. Local populations may show different morphological adaptations based on predator taxa, although this response may be masked by high gene flow among populations (Urban 2010). While winter hypoxia may limit or locally extirpate fish populations in some lakes, many historically fishless lakes in Maine now possess reproductive or annually stocked fish populations (Schilling et al. 2008). Stocked
historically fishless lakes in Maine are located in landscapes containing multiple breeding habitats, and lakes may be periodically recolonized by dispersing juveniles from other wetlands. While female breeding site fidelity may reach 100% in Maine (Vasconcelos and Calhoun 2004), long-distance juvenile movement to new breeding sites results in genetic similarities among breeding sites at distances up to 5 km (Zamudio and Wieczorek 2007). Amphibian populations in fishless ecosystems may be resilient to fish introduction as long as sources of re-colonizers (e.g., vernal pools) are locally available (Knapp et al. 2001). Unlike species that require permanent breeding habitats (e.g., L. muscosa), extirpations due to fish introductions may be lower for A. maculatum due to lower cumulative predation for the entire populations among multiple, fish-free habitats (Knapp et al. 2001).

Small, ephemeral waterbodies are among the most threatened wetland type, and losses of these ecosystems may threaten amphibian conservation through disruption of source-sink dynamics (Semlitsch and Bodie 1998; Gibbs 2000). While our results suggest that predation rates are likely high for A. maculatum larvae in permanent waterbodies containing fish and predatory amphibians, conservation of fish-free permanent habitats may be potential sources of juvenile recruitment in years of drought (Karraker and Gibbs 2009) and warrant further investigation into their role in long-term and landscape-scale amphibian conservation. Our results indicate that A. maculatum larvae differentially respond behaviorally and morphologically to predators characteristic of three breeding habitats in Maine. Future studies are needed to examine how these characteristics affect larval survival in field settings. This information, coupled with breeding population estimates (e.g., egg mass counts, Egan and Paton 2004) will assist
managers in assessing the importance of amphibian recruitment from diverse breeding habitats to landscape-scale and long-term amphibian conservation.

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