Amphibian Metamorphosis in Created and Natural Wetlands

Lauren A. McPherson

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Amphibian Metamorphosis
in Created and Natural Wetlands

Lauren A. McPherson

Thesis submitted to the Davis College of Agriculture, Natural Resources and
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Alphanumeric tag, Water quality, Wetland function

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ABSTRACT

Amphibian Metamorphosis in Created and Natural Wetlands

Lauren McPherson

Functional assessment of created wetlands is an important factor in monitoring the success of wetland mitigation projects. Determining the ability of a created wetland to replace lost wildlife habitat and to support productive wildlife populations should be a priority in the assessment of created wetland success. We used a mesocosm design featuring water collected from 3 created wetlands and 3 natural wetlands in West Virginia to evaluate how the water quality from the 2 wetland types were able to support metamorphosis in larval spring peepers (*Pseudacris crucifer*) and wood frogs (*Rana sylvatica*). Spring peepers displayed similar metamorphosis rates in created and natural wetlands in both years of the study. Wood frogs displayed similar metamorphosis rates in 2015, but in 2014 wood frogs reached metamorphosis in less time and at a larger body size in the natural wetlands than in the created wetlands. These results suggest that created wetlands may provide partial mitigation in terms of water quality for amphibian development. We recommend that future monitoring of created wetlands include measures of juvenile amphibian recruitment.

Monitoring larval amphibians through metamorphosis and into adulthood would benefit from an individual marking technique. In an effort to identify a reliable tagging method, we evaluated the retention rate of visible implant alphanumeric (VIAlpha) tags in larval green frogs (*Rana clamitans*) in 3 body locations (dorsal, ventral, lateral) and with 2 incision treatments (surgical glue, no glue). We found that 100% of ventrally tagged tadpoles lost their tags during our study and that surgical glue did not improve retention in any of the body locations. The retention rate of dorsally tagged tadpoles was 64% and the retention rate of laterally tagged tadpoles was 68%. These retention rates are not sufficient for reliable use, but they are an improvement upon the 4% retention rate seen in a previous study. Further research and practice with VIAlpha tags may improve tag retention and readability. Future studies could then individually mark larval amphibians in a field study comparing created and natural wetlands and more accurately monitor juvenile recruitment, along with other habitat variables, to determine the ability of created wetlands to function as amphibian habitat.
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CHAPTER 1

Introduction to the Use of Amphibians to Assess Wetland Function

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Introduction

Background

The regulatory definition used by the U.S. Environmental Protection Agency (EPA) and the U.S. Army Corps of Engineers (USACE) defines wetlands as “those areas that are inundated or saturated by surface or ground water at a frequency and duration sufficient to support, and that under normal circumstances do support, a prevalence of vegetation typically adapted for life in saturated soil conditions” (Environmental Laboratory 1987). The U.S. Fish and Wildlife Service (USFWS) defines wetlands as transitional lands between terrestrial and aquatic systems that have at least one of the following attributes: (1) the land supports predominantly hydrophytic vegetation; (2) the substrate is predominantly made up of hydric soil; and (3) the area is saturated or inundated by water at some time during the growing season (Cowardin et al. 1979). Wetland delineations follow the jurisdictional definition provided by the EPA and USACE, which requires all three of the above attributes (hydrology, hydric soils, and hydrophytic vegetation) to be present.

Wetlands are found in nearly every climatic zone and on every continent except Antarctica, and therefore vary widely due to differences in soils, topography, climate, hydrology, water chemistry, vegetation, and human disturbance (USEPA 1995). Wetlands are highly diverse across the United States, which has resulted in the development of a classification system to identify and classify wetlands (Cowardin et al. 1979). This classification can be used to describe and arrange ecological units in a system that will aid resource management decisions, provide units for inventory and mapping, and to provide consistency in concepts and
terminology (Cowardin et al. 1979). Because wetlands range from fully submerged marine systems to terrestrial palustrine systems dominated by trees and other emergent vegetation, wetlands as a whole provide a wide range of ecological functions and services.

The Ramsar Convention on Wetlands lists the ecosystem services provided by wetlands as flood control, groundwater replenishment, shoreline stabilization and storm protection, sediment and nutrient retention and export, water purification, reservoirs of biodiversity, wetland products, cultural values, recreation and tourism, and climate change mitigation and adaptation (Hails 2011). These valuable ecosystem services are driven by specific wetland functions, such as surface-water storage, soil retention, and the storage, internal cycling, processing, and acquisition of nutrients (Costanza et al. 1997). High rates of primary productivity and plant decomposition support a large variety of species of microbes, plants, insects, fish, amphibians, reptiles, birds, and mammals (USEPA 1995).

Wetlands provide critical habitat to both plants and wildlife in the United States. Although wetlands only compose 5.5% of the surface area in the lower 48 states, nearly half of the federally endangered wildlife species use wetlands at some point in their lives (Dahl 2011, Mitsch and Gosselink 2007, USEPA 1995). The high biological productivity of wetlands produces a rich biota associated only with wetlands (Gibbs 2000). Wetland plants provide food and shelter to the wetland ecosystem. The decomposition of plant matter in the water provides detritus and microalgae to feed many small aquatic insects, larval amphibians, shellfish, and small fish (Delgado and Stedman 2004). These small animals are then used as a
food source for larger predatory fish, reptiles, amphibians, birds, and mammals that exploit the abundant food supplies offered by wetlands. Wetlands provide essential nesting, migratory, and wintering areas for more than 50 percent of the United States’ migratory bird species (Dahl and Johnson 1991). Additionally, most amphibians, particularly anurans, rely exclusively on wetland habitat for breeding, larval development, foraging, and dispersal (Balcombe et al. 2005c).

*Wetland Loss*

Despite these tremendously valuable ecosystem services and functions, wetlands have historically faced considerable destruction and loss. Prior to the 1970s, wetland destruction was a common practice and was even encouraged by some government policies (Mitsch and Gosselink 2007). At the time of European settlement in the early 1600s, the area that now constitutes the lower 48 of the United States contained an estimated 89.4 million hectares of wetlands (Dahl 1990). As of the mid-1980s, about 41.7 million hectares remained (Dahl and Johnson 1991). This amounts to a nearly 53% loss from the original wetland area (Dahl 1990). Twenty-two states have lost 50% or more of their wetlands, and six states have lost 85% or more of their wetland area, including California, Iowa, Missouri, Illinois, Indiana, and Ohio (Dahl 1990, Dahl and Allord 1996). Wetland loss has occurred in every state in the country: West Virginia has lost at least 24% of its original 54,000 hectares of wetland from the 1780s to the 1980s (Dahl 1990, Herbst 2002). Based on estimates from different survey techniques, the current total wetland area in West Virginia is between 23,000 and 41,000 hectares, representing
less than 1% of land cover (Tiner 2009). Wetland loss across the United States has slowed since the 1980s, but recent analysis shows that national wetland loss still outweighs wetland gains (Dahl 2011).

This history of wetland loss is due to the misconception that wetlands are wastelands that should be avoided or, better yet, drained and filled in order to make the land more useful (Mitsch and Gosselink 2007, USEPA 1995). Because wetlands were thought to restrict travel and impede the production of food and fiber, wetlands were quickly drained and the land was reclaimed for other purposes, such as agriculture (Dahl and Allord 1996). Frayer et al. (1983) estimated that 87% of the wetland losses from the mid-1950s to the mid-1970s were due to agricultural conversion. From the mid-1970s to the mid-1980s, wetland losses due to agricultural conversion had decreased to 54% (Dahl and Johnson 1991). From 1986–1997, 26% of wetland loss was due to agriculture while 74% of wetland loss was due to nonagricultural uses, which include silviculture and urban and rural development (Dahl 2000, National Research Council 2001). Forest conversion for silviculture operations accounted for 56% of wetland losses from 2004 to 2009 (Dahl 2011). Other significant sources of wetland alteration include hydrologic modifications (ditching, draining, levee building), highway construction, mining and mineral extraction, and water pollution (Mitsch and Gosselink 2007).

*Compensatory Mitigation*

Interest in wetland protection began in the 1970s as scientists began to identify the values of these ecosystems (Mitsch and Gosselink 2007). Although
there is still no comprehensive federal legislation in the United States that is specific
to wetland conservation, wetlands are given some protection under certain federal
regulations. The Clean Water Act (CWA, as amended in 1977) is the primary
regulatory legislation to protect wetlands in the United States (Gardner et al. 2009).
The principle objective of the CWA is to “restore and maintain the chemical,
physical, and biological integrity of the Nation’s waters” (National Research Council
2001). Section 404 of the CWA instructs that before proceeding with a project that
could damage a wetland, a property owner must obtain a permit from the USACE
(Gardner et al. 2009). The USACE has the authority to regulate the discharge of
dredged or fill material into wetlands. The three-part cornerstone of section 404 of
the CWA is to avoid (through alternative processes with less adverse impacts),
minimize (through modification of project designs to reduce impacts), and then
mitigate for impacts to waters of the United States (Adusumilli 2015).

President George H.W. Bush publicly launched the “no net loss” policy, which
states that net wetland gains should outweigh wetland losses, during his 1988
presidential campaign (Turner et al. 2001). The goal of “no net loss” of wetlands is
to promote compensatory mitigation through wetland construction and restoration
to replace destroyed wetlands, if the damage is unavoidable (Mitsch and Gosselink
2007, Zedler 1996). Compensatory mitigation is defined as the creation,
restoration, enhancement, or preservation of a wetland that is designed to offset the
Mitigation ratios are the “proportional requirements for replacing wetlands that are
permitted for fill” and are expressed as a ratio of wetland area mitigated to wetland
area lost (National Resource Council 2001). Mitigation ratios aim to reflect the functional values of the lost wetland so that a pristine wetland with a high functional value will have a high ratio and will therefore require more substantial mitigation than a poor-quality wetland would require (National Resource Council 2001). The mitigation ratios for West Virginia, for example, are 1:1 for open water wetlands, 2:1 for emergent wetlands, and 3:1 for scrub-shrub and forested wetlands (§47CSR5A 2010).

Establishing the desired area to be created or restored is only one step of the mitigation process. The policy of “no net loss” refers to wetland functions and values, not simply wetland area. The compensatory wetland should equal or exceed the performance of the damaged wetland (Turner et al. 2001). Although useful, the simple mitigation ratios fail to account for the differences in wetland function that may have been present at the original wetland sites. It is difficult to measure most wetland functions, and this difficulty has resulted in a lack of permit requirements for functional success (Cole and Shafer 2002). The oversight in accounting for wetland function has led to many studies investigating the true ecological success of wetland mitigation.

Approaches for Wetland Assessment

Achieving success and measuring the success of a created wetland are both difficult aspects of assessing wetland mitigation. Mitsch and Wilson (1996) suggest the definition of success as the “establishment of a biologically viable and sustainable wetland ecosystem”, but that still leaves room for interpretation. There
are three main ways to gauge the success of a restored or created wetland. One way to gauge the success of mitigation is to judge whether the project meets its administrative performance measures, which refers to the degree to which they meet their permit requirements (Kihslinger 2008). The rate at which mitigation projects meet their permit conditions is often low. The criteria that most often go unmet include monitoring, submission, and long-term maintenance requirements (Kihslinger 2008, Turner et al. 2001). Although permitting is an important component of wetland mitigation, permit compliance is a poor indicator of the ecological functions of wetlands, especially considering that permits contain little or no ecological criteria for wetland function (Cole and Shafer 2002, Kihslinger 2008, Turner et al. 2001).

The second means of gauging the success of created wetlands is to determine whether or not they replace the functions as the original wetland that is being replaced (Mitsch and Wilson 1996). This can be a difficult process because mitigated wetlands are often constructed in a different geological area or take on a completely different wetland form than their lost wetland counterparts, which may result in different functions. Mitigation has resulted in a shift from scrub-shrub and forested wetlands to less vegetated, open water ponds (Cole and Shafer 2002). These mitigated open water ponds are typically easier to construct and have a higher “success” rate than mitigated forested wetlands (Robb 2001). Replacing a forested wetland with an open water pond is not likely to provide the same level of wetland function to the environment (Cole and Shafer 2002). A study by Pechmann et al. (2001) compared four created ponds with conditions at the original site as
Functional Assessment of Created Wetlands

To date, many of the studies evaluating the success of created wetlands focus on the three important wetland characteristics: hydric soils, hydrophytic vegetation,
and hydrology. A comparison of created and natural wetlands in Pennsylvania yielded less organic matter and more rock fragments in created wetland soil than in reference wetlands, while natural reference wetlands supported greater vegetation species richness and total cover (Campbell et al. 2002). Other studies support these findings of higher quality soil occurring in natural wetlands than in created wetlands, specifically in terms of more soil organic matter content (Bruland and Richardson 2006) and total organic carbon (Stapanian et al. 2013). Measures of vegetation between created and natural wetlands vary greatly. Some studies found greater vegetation species richness and different vegetative community structure in natural wetlands (Campbell et al. 2002, Delphey and Dinsmore 1993, Hartzell et al. 2007, Moore et al. 1999), while other studies saw greater species richness, evenness, and diversity in created wetlands (Balcombe et al. 2005d). The overall trend is that plant communities differ between created and natural wetlands. These differences in vegetative composition may decrease over time (Balcombe et al. 2005d, Desrochers et al. 2008), but that is not always the case (Dee and Ahn 2012, Kearney et al. 2013, Stefanik and Mitsch 2012).

Created wetlands tend to have a longer hydroperiod and contain larger areas of open water than natural wetlands (Cole and Brooks 2000). Wetland mitigation permits require evidence of hydrology, and it is easier and faster to create ponded wetland conditions than to replicate the more temporary inundation patterns of a natural wetland (Cole and Brooks 2000, Robb 2001). Gingerich (2010) found similar hydroperiod (measured in the number of transitions between flooded and exposed conditions per day) between created and natural wetlands in West Virginia.
Hydroperiod is one of the most important factors driving all other wetland functions, but it is perhaps the most difficult parameter to reproduce (Calhoun et al. 2014). A study of the hydrology and physiochemistry in created and natural vernal pools in Ohio found greater surface inundation duration (longer hydroperiod), as well as higher dissolved oxygen and hourly temperature in created vernal pools, while conductivity was higher in the natural vernal pools (Korfel et al. 2010). Differences in physiochemistry may indicate differences in hydrologic sources. The higher conductivity in the natural wetlands may be caused by receiving surface run-off or groundwater input, while the lower conductivity in the created pools indicates that precipitation is the likely water source (Korfel et al. 2010). Wolf et al. (2013) also found greater hydrologic inputs and cycling of ammonium and nitrogen in wetlands with stream water as their primary water source compared to wetlands with precipitation or groundwater as their main source.

Some studies have looked beyond the measures of hydrology, soil, and vegetation to assess the ability of created wetlands to support ecological communities and to gain a deeper understanding of the functional success of these wetlands. Litter decomposition plays an important role in wetland development, and it influences many wetland functions and services through the accumulation of organic matter (Gingerich and Anderson 2011). Some studies have seen higher rates of decomposition in natural wetlands compared to created wetlands (Fennessy et al. 2008, Spieles and Mora 2007), while others found similar litter decomposition potential in both wetland types (Gingerich and Anderson 2011). Soil temperature and air temperature tend to be important driving factors controlling
rates of decomposition, as well as varying periods of flooded conditions, where some litter species decompose faster when there are frequent transitions between flooded and exposed conditions (Gingerich et al. 2014). Taylor and Middleton (2004) also suggest that pH and conductance have an affect on the decomposition of litter. Invertebrates may also be used as indicators of wetland function, as they are sensitive to habitat quality and they influence other wetland functions. Balcombe et al. (2005a) found similar invertebrate taxa richness, diversity, density, and biomass between created and natural wetlands in West Virginia, as did a study on benthic invertebrates in Ohio (Stanczack and Keiper 2004). These findings suggest that created wetlands provide adequate food resources for wildlife, but a healthy invertebrate community alone does not guarantee that wildlife are supported equally in created and natural wetlands (Strain et al. 2014).

Most compensatory mitigation projects do not include wildlife criteria in their design and performance standards, but the ability to replace wildlife habitat should be considered when evaluating wetland mitigation sites (Kihslinger 2008, National Resource Council 2001). Several studies have found that created wetlands failed to compensate for wildlife habitat services lost due to wetland destruction (Kihslinger 2008, Race and Fonseca 1996). Delphey and Dinsmore (1993) found lower species richness and abundance of breeding birds at restored prairie potholes, where emergent vegetation abruptly transitioned to upland vegetation, compared to natural wetlands, where there were areas of distinct low prairie and wet meadow zones that the breeding birds prefer. Desrochers et al. (2008) saw a
similar trend in avian communities in created salt marshes in Virginia, also due to the differing vegetation communities between created and natural wetlands.

Balcombe et al. (2005c) found higher anuran richness and abundance at created wetlands, likely due to higher heterogeneity, larger size, and more submerged aquatic vegetation compared to natural wetlands in West Virginia. Denton and Richter (2013) found that created wetlands had similar species richness to natural wetlands, but that the community composition differed. Pechmann et al. (2001) also saw differences in the community structure of amphibians between created and natural wetlands in South Carolina, and they concluded that created wetlands provided “partial mitigation” for the loss of the natural breeding habitat. They noted that anuran development and communities are largely influenced by hydrologic regimes, wetland size, substrates, vegetation, and surrounding terrestrial habitats. Calhoun et al. (2014) identified hydrology, hydrogeomorphic setting, vegetation, slope, and soil development as the factors that affected created wetland success from the perspective of vernal pool-breeding amphibians. All of these factors tend to be difficult to replicate, which makes it likely that the anuran communities in a created wetland would differ from a natural wetland.

Estimating the ecological success of created wetlands is complex and site-specific. Useful analyses of ecological function require considerable time, financial resources, and ecological expertise (Brown et al. 2012). Due to these limitations, there is little information on whether the ecosystem functions of created or restored wetlands adequately compensate for those lost in the original wetland sites. Monitoring of created wetlands, limited as it may be, primarily focuses on wetland
hydrology, biogeochemistry, and vegetation, with little emphasis on wildlife use and abundance (Balcombe et al. 2005c, Pechmann et al. 2001). Considering the role of wildlife in healthy wetland function highlights the need for evaluative studies of wildlife use of created wetlands. It is important to understand how created wetlands function to provide habitat for wildlife communities so that biologists can develop proper monitoring protocols and plan future wetland construction to best support a diversity of wetland wildlife (Balcombe et al. 2005c, Calhoun et al. 2014).

Role of Amphibians

Amphibians can comprise a large proportion of the vertebrate biomass in temperate wetlands (Whiles et al. 2006). Anurans in particular spend some or all of their life cycle in wetlands, relying on wetlands for breeding, larval development, foraging, and hibernation (Balcombe et al. 2005c, Hecnar and M’Closkey 1998, Houlanhan and Findlay 2003). Anurans play an important role within a wetland ecosystem by acting as primary consumers and altering algal communities, as well as by serving as a food source for higher trophic levels (Pough 1980, Whiles et al. 2006). The ability of amphibians to use both aquatic and terrestrial habitats makes them vulnerable to a wide range of biotic and abiotic stressors (Korfel et al. 2010, Whiles et al. 2006). Due to this substantial environmental sensitivity, amphibians are facing dramatic global declines (Hof et al. 2011, Houlanhan et al. 2000, Stuart et al. 2004). Amphibians are considered the most imperiled taxonomic class of vertebrates (Wake and Vredenburg 2008). Numerous causes for these severe declines in population sizes and diversity have been proposed, including land-use
change and habitat loss, commercial overexploitation, introduced species, emerging infectious disease, global climate change, and environmental contaminants (Collins and Storfer 2003). In the U.S., habitat loss and alteration are likely the most significant contributors to amphibian decline (Denton and Richter 2013). However, emerging diseases are causing tremendous impacts and local extinctions on amphibian populations in remaining habitats, such as ranavirus (Earl and Gray 2014) and *Batrachochytrium dendrobatidis* (Olson et al. 2013). Most local declines are caused by interactions of multiple factors rather than one single factor acting alone, making it challenging to combat these amphibian declines (Hayes et al. 2010).

Biologists often use amphibians as indicators of environmental condition and to assess ecosystem function and habitat quality (Korfel et al. 2010). Because anurans are important components of wetland ecosystems and have the ability to function as bioindicators, evaluating the effects of wetland mitigation on anuran species provides insight into the function of these created wetlands. The ability of anurans to sustain a viable population in wetlands depends on their ability to hatch, grow, undergo metamorphosis, and then survive until the following spring so they can breed (Pollet and Bendell-Young 2000). Tadpoles in the larval development stage are particularly sensitive to aquatic habitat quality, so water quality in breeding pools can be an important determinant of amphibian species composition, richness, and abundance beyond the larval stage (Brown et al. 2012).

The success of created wetlands has been evaluated based on the presence (or absence) of certain wetland factors, such as wetland vegetation and amphibians, during the first three years after mitigation (Lichko and Calhoun 2003). However,
the presence of amphibians in a wetland does not necessarily translate into reproductive success, as seen in a long-term study of created wetlands that showed low levels of amphibian reproductive success following a strong initial colonization of some created vernal pools (Vasconcelos and Calhoun 2006). Many of the studies using amphibians to evaluate created wetland success do so through the use of anuran call surveys (Balcombe et al. 2005c, Brand and Snodgrass 2009). Although a relatively fast and easy method of determining anuran presence, species richness, and relative abundance, anuran call surveys may not be the best method for the functional assessment of a wetland. Anuran call surveys tell the observer which adult male frogs are calling, but the presence of these calling frogs does not indicate successful breeding or recruitment (Berkey and Phillips 2010). The presence of amphibians alone cannot be accurately used to indicate the ability of a created wetland to function as a habitat that can support a persistent amphibian population. Intensive monitoring, including measures of reproductive success and juvenile recruitment, is essential for the proper assessment of the functional success of created wetlands (Balcombe et al. 2005c, Vasconcelos and Calhoun 2006). Calhoun et al. (2014) stressed that recruitment (number of tadpoles that successfully complete metamorphosis and contribute to the breeding population) is a better predictor of population health than reproductive effort (number of egg masses laid) because created wetlands could be ecological traps where adults breed, but larvae are unable to properly develop.

A variety of pollutants occur in wetland habitats that may have impacts on amphibian survival and growth (Egea-Serrano et al. 2012). The nitrogen cycle that
occurs in wetlands transforms the naturally occurring nitrogen into other forms, including ammonia, nitrate, and nitrite (Rouse et al. 1999). Excess nitrates, nitrites, and phosphorus are often added to wetlands from runoff water from developed and agricultural uplands. Wetlands function to lower the concentration of these potentially harmful pollutants from the water and from the landscape through nitrification-denitrification (nitrates and nitrites) and adsorption by soil particles (phosphorus), although the performance of these functions varies greatly (Woltemade 2000). These nitrogenous and phosphorus compounds can be lethal to aquatic organisms at very high levels, and often there are sublethal effects at intermediate concentrations of these pollutants in aquatic habitats that impact tadpole survival, growth, development, behavior, and morphology (Burgett et al. 2007, Egea-Serrano et al. 2012, Marco and Blaustein 1999). The water quality guidelines for healthy drinking water for humans sets a maximum limit of 10 mg/L for nitrates and 1 mg/L for nitrites (USEPA 2013). However, no such water quality criteria exist for the protection of wildlife, and nitrogen and phosphorus levels may vastly exceed the human drinking water limits in agricultural areas (Rouse et al. 1999).

Aside from potentially harmful pollutants, amphibians tend to be sensitive to varying levels of other water chemistry characteristics such as temperature, dissolved oxygen, and temperature. Harkey and Semlitsch (1988) observed more rapid development (shorter larval stage) in *Pseudacris ornata* tadpoles at high temperatures, which is well supported throughout the literature. However, the *P. ornata* tadpoles reached a larger body size at metamorphosis under lower
temperature conditions, because they were channeling a greater proportion of their energy into tissue growth than to maintenance metabolism (Harkey and Semlitsch 1988, Smith-Gill and Berven 1979). Levels of dissolved oxygen are also known to have variable effects on tadpole development, depending on the species being studied and their behavioral and physiological adaptations. Species that develop lungs in the late stages of development (*Bufo* spp.) tend to prefer waters with higher concentrations of oxygen, while some species of tadpoles have been observed gulping air in the early stages of development in oxygen-deficient water (Martin et al. 2015, Noland and Ultsch 1981). Tadpoles that spend more time gulping air at the surface may be spending less time foraging for food, and they may be allotting more energy to breathing rather than to growth and development (Smith 1997). For these reasons, lower oxygen concentrations may decrease the growth rate of tadpoles. Responses to pH conditions tend to vary based on site and species, but typically low pH (< 5.0) has negative effects on amphibian larvae with prolonged exposure, either by causing mortality or by decreasing growth rates (Cummins 1986, Rowe et al. 1992).

The effects of these water quality characteristics on larval development are important at the population level of amphibians. Alteration of water quality in the larval habitat can lead to phenotypic variation in traits that affect metamorphosis and fitness (Harkey and Semlitsch 1988, Newman 1998). For example, larger body size at metamorphosis may result in larger size at first reproduction, higher fecundity, and earlier time to first reproduction. However, having a longer larval period puts the tadpole at risk of high levels of predation and other potential
threats, such as pond drying (Semlitsch et al. 1988). The ideal larval habitat conditions for an amphibian population would allow tadpoles to grow to a large body size in a short period of time. Wilbur and Collins (1973) suggested that poor conditions in the aquatic environment would cause tadpoles to metamorphose earlier and at a smaller size than tadpoles under high quality conditions, to which Newman (1998) responded that age and size metamorphosis may not respond equally to environmental factors. It is important to investigate how parameters of water quality affect the mechanisms of tadpole growth, development, and survival so that the effects on the overall population can then be fully understood.

*Marking Larval Amphibians*

To study the long-term success of created wetlands as amphibian habitat, it would be valuable to assess the ability of larval amphibians that hatch in a created wetland to survive to sexual maturity and to reproduce successfully. It may also be valuable to see if amphibians are selecting to breed in natural wetlands more or less frequently than in created wetlands, and if this preference is influenced by the type of wetland in which they developed as larvae. To accomplish these goals, it would be most effective to individually tag the amphibians during their larval stage and monitor their survival and dispersal throughout their lifespan.

Marking larval amphibians is particularly difficult due to their small size, fragility, and rapid development (Martin 2011). According to Ferner (2007), the ideal marking technique should be permanent, easily identifiable, usable across a range of organism sizes, relatively inexpensive, and have no impact on survivorship,
performance, and behavior. Many techniques have been used to mark tadpoles, each with their own set of potential problems. Some of these techniques include tail-clipping (Turner 1960), staining the entire tadpole (Travis 1981), and photographic identification (Kenyon et al. 2009). These techniques lose their strength after the tadpole undergoes metamorphosis and are best used for identifying cohorts rather than individuals, so they are unsuitable for accurately monitoring individual tadpoles throughout their lifespan.

Passive Integrated Transponder (PIT) tags have proved to be successful in terms of retention on adult amphibians but the tags are too large and heavy for most species of tadpoles (Brown 1997). Coded wire tags (CWT; Northwest Marine Technology Inc., Shaw Island, Washington, USA) have been used to mark Bufo calamita metamorphs (Sinsch 1997), but only one study has investigated the use of CWTs on tadpoles (Martin 2011). CWTs are sections of stainless steel wire as small as 0.5 mm in length that are etched with a number sequence that is viewed under a low-powered microscope (Martin 2011). Although the CWT offers the potential to mark millions of individual tadpoles, Martin (2011) found that they only had a retention rate of 80%. Magnification was needed to identify the presence of the CWT (the tag was often not visible with the naked eye), and often times the tag had to be removed from the ventral tail membrane in order to read the number sequence. If a CWT has to be removed from the tadpole in order to be read, it may not be the best marking technique to use to follow individuals throughout their lifetime. Visible implant elastomer (VIE; Northwest Marine Technology Inc., Shaw Island, Washington, USA) is a silicone material that is injected as a liquid and cures
into a solid under the organism’s skin. Each organism may receive multiple injections of different colors in various spots on the body, but there is still a limit to the number of combinations of colors. Brannelly et al. (2013) tested the effectiveness of VIE tags in adult anurans and found that tag movement occurred in 50% of the implanted tags which caused about 70% of individuals to be potentially misidentified. Grant (2008) performed a similar study on larval *Rana sylvatica* and found that after completing metamorphosis, 67% had lost at least one of the two marks that were implanted. With the high risk of misidentification through tag movement or loss, the use of VIE tags to individually mark tadpoles may be highly unreliable.

An alternative technique that may solve some these problems is the use of visible implant alphanumeric tags (VIAlpha tags, Northwest Marine Technology Inc., Shaw Island, WA). VIAlpha tags are small, fluorescent rectangles of a biocompatible elastomer inscribed with an alphanumeric code. The tags, which contain one letter (A–Z) and two numerals (00–99), are available in four colors, which provide the capability of marking 10,400 individuals. VIAlpha tags have been used successfully with several organisms including fish (Olsen et al. 2004), lobsters (Woods and James 2003), seahorses (Woods 2005), caecilians (Measey et al. 2001), and salamanders (Osbourn et al. 2011). VIAlpha tags have also been used to successfully mark adult anurans (Chelgren et al. 2006, Heard et al. 2008, Pittman et al. 2008). Despite the growing popularity of the VIAlpha tagging method, success has yet to be reached in tagging larval amphibians. Two recent studies have investigated tag retention in larval anurans (Courtois et al. 2013, Strain et al. 2013), but neither was entirely...
successful. Strain et al. (2013) found that *Rana clamitans* tadpoles had an 82% loss rate within two weeks after tag implantation, while only 25% of *Alytes obstetricans* lost their tags in the Courtois et al. (2013) study. Both studies recommend future research on this potential tagging technique, with additional suggestions including using glue to seal the incision site, trying different tagging locations on the tadpole’s body, attempting to mark species with smaller bodies, and monitoring the metamorphosed anurans to see if marking affects their behavior and survival.

**Conclusion**

Wetlands are unique ecosystems that provide a wide range of valuable services to humans, wildlife, and the surrounding environment. As the human population grows and the subsequent anthropogenic effects of development continue to impact the environment, wetlands are often targeted for destruction to make way for new land use. Compensatory mitigation aims to create or restore wetlands in the unavoidable event that a wetland is lost, so that the ecological function of the lost wetland is not permanently eliminated from the landscape. Research is lacking to determine the ability of a created wetland to replace the function of a lost wetland, especially in terms of providing critical habitat to wetland-dependent wildlife species. It is important to understand how wildlife species, such as amphibians, are using created wetlands across all life cycle stages so that managers can improve the planning and construction of future wetland mitigation to more successfully support amphibian communities. Including a long-term monitoring protocol following a wetland creation will provide the most
detailed information on how ecologically successful the creation is. The monitoring of amphibians would benefit greatly from the development of a technique to individually mark larval amphibians. Tracking a tadpole from a created wetland through metamorphosis and throughout adulthood would allow researchers to study how the larval wetland conditions affect individual fitness, as well as overall amphibian populations.

Objectives

This research project consisted of two studies. The objective of the first study was to evaluate the functional success of created wetlands by comparing water quality and rates of anuran metamorphosis between created wetlands and natural reference wetlands in West Virginia. The more specific objectives for this study were:

1. To rear two species of tadpoles (*Rana sylvatica, Pseudacris crucifer*) through metamorphosis in outdoor mesocosms (Figure 1) filled with water from created and natural wetlands and measure development, body size, and time to metamorphosis, and to compare anuran metamorphosis rates between created and natural wetlands.

2. To determine the effect of anuran egg hatching location (whether an anuran egg mass was laid in a created wetland or a natural wetland) on the hatchlings’ metamorphic success (in 2015).
3. To measure dissolved oxygen, pH, conductivity, water temperature, ammonia, nitrate, nitrite, and total nitrogen concentrations in the mesocosms as well as in the source wetlands, and to compare the water quality between created and natural wetlands (in 2014).

4. To measure dissolved oxygen, pH, conductivity, water temperature, total nitrogen, phosphorus, and alkalinity in the mesocosms and the source wetlands, as well as rates of litter decomposition in the mesocosms, and to compare the water quality between created and natural wetlands (in 2015).

5. To determine the effect water quality has on litter decomposition rates, and to compare results from created and natural wetlands (in 2015).

6. To determine the effect water quality has on anuran metamorphosis rates, and to compare results from created and natural wetlands.

I hypothesized that water quality would differ between created and natural wetlands. I hypothesized that the quality of water in the natural wetlands would cause higher rates of litter decomposition, as well as higher metamorphic success in the anuran species (shorter larval stage, larger body size) than the water in the created wetlands. Because litter decomposition provides a potential food source for larval anurans, I hypothesized that metamorphic success will be positively correlated with litter decomposition. I also hypothesized that the type of wetland (created or natural) that the tadpoles spend the majority of their larval stage in (the
mesocosms) will have a greater impact on their metamorphic success than where they were laid as eggs.

The objective of the second study was to assess the suitability of VIAlpha tags (Figure 2, Figure 3) as a means of individually marking tadpoles (*Rana clamitans*).

The specific objectives of this study were:

1. To inject VIAlpha tags dorsally, laterally, or ventrally on the body of a tadpole, and either treat the incision site with surgical glue or with no glue.
2. To determine the effect of tagging location (dorsal, ventral, lateral) and incision treatment (glue, no glue) on survival, tag retention, tag readability, and metamorphic success (Figure 4).
3. To evaluate which tagging technique was the most successful.

I hypothesized that VIAlpha tags could be a successful way to mark larval amphibians once the best technique was determined. I hypothesized that treating the incision site with surgical glue would improve tag retention, and I did not expect any differences in survival between the tagging techniques.

**Study Sites**

The first study included six wetlands in north central West Virginia, USA. Three of the wetlands were created (Sugar Creek, Pleasant Creek WMA, Upper Deckers Creek WMA) and three were natural reference wetlands (Meadowville, Pleasant Creek, Upper Deckers Creek) (Figure 5). Created wetlands and natural wetlands were selected in pairs, so that each pair contained one created wetland.
and one natural wetland (Sugar Creek-Meadowville; Pleasant Creek WMA-Pleasant Creek; Upper Deckers Creek WMA-Upper Deckers Creek). Each wetland pair was selected to have a similar location, elevation, underlying geology, and watershed (Table 1). All of the wetlands had some level of disturbance adjacent to them, caused by paved roads, gravel roads, hiking trails, housing, or mowing.

**Created wetlands**

**Sugar Creek (Sugar)**

The wetland at Sugar Creek was built in 1995 by the West Virginia Division of Highways (DOH) as mitigation for the Appalachian Corridor H highway project. It is located in Barbour County near WV State Route 92, but it sits further back in wooded hills. The wetland is 6.8 ha in size and is comprised of open water ponds, scrub-shrub patches, and wet meadows (Figure 6). A series of berms were installed to control surface runoff (Copen 2004). It sits at an elevation of 479 m. This wetland is located in the Tygart Valley River Watershed and is comprised of shale and sandstone.

**Pleasant Creek WMA (PLC)**

The wetland at the Pleasant Creek Wildlife Management Area, located in Barbour County, was built in 2001 by the Natural Resources Conservation Service. It sits directly adjacent to US Routes 119 and 250, just north of Phillipi and south of Grafton. It is at an elevation of 490 m and is 7.5 ha in size. It is a palustrine emergent wetland with large areas of open water (Figure 7). This wetland is in the Tygart
Valley River watershed and the underlying geology is shale.

**Upper Deckers Creek WMA (UDWM)**

The Upper Deckers Creek Wildlife Management Area wetland is located in Preston County (Figure 8). It was built in 1968 as mitigation for channelization work done on a portion of Deckers Creek. It is adjacent to WV State Route 7, just north of Reedsville, and is surrounded by forest patches, residential houses, roads, and farmland. The berm on the northwest corner of the upper wetland cell that has a water level control structure. The wetland is at an elevation of 520 m and the two cells of the wetland comprise 6.5 ha. It is dominated by open water and aquatic bed. It is in the Monongahela River watershed and has underlying shale and sandstone.

**Natural wetlands**

**Meadowville (Mead)**

The Meadowville wetland is a natural wetland in Barbour County (Figure 9). It is directly adjacent to WV State Route 92 and sits at an elevation of 468 m. The Meadowville wetland is located northeast of its corresponding created wetland, Sugar Creek. The site is 6.6 ha in size and is part of a bottomland wetland complex along Glady Fork, a tributary of Sugar Creek (Gingerich 2010). It is comprised of both emergent persistent and scrub-shrub habitat. This site is in the Tygart Valley River Watershed and is comprised of shale and sandstone.
Pleasant Creek (PLN)

The natural Pleasant Creek wetland is located within the Pleasant Creek Wildlife Management Area in Taylor County at an elevation of 355 m (Figure 10). This natural wetland is located to the east of its corresponding created wetland, Pleasant Creek WMA. It sits along a paved road through the Wildlife Management Area, close to US Routes 119 and 250. It is 3.0 ha in size and is a palustrine scrub-shrub wetland. It is in the Tygart Valley River watershed and the underlying geology is shale.

Upper Deckers Creek (UDC)

The Upper Deckers Creek wetland is a natural wetland located southwest of Masontown in Preston County (Figure 11). Its corresponding created wetland, Upper Deckers Creek WMA, is to the southeast. This site is an oxbow wetland of Deckers Creek at an elevation of 512 m. The wetland is 2.6 ha in size and consists of aquatic bed, emergent persistent, and scrub-shrub habitat. There is a field to the east of the wetland, and forested land to the west. It is in the Monongahela River watershed and is comprised of shale and sandstone.

Study Species

Two anuran species were used in the first study: wood frog (*Rana sylvatica*) and spring peeper (*Pseudacris crucifer*). These species were selected because they are abundant in north central West Virginia, and collecting several hundred
individuals (240 of each species in 2014, 360 of each species in 2015) was not likely to have any impact on their population. Additionally, both species have relatively early breeding times, so tadpoles of each species were collected at similar times.

Due to their freeze-tolerance and adaptation to cold temperatures, wood frogs have a range extending from the southern Appalachians in the northeastern U.S. through Canada to Alaska (Storey and Storey 1984). Adult wood frogs are entirely terrestrial and even hibernate in forested habitats rather than in water. They do, however, seek water during their brief breeding season. In late February to early April, wood frogs migrate to vernal pools that are relatively small, temporarily hold water, have sufficient canopy cover, abundant leaf litter and woody material, and that lack fish (Calhoun et al. 2014). Wood frogs are explosive breeders, and adults typically arrive at the breeding pool, mate, lay eggs, and leave the pool all within a span of one to two weeks (Hunter et al. 1999). Individual egg masses contain up to 3,000 eggs. Eggs hatch within 10 to 30 days, and tadpoles metamorphose in 42 to 105 days, depending largely on temperature and hydroperiod (DeGraaf and Rudis 1983). Wood frog tadpoles feed on algae, detritus, and microorganisms during their aquatic larval stage (Hunter et al. 1999). A mark-recapture study in Appalachian ponds showed that adult wood frogs were 100% faithful to the ponds in which they first bred, but 18% of juveniles dispersed away from their larval ponds to breed in different ponds (Berven and Grudzien 1990).

Spring peepers occur throughout the eastern U.S. into southern Canada. Spring peepers are habitat generalists. Their non-breeding habitat ranges from old growth forest to field habitats, though they tend to be most commonly found in
moist deciduous forests. They use a broad array of water sources for breeding habitat, as long as there is enough surface water to support successful breeding (Wright 1914). They tend to prefer ponds with longer hydroperiods. From March to May, adults migrate to these aquatic habitats to breed and may remain in the breeding pools for about a month. They lay their eggs singly at the bottom of the breeding pool rather than in a mass, and females may deposit up to 900 eggs (Duellman and Trueb 1986). Eggs hatch in 4 to 14 days and larvae reach metamorphosis in 80 to 100 days (Wright 1914). Spring peeper tadpoles feed on algae, detritus, bacteria, and fungi (Skelly and Golon 2003).

One anuran species was used in the VIAlpha tagging study: green frog (*Rana clamitans*). Green frogs are found throughout the eastern U.S. and southeastern Canada. Green frogs are one of the larger anurans in North America, second only to the bullfrog (*Rana catesbeiana*). Adult green frogs are rarely seen far from water and they will utilize nearly any type of freshwater habitat. In late spring to early summer, green frogs typically breed in semi-permanent or permanent aquatic habitats such as lakes, ponds, streams, and swamps (DeGraaf and Rudis 1983). The female green frog lays an egg mass with up to 4,000 eggs that hatch within 6 days (Wright and Wright 1949). Green frog tadpoles are also larger than many other species, reaching 60–80 mm total length (or 18–45 mm snout-vent length) by the time metamorphosis begins (Lannoo 2005, Warny et al. 2012). It was important to select a large anuran species in the hopes that a large tadpole body size would allow the highest possible success in VIAlpha tag retention. The southern U.S. provides warm enough temperatures such that tadpoles can complete metamorphosis within
two to three months without overwintering. Northern populations of green frogs have a shorter growing season after hatching, so they overwinter in their natal ponds and continue with development and metamorphosis the following year (Lannoo 2005, Warny et al. 2012). In West Virginia, both overwintering and same-season metamorphosis may occur depending on the date the eggs are laid (Meshaka 2011, Warny et al. 2012).
References

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Table 1. List of three created wetlands and three natural wetlands in West Virginia, including whether it is a created or natural wetland, age (years), size (ha), elevation (m), watershed, underlying geology, and Universal Transverse Mercator (UTM) coordinates.

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<th>Name</th>
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<th>Elevation</th>
<th>Watershed</th>
<th>Geology</th>
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Figure 1. Outdoor mesocosms (plastic wading pools) filled with water from created wetlands and natural wetlands and located in Morgantown, West Virginia.
Figure 2. Visible Implant Alphanumeric (VIAlpha) tags are small plastic tags (1.2 mm × 2.7 mm) inscribed with a letter (A–Z) followed by two numbers (00–99).
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CHAPTER 2

The Use of Visible Implant Alphanumeric Tags in Green Frog
(*Rana clamitans*) Tadpoles

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ABSTRACT

The ability to mark and identify individual organisms is an important component in monitoring the growth, dispersal, reproduction, and survival of individuals and to study the life history and behavioral ecology of a population or species. The accuracy of estimating these demographic parameters assumes that there is no loss of marks, that there is no misidentification of marks, and that the marks do not alter survival or recapture probabilities. Marking larval amphibians is particularly difficult due to their small size, fragility, rapid development, and drastic morphological changes during metamorphosis. In an effort to identify a reliable individual marking method for larval amphibians, we evaluate the retention and readability of visible implant alphanumeric (VIAlpha) tags in larval *Rana clamitans*.

We injected VIAlpha tags in 3 body locations (dorsal, lateral, ventral) and with 2 incision treatments (surgical glue, no glue). We found that 100% of ventrally tagged tadpoles lost their tags during our study. The dorsal tagging location had a 64% retention rate and the lateral location had a 68% retention rate (p = 0.87). Surgical glue applied to the incision site did not improve tag retention (68%) compared to those not treated with the glue (64%; p = 0.99). Of the dorsal and lateral tags, 66% were visible at metamorphosis and 27% were readable. The combined 66% retention rate and low readability of the dorsally and laterally tagged tadpoles is not sufficient for reliable use, but further research and practice with VIAlpha tags may improve tag retention and readability in future studies.
The ability to mark and identify individual organisms is important for many areas of research. Monitoring the growth, dispersal, reproduction, and survival of individuals allows researchers to study the life history and behavioral ecology of a population or species (Courtois et al. 2013; Ringler et al. 2014). Amphibians are facing dramatic global declines and are considered the most imperiled taxonomic class of vertebrates (Hof et al. 2011; Houlanhan et al. 2000; Stuart et al. 2004; Wake and Vredenburg 2008). To fully understand and combat these massive declines, it is important to develop marking methods as a tool to study the demography and ecology of amphibian species. The accuracy of estimating these demographic parameters relies on 3 fundamental assumptions about marking: (1) there is no loss of marks; (2) there is no misidentification of marks; and (3) the marks do not alter survival or recapture probabilities (Heard et al. 2008). It is unlikely that all of these assumptions will be satisfied in every mark-recapture study, but efforts should be made to reduce violations of the assumptions.

Marking larval amphibians is particularly difficult due to their small size, fragility, rapid development, and drastic morphological changes during metamorphosis (Martin 2011; Ringler et al. 2014). According to Ferner (2007), the ideal marking technique should be permanent, easily identifiable, usable across a range of organism sizes, relatively inexpensive, and have no impact on survivorship, performance, and behavior. Many techniques have been used to mark tadpoles, each with their own set of potential problems. Some of these techniques include tail-fin notching (Turner 1960), staining the entire tadpole (Jung et al. 2002; Travis 1981), and photographic identification (Clemas et al. 2009, Ribeiro and Rebelo 2011).
These techniques lose their strength after the tadpole undergoes metamorphosis and are best used for identifying cohorts rather than individuals, so they are unsuitable for accurately monitoring individual tadpoles throughout their lifespan.

More advanced tagging techniques have been used to individually mark amphibians, each with their own set of advantages and disadvantages. Passive Integrated Transponder (PIT) tags have proved to be successful in terms of retention in adult amphibians, but the tags are too large and heavy for most species of tadpoles (Brown 1997; Courtois et al. 2013; Osbourn et al. 2011). Coded wire tags (CWT; Northwest Marine Technology Inc., Shaw Island, Washington, USA) have been used to mark *Bufo calamita* metamorphs (Sinsch 1997), but only one study has investigated the use of CWTs on younger tadpoles (Martin 2011). Martin (2011) found that CWTs had a retention rate of 80% in *Spea multiplicata*, but the tags had to be removed from the ventral tail membrane in order to read the number sequence. The use of visible implant elastomer (VIE; Northwest Marine Technology Inc., Shaw Island, Washington USA) has been evaluated in adult *Nectophrynoides asperginis* and *Rana pipiens* (Brannelly et al. 2013), larval *Rana sylvatica* (Grant 2008), and larval *Litoria aurea* (Bainbridge et al. 2015). VIE offers varying levels of tag retention, but a common issue throughout these studies is a high risk of misidentification through tag movement or loss.

An alternative technique that may solve some of these problems is the use of visible implant alphanumeric tags (VIAlpha tags, Northwest Marine Technology Inc., Shaw Island, Washington USA). VIAlpha tags are flat, fluorescent rectangles of a biocompatible elastomer inscribed with an alphanumeric code. The tags, which
contain one letter (A–Z) and two numerals (00–99) and are available in 4 colors, provide the capability of marking 10,400 individuals. VIAlpha tags have been used successfully with several organisms, including adult anurans (Chelgren et al. 2006; Clemas et al. 2009; Heard et al. 2008; Pittman et al. 2008). These studies of VIAlpha tag efficiency in adult anurans generally report high rates of tag retention, successful readability, and no negative impacts on the health or survival of marked individuals, which largely satisfies the fundamental assumptions of estimating demographic parameters using mark-recapture (Heard et al. 2008). To date, 2 studies have investigated tag retention in larval anurans (Courtois et al. 2013; Strain et al. 2013). Strain et al. (2013) found that *Rana clamitans* tadpoles had an 82% loss rate within 2 weeks after tag implantation, with all tadpoles tagged dorsally. Courtois et al. (2013) marked larval *Alytes obstetricans* ventrally and dorsally and found that overall, only 25% of the tadpoles lost their tag. Both studies recommend future research on this potential tagging technique, with additional suggestions including using glue to seal the incision site, injecting the tag in different locations on the body of the tadpole, and attempting to mark tadpole species with smaller bodies.

In this study, we evaluated the retention of VIAlpha tags in larval *Rana clamitans* through metamorphosis. Three injection locations (dorsal, lateral, and ventral) and 2 incision site treatments (presence or absence of surgical glue) were tested to determine the most effective tagging technique. Our objective was to measure both retention and readability of each tagging technique (3 locations × 2
glue treatment) to assess whether VIAlpha tags could be a reliable method for individually marking anuran tadpoles.

**Materials and Methods**

*Tadpole rearing.*— On July 5, 2014 we collected 1 green frog egg mass from a shallow puddle near a wetland in Grant County, West Virginia (39.217814°N, 79.429066°W). We transported the egg mass to the laboratory and housed it indoors in a 55 L glass aquarium oxygenated with an air pump and an airstone. The aquarium initially contained water collected from the source wetland and treated tap water (EasyBalancePlus Aquarium Water Treatment; Tetra, Blacksburg, Virginia USA) was gradually used to replace lost water. After the eggs hatched (at about 2 days), the tadpoles remained in the tank until they reached Gosner stage (GS) 25 (Gosner 1960). At that point, tadpoles were randomly added to 6 L plastic aquaria (N = 35) at a density of 3 tadpoles per aquarium (total N = 105). The aquaria were filled with 3 L of treated tap water and were oxygenated with an air pump and airstone. The tadpoles were maintained at about 22°C and at a photoperiod of 14 h light: 10 h dark. Every 3 days, we siphoned uneaten food, feces, and debris from the bottom of the aquaria, replaced half the volume of water with fresh treated water, and provided fresh food ad libitum. The tadpole diet consisted of a 3:1 mixture of rabbit chow (Purina Rabbit Chow; Purina Animal Nutrition Center, Gray Summit, Missouri USA) and fish flake food (Tetramin Tropical Flakes; Tetra, Blacksburg, Virginia USA).

*VIAlpha tags.*— Once the tadpoles reached GS 37 (at 186–262 days), up to 3 tadpoles were anesthetized together in an immersion bath with 0.5g/L tricaine
methylsulfonate (MS-222, Green 2001). The earliest tadpoles were tagged on January 8, 2015 and the latest tadpoles were tagged March 25, 2015. When a tadpole failed to respond to light touches to the eyes or a gentle pinch to a toe, it was suitably anesthetized to receive a tag (minutes for full anesthesia: $\bar{x} = 5.46, SE = 0.14$; Green 2001). Once immobilized, tadpoles were handled with latex gloves and gently held between the thumb and index finger of one hand. An injector with a hollow needle (19 µm) specifically designed to accommodate a 1.2mm x 2.7mm VIAlpha tag was used to implant the tags. The tags used in this study had black text on a fluorescent orange background. The injector needle was angled downwards (45°) towards the body of the tadpole until it pierced the first layer of skin. Once the injector tip was under the skin, we slid it parallel to the body as far as it would go and ejected the tag through the hollow needle into the skin. This handling and injecting period typically took less than 20 seconds. The tadpoles then recovered in a container of clean distilled water until they resumed normal behavior before returning to their aquaria (minutes for full recovery: $\bar{x} = 17.58, SE = 0.88$). Normal behavior was determined when the tadpole was able to swim normally and retracted when poked (Green 2001).

We tagged 17 tadpoles ventrally (8 with glue and 9 without), 25 tadpoles dorsally (11 with glue and 14 without), and 19 tadpoles laterally (11 with glue and 8 without). These sample sizes varied and were smaller than planned because many of the tadpoles stopped developing around GS 35, reducing the total number of tadpoles that grew to GS 37 (and to metamorphosis) to $N = 61$. The tadpoles in each of these location groups that were treated with glue received 1 to 2 drops of surgical
glue (Histoacryl Topical Skin Adhesive; TissueSeal, Ann Arbor, Michigan USA) on the incision hole. The glue hardened quickly (about 5 seconds), so these tadpoles were not exposed to the air for much longer than those without the glue treatment. The injection site on the dorsally tagged tadpoles was at the base of the tail and the tag was aimed at the center of the dorsum, posterior to the eyes (Fig. 1a). The injection site on the ventrally tagged tadpoles was adjacent to the base of the underside of the tail and cloaca, and the tag was aimed at the center of the belly (Fig. 1b). The injection site on the laterally tagged tadpoles was also at the base of the tail, but instead of aiming the tag towards the eyes, it was aimed to the side of the body between where the arms and legs would be (Fig. 1b).

The tadpoles were monitored daily and the date of any lost tags was recorded. Any tadpoles that lost their tag were kept in the aquaria to maintain constant densities. As tadpoles neared metamorphosis, they were checked daily for the emergence of front limbs (GS 42). At GS 42, tadpoles were removed from aquaria, weighed, and transferred to individual plastic cups with shallow water and a clean paper towel to allow them to climb out of the water during tail resorption. Metamorphs in plastic cups were also checked daily for completion of metamorphosis and were weighed upon full tail resorption (GS 46). In addition to measuring the mass of each subject at GS 42 and GS 46, we also recorded the time required to complete metamorphosis (number of days between hatching and GS 46). We recorded the degree of readability for all present tags (0: could not see the tag at all, 1: could see the tag under the skin, but could not read the code, 2: could see and read the code) at the time of tag injection, at GS 42, and at GS 46. A deep
violet visible implant flashlight (VI Light, Northwest Marine Technology Inc., Shaw Island, Washington USA) was used to improve readability of the fluorescent tags. At the end of the study, all remaining metamorphs and tadpoles were euthanized with 5 g/L MS222 to eliminate the risk of spreading any diseases contracted in the laboratory to wild populations.

Another 7 tadpoles did not receive tags and served as a reference group. The reference group underwent the same anesthesia and recovery as the experimental tadpoles to keep potential effects of the handling process constant across all study animals. The reference group was compared with the treatment groups to see how the VIAlpha tags affected growth and development.

Statistical analysis.— The retention rate was reported as a percentage of tadpoles in each treatment group that retained their tag throughout metamorphosis. The null hypothesis that tag retention was independent of the tagging location (ventral, dorsal, lateral) was tested using Fisher’s exact test to accommodate small numbers (Kaps and Lamberson 2004). The effect of tagging location (ventral, dorsal, lateral) and surgical glue (glue, no glue) on retention rate was analyzed using the Mantel-Haenszel test for categorical variables (Kaps and Lamberson 2004). Fisher’s exact test and the Mantel-Haenszel (CMH) test were also used to analyze survival (reported as percentage of tadpoles in each treatment group that survived through metamorphosis) and readability of the tags at metamorphosis (0, 1, or 2).

Mass at GS 46 and time to GS 46 were found to be normally distributed using Shapiro-Wilk W test and were found to have homogeneity of variance using Bartlett’s test. Mass at and time to GS 46 were then analyzed by analysis of variance
ANOVA) with main effects of tagging location (dorsal, lateral, reference) and glue treatment (glue, no glue), and their interactions. Significant relations were further tested by post-hoc Tukey-Kramer HSD (honestly significant difference) multiple comparison tests (Kaps and Lamberson 2004).

We performed ANOVAs using the software package R ([http://cran.r-project.org/](http://cran.r-project.org/)) and frequency analyses using Program PROC FREQ (SAS® v9.1.3). Tests were significant at P < 0.05.

**Results**

Of the 61 tadpoles tagged with a VIAlpha tag (regardless of tagging location and glue treatment), 29 kept their tag through metamorphosis (48%), 21 lost their tag prior to metamorphosis (34%), 3 died (5%), and 8 tags were unknown (were not found in the aquaria, but also were not seen on the body of the tadpole, unable to accurately categorize as “lost” or “kept”; 13%) (Table 1).

Of the 21 tags that were lost, 15 were lost within 24 hours after tag injection (71%) and 5 more were lost within 2 weeks of tagging (95%). Of those 21 lost tags, 17 had been tagged ventrally (81%), 3 had been tagged dorsally (14%), and 1 had been tagged laterally (5%). All (100%) of the ventrally tagged tadpoles lost their tags (Fig. 2), so tag retention was highly dependent on tagging location (p < 0.0001).

Of the 25 dorsally tagged tadpoles (Fig. 2), 16 retained their tag (64%). Of the 19 laterally tagged tadpoles, 13 kept their tag (68%). Tag retention was similar between the dorsal and lateral tagging location (CMH$_3$ = 0.713, p = 0.87) as well as between the presence or absence of glue (CMH$_3$ = 0.018, p = 0.99). Of the 3 tadpoles that died, 2 were tagged dorsally with no glue and 1 was tagged ventrally with glue,
but neither tagging location \((\text{CMH}_2 = 1.313, p = 0.52)\) nor the use of glue \((\text{CMH}_1 = 0.294, p = 0.59)\) affected tadpole survival (Table 1).

At the time of injection, 89\% of dorsal and lateral tags combined (regardless of glue treatment) were at least visible (readability score of 1 or 2) and 26\% were readable (readability score of 2). At metamorphosis (GS 46), 66\% of dorsal and lateral tags combined were at least visible and 27\% were readable. At metamorphosis, none of the ventrally tagged tadpoles retained their tags so only the dorsally and laterally tagged tadpoles were included in readability analysis. Of the 16 dorsally tagged tadpoles that kept their tag through metamorphosis, 6 (38\%) were readable and 10 (63\%) were visible, but not readable (Fig. 3). Of the 13 laterally tagged tadpoles that retained their tag, 6 (46\%) were readable and 7 (54\%) were visible, but not readable (Fig. 3). Tag readability was not statistically different between dorsal and lateral tagging location \((\text{CMH}_3 = 0.93, p = 0.82)\) or between the presence and absence of glue \((\text{CMH}_3 = 0.147, p = 0.99)\).

The number of days it took the tadpoles to reach GS 46 was affected by tagging location \((F_{2,39} = 8.896, p < 0.001)\), but not by the use of glue \((F_{1,40} = 0.135, p = 0.71)\) and there was no interaction \((F_{1,37} = 0.328, p = 0.57)\). Tukey Kramer’s HSD test showed that the dorsally tagged tadpoles took significantly less time to reach GS 46 (days: \(\bar{x} = 257, \text{SE} = 5.47, \text{Fig. 4}\) than the laterally tagged tadpoles \((\bar{x} = 276, \text{SE} = 4.75, p = 0.025)\) and the reference tadpoles \((\bar{x} = 299, \text{SE} = 6.92, p = 0.001)\). The mass at GS 46 was not affected by location \((F_{2,39} = 0.758, p = 0.47)\), use of glue \((F_{1,40} = 0.711, p = 0.40)\), or their interaction \((F_{1,37} = 0.119, p = 0.73)\). There was not a significant difference in mass at GS 46 between dorsally tagged tadpoles \((\bar{x} = 1.81 \text{ g}, \text{Fig. 5})\).
SE = 0.07), laterally tagged tadpoles ($\bar{x} = 1.85$ g, SE = 0.06), and reference tadpoles ($\bar{x} = 1.67$, SE = 0.08).

**Discussion**

Omitting the unsuccessful ventral tags and considering the partially successful dorsal and lateral tags, VIAlpha tag retention in *R. clamitans* tadpoles was too low to reliably use for mark-recapture studies (66%). This retention rate is lower than the 75% retention rate in *A. obstetricans* in Courtois et al. (2013), but substantially higher than the 4% retention in *R. clamitans* in Strain et al. (2013). With this low retention rate we do not recommend the use of VIAlpha tags on *R. clamitans* tadpoles due to the violation of the mark-recapture assumptions (Heard et al. 2008), but we do see the potential for further improvements and research on this tagging method. We increased tag retention from the Strain et al. (2013) study by 62% by trying new tagging locations and techniques, and we offer suggestions that may further increase tag retention in larval amphibians.

Courtois et al. (2013) achieved the highest success when they tagged *A. obstetricans* tadpoles ventrally: retention rate and readability were higher on the ventral side than on the dorsal side. In our study, it was nearly impossible to tag *R. clamitans* on the ventral side. The outer layer of skin on the ventral side of the *R. clamitans* tadpole is fused to the body cavity, so it was not possible to position the tag under the skin without penetrating the body cavity. The ventral side of *R. clamitans* is unpigmented (Fig. 1b), which would have likely provided high levels of readability if the tag could have been injected there. Ventral insertion of VIAlpha tags should be avoided for *R. clamitans*. However, due to the high success of the
ventral tagging location in *A. obstetricans*, it seems that VIAlpha tag retention is dependent on the tadpole species being studied. Anatomical differences between species influence the success of tagging methods (Clemas et al. 2009). The results of this study on *R. clamitans* may not apply directly to other anuran species and this study should be repeated on other species before attempting to use VIAlpha tags as a marking method.

This study and the Strain et al. (2013) study both evaluated *R. clamitans*, but this study resulted in a 66% retention rate while only 4% of tagged tadpoles retained their tags through metamorphosis in Strain et al. (2013). Strain et al. (2013) tagged all of their tadpoles dorsally, but they aimed their tag toward the base of the tail while we aimed dorsal tags toward the middle of the back. The tail movement of the tadpoles gradually pushed the tags out of the incision site in the Strain et al. (2013) study. Placing the tag further away from the tail, such as in the center of the back or closer to the eyes, may reduce movement of the tag and could explain the improved tag retention in our study. Additionally, Strain et al. (2013) housed tadpoles at a density of 5 tadpoles per 3 L of water, did not aerate the tanks, and cleaned tanks once a week. We housed tadpoles at a lower density, aerated the tanks, and cleaned tanks every 3 days. It is possible that the conditions, in terms of crowdedness and cleanliness, may have effected the retention of the tags in the larval *R. clamitans*.

Inserting the tag dorsally on *R. clamitans* was the easiest method compared to tagging ventrally, which was nearly impossible, and tagging laterally, which required some maneuvering. However, the dorsal side of *R. clamitans* is heavily
pigmented (Fig. 1a). The dark pigmentation made it difficult to read the dark code printed on the fluorescent tag under the skin, and in some cases the tag was not visible at all. On the side of the tadpole body where the ventral side and dorsal side converge, the pigmentation lightens (Fig. 1b). When tagging in the lateral position, this is the spot that we aimed for in the hopes that the diluted pigmentation would improve readability. The success of this technique varied from individual to individual. If the incision site was close enough to the base of the tail, the tag could reach across the body to the lightly pigmented lateral side. If the incision site was posterior to the body or if it was a large tadpole, the tag injector would not reach the area with the lighter pigmentation.

The use of surgical glue did not have an effect on tag retention. Courtois et al. (2013) saw lower tag retention in those tadpoles that received glue and they reported signs of discomfort in their tadpoles that were treated with glue. We did not see any differences in behavior between glue treatments. In most cases, the glue came off the body within 24 h. Heard et al. (2008) also reported that the adhesive used on juvenile *Litoria raniformis* in their study (Nexaband, Abbot Animal Health, Abbot Park, Illinois USA) was generally sloughed within 48 h of application. While the glue did not appear to have any negative affect on the tadpole or on tag retention in our study, it also did not improve tag retention. The brand of surgical glue that we selected (Histoacryl® Topical Skin Adhesive, TissueSeal, Ann Arbor, Michigan USA) is a cyanoacrylate tissue adhesive which is suggested for amphibian surgical procedures (Gentz 2007), but it is not meant for wet wounds. We do not recommend
the use of surgical glue because it does not positively affect VIAlpha tag retention in our study or the Courtois et al. (2013) study.

Overall, survival was high (95%) in the VIAlpha tagged tadpoles. Two of the 7 reference tadpoles died, resulting in only 71% survival in the untagged tadpoles. This suggests that any mortality that occurred in this study was likely due to housing conditions or natural mortality rather than the effects of the tags.

We used a common anesthesia concentration of 0.5g/L MS-222 (Green 2001; Osbourn et al. 2011), while other studies used lower concentrations of MS-222 (0.25g/L in Chelgren et al. 2006; 0.44g/L in Strain et al. 2013). We found that allowing tadpoles to become fully anesthetized (3–8 minutes) allowed us to inject the tags with seemingly no response from the tadpoles. Although properly anesthetizing each tadpole and allowing them to recover takes up to 40 minutes total (6–30 minutes for recovery), we recommend including anesthesia in the tagging procedure. Using a lower concentration of anesthetic (< 0.5g/L) may reduce the total time of this procedure, as long as it is determined that the tadpoles are immobilized prior to injection (Green 2001). Juvenile *L. raniformis* tagged with VIAlpha tags without anesthesia showed signs of distress in the form of vocalization and flinching during tag insertion, indicating that this may be an uncomfortable procedure for amphibians (Heard et al. 2008). Although tag insertion with anesthesia may be tedious and requires considerable initial handling time, properly inserted tags can be easily read which allows for a quick handling time when recaptured (Clemas et al. 2009).
As mentioned in other tagging studies (Clemas et al. 2009; Osbourn et al. 2011; Sinsch 1997), the success of the tags depends largely on the skill of the operator. Tagging requires some initial practice and the skill of tag insertion improves over time (Osbourn et al. 2011; Sinsch 1997). It can take several attempts before finding the right amount of pressure, angle of insertion, and location of insertion when injecting the VIAlpha tags for the first time. We recommend practicing the use of the tag injector on a subsample of tadpoles before marking the study tadpoles with a tag. Increased practice may improve the tag retention and readability seen in this study.

To evaluate the efficacy of VIAlpha tags on marking individual larval *R. clamitans*, we return to the 3 fundamental assumptions for mark-recapture: (1) there is no loss of marks; (2) there is no misidentification of marks; and (3) the marks do not alter survival or recapture probabilities (Heard et al. 2008). The results of our study violate the first 2 assumptions. The retention rate for dorsal and lateral tags (65.91%) is too low for reliable estimates of demographic parameters. High rates of tag loss result in overestimation of population size and underestimation of survival and dispersal (Heard et al. 2008). Additionally, of the dorsal and lateral tags that were retained, less than half were readable, which led to low rates of accurate identification of individuals. The third assumption was satisfied by the high survival rate (95%) seen in our study. While our study shows that VIAlpha tags violate 2 of the 3 assumptions for an effective tagging method, there are possible remedies that may improve tag retention and readability in tadpoles. There are pros and cons of each individual tagging method for larval
amphibians (VIAlpha, CWT, VIE), but a focus on improving VIAlpha tag retention and readability may highlight VIAlpha tags as a reliable tagging option (Table 2).

To improve tag readability, future studies could use black tags inscribed with fluorescent lettering (Clemas et al. 2009) to see if they are easier to read through the pigmentation on a tadpole's skin compared to black lettering against a fluorescent background. Additionally, Heard et al. (2008) had observers read tags in a dimly lit room to mimic conditions of nocturnal field surveys. The VI Light used in this study definitely improved tag readability, and it is likely that using the VI Light in a dark environment would further enhance readability. Due to the small size of these tags (1.2mm x 2.7mm), magnification may also improve the readability of the tiny alphanumeric code.

One potential way to overcome the high rate of VIAlpha tag loss is by double-tagging, either by marking a tadpole with 2 VIAlpha tags or by marking with 1 VIAlpha tag and 1 VIE tags (Courtois et al. 2013; Heard et al. 2008). If a tadpole is marked with 2 VIAlpha tags, the loss of 1 tag would still allow the individual to be identified as long as the second tag remains. If a tadpole is marked with 1 VIAlpha tag and 1 VIE tag but loses the VIAlpha tag, the remaining VIE tag would indicate that the tadpole lost its tag and would allow the researcher to account for tag loss (Heard et al. 2008). The risk of losing both tags and the added stress of doubly tagging a tadpole are considerable disadvantages to the double tagging remedy. We agree with Heard et al. (2008) that developing techniques to diminish tag loss of a single VIAlpha tag should be the priority. Chelgren et al. (2006) allowed a full 24 h period after injecting individual *Rana aurora* with VIAlpha tags to ensure full
recovery from anesthesia and to monitor tag retention. Eliminating tadpoles that lose their tag during this period of high risk of tag loss and releasing only the tadpoles that retained their tag through the first 24 h may reduce overall tag loss throughout the rest of a study. Keeping all marked tadpoles for a 24 h recovery period may be more feasible in a laboratory setting than in the field, so we offer it as a suggestion if the tagging procedure is planned to occur in a laboratory.

Modification of tagging techniques should focus on: (1) inserting the tag far enough away from the injection site so that they are not able to exit the injection site before the wound heals; and (2) positioning the tag below a section of the skin that is translucent enough to promote readability (Heard et al. 2008; Osbourn et al. 2011). In our study, we were not successful at massaging the tag under the skin further away from the injection site once the tag was implanted: doing so would sometimes cause the tag to disappear under the musculature of the tadpole. However, Chelgren et al. (2006) were able to subcutaneously move tags in juvenile *R. aurora* so this technique warrants practice in other species. While tagging ventrally was unsuccessful in our study, there seems to be potential in injecting the tag laterally to get the tag to reach the area of lighter pigmentation close to the ventral side. Maximizing the distance between the tag and the injection site and positioning the tag under the lightly pigmented lateral side of the body are 2 recommendations we have for future tagging of *R. clamitans* tadpoles.

Future studies should monitor tag retention and the effects of tags past the juvenile stage through adulthood. A long-term field study could determine changes in tag retention and readability, changes in behavior, and affects on susceptibility to
predation and disease over time caused by the injection of a VIAlpha tag during the larval stage (Courtois et al. 2013). VIAlpha tags have the potential to be used in many demographic studies on amphibians, but we must first understand all of the effects that this tagging method has on an amphibian before using it to make assumptions about their life history.

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Table 1. Number of *Rana clamitans* tadpoles (and percentage) that died, lost their tag, had an unknown fate (unable to determine if tag was kept or lost), had a readability score of 1 at metamorphosis (visible, not readable) or had a readability score of 2 at metamorphosis (readable) in each tagging treatment (3 tagging locations, 2 glue treatments), 2014–2015.

<table>
<thead>
<tr>
<th>Location</th>
<th>Glue Trt.</th>
<th>Died</th>
<th>Tag Loss</th>
<th>Unknown</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral</td>
<td>Glue</td>
<td>0</td>
<td>8 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Ventral</td>
<td>No Glue</td>
<td>0</td>
<td>9 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Ventral</td>
<td>Total</td>
<td>0</td>
<td>17 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>Dorsal</td>
<td>Glue</td>
<td>0</td>
<td>2 (18%)</td>
<td>2 (18%)</td>
<td>5 (45%)</td>
<td>2 (18%)</td>
<td>11</td>
</tr>
<tr>
<td>Dorsal</td>
<td>No Glue</td>
<td>2</td>
<td>1 (7%)</td>
<td>2 (14%)</td>
<td>5 (36%)</td>
<td>4 (29%)</td>
<td>14</td>
</tr>
<tr>
<td>Dorsal</td>
<td>Total</td>
<td>2</td>
<td>3 (12%)</td>
<td>4 (16%)</td>
<td>10 (40%)</td>
<td>6 (24%)</td>
<td>25</td>
</tr>
<tr>
<td>Lateral</td>
<td>Glue</td>
<td>1</td>
<td>0</td>
<td>2 (18%)</td>
<td>4 (36%)</td>
<td>4 (36%)</td>
<td>11</td>
</tr>
<tr>
<td>Lateral</td>
<td>No Glue</td>
<td>0</td>
<td>1 (13%)</td>
<td>2 (25%)</td>
<td>3 (38%)</td>
<td>2 (25%)</td>
<td>8</td>
</tr>
<tr>
<td>Lateral</td>
<td>Total</td>
<td>1</td>
<td>1 (5%)</td>
<td>4 (21%)</td>
<td>7 (37%)</td>
<td>6 (32%)</td>
<td>19</td>
</tr>
</tbody>
</table>
Table 2. Comparison of three individual tadpole tagging methods: visible implant alphanumeric (VIAlpha) tag, coded wire tag (CWT), and visible implant elastomer (VIE).

<table>
<thead>
<tr>
<th>Tag Factor</th>
<th>VIAlpha</th>
<th>CWT</th>
<th>VIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tag Size</td>
<td>1.2 × 2.7 mm</td>
<td>0.5 mm</td>
<td>“small dot”</td>
</tr>
<tr>
<td>Injector Size</td>
<td>Large (19 µm)</td>
<td>Small (~4 µm)</td>
<td>Small (4 µm)</td>
</tr>
<tr>
<td>Tag Cost</td>
<td>$1.00</td>
<td>$1.35</td>
<td>$0.15</td>
</tr>
<tr>
<td>Retention Rate in Larval Anurans</td>
<td>65.91% - our study</td>
<td>80% - (Martin 2011)</td>
<td>~88% - (Bainbridge et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>75% - (Courtois et al. 2013)</td>
<td></td>
<td>33% - (Grant 2008)</td>
</tr>
<tr>
<td></td>
<td>4% - (Strain et al. 2013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Disadvantages</td>
<td>Tags must be removed from the body in order to be read.</td>
<td>High rate of misidentification (~70%) due to tag migration.</td>
<td>A unique pattern of multiple tags is needed to identify individuals.</td>
</tr>
</tbody>
</table>
Fig. 1a. Photograph of green frog (*Rana clamitans*) tadpole showing a dorsal VIAlpha tag (A) and the incision site for the tag that has been treated with surgical glue (B).

Fig. 1b. Photograph showing where a lateral VIAlpha tag would be positioned (C) and where a ventral tag would be positioned (D).
Fig. 2. Mosaic plot representing the contingency table of the frequencies of each *Rana clamitans* tadpole outcome (died, unknown tag fate, lost tag, retained tag) in each tagging location (dorsal, lateral, ventral) regardless of glue treatment, 2014–2015.
Fig. 3. Percentage of *Rana clamitans* tadpoles tagged dorsally and tagged laterally (regardless of glue treatment) that retained their tags through metamorphosis that had poor readability (Score 1: visible but not readable) or good readability (Score 2: visible and readable) at metamorphosis, 2014–2015.
Fig. 4. Boxplot showing the relation between time to Gosner Stage (GS) 46 (Gosner 1960) and the tagging location (regardless of glue treatment), where the average number of days to GS 46 in the dorsally tagged tadpoles was significantly lower than in the laterally tagged tadpoles and in the reference tadpoles. A,B - groups that do not share the same letter are significantly different (P < 0.05).
CHAPTER 3

Functional Equivalence of Created Wetland Water Quality: A Comparison of Amphibian Metamorphic Success

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Abstract

Wetlands are often created through wetland mitigation to replace lost natural wetlands, but further evaluation is needed to determine the ability of a created wetland to replace lost wetland functions, especially providing wildlife habitat. We used a mesocosm design to compare the water quality and decomposition potential between three created wetlands and three natural wetlands in West Virginia and to evaluate how the water quality from the two wetland types were able to support metamorphosis in larval spring peepers (*Pseudacris crucifer*) and wood frogs (*Rana sylvatica*) across two years (2014–2015). Responses in metamorphosis rates differed between species and between years. Spring peepers displayed similar metamorphosis rates in the created and natural wetlands in both years of the study. Wood frogs displayed similar metamorphosis rates in created and natural wetlands in 2015, but in 2014 wood frogs reached metamorphosis in less time and at a larger body size in the natural wetlands, suggesting that the wood frogs that developed in the natural wetlands may have higher fitness than those that developed in the created wetlands. Water quality did not differ significantly between created wetlands. Our study suggests that created wetlands may be providing partial mitigation in terms of water quality for amphibian development. We recommend that future monitoring of created wetlands include measures of juvenile amphibian recruitment as well as additional habitat variables to better determine the ability of created wetlands to function as amphibian habitat.
Introduction

Wetland mitigation aims to replace lost wetlands through the creation or restoration of new wetlands, although it is unclear whether mitigation projects adequately replace wetland function. Functional assessment of created wetlands has been the subject of many studies, typically comparing the functional attributes of created wetlands to those seen in natural reference wetlands to evaluate if successful wetland mitigation has occurred. Because wetlands are capable of so many complex ecological functions, studies tend to focus on a few attributes at a time, including soil characteristics, vegetative cover, hydrology, physiochemistry, plant litter decomposition rates, and wildlife species richness and diversity. To date, many of the studies evaluating the success of created wetlands focus on the three primary wetland characteristics (Cowardin et al. 1979): hydric soils (Bruland and Richardson 2006; Campbell et al. 2002; Stapanian et al. 2013), hydrophytic vegetation (Balcombe et al. 2005a; Campbell et al. 2002; Delphey and Dinsmore 1993; Hartzell et al. 2007; Moore et al. 1999), and hydrology (Cole and Brooks 2000; Robb 2001). Each of these studies result in differing conclusions about the functional success of wetland mitigation, and relatively few studies include direct measures of how wildlife are able to use these newly created habitat options. The ability of a created wetland to replace lost wildlife habitat and to support wildlife populations are important factors to include in the assessment of created wetland success.

Amphibians can comprise a large proportion of the vertebrate biomass in temperate wetlands (Whiles et al. 2006). Anurans in particular spend some or all of their life cycle in wetlands, relying on wetlands for breeding, larval development, foraging, and hibernation (Balcombe et al. 2005b; Hecnar and M’Closkey 1998; Houlanah and Findlay 2003).
Anurans play an important role within a wetland ecosystem by acting as primary consumers and altering algal communities, as well as by serving as a food source for higher trophic levels (Pough 1980; Whiles et al. 2006). The ability of amphibians to use both aquatic and terrestrial habitats makes them vulnerable to a wide range of biotic and abiotic stressors (Korfel et al. 2010, Whiles et al. 2006). Due to this substantial environmental sensitivity, amphibians are facing dramatic global declines (Hof et al. 2011, Houlahan et al. 2000, Stuart et al. 2004) and are considered the most imperiled taxonomic class of vertebrates (Wake and Vredenburg 2008). In the U.S., habitat loss and alteration are likely the most significant contributors to amphibian decline (Denton and Richter 2013). With the high frequency of wetland loss, which is critical habitat for these threatened amphibians, it is crucial that wetland mitigation successfully functions to replace any lost wetland habitat.

The ability of anurans to sustain a viable population in wetlands depends on their ability to hatch, grow, undergo metamorphosis, and then survive until the following spring so they can breed (Pollet and Bendell-Young 2000). Tadpoles in the larval development stage are particularly sensitive to aquatic habitat quality, so water quality in breeding pools can be an important determinant of amphibian species composition, richness, and abundance beyond the larval stage (Brown et al. 2012). The presence of amphibians alone cannot be accurately used to indicate the ability of a created wetland to function as a habitat that can support a persistent amphibian population. Intensive monitoring, including measures of reproductive success and juvenile recruitment, is essential for the proper assessment of the functional success of created wetlands (Balcombe et al. 2005b, Vasconcelos and Calhoun 2006). Calhoun et al. (2014) stress that recruitment is a better
predictor of population health than reproductive effort because created wetlands could be ecological traps where adults breed, but larvae are unable to properly develop.

The effects of wetland water quality characteristics on larval development are important at the population level of amphibians. Alteration of water quality in the larval habitat can lead to phenotypic variation in traits that affect metamorphosis and fitness (Harkey and Semlitsch 1988, Newman 1998). For example, larger body size at metamorphosis may result in larger size at first reproduction, higher fecundity, and earlier time to first reproduction (Semlitsch et al. 1988). However, having a longer larval period puts the tadpole at risk of high levels of predation and other potential threats, such as pond drying. The ideal larval habitat conditions for an amphibian population would allow tadpoles to grow to a large body size in a short period of time. Wilbur and Collins (1973) suggested that poor conditions in the aquatic environment would cause tadpoles to metamorphose earlier and smaller than tadpoles under high quality conditions, to which Newman (1998) responded that age and size metamorphosis may not respond equally to environmental factors. It is important to investigate how parameters of water quality affect the mechanisms of tadpole growth, development, and survival so that the effects on the overall population can then be fully understood. In this study, we used mesocosms to highlight the effects of water quality on wood frog (Rana sylvatica) and spring peeper (Pseudacris crucifer) larval development and to evaluate the functional success of created wetlands by comparing water quality and rates of anuran metamorphosis between created wetlands and natural reference wetlands in West Virginia.
Methods

Study Area

Our study evaluated three created (Sugar Creek, Pleasant Creek Wildlife Management Area (WMA), Upper Deckers Creek WMA) and three natural (Meadowville, Pleasant Creek, Upper Deckers Creek) wetlands in north-central West Virginia, USA (Fig. 1). Created wetlands and natural wetlands were selected in pairs, so that each pair contained one created wetland and one natural wetland (Sugar Creek-Meadowville; Pleasant Creek WMA-Pleasant Creek; Upper Deckers Creek WMA-Upper Deckers Creek). Each wetland pair was selected to have a similar location, elevation, underlying geology, and watershed (Table 1). Most of the created wetlands contained large areas of open water while all of the natural wetlands contained areas of scrub-shrub habitat. All of the wetlands had some level of disturbance adjacent to them, caused by paved roads, gravel roads, hiking trails, housing, or mowing.

The tadpoles in this study were housed in outdoor mesocosm aquaria (~150 L plastic wading pools) at the West Virginia University Organic Research Farm in Morgantown, West Virginia. The created \((n = 3)\) and natural \((n = 3)\) wetlands were represented by mesocosms in this experiment. Several weeks prior to tadpole introduction, pools were filled with 100 L of water from respective wetlands. We added 100 g of dried leaf litter comprised largely of American sycamore \((Platanus occidentalis,\) Stoler and Relyea 2013) and 1 g of rabbit chow to support the establishment of periphyton. Each pool was covered with a 121 cm x 121 cm sheet of fiberglass window screening secured with bungee cords and binder clips to prevent colonization of insects or other amphibians. To mimic realistic temporal changes in wetland water chemistry and to
replace water lost through evaporation, ~12 L of water from created and natural wetland sites were collected and added to respective pools each week. The pools were positioned on an open grass field so that all pools received the same amount of sunlight. Keeping all other habitat factors constant between pools (shade, water depth, size, density, leaf litter, elevation) allowed us to focus exclusively on water quality effects on metamorphosis.

*Larval Development*

Two anuran species were used in this study: wood frog and spring peeper. These species were selected because they are abundant in north-central West Virginia and collecting several hundred individuals (240 of each species in 2014, 360 of each species in 2015) was not likely to have any impact on their population. Additionally, both species have relatively early breeding times (late February through March in wood frogs; March through May in spring peepers), so tadpoles of each species were collected at similar times.

On April 10, 2014, we collected a single wood frog egg mass from one natural palustrine emergent wetland near Morgantown, West Virginia. The egg mass was transferred to a glass aquarium in the laboratory and housed in 25°C wetland water oxygenated with an air pump. Once the eggs hatched, the tadpoles were fed rabbit chow (Purina Rabbit Chow; Purina Animal Nutrition Center, Gray Summit, Missouri USA) ad libitum until they reached Gosner stage (GS, Gosner 1960) 25 (April 30, 2014), at which point 240 randomly selected tadpoles were transferred to the experimental mesocosm pools. On May 17, 2014, early stage spring peeper tadpoles were collected from one created wetland (palustrine unconsolidated shore with mud) near Morgantown (because spring peepers lay their eggs singly, it is easier to collect them after they hatch). The
tadpoles were transferred to the laboratory, housed in 25°C oxygenated wetland water and fed rabbit chow ad libitum for a 48-hour acclimation period, and then 240 spring peeper tadpoles were randomly selected for the same experimental pools (added May 19, 2014). Each of the six wetland study sites were represented by four mesocosm pools, with a total of 24 pools each containing 10 wood frogs and 10 spring peepers.

In the second year of the study (2015), we accounted for potential inherent differences between frogs collected from created wetlands and frogs collected from natural wetlands (differences based on which wetland type embryos developed in). On April 5, 2015, we collected one wood frog egg mass from a natural palustrine emergent wetland and on April 23, 2015, we collected recently hatched wood frog tadpoles from a created palustrine scrub-shrub wetland near Morgantown, West Virginia (by the time we were able to find wood frog eggs in a created wetland, they had already hatched). Both batches of wood frogs were transported to the laboratory, kept under the same conditions as in 2014, and then 180 tadpoles from each wetland source were randomly selected to be placed in the mesocosm pools (added May 1–3, 2015). On May 11, 2015, early stage spring peeper tadpoles were collected from a natural palustrine scrub-shrub wetland and on May 19, 2015, early stage spring peeper tadpoles were collected from a created palustrine emergent wetland near Morgantown, West Virginia. Tadpoles were transported to the laboratory, kept under the same conditions as in 2014, and then 180 tadpoles from each wetland source were randomly selected to be placed in the mesocosm pools (added May 11–19, 2015). Tadpoles from created and natural wetlands were housed in separate pools so that source wetland effects could be evaluated. Each of the 6 wetland study sites were represented by 6 mesocosm pools: 3 pools contained wood frogs and spring peepers that
were collected from natural wetlands; 3 pools contained wood frogs and spring peepers that were collected from created wetlands. There were a total of 36 pools each containing 10 wood frogs and 10 spring peepers.

As larvae neared metamorphosis, they were checked daily for the emergence of front limbs (GS 42). At the time of front limb emergence, metamorphs were removed from pools, transported to the laboratory, weighed (to the nearest 0.001 g), and transferred to individual 500 mL plastic cups with ~20 mL water and a clean paper towel to allow them to climb out of the water during tail resorption. These cups were covered with fiberglass window screen to prevent the metamorphs from escaping their cups. Metamorphs in cups were also checked daily for completion of metamorphosis and weighed once they did complete metamorphosis (full tail resorption, GS 46). In addition to measuring the mass of each subject at GS 42 and GS 46 and the snout-vent length (SVL: tip of snout to posterior end of the vent) at GS 46, we also determined the length of the larval period from the day they entered the mesocosm until they completed metamorphosis. Frogs surviving through metamorphosis were euthanized with 5 g/L tricaine methane sulfonate (MS-222) to eliminate the risk of spreading any diseases contracted in the laboratory to wild populations.

*Water Quality*

In 2014, we measured dissolved oxygen (% ±2% accuracy) from three samples of each pool once a week using a YSI™ Model 55 meter (YSI Inc., Yellow Springs, OH) and pH (±0.01 accuracy), conductivity (µS, ±2% full scale accuracy), and water temperature (°C, ±1°C accuracy) from three samples of each pool once a week using an ExStik® EC500
These meter measurements were taken throughout the day from 0900 h to 1600 h. We also collected water samples weekly (the same day as the meter measurements) from each pool at 0830 h, stored them in a cooler, and transported them to the laboratory. There, we used a Hach Multimeter (DR 3900 Benchtop Spectrophotometer; Hach® Company, Loveland, CO) to measure total nitrogen (range: 1–16 mg/L N, ±0.1 mg/L accuracy), ammonia (range: 1–12 mg/L NH₃-N, ±0.03 mg/L accuracy), nitrate (range: 0.23–13.50 mg/L NO₃-N, ±0.3 mg/L accuracy), and nitrite (range: 0.015–0.600 mg/L NO₂-N, ±0.01 mg/L accuracy) of each pool sample. The mutimeter measurements were taken from one sample from each mesocosm pool each week. We collected the same water quality measurements weekly from each wetland site, where all measurements were taken and samples were collected throughout the day from 0900 h to 1800 h. The water quality measurements at the wetland sites were taken the same day that water was collected to bring back to the mesocosms. The water quality measurements in the mesocosms were taken the day after new wetland water was added. Water quality was measured May 18–June 7, 2014. Ammonia, nitrate, and nitrite were eliminated from analysis because the measurements fell below the reading range of the test kits being used.

In 2015, we performed the same water quality procedures as in 2014. However, we eliminated ammonia, nitrate, and nitrite and we added measurements of phosphorus (range: 0.15–4.5 mg/L PO₄, ±0.02 mg/L accuracy), alkalinity (range: 25–400 mg/L CaCO₃, ±0.09 mmol/L accuracy), and leaf litter decomposition potential (percent ash-free dry mass remaining). Phosphorus and alkalinity were each measured once in each mesocosm pool.
and each wetland site per week. Water quality was measured May 10–June 20, 2015. Decomposition was only measured in the mesocosm pools.

**Decomposition**

In 2015, we measured leaf litter decomposition potential in the pools using the litter bag method (Benfield 1996). Broadleaf cattail (*Typha latifolia*) was chosen for the decomposition study because it is a common wetland species that grows throughout most of North America (Gingerich and Anderson 2011). All broadleaf cattail was collected from the Meadowville natural wetland to help minimize differences in litter quality. Because we used litter from one site rather than collecting litter from each wetland study site, we refer to the decomposition that occurs as decomposition potential rather than decomposition rate (Gingerich and Anderson 2011). Broadleaf cattail stalks were clipped and collected in April 2015 and the leaves and stems air-dried in the laboratory for a minimum of 10 days before being processed.

Litter bags measuring 20 × 20 cm were constructed from fiberglass window mesh with one folded side, two sides stitched closed at 5 cm intervals with fishing line, and one side stapled shut at 5 cm intervals. A plastic laminated tag with an identifying number was placed in each bag along with 13 g of broadleaf cattail litter. Litter bags (n = 2) were placed in each pool on May 15, 2015. An additional set of 10 bags was placed in a pool, immediately collected, taken to the laboratory, and processed to correct for handling losses and to calculate the initial ash-free dry mass (AFDM; Benfield 1996). The rest of the bags were collected June 25, 2015 (42 days). Litter was removed from the bag, rinsed off, placed in brown paper bags labeled with the same identifying number as the mesh litter bags, and
oven-dried at 65°C for 5 days. We recorded the dry mass (DM) and the litter was ground to a powder in a 2-mm mesh power cutting mill. Three subsamples of 250 mg of powder were weighed out (sample dry mass; SDM) and placed in small aluminum pans and ashed at 550°C for 30 minutes, cooled, and weighed (sample ash mass; SAM). We computed the percent organic matter (OM) of the milled samples as follows:

\[
\%OM = \left( \frac{SDM - SAM}{SDM} \right) \times 100.
\]

We converted DM values of each litter bag to AFDM as follows:

\[
\text{Final AFDM} = DM \times \%OM.
\]

We computed the %AFDM remaining for each litter bag as follows:

\[
\%AFDM = \left( \frac{\text{Final AFDM}}{\text{Initial AFDM}} \right) \times 100.
\]

**Statistical Analysis**

The means of days to GS 46, mass at GS 46, and SVL at GS 46 from each mesocosm pool were used in analyses. The time to GS 46, mass at GS 46, and SVL at GS 46 were found to be normally distributed using Shapiro-Wilk W test. The water quality measurements of dissolved oxygen, pH, conductivity, temperature, and total nitrogen (as well as phosphorus and alkalinity in 2015) were measured weekly as continuous variables. The water quality variables were found to be normally distributed using Shapiro-Wilk W test, except for total nitrogen which was log transformed. The means of each water quality variable from each mesocosm pool were averaged across weeks for analyses. To compare the water quality from the mesocosm pools to the water quality taken directly from the wetland sites, we used a matched pairs analysis of variance (Anova) with water source (mesocosm, wetland) as the paired columns and wetland type (created, natural) as the grouping variable. We
used the pool means and then averaged the pool means and wetland means across weeks for the paired t-test. We did the paired t-tests separately for each year because the metamorphosis data were analyzed separately by year.

We used canonical correspondence analysis (CCA; ter Braak and Verdonschot 1995) using the software package R (http://cran.r-project.org/) to correlate water quality variables to metamorphosis endpoints. Canonical correspondence analysis is a multivariate direct ordination method that incorporates linear regression to summarize variation in a response related to environmental variables (Snodgrass et al. 2000, ter Braak 1987). We used CCA separately for each species and for each year. In the first CCA, we used metamorphosis endpoints as the dependent variables and wetland type as the environmental (independent) variable (created, natural). The second CCA used water quality variables as the dependent variables and wetland type as the environmental variables. The third CCA used metamorphosis endpoints as the dependent variables and water quality as the environmental variables. We used eigenvalues ($\lambda$; relative ability of an axis to separate response distribution; Balcombe et al. 2005c) and percentage of variation explained in the dependent variables to assess the relative importance of environmental variables in structuring the responses, and we used correlation coefficients to assess the relative importance of water quality variables on metamorphosis. We performed an ANOVA with 999 permutations to test the null hypothesis that there was no relation between dependent variables and environmental variables for each CCA. We determined the significance of correlations between matrices only by axis one p-values, because axis one accounted for the most variation in all analyses (Balcombe et al. 2005c).
We conducted additional univariate analyses on the data to investigate relations between dependent variables and environmental variables when they appeared to have a strong response in the CCA. ANOVAs were performed when wetland type was the environmental variable, and forward stepwise regression was used when water quality was used as the environmental variables. Analyses of covariance (ANCOVA) were conducted with Program PROC GLM (SAS® v9.1.3) on significant water quality factors from regression to identify interactions between water quality variables and wetland type on metamorphosis. In the ANCOVAs, each water quality variable was the covariate and wetland type was the grouping variable. Results for all tests were considered significant when p < 0.1.

Results

Year 2014

When we compared water quality between the mesocosm pool and the source wetlands, we found that pH, temperature, and nitrogen were similar between the pools and the wetlands (Table 2). Dissolved oxygen (p = 0.002) and conductivity (p = 0.022) were higher in the wetlands than in the pools.

The results of the first CCA relating variation in metamorphosis data to wetland type indicated that spring peepers were not significantly affected by wetland type (F_{1,22} = 0.885, p = 0.375, \lambda = 0.00005, 3.87% variance explained; Table 3). However, wood frog metamorphosis was influenced by wetland type (F_{1,22} = 13.445, p = 0.001, \lambda = 0.0002, 37.93% variance explained; Figure 2). Indicated by the results of the ANOVA, the wood frog tadpoles raised in natural wetland water reached metamorphosis in less time (x = 39.1
days, SE = 0.203) than those in created wetland water (\(\bar{x} = 40.5\) days, SE = 0.378)\((F_{1,22} = 10.55, p = 0.004)\) (Figure 3A). Additionally, wood frogs in natural wetland water reached an SVL (\(\bar{x} = 15.2\) mm, SE = 0.123) that was longer than those in created wetland water (\(\bar{x} = 14.8\) mm, SE = 0.170)\((F_{1,22} = 4.273, P = 0.050)\) (Figure 3C). A similar relation occurred in body mass, where wood frogs in natural wetlands reached a body mass (\(\bar{x} = 0.662\) g, SE = 0.020) that was heavier than those in created wetlands (\(\bar{x} = 0.598\) g, SE = 0.024)\((F_{1,22} = 4.227, P = 0.052)\) (Figure 3B).

The results of the second CCA relating variation in water quality data (including leaf litter decomposition potential) to wetland type indicated that wetland type did not influence water quality \((F_{1,22} = 0.106, p = 0.844, \lambda = 0.0001, 0.48\%\) variance explained). The results of the third CCA relating variation in metamorphosis data to water quality (including leaf litter decomposition potential) indicated that water quality did not influence metamorphosis in spring peepers or in wood frogs (Table 4).

**Year 2015**

The initial mass of the spring peeper tadpoles collected from created wetlands (\(\bar{x} = 0.081, SE = 0.006\)) was significantly higher than the initial mass of those collected from natural wetlands (\(\bar{x} = 0.051, SE = 0.006\)) \((F_{1,47} = 12.63, p = 0.001)\). This same significant trend was observed between wood frogs collected from created wetlands (\(\bar{x} = 0.173, SE = 0.012\)) and wood frogs collected from natural wetlands (\(\bar{x} = 0.128, SE = 0.009\)) \((F_{1,43} = 8.89, p = 0.005)\). These differences in initial mass were likely due to the collection of individuals at different developmental stages rather than other effects from embryos developing in different wetland types. Because both species contained an equal number of individuals
with a smaller mass and individuals with a larger mass, we performed all analyses combining both frog sources and evaluating the species as a whole. Results from analyses including effects of frog source (wetland type where frogs were collected from) can be found in Appendices A–G. When we compared water quality between the mesocosm pool and the source wetlands, we found that all water quality variables were similar between the pools and the wetlands except for pH, which was higher in the pools than in the wetlands (p = 0.008), and conductivity, which was higher in the wetlands than in the pools (p = 0.053)(Table 2).

The results of the first CCA relating variation in metamorphosis data to wetland type indicated that spring peepers (F_{1,28} = 0.076, p = 0.819, \lambda = 0.000007, 0.27\% variance explained) and wood frogs (F_{1,25} = 2.086, p = 0.16, \lambda = 0.002, 7.7\% variance explained) were not significantly affected by wetland type (Table 3). The results of the second CCA relating variation in water quality data to wetland type indicated that wetland type significantly influenced water quality (F_{1,30} = 2.991, p = 0.07, \lambda = 0.0038, 9.07\% variance explained). Results of the ANOVA indicated that conductivity in natural wetland water (\bar{x} = 126.6 \mu S, SE = 4.600) was lower than the conductivity in created wetland water (\bar{x} = 165.9 \mu S, SE = 21.667)(F_{1,30} = 3.989, P = 0.055)(Figure 4).

The results of the third CCA relating variation in metamorphosis data to water quality (including leaf litter decomposition potential) indicated that water quality did not influence metamorphosis in spring peepers, but wood frog metamorphosis was influenced by water quality (p = 0.004) (Table 4, Figure 5). Specifically, the results from stepwise regression indicated that days to metamorphosis for wood frogs were positively influenced by pH (F_{1,24} = 4.68, p = 0.041)(Figure 6). Mass at metamorphosis was negatively influenced
by dissolved oxygen ($F_{1,24} = 19.49, p = 0.0002$)(Figure 7). SVL at metamorphosis was negatively influenced by both dissolved oxygen ($F_{2,23} = 11.57, p = 0.002$)(Figure 8A) and phosphorus ($F_{2,23} = 6.85, p = 0.015$)(Figure 8B). ANCOVA (SVL = phosphorus + wetland type + interaction between phosphorus and wetland type) resulted in a significant interaction between phosphorus and wetland type on wood frog SVL at metamorphosis ($F_{1,23} = 5.38, p = 0.030$)(Figure 9). In created wetlands, phosphorus did not have a significant effect on SVL ($t_{23} = 1.57, p = 0.130$), but phosphorus did have a significantly negative effect on SVL in natural wetlands ($t_{23} = -2.29, p = 0.031$).

Discussion

*Rates of Metamorphosis*

Rates of spring peeper metamorphosis were similar between created and natural wetlands in both years of the study. Wood frog metamorphosis was similar between created and natural wetlands in 2015, but in 2014 wood frogs tended to reach metamorphosis more quickly and at a larger body size in natural wetlands. In 2015, we studied wood frogs from two wetlands: one egg mass from a natural wetland and recently hatched tadpoles from a created wetland. The high variance in the 2015 wood frogs could explain why we did not see effects of wetland type on their development. In 2014, we studied wood frogs from a single egg mass, where variation was more limited and effects of wetland type were more noticeable.

Few other studies that assess the functional status of created wetlands include a measure of amphibian metamorphic rates. Pechmann et al. (2001) found significantly
smaller mean SVL at metamorphosis for spring peepers and ornate chorus frogs 
(*Pseudacris ornata*) in created ponds than at a reference pond, but no significant difference occurred for the other six anuran species in their study. Although our study did not find an effect of wetland type on spring peeper metamorphosis, together our study and the Pechmann et al. (2001) study suggest that frogs that metamorphose in created wetlands may have slightly reduced fitness compared to those in natural wetlands. Our study suggests that created wetlands may be providing partial mitigation in terms of water quality for amphibian development.

*Effects of Water Quality*

The 2014 wood frogs reached a larger body size in less time in the natural wetlands than in the created wetlands, but the water variables measured in this study were not able to explain this difference in metamorphosis between wetland types. The 2015 wood frogs had similar rates of metamorphosis in the created and natural wetlands, but overall their metamorphosis was affected by the water variables, specifically pH, dissolved oxygen, and phosphorus.

Dissolved oxygen had a negative effect on the size of wood frogs at metamorphosis in 2015, where both mass and SVL decreased with increasing levels of dissolved oxygen. The opposite trend is often seen in other studies, where higher oxygen is typically preferred by larval amphibians (Martin et al. 2014; Schmutzer et al. 2008; Smith 1997; Stevens et al. 2006). Gerlanc and Kaufman (2005) observed larger body mass of western chorus frogs (*Pseudacris triseriata*) at lower levels of dissolved oxygen in 1 year of their study, but the reverse was true in the second year of their study. Helff and Stubblefield
(1931) and Mann and Bidwell (2001) suggest that 20% saturation of dissolved oxygen is considered low enough for negative effects to occur on tadpoles. The dissolved oxygen measured in our study in 2015 ranged from 18.6–58.6%, with a mean of 36.3 (± 2.2). It is difficult to explain why the lowest values of dissolved oxygen in our study, which were as low as the “harmfully” low values seen in other studies, corresponded with the highest body sizes in wood frogs. Noland and Ultsch (1981) found northern leopard frogs (*Rana pipiens*) (same genus as wood frogs) in < 50% oxygen-saturated waters and explained this tolerance of lower oxygen environment by the early development of lungs in these species. In our study, low levels of dissolved oxygen and pH were related to a larger body size and faster development of 2015 wood frogs, respectively. This may be explained by high microbial respiration associated with decaying plants (Bidwell 2013). Decaying plant biomass reduces oxygen and pH in the water, but larval wood frogs may benefit from the food source provided by decaying plant matter. The percent decomposition measured in our study did not appear to affect wood frog metamorphosis, although the litter bags were only left in the mesocosms for 42 days which may not have been long enough to measure the true decomposition potential of each wetland type.

Phosphorus had an overall negative effect on SVL of 2015 wood frogs at metamorphosis, and this trend specifically occurred in natural wetlands. Depending on the concentration, phosphorus can act as a limiting nutrient or a harmful pollutant. Kapfer et al. (2007) found higher phosphorus in constructed agricultural ponds that allowed grazing of livestock than in natural ponds. They also found that African clawed frog (*Xenopus laevis*) survival was negatively correlated with phosphorus. We did not find differences in phosphorus concentrations between created and natural wetlands, and none of our study
wetlands were grazed agricultural ponds, but we did find a difference in phosphorus effects between created and natural wetlands, where the negative effect of phosphorus on wood frogs was significantly apparent in natural wetlands. The phosphorus concentrations in our study in 2015 were relatively low, with 82% of measures falling under 0.5 mg/L. This suggests that wood frogs are particularly sensitive to phosphorus in their larval environments.

*Environmental Applications*

This study focused on the effects of water quality on metamorphosis, and aimed to identify differences in water quality between created and natural wetlands. However, there tends to be high natural variability in wetland water quality. Trebitz et al. (2005) found within-wetland differences in wetland water quality that were as large or larger than differences between wetlands. Batzer et al. (2004) measured a suite of water quality parameters and reported variation of up to two orders of magnitude in a series of forested wetland ponds. There are many factors that can influence the water quality of a wetland, regardless of whether it is a created or a natural wetland. Landscape influences such as surrounding land use (Trebitz et al. 2007), the size and slope of watersheds that feed wetlands (deCatanzaro and Chow-Fraser 2011), and buffer zone quality (Houlahan and Findlay 2004) can affect water quality in a wetland. Water quality can also be affected by internal biological processes driven by aquatic plant photosynthesis and microbial respiration. Variation in water quality could be due to a wide range of landscape, internal, or temporal factors beyond whether a wetland is natural or manmade.
Some studies evaluate the effects of “extreme” water quality factors on larval amphibians, concluding that in conditions with water quality falling on extreme ends of a gradient, larval development may be negatively affected (Costa 1967; Cummins 1986; Egea-Serrano et al. 2012). Although variation in water chemistry in our mesocosm pools was high, none of the variables reached extremely high or low values. The water chemistry observed in this study was similar to values seen in other studies of typical wetlands (Batzer et al. 2004; Vasconcelos 2003). The variation seen in water quality in our study was not sufficient to influence most metamorphosis endpoints. It is likely that the resident anurans of north-central West Virginia are tolerant of the natural range of water chemistry variation (Batzer et al. 2004; Calhoun et al. 2014).

Spring peepers in particular were not affected by wetland type or by the environmental variables measured in this study. Denton and Richter (2013) found that spring peepers had a high abundance in both constructed and natural wetlands. Pechmann et al. (2001) also found that spring peepers had high reproductive success in created wetlands due to their tendency to breed in both permanent and temporary ponds. However, wood frogs may be more sensitive to changes in habitat quality. Denton and Richter (2013) found wood frogs exclusively in natural wetlands; no wood frogs were found in the 14 created wetlands in their study. Vasconcelos and Calhoun (2006) found breeding wood frogs in created wetlands, but reproductive success was often low. Many studies agree that the exclusion of wood frogs from created wetlands is due to the large size and long hydroperiod of many created wetlands which support populations of predatory amphibian species, such as green frogs (Rana clamitans) and bullfrogs (Rana catesbeiana) (Calhoun et al. 2014; Denton and Richter 2013; Vasconcelos and Calhoun
Spring peepers can breed in, and larvae can develop in, temporary and permanent ponds that are natural or constructed, so they are likely to be tolerant of a range of environmental conditions. Wood frogs typically breed in forested vernal pools, which is a less variable habitat preference than that of the spring peepers, suggesting that wood frogs may be more sensitive to environmental variability.

This mesocosm design allowed us to focus on the effects of water quality. Abiotic factors, such as water chemistry, are known to affect growth and developmental rates of larval amphibians (Gerlanc and Kaufman 2005), although the strength and direction of the effects are highly variable and vary between sites and species. Focusing on water quality allowed us to address specific questions about the performance of created wetlands to support aquatic wildlife and to assess the phenotypic plasticity of larval amphibians in response to variation in water quality. However, the applications of our results to real wetland ecosystems are limited. The obvious next step would be to continue seeking answers to these research questions in a field-based study. While it is true that water chemistry affects rates of larval development, there are many other habitat variables that have proven to be important influences on amphibians, such as hydrology (Calhoun et al. 2014; Denton and Richter 2013; Pechmann et al. 2001; Snodgrass et al. 2000), canopy cover (Denton and Richter 2013; Skelly et al. 2002; Stephens et al. 2013), quality of surrounding habitat (Babbitt et al. 2006), and aquatic vegetative communities (Martin et al. 2014). Water quality is driven by all of these habitat variables. If significant differences are found in water quality between a created and a natural wetland, it may be difficult to develop management recommendations for future wetland creation without knowing which habitat features are attributing to the differing water quality. Including habitat
variables along with water chemistry measures will provide the clearest explanation of how created wetlands are supporting larval amphibian development compared to natural wetlands.

Of the studies that evaluate amphibian use of created wetlands, several aim to compare community structure or species diversity of anurans between created and natural wetlands (Balcombe et al. 2005a; Denton and Richter 2013; Korfel et al. 2010; Pechmann et al. 2001; Porej and Hetherington 2005). Assessing anuran communities can be useful in determining the potential for created wetlands to replace lost wetland habitat. However, seemingly functional created wetlands may attract anurans to breed there, but conditions may be such that embryos and larvae are unable to develop (known as an ecological trap) (Brand and Snodgrass 2009; Calhoun et al. 2014). Measuring the reproductive success and metamorphic success (juvenile recruitment) in a wetland would be the best predictor of the success of created wetlands as habitat for pond-breeding amphibians (Calhoun et al. 2014; Vasconcelos and Calhoun 2006). Between the two years of our study, we observed variation in both metamorphosis rates and water quality. Due to variability within individual pools, between pools, and between years (Batzer et al. 2004; Calhoun et al. 2014; Trebitz et al. 2005), long-term monitoring of wetland conditions is critical. While the results of this study are valuable, the study could be improved upon by including more years in the assessment. We recommend that the monitoring of created wetlands include measures of juvenile amphibian recruitment for at least five years to better determine the ability of created wetlands to function as amphibian habitat.
Acknowledgments

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Literature Cited


Table 1. Characteristics of three created wetlands and three natural wetlands in West Virginia, including whether it is a created (C) or natural (N) wetland, age of wetland (years), size (ha), elevation (m), dominant Cowardin class (all wetlands are palustrine), watershed, underlying geology, and Universal Transverse Mercator (UTM) coordinates used in an amphibian metamorphosis study, 2014-2015.

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<tr>
<th>Name</th>
<th>Type</th>
<th>Age</th>
<th>Size</th>
<th>Elevation</th>
<th>Palustrine Classa</th>
<th>Watershed</th>
<th>Geology</th>
<th>UTM Y</th>
<th>UTM X</th>
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aBased on Cowardin et al. (1979)
Table 2. Average values of each water quality variable in created and natural mesocosm pools and created and natural source wetlands, as well as the results from the matched pairs analysis of variance with wetland source as the paired columns (t test; df = 5) and wetland type as the grouping variable (F ratio; df = 1.4), in West Virginia, 2014–2015.

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<th>p-value</th>
<th>F ratio</th>
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* Significant (α = 0.1)
Table 3. Average values of metamorphosis endpoints, specifically days to Gosner stage (GS) 46 (Gosner 1960) and mass and snout-vent length (SVL) at GS 46, for spring peepers and wood frogs in created and natural wetlands of West Virginia, as well as the analysis of variance results comparing created and natural means, in 2014 and 2015.

<table>
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<tr>
<th>Year</th>
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<th>Wetland type</th>
<th>Days to GS 46</th>
<th>P value</th>
<th>Mass at GS 46</th>
<th>p-value</th>
<th>SVL at GS 46</th>
<th>P-value</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>x</td>
<td>SE</td>
<td>x</td>
<td>SE</td>
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<tr>
<td>2014</td>
<td>Spring Peeper</td>
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* Significant (α = 0.1). \(^a\)F\(_{1,22}\) = 1.365; \(^b\)F\(_{1,22}\) = 0.149; \(^c\)F\(_{1,22}\) = 0.002; \(^d\)F\(_{1,28}\) = 0; \(^e\)F\(_{1,28}\) = 0.076; \(^f\)F\(_{1,28}\) = 0.245; \(^g\)F\(_{1,22}\) = 10.55; \(^h\)F\(_{1,22}\) = 4.227; \(^i\)F\(_{1,25}\) = 4.273; \(^j\)F\(_{1,25}\) = 0.803; \(^k\)F\(_{1,25}\) = 0.507; \(^l\)F\(_{1,25}\) = 2.242
Table 4. Summary of results from canonical correspondence analyses with water quality from West Virginia wetlands as the environmental variable and metamorphosis data for spring peepers and wood frogs as the dependent variable, 2014–2015. Correlation coefficients are reported for all environmental variables.

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<th>Axis 2</th>
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<td>-0.2081</td>
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</table>

* Significant (α = 0.05).
Figure 1. Six wetland sites in West Virginia were included in the wetland comparison study that ran from March 2014 through July 2015. Three of these wetlands were created and three were natural wetlands.
Figure 2. Biplot of canonical correspondence analysis (CCA) results relating metamorphosis endpoints, specifically days to Gosner stage (GS) 46 (Gosner 1960) and mass and snout-vent length (SVL) at GS 46, of wood frogs to wetland type (created, natural) in 2014. The arrow represents the direction of the created wetland type.
Figure 3. Boxplots showing the effects of wetland type (created, natural) on: (A) wood frog days to metamorphosis (p = 0.004); (B) mass at metamorphosis (p = 0.052); and (C) snout-vent length (SVL) at metamorphosis (p = 0.050) in 2014.
Figure 4. Boxplots showing the effects of wetland type (created, natural) on conductivity ($\mu$S) in mesocosm pools in 2015 ($p = 0.060$).
Figure 5. Biplot of canonical correspondence analysis (CCA) results relating metamorphosis endpoints, specifically days to Gosner stage (GS) 46 (Gosner 1960) and mass and snout-vent length (SVL) at GS 46, of wood frogs to water quality of West Virginia wetlands in 2015. The arrows represent the direction of the water quality variables.
Figure 6. Effect of pH in West Virginia wetlands on days to metamorphosis in wood frogs in 2015 (p = 0.041).
Figure 7. Effect of dissolved oxygen in West Virginia wetlands on mass at metamorphosis in wood frogs in 2015 (p = 0.0002).
Figure 8. Effect of (A) dissolved oxygen (p = 0.002) and (B) phosphorus (p = 0.015) in West Virginia wetlands on snout-vent length (SVL) at metamorphosis in wood frogs in 2015.
Figure 9. Effects of phosphorus on snout-vent length (SVL) of wood frogs in 2015 between created wetlands ($p=0.130$) and natural wetlands ($p=0.031$) in West Virginia. Filled circles and the solid line represent natural wetlands, open circles and the dashed line represent created wetlands.
Appendix A. Statistical analysis results from a 2015 wetland mesocosm study when spring peeper and wood frog metamorphosis were compared to wetland type and water quality separately based on frog source (collected from created wetland, collected from natural wetland) in West Virginia.

**Spring Peepers collected from created wetland**

The results of the first CCA relating variation in metamorphosis data to wetland type indicate that spring peepers were not significantly affected by wetland type ($F_{1,13} = 3.21$, $p = 0.058$, $\lambda = 0.00004$, 19.8% variance explained). The results of the third CCA relating variation in metamorphosis data to water quality (including leaf litter decomposition potential) indicate that spring peeper metamorphosis was not significantly influenced by water quality ($p = 0.113$) (Appendix B).

**Spring Peepers collected from natural wetland**

The results of the first CCA relating variation in metamorphosis data to wetland type indicate that spring peepers were not significantly affected by wetland type ($F_{1,13} = 1.56$, $p = 0.251$, $\lambda = 0.0002$, 10.69% variance explained). The results of the third CCA relating variation in metamorphosis data to water quality (including leaf litter decomposition potential) indicate that spring peeper metamorphosis was not significantly influenced by water quality ($p = 0.151$) (Appendix B).

**Wood Frogs collected from created wetland**

The results of the first CCA relating variation in metamorphosis data to wetland type indicate that wood frogs were not significantly affected by wetland
type \(F_{1,11} = 0.47, p = 0.55, \lambda = 0.00008, 4.1\% \) variance explained. The results of the third CCA relating variation in metamorphosis data to water quality (including leaf litter decomposition potential) indicate that wood frog metamorphosis was not significantly influenced by water quality \( (p = 0.223) \) (Appendix C).

Wood Frogs collected from natural wetland

The results of the first CCA relating variation in metamorphosis data to wetland type indicate that wood frogs were not significantly affected by wetland type \( F_{1,12} = 1.16, p = 0.311, \lambda = 0.0002, 8.79\% \) variance explained. The results of the third CCA relating variation in metamorphosis data to water quality (including leaf litter decomposition potential) indicate that wood frog metamorphosis was influenced by water quality \( (p = 0.042) \) (Appendix C, Appendix D). Specifically, the results from stepwise regression indicate that mass at metamorphosis for wood frogs was negatively influenced by dissolved oxygen \( (t_{11} = -3.71, p = 0.0035) \) and phosphorus \( (t_{11} = -2.59, p = 0.0253) \) (Appendix E).

ANCOVA resulted in a significant negative effect of dissolved oxygen on all four groups of wood frogs: FC_TC: collected from created wetland-raised in created wetland; FC_TN: collected from created wetland-raised in natural wetland; FN_TC: collected from natural wetland-raised in created wetland; and FN_TN: collected from natural wetland-raised in natural wetland. The effect of dissolved oxygen was significant \( F_{1,19} = 12.73, p = 0.002 \), but there was no interaction between dissolved oxygen and frog source/wetland group \( F_{5,19} = 0.86, p = 0.477) \) (Appendix F). ANCOVA on the effect of phosphorus on all four groups of wood frogs resulted in a
significant interaction between phosphorus and group (F_{3, 19} = 4.46, p = 0.016) (Appendix G). Phosphorus had a positive effect on the mass of wood frogs raised in created wetlands (collected from created wetland: t_{19} = 2.33, p = 0.031; collected from natural wetland: t_{19} = 2.18, p = 0.042), and there was a weaker negative effect of phosphorus on the mass of wood frogs raised in natural wetlands (collected from created wetland: t_{19} = -0.51, p = 0.616; collected from natural wetland: t_{19} = -1.82, p = 0.084).
Appendix B. Summary of results from canonical correspondence analyses with water quality from West Virginia wetlands as the environmental variable and metamorphosis data for spring peepers (by collection source) as the dependent variable, 2014–2015. Correlation coefficients are reported for all environmental variables.

<table>
<thead>
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<th>Variable</th>
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<th>Axis 2</th>
</tr>
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<tr>
<td><strong>Metamorphosis~Water Quality in Spring Peepers from Natural Wetland</strong></td>
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<tr>
<td>Eigenvalue (λ)</td>
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Appendix C. Summary of results from canonical correspondence analyses with water quality from West Virginia wetlands as the environmental variable and metamorphosis data for spring peepers (by collection source) as the dependent variable, 2014–2015. Correlation coefficients are reported for all environmental variables.

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Metamorphosis~Water Quality in Wood Frogs from Created Wetlands

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Appendix D. Biplot of canonical correspondence analysis (CCA) results relating metamorphosis endpoints, specifically days to Gosner stage (GS) 46 (Gosner 1960) and mass and snout-vent length (SVL) at GS 46, of wood frogs collected from a natural wetland, to water quality of West Virginia wetlands in 2015. The arrows represent the direction of the water quality variables.
Appendix E. Effect of (A) dissolved oxygen ($p = 0.0035$) and (B) phosphorus ($p = 0.0253$) in West Virginia wetlands on mass at metamorphosis in wood frogs collected from a natural wetland in 2015.
Appendix F. Effects of dissolved oxygen on mass of wood frogs in 2015 between those collected from created and natural wetlands and raised in created and natural wetlands in West Virginia. FC_TC: collected from created wetland, raised in created wetland; FC_TN: collected from created wetland, raised in natural wetland; FN_TC: collected from natural wetland, raised in created wetland; FN_TN: collected from natural wetland, raised in natural wetland.
Appendix G. Effects of phosphorus on mass of wood frogs in 2015 between those collected from created and natural wetlands and raised in created and natural wetlands in West Virginia. FC_TC: collected from created wetland, raised in created wetland; FC_TN: collected from created wetland, raised in natural wetland; FN_TC: collected from natural wetland, raised in created wetland; FN_TN: collected from natural wetland, raised in natural wetland.