Assessment of Environmental DNA for Detecting Presence of Imperiled Aquatic Amphibian Species in Isolated Wetlands

Anna M. McKee,* Daniel L. Calhoun, William J. Barichivich, Stephen F. Spear, Caren S. Goldberg, Travis C. Glenn

Abstract

Environmental DNA (eDNA) is an emerging tool that allows low-impact sampling for aquatic species by isolating DNA from water samples and screening for DNA sequences specific to species of interest. However, researchers have not tested this method in naturally acidic wetlands that provide breeding habitat for a number of imperiled species, including the frosted salamander (*Ambystoma cingulatum*), reticulated flatwoods salamanders (*Ambystoma bishopi*), striped newt (*Notophthalmus perstriatus*), and gopher frog (*Lithobates capito*). Our objectives for this study were to develop and optimize eDNA survey protocols and assays to complement and enhance capture-based survey methods for these amphibian species. We collected three or more water samples, dipnetted or trapped larval and adult amphibians, and conducted visual encounter surveys for egg masses for target species at 40 sites on 12 different longleaf pine (*Pinus palustris*) tracts. We used quantitative PCRs to screen eDNA from each site for target species presence. We detected flatwoods salamanders at three sites with eDNA but did not detect them during physical surveys. Based on the sample location we assumed these eDNA detections to indicate the presence of frosted flatwoods salamanders. We did not detect reticulated flatwoods salamanders. We detected striped newts with physical and eDNA surveys at two wetlands. We detected gopher frogs at 12 sites total, three with eDNA alone, two with physical surveys alone, and seven with physical and eDNA surveys. We detected our target species with eDNA at 9 of 11 sites where they were present as indicated from traditional surveys and at six sites where they were not detected with traditional surveys. It was, however, critical to use at least three water samples per site for eDNA. Our results demonstrate eDNA surveys can be a useful complement to traditional survey methods for detecting imperiled pond-breeding amphibians. Environmental DNA may be particularly useful in situations where detection probability using traditional survey methods is low or access by trained personnel is limited.

Keywords: *Ambystoma cingulatum*; eDNA; *Lithobates capito*; *Notophthalmus perstriatus*; quantitative PCR

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Introduction

Over the last several hundred years, the southeastern United States has undergone major habitat alteration, most notably the loss of longleaf pine (*Pinus palustris*) forest due to conversion to agricultural land, fire suppression, and other factors (Frost 1993). Concurrent with the loss of longleaf pine forest has been the reduction and modification of ephemeral isolated wetlands that contribute to the high degree of biodiversity and endemism of the region (Hefner and Brown 1984; Kirkman et al. 1999; Kirkman et al. 2012). A number of endemic aquatic species, including several imperiled amphibian species, have evolved to rely on these ponds for breeding, as the seasonal hydrologic inundation and variable hydroperiods reduce the likelihood of persistence of fish and other predators. The majority of these imperiled amphibian species also inhabit the terrestrial habitats surrounding these wetlands during the non-breeding season. Alterations to the terrestrial environment may be detrimental to these species even in instances where ephemeral wetlands have remained intact (Semlitsch 1998).

Most standard methods for surveying pond-breeding amphibians require the physical capture or visual observation of the individuals (Heyer et al. 1994). Life history traits of some amphibians (e.g., cryptic behavior, small population sizes, high variation in annual reproductive success, and nocturnal activity; Dodd and LaClaire 1995; Palis 1997; Bevelhimer et al. 2008) can contribute to low detectability and make many survey methods ineffective (Liner 2006; Smith et al. 2006). Dipnetting, which is one of the least expensive (Liner 2006) and most commonly used methods for physically surveying aquatic amphibians can be time-consuming and is dependent on surveyor experience level (Skelly and Richardson 2010); moving nets and traps among wetlands may result in pathogen or invasive species transfer across wetlands (Gates et al. 2008).

Researchers have recently developed a molecular method for detecting aquatic animals that amplifies species-specific DNA from water samples (i.e., environmental DNA [eDNA]) and have been successful in detecting rare and cryptic aquatic species across several ecosystem types (Goldberg et al. 2011; Thomsen et al. 2012a; Thomsen et al. 2012b). This method for detecting aquatic species has several advantages that may complement and enhance traditional aquatic amphibian survey methods (Dejean et al. 2012; Pilliod et al. 2013). Researchers conduct the sampling by collecting water samples, making eDNA surveys low impact to habitat and minimally invasive to target and nontarget species. Additionally, for species that are difficult to physically detect because the species is elusive, occurs at low density, or the habitat is difficult to survey, eDNA surveys may be more time- and resource-efficient (Darling and Mahon 2011; Goldberg et al. 2011; Pilliod et al. 2013) or may reduce the uncertainty of detection probability when used to complement physical surveys. Several studies have also suggested that estimates of DNA concentration obtained from this molecular method are correlated with relative abundance or densities of target species (Takahara et al. 2012; Thomsen et al. 2012a; Goldberg et al. 2013), although the relationship, if there is one, under field conditions may be casual.

Studies have demonstrated the ability to use eDNA to detect aquatic amphibians in a variety of habitats, including streams, ponds, and aquaria (Ficetola et al. 2008; Goldberg et al. 2011; Dejean et al. 2012; Thomsen et al. 2012a). However, each species and system presents its own unique set of conditions affecting detection, such as species biology, system hydrology, and water chemistry. For example, reproductive timing may differ among pond-breeding amphibian species and must be taken into account when collecting eDNA samples. The effect of directional flow on detection will differ between lentic and lotic systems, and DNA degradation rates and levels of PCR inhibition may be greater for sites with greater acidity and organic compounds. Environmental DNA surveys must therefore be optimized for focal taxa by system.

In addition to containing high levels of organic matter, temporary wetlands in the southeastern United States are often naturally acidic (Kirkman et al. 2000), which may cause DNA to break down faster than in less acidic environments (Strickler et al. 2015). These wetlands provide important breeding habitat for a number of species, including several amphibians of conservation concern, such as frosted flatwoods salamanders (*Ambystoma cingulatum*; Figure 1a) and reticulated flatwoods salamanders (*Ambystoma bishopi*; Figure 1a), which are listed as threatened and endangered, respectively, pursuant to the U.S. Endangered Species Act (64 FR 15691 and 74 FR 6700, respectively; ESA 1973, as amended); the striped newt (*Notophthalmus perstriatus*; Figure 1b), which is classified as threatened by the state of Georgia (Stevenson et al. 2007a); and the gopher frog (*Lithobates capito*, formerly referred to as *Rana capito*; Figure 1c), which is classified as threatened in Georgia (Stevenson et al. 2007b) and is a state species of special concern in Florida (Enge et al. 2011). All four species
Figure 1. Target species from the southeastern United States for which we developed and optimized environmental DNA assays. (a) Adult frosted flatwoods salamander (*Ambystoma cingulatum*) from northern Florida. Photo credit: Todd Pierson, 2009. Reticulated flatwoods salamanders (*Ambystoma bishopi*), also a target species of the study, are visually indistinguishable from frosted flatwoods salamanders. (b) Paedomorphic striped newt (*Notophthalmus perstriatus*) from northern Florida. Photo credit: Todd Pierson, 2010. (c) Juvenile gopher frog (*Lithobates capito*) from the Georgia Fall Line. Photo credit: Todd Pierson, 2011.
require temporary wetlands for breeding habitat; however, their reproductive strategies differ.

Gopher frogs migrate to breeding ponds in association with heavy rains in the fall, winter, spring, and occasionally summer (Jensen and Richter 2005). Females deposit large eggs masses of 2,000 or more eggs that they attach to emergent vegetation in the wetland. Eggs hatch into tadpoles, which metamorphose in 90 to 215 d. Frosted and reticulated flatwoods salamanders also migrate to breeding wetlands in association with major rain events in the fall and winter; however, breeding generally occurs before the wetland has filled (Palis and Means 2005). Females can deposit up to 222 eggs in the wetland basin or around its margins (Anderson and Williamson 1976). Larvae begin to develop in the eggs, which hatch when inundated as the wetland fills. Larvae generally metamorphose between 77 and 126 d. Similarly, striped newts also breed in correspondence with rain events in the winter (Dodd et al. 2005). However, their life cycle is more complex than those of gopher frogs or flatwoods salamanders. Female striped newts lay eggs singly or in clumps, and may lay eggs over a period of several months. They have an aquatic egg and larval stage followed either by a terrestrial juvenile stage (i.e., eft) or, if sufficient water remains, an aquatic paedomorphic stage. Paedomorphs retain their gills and become sexually mature. Both efts and paedomorphs may metamorphose into terrestrial adults, but only paedomorphs may alternatively become sexually mature in an aquatic form (Dodd et al. 2005). Development time for striped newts depends on the developmental pathway. Metamorphosis to eft stage may take approximately 180 d whereas it may take a year for a larva to mature as a paedomorph (Johnson 2005). Therefore, a late-winter to early-spring sampling time is needed to have the highest probability of species presence in breeding areas. These differences in breeding strategy suggest that gopher frogs may have high detectability due to a wide breeding season and high fecundity, whereas flatwoods salamander life history likely limits its detectability using eDNA methods. Factors such as a limited number of eggs laid at a single time may decrease the detectability of the striped newt with eDNA; however, the longer larval stage and the possibility of a paedomorphic stage may increase its detectability in aquatic samples.

Effective conservation and management requires the ability to monitor species over time. The ease and minimally invasive nature of eDNA collection makes it an appealing potential method to compliment traditional survey methods. Our objectives for this study were to develop and optimize eDNA survey protocols and assays for four species of conservation concern: the frosted and reticulated flatwoods salamanders, gopher frog, and striped newt.

Methods

Between January and May 2013, we visited 35 wetlands on 12 different public and private longleaf pine tracts across Georgia and northern Florida (Figure 2).
Table 1. Positive detection results for the frosted flatwoods salamander, *Ambystoma cingulatum*; striped newt, *Notophthalmus perstriatus*; and gopher frog, *Lithobates capito* from physical and environmental DNA surveys of wetlands in longleaf pine tracts in the southeastern United States between January and May 2013. Reticulated flatwoods salamanders (*Ambystoma bishopi*) were not detected from any surveys. Species of flatwoods salamanders detected were determined based on sample location and species’ ranges, which do not overlap. Volume is the average volume of water filtered for the sample replicates. Proportion positive samples is the number of eDNA samples that tested positive based on the criteria of at least two positive quantitative PCR replicates, divided by the total number of samples collected at the given site. Proportion positive qPCRs is for positive samples, the number of qPCR replicates that were positive, divided by the number of qPCR replicates run for those samples. *nd* indicates the species was not detected.

<table>
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<th>Species and sites</th>
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<th>eDNA detection</th>
<th>Proportion positive samples</th>
<th>Proportion positive qPCRs</th>
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a Longleaf pine tracts with positive detections included Fort Stewart (FS), Fort Benning (FB), Apalachicola National Forest (ANF), Fall Line Sandhills Wildlife Management Area (FLSH), Ohooppee Dunes Natural Area (OD), Joseph W. Jones Ecological Research Center at Ichauway (ICH), Williams Bluff Preserve (WB), and a private tract located in Irwin County, Georgia (IRW). Site names are composed of the longleaf pine tract where the site was located, the name of the wetland, and the visit number if the wetland was surveyed multiple times during the study (e.g., ANF.P1.1 indicates the site was the first visit to wetland P1, which was in the Apalachicola National Forest).

b Six water samples collected instead of three.

c Wetland visited more than one time over sampling period.

d Species was sighted within 2 wk prior to sampling but not detected during physical surveys.

e Physical survey was performed 5 d prior to water sample collection.

f At least one qPCR amplified target species DNA despite partial inhibition.

g + indicates species was detected with associated detection method; — indicates species was not detected with eDNA and proportion of positive samples could not be calculated.

In general, we collected eDNA samples from 40 sites. In the southeastern United States between January and May 2013. Reticulated flatwoods salamanders (*Ambystoma bishopi*) were not detected from any surveys. Species of flatwoods salamanders detected were determined based on sample location and species’ ranges, which do not overlap. Volume is the average volume of water filtered for the sample replicates. Proportion positive samples is the number of eDNA samples that tested positive based on the criteria of at least two positive quantitative PCR replicates, divided by the total number of samples collected at the given site. Proportion positive qPCRs is for positive samples, the number of qPCR replicates that were positive, divided by the number of qPCR replicates run for those samples. *nd* indicates the species was not detected.
Detecting Imperiled Amphibian Species in Isolated Wetlands With Environmental DNA

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Quantitative PCR primer and probe development

We used DNA target species’ mitochondrial DNA sequences from GenBank and unpublished mitochondrial DNA sequences from S. Richter for quantitative PCR (qPCR) primer and probe development. As the frosted and reticulated flatwoods salamanders are the only known extant flatwoods salamander species and do not occur in the same range (Figure 2), we developed a qPCR primer and probe set from a consensus DNA sequence for the two species such that the assay could detect both species and we used geographic location of the sample to determine which species was detected (this qPCR primer and probe set is from here on referred to as the flatwoods salamander qPCR primers and probe). We used a qPCR assay with dual-labeled probes, which has been found to be more sensitive and specific for detecting low quantities of target species DNA than standard PCR (Wilcox et al. 2013). We developed qPCR primers and probes with Applied Biosystems® Primer Express Software v3.0 (Life Technologies) for the flatwoods salamanders and striped newt, and with Biosearch Technologies online software (available at https://www.biosearchtech.com/bhqprobes) for the gopher frog (Table 2). We tested all primer and probe combinations in silico against the GenBank database for potential amplification of non-target co-occurring species and had at least two mismatches per primer and at least six total mismatches among the primers and probe from available corresponding sequences in potentially co-occurring species. We then tested primers and probes that were specific for our target species based on GenBank comparisons in the lab on DNA samples of closely related (same genera), potentially syntopic species to ensure target species specificity (Table S2, Supplemental Material). We developed two sets of striped newt qPCR primers and probes (Table 2) because the first set amplified eastern newt (Notophthalmus viridescens) DNA. With the exception of the first set of striped newt primers and probe, which detected both striped and eastern newts, none of the nontarget species DNA tested positive with qPCR (see explanation below) using the developed target species primers and probes (Table S2, Supplemental Material).

Quantitative PCR and data analysis

We extracted eDNA from half of each filter in a low-DNA copy lab as described in Goldberg et al. (2011) with a Qiagen DNeasy Tissue and Blood Kit (Qiagen, Inc.) and a QIAshredder Kit (Qiagen, Inc.). We performed one DNA extraction negative control for approximately every 23 samples, including field negative controls. We retained remaining filter halves in case sample re-extractions were necessary. We also set up qPCRs in a low-DNA copy lab. Primers and probes were either multiplexed (flatwoods salamander, striped newt and gopher frog; striped newt and gopher frog; or flatwoods salamander and gopher frog) or run separately by species using Qiangen QuantiTect Multiplex PCR NoROX Kit (Qiagen, Inc.) with an internal positive control (IC) from the Qiagen QuantiFast Pathogen PCR +IC Kit (Qiagen, Inc.; Table S3, Supplemental Material). We ran reactions in 96-well optical qPCR plates on an Applied Biosystems StepOnePlus™ (Life Technologies Corp.; Table S4, Supplemental Material). We set up three replicate qPCRs for each water sample and field negative control; we ran three replicate no-template negative controls, and three replicates for each standard (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ ng/µL from genomic extractions of tissue samples) on each qPCR plate. A lack of or delayed amplification of the IC was indicative of qPCR inhibition or partial inhibition, respectively (Hartman et al. 2005). We determined samples to be inhibited if the threshold cycle (i.e., the qPCR cycle at which the amplification curve crosses a given quantification threshold; Cq) of the IC in at least one sample qPCR replicate was three or more Cq greater than the average Cq of the IC in the three no-template negative controls replicates for the associated qPCR plate, or if the IC of at least one sample replicate did not amplify.

We used exponential amplification in a target species assay for a qPCR replicate to be a positive qPCR result. We reran samples with a single positive qPCR (14 samples for the flatwoods salamanders, 2 for the striped newt, and 6 for the gopher frog). If we reran a sample because of an initial single positive qPCR replicate and none of the resulting qPCR replicates were positive, we assumed positive results from the initial qPCR were false and caused by contamination or mismatching that occurred while setting up or running the qPCR (10 samples for the flatwoods salamanders, 1 sample for the striped newt, and 4 samples for the southern leopard frog [Lithobates sphenocephalus]). However, if one or more qPCR replicates from the reruns were positive, we assumed the initial positive qPCR results were evidence of presence. We screened some samples with the first striped newt assay (Table S5, Supplemental Material) that also detected eastern newts. We reran samples that tested positive with the first striped newt assay with the redeveloped assay (22 samples; Table S5, Supplemental Material) to ensure the initial detection was not due to eastern newt DNA. We interpreted lack of amplification of an assay as no evidence of presence, unless the qPCR replicate was inhibited (Hartman et al. 2005), in which case results were inconclusive. We considered qPCR replicates with positive target species detection but partial or full inhibition of the ICs to be positive detections. Of the 126 water samples surveyed, 21
samples from 13 different sites exhibited inhibition in at least one qPCR replicate (Table S1, Supplemental Material). We treated these samples for qPCR inhibition with a 10-fold dilution with PCR-grade water, postextraction spin-column purification (OneStep™ PCR Inhibitor Removal Kit, Zymo Research, Irvine, CA), or postextraction spin-column purification and a subsequent fivefold dilution (Table S5, Supplemental Material; McKee et al. 2015). The particular treatment a sample received for qPCR inhibition was based on the amount of remaining sample.

We detected evidence of contamination during DNA extraction or qPCR setup in eight samples for the flatwoods salamanders, one sample for the striped newt, and eight samples for the gopher frog (Table S1, Table S5, Supplemental Material). We resolved this issue by decontaminating the low-DNA copy lab with 10% bleach and re-extracting DNA from samples that tested positive for the contaminating target species. We reran qPCRs with the newly extracted DNA and we interpreted initial qPCR results for the potentially contaminated qPCR replicates similarly to uncontaminated samples. If one or more qPCR replicates for a re-extracted sample were positive we assumed the initial positive qPCR results were not caused by contamination and considered them evidence of presence (ratio of potentially contaminated positive qPCRs to uncontaminated positive qPCRs for positive

Table 2.  Frosted flatwoods and reticulated flatwoods salamander, (Ambystoma cingulatum and Ambystoma bishopi, respectively), striped newt (Notophthalmus perstriatus); and gopher frog (Lithobates capito) quantitative PCR (qPCR) primer and probe sequences, regions of the mitochondrial genome from which the primers and probes were designed, and suppliers for analysis of environmental DNA samples collected from isolated wetlands in the southeastern United States between January and May 2013. The frosted and reticulated flatwoods salamander qPCR primers and probe were designed from a consensus DNA sequence of the two species. Sequences are 5′→3′. Probe sequences are presented as 5′ dye – sequence – 3′ quencher.

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<th>Primer/probe sequence</th>
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<th>Supplier</th>
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<td>CAL Fluor Red 610 – TYC ATT GAC TCT TAG CCT GAG TAG GCY TA - BHQ-2</td>
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</table>

a We redesigned striped newt primers and probe partway through the study due to nonspecificity. We reran all samples that tested positive with the original primers (forward: CTC CCA TGA GGC CAA ATA TCA; reverse: TGA AAA TCC GCC CCA AAT T) and probe (NEO – TTT GAG GCG CTA CAG TTA – MGBNFFQ) with the redesigned primers and probe. We ran all samples not yet screened prior to the redesign solely with the new primers and probe.
samples in Table S1, Supplemental Material). However, if all qPCR replicates for a re-extracted sample were negative, we assumed the initial positive qPCR results were caused by contamination. We calculated eDNA detection rates for each occupied site as the number of positive samples divided by the number of samples collected from that site. We were not able to filter 250 mL for 43 of the 126 water samples; therefore we tested the relationship between the average water sample volume for a site and the eDNA detection rate for that site using a Spearman correlation test in Spatial Analysis in Macroecology (SAM version 4.0; Rangel et al. 2006; Rangel et al. 2010).

Results

Sites where we measured pH ranged from slightly acidic (pH 6.1; Table S1, Supplemental Material) to extremely acidic (pH 3.5; Table S1, Supplemental Material). The only locations where we detected flatwoods salamanders with either eDNA or physical surveys during the study were within the frosted flatwoods salamander range (Figure 2). Therefore we assumed all flatwoods salamander detections were from frosted flatwoods salamander presence as opposed to reticulated flatwoods salamander presence.

Environmental DNA detection

We detected target species with eDNA surveys at 14 of 40 sites; three sites showed the frosted flatwoods salamander, nine sites showed the gopher frog, one site showed the striped newt, and one site showed the gopher frog and striped newt. The only site where we detected multiple target species (striped newt and gopher frog) with eDNA was FLSSH.P1.3 (Table 1). Positive eDNA detection rates ranged from 0.33 to 0.67 for the frosted flatwoods salamander and striped newt, and 0.33 to 1.00 for the gopher frog (Table 1). Nine of the 15 instances with positive eDNA detection had detection rates less than 1.0, indicating that multiple samples per site are often necessary to identify species presence (Table 1).

We detected qPCR inhibition in 92 of 824 qPCR replicates. The majority of inhibited reactions were fully inhibited (no amplification of the IC; 66 reactions). There were eight partially inhibited qPCR reactions (delayed amplification of the IC) from three samples that tested positive for gopher frogs, and one partially inhibited qPCR reaction that tested positive for striped newts (Table S5, Supplemental Material). Five fully inhibited qPCR reactions tested positive for gopher frogs, and one fully inhibited qPCR reaction tested positive for striped newts (Table S5, Supplemental Material). Six of the 18 samples that we treated for qPCR inhibition tested positive for gopher frogs after treatment, including the three samples that tested positive for gopher frogs prior to treatment as well. None of the inhibited samples tested positive for striped newts or frosted flatwoods salamanders after treatment.

Physical detection

We detected at least one target species with physical surveys at 10 of the 40 sites surveyed (Table 1). We did not detect either species of flatwoods salamander during the physical surveys. We detected striped newts during dipnet surveys at two sites with previously documented breeding populations (Table 1). Relative to the three other target species, gopher frogs were the most commonly encountered species across sites that we surveyed. We also detected gopher frog tadpoles during dipnet surveys at nine sites (Table 1). In agreement with eDNA surveys, there was only one instance where we detected multiple target species at the same site (striped newt and gopher frog at FLSSH.P1.3; Table 1).

Comparison of eDNA detections vs. physical survey detections

Results for DNA evidence of target species presence were identical to our physical detection results for the striped newt; however, there were a number of differences between the two methods for frosted flatwoods salamanders and gopher frogs. In two sites physical surveys detected one of our target species that eDNA did not detect (gopher frogs at ANF.P5 and FLSSH.P2.1; Table 1). The remaining six differences in presence results between eDNA and physical surveys were due to detection of frosted flatwoods salamanders or gopher frogs with eDNA but not physical surveys (Table 1). With the exception of gopher frogs at OD.P1, all wetlands where we detected our target species with only eDNA were known historic breeding sites for the target species detected and were believed to have contemporary breeding populations (J. Jensen, Georgia Department of Natural Resources; J. Macey, Fort Stewart Department of Public Works; P. Hill, Florida Fish and Wildlife Conservation Commission; R. Means, Coastal Plains Institute and Land Conservancy; and R. Thornton, Fort Benning Conservation Branch; personal communication). We did not detect frosted flatwoods salamanders with physical surveys at any of the three sites where we detected them with eDNA (Table 1). However, frosted flatwoods salamanders were seen at one of these sites approximately 2 wk prior to our survey (ANF.P2.1; P. Hill, personal communication; Table 1), as well as at an additional site where we did not detect them with either method (ANF.P1.1; P. Hill, personal communication; Table 1) even though we anticipate that larvae would not have metamorphosed over that time period. In three sites we detected gopher frogs with eDNA but not with physical surveys (Table 1). Gopher frog egg masses were seen within 2 wk prior to our survey at one additional site (Fort Stewart.P3; J. Macey, personal communication; Table 1) where we did not detect them with either eDNA or physical surveys.

Effect of water volume on detection

We filtered 250 mL of water from each sample collected from sites where frosted flatwoods salamanders were detected; therefore we did not include frosted flatwoods salamander detection rates in the analysis of the effect of water volume on detection because of the lack of variation in sample volume. We detected striped newts at two sites, which was an insufficient sample size to account for the potential differences in species’ detection rates. We therefore excluded detection rates of striped newts from this analysis as well. Average water
volumes that we filtered for sites where gopher frogs were detected with eDNA ranged from 75 to 250 mL. We did not find evidence of a relationship between average water sample volume and detection for gopher frogs ($r = -0.51, P = 0.082$).

**Discussion**

We developed eDNA survey protocols to detect both the frosted and reticulated flatwoods salamanders, the striped newt, and the gopher, all of which are imperiled amphibian species endemic to the longleaf pine forests of the southeastern United States. We compared this method to traditional physical survey methods (dipnetting, trapping, and visual encounter surveys) for detecting amphibians. With the exception of the reticulated flatwoods salamander, which was not detected by eDNA or physical surveys, we were generally able to detect our target species in isolated wetlands with relatively low pH (e.g., pH 3.9 at OD.P1). Nine of the 11 physical detections of our target species were also positive for the same species based on eDNA detection, suggesting eDNA would be a viable method for amphibian detection in this system. Environmental DNA also detected target species presence at an additional six sites where they were not detected with physical surveys.

There were two instances in which we detected gopher frogs with physical surveys but not eDNA surveys. Environmental factors such as pH, temperature, and ultraviolet radiation influence eDNA degradation rates (Strickler et al. 2015), and the conditions at these sites such as low pH or conditions conducive to microbial growth may have caused higher rates of degradation. While it is likely that we simply did not detect gopher frogs with eDNA at either site, an alternative possibility is that tadpoles identified as gopher frogs at this site were actually southern leopard frogs, a species common to the area. Gopher frogs and southern leopard frog tadpoles generally have physical characteristics that help distinguish them from each other. However, these physical characteristics may not be pronounced enough when tadpoles are still relatively small to confidently discern the two species.

Several eDNA studies have found evidence of target species presence at sites where they were not detected with traditional survey methods (Jerde et al. 2011; Dejean et al. 2012; Pilliod et al. 2013; Takahara et al. 2013). False positives with eDNA surveys may occur if assays are not sufficiently specific to target species, such that DNA of closely related species may be detected; if a vector introduces target species DNA to the surveyed habitat, such as by the defecation of predators (Merkes et al. 2014); or if decontamination of field gear between sites is not sufficient. An alternative explanation for discrepancies in detections between survey methods is that eDNA may be more sensitive than physical surveys for detecting target species when densities are low (Pilliod et al. 2013), based on the idea that diffusion and mixing of eDNA within a water body increases the area from which the target species may be detected (Darling and Mahon 2011). Although there were two instances in which physical surveys detected a target species and eDNA surveys did not, we detected presence with eDNA at more sites than with physical surveys. Assuming there were no false positives in our results, these results suggest that under our survey protocols, eDNA surveys were more sensitive than physical surveys.

Less than half of our eDNA detections were positive across all samples for the associated site, reaffirming that collecting multiple samples per wetland is often necessary for detection (Thomsen et al. 2012a). Of the 15 instances where we detected our target species with eDNA, only six sites had positive detections in all of the water samples collected, all of which were gopher frog detections. The life histories of the target species suggest gopher frogs may be easier to detect across a wetland with eDNA than the other target species for reasons such as their greater fecundity and longer breeding season. While our results reinforce the need to collect multiple water samples per site for eDNA detection, the number of and volume of water samples necessary to reliably detect eDNA may differ among species depending on factors such as life history. Applying an occupancy modeling framework to future surveys would help provide estimates of detection probabilities as well as minimize the number of samples necessary to reliably estimate presence (MacKenzie and Royle 2005).

**Utility of eDNA for monitoring threatened pond-breeding amphibians in the southeastern United States**

If the benefits of eDNA as a survey tool are maximized when target species densities are low (Pilliod et al. 2013), eDNA may be best utilized by researchers as a complement to conventional survey methods for striped newt and frosted and reticulated flatwoods salamander surveys because of the smaller clutch sizes and shorter breeding seasons relative to gopher frogs. Although eDNA may be more sensitive than traditional surveys for detecting presence of target species under certain conditions, the information that can be obtained from eDNA surveys is limited essentially to presence (and thus presence-derived parameters) and, potentially, relative density or relative biomass (Takahara et al. 2012; Thomsen et al. 2012a). A possible strategy for maximizing the strengths of both eDNA and physical surveys would be to conduct eDNA surveys across unknown or historic wetlands, and conduct targeted physical surveys at wetlands with known presence to more precisely assess population abundances or population trends.

Environmental DNA surveys may have an additional role in assisting with aquatic species monitoring at locations where physical surveys are not possible, such as high-security areas on military bases. In the southeastern United States, military bases contain a large portion of the few remaining substantial and properly maintained longleaf pine forests (Frost 2006). Sample collection for eDNA surveys requires nonspecialized training and a brief amount of time at field sites. In circumstances where trained personnel are unable to physically survey wetlands because of security restrictions, military personnel with security clearance may be trained to conduct eDNA surveys, military personnel with security clearance may be trained to conduct eDNA surveys.
surveys. However, eDNA sampling may require more attention to detail than other sampling methods in order to avoid sample contamination, and any personnel who will be collecting eDNA samples should be properly trained in quality assurance and quality control measures prior to collecting samples.

**Future directions**

At least one qPCR for samples from more than 30% of our sites was inhibited, as inferred from a lack of or delayed amplification of the IC. In addition to the effect PCR inhibition may have on assessing target species presence, inhibition may also affect estimates of eDNA concentration and resulting inferences about relative density and biomass (Takahara et al. 2012; Thomsen et al. 2012a; Goldberg et al. 2013). Assessment of different techniques for reducing inhibition, such as use of dilutions and commercial kits, has demonstrated that these methods are generally effective, although they may also reduce sensitivity (McKee et al. 2015).

Although we sampled several wetlands multiple times over the study period, our study was not intended to address temporal changes in detection or to estimate occupancy. The temporal changes in physiological and environmental factors that influence the amount of DNA available to be detected in aquatic systems may improve or reduce detection independent of target species abundance (Pilliod et al. 2014). Therefore, areas for future investigation include measuring how eDNA detection rates of our target species change over time and under various environmental conditions. Given how much uncertainty remains regarding how these and other factors affect eDNA detection probability, incorporating an occupancy modeling framework to account for imperfect detection (MacKenzie et al. 2002) in future studies will be necessary to obtain the robust information needed to inform management decisions in these environmental settings.

**Supplemental Material**

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**Table S1.** Date of sample collection, wetland area, sample volume, site pH, possible target species DNA contamination, and quantitative PCR (qPCR) inhibition of environmental DNA (eDNA) samples collected from isolated wetlands in the southeastern United States between January and May 2013 and screened for frosted and reticulated flatwoods salamanders (*Ambystoma cingulatum* and *Ambystoma bishopi*, respectively), striped newts (*Notophthalmus perstriatus*), and gopher frogs (*Lithobates capito*). We designed frosted and reticulated flatwoods salamander qPCR primers and probe from a consensus DNA sequence of the two species, and results are presented for which species’ range the site is contained in. For instances in which the site was not within the range of either flatwoods salamander species, we use the generic term “flatwoods salamander.” Average sample volume is the average volume of three water samples filtered for each site, except when noted otherwise. Area is the approximate wetland size, estimated from the National Wetlands Inventory (NWI; http://www.fws.gov/wetlands/); ne indicates no wetland was delineated by the NWI for the site. Max pH is the highest (least acidic) of three (except when noted otherwise) pH measurements. The ratio of potential contamination is for positive samples (i.e., samples with at least two positive qPCR replicates), the ratio of the number of positive qPCR replicates from qPCR plates without evidence of contamination to the number of positive qPCR replicates from plates with evidence of contamination. When qPCR results indicated evidence of target species DNA contamination in negative controls, as inferred by qPCR amplification of a target species assay in a negative control, we made the following inferences: 1) if contamination was present in a field negative control, it could have occurred during DNA extraction, qPCR setup, or in the field; 2) if a DNA extraction negative control was contaminated, this may have occurred during DNA extraction or during qPCR setup; 3) if a no DNA template control was contaminated, this occurred during qPCR setup. The ratio of qPCR inhibition is the ratio of the number of inhibited qPCR replicates, as indicated by a lack of or delayed amplification of the qPCR internal control, to the total number of qPCR replicates for the target species and site. nm indicates no pH measurements were collected. – indicates the eDNA sample did not test positive for associated species presence.

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**Table S2.** Nontarget amphibian species and the number of DNA samples tested between December 2012 and June 2013 for amplification with quantitative PCR primers and probes developed for four imperiled pond-breeding amphibian species from the southeastern United States (frosted flatwoods salamander, *Ambystoma cingulatum*; reticulated flatwoods salamander, *Ambystoma bishopi*; striped newt, *Notophthalmus perstriatus*; and gopher frog, *Lithobates capito*).

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**Table S3.** Recipe per quantitative PCR replicate for assays developed to detect four imperiled pond-breeding amphibian species from isolated wetlands in the southeastern United States (frosted flatwoods salamander, *Ambystoma cingulatum*; reticulated flatwoods salamander, *Ambystoma bishopi*; striped newt, *Notophthalmus perstriatus*; and gopher frog, *Lithobates capito*), collected between January and May 2013.

Found at DOI: http://dx.doi.org/10.3996/042014-JFWM-034.S3 (13 KB DOCX).

**Table S4.** Quantitative PCR cycling protocol for assays developed to detect for four imperiled pond-breeding amphibian species from the southeastern United States (frosted flatwoods salamander, *Ambystoma cingulatum*; reticulated flatwoods salamander, *Ambystoma bishopi*; striped newt, *Notophthalmus perstriatus*; and gopher frog, *Lithobates capito*).

**Table S5.**
frog, *Lithobates capito* from water samples collected between January and May 2013.

Found at DOI: http://dx.doi.org/10.3996/042014-JFWM-034.S4 (13 KB DOXC).

**Table S5.** Quantitative PCR data for frosted and reticulated flatwoods salamanders (*Ambystoma cingulatum* and *Ambystoma bishopi*, respectively), striped newt (*Notophthalmus perstriatus*), and gopher frog (*Lithobates capito*) from environmental DNA samples collected between January and May 2013 from isolated wetlands in the southeastern United States. We designed flatwoods salamander qPCR primers and probe from a frosted and reticulated flatwoods salamander consensus DNA sequence; however, none of the flatwoods salamander detections were within the reticulated flatwoods salamander range, indicating the flatwoods salamander *C*<sub>s</sub> (the qPCR cycle at which the amplification curve crosses a given threshold) results are for the frosted flatwoods salamander.

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**Data S1.** Table S5 variable descriptions.

Found at DOI: http://dx.doi.org/10.3996/042014-JFWM-034.S6 (13 KB DOCX).

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**References**


