Development of Environmental DNA Assay for Screening of Blanchard's Cricket Frog (*Acris blanchardi*) Via Laboratory and Field Methods in Oklahoma, USA

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Abstract: Amphibians represent one of the most threatened vertebrate groups, and although monitoring amphibian population dynamics is critical for conservation, most traditional survey efforts depend on time-consuming, often invasive monitoring activities and visual surveys. Screening environmental DNA (eDNA), a non-invasive monitoring technique, has the potential to identify species presence at a site, even in the absence of visual confirmation. In this study, we developed an aquatic eDNA detection protocol for a common and widespread frog species in Oklahoma (*Acris blanchardi*). We first conducted three laboratory tests to examine assay specificity and sensitivity. Once the primer-probe assay was confirmed to discriminate the target species from others consistently, we then sampled eDNA in four of Oklahoma's six ecological regions to assess how the variation of abiotic factors impact assay sensitivity. In field testing of over 500 samples, we were able to detect *A. blanchardi* eDNA at 60% of the waterbodies sampled, at nearly all field sites across all sampled ecoregions. The proportion of negative eDNA assay results in the waterbodies where the target species were visually observed underscore the importance of continuing traditional surveys alongside newer genetic screening techniques to improve species detection and occupancy modeling.

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Introduction

Our planet is facing a biodiversity crisis, with extinctions increasing at unprecedented rates due to anthropogenic activities (Campbell Grant et al. 2020, Ford et al. 2020, Green et al. 2020). Among vertebrate taxa, amphibians are among the most impacted groups of species—it is currently estimated that 40% of the world's amphibians are threatened (Bolochio et al. 2020). Factors synergistically affecting amphibian populations include climate change, habitat loss, pollution, invasive species, and the proliferation of diseases such as the fungal pathogen *Batrachochytrium dendrobatidis* (Cohen et al. 2019; Campbell Grant et al. 2020; Ford et al. 2020). Therefore, it is critical to closely monitor changes in amphibian populations at a variety of geographic and temporal scales to observe population trends and develop effective conservation strategies (Canessa et al. 2019). However, nearly all amphibian conservation efforts depend on time-consuming visual surveys or invasive capturing activities, such as trapping, seining, dip netting, or hand-capture (Goldberg et al. 2015, 2016; McGrath et al. 2015; Thomsen and Willerslev 2015). These traditional methods require special equipment, training, and permits, making field work logistically challenging (Ficetola et al. 2019; Ruppert et al. 2019). Additionally, these methods often fail to locate rare or cryptic species, resulting in false negatives where species are undetected but actually present (McGrath et al. 2015; Ruppert et al. 2019). Therefore, many researchers have begun investigating and implementing innovative methods for species monitoring, including surveys by unmanned aerial vehicles (UAVs or drones), automated acoustic identification, and environmental DNA (eDNA) detection from air, water, or soil samples (e.g., Chabot and Bird 2015; Goldberg et al. 2015; Russo and Voigt 2016; Lynggaard et al. 2022). The use of eDNA to detect species in an environment is a particularly exciting development in conservation biology. Over the last decade, research efforts have focused on developing and refining eDNA assays, lab methods, and field methods to increase the efficacy of this tool for species monitoring (Goldberg et al. 2016; Harper et al. 2019; Ruppert et al. 2019; Thalinger et al. 2021).

We define eDNA as genetic material that organisms shed into their environment (e.g., within urine, feces, hair, skin, etc.). These eDNA can be collected from a study site and analyzed to determine the presence of target species in the environment without relying on traditional survey methods or disturbing the focal habitats (Wilcox et al. 2013; Diaz-Ferguson and Moyer 2014; Goldberg et al. 2015, 2016; Ficetola et al. 2019). The use of eDNA is a non-invasive approach for monitoring biodiversity that can be standardized and applied broadly across taxa and ecosystems (Goldberg et al. 2015; Ficetola et al. 2019; Ruppert et al. 2019) for a variety of purposes including determining community composition (Yu et al. 2012; Valentini et al. 2016; Lopes et al. 2017; Bálint et al. 2018), detecting cryptic or rare target species at sites where they are not detected by traditional survey methods (Hobbs et al. 2019; Wineland et al. 2019), detecting invasive species (Darling and Mahon 2011; Dejean et al. 2012; Goldberg et al. 2013; Thomas et al. 2019), and screening for infectious diseases within an ecosystem (Baker et al. 2020). Additionally, eDNA tools have the potential to be applied beyond species detection; for example, eDNA has been used to estimate species abundance or biomass of individuals of a target species at a site (Takahara et al. 2012), to determine whether a site has a viable population of a target species (Kamoroff and Goldberg 2018), and to examine within-species genetic variation (Adams et al. 2019a). Therefore, eDNA tools have enormous potential to be applied towards a wide range of biodiversity- and conservation-related research questions.

To use species-specific eDNA tools to detect and monitor a target species, a genetic assay must first be developed specifically for the species and tested to ensure specificity. The ultimate goal of an eDNA assay is to detect a target species across the species' geographic range without falsely detecting non-target species. Therefore, before an assay can be used for research or species monitoring, it must be rigorously tested against the target species, its congenerics

(regardless of sympatry), and additional non-related sympatric species (Thalinger et al. 2021). Evaluating eDNA assays provides a resultant value scale from Level 1 (incomplete) to Level 5 (operational), and is composed of three parts: 1) *in silico*, where software is used to analyze assay specificity using genetic databases; 2) *in vitro*, where the assay is tested and optimized under controlled laboratory conditions; and 3) *in situ*, where the assay is thoroughly tested in the field encompassing a variety of habitats and abiotic conditions (Thalinger et al. 2021). Here we report on protocols of the development of *in silico*, *in vitro*, and *in situ* assessments of an eDNA assay for Blanchard's Cricket Frog, *Acris blanchardi* (Family Hylidae), whose North American distribution covers the central great plains regions of the United States and Canada (McCallum et al. 2011), and in some areas, is experiencing decline (Lehtinen and Skinner 2006). We selected *A. blanchardi* for assay development because this species is common and widespread throughout a variety of aquatic habitats in most ecoregions across Oklahoma (Figure 1; Sievert and Sievert 2021), allowing us to assess the efficacy of the assay in the lab and in the field across multiple local geographic/ecological regions. Additionally, since *A. blanchardi* are found in high densities along shorelines regardless of water flow levels and do not have a strong preference for specific substrates, light levels, or temperatures (Smith et al. 2003; Sievert and Sievert 2021), their genetic material is expected to be found in the majority of sampling locations within their distributed range. Although we focus on *A. blanchardi* in the current study, our ultimate goal is to create a workflow for developing eDNA assays for a diverse group of amphibians across Oklahoma.

Methods

qPCR assay development and* in silico *testing of the primer-probe pair.—The *Acris*

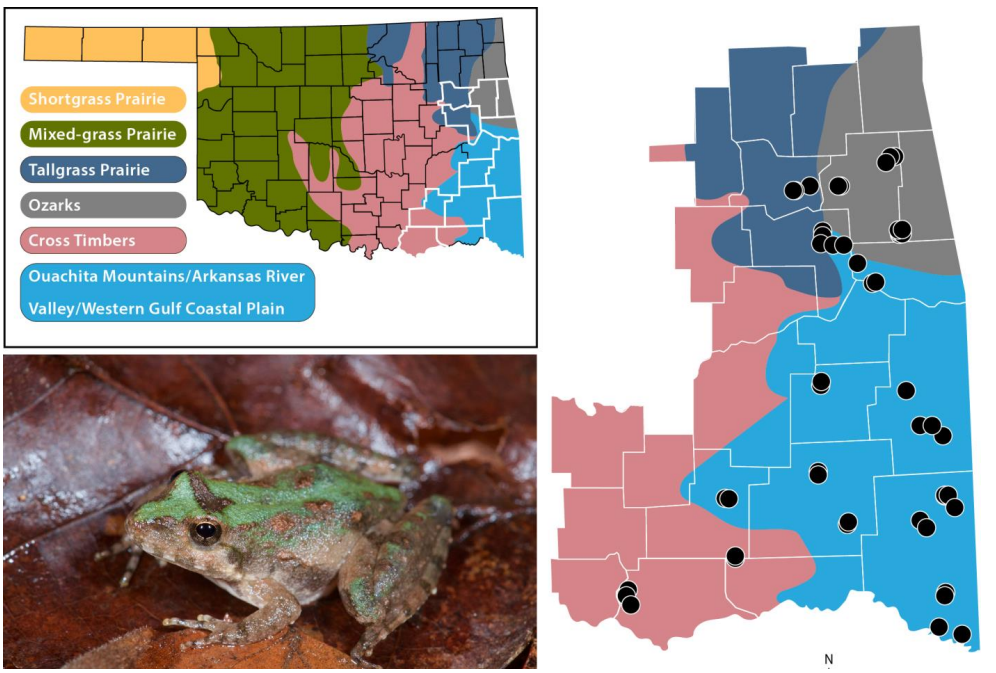

Figure 1. (Top Left) County map of Oklahoma showing the distribution of the six recognized ecoregions in Oklahoma, with counties included in the field sampling (*in situ*) portion of the study outlined in white for reference. (Bottom Left) Photograph in life of *Acris blanchardi* (Photo by K. Wang). (Right) Closeup view of aquatic waterbodies sampled in study (black circles) in eastern Oklahoma (counties outlined in white, ecoregions shown in color).

blanchardi-specific quantitative PCR (qPCR) assay was designed for the mitochondrial gene (mtDNA) cytochrome b (cytb). Mitochondrial genes are better for assay development than nuclear DNA because their faster rate of evolution when compared to nuclear DNA (Moriyama and Powell 1997) results in more species-specific single nucleotide polymorphisms (SNPs) that can distinguish one species from closely related congeneric species. We specifically chose cytb for assay development because of the availability of sequence data on GenBank (NCBI, Bethesda, MD) for multiple individuals of *A. blanchardi* and other North American anurans across a wide geographic range. Initial investigations into cytb suggested the presence of multiple *A. blanchardi*-specific single nucleotide polymorphisms (SNPs) that distinguished the species from other anurans. Other mtDNA genes, NADH dehydrogenase subunit 2 (ND2) and cytochrome c oxidase subunit I (COI), were also considered for assay development, but these genes did not have the needed *A. blanchardi*-specific SNPs.

We obtained cytb sequences of four *A. blanchardi* individuals from GenBank, from across the geographic range of the species (GenBank accession numbers: EF988109 [Illinois]; EF988144 [Mississippi]; EF988127 [Missouri]; EF9881260 [Oklahoma]) and four other anuran species that are often found in the same waterbodies as *A. blanchardi* in Oklahoma (*Anaxyrus americanus* EU938446 [Kansas]; *Pseudacris crucifer* EF988160 [Minnesota]; *Lithobates catesbeianus* AY083293 [Ohio]; *L. clamitans* AY083282 [Missouri]). We aligned the four *A. blanchardi* sequences using Geneious v9.0 (San Diego, CA) and created a consensus sequence (683 bp). The four sequences had a pairwise identity of 98.9% and were identical for 668/683 nucleotides for a site-wide identity of 97.8% (Figure 2). The consensus sequence was imported into the program Primer Express Software v3.01 (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA).

We then aligned the *A. blanchardi* consensus sequence with the outgroup anuran sequences in Geneious and identified SNPs that distinguish *A. blanchardi* from the other species. For each SNP site, we used Primer Express to find acceptable sets of probe and primer sequences surrounding the SNP site, with default optimization settings (probe length = 13–25 base pairs, Tm = 68–70ºC, %GC = 30–80%). From those sets, we selected the one set with the lowest penalty score and shortest amplicon size for our assay. The resulting primers were AB_CytB_F2: 5'–CCTTTCTGCTGCCCCTTA–3' (18 bp) and AB_CytB_R1: 5'–GGTGGCGTTGTCTACTGAA–3', (19 bp) and the custom TaqMan MGB probe was 5'–CTGAGCTAGTCCAATG–3' (16 bp), with FAM reporter dye (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA) (Figure 2).

To confirm that the selected primer-probe set worked across the geographic range of *A. blanchardi* beyond the four samples that were used to develop it, we aligned all 34 *A. blanchardi* cytb sequences available in GenBank as of 18 October 2021 (excluding those that were used in primer-probe design; Table S1) and compared the primer and probe to the alignment. To check if the identified primers were specific for *A. blanchardi*, we used Primer-BLAST (NCBI, Bethesda, MD; Ye et al. 2012) to compare them to additional anuran species in the GenBank database. The Primer-BLAST parameters were set as follows: Search mode

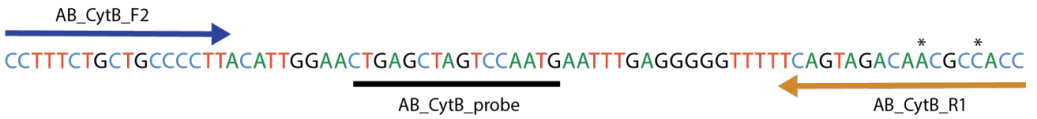

Figure 2. Quantitative PCR assay site covering 76 bp of mitochondrial cytochrome b (cytb) with the locations of forward and reverse primers, and probe. The sequence shown is the majority-rule consensus of the four *Acris blanchardi* sequences used for designing the assay. Two of four sequences include one transitional substitution in the reverse primer sequence (sites indicated by *).

= automatic; Database = nr; Primer specificity stringency = 2 total mismatches including at least 2 mismatches within the last 5 bps at the 3' ends and ignore targets that have 6 or more mismatches to the primer. The results of the Primer-BLAST search returned 57 sequences from two anuran families: Family Hylidae (*Hyla cinerea*, *Pseudacris clarkii*, *P. fouquetti*, *P. maculata*, *P. regilla*) and the Family Ranidae (*Lithobates palustris*, *L. sylvaticus*). Because Primer-BLAST only has the option to check two primers for specificity, all sequences returned in the Primer-BLAST search were downloaded into Geneious and manually compared to the probe sequence to determine the number of mismatches between those sequences and the probe sequence (Table S1). This analysis indicated that the primers were specific to *A. blanchardi* and would not cross-amplify other species.

In vitro *testing of the qPCR assay*.—To further evaluate the *A. blanchardi* eDNA assay, we ran three *in vitro* laboratory experiments using DNA extracted from tissues of the target and non-target anuran species or from dilute lab-created aquatic eDNA solutions to assess assay specificity and efficacy. All frogs used in *Experiment 2* and *Experiment 3*, described below, were euthanized via aqueous chloretone and prepared as voucher specimens for the SNM Herpetology Collection within a few hours of experiment completion, following Simmons (2015). All live anurans captured were collected under applicable Oklahoma Department of Wildlife Scientific Collecting Permits to CDS and JLW, with protocols approved by the University of Oklahoma Institutional Animal Care and Use Committee (IACUC #R14-026).

Experiment 1—testing assay using DNA extracts: The primer-probe set was first tested to ensure that it worked as expected under standard qPCR conditions and to determine validation estimates for the assay. To do this, we extracted DNA from five vouchered *A. blanchardi* tissue samples from Oklahoma (OMNH 46229 [Adair County (Co.)], 46414 [Delaware Co.], 46421–23 [Delaware Co.]; Fig. 1; Table 1) using the high salt DNA extraction method (Esselstyn et al. 2008) and created the following serial dilutions of each extraction starting from a standardized concentration of 20 ng/µl: 20.0, 2.0, 0.2, 0.02, 2×10^{-3}, 2×10^{-4}, 2×10^{-5}, 2×10^{-6} ng/µl. Each dilution was tested against the assay in triplicate using a Quant Studio 3 (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA) qPCR machine following standardized protocols (Siler et al. 2020). Results of this test were used to obtain performance validation estimates for the assay, including values for the r^2, slope, efficiency, and the Limit of Detection (LoD; Klymus et al. 2020; https://github.com/cmerkes/qPCR_LOD_Calc).

Next, the qPCR assay designed for *A. blanchardi* was tested for specificity using DNA from nine anurans from Oklahoma—five individuals of *A. blanchardi* (Ellis Co., Marshall Co., McCurtain Co., Oklahoma Co., Sequoyah Co.; Table 1) and four outgroup co-distributed species (*Hyla chrysocelis/versicolor* [Latimer Co.], *Pseudacris clarkii* [Oklahoma Co.], *P. streckeri* [Le Flore Co.], *Lithobates catesbeianus* [McCurtain Co.]; Table 1). The DNA extracted from liver tissues for this test were obtained from specimens deposited at SNM using the high salt extraction method and were serially diluted to concentrations of 0.02 ng/µl and 0.002 ng/µl to simulate the low concentrations of eDNA that might be encountered in nature. We tested the qPCR assay on four of the five *A. blanchardi* extracts (excluding the individual from Sequoyah Co.) and on all four non-*Acris* species individually to confirm that the assay could detect *A. blanchardi* from multiple populations across Oklahoma without detecting the non-target species. We then tested the qPCR assay using combinations of extracts from the target and non-target species to determine whether the presence of DNA from multiple species would confound the assay and generate either false positive or negative results. To do this, we combined equal volumes of DNA of all four non-target species with and without DNA from the *A. blanchardi* individual from Sequoyah Co. (Table 1). These qPCR assay evaluation runs were conducted in duplicate on a single plate using a BioRad CFX96 Connect (Hercules, CA) following standard qPCR protocols, and

Table 1. *In vitro* testing for specificity of the *Acris blanchardi* qPCR assay, via three experimental approaches. Amplification by qPCR is represented as positive (+) or negative (–). Tissue sample acronyms are as follows: non-vouchered specimens collected by grants funded the Oklahoma Department of Wildlife Conservation (ODWC), vouchered species from Sam Noble Oklahoma Museum of Natural History (OMNH), and vouchered specimens from Louisiana State University (LSU).

Experiment 1—testing assay using DNA extracts		
Individual Species/Community Pools	Sample Numbers	Amplification
Acris blanchardi (Adair Co., OK)	OMNH 46229	+
Acris blanchardi (Delaware Co., OK)	OMNH 46414	+
Acris blanchardi (Delaware Co., OK)	OMNH 46421	+
Acris blanchardi (Delaware Co., OK)	OMNH 46422	+
Acris blanchardi (Delaware Co., OK)	OMNH 46423	+
Acris blanchardi (Ellis Co., OK)	OMNH 41666	+
Acris blanchardi (Marshall Co., OK)	OMNH 44270	+
Acris blanchardi (McCurtain Co., OK)	OMNH 44285	+
Acris blanchardi (Oklahoma Co., OK)	OMNH 44297	+
Hyla chrysocelis/versicolor (Latimer Co., OK)	OMNH 44340	–
Pseudacris clarkii (Oklahoma Co., OK)	OMNH 44432	–
Pseudacris streckeri (Le Flore Co., OK)	OMNH 44439	–
Lithobates catesbeianus (McCurtain Co., OK)	OMNH 44509	–
Mixture of *H. chrysocelis/versicolor*, *P. clarkii*, *P. streckeri*, *L. catesbeianus*, and *A. blanchardi* (Sequoyah Co., OK)	OMNH 44340, OMNH 44432, OMNH 44439, OMNH 44509, OMNH 44324	+
Mixture of *H. chrysocelis/versicolor*, *P. clarkii*, *P. streckeri*, and *L. catesbeianus* extracts	OMNH 44340, OMNH 44432, OMNH 44439, OMNH 44509	–
Acris crepitans (Tallapoosa Co., AL)	LSU H-18789	–
Acris crepitans (East Baton Rouge Parish, LA)	LSU H-20744	+
Acris gryllus (Macon Co., AL)	LSU H-18811	–
Acris gryllus (Livingston Parish, LA)	LSU H-20578	–
Experiment 2—testing assay using eDNA generated in the lab		
Individual Species/Community Pools	Sample Numbers	Amplification
Acris blanchardi (Nowata Co., OK); 250 mL	OMNH 46424	+
Acris blanchardi (Nowata Co., OK); 50 mL each, pooled to 250 mL total	OMNH 46361, OMNH 46362, OMNH 46366, OMNH 46370, ODWC 46371	+
Acris blanchardi, *Hyla chrysocelis/versicolor, Pseudacris maculata*, *Lithobates catesbeianus*, *Lithobates sphenocephalus* (all Nowata Co.); 50 mL each, pooled to 250 mL total	OMNH 46368, OMNH 46454, ODWC 46505, OMNH 46693, OMNH 46689	+
Hyla chrysocelis/versicolor, *Pseudacris maculata, Lithobates catesbeianus*, *Lithobates sphenocephalus* (all from Nowata Co., OK); 62.5 mL each, pooled to 250 mL total	OMNH 46447, OMNH 46508, OMNH 46531, OMNH 46698	–
250 mL ddH_2O water (no frogs)	---	–
Experiment 3—final efficacy testing of the assay using field and lab-generated eDNA samples		
Individual Species/Community Pools	Sample Numbers	Amplification
500 mL of pond water (N = 3) collected from Sutton Urban Wilderness (Cleveland Co., OK), within 1 ft. of live *Acris blanchardi*	---	+
Acris blanchardi (N = 1); 1 L	OMNH 47567	+
Acris blanchardi (N = 1); 2 L	OMNH 47567	+
Acris blanchardi (N = 1); 5 L	OMNH 47567	+
Acris blanchardi (N = 1); 10 L	OMNH 47567	+

the plate included a no-DNA control, also run in duplicate.

Finally, there are two additional species of *Acris* in North America; the ranges of these congenerics do not overlap with the range of *A. blanchardi* in Oklahoma although they do appear to overlap east of Oklahoma along the Mississippi River (Gamble et al. 2008). Therefore, we were interested in whether the assay could be used to distinguish *A. blanchardi* from its congenerics. We tested the assay for specificity against the species *A. crepitans* (from Tallapoosa Co., Alabama and East Baton Rouge Parish, Louisiana) and *A. gryllus* (from Macon Co., Alabama and Livingston Parish, Louisiana), obtained from tissue stored at Louisiana State University (LSU). Liver tissue for these four individuals were extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and the extracted DNA was quantified and serially diluted to 1:10 and 1:100 for all four samples. A qPCR run was conducted in triplicate using standard protocols (Siler et al. 2020) on a Quant Studio 3 (Applied Biosystems/ Thermo Fisher Scientific, Waltham, MA).

Experiment 2—testing assay eDNA samples generated in the lab: The qPCR assay was tested using water samples obtained after submerging live frogs in water in a controlled laboratory setting, to determine if the assay could detect DNA that had been shed externally by a living organism into water. Frogs were collected three days before the test from Oologah Wildlife Management Reserve in Oklahoma (Nowata Co.) and were kept in captivity at the SNM. Each frog was placed individually in a separate sterilized glass jar with molecular grade distilled and deionized water (ddH_2O) and left for one hour. The following five samples of 250 mL water were created in the lab, and no individual frog was used more than once, for a total of one hour each: (1) one *A. blanchardi* (in 250 mL); (2) five *A. blanchardi* in separate jars (50 mL of ddH_2O each), then combined to form 250 mL in total; (3) one *A. blanchardi*, plus four co-distributed non-target species (*H. chrysocelis/ versicolor*, *P. maculata*, *L. catesbeianus*, *L. sphenocephalus*) in separate jars (50 mL of ddH_2O each), then combined to form 250 mL in total; (4) one each of the four non-target species in separate jars (62.5 mL of ddH_2O), then combined to form 250 mL in total; and (5) control sample of 250 mL ddH_2O and no frogs, as a negative control (Table 1). Each water sample was filtered on a separate 0.45µm pore PES filter (75 mm filter diameter) immediately after the experiment ended. The filter was stored overnight in 95% EtOH in a -20ºC freezer until the time of DNA extraction. Each experimental extraction was used in duplicate to test the qPCR assay on a QuantStudio 3 using standard qPCR protocols (Siler et al. 2020).

Experiment 3—final efficacy testing of the assay using field and lab-generated eDNA samples: An additional set of tests was conducted to evaluate the qPCR assay as well as the robustness of our filtration and extraction methods using more dilute water samples than those used in the experiment above to simulate sampling conditions in the field. Three separate 500 mL water samples were collected from the Sutton Urban Wilderness Area (Cleveland Co., Oklahoma; using the methods described below), from within one foot of a single live *A. blanchardi*. Additionally, one *A. blanchardi* individual each was collected by hand and placed sequentially into sterilized containers with 1 L, 2 L, 5 L, and 10 L of ddH_2O within four hours of capture, for a total of one hour each, to further mimic the small quantities of eDNA expected in a natural water body setting (Table 1). For all seven water samples (three from Sutton Urban Wilderness area, four from lab testing), 500 mL was filtered individually on separate 0.45µm pore PES filters (75 mm filter diameter), processed for DNA extraction and screened in triplicate on a QuantStudio 3, using standard qPCR protocols (Siler et al. 2020).

In situ *evaluation of the qPCR assay.*—We conducted preliminary *in situ* evaluation of the *A. blanchardi* qPCR assay using water samples collected across Oklahoma in spring–summer in years 2017–2018 to determine the efficacy of the assay when applied in the field. At all large-scale field sites sampled (Figure 1; Table S2), we had historical visual and/or auditory confirmation of

A. blanchardi presence and therefore expected our qPCR assay to return positive results.

Field eDNA collection and filtration.—Water samples were collected in aquatic habitats across four recognized ecoregions in eastern Oklahoma (Fig. 1; Table S2): Crosstimbers (July 2017), Ouachita Mountains/Arkansas Valley/West Gulf Coastal Plains (April–May 2017; March–May 2018), Ozarks (April–May 2018), and Tallgrass Prairie (May 2018). To minimize any seasonal factors that could impact eDNA detection (De Souza et al. 2016; Takahara et al. 2020), all field samples were collected in spring/early summer, which coincides with the active breeding season of *A. blanchardi* (McCallum et al. 2011). Our sampling scheme used the following hierarchy, represented from largest category to smallest: ecoregion (N = 4), counties (N = 11), field sites (N = 24), unique waterbodies within each field site (N = 79 total; 1–9 per field site) (Fig. 1; Table S2; Siler et al. 2020). The field sites included USDA national forests, USFWS national wildlife refuges (NWR), state parks (SP), state-run wildlife management areas (WMA), The Nature Conservancy preserves (TNCP), and public access points (PUA) affiliated with U.S. Army Corps of Engineers lakes (Table S2).

For each waterbody, 2–8 samples of 500–600 mL of water was collected 1–2 m from shore at a 5–10 cm water depth (N = 565 samples in total), using sterile 1,065 mL one-time use Whirl-pak sampling bags (Nasco, Madison, WI; Wineland et al. 2019; Siler et al. 2020). The number of samples collected per waterbody was determined based on the waterbody size and capacity to allow a minimum distance of 10 m between samples, with a maximum of eight samples regardless of waterbody size. Additionally, we created a negative control for each waterbody by filling a water sample bag with dH_2O, sealing it, and dipping the sealed bag into the water for approximately 30 seconds (N = 79 controls). Samples were stored cold, but not frozen, in a dark cooler to prevent potential DNA degradation by UV light or warm temperatures (Pilliod et al. 2013a; Strickler et al. 2015) and detection difficulties due to freezing samples prior to filtration (Takahara et al. 2014).

We filtered all samples within 24 hours of collection, maintaining strict sterility protocols, whether filtration occurred in the field or in the lab; no filtration occurred within the same space as *A. blanchardi* tissue extraction. Prior to sample filtration, all work surfaces were sterilized with ELIMINase (Decon Labs, King of Prussia, PA) or 10% bleach, and nitrile gloves were changed between each sample. Water was homogenized in the sample bag, then poured into a sterile, one-time use 500 mL polyethersulphone (PES) membrane filters, with a 75 mm filter diameter and a 0.45μm pore size (various vendors: Thermo Fisher Scientific, Waltham, MA; VWR, Radnor, PA; Foxx Life Sciences, Salem, NH). We vacuum-filtered both experimental samples and negative controls until the membranes became clogged or until 500 mL was filtered (whichever came first), cut out the filter membrane using a sterile from the sterile housing using a one-time use 11-blade scalpel, placed the membrane into a 10 mL cryovial with 95% ethanol, and stored the vial in a -20ºC freezer until the time of extraction (less than six months) (Siler et al. 2020).

Extraction and screening of eDNA samples.—All lab procedures for DNA extraction and qPCR screening were conducting at the SNM Genomics Core Facility using strict sterility protocols as described previously. For each batch of eDNA extractions, a second negative control sample was created by placing a PES membrane filter into sterile ddH_2O and extracted along with the experimental filters. We isolated total genomic eDNA using a modified protocol based on the DNeasy Blood and Tissue Kit/QIAshredder (Qiagen, Hilden, Germany; Buxton et al. 2017; Pilliod et al. 2013a; Siler et al. 2020), with additional steps for removal of PCR inhibitors using Zymo OneStep PCR Inhibitor Removal Kits (Zymo Research Products, Irvine, CA; McKee et al. 2015; Turner et al. 2015; Adams et al. 2019b; Baker et al. 2020; Siler et al. 2020). For these extractions, only one-half of each vouchered filter membrane was used, and the remaining one-half of the filter was returned to the freezer for archival purposes. The filter membrane was first diced into small pieces using forceps and scissors sterilized with

10% bleach. Because these membrane pieces did not fit in one 1.5mL microcentrifuge tubes, they were divided equally into two sterile 1.5mL microcentrifuge tubes and later combined during the QIAshredder process (Siler et al. 2020).

All eDNA extracts, including both field and laboratory created negative controls, were screened with the *A. blanchardi*-specific qPCR assay described above, following the protocols of Siler et al. (2020). All eDNA samples were tested in triplicates, which is a common protocol for samples collected from the field (Pilliod et al. 2013a,b; Barnes et al. 2014; Strickler et al. 2015; Adams et al. 2019b). A well was considered positive if it crossed the call threshold determined by the QuantStudio Design and Analysis software (v1.5.1) using the presence/absence experiment protocol. We considered *A. blanchardi* eDNA as present if two or more of the triplicate wells were called positive, or one of the triplicate wells crossed the threshold on two successive qPCR runs, following established protocols (e.g. Strickler et al. 2015; Siler et al. 2020). *Acris blanchardi* is ubiquitous across Oklahoma, and we expected each plate to have at least one positive, which acted as a positive control, and as such, these qPCR were run without additional positive controls.

Results and Discussion

Overall, our *A. blanchardi* eDNA successfully detected the presence of *A. blanchardi* in both lab (*in silico*, *in vitro*) and field (*in situ*) settings and did not detect non-target species under a variety of lab (*in silico*, *in vitro)* experiments. The *in silico* experiments, in which software was used to assess the efficacy of the primer-probe set using sequences of target and non-target species that were not used in the initial assay design, suggested that the assay was specific to multiple populations of *A. blanchardi* across its geographic range. Aligning the primers and probes to the available 34 *A. blanchardi* cytb sequences in GenBank indicated that across all sequences, the probe was a 97% match, the forward primer was a 100% match, and the reverse primer was a 94% match (Table S1). Furthermore, testing the primers against sympatric non-target anurans using BLAST indicated that there were 2–4 mismatches between all individuals tested and the forward primer and 0–3 mismatches between all individuals tested and the reverse primer. These mismatches included both transitions and transversions. Therefore, *in silico* testing validated the assay as being able to detect *A. blanchardi* across the known sequence diversity of the species while not detecting closely and distantly related non-target species that co-occur in the same habitats as *A. blanchardi.*

The second step of assay validation includes *in vitro* laboratory testing to determine if the assay works as expected under controlled conditions with known positive and negative samples. In this *in vitro* testing, the assay performed as expected in all three experiments (Table 1). In Experiment 1, we first tested the assay against extracted DNA from five Oklahoma *A. blanchardi* tissues to assess the performance of the assay under standard qPCR protocols and to measure the limit of detection (LOD). The LOD for our assay was 2×10^{-4} ng/µl. However, the assay detected *A. blanchardi* DNA down to a concentration of 2×10^{-5} ng/µl in 13/15 replicates and 2×10^{-6} ng/µl in 6/15 replicates. The r^2 was 0.995, the slope was -3.481, and the efficiency was 93.76%. We then examined the assay for specificity using extracted DNA from multiple Oklahoma populations of *A. blanchardi* and multiple closely and distantly related non-target anurans, some species of which were not used in the initial assay development or *in silico* testing. Results suggested that the assay was specific to *A. blanchardi* across a broad geographic range and would not provide false negatives when *A. blanchardi* DNA was present or false positives in the absence of *A. blanchardi* (Table 1). However, in Experiment 1, when testing the assay against *A. blanchardi* congenerics, a single sample of the closely related *A. crepitans* resulted in a positive result (LSU H-20744 from East Baton Rouge Parish, LA), but no *A. gryllus* tested positive (Table 1). *Acris blanchardi* and *A. crepitans* are sister species which were only recently segregated into two species and are also difficult to distinguish visually (Gamble et al. 2008). Therefore, this result could have (1) been caused by human error if the frog was

misidentified as *A. crepitans* upon capture and was actually *A. blanchardi* or (2) a result of the two species' cytb genes being similar due to recent divergence. Furthermore, although hybridization between *Acris* species has not been studied, hybridization is known to occur between other species in the family Hylidae, including, famously, historical and ongoing hybridization in the *H. versicolor/chrysocelis* species complex (e.g. Booker et al. 2020). This result indicates that any future researchers wishing to work on *A. blanchardi* versus *A. crepitans* specifically should further develop a more refined primer-probe assay, especially if they are working in areas where the two species may be sympatric (e.g. along the Ohio River; Gamble et al. 2008). This level of specificity was beyond the goals of our particular project. In Experiments 2 and 3 we further tested the assay against eDNA from a single population of *A. blanchardi* and several species of sympatric non-target anurans, one of which had not been used for assay development, *in silico* testing, or in previous *in vitro* testing to determine if the assay would work using standard filtration and filter extraction protocols. Once again, all experimental samples were positive in the presence of *A. blanchardi* DNA, with no false negatives or false positives (Table 1).

In contrast to the complete success of the *in silico* and *in vitro* experiments, *in situ* field validation of the *A. blanchardi* eDNA assay had varying results. The *in situ* screening of the *A. blanchardi* qPCR assay suggested that it was able to detect species-specific DNA in a wide variety of field conditions and locations across Oklahoma, but not all waterbodies returned positive results despite historical (within the last year), visual and/or auditory confirmation of *A. blanchardi* at the waterbody specifically and/or at the broader site more generally. Out of a total of 565 samples (excluding the field-based negative controls), *A. blanchardi* DNA was detected in 120 samples (21.24%; Table 2). These positive samples came from all four eastern Oklahoma ecoregions surveyed, in 21 of 24 field sites (87.5%), and in 48 of 79 waterbodies (60.8%; Table 2; Table S2). We found no *A. blanchardi* false positives in either the lab or field negative. All qPCR plates contained at least one positive well, with a single exception, which was likely a sample size artifact since only four eDNA extracts were screened on that plate (0.6% of all samples). Therefore, reagent or equipment failure or human error during the qPCR setup were unlikely to be the cause of negative results. Although we did not detect *A. blanchardi* in the field as often as we had originally expected given the success of our previous laboratory testing, our knowledge of our sampling sites, and well-documented information about the species in Oklahoma (Sievert and Sievert 2021), we did detect *A. blanchardi* in every ecoregion and at nearly every field site sampled. Overall, our results suggest that the assay is successful in the field, but continued refinement of field protocols is needed to ensure that false negatives, in which *A. blanchardi* is confirmed present at a site but not detected, are not obtained via eDNA screening.

Our results suggest that false negatives were a common result of our *in situ* testing. For the 31 waterbodies at which field crews made concrete notes about *A. blanchardi* presence at the time of eDNA sample collection and/or a specimen from the waterbody was vouchered on the same date as eDNA sample collection, qPCR screening failed to detect the species in 16 waterbodies within this subset (52% false negatives within this subset of sites with known presence; Table S2). These false negatives may be attributed to several unforeseen challenges related to water turbidity and stagnation at our sampling sites. High levels of turbidity observed at our sites may have reduced *A. blanchardi* eDNA detection rates, through direct effects (filter clogging) or indirect effects (introduction of PCR inhibitors). Oklahoma waters are known to be highly turbid (Penfound 1953), which we also observed, with the exception of a handful of sampled streams. Approximately one-third of our field samples clogged before reaching the desired filter volume of 500 mL, presumably resulting in less overall captured eDNA. We considered changing to a larger pore size (i.e. 0.8 µm), but filters with a 0.45 µm pore size, such as those used in our research, were the most commonly used at the time we began sampling

Table 2. Summary qPCR results of eDNA field surveys screening using the *Acris blanchardi* qPCR assay. The total number of waterbodies and samples by county are shown (excluding field-based negative controls), followed by the number and percentage (%) of positive (+) samples detected by Oklahoma ecoregion.

Ecoregion	No. waterbodies (No. samples)	No. + waterbodies (No. + samples)	% + waterbodies (% + samples)
Crosstimbers	7 (42)	4 (10)	57.14% (23.81%)
Ouachita Mountains/Arkansas River Valley/Western Gulf Coastal Plain	54 (383)	34 (87)	62.96% (22.72%)
Ozarks	14 (126)	8 (20)	57.14% (17.86%)
Tallgrass Prairie	4 (32)	2 (3)	50.00% (10.71%)
TOTAL	**79 (565)**	**48 (120)**	**60.76% (21.24%)**

in 2017 (e.g. Goldberg et al. 2013; Pilliod et al. 2013a,b). More recent studies have shown that either there is no statistical difference in eDNA collection between the two pore sizes (Li et al. 2018) or that the 0.45 µm pore size is still optimal (Capo et al. 2019). It is generally accepted that large pore sizes, above those mentioned, often result in a lack of DNA particulate collection (Turner et al. 2014). In addition to filter clogging, the high turbidity we observed could be linked to an increase in PCR inhibitors that can decrease the ability to detect eDNA in qPCR screening and interfere with various extraction steps, potentially leading to an increase in false negatives (Buxton et al. 2017; Williams et al. 2017; Li et al. 2018). These PCR inhibitors tend to build up in stagnant water systems (Harper et al. 2019), such as in those in which we primarily sampled. Inhibitor reduction through dilutions of the eDNA extracts with ddH_2O or buffers was not feasible as it would result in an over-dilution of samples, to the point of undetectability of target species DNA (Williams et al. 2017; Harper et al. 2019). Instead of employing dilution, we incorporated OneStep PCR Inhibitor Removal Kits (Siler et al. 2020), to remove potential PCR inhibitors from turbidity and/or other elements in the water, as these kits have successfully been used for removing PCR inhibitors (McKee et al. 2015; Turner et al. 2015; Adams et al. 2019b; Baker et al. 2020). In fact, McKee et al. (2015) confirmed that samples treated with OneStep PCR Inhibitor Removal Kits performed better in qPCR than samples that were diluted 5- or 10-fold, resulting in more precise DNA concentration estimates, particularly in samples obtained from sediment-laden wetlands.

Therefore, although *in silico* and *in vitro* testing of our *A. blanchardi* eDNA assay validated the efficacy of our assay for detecting and monitoring populations of *A. blanchardi* across a wide geographic range, our *in situ* testing suggests that further refinement of the field and laboratory extraction and/or screening protocols are necessary before the assay can be implemented successfully (Burian et al. 2021). These refinements might include increasing the number of samples that are collected from a site and increasing the number of replicates on a qPCR run. Recent research has explored optimal sample and replicate number for eDNA field protocols and has shown that to optimize eDNA detectability, field sample sizes and qPCR replicate number should be adjusted according to species-specific factors, including whether the focal taxa are rare vs. common (Akre et al. 2019; Erickson et al. 2019; Ficetola et al. 2019), and the type of habitat (Goldberg et al. 2018). Specifically, Akre et al. (2019) propose a minimum of four water samples per waterbody, whereas Goldberg et al. (2018) and Erickson et al. (2019) state that up to 15 water samples may be required for detection of a common species and over 45 samples may be required for detection of a rare species. Additionally, according to Ficetola et al. (2019), a minimum of four qPCR replicates is required to ensure eDNA detection and eight is recommended, whereas Erickson et al. (2019) suggest up to 16 qPCR replicates per sample if the species is rare. Based on this recent research, both the number of water samples collected in our field sampling regime and the number of qPCR replicates used for each site were lower than what is necessary to ensure detection of *A. blanchardi*. Nevertheless, despite our suboptimal field and laboratory protocol, we were able to detect *A. blanchardi* in more

than 60% of all waterbodies tested, comprising more than 87% of all our field sites. These results indicate that our *A. blanchardi* eDNA assay is viable and with the refinement of field and lab protocols, can be applied to monitoring and conservation efforts Oklahoma. Given the results of our *in silico* and *in vitro* testing, the assay also will likely be successful across the full geographic range of *A blanchardi* but *in situ* testing needs to be done in locations beyond Oklahoma to confirm this.

Finally, additional research has argued for different criteria employed across studies to determine whether a positive well in a qPCR run reflects the presence of a species at a site. As stated above, we considered an eDNA sample positive for the target species if two or more of the triplicate wells crossed this experimental threshold in a single qPCR run, or if one of the triplicate wells crossed the threshold on two successive qPCR runs, but compared to other published studies, our value could be considered either too conservative (Raemy and Ursenbacher 2018) or too liberal (Kamoroff and Goldberg 2018; Wineland et al. 2019). Furthermore, some studies have suggested follow-up Sanger sequencing for any positive qPCR sample to confirm target DNA was accurately detected (e.g. De Souza et al. 2016); however Goldberg et al. (2016) suggest that this is only necessary when an assay consists of primers only and not a primer-probe set, as in our assay. Ruppert et al. (2019) argue that although field and laboratory methods vary greatly in the realm of eDNA research, the most important component is primer-probe specificity to the target species; given the specificity of our assay we are confident that our *in situ* testing reflects true positives, true negatives, and false negatives, and not false positives.

Therefore, based on the eDNA assessment scale developed by Thalinger et al. (2021) we suggest that our *A. blanchardi* eDNA assay meets Level 4 out of five levels of validation for routine species monitoring for *A. blanchardi* in Oklahoma, and Level 2 for routine species monitoring across the geographic range of *A. blanchardi* more broadly. Our *in silico* and *in vitro* testing of the assay indicated that the assay is specific to *A. blanchardi* and works effectively in controlled laboratory conditions. Under field conditions, our assay was shown to be successful but not completely effective. Additional *in situ* testing and optimization, including determining and minimizing the rate of false negatives, needs to be done before the sampling protocols and eDNA detection assay are fully validated and field ready.

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