EVALUATING THE DETECTION OF SEASONALLY PRESENT, POND-BREEDING AMPHIBIANS USING ENVIRONMENTAL DNA: A CASE STUDY WITH THE HOUSTON TOAD (*BUFO* [=*ANAXYRUS*] *HOUSTONENSIS*)

by

William W. Keitt, B.Sc.(AG.)

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Committee Members:

Michael R.J. Forstner, Chair

Dittmar Hahn

David Rodriguez

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DEDICATION

To Mom, Dad, and Amy

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ABSTRACT

Molecular survey techniques focusing on the detection environmental DNA (eDNA) are increasingly being implemented for a wide breadth of animal taxa in order to document occurrence across various habitats. Despite increasing instances of its use, relatively few studies have evaluated this method for seasonally present, pond-breeding amphibians. This study seeks to provide an evaluation of the efficacy of eDNA surveys for the detection of one such species, the Houston toad (*Bufo* [=*Anaxyrus*] *houstonensis*). The Griffith League Ranch (GLR), a primary recovery site in Bastrop county Texas, was sampled weekly during the Houston toad breeding season from February to June of 2016, and sporadically in the spring of 2017. Nine perennial ponds on the GLR were surveyed, and 557 water samples were collected for eDNA analysis, with 217 representing known positive captive controls, collected from buckets with each of the primary life stages of this amphibian. Samples were collected following a USGS approved protocol (Goldberg et al. 2011). Both PCR, and nested PCR assays were used to assess the utility of this technique in the detection of the Houston toad based on positive amplification of a diagnostic fragment of mitochondrial DNA. PCR assays successfully showed amplification of Houston toad eDNA in 82% of known positive captive controls, while only 1.1% of eDNA samples from the sampled ponds, and none of the eDNA samples of known positive pond controls showed amplification. Nested PCR assays proved more efficient, detecting Houston toad eDNA in 86% of all known positive captive controls, 7.4% of all pond samples, and 14% of the samples collected from known positive pond

controls. Our results suggest that these PCR-based detection methodologies are unreliable in the detection of eDNA as they incur routine false negative detections, and therefore, are likely less reliable than current survey approaches. The inability of eDNA surveys to accurately detect species presence may be impacted by a variety of factors ranging from environmental inhibitors, to the limitations of the detection assay. Therefore, I offer many critical considerations for the effective implementation of this monitoring strategy, as a USGS approved protocol appears inadequate in the detection of elusive, seasonally present, pond-breeding amphibians, like the Houston toad.

1. AN INTRODUCTION TO THE CONSERVATION STATUS, AND LIFE HISTORY, OF THE HOUSTON TOAD (*BUFO* [=*ANAXYRUS*] *HOUSTONENSIS*)

INTRODUCTION

The Houston toad (*Bufo* [=*Anaxyrus*] *houstonensis*) was first described in Houston, Texas in 1953 (Sanders 1953; Peterson et al. 2004). Since this first description, Houston toads have experienced significant population declines, even within suitable habitat (Allison and Wilkins 2001). While there are believed to be multiple contributing factors leading to the drastic population, and subsequent habitat reduction of the Houston toad, many believe the main causes to be prolonged drought (2007 – 2009), and land-use changes associated with urbanization (U.S. Fish and Wildlife 1984; Seal 1994; Duarte et al. 2014). Thus, only 17 years after its first description, *B.* [=*A.*] *houstonensis* became the first Texas species to be state listed (Allison and Wilkins 2001), as well as federally listed under the Endangered Species Conservation Act of 1970 (Gottschalk 1970; United States List of Endangered Native Fish and Wildlife 1970; Peterson et al. 2004).

The restricted range of Houston toads was assumed to be one of the leading concerns that led to its federal listing (Allison and Wilkins 2001). Historically found in thirteen counties across central, and coastal Texas, the current range has been reduced to nine currently occupied counties (Fig. 1) (U.S. Fish and Wildlife 2011). The Lost Pines, and Rolling Plains regions of Texas, and the surrounding counties are meant to exhibit the optimal habitat for the Houston toad, i.e. the presence of deep, sandy loam soils (U.S. Fish and Wildlife 2011). Outside of the Lost Pines region, large chorusing populations were detected in Robertson county, with lesser chorusing populations in Leon, and Milam

counties (Forstner and Dixon 2011). A review of the Houston toad reported from Freestone county has been confirmed (Forstner and Dixon 2011), however it did not inspire adequate surveys for the species there, nor in the entire area characterized, geologically, by the Simsboro formation, which is comprised of sands very analogous to those supporting the species in Bastrop, and other known occupied counties. Thus, currently, detection of this taxon has been restricted to as few as nine Texas counties (Duarte et al. 2014), without adequate surveys in adjacent counties to document occupancy. This restriction to sandy habitats for adults, and the habitat fragmentation that has been documented for all Houston toad habitats, even within federally designated critical habitat units, remains one of the most pressing difficulties facing the Houston toad.

Other concerns affecting Houston toad distribution, and abundance are its short reproductive period (Hillis et al. 1984), hybridization with other toad species (Hillis et al. 1984), and environmental stressors such as extreme weather conditions, (i.e., drought, and wildfires), which have culminated in continued population decline of this taxon (Duarte et al. 2014). A perceived rapid reduction in suitable habitat led to the creation of critical habitat units for the Houston toad in two of the aforementioned counties. In 1978, the U.S. Fish and Wildlife Service (USFWS) designated areas of Bastrop, and Burleson counties as critical habitat units (CHUs), with the hope that it would lead to zones of protection, and spur a population increase of the critically endangered amphibian (U.S. Fish and Wildlife 1984). The CHUs were established to help mitigate urbanization, and land-use changes, two of the most prominent threats to this amphibian. It is of note that the original recommendation for the development of these CHUs included areas of

Bastrop, Burleson, and Harris counties (Lannoo 2005). While the CHU in Bastrop county remains for the Houston toad, the proposed area in Harris county was excluded from the final ruling once commercial interests protested the loss of prime land for future development (Lannoo 2005). The ruling highlighted the lack of priority in establishing, and managing, suitable habitat for the Houston toad, thus embodying the dangers urbanization imposes on such a threatened species.

Despite the loss of the Harris county CHU, and the fact that the Burleson CHU represents one singular locality (Lannoo 2005), the CHU established in the Lost Pines region has continued to provide some level of regulatory oversight for the threatened species. The CHU in the Lost Pines region is predominantly comprised of loblolly pine (*Pinus taeida*), and oak (*Quercus* sp.) trees, with deep, sandy loam soils (Campbell 2003; Al-Rabab'ah and Williams 2004). Houston toads are poor burrowers (Bragg 1960), and sandy soils provide a substrate that allows easier burrowing for refugia, and temperature regulation (Campbell 2003). The survivability of the Houston toad in the Lost Pines region has since sparked a debate about the true habitat limitations of the Houston toad. Some have noted that the species is not restricted by the Lost Pines forest region, but by the availability of soft soils in which it is capable of burrowing (Brown and Thomas 1982). While suitable habitat is still often reported to be restricted to the Lost Pines, and Rolling Plains regions, the debate to define the most important environmental covariate(s) to the species' survival remains ongoing.

However, the argument about optimal habitat, and causes of range restriction of the Houston toad became more critical after 2011, when a wildfire rapidly spread throughout Bastrop County. The Lost Pines region sustained devastating damage before

the wildfire was finally contained. An estimated 36,000 acres, or 40%, of proposedly optimal Houston toad habitat was impacted (Wallace et al. 2011; Brown et al. 2013).

The habitat changes wrought by the 2011 wildfires have highlighted the importance of identifying, and maintaining optimal habitat units for the Houston toad in the Lost Pines region, and outside of it. Intensive monitoring surveys, including those that have used automated recording devices (ARDs), have been implemented to better identify currently occupied breeding habitat throughout the region. Recently, more research focused on examining potentially more sensitive methods to identify, and locate threatened, and endangered amphibians such as the Houston toad (Acevedo and Villanueva-Rivera 2006; Jackson et al. 2006; MacLaren 2015). These proposed alternative survey methodologies must not only prove wholly reliable, but must minimize false negative detections (i.e., stating that the species is absent, when it is present but the survey method failed to detect it). The detection of presence of such a critically threatened species demands the implementation of a survey methodology that is not only exhaustive, accurate, and robust, but relatively simple to implement, and cost effective.

Owing to a demanding list of requirements for the effective implementation of alternative survey methodologies, evaluations of molecular techniques aimed at the detection of environmental DNA (eDNA) have quickly grown in popularity (Goldberg et al. 2011; Dejean et al. 2012; Thomsen et al. 2012 and Willerslev 2015). The extensive global decline in biodiversity has led to a push for new ways to provide biodiversity monitoring that are both efficient, and inexpensive, but also non-invasive (Goldberg et al. 2015; Thomsen and Willerslev 2015). The advances in molecular techniques have opened the way for determinations of presence/absence to be made via successful amplification

of eDNA in a variety of organisms, including vertebrates (Ficetola et al. 2008), various aquatic species (Ficetola et al. 2008; Dejean et al. 2011; Goldberg et al. 2011; Jerde et al. 2011; Takahara et al. 2012), invasive species (Dejean et al. 2012; Mahon et al. 2013; Takahara et al. 2013; Pilliod et al. 2013a), and even rare or elusive species (Jerde et al. 2011; Pilliod et al. 2013b; Piaggio et al. 2014). The ability, and efficiency, in which eDNA can be used to catalog biodiversity in a given area is one of the reasons for the quick adoption of eDNA analyses as emerging survey methodologies in conservation biology (Goldberg et al. 2015; Goldberg 2016).

While many conservation biologists are championing the use of this powerful new molecular survey method for its use in biodiversity monitoring, there have been relatively few tests of taxa that are not fully aquatic, and relatively small (Thomsen et al. 2009; Goldberg et al. 2011; Willerslev et al. 2014). The methods approved by the United States Geological Service (USGS) for eDNA monitoring have been tested on numerous anurans (Ficetola et al. 2008; Dejean et al. 2011; Goldberg et al. 2011), Caudata (Goldberg et al., 2011; Biggs et al., 2015), Squamata (Piaggio et al. 2014; Hunter et al. 2015), and a variety of marine life including various species of *Cyprinus*, and crustaceans (Jerde et al. 2011; Takahara et al. 2012; Thomsen et al. 2012).

From this point of view, the Houston toad is an interesting object for the evaluation of the efficiency, and reliability of this emerging molecular survey technique. This taxon lays eggs in a pond which hatch within seven days, with tadpoles metamorphing into terrestrial juveniles after around 53 to 65 days, depending on environmental conditions (Hillis et al. 1984). These juveniles occupy upland burrows near a perennial pond, before returning to the pond edge for chorusing events throughout

the breeding season. As the adult life stage is not fully aquatic, and only utilizes the pond edge during the breeding season, the Houston toad offers some interesting challenges to the viability of this molecular survey technique. To be proven a reliable alternative survey methodology, eDNA analyses must be shown to be exhaustive, accurate, robust, cost effective, and limit the incursion of Type I, and II errors in detection.

2. Evaluating the Detection of Seasonally Present, Pond-Breeding Amphibians Using Environmental DNA: A Case Study With the Houston toad (*Bufo* [=*Anaxyrus*] *houstonensis*)

Introduction

The Houston toad (*Bufo* [=*Anaxyrus*] *houstonensis*) was first described from a Harris county, Texas specimen in 1953 (Sanders 1953; Peterson et al. 2004). Historically present in thirteen Texas counties, the range of the Houston toad has been reduced to nine currently occupied counties (Fig. 1) (Peterson et al. 2004; U.S. Fish & Wildlife 2011). While not explicitly known, it has been suggested that this large-scale reduction in suitable habitat, brought about by prolonged drought, and land-use changes associated with urbanization, has played a key role in the reduction of population sizes across Texas (Sanders 1953; Brown 1971; U.S. Fish & Wildlife 1984; Seal 1992; U.S. Fish & Wildlife 2011; Duarte et al. 2014). Thus, only 17 years after its first description, the Houston toad became the first Texas species to be state listed (Allison and Wilkins 2001), as well as federally listed under the Endangered Species Conservation Act of 1970 (Gottschalk 1970; U. S. List of Endangered Native Fish & Wildlife 1970; Peterson et al. 2004).

The threats currently facing the Houston toads highlight the need for the implementation of effective, and robust, monitoring practices to better identify, and protect occupied habitat throughout this narrow range (Jackson et al. 2006; MacLaren 2015). The U.S. Fish and Wildlife Service (USFWS) mandates the implementation of call surveys to monitor for the Houston toad (U.S. Fish & Wildlife 1984). Despite manual call surveys being the primary monitoring effort for over 30 years, recent research suggests

that the 5-minute window in which these call surveys take place is susceptible to the inclusion of false negative detections (Bridges and Dorcas 2000; Jackson et al. 2006; MacLaren 2015). As the reduction of false negative detections is imperative when surveying for federally listed species, this research has led to the evaluation of more robust detection methodologies, like automated recording devices (ARDs) (MacLaren 2015). While ARDs are more effective in species detection (Heyer et al. 1994; Clark et al. 2010; MacLaren 2015; Crump and Houlahan 2016), and pose no risk of generating false negative detections (MacLaren 2015; Crump and Houlahan 2016), they are only capable of detecting chorusing individuals (MacLaren 2015; Crump and Houlahan 2016). Therefore, ARDs are still susceptible to false negative detections of the other primary life stages of seasonally present pond-breeding amphibians, including tadpoles, juvenile metamorphs, females, and non-chorusing adult males. These possible false negatives could potentially skew evaluations of habitat use, and selection preference of currently occupied geographic range. So, evaluations of additional survey techniques are needed to supplement call surveys, and ARDs, to reduce false negative detections as well as identify non-chorusing (eggs, tadpoles, juvenile metamorphs, females, and some males) individuals.

The implementation of molecular techniques, such as surveying for environmental DNA (eDNA), offer the ability to detect a broader range of amphibian life stages. This survey methodology seeks to assess presence of a target taxon by detecting DNA that is released from an organism into the environment through multiple means including shed skin, mucus, hair follicles, fecal matter, urine, sexual byproducts, or even decaying carcasses (Pilliod et al. 2013). The monitoring of eDNA has quickly gained popularity as

a relatively inexpensive, and reliable tool for cataloging biological inventories of aquatic systems, and determining occupancy of rare, and/or threatened species in near real-time (Taberlet et al. 2012; Rees et al. 2014; Goldberg et al. 2015; Goldberg et al. 2016). Unlike traditional anuran call surveys that require many nights of call surveys to determine presence or absence, and ARDs that are expensive to purchase, and monitor, the focus on eDNA for detection may only require a single trip to a given field site in order to obtain water samples for eDNA analysis (Rees et al. 2014).

Further, while implemented in a wide breadth of taxa (Ficetola et al. 2008; Dejean et al. 2011; Goldberg et al. 2011; Jerde et al. 2011; Dejean et al. 2012; Takahara et al. 2012; Mahon et al. 2013; Takahara et al. 2013; Pilliod et al. 2013a; Pilliod et al. 2013b; Piaggio et al. 2014; Biggs et al. 2015), eDNA has been proven successful in the detection of various amphibians (Goldberg et al. 2011; Dejean et al. 2012; Thomsen & Willerslev 2015). Studies of eDNA have tested samples of as little as 15 mL of water, and successfully detected the presence of the common Spadefoot toad (*Pelobates fuscus*) 91 – 100% of the time in verified positive bodies of water (Thomsen et al. 2012). The protocol proved equally reliable in accurately detecting the target species with a larger sample of water (Pilliod et al., 2013a). When a 1L sample was taken, the reported detection rate was over 90% for the Idaho Giant Salamander (*Dicamptodon aterrimus*), with the added benefit of demonstrating no false positives from unoccupied streams (Pilliod et al. 2013). Other studies show eDNA analyses to be more effective than traditional call surveys, detecting the American bullfrog (*Rana* [=*Lithobates*] *catesbeianus*) in 63% more of the surveyed ponds (Dejean et al. 2012).

Despite increasing instances of its use, relatively few studies have evaluated this

method for the detection of seasonally present, pond-breeding amphibians (Thomesen et al. 2012; Lodge et al. 2012; Rees et al. 2014; Biggs et al. 2015). This class of amphibians pose an interesting challenge for the implementation of eDNA analyses to reliably detect taxa that are not habitually present, or do not utilize a body of water for an extended period of time. The Houston toad itself offers an interesting case study. Primarily living upland of perennial ponds in burrows (Brown & Thomas 1982; Campbell 2003), the Houston toad, emerges sporadically throughout the breeding season (February – June) (Campbell 2003) and moves to the edge of nearby ponds to chorus. If successful, the female Houston toad will lay an egg strand which will develop into tadpoles within seven days (Hillis et al. 1984) before metamorphed juveniles begin emerging from the pond between 53 – 65 days later depending on environmental conditions (Hillis et al. 1984). This narrow range in which the Houston toad is regularly present in a fixed water body, combined with the quick developmental rate to terrestrial juveniles make this species an ideal proxy to evaluate the utility of using eDNA amplifications to detect other seasonally present, pond-breeding amphibians.

While the USFWS still recommends manual call surveys for the Houston toad, recent research has pointed to glaring problems with its approach (MacLaren 2015). Despite the implementation of ARDs in monitoring efforts, further evaluations of more robust alternative survey methodologies are still essential in the stewardship of seasonally present amphibians. The utility of eDNA analyses as a survey methodology to detect a target species without direct observation, identify isolated populations, and thus better define suitable habitat for the individual while simultaneously reducing the per person effort required to survey makes eDNA analysis an appealing alternative for monitoring

rare, and/or elusive taxa.

This study seeks to provide a vigorous evaluation of the efficacy, and sensitivity of eDNA monitoring for seasonally present, pond-breeding amphibians, using targeted, polymerase chain reaction (PCR)-based DNA analyses. I will also directly compare the use of eDNA monitoring to comparative monitoring strategies (ARDs and manual call surveys) that were conducted throughout the same time period, allowing us to subsequently evaluate an approved USGS protocol. Further this study is distinct in that it will routinely sample ponds known to be present for Houston toad activity, documented through detection of chorusing activity, as well as egg strands and tadpoles released at these sites as part of the head-starting program run in conjunction with the Houston Zoo. Finally, I also evaluate the possible relationship(s) of water quality parameters, including temperature, pH, dissolved oxygen (DO), turbidity, and conductivity, as well as time before sampling of known positive controls, and successful amplification of eDNA.

METHODOLOGY

Study Area. — I conducted eDNA surveys on the Griffith League Ranch (GLR), a primary recovery site for the Houston toad in Bastrop county, Texas (Fig. 2). The GLR is also the primary release site for the Houston Zoo's captive release program for the Houston toad. Each year, approximately 300,000 – 600,000 Houston toad eggs, and 15,000 tadpoles are released in select ponds (i.e., ponds 2, 5, and 12) on the GLR weekly throughout the breeding season (February – June). Historic Houston toad occurrence, and distribution at this location is well monitored through call surveys done exhaustively throughout the range by Jim Yantis, and collaborators (Yantis & Price 1989; 1990; 1991;

1992; 1993). Houston toad surveys on the GLR have been completed annually for the past 16 years, as well as four years with ARDs, including egg strand sampling, metamorph emergence analyses, and pitfall trap captures with associated drift fence data.

I collected water samples for eDNA analyses at nine ponds on the GLR (Fig. 2) in a stratified sampling design that included two Houston toad occupancy categories: 1) known occupied ponds, and 2) ponds with an unknown occupancy status. Known positive ponds (i.e., ponds 2, 5, and 12) had verified chorusing activity, and captured individuals during the course of the sampling timeframe, in addition to serving as egg strand, and tadpole release sites for the Houston Zoo's captive head-starting program. The remaining surveyed ponds (i.e., ponds 1, 9, 10, 11, 15, and 16) represent locations with an unknown occupancy status. Previous survey years have had observations of chorusing activity at these localities (Jones 2006; Duarte et al. 2012), however no chorusing activity, or individuals, were observed utilizing these ponds during this study. The determination of pond occupancy was completed through both manual call surveys conducted according to the USFWS protocol (U.S. Fish & Wildlife 1984), with supplemented ARD surveys (120 – 708 minutes of recording per night during the breeding season) conducted over the past four breeding seasons.

Sample Collection. — I collected two distinct classes of water samples to evaluate the efficacy of monitoring for the Houston toad via detection of eDNA, including: 1) known positive controls, and 2) samples collected from ponds with an unknown occupancy status (i.e., ponds 1, 9, 10, 11, 15, and 16) (Table 1) (Goldberg et al. 2011).

The known positive controls fall into two categories: 1) known positive pond

controls, and 2) known positive captive controls. The known positive ponds controls represent samples collected from ponds 2, 5, and 12, in which Houston toad chorusing activity was detected by our comparative monitoring strategies, and/or had egg strands, and tadpoles that were released as part of the head-starting program conducted on the property. While the known positive captive controls represent evaluations of eDNA detection viability in each of the three primary life stages of this amphibian (egg strands, tadpoles, and adults). Captive born egg strands, and tadpoles were sampled on the GLR weekly during their release throughout the breeding season (late January to early May) (Hillis et al. 1984; Swannack 2007). These samples were transported to the site in 15L buckets containing approximately 9 – 14L of water with 1,000 or more eggs or tadpoles. A 1L sample was collected from each bucket that was released on the GLR. I collected known positive controls for the adult life stage by sampling 1L of standing water from aquariums containing 3 – 4 adult Houston toads from the Houston Zoo's captive insurance colony.

I surveyed the nine ponds on the GLR weekly from February – June of 2016 in order to bracket peak breeding activity. I collected a total of 340 water samples during this period with 132 of those samples collected from known positive pond controls (ponds 2, 5, and 12) (Table 1). Houston toad chorusing activity was verified at these ponds during the entire course of the monitoring via call surveys, ARDs, and the release of captive bred egg strands, and tadpoles from March to April (Table 1). The collected water samples (n = 557) were evaluated using a standard USGS method approved for eDNA isolation with 0.45 μm cellulose nitrate filter paper (Goldberg et al. 2011). I collected 1L water samples for both the stratified ponds, and known positive controls

using separate, sterile, autoclaved 1L Pyrex™ bottles to ensure no cross contamination. I used a negative control, 1L of distilled water, to evaluate potential contamination issues (Pilliod et al. 2013; Goldberg et al. 2016). The water samples were vacuum pumped through the cellulose nitrate filter paper, according to USGS specifications for collecting eDNA, immediately after collection (Goldberg et al. 2011). A vacuum pump was used instead of a peristaltic pump in order to reduce the risk of cross contamination among the replicates, with sterile funnels, and filters being exchanged between each sample (Fig. 3) (Gregorski, 2003). I stored each filter in a Nunc™ cryogenic tube with 95% ethanol on ice in the field, before they were stored at, or below, -20°C in the lab until analysis (Thomsen et al. 2012). I conducted a comparative evaluation of eDNA collection techniques in which a subset of the samples (n = 31), including samples of ponds with unknown occupancy, along with known positive pond, and captive controls, were preserved in a 50:50 solution with 99% isopropyl alcohol, and subsequently spun down in a centrifuge to form a pellet of DNA, and cell-tissue debris (Edwards et al. 1991; Lever et al. 2015). Water chemistry parameters including temperature, pH, DO, turbidity, and conductivity were collected for each sample using a Eureka Manta Model 3.5 water probe with an Amphibian2 handheld device.

DNA Extraction. — Samples were dried overnight to remove excess ethanol (Goldberg et al. 2011). I compared the efficiency of Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA), and Prepman™ Ultra DNA extraction (Thermo-Fisher Scientific) protocols on a subset of the filters (n = 30). I extracted the remaining samples with a Prepman™ Ultra DNA extraction (Thermo-Fisher Scientific) protocol, diluting all

samples 10-fold before conducting genetic analyses. A subset of samples were suspended using a 50:50 solution of isopropanol (n = 31), and subsequently extracted using a Fisher BioReagents™ SurePrep™ Soil DNA isolation kit as per the manufacturer's protocol.

Genetic Analyses. — To evaluate PCR inhibition of eDNA amplification a subset (n = 30) samples were cleaned using phenol-chloroform precipitation (Sambrook and Russell 2006), Wizard SV gel and PCR clean-up system (Promega), and Zymoclean™ gel DNA recovery Kit (Zymo Research).

In order to determine if the final extracted DNA samples could be positively amplified if significant concentrations of target DNA were present in the final sample, 3µL of 1,967 ng / µL *Salmonella enterica* DNA was diluted 10-fold, and then added to 97 µL of a subset of water samples (n = 30) and then amplified using the primers 139F (5' – GTGAAATTATCGCCACGTTCGGGCAA – 3'), and 141R (5' – TCATCGCACCGTCAAAGGAACC – 3') (Rahn et al. 1992) in reactions with Go-Green *Taq* mix (Promega). Thermal cycling for all reactions included an initial denaturing period at 95 ºC for 5 minutes followed by 40 cycles consisting of a denaturing period of 30 seconds at 95 ºC, an annealing step at 60 ºC for 1 minute, and extension at 72 ºC for 1 minute. Following these cycles, a final extension period was run at 72 ºC for 5 minutes before the reactions were held to 4 ºC. Successful amplifications were determined based on fluorescence of the sample in a 2% gel electrophoresis under an Enduro gel documentation system (GDS). The spiked samples represented two distinct categories: known positive controls (i.e., ponds 2, 5, and 12, as well as positive captive controls of egg strands, tadpoles, and adults), as well as samples collected from unknown occupied

pond (i.e., ponds 1, 9, 10, 11, 15, and 16.)

I evaluated samples for eDNA detection of the Houston toad with both PCR, and nested PCR assays, with each reaction run in triplicate. Occurrence of Houston toad eDNA in a sample was verified via amplification of a diagnostic 533 base pair (bp) fragment of the control region (D-loop) of the mitochondrial genome (mtDNA) using the primers BHDL1 (5' – TGCATATCATCACCAATCC – 3'), and BUFOR1 (5' – CTGAGGCCGCTTTAAGGTACGATAG – 3') (McHenry and Forstner 2009) in reactions with Go-Green *Taq* Mix (Promega). A sample was deemed positive for Houston toad eDNA based on successful amplification of the diagnostic fragment in at least one of the three replicates. Positive (DNA extracted from a toe clip, or blood sample taken from a Houston toad), and negative (distilled water) controls were used to assess any potential contamination issues in the reactions.

I ran two amplifications for the nested PCR assays, and again ran each reaction in triplicate. The first step amplified an approximately 1,100 bp fragment of the mtDNA genome that spans the diagnostic control region using the primers BUFOF1 (5' – TCTTACTTTGCTATTTTCATTATTTTTATYCC – 3'), and BUFOR1 (5' – CTGAGGCCGCTTTAAGGTACGATAG – 3') (McHenry and Forstner 2009) in reactions with Go-Green *Taq* Mix (Promega). The second reaction amplified the 533 bp diagnostic fragment of the D-loop using the primers BHDL1 (5' – TGCATATCATCACCAATCC – 3'), and BUFOR1 (5' – CTGAGGCCGCTTTAAGGTACGATAG – 3') (McHenry and Forstner 2009) in reactions with Go-Green *Taq* Mix (Promega). Again, sample was deemed positive for Houston toad eDNA based on successful amplification of the diagnostic fragment in at

least one of the three replicates. Positive (DNA extracted from a toe clip, or blood sample taken from a Houston toad), and negative (distilled water) controls were used to assess any potential contamination issues in the reactions.

Thermal cycling for all reactions included an initial denaturing period at 95ºC for 5 minutes followed by 40 cycles consisting of a denaturing period of 30 seconds at 95ºC, an annealing step at 55 ºC for 1 minute, and extension at 72 ºC for 1 minute. Following these cycles, a final extension period was run at 72 ºC for 5 minutes before the reactions were held to 4 ºC. Successful amplifications were determined based on fluorescence of the sample in a 2% gel electrophoresis under an Enduro GDS. PCR products were purified with an ExoSAP-IT™ PCR product cleanup reagent (Applied Biosystems), and then cycle sequenced with the above primers using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer's instructions. Thermal cycling was 30 cycles of 96 ºC for 20 seconds, 50 ºC for 20 seconds, and 60 ºC for 4 minutes. Cycle sequencing products were cleaned using a Centri-Sep spin column procedure (Princeton Separations) following protocol. All positive amplifications were verified using nucleotide sequencing with an ABI 3500 XL genetic analyzer, before being edited, and aligned using Geneious v6.0.1 software (Kearse et al. 2012).

Water Chemistry Data Analyses. — I ran two, separate generalized linear models (GLM) with a binomial distribution family of known positive captive controls, and known positive ponds (i.e., 2, 5, and 12) to analyze possible relationships among the collected water chemistry parameters, temporal range a known positive control was present in a sampled water body and successful eDNA detections. The analyses were

performed using Program R v3.2.2 (R Core Team 2016), and the package "glmmADMB" (Skaug et al. 2015). Significance of the fixed effects were tested using the Wald Z-test (Bolker et al. 2008).

RESULTS

DNA Extraction. — I evaluated the efficacy of the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA), and Prepman™ Ultra DNA extraction (Thermo-Fisher Scientific) to extract eDNA on a subset of samples (n = 30). Using Qubit Fluorometric Quantification no observable difference was detected between the extraction protocols (Table 2). Further, of the samples suspended using a 50:50 solution of isopropanol (n = 31), and extracted with a Fisher BioReagents™ SurePrep™ Soil DNA isolation kit as per the manufacturer's protocol, none were found to have a quantifiable concentration of DNA using Qubit Fluorometric Quantification (dsDNA high sensitivity assay) (<0.50 ng/μL) (Table 2).

In addition to evaluating the extraction efficiency of two extraction protocols, I also tested whether a 10-fold dilution of the DNA extracted via the Prepman Ultra protocol, interfered with detection of Houston toad eDNA in the sample by diluting the copy numbers of the targeted amplicon. A side by side comparison of undiluted samples, and those run at a 10-fold dilution showed no change in my ability to detect amplification. Therefore, all remaining samples were diluted 10-fold before final genetic analyses.

PCR. — Of the known positive captive controls, the diagnostic fragment successfully amplified in 84% of the sampled egg strands (n = 155), 100% of the sampled tadpoles (n = 3), and 63% of the samples collected from the adult life stage captive control (n = 19). A total of 1.1% (n = 4) of the 340 samples collected from the nine GLR ponds showed successful amplification, with none of the 132 samples from known positive pond controls (i.e., ponds 2, 5, and 12) amplifying. Further these ponds failed to show successful amplification despite verified occurrences of chorusing, detected via call surveys, and ARDs, as well as observations of egg strands, and tadpoles from release efforts, and eventual metamorph emergence, and dispersal. From the ponds with unknown occupancy on the GLR, 2.86% of the samples collected from both ponds 10 (n = 1), and 16 (n = 1) showed successful amplification, as well as 5.13% of the samples from pond 1 (n = 2) (Fig. 4, Fig. 5). As these samples of unknowns appeared to contain an amplicon approximating the diagnostic fragment observed across the positive controls, they were sequenced to ensure their genetic identity matched that of a Houston toad (Fig. 6). DNA concentration of samples ranged from too low to detect (<0.50 ng/µL) to 1.64 ng/µL (Table 2). Samples spiked with *S. enterica* (n = 30) all showed positive amplification of the diagnostic fragment, suggesting PCR amplification of the sample was possible.

Nested PCR. — Using nested PCR, the diagnostic fragment successfully amplified in 87% of the known positive captive control egg strand samples (n = 160), while no change was observed in detection of the tadpole life stage (n = 3), and 77% of the adult positive control water samples (n = 23) showed positive amplifications. Of the 340

samples collected from the ponds, 7.4% (n = 25) showed amplification of the diagnostic fragment, while 14% (n = 19) of our samples collected from known positive control ponds showed amplification.

Single detections were observed once again at both ponds 10, and 16, as well as 10.26% of the samples collected from pond 1 (n = 4) (Fig. 7). These ponds were not known to be currently active via ARD or call surveys conducted throughout the season (Fig. 7). Samples from known positively occupied ponds (i.e., ponds 2, 5, and12) showed successful amplification of the diagnostic fragment in 2.44% of the samples collected from pond 2 (n = 1), 21.95% of samples collected from pond 5 (n = 9), and 21.95% of the samples from pond 12 (n = 9) (Fig. 7, Fig. 8). As these samples of unknowns appeared to contain an amplicon approximating the diagnostic fragment observed across the positive controls, they were sequenced to ensure their genetic identity matched that of a Houston toad (Fig. 6). The DNA concentration of samples ranged from too low to detect (<0.50 ng/µL) to 1.64 ng/µL (Fig. 5). Of the samples spiked with *S. enterica* (n = 30), all showed positive amplification of the diagnostic fragment, suggesting PCR amplification of the sample was possible.

Water Chemistry, and Degradation Rate Analyses. — The GLM conducted on all known positive captive controls suggested that there were no significant predictors of eDNA amplification. Despite known positive controls persisting in the sampled body of water between 204 – 4320 minutes, no correlation was observed with successful detection.

However, significant predictors of eDNA amplification were observed among the

known positive control ponds (i.e., ponds 2, 5, and 12). The results of the logistic regression suggested that an inverse relationship exists between successful eDNA amplification, and pH (β = -1.376606, SE = 0.463797, Z = -2.968, p = 0.00300). I observed a positive relationship among successful eDNA amplification, and dissolved oxygen (β = 0.960577, SE = 0.319807, Z = 3.004, p = 0.00267), turbidity (β = 0.014818, SE = 0.005815, Z = 2.549, p = 0.01081), and conductivity (β = 0.032722, SE = 0.006309, Z = 5.187, p = 2.147e-07).

DISCUSSION

I evaluated the efficacy of the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA), and Prepman™ Ultra DNA extraction (Thermo-Fisher Scientific) to extract eDNA on a subset of samples (n = 30). Using Qubit Fluorometric Quantification no observable difference was detected between the extraction protocols, consistent with previous evaluations (Goldberg et al. 2011) (Table 2). Therefore, I extracted all remaining samples with a Prepman™ Ultra DNA protocol (Thermo-Fisher Scientific). While no observable difference was seen between the methodologies evaluating the extraction of eDNA from filter paper, none of the samples collected using isopropanol suspension showed detectable concentrations of DNA (<0.50 ng/μL) via Qubit Fluorometric Quantification. Further none of these samples were able to successfully amplify the diagnostic fragment, despite amplifying all samples spiked with *S. enterica*. This suggests that isopropanol suspension techniques may be less effective in the detection of seasonally present, pond-breeding amphibians compared to filtering based methodologies. As all samples spiked with *Salmonella enterica* showed positive

amplification, I concluded that the failure of eDNA was most likely due to low DNA concentrations, and not PCR inhibition (McKee et al. 2015).

The PCR results show detections in three ponds in which Houston toads were not detected using our comparative monitoring strategies (ponds 1, 10, and 16), while simultaneously failing to detect Houston toad presence in known occupied ponds (ponds 2, 5, and 12). These false negative detections represent a critical failure of the monitoring strategy to detect the Houston toad, despite verified detection of the taxon via both comparative monitoring strategies, as well as observing egg strands, and tadpoles being released at these ponds. Further this monitoring strategy failed to reliably amplify eDNA from samples of known positive captive controls of the egg strand, and adult life stages. However, the amplifications at ponds in which our comparative monitoring strategies (ARDs and manual call surveys) failed to detect the Houston toad represent could represent a variety of outcomes including: a false positive detection due to laboratory contamination, amplification of a hybridized individual (Hillis et al. 1984; Vogel & Johnson 2008), or the detection of a non-chorusing Houston toad that our comparative monitoring strategies would be unable to detect, with the latter proving an intriguing possibility.

Nested PCR assays were used to increase the copy number of the diagnostic mtDNA fragment in the samples. Of the 340 samples collected across the nine ponds on the GLR, 7.4% showed positive amplification of the diagnostic Houston toad mtDNA fragment. This translates to the amplification of Houston toad eDNA in 21 more samples, then traditional PCR assays. Further the use of this assay led to the detection of Houston toad eDNA in 14% of the samples collected from known positive pond controls (n = 19).

In addition to increasing the ability to detect Houston toad eDNA in the wild, the use of this assay increased the detection probability of the known captive positive controls, successfully amplifying in 5 more samples of the egg strand life stage control, and 4 more of the samples of the adult life stage. Despite increased detection probabilities, both assays failed to reliably detect both known positive controls, and known occupied ponds, raising questions about the efficacy, and sensitivity of eDNA for this taxon. Further we are still unable to ascertain if the amplifications seen in samples from ponds 1, 10, and 16 represent true, or false positive detections.

I observed stark differences in the detection capability of eDNA between known positive captive controls, and known positive pond controls. As egg strands, tadpoles, juveniles, and adult Houston toads were observed at these known positive ponds throughout the course of the study, the detection probabilities should be more consistent. Variation of the detection probability could stem from the influence of water quality parameters affecting amplification, the use of a large, mtDNA diagnostic fragment, or PCR assay sensitivity.

Water quality parameters can impact eDNA detection by increasing the degradation rate (Pilliod et al. 2013b; Goldberg et al. 2016), and/or interfering with laboratory protocol in amplification (Liang 2013; Barnes et al. 2014). My evaluation of this possible relationship focused on the covariates of temperature, pH, DO, conductivity, and turbidity. I observed an inverse relationship between eDNA detection, and pH, which is contradictory to predicted expectations. Publications have shown that eDNA is highly degraded in environments with factors favorable to microbial growth (neutral pH) (Ageno et al. 1969; Strickler et al. 2014). My study shows positive detections throughout

a pH gradient of 5.43 – 9.14; possibly due to the high concentration of the target taxon in the sampled area. eDNA decay beyond detectable limits is known to be dependent on concentration, and environmental conditions, including DO (Weltz et al. 2017). Therefore, the positive relationship among this covariate, and successful detection is expected. Increased turbidity could potentially represent higher levels of silt, and other organic compounds, like tannic acids, which are known PCR inhibitors (Schrader et al. 2012), but could potentially help reduce direct exposure of eDNA to ultraviolet light (UV), and subsequent degradation (Pilliod et al. 2013). A higher conductivity is correlated to a higher concentration of salts, which are capable of conducting an electrical current that can inhibit DNA extraction by precipitating DNA from solution, and thus hinder amplification (Weyant et al. 1990; Schrader et al. 2012). However, it should be noted that the highest conductivity level observed among these samples, 203.4 μS/cm, is unlikely to have a significant impact on DNA extraction. These relationships play a significant role in overall detection capability of eDNA, and remain an understudied area of this survey methodology (Eichmiller et al. 2016). Despite the verified observations of the minimum time of amplexus observed resulting in an egg strand being laid in a pond being 180 minutes, the temporal range analyses of our known positive controls saw no correlation between time of the amphibian life stage in the sampled water, and positive eDNA detection.

The use of a large, mtDNA diagnostic fragment may also have hindered eDNA detection in known occupied pond samples. Two threats to the Houston toad are widespread hybridization (Blair 1963; Brown 1971; Hillis et al. 1984; Pauly et al. 2004; McHenry 2010), and genetic introgression observed throughout Bufonidae (Vogel &

Johnson 2008). The transmission processes of eDNA to the environment highlight the fact that it will likely be degraded upon collection. Therefore, the use of a larger fragment (greater than 250 bp) may prove less than ideal for accurate eDNA detection. In addition to the size of the fragment, the use of mtDNA is less than ideal in highly hybridized species. As mtDNA is maternally inherited, it could potentially detect all individuals descending from a Houston toad maternal lineage, and not only pure breeding individuals (Vogel & Johnson 2008; McHenry 2010). This could generate false positive detections.

Disparities in detection probabilities could also potentially be explained by the choice of PCR assay used. While some have argued that traditional PCR assays are adequate for eDNA detection (Nathan et al. 2014), the use of more sensitive detection methodologies, such as quantitative real-time PCR (qPCR), and digital PCR (dPCR), have become the standard in eDNA studies due to their restriction to the detection of smaller amplicons (less than 150 bp), and the fact that these assays are more tolerant to environmental inhibitors in a final sample (Thomsen et al. 2012; Treguier et al. 2014; Doi et al. 2015; Goldberg et al. 2015; McKee et al. 2015; Spear et al. 2015; Piggiott et al. 2016). With most studies in 2017 utilizing qPCR for eDNA detection (Andruszkiewicz et al. 2017; Erickson et al. 2017; Gingera et al. 2017; Gomes et al. 2017; Klobucar et al. 2017; Niemiller et al. 2017; Smith 2017; Song 2017), and only a few evaluating dPCR (Evans et al. 2017; Hunter et al. 2017; Shogren et al. 2017; Song 2017). However, as a species-specific primer to identify Houston toads has yet to be developed for use in qPCR or dPCR, I had to evaluate eDNA using traditional PCR techniques. This could potentially explain the high number of false negative detections in known occupied ponds.

While many problems have been discussed about the implementation of eDNA in detection of the Houston toad, it should be noted that these are broadly applicable considerations for implementation in all seasonally present, pond-breeding amphibians. Further, in future evaluations of eDNA for implementation in this class of amphibian, the likelihood of finding the target taxon must be considered, as detection by eDNA is directly correlated to the detection probability of the target taxon (Goldberg et al. 2016). So, eDNA surveys must be initiated during a time, and in a place, that make relevant biological sense for the target taxon (Goldberg et al. 2016). Finally, future evaluations must take capture, and DNA extraction efficiency, probability of sample interference from various inhibitors, and even assay sensitivity into account (Ficetola et al. 2008; Schmidt et al. 2013; Schultz & Lance 2015).

CONCLUSIONS

While eDNA is a rapidly growing molecular survey technique, this study demonstrates that it is an ineffective monitoring alternative for seasonally present pond-breeding amphibians, such as the Houston toad, using traditional DNA sequencing techniques and PCR assays. In addition, this study highlights concerns about the utility of this method in the evaluation of other congeneric taxa with a highly conserved genome, such as Nearctic Bufonids, and necessitates identification of a diagnostic fragment compatible with more sensitive assay, ideally for *d*PCR. Further, this study offers many critical considerations for the effective implementation survey strategies aimed at the detection of eDNA including the consideration of the detection probability of the target taxon, capture, and extraction efficiency, access to the most sensitive detection

methodologies (qPCR or dPCR) (Freeland 2016; Goldberg et al. 2016), and the probability of sample interference from various inhibitors (MacKenzie et al. 2002; Ficetola et al. 2008; Schmidt et al. 2013; Schultz & Lance 2015; Goldberg et al. 2016) to determine if eDNA is appropriate. The routine inclusion of false negative detections of samples collected from known positive pond, and captive controls, as well as the potential false positive detections observed, highlight that PCR assays are an unreliable detection methodology for eDNA monitoring. In addition, monitoring for seasonally present, pond-breeding amphibians, such as the Houston toad with the USGS protocol is ineffective, and should be re-evaluated before its continued implementation in the monitoring of elusive, and/or endangered taxa.

APPENDIX

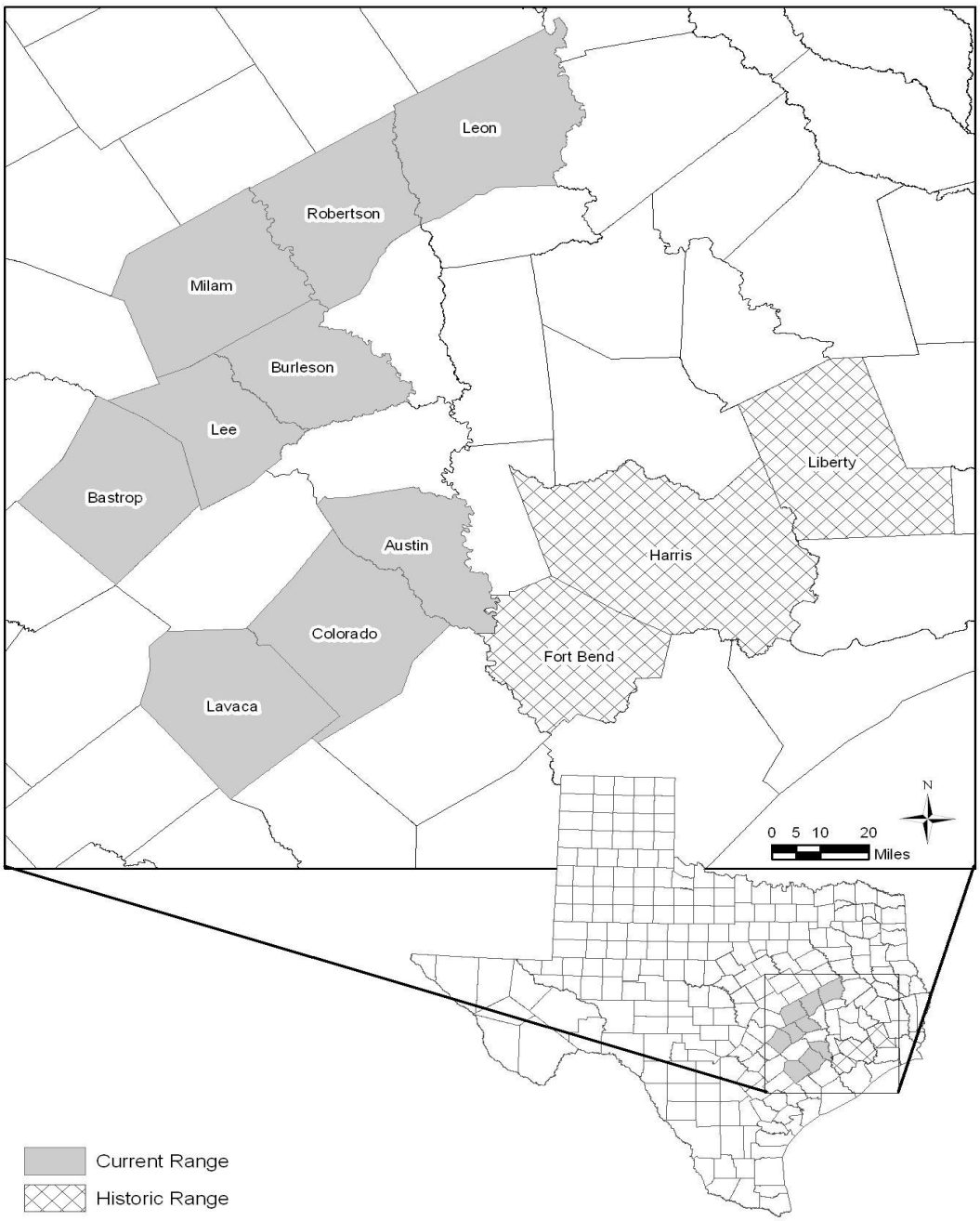


Figure 1. The historic, and current range of the Houston toad (*Bufo* [=*Anaxyrus*] *houstonensis*) in Texas. This figure is reprinted from USFWS (2011).

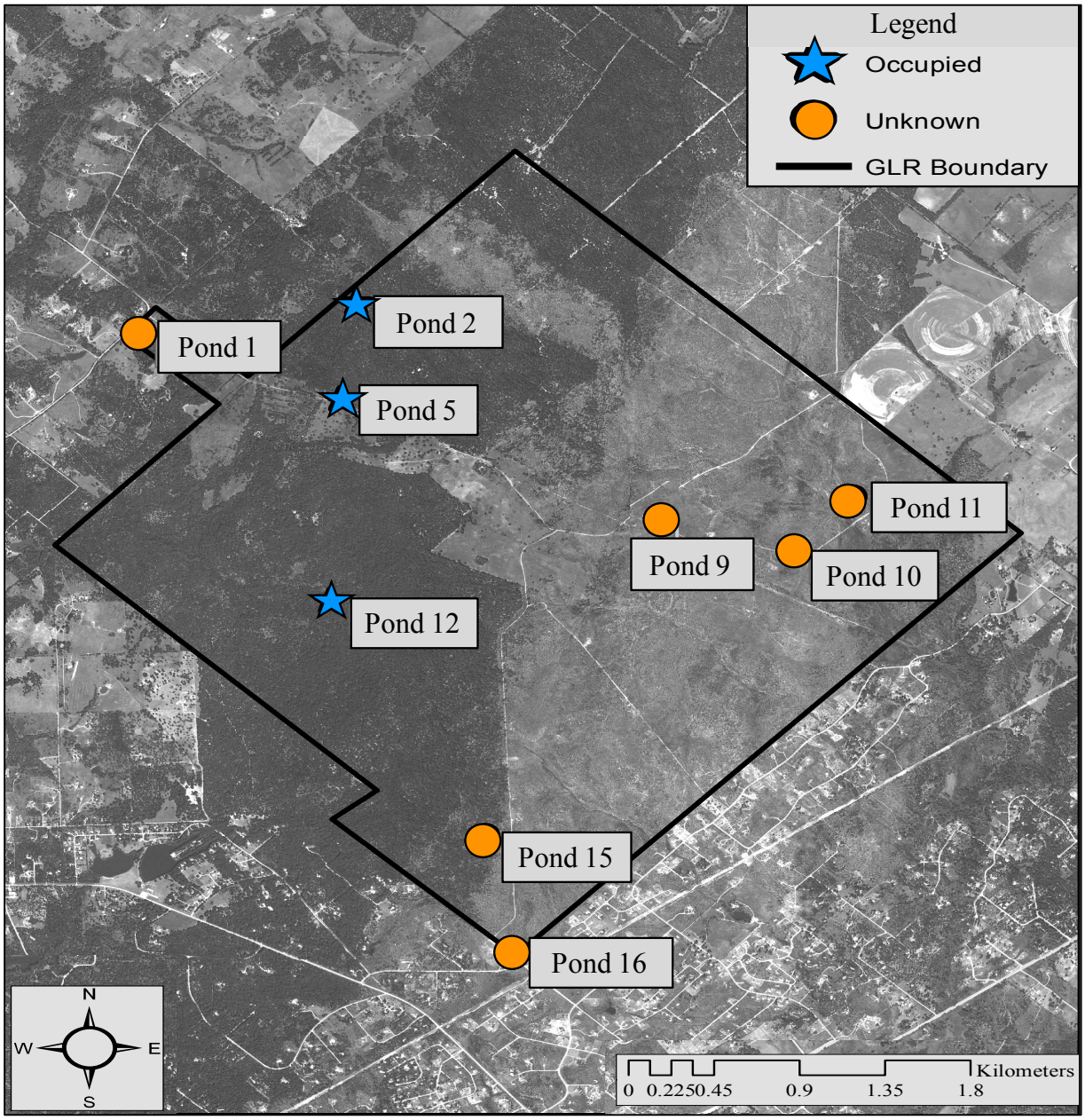
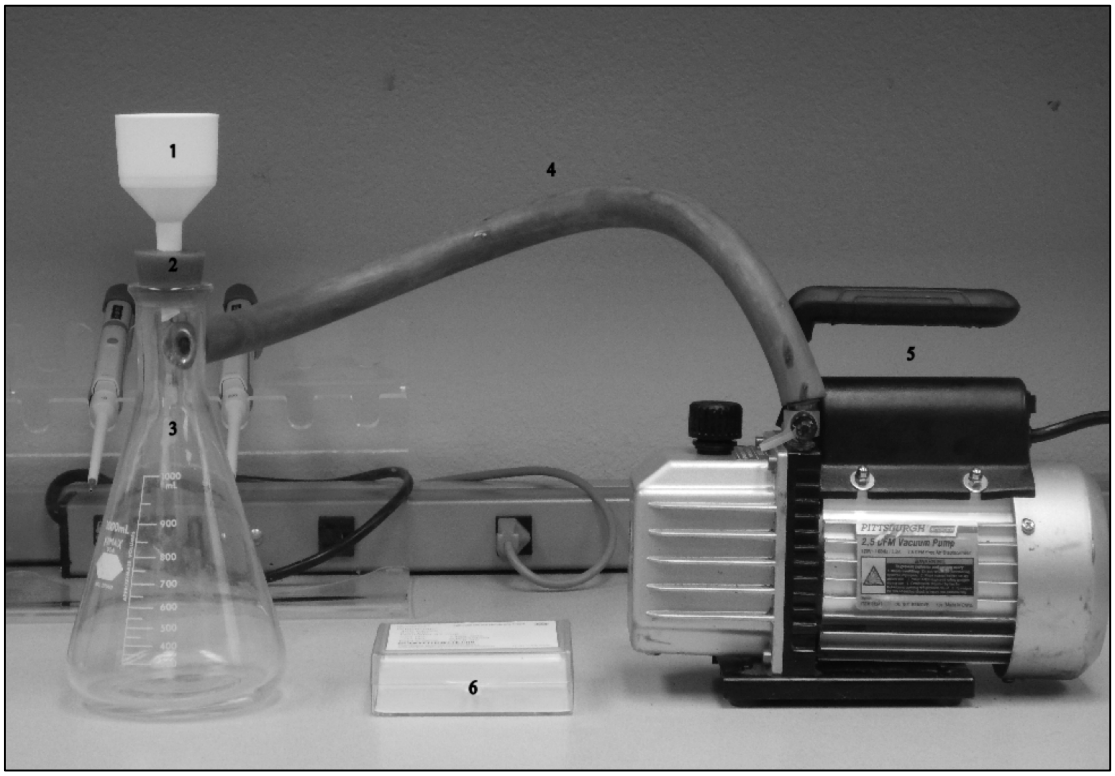


Figure 2. The Griffith League Ranch (GLR), Bastrop County, Texas, and all surveyed ponds. The GLR has been a primary recovery site of the Houston toad in Bastrop County, Texas for over 15 years. The nine surveyed ponds in this study fall into two categories: known occupied ponds (i.e., ponds 2, 5, and 12), and ponds with an unknown occupancy status (i.e., ponds 1, 9, 10, 11, 15, and 16).



1. 55 mm Buchner Funnel
2. #8 Silicone Stopper
3. Kimble KIMAX 1L Filtering Flask with Side Arm
4. Rubber Hose
5. Pittsburgh 2.5 CFM Vacuum Pump
6. Whatman 0.45µm Cellulose Nitrate Membrane Filters (47 mm)

Figure 3. Filtering apparatus used to collect samples for environmental DNA analysis. Each sample was filtered through the apparatus using a sterile, plastic Buchner funnel, and Whatman 0.45µm cellulose nitrate membrane filters to prevent cross contamination among samples. All samples were collected following a USGS approved protocol (Goldberg et al. 2011). All equipment was cleaned with a 50% by volume bleach solution between samples to prevent cross contamination (Pilliod et al. 2013).

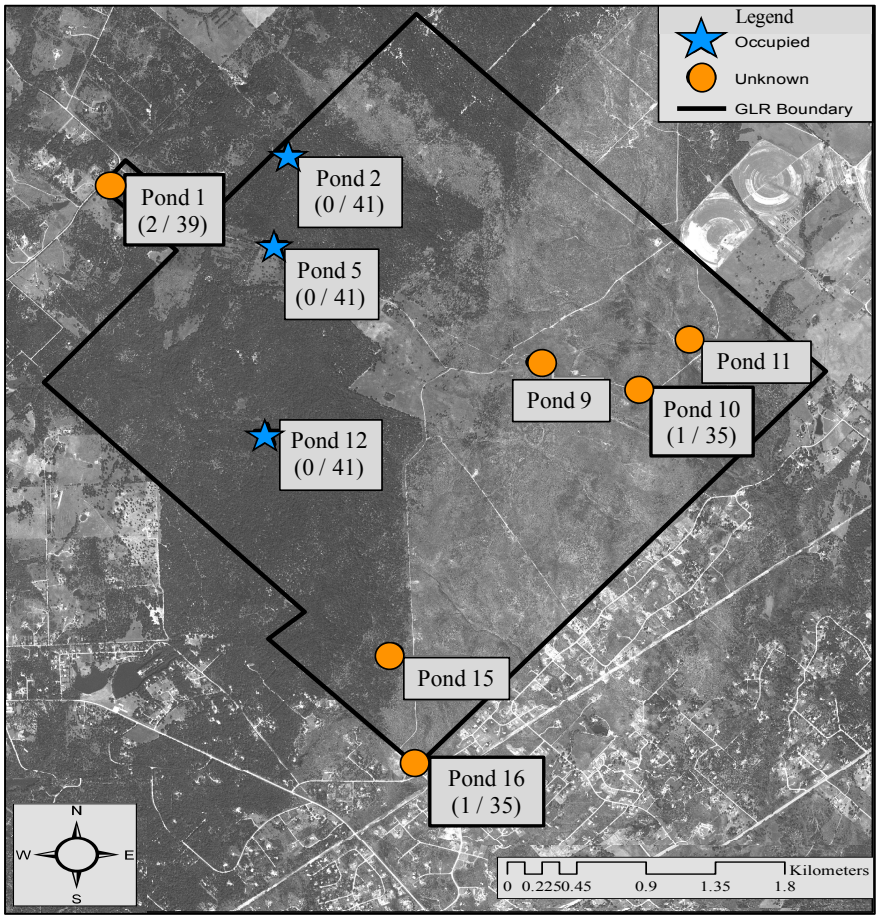


Figure 4. A map of all positive amplifications of *Bufo* [=*Anaxyrus*] *houstonensis* environmental DNA from samples of the nine surveyed ponds on the Griffith League Ranch, Bastrop County, Texas using traditional Polymerase Chain Reaction assays. Of the nine surveyed ponds, 1.1% showed positive amplifications of the diagnostic fragment. However, none of the samples from known positive control ponds (i.e. ponds 2, 5, 12) showed positive amplifications. I observed a single detection in samples from both ponds 10, and 16, as well as two separate amplifications in pond 1. These ponds had an unknown occupancy status during the course of our surveying, and were not verified to have chorusing activity of the Houston toad via our comparative monitoring strategies (ARDs and manual call surveys). The number of detections by pond are labeled.

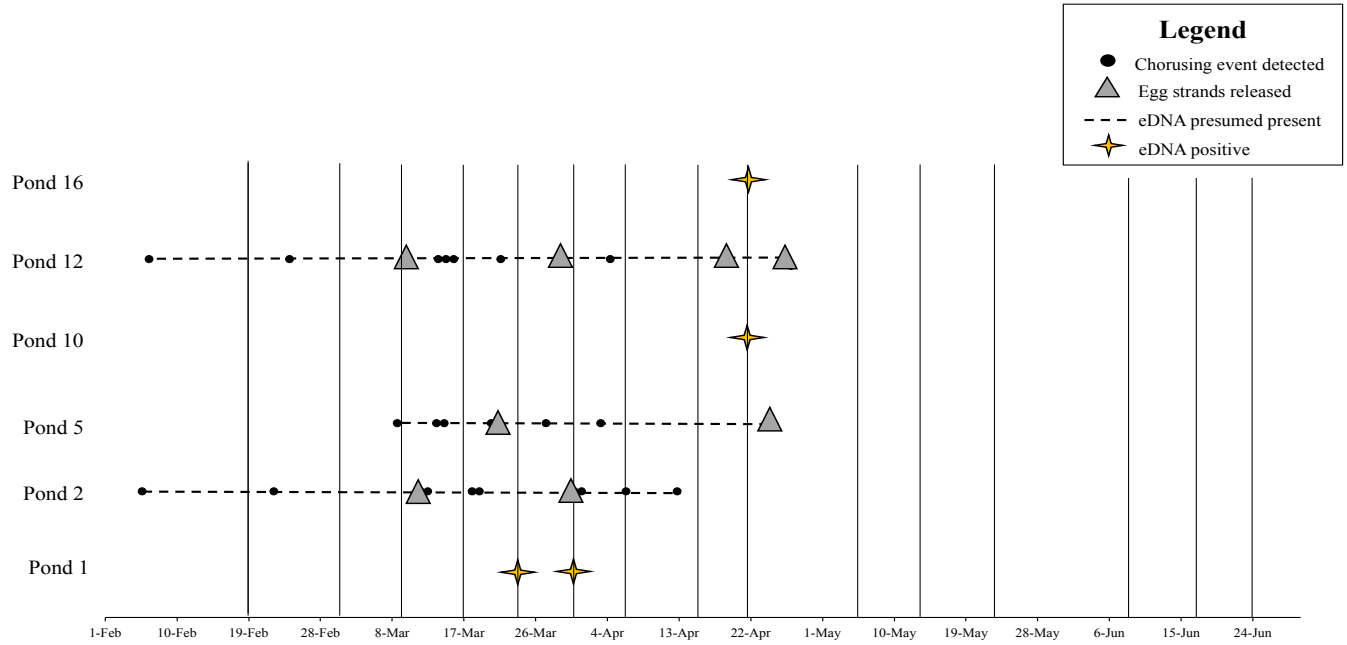


Figure 5. A time series map of chorusing events, egg strand releases, and positive amplifications of *Bufo* [=*Anaxyrus*] *houstonensis* environmental DNA from samples collected across the Griffith League Ranch, Bastrop County, Texas using traditional Polymerase Chain Reaction assays. eDNA surveys were conducted weekly across the property from 19 February – 23 June 2016, and are shown as the vertical, black bars. Despite chorusing activity detected at ponds 2, 5, and 12, as well as egg strands being released weekly at these three ponds, no detections of eDNA were observed during the course of our survey.

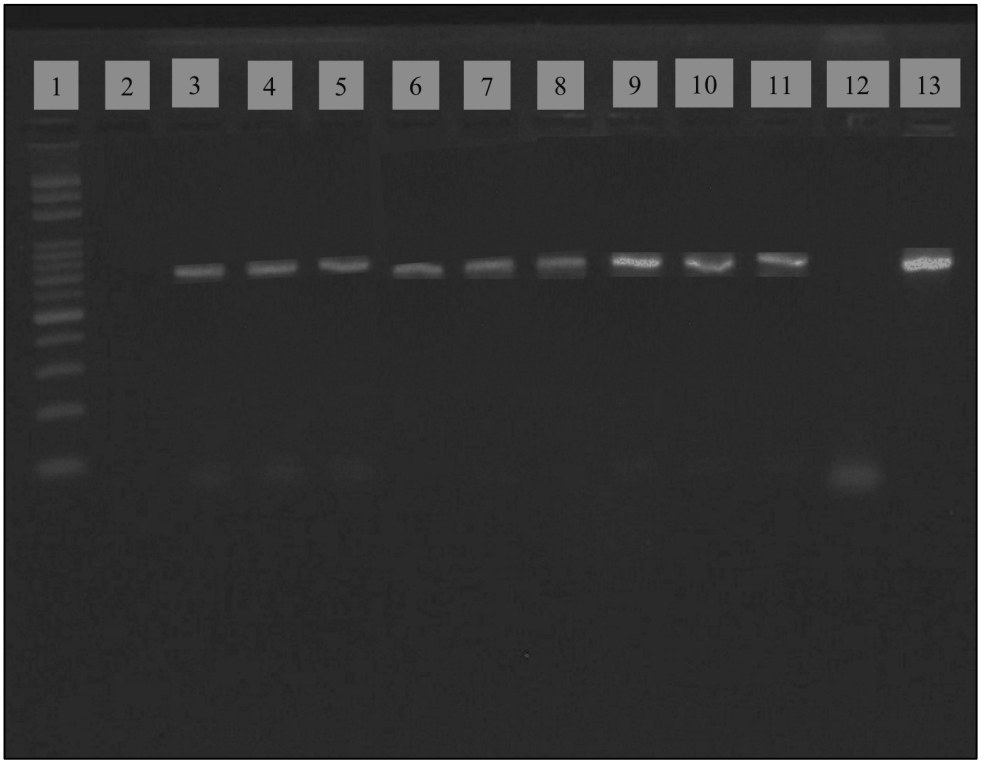


Figure 6. A gel image showing the overall amplicon length observed in nested Polymerase Chain Reactions across all types of samples. Well 1 of the gel shows a 2,000-base pair (bp) ladder with each rung demarcating 100 bp, while well 2 represents a negative control for the gel. Wells 3 – 5 show representative amplifications observed from samples of ponds with an unknown occupancy status (i.e., ponds 1, 10, and 16 respectively). While wells 6 – 8 show amplifications of the diagnostic fragment detected in known positive pond controls (i.e., ponds 2, 5, and 12 respectively). Representative amplifications of the known positive captive controls can be seen in wells 9 – 11 with each one representing a different life stage (i.e., egg strands, tadpoles, and adults respectively). Wells 12 (negative control), and 13 (positive control extracted from a toe clip of a Houston toad) were used to verify that no contamination was observed during the reaction.

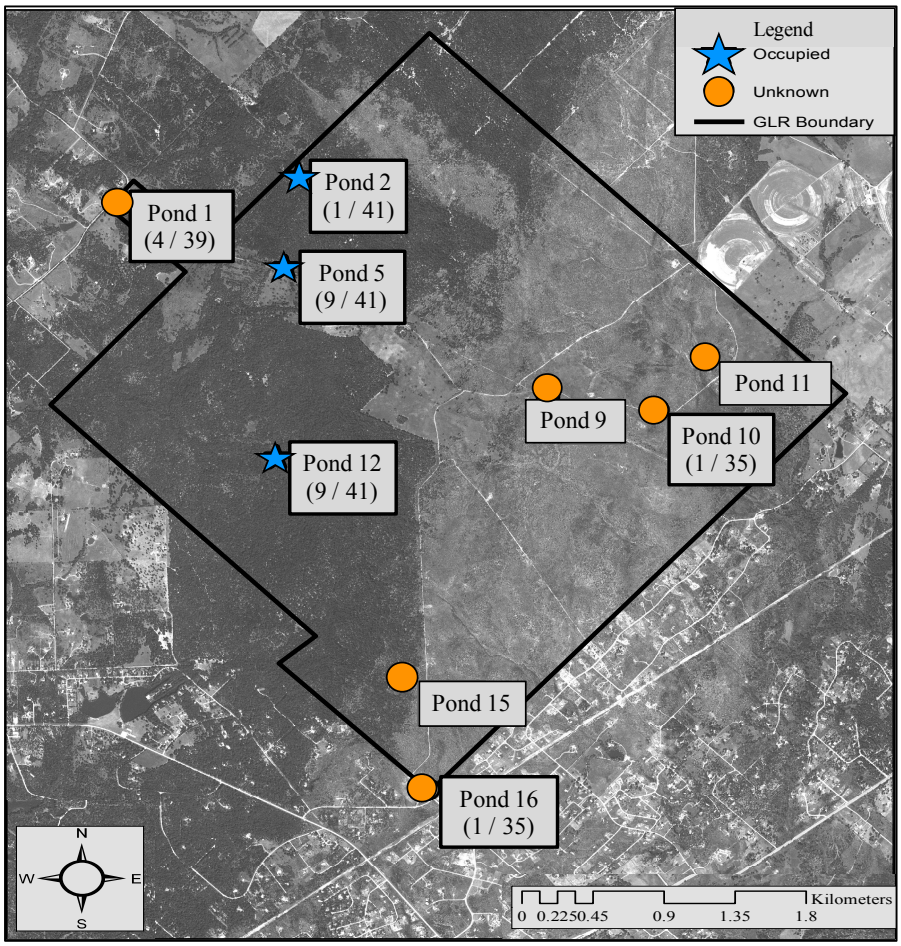


Figure 7. A map of all positive amplifications of *Bufo* [=*Anaxyrus*] *houstonensis* environmental DNA from samples of the nine surveyed ponds on the Griffith League Ranch, Bastrop County, Texas using nested Polymerase Chain Reaction assays. The use of nested PCR assays showed 21 more amplifications of Houston toad eDNA across the nine surveyed ponds compared to traditional assays. Further, 19 detections were observed in samples of known positive control ponds 2, 5, and 12. Detections were also observed in samples from ponds 1, 10, and 16. These ponds had an unknown occupancy status during the course of our survey, and were not verified to have chorusing activity of the Houston toad via our comparative monitoring strategies (ARDs and manual call surveys). The number of detections by pond are labeled.

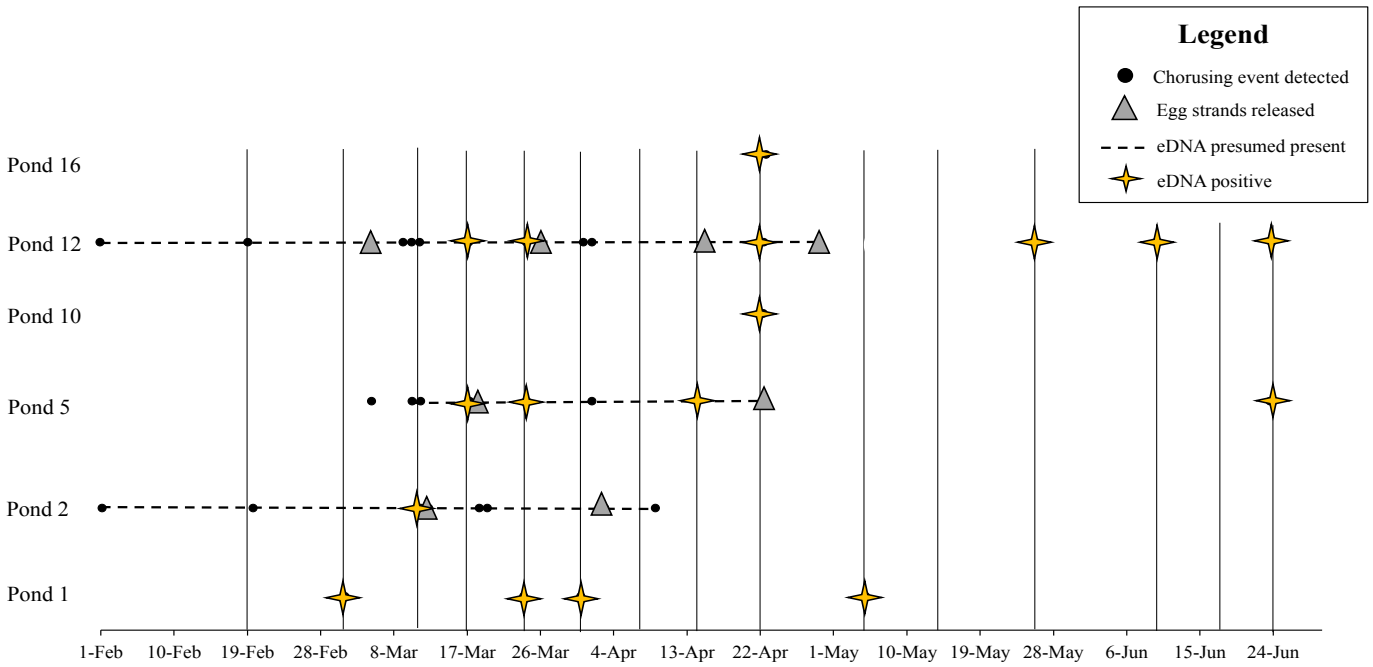


Figure 8. A time series map of chorusing events, egg strand releases, and positive amplifications of *Bufo* [=*Anaxyrus*] *houstonensis* environmental DNA from samples collected across the Griffith League Ranch, Bastrop County, Texas using nested Polymerase Chain Reaction assays. eDNA surveys were conducted weekly across the property from 19 February – 23 June 2016, shown as vertical, black bars. Despite chorusing activity detected at ponds 2, 5, and 12, as well as egg strands being released weekly at these three ponds, no detections of eDNA were observed during the course of our survey.

Table 1. The sampling strategy focused on the collection of two distinct classes of water samples, including: 1) known positive controls, and 2) unknowns, where the occupancy status was not readily known.

	Types of Samples	Number of Samples	Volume of Water Sampled	Time Series (minutes)	First Detected Chorusing Event	First Egg Strands Released
Known Positive, Captive Controls (9 – 14 L)	Egg Strands	184	1 L	204 – 387	-	-
	Tadpoles	3	1 L	250 – 355	-	-
	Adults	30	1 L	4080 – 4320	-	-
		n = 217				

	Types of Samples	Number of Samples	Volume of Water Sampled	First Detected Chorusing Event	First Egg Strands Released
Known Positive, Pond Controls	Pond 2	44	1 L	1 Feb 2016 17 Jan 2017	11 Mar 2016 23 Feb 2016
	Pond 5	44	1 L	9 Mar 2016 20 Feb 2017	18 Mar 2016 2 Mar 2017
	Pond 12	44	1 L	1 Feb 2016 21 Feb 2017	5 Mar 2016 16 Mar 2017
		n = 132			
Unknown Occupancy	Pond 1	39	1 L	12 Apr 2017	-
	Pond 9	33	1 L	-	-
	Pond 10	35	1 L	-	-
	Pond 11	33	1 L	-	-
	Pond 15	33	1 L	-	-
	Pond 16	35	1 L	-	-
		n = 208			

Table 2. A comparison of DNA extraction efficiency of the Qiagen DNeasy Blood, and Tissue Kit (Qiagen Inc., Valencia, CA) and Prepman™ Ultra DNA extraction (Thermo-Fisher Scientific) protocol.

Sample Location	DNA Concentration via Qiagen DNeasy Blood and Tissue Kit Extraction	DNA Concentration via Prepman Ultra Extraction Protocol	DNA Concentration of Samples Suspended in 50:50 solution of Isopropanol
Pond 1	< 0.50 ng/µL	< 0.50 ng/µL	< 0.50 ng/µL
Pond 2	0.68 ng/µL	0.65 ng/µL	< 0.50 ng/µL
Pond 2	0.98 ng/µL	1.04 ng/µL	< 0.50 ng/µL
Pond 5	0.77 ng/µL	0.68 ng/µL	< 0.50 ng/µL
Pond 5	< 0.50 ng/µL	< 0.50 ng/µL	< 0.50 ng/µL
Pond 9	0.83 ng/µL	0.90 ng/µL	< 0.50 ng/µL
Pond 10	< 0.50 ng/µL	< 0.50 ng/µL	< 0.50 ng/µL
Pond 11	0.88 ng/µL	0.90 ng/µL	< 0.50 ng/µL
Pond 12	1.13 ng/µL	1.64 ng/µL	< 0.50 ng/µL
Pond 12	< 0.50 ng/µL	< 0.50 ng/µL	< 0.50 ng/µL
Pond 15	0.57 ng/µL	0.61 ng/µL	< 0.50 ng/µL
Pond 16	< 0.50 ng/µL	< 0.50 ng/µL	< 0.50 ng/µL
Egg strand	0.99 ng/µL	1.03 ng/µL	< 0.50 ng/µL
Egg strand	0.57 ng/µL	0.94 ng/µL	< 0.50 ng/µL
Egg strand	0.57 ng/µL	0.76 ng/µL	< 0.50 ng/µL
Egg strand	0.66 ng/µL	0.64 ng/µL	
Egg strand	1.32 ng/µL	1.35 ng/µL	
Egg strand	< 0.50 ng/µL	< 0.50 ng/µL	
Egg strand	1.07 ng/µL	0.99 ng/µL	
Tadpole	0.56 ng/µL	0.61 ng/µL	
Tadpole	0.72 ng/µL	0.88 ng/µL	
Tadpole	< 0.50 ng/µL	< 0.50 ng/µL	
Adult	0.98 ng/µL	0.93 ng/µL	
Adult	1.01 ng/µL	1.09 ng/µL	
Adult	< 0.50 ng/µL	< 0.50 ng/µL	
Adult	< 0.50 ng/µL	< 0.50 ng/µL	
Adult	0.60 ng/µL	0.61 ng/µL	
Adult	0.77 ng/µL	0.73 ng/µL	
Adult	< 0.50 ng/µL	< 0.50 ng/µL	
Adult	0.56 ng/µL	0.58 ng/µL	

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