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Baseline Surveys and eDNA Protocol for *Eurycea* Salamanders

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Summary

The Edwards Plateau of west central Texas is home to a growing human population as well as many endemic species that inhabit the region's karst environments. Monitoring of these species, many of which are of conservation concern, is of critical importance in maintaining the region's water quality and ecosystem health. Many of these species, however, pose challenges when surveying with traditional methods because of their low abundance and subterranean habitats. Here, we developed a survey method using environmental DNA (eDNA) and quantitative polymerase chain reaction (qPCR) to detect endemic groundwater salamanders of the genus *Eurycea*. We collected 192 samples from 74 sites across the Edwards Plateau and used species and clade-specific qPCR assays to determine presence or absence at the species level. The goal of this work was to better understand the distribution of species in this group and aid in the designation of critical habitat. Based on our survey results, we conclude that eDNA surveys can be a useful tool in identifying salamander presence, but we recommend repeated testing and other survey methods before determining that salamanders are absent from a site. We also propose areas of future investigation that could improve detection rates.

Introduction

Freshwater systems have been described as hotspots for biodiversity and endemism (Strayer et al. 2010), with freshwater fauna experiencing high rates of decline in the twentieth century (Ricciardi et al. 1999). Moreover, fresh water is a vital resource to many organisms, and the growing demand for water from an increasing human population poses a threat to many endemic species (Jackson et al. 2001). The Edwards-Trinity aquifer system is an important source of fresh water to many residents of central Texas, and it directly or indirectly supplies water to major cities including San Antonio, San Marcos, and Austin (Devitt et al. 2019).

The Edwards-Trinity Aquifer supports a diversity of aquatic habitats that harbor a multitude of endemic species. The species that inhabit the inaccessible regions of the aquifer have a relatively low detection rate and present challenges for sampling to fully understand their abundance and distribution. Many of these species are federally endangered or threatened and are only sporadically found via spring outflows and wells (Tovar et al. 2012, Hutchins et al. 2013). The relatively low detection rate, combined with the inaccessibility of the habitat, make sampling difficult and warrant investigation of alternative methods for detecting and studying aquifer-limited species. Environmental DNA (eDNA) has been shown to be a sensitive and efficient way of detecting aquatic vertebrates in rivers, canals, and fast flowing streams (Gorički et al. 2017, Thomsen et al. 2011, Ficetola et al. 2008), but there has only been limited testing and use of eDNA in karst aquifer systems (Lyons and Hillis, 2019, Adcock et al. 2023). Our previous work on the Edwards-Trinity Aquifer has shown that eDNA methods can be used to detect the presence of groundwater salamanders of the genus *Eurycea* in this system (Lyons and Hillis, 2019).

Subterranean organisms are among the most cryptic and least understood of all Texas species (Chippindale et al. 2000). Species that inhabit caves and aquifers are more difficult to detect and collect than their surface counterparts. Particular subterranean groups of conservation concern include the salamander genus *Eurycea*, which contains at least 15 species unique to Texas, all listed as Species of Greatest Conservation Need in the Texas Conservation and Action Plan (TPWD, 2012); two are federally listed as endangered, four as threatened, and five are under review (USFWS, 2013a). Critical habitat has been established for three species but remains to be finalized for others, due to the paucity of information on distribution and habitat requirements. Although numerous caves and springs are potentially occupied habitat, the subterranean nature of these species precludes straightforward sampling. Relatively few portals to the underground aquifer are accessible to researchers, and groundwater vertebrates typically have low detection probabilities when traditional sampling methods are used (Gluesenkamp and Krejca 2007, McDermid 2015). What is clear, however, is that threats to these organisms are increasing in extent and severity: direct and indirect impacts to habitat, decreasing water quality and quantity, and non-native predators and pathogens. (USFWS, 2013a, TPWD, 2012).

Here, we developed a method for detecting eDNA to better understand the distribution of the enigmatic salamander genus *Eurycea* from Central Texas. We designed ten distinct assays targeting individual species or clades of closely related species in an effort to delineate range boundaries of Central Texas *Eurycea*. The application of eDNA to the cave salamanders of west central Texas has the power to detect species presence, aid in the designation of critical habitat, and produce more robust conservation and recovery assessments and plans.

Methods

Collection and Filtering

Water samples were collected from field sites in 1-L plastic Nalgene containers. Field sites were chosen based on known presence of salamanders or suitable salamander habitat. Collected samples were filtered using a Geopump II Peristaltic Pump (Geotech Environmental Equipment, Inc.) to pump water through self-preserving filter packs containing 1.2-µM polyethersulfone filter papers (Smith-Root, Inc.). The amount of water filtered through one filter pack varied from

1-L to 9-L. Filter packs were stored at room temperature until ready for DNA extraction. All containers used for collection were sanitized with bleach and rinsed thoroughly with water before reuse.

DNA Extraction

DNA was extracted from each filter using a customized protocol with reagents from a Qiagen DNeasy Blood and Tissue Kit. Filters were transferred to 5-ml centrifuge tubes with 720-µl Buffer ATL and 20-µl proteinase-K and incubated overnight at 56 C. Following incubation, we transferred the filter and lysate to a Qiagen QIAShredder with collection tube and centrifuged at 16,000 RCF for one minute. We then discarded the filter and returned the lysate to its original 5-ml tube with 800-µl Buffer AL and incubated for 10 minutes at 70 C. Following the second incubation, we added 100% ethanol and transferred 600-µl solution to a DNeasy mini spin column with collection tube. The spin column and collection tube were centrifuged for one minute at 16,000 RCF. Flow through was discarded and this step was repeated until all solution from the 5-ml tube was used. We then added 500-µl Buffer AW1 and centrifuged again for one minute at 16,000 RCF. We discarded flow through, added 500-µl Buffer AW2, and centrifuged for three minutes at 16,000 RCF. The collection tube was discarded and the spin column was placed over a 1.5-ml centrifuge tube. We eluted DNA from the spin column by adding 80-µl nuclease free water and centrifuging for one minute at 16,000 RCF. The spin column was then discarded.

qPCR Primer and Probe Design

We used quantitative polymerase chain reaction (qPCR) to detect the presence or absence of salamander DNA in each sample. Primer and probe design are crucial for detecting target species in that the correct probes must amplify any member of the group without the false positives of other closely related species (Wilcox, et al., 2013). For all *Eurycea* species, the target gene was the mitochondrial *cytochrome b*. Mitochondrial genes are found in eDNA at an order of magnitude greater than nuclear genes, and the *cytochrome b* (*cytb*) gene is often one of the first to be sequenced and published to repositories like GenBank. The goal of probe design was, for each target species or closely related clade, to develop a forward primer, reverse primer, and qPCR probe that would amplify a short 70-150 base pair region of the cytb gene. The region, or amplicon, is short because eDNA is most likely to be degraded and fragmented before collection (Turner et al. 2014). Primers and probes used in this study were designed according to methods described by Lyons and Hillis (2019), except for the primer-probe set for the more recently discovered *E.* sp. Georgetown. For this species, a *cytb* region was sequenced in house using previously published primers (Hillis et al. 2001). The sequence was aligned with syntopic species (*E. naufragia*, *E. tonkawae*, *E. chisholmensis*) using Geneious Prime 2019.0.4 (https://www.geneious.com) to identify a variable region unique to *E.* sp. Georgetown. The sequence was imported into Integrated DNA Technologies (IDT) Primer Quest Tool for creating an optimal assay that was then ordered from IDT.

Name	Forward Primer	Reverse Primer	Probe
sosorum-nana	AGGTGGAGTAATAGCCTTATTAGCCTCTAT	GAAATGACATGCTTCGGTGCT	CTATTCCAGCTATTCATAC

tonkawae-chisholmensis	TCTTCACGAAACCGGATCAAG	TGGGTGAAACGGGATTTTGT	AACCCAACAGGACTAAA
rathbuni-waterlooensis	CACATTTGCCGCGATGTAAA	TGACGCTCCGTTAGTGTGAATATT	TACGGCTGACTTATGC
naufragia	CACGAAACCGGATCAAGCA	AACGGGATTTTGTCTGGGTTAG	CCCAACAGGACTAAAC
sp. 2	CACTAATTACACCCCCACACATTC	CGAAGGATTGCGTAGGCAAA	ACCAGAGTGATACTTCT
sp. 3	GCAACACTCACCCGGTTTTT	TGCTGACCCCTGCAATTATAAA	CTTTCCACTTTATTTTGCC
sp. 1	TCCATGAAACCGGATCAAACA	GAATGGGATTTTGTCTGGGTTAGA	CCCAACAGGAATTAA
troglodytes	ACTAACTACACCCCCACACATTCA	CGAAGGATTGCGTAGGCAAA	CCAGAGTGATACTTCTT
latitans-neotenes-pterophila	CACGAAACCGGGTCAAACA	GAATGGGATTTTGTCTGGGTTAGA	CCCAACAGGAATTAA
E. sp. Georgetown	TTCACGAAACCGGGTCAA	GAATAATATGGGTGGAATGGGATTT	TAGGTTCGAGTTTAATCCTGTTGGGTTGC

Table 1. Primer and probe sequences for all Central Texas *Eurycea* species and clades.

qPCR

Total reaction volumes were 20-µl and consisted of 10-µl TaqMan Fast Advanced Master Mix, 1.6-µl forward primer, 1.6-µl reverse primer, 2-µl probe, 2.8-µl nuclease free water, and 2-µl DNA sample. We created a five-point standard curve for each assay using 1:10 serial dilutions made from extracted salamander DNA samples that were standardized to ~10 ng/µl before dilution. Assays for each sample were chosen based on geographic locality. If a sample was collected at a site in a geographically ambiguous area, we ran multiple reactions to target any possible species that might be present. Reactions were thermocycled on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with initial denaturation at 95 C for 20 seconds and 50 cycles with 1 second of denaturation at 95 C and 20 seconds of annealing at 60 C. Samples with a reported cycle threshold value were considered positive.

Results

We collected a total of 192 test samples (any amount of water collected from one site on the same day and tested with one qPCR assay; see Supplementary Materials), from 74 sites (counted by number of distinct GPS coordinates recorded) including springs, streams, wells, and caves across the Edwards-Trinity aquifer system of west central Texas (See Figure 1 for known occurrences of salamanders prior to sampling and Figure 2 for collection sites). Water quality data was collected from a subset of sites and is included in the Supplementary Materials. Using qPCR, we had positive detections of *Eurycea* DNA in 24 of these samples (12.5% detection rate by sample). These included positive detections at 20 of the unique sites (Table 2) that we sampled from (27.0% detection rate by site).

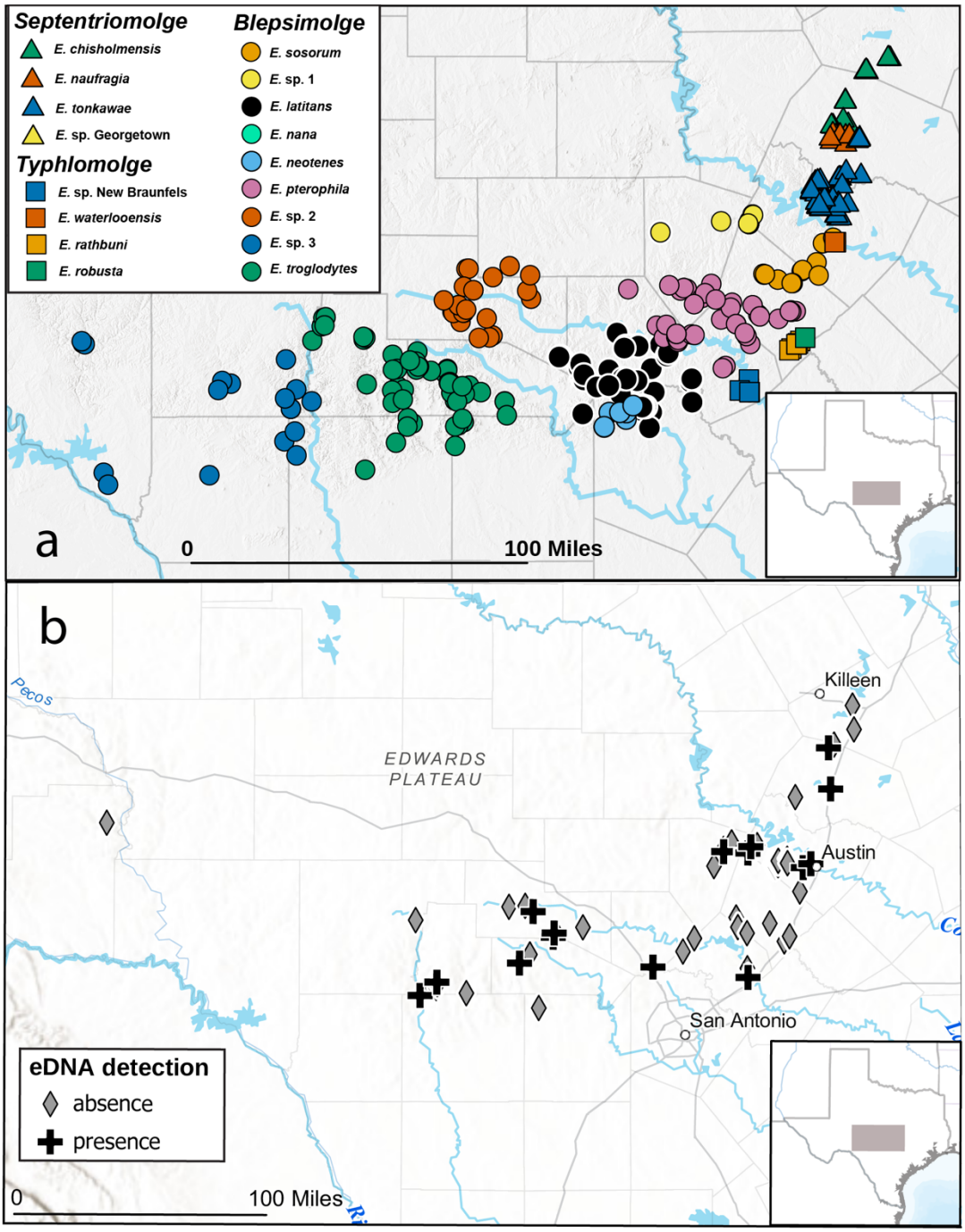

Figure 1. Occurrence records (a) and eDNA detection results (b) for groundwater salamanders (*Eurycea*) in the Edwards-Trinity aquifer system of west central Texas. Occurrence records have been compiled from several sources, including published records (Longley 1978; Stejneger 1896; Uhlenhuth 1921; Baker 1961; Sweet 1978; Chippindale et al. 2000; Hillis et al. 2001; Bendik et al. 2013; Wray and Steppan 2017; Devitt and Nissen 2018 and Devitt et al. 2019) and unpublished observations. Some of the occurrence records—particularly for *E. latitans*, *E. neotenes*, and *E. pterophila*—have been assigned to species based on proximity to samples analyzed by Devitt et al. (2019). Further work is needed to delimit the boundaries of these taxa. Samples included in Devitt et al. 2019 are indicated in the associated attribute table. An online version of this figure as an ArcGIS project package is available at https://ut-austin.maps.arcgis.com/home/item.html?id=fc471a22dd2246fd99a54843bf870db8#overview.

All assays detected salamander DNA extracted from tissue, as evidenced by successful amplification of all dilution series, and seven of the ten assays had positive field detections. Assays with the greatest number of field detections included those for *E.* sp. 1 (6 detections at 5 sites), *E. naufragia* (6 detections at 4 sites), *E.* sp. 2 (3 detections at 3 sites), and *E. troglodytes* (3 detections at 3 sites). The three assays with no field detections were those for *E.* sp. 3, the subterranean subgenus *Typhlomolge* (including *E. waterlooensis* and *E. rathbuni*), and the subterranean *E.* sp. Georgetown. Subterranean species may be particularly difficult to detect due to low copy number. When excluding samples using these assays, our overall detection rate increased to 17.6% (24 of 136 samples).

Volume of water pumped through one filter had an impact on detection rate (Figure 2). Among samples in which at least three liters of water were pumped through one filter, 18.8% (9 of 48) had positive detections, compared to a 10.4% detection rate among all other samples. This detection rate increased to 25.7% (9 of 35 samples) when excluding subterranean species.

Site	County	Species	Samples Collected	Positive Samples	Detection Rate
Pecan Spring	Williamson	*E. chisholmensis*	1	1	100.00%
Cascade Caverns	Kendall	*E. latitans*	2	1	50.00%
Comal Springs	Comal	*E. pterophila*	1	1	100.00%
Beaver Spring	Williamson	*E. naufragia*	38	2	5.30%
Jacob's Seep	Williamson	*E. naufragia*	18	2	11.10%
Fissure Spring	Williamson	*E. naufragia*	1	1	100.00%
San Gabriel Springs (West)	Williamson	*E. naufragia*	3	1	33.30%
Kerr Ecolab Site 1	Kerr	*E.* sp. 2	1	1	100.00%
Lange Ravine	Kerr	*E.* sp. 2	1	1	100.00%
Natives of Texas Site 3	Kerr	*E.* sp. 2	3	1	33.30%
Cold Spring	Travis	*E. sosorum*	1	1	100.00%
Backdoor Spring	Travis	*E. sosorum*	1	1	100.00%
Medina Tributary Spring	Bandera	*E. troglodytes*	2	2	100.00%
Piñon Springs	Real	*E. troglodytes*	2	1	50.00%

Nueces Hwy 55	Uvalde	*E. troglodytes*	1	1	100.00%
Red's Spring Box	Hays	*E.* sp. 1	1	1	100.00%
Herschel's Spring	Hays	*E.* sp. 1	3	2	66.70%
Anonymous Spring 1	Hays	*E.* sp. 1	4	1	25.00%
House Well	Blanco	*E.* sp. 1	1	1	100.00%
Reimer's Ranch	Travis	*E.* sp. 1	1	1	100.00%

Table 2. All sites with positive detections, species detected (based on assay used and geographic distribution of species), total samples collected, and total number of positive detections at each site. Data for all samples collected in this study can be found in the Supplementary Materials.

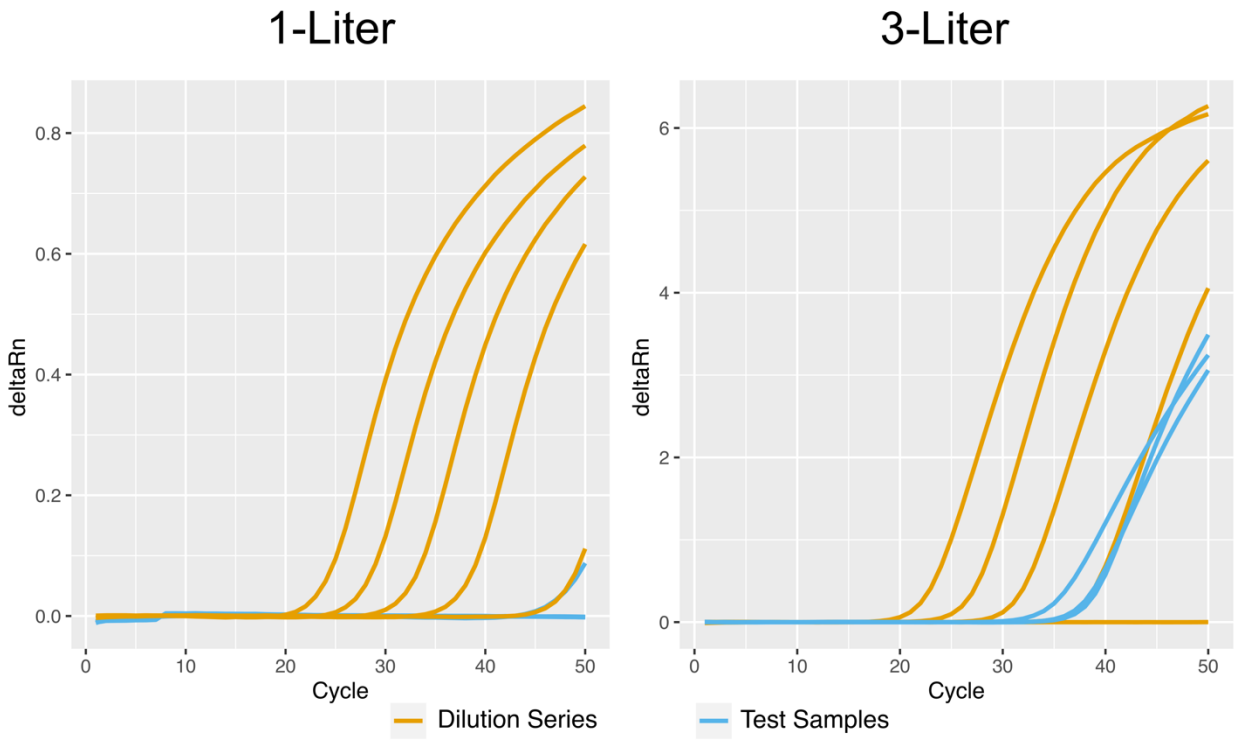

Figure 2. A comparison of samples collected from Medina Tributary Spring in Bandera County, TX on two different days, both times with salamanders visibly present. qPCR amplification curves for a sample consisting of 1-L collections (from September 18, 2022) along with the *E. troglodytes* dilution series show a faint positive that amplified similarly to the last of the dilution series (left). A sample of 3-L collections (from May 21, 2023), all positive detections, showed much stronger amplification in comparison to the dilution series (right). This serves as an illustrative example of our increased detection capability when collecting higher volume samples.

Discussion

Our objective was to develop species and clade-specific assays to detect species presence of *Eurycea* salamanders from sites in west central Texas and to better understand species

distribution in the region. We were successful in designing assays that can detect salamander presence, particularly among surface-dwelling species, and we identified methods that can improve rate of detection, such as increasing water volume per sample. Our assays also proved to be capable of distinguishing between closely related species, at least in some cases. For example, among samples collected in the western Edwards Plateau in Real and Uvalde counties, we detected the presence of *E. troglodytes* but not *E.* sp. 3, helping to delineate range boundaries of these parapatric species.

Positive detections for this study were relatively low compared to some other amphibian eDNA studies (Plante et al. 2021; Ruppert et al. 2022; Adcock et al. 2023), and there are several factors that may have contributed to this. These groundwater-dwelling salamanders are small organisms that often occur in low abundance, decreasing the probability of DNA capture in each water sample. Compounding this, salamanders have the lowest metabolic rates among tetrapods (Chong and Mueller, 2013), meaning that shed DNA in the form of metabolic waste may be particularly low for these animals. These factors highlight the importance of maximizing water volume pumped through one filter to increase the chances of DNA capture per sample. Our results support this, as samples consisting of at least three liters of water had a much higher rate of detection compared to smaller volume samples. We note, however, that there are tradeoffs involved in passing greater volumes of water through one filter. Filters become increasingly obstructed with debris with greater volumes of water, slowing filtering time dramatically in some cases. Although most of our 3-L samples filtered quickly, filtering time can be highly variable depending on amount of sediment and other debris in the sampled water. An alternative method for filtering greater volumes of water or water with large amounts of sediment is to use multiple filters for one sample (Hunter et al. 2019).

Based on these data, we emphasize that a single negative result, or even multiple negative results, is not necessarily indicative of salamander absence. Of the 20 sites at which we obtained a positive detection, 8 of these also yielded samples with negative detections. Most notably, we sampled from Beaver Spring on the San Gabriel River (Williamson County) 38 times while obtaining positive detections on only two dates. Similarly, sampling from Jacob's Seep on the San Gabriel River (11.1% detection rate) also required collecting multiple samples to obtain a small number of positive detections. Thus, repeated testing along with visual surveying and trapping are needed before concluding that salamanders are absent from a site.

Future Work and Conclusions

Future work is needed to address issues of false negatives when sampling from sites where salamanders are known to be present. False negatives may result from low copy number as well as the presence of compounds in water samples that act as PCR inhibitors. Methods that could mitigate the presence of these inhibitors include inhibitor removal kits and the addition of bovine serum albumin to the PCR Master Mix (Kreader 1996; Hunter et al. 2019). Perhaps the most promising avenue of study, however, involves the use of a semi-nested PCR protocol to amplify signal and overcome the presence of inhibitors (Ruppert et al. 2022). A potential follow-up study could include an adaptation of this nested qPCR strategy to Central Texas *Eurycea* and a retesting of likely false negative samples.

In conclusion, this eDNA methodology is a useful component of a larger survey strategy to detect the presence of surface-dwelling salamanders in the Edwards Plateau of Texas. Repeated testing at different times of year and under different flow conditions will likely yield positive results where salamanders are present. Additional sampling is needed at sites in the western Edwards Plateau to confirm the effectiveness of the *E.* sp. 3 assay. Further experimentation with methods to improve assay sensitivity in samples with low copy number and/or PCR inhibitors may be needed to improve detection rates, particularly for subterranean species.

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