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Share with Wildlife Program
Year 2 Interim Report – June 2026

Using Environmental DNA (eDNA) to Survey for Imperiled Snakes in New Mexico


Nerodia erythrogaster Drew R. Davis


Thamnophis eques Jeff Servoss

Agreement #250404

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OBJECTIVES

This project aims to detect two imperiled semi-aquatic snakes in New Mexico, the Plain-bellied Watersnake (*Nerodia erythrogaster*) and the Mexican Gartersnake (*Thamnophis eques*), by developing and validating environmental DNA (eDNA) assays and analyzing water samples for target species eDNA. The proposed work aims to (I) develop and validate species-specific eDNA assays for *N. erythrogaster* and *T. eques*, (II) standardize and implement field sampling protocols to detect target species in potential habitats, and (III) identify habitat characteristics associated with positive eDNA detections.

INTRODUCTION

This document reports progress and activities from January–June 2026. Background information and rationale for this project are provided in the Year 1 report (Kuschel and Davis 2025). During the current project period, our goals were to (1) re-analyze a subset of *N. erythrogaster* eDNA samples from 2025, (2) develop and validate a *T. eques* eDNA assay, (3) analyze an initial round of *T. eques* eDNA field samples, (4) continue to examine potentially misidentified occurrence records of these species across New Mexico, (5) create maps of species distributions from both eDNA results and historic occurrences, and (6) begin eDNA sampling for the 2026 field season. The progress on each of these goals is further described below.

RESULTS TO DATE

Re-analyzing *Nerodia erythrogaster* eDNA samples.—We selected a subset (N = 8) of eDNA samples from 2025 to re-analyze to increase focal species detection probabilities and confirm results of the first round of analyses that were conducted in Fall 2025. We re-analyzed site samples where *N. erythrogaster* individuals or shed skins were detected visually but did not amplify during the first round of eDNA analyses, in addition to our two positive detections from the first round of analyses. In all eight re-analyzed samples, we failed to amplify *N. erythrogaster* eDNA. These results suggest that very low eDNA concentrations were present in the samples that tested positive during the first round and confirms the lack of detectable eDNA at sites where the focal species were visually observed. Although we found visual evidence of *N. erythrogaster* presence at these sites (e.g., individuals or shed skin), the false negative results from the eDNA analyses highlight recognized drawbacks to eDNA surveys (e.g., Bajaffer et al. 2026).

***Thamnophis eques* eDNA assay development and validation**.—In the lab, eDNA filter extraction followed an adapted Epoch GenCatch Blood & Tissue Genomic Prep Kit protocol. Inhibitor removal kits have been shown to be an essential step for eDNA surveys (Jane et al. 2015; Hunter et al. 2019) and a commercial inhibitor removal kit (Zymo OneStep™ PCR Inhibitor Removal Kit) was used following DNA extraction. Primers were designed to amplify a small fragment targeting the ND4 mitochondrial gene (forward sequence: 5'-CGAAAAGGCCCCTAACCTGG-3'; reverse sequence: 5'-

CTATGATTGGTGAYAGGGTGAACAGT-3'; Sleeting et al. 2014). The forward and reverse primers had similar melting temperatures (forward primer: 62.3°C; reverse primer: 63.8°C). The initial, 25-µL PCR reaction consisted of 12.5 µL Promega GoTaq G2 Hot Start Master Mix, 0.5 µL each of 10 µM forward and reverse primers, 1.5 µL nuclease-free water, and 10 µL of DNA extract (or nuclease-free water for control samples). The second round of PCR was a 50-µL reaction, consisting of 25 µL Promega GoTaq G2 Hot Start Master Mix, 1 µL each of 10 µM forward and reverse primers, 13 µL nuclease-free water, and 10 µL of PCR product from the initial reaction. To detect lab contamination, a no-template control in addition to a field blank (i.e., DI water run through the filter) were run in conjunction with the samples. All samples went through two rounds of PCR to increase the probability of amplifying even low concentrations of eDNA that might have been present in water samples. To avoid any potential contamination of samples due to the sensitivity of the two rounds of PCR, no internal positive control was included. PCR conditions were specific to the primers being used. PCR reactions occurred using a BIO-RAD T100 Thermocycler, with the heated lid set to 105°C. The cycling conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, and a final extension at 72°C for 30 seconds. Following amplification, reactions were held at 10°C indefinitely until removed from the thermocycler. Following the completion of the second round of PCR, the PCR product was run on a 2% agarose gel for 40 min at 100 V alongside a 50-bp ladder, and the gel was visualized using a UV transilluminator. If samples produced at least two bands of the appropriate size, the remaining 5 µL of PCR product from each technical replicate that produced a band of the correct size was purified using a New England Biolabs Exo-CIP™ Rapid PCR Cleanup Kit. Then, 5 µL of purified PCR product and 5 µL of the forward primer were sent to Eurofins Genomics for Sanger sequencing. Sequences >95% identical to published target species sequences on NCBI BLAST were interpreted to be positive species detections. If only one band of the correct size was produced after completing a nested PCR, samples were re-run.

For *T. eques*, we initially tested primers from Sleeting et al. (2024), as referenced in Fremier et al. (2019), which were developed for *T. eques* in Arizona, USA. Unfortunately, these primers were non-specific and amplified DNA from other sympatric and congeneric species, including *T. cyrtopsis* which occurs in the Cliff–Gila Valley. We conducted bioinformatic primer design efforts using all available *Thamnophis* mitochondrial sequences (cytb, ND1, ND2, ND4) from NCBI, implementing Primer3 in Geneious software for assistance and prioritizing short primer lengths (ca. 18 bp) to increase eDNA amplification success in degraded samples. However, due to low sequence divergence between *T. eques* and its congeners and biochemical constraints (e.g., primer dimers, GC content, melting temperature), no suitable primer pair could be identified. As a result, we began exploring restriction fragment length polymorphism (RFLP) assays. This approach uses shared primers to amplify ND4 and then digests the amplicons with species-specific restriction enzymes to produce differing band patterns during gel electrophoresis. We identified enzymes capable of uniquely cutting ND4 amplicons of each target species. In addition to Sanger sequencing results, this is a promising direction for differentiating *T. eques* from congeners in field samples.

eDNA assay development included creating a serial dilution using *T. eques* DNA extract to determine the limit of detection. Genomic DNA was extracted from a *T. eques* tissue sample from an individual collected in Arizona (provided by D. Wood, USGS) and serially diluted in ten-fold increments. Initial DNA extract concentrations were quantified using a Qubit fluorometer. Across three replicates, initial DNA concentration averaged 145 ng/μL. We created serial dilutions down to 1.45×10^{-6} ng/μL. Each dilution was amplified in triplicate using a two-round PCR protocol with the ND4 primer set from Sleeting et al. (2024). A negative PCR control was included at all steps. Products were visualized using gel electrophoresis. The limit of detection (LOD) is defined as the lowest DNA concentration that consistently produced visible amplification in all three replicates. Using these criteria, our assay demonstrated a LOD of ca. 1.45×10^{-3} ng/μL, with consistent amplification across replicates at this concentration using our protocol. At lower concentrations amplification was variable, with faint bands observed in one or two (of three) replicates down to ca. 1.45×10^{-3} ng/μL, not meeting the threshold to define the LOD but demonstrating assay sensitivity at low DNA concentrations.

To validate our *T. eques* eDNA assay, we conducted a mock eDNA trial under environmental conditions resembling field conditions. A known concentration of *T. eques* DNA extract (1.46 ng) was added to 1000 mL of field water, creating a concentration of 1.45×10^{-6} ng/μL. We then passed the eDNA-spiked water through a Whatman Grade 4 cellulose filter and proceeded with filter extraction, PCR amplification, and gel electrophoresis. Although the negative control showed no amplification, we also failed to amplify *T. eques* eDNA from the spiked sample. Because the eDNA concentration in the spiked sample was 1000× lower than the DNA concentrations that were reliably detectable from tissue samples, we plan to run this test again with increased eDNA concentrations. Our eDNA assay reliably amplifies low concentrations of target DNA using tissue samples, but further testing is required to establish the lower limit of eDNA concentration detectability in field-quality water samples.

Analyzing *Thamnophis eques* eDNA samples.—During the 2025 sampling season, we visited a total of 24 field sites. We collected approximately 1L of water from each of three separate locations within selected microhabitats of a specific site, which were poured into a 5-gal bucket as they were collected. After mixing the pooled samples in the bucket, three 1L replicates were filtered from the pooled mixture. We aimed to filter 1L of water per replicate, resulting in three field replicates per site sample. When high turbidity limited the volume of water that we were able to pass through each filter for each of the three original replicates, we added one or two replicates to reach the targeted 3L of water filtered. Field surveys during 2025 resulted in 24 total sampling points along the Gila River and associated creeks within the USFWS Critical Habitat (CH) designated for *T. eques* in the Cliff–Gila Valley (USFWS 2021), in addition to points at or near historical *T. eques* localities outside of the USFWS CH. Several historical localities for this species, such as Mule Creek and Duck Creek, have since experienced major water loss and habitat degradation (Degenhardt et al. 1996; USFWS 2020) or occur on private land, and as a result are currently inaccessible for sampling (Hotle et al. 2012). One record of *T. eques* was documented on the Gila River “5 mi E of Virden” in Hidalgo County (NMSU 5377); although *T. eques* is currently considered to

have been extirpated from this area (USFWS 2024), we visited this location regardless. We also decided to focus most of our 2025 sampling efforts in the Cliff–Gila Valley, the only locality in New Mexico where this species has been detected during the past three decades (USFWS 2006, 2024; Geluso 2023; NMDGF 2024).

From January–May 2026, we analyzed samples collected for *T. eques* from the 2025 field season. We detected *T. eques* eDNA at three of 24 sites (12.5%). All three positive eDNA detections came from samples taken from the Gila River on NMDGF property (Fig. 1). All other site samples either produced no visual bands or produced weak bands, and/or generated inconclusive sequencing data. No amplification occurred in any laboratory (e.g., no-template control) or field controls. No sympatric *Thamnophis* individuals were observed on the NMDGF Gila River property during surveys. All three positive sites (IB1, IB2, and IB3) were sampled in late May–early June 2025, from mornings to late afternoon. These detections represent three separate points on the Gila River: (1) near the southern border of the NMDGF property, (2) situated near the midpoint of the property, and (3) at the northern edge of property. Our eDNA survey results suggest that this small section of the Gila River may harbor one of the only remaining populations of *T. eques* in New Mexico. We observed that eDNA detection strength (e.g., relative band brightness, number of positive replicates, NCBI BLAST sequencing strength) sequentially increased across these three sites in the downstream direction with the strongest detection in the most-downstream location. We collected eDNA samples at intermittent points along a longitudinal section of the Gila River through the Cliff–Gila Valley, spanning from the lower portion of the valley upstream toward the upper valley. Because eDNA in flowing systems can reflect both the presence of nearby organisms and downstream transport from upstream populations, sampling across this gradient can help evaluate whether *T. eques* eDNA is restricted to isolated points or detectable across a larger portion of the river.

Based on the lack of visual detections of this species during surveys and the overall rarity of *T. eques* in New Mexico, we expected to find few positive detections. To conserve both time and resources, the goal of our analyses for *T. eques* over this period was to complete an initial round of analysis on each of the 24 samples and select a subset of samples for further analysis and/or the use of RLFP assays. Currently, we have not utilized RLFP assays for any samples, but we still plan to test these assays for further validation using the current NMDGF Gila River site results and any future 2026 positive *T. eques* eDNA samples. During the initial round of analysis, all three NMDGF Gila River sites resulted in a band of the appropriate size in at least two (of three) replicates.

Next, as part of our normal eDNA assay workflow, we sent all three NMDGF Gila River samples to be sequenced. Sanger sequencing results were interpreted with caution, as the primers used are not species-specific and amplify eDNA of congeners. Surprisingly, in all three samples, Sanger sequencing resulted in *T. eques* as the top species match, matching multiple *T. eques* voucher sequences published on GenBank. *Thamnophis cyrtopsis* did not occur in the top five species matches in any of the NMDGF Gila River samples. For all three sites, Sanger sequencing resulted in a >95% match to *T. eques*. For further validation of our sequencing results, we aligned the Sanger sequences from each field sample with sequences from (1) *T. eques* and *T. cyrtopsis* GenBank data, (2) *T. eques* tissue samples, and (3) a *T. cyrtopsis* tissue sample from the Cliff–Gila Valley. For all three field samples, the

sequence alignments with *T. eques* GenBank data and tissue samples sequences demonstrated a very strong match across overlapping regions. In contrast, the Cliff–Gila Valley *T. cyrtopsis* tissue sequence differed from the *T. eques* eDNA sequences through multiple substitutions within the same region, including diagnostic differences throughout the sequence. These results indicate that the NMDGF Gila River eDNA detections are consistent with *T. eques* rather than *T. cyrtopsis* and represent genuine detections of *T. eques* eDNA. Separately, we also amplified low concentrations (1.92×10^{-3} ng/µL) of *T. eques* and *T. cyrtopsis* (from the Cliff–Gila Valley) DNA extracted from tissue samples and sequenced these results. When analyzing these sequences using NCBI BLAST, results accurately confirmed the identification of each species, strengthening the confidence in our methodology's ability to differentiate *T. eques* from *T. cyrtopsis*.

Mis-identified museum specimens.—We have corrected the identity of three supposed *T. eques* specimens from the Rio Hondo in Lincoln County (MVZ:Herp:280125–280127) which have been identified as *T. elegans*. Additionally, we have corrected the identity of two *T. eques* specimens from the Mimbres River, Grant County, that were collected in 1944 (CHAS 12445, 12446) which have been identified as *T. cyrtopsis*. Locality data was received for two additional *T. eques* specimens (LSUMZ 7108, 7114), which were from Mule Creek. An additional *T. eques* specimen (LSUMZ 7838) identity was corrected to *T. elegans*.

Visualizing Species Distributions.—A map showing results from *T. eques* eDNA sampling is provided below (Fig. 1). Utilizing the historic species occurrence database we created for both species, we have included a combined (both *N. erythrogaster* and *T. eques*) occurrence map (Fig. 2). For *N. erythrogaster*, most records of this species occur in Eddy County (Fig. 3A); however, a single occurrence of this species is known from Quay County (Fig. 3B). For *T. eques*, records have been restricted to Grant and Hidalgo counties (Fig. 4), largely through our efforts to validate and confirm identifications of museum occurrence records.

2026 summer fieldwork.—From 20–25 May 2026, we collected 16 eDNA samples for *T. eques* in the Cliff–Gila Valley (Table 1; Fig. 5). This field season, we aim to repeat sampling at our 2025 detections of *T. eques* and further investigate the spatial detection limits of this population along the Gila River. The early summer period from May–June represents a critical period for eDNA sampling because the Gila River during this period has not yet experienced flooding and monsoonal fluxes that typically result in high levels of water turbidity and sediment suspension. This turbidity limits the volume of water we can pump through our filters. Previous surveys during late July and September resulted in very low volumes of water passed through filters at sampling locations on the Gila River. The goal of the trip in May 2026 was to repeat sampling at locations from the NMDGF Gila River site where we detected *T. eques* eDNA in May 2025, revisit other previously-sampled locations that contain suitable habitat, and sample a few additional sites we did not previously visit. We also set out to collect additional samples upstream and downstream from the NMDGF Gila River site. We collected samples from four locations on the NMDGF Gila River site,

including at all three 2025 locations and an additional sample upstream of our northernmost positive detection at the NMDGF Gila River site. We also resampled locations from the 2025 season on the TNC Runyan, Agnew, and Lichty properties, in addition to the NMDGF Double E Wildlife Management Area site. We investigated potential habitat in the form of seasonal streams and stock tanks in the San Francisco River drainage system with hope of collecting samples in the vicinity of the historical Mule Creek locality, but no viable habitat was located near this locality. During this trip, 11 of our 16 sampling locations fell within designated USFWS Critical Habitat for *T. eques* (USFWS 2021).

All samples were collected from early morning (approximately 0900 hours) to late afternoon (approximately 1830 hours). The water on the Gila River was generally clear, with moderate flow and low turbidity, allowing us to filter large volumes of water (1000 mL) across almost all replicates during surveys. We also recorded conductivity data and created habitat plots in three locations across all 16 sites and will analyze this data throughout the summer into the fall. We did not visually observe any *T. eques* during eDNA surveys. We observed additional snake species, including the Mountain Patch-nosed Snake (*Salvadora grahamiae*), Desert Kingsnake (*Lampropeltis splendida*), Striped Whipsnake (*Masticophis taeniatus*), Prairie Rattlesnake (*Crotalus viridis*), and Gophersnake (*Pituophis catenifer*), but did not observe any other *Thamnophis* during eDNA surveys. Lizard species observed include Clark's Spiny Lizard (*Sceloporus clarkii*), Common Side-blotched Lizard (*Uta stansburiana*), Common Lesser Earless Lizard (*Holbrookia maculata*), Ornate Tree Lizards (*Urosaurus ornatus*), Madrean Alligator Lizard (*Elgaria kingii*), and whiptails (*Aspidoscelis* spp.). American Bullfrog (*Rana catesbeiana*) observations were numerous throughout the Gila River in the Cliff–Gila Valley during our visit. Although there were some sections (<2 km) where *R. catesbeiana* populations were extremely dense, some sections were nearly devoid of *R. catesbeiana*. Bullfrogs were detected in all life stages across sites. During this sampling period, the highest abundance of *R. catesbeiana* was observed on the Gila River, downstream of the NM Hwy 211 bridge crossing (Fig. 6); we detected fewer *R. catesbeiana* on the Gila River between the US Hwy 180 bridge crossing to the southern portion of the NMDGF Iron Bridge site. We did not detect *R. catesbeiana* along Bear Creek. Other amphibians observed include Arizona Toads (*Anaxyrus microscaphus*) and Woodhouse's Toads (*Anaxyrus woodhousii*) in all life stages. We also noted numerous populations of small fish and tadpole communities across sites within designated USFWS Critical Habitat.

During our sampling trip, we noticed higher levels of human activity and recreation along the Gila River in the Cliff–Gila Valley than we observed in 2025. Along with other signs of human activity, we observed widespread refuse, primarily in the form of glass and plastic bottles (usually alcohol bottles), at many locations along the Gila River within designated USFWS Critical Habitat. We also observed high road mortality for snakes throughout the Cliff–Gila Valley during our most recent visit in addition to during our 2025 visits; this has previously been noted as a concern for *T. eques* (Geluso 2023). We also observed evidence of cattle grazing across sites during this visit that is reducing natural vegetation along the shoreline of the Gila River and its adjacent waterbodies. Despite these habitat quality issues, many portions of the Gila River along the designated USFWS Critical Habitat still contain viable habitat for *T. eques* with an abundant prey community, healthy riparian vegetation, and cover objects in the form of natural debris (Fig. 7). Although we were not able

to visually detect *T. eques* at sites that were positive for eDNA presence (from our 2025 samples), habitat along the Gila River contains a wide array of microhabitats that may provide cover for *T. eques*, such as thick piles of tree debris with extensive tunnel systems at their base, a heavily vegetated floodplain littered with fallen tree debris, thick piles of flotsam, and large floating aquatic plant masses (Fig. 7). We suspect that abundant availability of cover, coupled with low existing population numbers, may partly explain the elusiveness of *T. eques* in the area despite positive eDNA detections (from our 2025 samples). Nevertheless, we have demonstrated that eDNA surveys can be extremely useful for detecting snake populations that are imperiled, rare, occur in low abundances, or otherwise difficult to detect via visual encounter or trapping surveys. The eDNA samples that we collected in May 2026 will be analyzed during the Summer and Fall of 2026.

FUTURE PLANS

To further validate our results, we plan to use RLFP fragment assays to re-analyze all positive *T. eques* eDNA samples that resulted in bands at expected fragment length. Additional mock eDNA trials will be conducted to validate primer sensitivity in field-representative control samples. We will visit at least 20 sites each for both *T. eques* and *N. erythrogaster* in Summer 2026 and will prioritize our 2026 sampling efforts at those sites that are at or near those sites which tested positive for target species eDNA from 2025. Throughout Summer and Fall 2026, we will be analyzing eDNA samples from 2026 and updating distribution maps based on the eDNA results for both species.

ACKNOWLEDGMENTS

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FIGURES

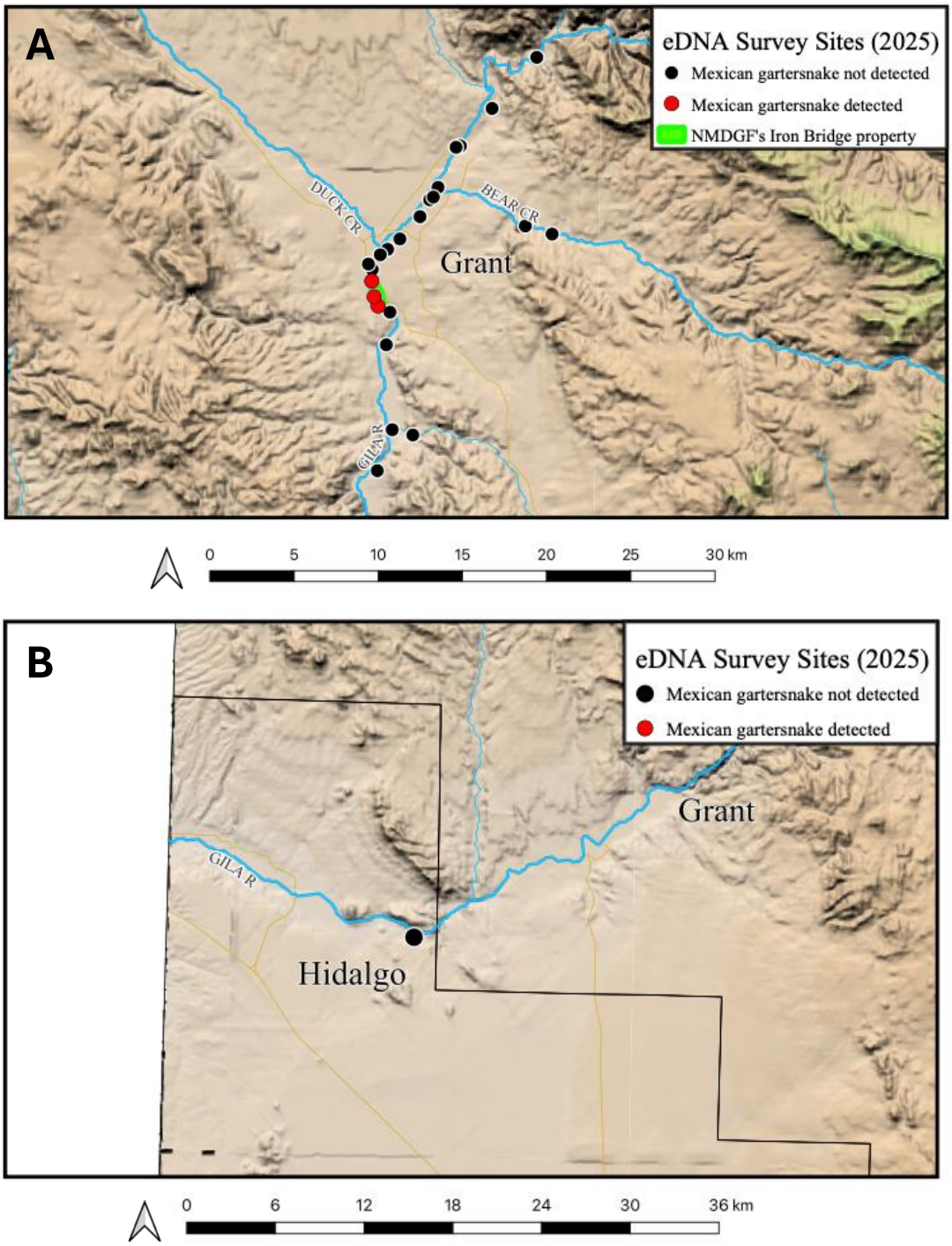

Figure 1. Mexican Gartersnake (*Thamnophis eques*) eDNA survey sites in Grant (A) and Hidalgo (B) counties, New Mexico, USA. Positive eDNA detections are indicated in red and negative eDNA detections are indicated in black.

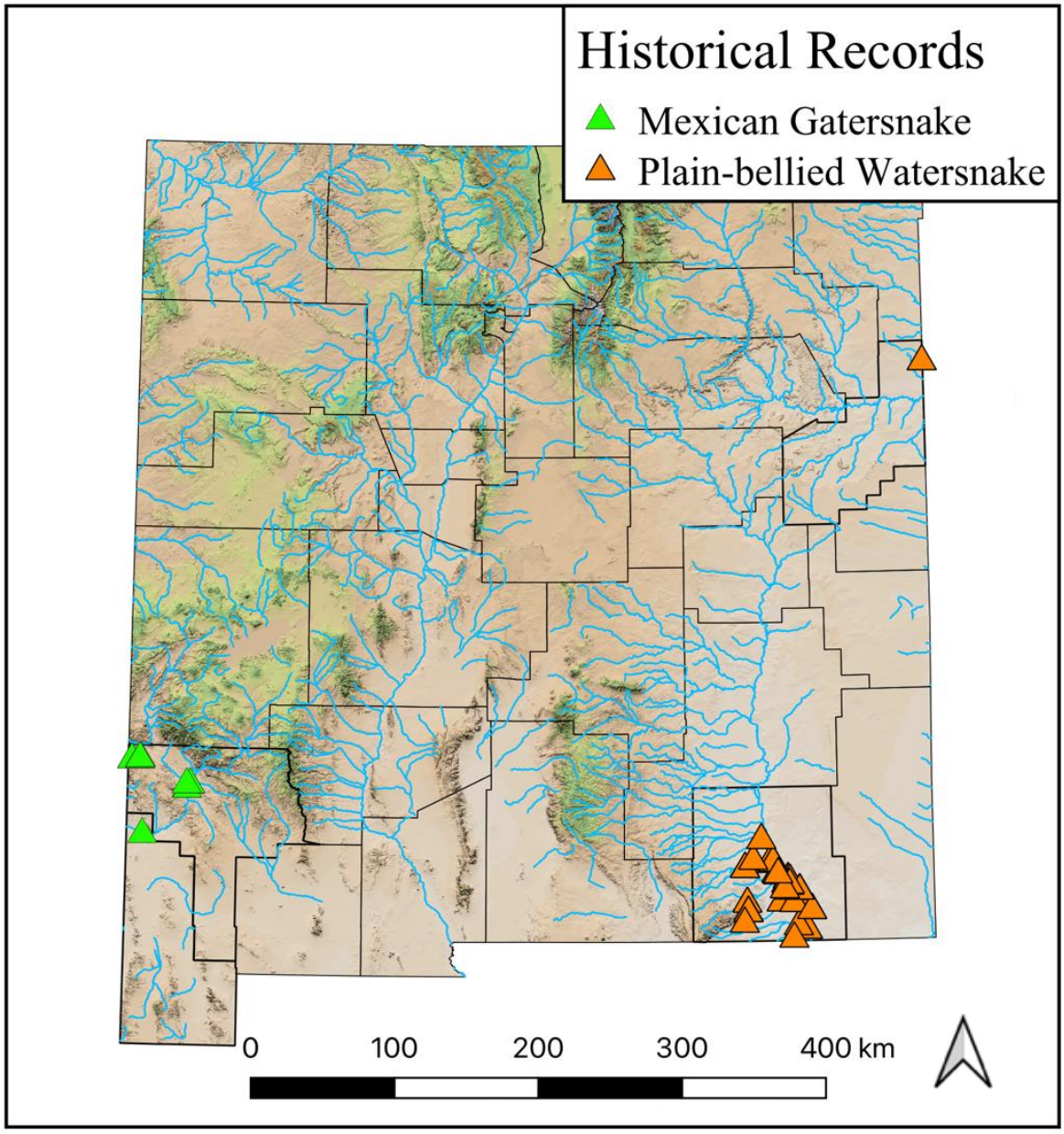

Figure 2. Historical occurrence records of Plain-bellied Watersnakes (*Nerodia erythrogaster*) and Mexican Gartersnakes (*Thamnophis eques*) across New Mexico, USA. Occurrence records are based off voucher specimens deposited in natural history collections and research-grade iNaturalist observations.

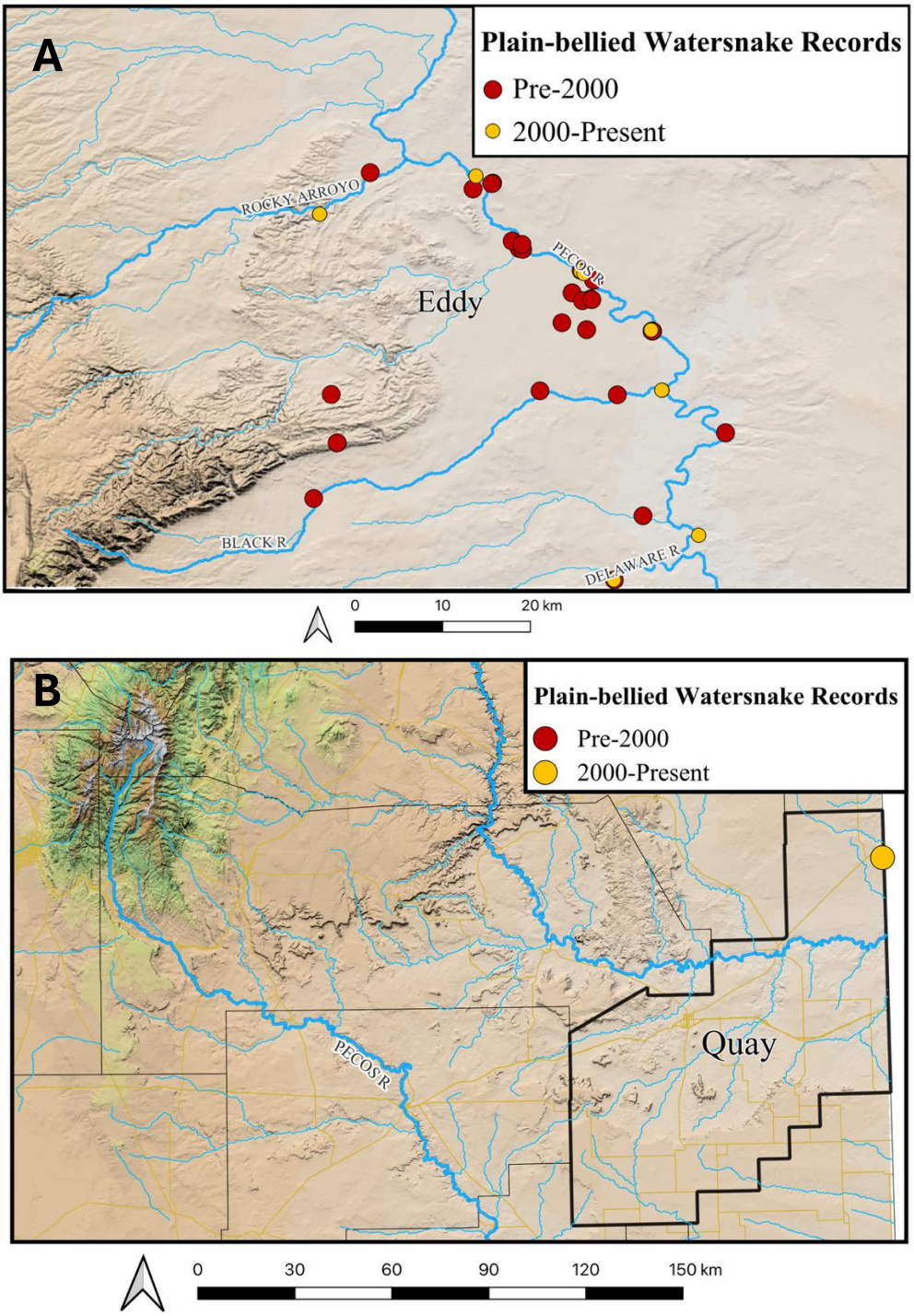

Figure 3. Historical occurrence records of Plain-bellied Watersnakes (*Nerodia erythrogaster*) from Eddy (A) and Quay (B) counties, New Mexico, USA. Occurrence records and separated by those from before 2000 (red) and those from 2000–present (yellow). Occurrence records are based off voucher specimens deposited at natural history collections and research-grade iNaturalist observations.

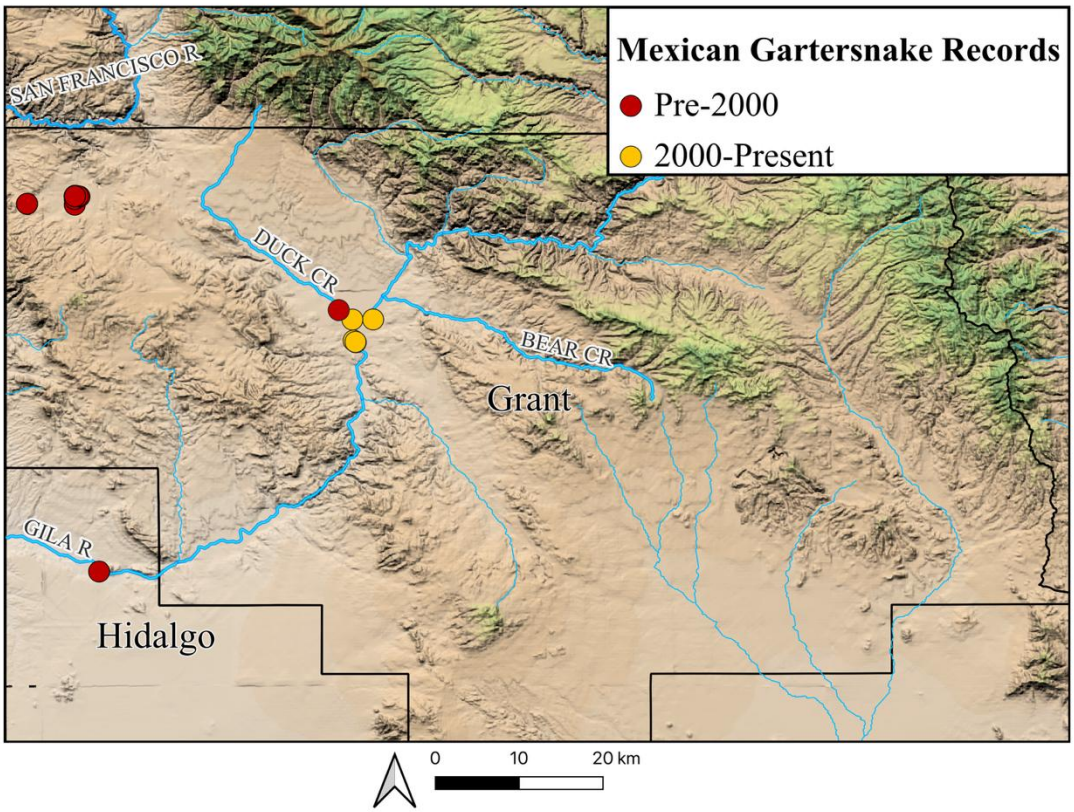

Figure 4. Historical occurrence records of Mexican Gartersnakes (*Thamnophis eques*) in Grant and Hidalgo counties, New Mexico, USA. Occurrence records and separated by those from before 2000 (red) and those from 2000–present (gold). Occurrence records are based off voucher specimens deposited at natural history collections.

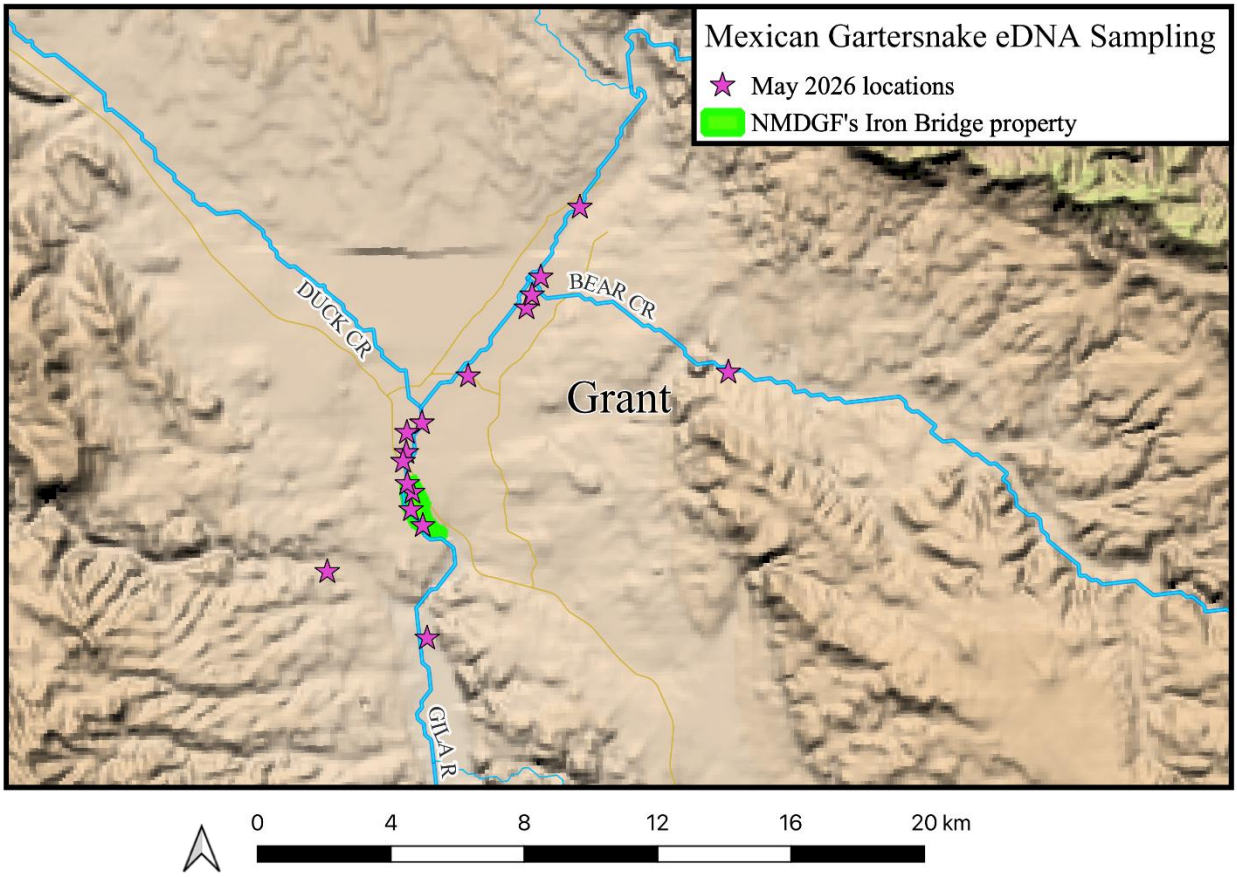

Figure 5. Map of May 2026 eDNA sampling locations for the Mexican Gartersnake (*T. eques*) in Grant County, New Mexico, USA.

Figure 6. Gila River in the Cliff–Gila Valley in Grant County, New Mexico, USA. High abundances of American Bullfrogs (*Rana catesbeiana*) were detected at this site during eDNA surveys. Photo by Jake E. Kuschel.

Figure 7. Debris piles and root systems may serve as potential refuge for *T. eques* throughout the Gila River. Photo by Jake E. Kuschel.

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