New Mexico Department of Game and Fish
Share with Wildlife Program
Interim Report – June 2025

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


Nerodia erythrogaster Drew R. Davis


Thamnophis eques Jeff Servoss

Agreement #250404

30 June 2025

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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 *T. eques* and *N. erythrogaster*, (II) standardize and implement field sampling protocols to detect target species across potential habitat, and (III) identify habitat characteristics associated with positive eDNA detections.

INTRODUCTION

The Plain-bellied Watersnake (*Nerodia erythrogaster*). *Nerodia erythrogaster* ranges throughout the central and southeastern USA, extending from southern Michigan and Delaware through the southeastern coastal plain and into northeastern Mexico (Degenhardt et al. 1996). The western populations of *N. erythrogaster* reach as far as southeastern New Mexico and western Oklahoma with isolated occurrences reported from Mexico in the states of Durango and Zacatecas (Rossman et al. 1996). This species reaches the westernmost portion of its distribution in New Mexico, occurring almost exclusively within the Pecos River drainage in Eddy County (Degenhardt et al. 1996), with one additional verified record from the Canadian River drainage in Quay County (Painter et al. 2011). This species prefers rocky ledge habitat and dense vegetation in riparian zones along permanent waterbodies (Degenhardt et al. 1996). Observations from Christman and Kamees (2007) suggest that *N. erythrogaster* individuals in New Mexico are "less likely to be encountered in habitats with deep water (> 2 m) or at least those lacking shallows, and there seems to be some preference to moving water with rocky retreats or foraging areas." This species is both diurnal and nocturnal and often uses branches or ledge habitat to forage or bask, escaping into the water from predators. *Nerodia erythrogaster* is confined to rivers, irrigation channels, or intermittent streams that contain deep pools with abundant prey items (Degenhardt et al. 1996). This species has not been recorded in headwater habitats, such as those of the Black River where pools may be too deep and cold (Degenhardt et al. 1996). Christman and Kamees (2007) examined both field-captured and museum specimens of *N. erythrogaster* as part of a dietary study and found a strong preference for fish, with amphibians occasionally consumed. This species relies heavily on water for foraging and thermoregulation; thus, aquatic habitat loss and degradation are likely to contribute to the decline of this species in this western portion of its range. Factors such as oil drilling, water diversion, water withdrawal, and seasonal drought may be contributing to this decline, resulting in the New Mexico Department of Game and Fish (NMDGF) listing *N. erythrogaster* as state endangered (NMDGF 1996). Many areas of the Pecos River drainage in Eddy County no longer contain viable habitat for this species due to hydrological changes resulting from years of ongoing drought and multiple main channel diversions.

The Mexican Gartersnake (*Thamnophis eques*). *Thamnophis eques* is a semi-aquatic snake historically documented from the southwestern United States in New Mexico and Arizona, and throughout central and northern Mexico (Rossman et al. 1996). In New Mexico, *T. eques* is only known from a small number of localities with limited records, restricted to the Gila and San Francisco River systems in Grant and Hidalgo counties. *Thamnophis eques* occupies riparian habitat in aquatic environments including streams, drainage ditches, stock tanks, and ciénegas. It is a highly aquatic gartersnake that relies heavily on proximity to water bodies and is rarely encountered far from aquatic habitat (Degenhardt et al. 1996). Although *T. eques* is well documented as a riparian obligate, in New Mexico this species has been documented as far as 380 m from the main river channel near an irrigation ditch, which suggests that *T. eques* may also occupy more terrestrial habitat than previously thought and may use smaller water sources further from the main channels of the Gila River, such as diversion ditches or stock ponds, for foraging or cover (Geluso 2023). *Thamnophis eques* is diurnal and is generally most active in the morning and late afternoon hours, with peak activity influenced by seasonal temperature and water availability (Degenhardt et al. 1996). *Thamnophis eques* exhibits opportunistic foraging behavior, where its documented prey includes fish and frogs (both tadpoles and post-metamorphic individuals) which are actively hunted along shoreline vegetation and shallow portions of the aquatic habitat (Degenhardt et al. 1996; Emmons et al. 2016). *Thamnophis eques* may rely heavily on ephemeral aquatic habitats which explains its patchy distribution in New Mexico, as populations are highly vulnerable to hydrological changes, drought, water diversions, and the loss of wetlands. Additionally, the presence of invasive species such as the American Bullfrog (*Rana catesbeiana*) and non-native fish heavily alter the dynamics of *T. eques* through predation and/or competition. Due to the paucity of records for this species, *T. eques* was federally listed as endangered in 2014 under the USFWS Endangered Species Act (USFWS 2014). A critical habitat model was developed for *T. eques* in 2021, which includes nine miles of the Gila River in the Cliff–Gila Valley and four miles of Duck Creek (USFWS 2021; Fig. 1).

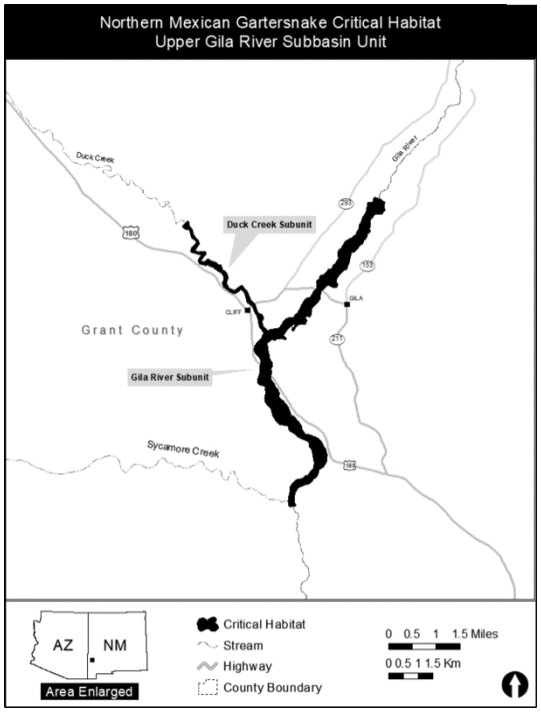

Fig. 1. USFWS Mexican Gartersnake (*Thamnophis eques*) critical habitat designation in the Upper Gila River Subbasin (USFWS 2021).

Environmental DNA (eDNA). Environmental DNA (eDNA) surveys are a rapidly advancing, non-invasive tool deployed to detect wildlife that has been particularly valuable for detecting cryptic, elusive aquatic species or species for which visual encounter surveys (VES) are time-consuming and costly, or in situations in which methods such as trapping yield poor results (Thomsen and Willerslev 2014; Ruppert et al. 2022). Because organisms

continuously shed DNA into their environment through the sloughing of skin cells and mucous and the excretion of urine and feces (Ficetola et al. 2008), this genetic material can be captured from water samples and analyzed to determine species presence (Goldberg et al. 2016; Robinson et al. 2022; Ruppert et al. 2022). For aquatic species, environmental water samples are filtered through fine pores in membrane filters to trap DNA, which is then extracted, amplified via polymerase chain reaction (PCR), purified, and sequenced to verify species identity (Goldberg et al. 2011, 2016; Turner et al. 2014). Species-specificity is ensured through the design of unique primers, often targeting short mitochondrial gene regions such as cytochrome b (*cytb*) or cytochrome c oxidase subunit 1 (*CO1*), which are favored due to high copy number and stability in environmental samples (Rees et al. 2014; Tsuji et al. 2019; Davis et al. 2020). eDNA is a particularly advantageous method for detecting species that are secretive, occupy fragmented distribution, or live in complex or difficult-to-access habitats that are otherwise not ideal for traditional survey methods. Traditional survey methods for snakes, such as VES or trapping, often result in low detection rates, especially in arid regions or during inactive periods such as seasonal drought when snake activity is low. eDNA is non-invasive, time and cost efficient, and can even detect species in the absence of direct visual or auditory encounters (Robinson et al. 2022; Ruppert et al. 2022). Although eDNA surveys do not provide abundance data, they have the potential to provide occurrence data rapidly across broad landscapes such as river systems with multiple drainages. Positive detections can identify sites to target for follow-up sampling with eDNA or sites where traditional methods should be utilized to generate in-hand detections. eDNA surveys are especially relevant for semi-aquatic, cryptic, and elusive species such as *N. erythrogaster* and *T. eques,* both of which are experiencing population declines and are understudied across New Mexico. The use of eDNA is particularly well suited for surveying these snake species in water bodies including rivers, pools, ciénegas, stock tanks, and agricultural drainages because these are habitats where snakes are likely to persist during dry periods and are where traditional surveys may be difficult or disruptive. Furthermore, the nature of these types of environments (e.g., low-medium flow, good cover availability, and high prey density) can lead to the conservation and thus detection of any shed eDNA.

To our knowledge, there has been no use of eDNA surveys to detect *N. erythrogaster* across its range. A report by Fremier et al. (2019) examined the use of eDNA assays as a monitoring tool for *T. eques* in Arizona. Using eDNA, the authors detected *T. eques* at two sites already documented to contain this species, which confirmed the technique's basic suitability for this species. For amphibians and other semi-aquatic vertebrates with aquatic life stages or habitat use, eDNA surveys have resulted in high detection probabilities (Olson et al. 2012; Ruppert et al. 2022). Although natricine snakes do not have aquatic developmental stages, their adults are highly associated with aquatic habitats, which increases the likelihood of eDNA shedding events and thus, the presence of eDNA in those habitats. The initial development of eDNA assays can involve significant investments in primer design, laboratory setup, and testing against non-target species (Smart et al. 2016), but the long-term suitability of this method outweighs its development costs. For this project, eDNA surveys provide a robust approach to detecting the target species and inform our current understanding of the distribution of *N. erythrogaster* and *T. eques* in New Mexico.

Utilizing eDNA surveys will allow us to potentially detect these species at both historically documented and previously unsampled locations, providing robust information to inform future management decisions.

METHODS

Species Occurrence Database. We conducted a comprehensive review of species occurrence records for *N. erythrogaster* and *T. eques* in New Mexico, utilizing data from natural history collections, community science platforms, and state and federal agencies. Databases that aggregate natural history collection specimen records such as VertNet, Arctos Database, Consortium of Vertebrate Collections (CVColl), and the Global Biodiversity Information Facility (GBIF), were queried for specimen holdings relevant to this study. The community science reporting platforms iNaturalist (www.inaturalist.org) and HerpMapper (www.herpmapper.org) were also queried; however, because of these species' imperiled status in New Mexico, iNaturalist records are automatically obscured so we had to follow up with individual users to request the specific details of their observations. Finally, occurrence data for these two species were requested from Natural Heritage New Mexico, the New Mexico Department of Game and Fish, and the U.S. Fish and Wildlife Service. Species occurrence data from these sources were then combined into a single Excel file and if needed, localities were georeferenced using GEOLocate (https://www.geo-locate.org) or Google Earth Pro v7.3.5 software.

Site Selection. Survey sites were chosen based on occurrence records (described above), habitat suitability, and site accessibility. Upon arrival at each site, a 200–500-m transect (depending on the site) was set along the length of the water body using GPS navigation software such as onX (Fig. 2). The full transect was walked to first assess the available habitat for eDNA sampling. If an accessible portion of the river or water body was > 500 m, the transect was split into two sub-sites. Along the transect, a microhabitat was chosen for eDNA sampling, based on habitat characteristics that indicate the likelihood of target species presence such as shallow areas with available cover, available basking spots, and prey presence. The chosen areas were optimized for these conditions that support the presence of the target species, but no one factor excluded a microhabitat from being sampled and the microhabitat was chosen by best judgement. The microhabitat was also chosen based on the likelihood of eDNA being present and limiting degradation factors. For example, we prioritized areas that have some level of flow in the

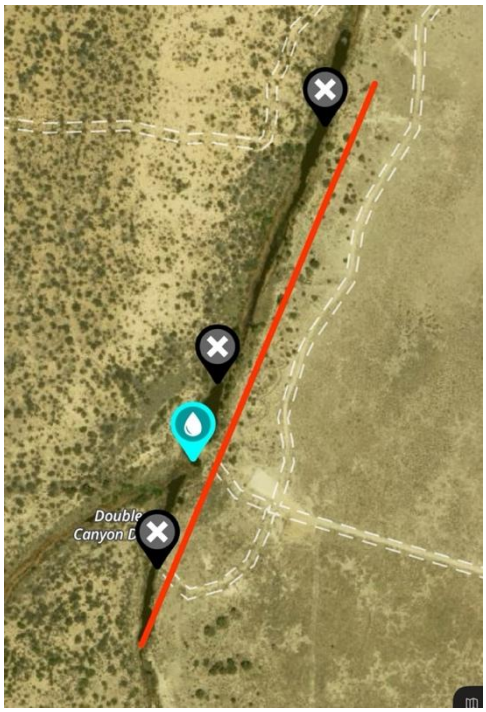

Fig. 2. Example of a survey site. The red line is a 500-m survey transect, the blue pin is where eDNA was sampled, and the black pins represent microhabitat survey plots.

water column and avoided areas that were stagnant, too shallow, highly turbid, and/or exposed to direct sunlight because these conditions may result in increased degradation of any eDNA present. After a microhabitat was chosen, we used a predefined sampling hierarchy: (1) thalweg (main channel), (2) side pool or backwater, (3) vegetated or open bank edge, and (4) emergent marshy zone. Each 1-liter water sample was collected at the safest and most accessible location within this hierarchy. Deviations from the hierarchy or transect design were documented in the site metadata.

Fig. 3. eDNA pump with filter cup attachment.

eDNA Sampling Protocol. A total of 26 sites were sampled for target snake eDNA. At each site, water was collected from five locations within a selected microhabitat to account for the heterogeneous distribution of eDNA and pooled in a sterilized bucket (Turner et al. 2014; Goldberg et al. 2016). The pooled water was slowly poured over a 47-mm diameter Whatman Grade 4 cellulose filter (25–30 µm pore size) placed inside of a 250-mL filter cup and pumped through using a hand-operated fluid extractor (as described in Ruppert et al. 2022; Fig. 3). Filtration occurred as follows: up to 1 L of field-collected water was filtered five separate times per field site as recommended by Fremier et al. (2014). Before filtering field-collected water, 1 L of deionized water was filtered at each field site as a field control (blank). In total, each site visit yielded 6 filters: one field blank and five field samples. Filters were stored in 2-mL tubes with 700 µL of DNAzol, a DNA isolation and buffering reagent. All filtration occurred on-site for immediate preservation. To prevent contamination among sites, nitrile gloves were worn and changed between sites, and all equipment was sterilized with a 50% bleach solution.

Laboratory Analyses. In the lab, eDNA filter extraction will follow an adapted GenCatch Blood and Tissue Genomic Prep Kit protocol. Inhibitor removal kits have been shown to be essential for many eDNA surveys, and a commercial inhibitor removal kit (Zymo) will be used following DNA extraction. Both initial and nested primers are designed to amplify a small segment (<200 base pairs) using a thermocycler. PCR reactions will include master mix, forward and reverse primers, nuclease-free water, and extracted samples. To detect lab contamination, a no-template control will be run in conjunction with the samples. No internal positive control will be included in order to avoid any potential contamination of samples due to the sensitivity of our nested PCR assay. The product from the initial round of PCR will be purified with a PCR Cleanup Kit prior to using it in the nested round. PCR conditions will be specific to the primers being used. Following the completion of the nested PCR, the PCR product will be run on a 1% agarose gel for 40 min at 100 V alongside a 50-bp

ladder, and the gel will be visualized using a UV transilluminator. If samples produce 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 will be pooled and purified. Then, 5 µL of purified PCR product and 5 µL of the reverse nested primer will be sent to Eurofins Genomics for Sanger sequencing. Sequences >95% identical to published target species sequences on NCBI BLAST will result in a positive species detection. If only one band of the correct size was produced after nested PCR, samples will be re-run. Detection results will be analyzed and then mapped using GIS software to visualize the results in a useful manner.

RESULTS TO DATE

***Nerodia erythrogaster* Occurrence Database**. Within New Mexico, *N. erythrogaster* is primarily found on the lower Pecos River and its drainages, including localities along the Rocky Arroyo, Black River, and Delaware River. A total of 72 occurrence records of *N. erythrogaster* from 1901-2024 have been added to our species occurrence database (Fig. 4). Major holdings of *N. erythrogaster* are primarily located at the University of New Mexico's Museum of Southwestern Biology (MSB); specimens are also in the holdings of the Biodiversity Collections at the University of Texas at El Paso (UTEP); the Biodiversity Institute at the University of Kansas (KU); Louisiana State University Museum of Zoology (LSUMZ); the Natural History Museum of Eastern New Mexico University (ENMU); New Mexico State University (NMSU); the National Museum of Natural History of the Smithsonian Institution (USNM); the University of Michigan's Museum of Zoology (UMMZ); and the Natural History Museum of Los Angeles County (LACM). We also used research grade community science records from iNaturalist (iNat).

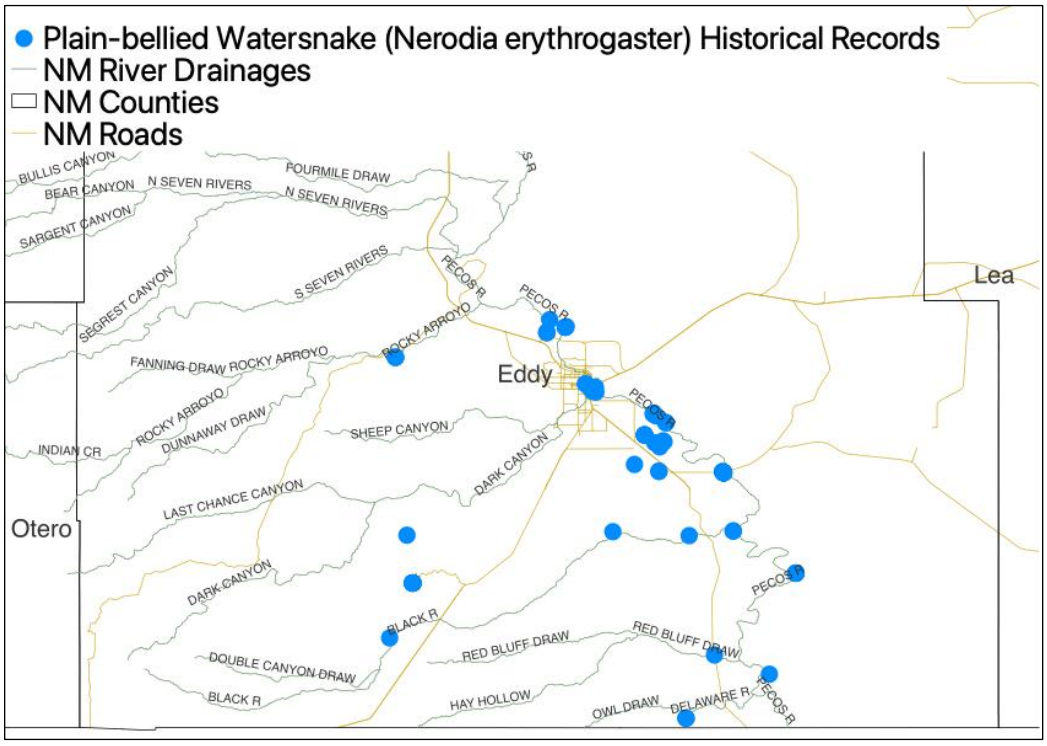

Fig. 4. Map of Plain-bellied Watersnake (*Nerodia erythrogaster*) museum specimens and research grade iNaturalist records from the Pecos River drainage in New Mexico, USA.

On the Pecos River, the northernmost putative occurrence of *N. erythrogaster* is from Major Johnson Springs along Brantley Lake, although no verifiable occurrence record (i.e., museum specimen or "Research Grade" iNaturalist record) exists at this site. W. G. Degenhardt observed *N. erythrogaster* at Major Johnson Springs in 1978 (pers. comm. to B. Christman) but Christman and Kamees (2007) mention that this site was under water at the time of their sampling in 2006, and further investigation of the locality is required to determine if there is continued species presence and/or if viable habitat still exists. Records of *N. erythrogaster* are known from Avalon Lake at or near the pools of the dam (MSB:Herp:23309, 23324, 26267; UTEP:Herp:1090, 1091) with the most recent record being from 2006 (MSB:Herp:73463). One *N. erythrogaster* anecdotal observation exists from 2006 at the spring near the flume at the north end of Carlsbad Municipal Lake (Christman and Kamees 2007). Further downstream on the Pecos River, specimen records exist from Six Mile Dam from 1993 (MSB:Herp:56464) and 2006 (MSB:Herp:73473) and a photographic research grade observation was recently reported in 2024 (iNat 219038777). The Pecos River upstream of Ten Mile Dam (MSB:Herp:19360, 19361) is reported as having the most robust *N. erythrogaster* population, featuring shallow waters and a "divided channel over bedrock" (Christman and Kamees 2007). Another specimen record from the Pecos River (UTEP:HerpOS:366) is located ca. 500 m from Pierce Canyon. There is also a specimen record from "ten miles south of US Highway 285 and junction to Black River Canyon" (MSB:Herp:19368). On the Rocky Arroyo, two *N. erythrogaster* specimens are known from one locality at the NM Hwy 137 (Queens Highway) crossing (UTEP:Herp:2770, MSB:Herp:73476) with the most recent museum specimen collected in 2006. *Nerodia erythrogaster* has also been recorded from Black River drainage upstream from Harkey Crossing on the Black River (UMMZ 121693), at the Higby Hole Road crossing (MSB:Herp:19369), and from Rattlesnake Springs (UMMZ 122941; KU 14177–14183). Records from Rattlesnake Springs are from 1961, and the presence of *N. erythrogaster* has not been confirmed at this locality since then despite extensive reptile and amphibian surveys from 2003–2004 at Rattlesnake Springs (Prival and Goode 2011). Specimen records also exist from 1931 from "2 mi from mouth of Carlsbad Caverns" (KU 13875, 13876, 14177), but finding this locality has proven challenging and suitable habitat to sample has not been located. From the Delaware River, there is only one locality where *N. erythrogaster* was present, which is at the old (destroyed) diversion dam where a single specimen was been collected in 1992 (MSB:Herp:55409) and a photographic research grade observation was reported in 2023 (iNat 166097518). In 2024, as part of surveys for Western Ribbonsnakes (*Thamnophis proximus*) conducted by Eastern New Mexico University researchers, five additional records of *N. erythrogaster* were collected along the Pecos River. The only specimen occurrence record outside of the Pecos River drainage was a single individual collected at an impoundment along Horse Creek in Quay County (MSB:Herp:75841), which is a part of the Canadian River drainage.

Unverified localities of *N. erythrogaster* appeared in my query, with localities such as Colfax and Lea counties. Degenhardt et al. (1996) mentioned that the Lea County records (from NMDGF 1979) are based on misidentified specimens. We are still attempting to track down this citation, as there is no mention of Lea County *N. erythrogaster* in subsequent editions (e.g., NMDGF 1988). Additionally, we submitted queries to verify the identification of a specimen record from Colfax County (MSB:Herp:98939), which was a misidentified *Thamnophis elegans* and not *N. erythrogaster* (T. Giermakowski, pers. comm.).

***Thamnophis eques* Occurrence Database**. Occurrence records of *T. eques* were collected from natural history collections, but there were no community science observations. A total of 62 occurrence records of *T. eques* from 1883–2013 were added to our species occurrence database (Fig. 5). As with *N. erythrogaster*, major holdings of *T. eques* are located at the Museum of Southwestern Biology, University of New Mexico (MSB). Additional holdings are present in the Los Angeles County Museum (LACM); California Academy of Sciences (CAS); Natural History Museum, University of Colorado Museum (UCM); Museum of Vertebrate Zoology, University of California at Berkeley (MVZ); University of Arizona (UAZ); Academy of Natural Sciences of Philadelphia (ANSP); New Mexico State University (NMSU); Louisiana State University Museum of Natural Science (LSUMZ); Peggy Notebaert Nature Museum of the Chicago Academy of Sciences (CHAS); National Museum of Natural History, Smithsonian Institution (USNM); and the Puget Sound Museum (PSM).

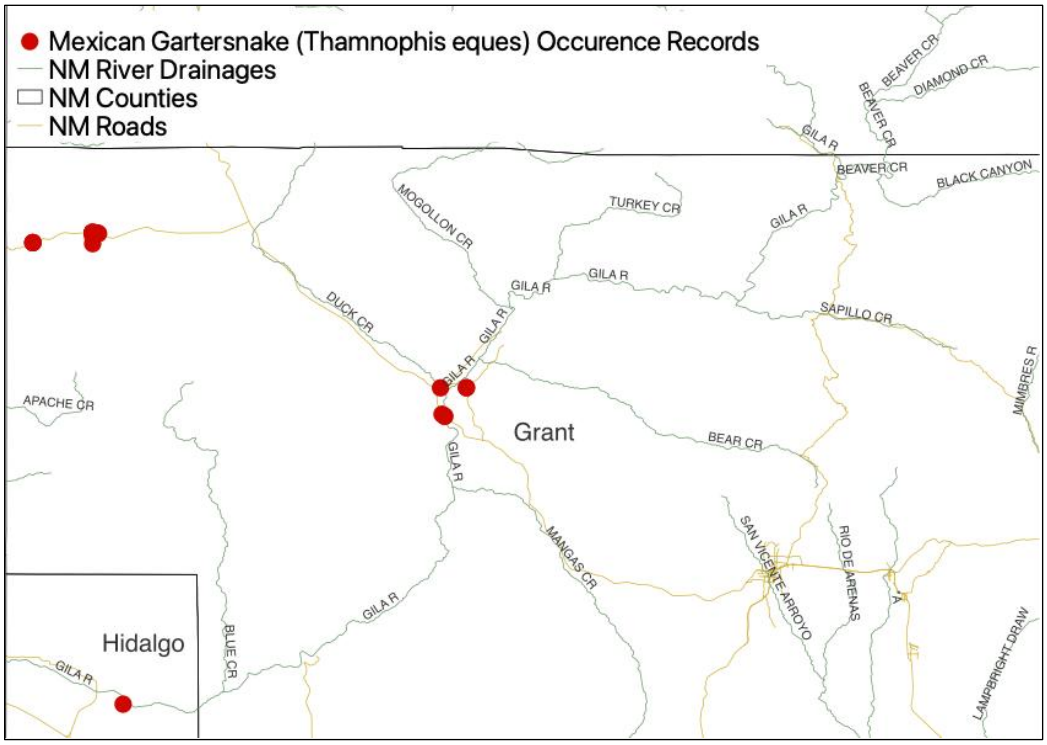

Fig. 5. Map of Mexican Gartersnake (*Thamnophis eques*) museum specimen records from the Gila River drainage in New Mexico, USA.

The earliest record of *T. eques* in New Mexico is from Duck Creek, near its confluence with the Gila River, collected by E. D. Cope in 1883 (ANSP 10688). The most robust historical population of *T. eques* in New Mexico is from Mule Creek, a drainage of the San Francisco River, including localities from "ca. 0.25 miles south of the intersection of Mule Creek and NM Highway 78 " (e.g., MSB:Herp:31991–32016), as well as adjacent localities along NM Hwy 78 (e.g., LACM 123800–123802; CAS 149699, 149700; UAZ 52614). The most recent specimens from this drainage are from 1983 (MSB:Herp:38939) and 1994 (MSB:Herp:57188), which document the species' continued presence in the area through the late 20th century. Currently, all of Mule Creek is privately owned, making updated presence data and habitat assessment difficult to obtain. *Thamnophis eques* has not since been recorded from Mule Creek. Two years of trapping and VES by Albuquerque BioPark staff members (Hotle et al. 2013) yielded no detections of *T. eques* at Mule Creek. In 2002, nearly 20 years after the last recorded *T. eques* occurrence in New Mexico, a researcher documented a photo of a subadult male along the Gila River on a pile of debris near U.S. Hwy 180 in Grant County in the Cliff–Gila Valley (P. Hill, in lit.). In 2013, three male *T. eques* specimens were found on private land along the Gila River in Cliff near the NMDGF Iron Bridge Property by Albuquerque BioPark staff members (MSB:Herp:94819–94821). In 2015, and once again in 2018, K. Geluso collected recently road-killed *T. eques* specimens in the Cliff–Gila Valley (MSB:Herp:99586, 99626; Geluso 2023). In Hidalgo County, a road-killed specimen collected "ca. 5 miles east of the town Virden" in 1973 (NMSU 5377) provides the only verified record from the lower Gila River region. These records confirm a scattered, but persistent, presence of *T. eques* in southwestern New Mexico, with the most recent verified records of this species concentrated in the Cliff–Gila Valley.

Additional specimens were documented from Catron, Chaves, Doña Ana, Lincoln, and Otero counties, but we viewed these putative records with skepticism. We have received photos of the questionable specimens from the Organ Mountains in Doña Ana County (PSM:Herp:04711, 06059, 06060) and discovered that these individuals were misidentified *T. cyrtopsis*. We have also received photos of a Chaves County specimen from "near the head of the North Spring River, ca. 2.5 miles northwest of Roswell" in Chaves County, New Mexico (UCM:Herp:879), but it is poorly preserved and a more confident identification from photographs is challenging; therefore, we have requested a loan of this specimen to examine it in person. A loan of a series of three specimens from the Rio Hondo in Lincoln County (MVZ:Herp:280125–280127) have been requested to re-identify them, but the loan has not yet arrived. Finally, we have requested photos of two specimens from the Mimbres River, Grant County, that were collected in 1944 (CHAS 12445, 12446) and are awaiting a response.

***Nerodia erythrogaster* Surveys**. From 17 May to 13 June 2025, we conducted eDNA surveys for *N. erythrogaster* at 11 sites across the lower Pecos River and adjacent drainages in Eddy County (Fig. 6; Table 1). Sites were selected based on historical museum records, habitat quality, and accessibility. Sampling locations included sites along the Pecos River and its drainages including the Black River and Rocky Arroyo. We targeted locations with historical records of *N. erythrogaster* and replicated sampling at some sites visited by Christman and Kamees (2007). Sites were characterized by a mix of riparian, rocky ledge, and emergent wetland habitats.

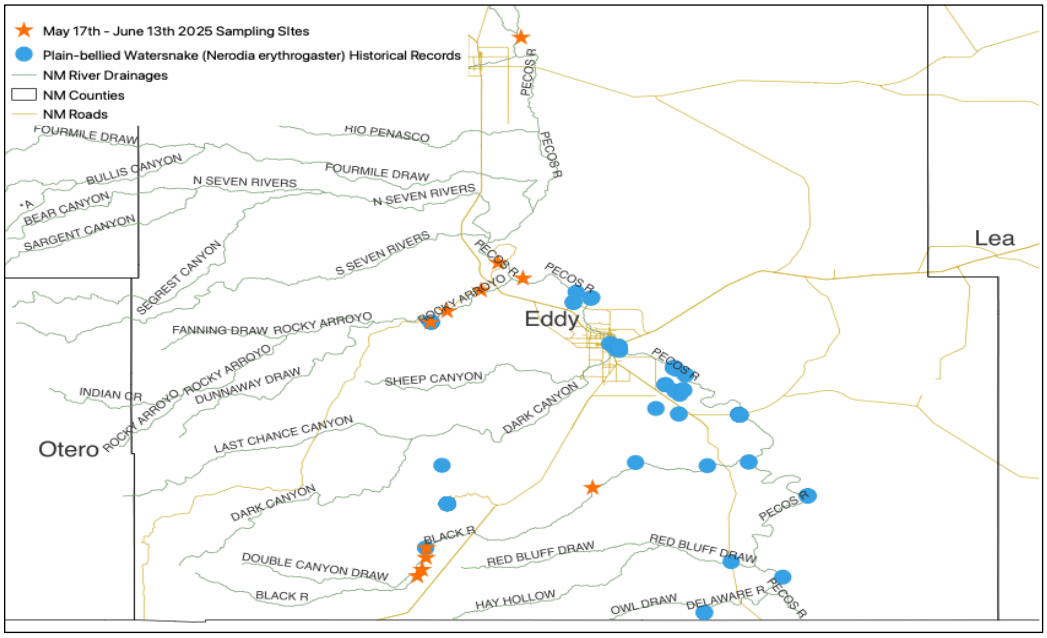

Fig. 6. eDNA survey sites from 2025 (stars) overlaid on a *Nerodia erythrogaster* occurrence record map.

Site #	Site Name	Ownership
1	Pecos River	Public
2	Rocky Arroyo 1	BLM
3	Rocky Arroyo 2	BLM
4	Rocky Arroyo 3	BLM
5	Pecos River, below Brantley Lake	BOR
6	W.S. Huey WMA	NMDGF
7	Black River, at Black River Village	Private
8	Rattlesnake Springs	Carlsbad Caverns NP
9	Black River, Cottonwood Day Use Area	BLM
10	Black River, Headwaters Forks	BLM
11	Black River, near Slaughter Canyon Draw	BLM

Table 1. Sites sampled for *N. erythrogaster* eDNA in Eddy County from 17 May – 13 June 2025, with site name and land ownership.

Fig. 7. Adult female *Nerodia erythrogaster* from Rocky Arroyo 3 (Site 4), Eddy County. Photo by Caden J. Myers.

Fig. 8. Rocky Arroyo 3 (Site 4), Eddy County, showing rocky ledge habitat used by *N. erythrogaster*. Photo by Jacob E. Kuschel.

Fig. 9. The Pecos River (Site 2), Eddy County. Photo by Jacob E. Kuschel.

Fig. 10. An adult *Thamnophis marcianus* from the Pecos River (Site 2), Eddy County. Photo by Caden J. Myers.

Several Black River sites contained cold, deep pools with steep embankments, but lacked rocky ledge structure. In contrast, sites on the section of the Pecos River between Brantley Lake and W.S. Huey WMA showed signs of degraded bank structure, high turbidity, and heavy anthropogenic use. During a scouting trip on 14 June 2025, we visited two

historical *N. erythrogaster* sites. The first site was the Pecos River site where Christman and Kamees (2007) recorded *N. erythrogaster*. This site contains viable habitat for *N. erythrogaster*. The habitat is characterized by flowing pools over a bedrock, with large rocks and concrete slabs, and bordered by riparian vegetation. No live *N. erythrogaster* were observed, but we did find a shed skin from an adult, confirming their continued presence. We also visited another historical site, the Pecos River Flume, that had a few small, shallow pools containing minimal suitable habitat features and a sparse prey base. This area has the potential to fill up after the monsoon season, but it seems unlikely that a population of *N. erythrogaster* exists here. The presence of fragmented habitat and the potential for hydrological rebound warrants further surveys for this location.

***Thamnophis eques* Surveys**. From 26 May–2 June 2025, we conducted eDNA surveys targeting *T. eques* at 15 total sites along the Gila River and its associated drainages in the Cliff–Gila Valley region of southwestern New Mexico (Fig. 11; Table 2). Sites were selected based on historical occurrence data, accessibility, and presence of viable habitat.

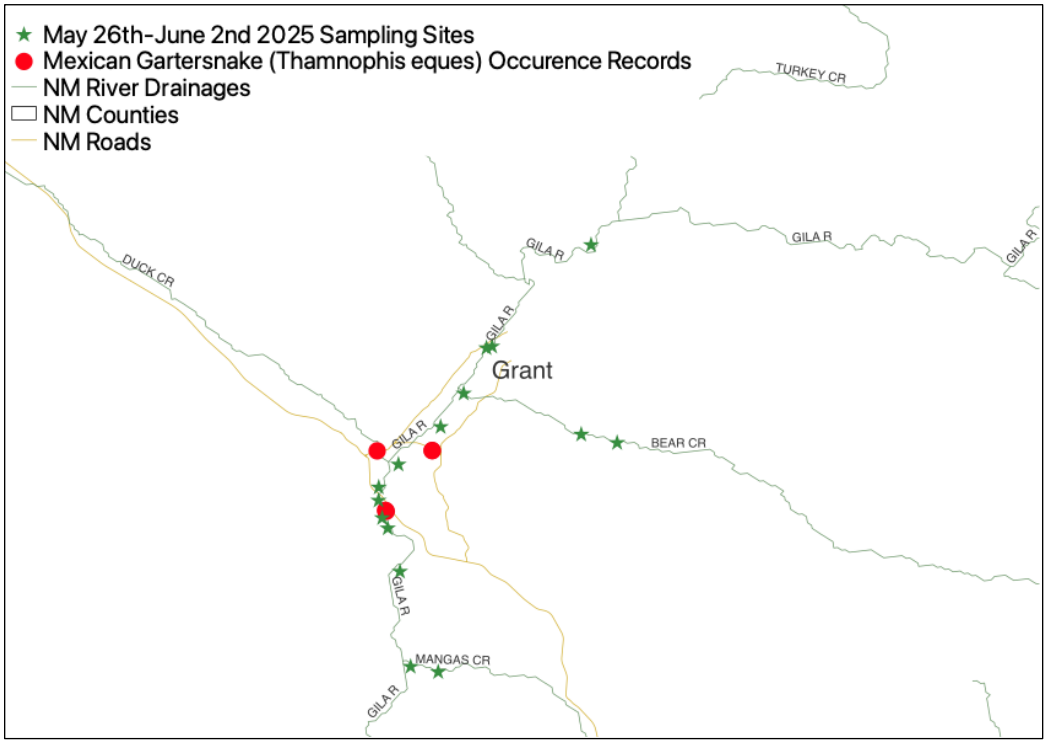

Fig. 11. eDNA survey sites from 2025 (stars) overlaid on a *Thamnophis eques* occurrence record map.

Site #	Site Name	Ownership
1	Gila River	NMDGF
2	Gila River	NMDGF
3	Gila River	NMDGF
4	Runyan Property, upstream	Pacific Western Land Co.
5	Gila River, Mangas Creek	Pacific Western Land Co.

6	upstream Mangas Creek, Bill Evans Lake	NMDGF
7	Gila River Preserve, pond	The Nature Conservancy
8	Upper Gila Diversion Ditch	The Nature Conservancy
9	Runyan Property, downstream	The Nature Conservancy
10	Gila River	US Forest Service
11	Gila River, upstream of Gila River Farm	The Nature Conservancy
12	Bear Creek, upstream	NMDGF
13	Bear Creek, downstream	NMDGF
14	Gila River	Pacific Western Land Co.
15	Gila River, at state land	Pacific Western Land Co.

Table 2. Sites sampled for *T. eques* eDNA in Grant County from 26 May – 2 June 2025. Site name and land ownership are included.

Surveyed sites included multiple locations along the Gila River, near the NMDGF Iron Bridge Property (Fig. 12) and three properties owned by The Nature Conservancy (TNC): Runyan (Fig. 13), Agnew, and Lichty. The Pacific Western Land Company gave permission to conduct eDNA surveys, allowing access to sites that had not been previously sampled for this species or otherwise surveyed for habitat data. Water samples were also taken from Gila River drainages such as Mangas Creek and Bear Creek, with no known occurrence records but viable habitat suitable for *T. eques*. We also sampled at smaller aquatic habitats such as a pond on the Gila River Preserve and an upstream diversion ditch that is also situated on this property (part of the Lichty Ecological Center), as these may be sites utilized by *T. eques* to forage when the main river channel is diverted throughout the summer months, especially during the dry season. Site transects ranged from 250–500 m in length and included five sampling points within one microhabitat per site, covering a wide range of microhabitats such as bank edges, emergent vegetation patches, side pools, and shallow thalweg river points. eDNA samples were collected at each randomized habitat plot along the transect. The sampled habitats included shallow, slow-moving water with emergent or overhanging vegetation in permanent water bodies with high prey availability. We prioritized sites near recent occurrences of *T. eques* that are included in the Designated Critical Habitat (Fig. 1). We visited Mule Creek to scout potential sites for eDNA sampling, but much of Mule Creek is now dry, and the only remaining portions of this creek with potential habitat are owned by private landowners who denied my requests to sample on their lands.

Fig. 12. The Gila River at The Nature Conservancy's Runyan Property in the Cliff–Gila Valley, Grant County. Photo by Jacob E. Kuschel.

Fig. 13. The Gila River at the NMDGF Iron Bridge Property in the Cliff–Gila Valley, Grant County. Photo by Jacob E. Kuschel.

During these recent surveys, survey sites exhibited signs of intense cattle grazing, including bank trampling, vegetation loss, and high turbidity. Heavy livestock use in riparian zones has been associated with habitat degradation for gartersnakes by reducing cover, compacting soils, and eliminating emergent vegetation for thermoregulation and foraging (Fleischner 1994; Rossman et al. 1996; Jones 2000). These compounded pressures, including predation by invasive species, loss of microhabitat, and grazing impacts may contribute to the observed scarcity of *T. eques* and other native snake species during this survey window.

eDNA Assay Validation. Successfully validating our eDNA assays for each of the target species is a crucial step, as we must ensure assay sensitivity and species specificity. For *N. erythrogaster,* we generated a comprehensive primer comparison matrix using mitochondrial gene regions (*cytb*, *ND1*, *ND2*, *ND4*, and *12S*) and targeted sequence differences between *N. erythrogaster* and both congeneric and sympatric species, all to ensure that the assay specificity. Out of multiple tested primer pairs, the *ND4* primer set successfully amplified *N. erythrogaster* DNA, but also DNA from *N. rhombifer*. However, because *N. rhombifer* does not occur in our study area, the *ND4* primer set may still serve as a valid detection tool. Other primer sets, including *cytb1*, *cytb2*, and *ND1*, failed to amplify *N. erythrogaster* or showed weak amplification of sympatric, non-target species. Based on these results, we selected *ND4* primers for further validation. To confirm assay sensitivity, we acquired a tissue-derived positive control from a captive *N. erythrogaster*, and plan to quantify DNA using a Qubit fluorometer. This quantified sample will be serially diluted to quantify the level at which the primers still amplify the target species DNA. We will then apply these results to an eDNA control gathered from water from a captive *N. erythrogaster* mixed with water from similar aquatic environments in which this species exists to simulate the degraded DNA levels that are typically found in aquatic environments. Amplification across dilution gradients will be assessed to establish the assay's detection threshold under field conditions, following protocols used to mimic eDNA in studies (Goldberg et a. 2011; Thomsen et al. 2012).

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. Unfortunately, these primers showed cross-amplification with other sympatric and congeneric species, including *T. cyrtopsis* and *T. elegans*, both of which occur in the Cliff–Gila Valley. A collaborator 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, two alternative pathways are being pursued. First, we plan to generate additional mitochondrial sequence data from *T. eques*, *T. cyrtopsis*, and *T. elegans* tissues in our collection to improve the resolution of primer design. Second, we are exploring restriction fragment length polymorphism (RFLP) assays. This approach uses shared primers to amplify *ND2* fragments, which are then digested with species-specific restriction enzymes to

produce patterns. We identified enzymes capable of uniquely cutting *ND2* amplicons of each target species, making this a promising direction for differentiating *T. eques* from congeners without sequencing.

FUTURE PLANS

We plan to continue to sample eDNA at additional sites for *T. eques* and *N. erythrogaster* to complete a robust sampling site list in the late summer (July/August) and early fall (September) of 2025 to improve the spatial and temporal coverage and strengthen the potential for species detection during different seasonal windows. In-between trips to collect eDNA samples, we are continuing to develop and validate these eDNA assays, focusing first on the *N. erythrogaster* assay. Future steps include testing primers against known eDNA-positive sites, determining the limit of detection for DNA concentrations, and validating a set of nested primers to increase eDNA detectability. Similar steps will be conducted for the *T. eques* eDNA assay in Fall 2025.

For *T. eques*, several priority sites in the Gila region have yet to be sampled and will be the focus of post-monsoonal surveys, including sites within the 2021 FWS critical habitat map. This includes the lower reaches of the Gila River near Virden, the Gila River downstream of the NMDGF Iron Bridge Property, the TNC Lichty Property, and downstream of the NM Hwy 211 bridge where habitat looks promising. We will be investigating additional irrigation ditches and stock tanks with abundant amphibian prey that run along the river on agricultural properties such as the TNC Agnew Farm. A trip is scheduled for September 2025 on the TNC Runyan Property.

For *N. erythrogaster*, additional surveys are planned along unsampled stretches of the Pecos River, Black River, and Delaware River in Eddy County. Known but unsampled habitats from historical records such as Six Mile Dam, Ten Mile Damn, Harkey Crossing, and crossings along Haroun Road, Potash Mines Road, Longhorn Road, and Higby Hole Road will also be prioritized. We have been told that the Delaware River may currently be dry due to fracking activities in Texas (B. Christman, pers. comm.), although we still plan to investigate this location. Post-monsoon sampling will offer the opportunity to revisit sites that contained little water during the dry season and target sites likely to benefit from increases in water availability. Seasonal timing for eDNA surveys is critical, and surveys will be planned shortly after monsoonal rainfall but before cold temperatures reduce snake activity and eDNA shedding rates. Late summer also coincides with increased amphibian and fish activity, potentially increasing snake detectability due to increased foraging.

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