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December 15, 2017

To: **Mr. John Roberts**
Executive Director
The Natomas Basin Conservancy
2150 River Plaza Drive, STE 460
Sacramento, CA 95833

Re: Giant Gartersnake (*Thamnophis gigas*) Environmental DNA and trapping surveys at the Natomas Basin Conservancy's Cummings Tract, Sacramento County, California

Dear Mr. Roberts,

This report summarizes surveys conducted during August and September of 2017 to assess the presence of giant gartersnakes (GGS) on the Natomas Basin Conservancy's (TNBC) Cummings Tract (tract) in Sacramento County, California. Methods included a mixed approach of visual encounter surveys (VES) and aquatic trapping surveys, the latter utilizing adaptive Bayesian protocols intended to address low detection probabilities associated with the species' cryptic and wary nature. However, because even rigorous trapping has failed to detect GGS occurring at low or diminishing densities in other parts of its range, we also applied recently-developed environmental DNA (eDNA) sampling techniques in the hope of increasing survey sensitivity.

Although the fully-executed trapping surveys failed to detect individual GGS, GGS DNA was detected in five of the eleven locations sampled on the tract. While eDNA techniques do not allow us to determine demographic characteristics such as population size, size/age or sex distribution, they do provide information regarding spatial distribution. Results of these surveys indicate GGS utilization on three of the four wetland cells and the southwestern well-water supply ditch at the time samples were collected. Because these samples were acquired simultaneously, and because DNA persistence in water is expected to be of relatively short duration, these results likely indicate utilization by multiple GGS. Finally, while it is possible to contaminate a site and provide misleading results with DNA from another source (e.g., via traps and equipment or source water occupied by the species of interest), the GGS DNA detected in the southwest well water supply channel was not exposed to any known contamination. Although measures were implemented to avoid contamination at all sites, this result demonstrates that some DNA is emanating from GGS utilizing features on the tract.

1. STATEMENT OF PURPOSE

Despite regular, annual efforts, standard aquatic trapping procedures have yet to verify GGS presence on the tract despite persistent regional presence and anecdotal information suggesting they may occur there. To assess occupancy, we have combined a newly-developed eDNA sampling approach with traditional trapping to increase the probability of detecting GGS in areas where animal density and probability of detection are extremely low. Not only is eDNA sampling more cost effective than intensive trapping, but probabilities of detection are substantially higher, thus providing the potential for achieving more comprehensive spatial coverage and increasing the likelihood of accurate results. Such results are needed to manage the tract for maximum species benefit under the Natomas Basin Habitat Conservation Plan.

2. METHODS

The sampling approach was two-tiered, designed to ascertain the presence, distribution, and relative abundance of GGS on or near the tract. Methods included a mixed approach of visual encounter surveys (VES) and aquatic trapping surveys, the latter utilizing adaptive Bayesian protocols and trap modifications intended to address low detection probabilities associated with the species' cryptic and wary nature (Halstead et al. 2011). Because even rigorous trapping can fail to detect GGS occurring at low or diminishing densities, we also applied recently-developed environmental DNA (eDNA) sampling techniques as a means of increasing survey sensitivity (Schumer et al. *in review*).

2.1. Trapping and VES

Both trapping and VES were conducted from August 24 to September 9, 2017. Visual encounter surveys included walking or kayaking along channels, wetlands, and nearby upland areas to search for basking and/or foraging snakes. These surveys were conducted incidental to all trap-checking activities and occurred primarily during the morning and early afternoon while snakes bask to attain active temperatures.

Aquatic trapping was conducted by placing 300 floating modified minnow traps divided into twelve, twenty-five-trap transects equally distributed within each of the four wetland units. Traplines were placed only where water was sufficiently high to ensure that traps were continuously wetted, thereby reducing the risk of desiccation or thermal stress for entrained snakes. We used galvanized 4-mesh eel pots (Tackle Factory [Cuba Specialty Manufacturing], Fillmore, New York, USA)

modified to float following the procedures in Casazza et al. (2000). Trapping methods were implemented to increase detection probability by decreasing escape rates. Flexible screen mesh covers extending below the waterline will be used on the aperture of each trap funnel to reduce escape rates (Hansen, unpublished data).

Data recorded for each trap location included UTM coordinates and environmental characteristics, (e.g., vegetation and substrate types). Water metrics including pH, specific conductivity (EC), and water temperature were measured at each wetland cell using a portable YSI 556 Multi-Probe unit. Wherever traps remained in place without interference, organisms within the traps were identified and counted at pre-determined intervals to compare prey composition between trap/transect sites.

Surveys were conducted per the terms and conditions of US FISH AND WILDLIFE SERVICE RECOVERY PERMIT 10(a) (1) (A) ESA TE-018177-7 (valid through 08/06/2019) and DEPARTMENT OF FISH AND GAME SCIENTIFIC COLLECTING PERMIT 003881 (valid through 04/16/2018).

2.2. eDNA

Water samples were collected for eDNA analysis on September 7, 2017. Environmental DNA field sampling and laboratory protocols followed procedures described in Bergman et al. (2016) and refined for this species through previous field trials (Schumer et al., *in review*). Sampling locations and timing were selected to: 1) maximize spatial coverage relative to aquatic feature extent and degradation distance established at other sites 2) allow ample degradation time to reduce the likelihood of contamination from equipment deployed as part of previous survey efforts, and 3) assess DNA presence in source water and inlets to all wetland cells to insure against potential DNA contamination emanating from off-site or upstream sources. For example, traps were deployed as part of a separate effort just before our trap surveys began. We attempted to reduce the likelihood of contamination from previously deployed equipment by waiting fifteen days to allow for DNA degradation before collecting water samples for eDNA analyses. For our own work, we used only new or bleached equipment to avoid transmitting genetic material from other sites. We also waited two weeks after surface water deliveries were curtailed and the system was flushed with well water before collecting samples. Finally, we assessed results for each wetland cell by testing its headwater, thus ensuring that any DNA detected downstream was not simply emanating from a single upstream point source.

For each sampling event, water was filtered directly from the water body at an approximate depth of six inches below the surface using sterile Saint Gobain XL-60 silicon tubing (Tygon®; internal diameter 6.3mm), and a portable Masterflex1

L/S Easy-Load II peristaltic pump (Cole-Parmer®) powered by a cordless hand drill. Water samples were filtered through a Millipore Sterivex™-GP 0.45µm sterile filter unit (EMD Millipore).

No water was transported or stored during sampling nor was any water transported between sampling sites; instead all filtration occurred *in situ* at each site. Sample filtrate was captured and measured in graduated flasks to verify the volume of each sample. Filtered water was poured out after completion of sampling at each site. To eliminate cross contamination between sites due to equipment or the investigator, sterile gloves and all sampling materials were pre-packaged and discarded after one use. Tubing and gloves were immediately disposed of after each use into a sealed trash bag. All filters were likewise considered single use. After filtration, the cylindrical filters were capped at each end, labelled with location ID, placed into a sterile secondary container, sealed, and immediately placed on ice. All filters were kept on ice in a cooler for the duration of the sampling event, after which they were transferred to a -20°C laboratory freezer. The filters were stored within individually sealed secondary containers at -20°C until DNA extraction.

DNA field controls were taken to ensure that field equipment was free of contamination. Each field control consisted of Sterivex™ filtered ultra-pure water processed in the same fashion as the field samples. The field controls were processed for the presence of GGS DNA in parallel with all samples. DNA extractions were conducted using PowerWater Sterivex™ DNA Isolation Kit (Mo Bio Laboratories, Inc.) following the manufacturer's recommended guidelines. A DNA extraction negative control was processed in parallel to ensure sample integrity throughout extraction procedure. The DNA extraction control consisted of Sterivex™ filtered ultrapure water only. DNA extraction controls were processed using the same equipment utilized to extract DNA from all samples. Each sample and all controls were analyzed in triplicate for the presence of the giant gartersnake DNA using a quantitative polymerase chain reaction (qPCR) primer (PCR is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude) and probe set developed by Cramer Fish Sciences (Schumer et al., *in review*).

Each qPCR replicate consisted of a 5 ul reaction volume. Each 5 ul qPCR reaction was composed of 1x Applied Biosystems TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems™), 900nm final primer concentration, 60nm final probe concentration, and 1 ul DNA template. Thermocycling was performed using a Bio-Rad CFX 96 Real time System (Bio-ad Laboratories, Inc.) with the following profile: 10 minutes at 95°C, 40 cycles of 15 second denaturation at 95°C and 1-minute annealing-extension at 60°C. Six template control (NTC) reactions were run on the plate with the samples template controls consisted of 1ul of ultrapure water replacing DNA template within reaction volume. Three

positive control reactions consisting of 20ng/ul giant gartersnake DNA template were also tested in parallel to ensure consistent PCR performance. All PCR master mixes were made inside a UV PCR enclosed workstation. DNA template was added to master mix outside of the UV PCR workstation on a dedicated PCR set up workbench. All PCR reactions were conducted on instruments located outside of the main lab in a separate portion of the building. Results of the qPCR reactions were analyzed using BioRad CFX manager v3.1 (Bio-Rad Laboratories, Inc.). A sample was considered positive for the presence of GGS DNA if any one of the three replicates showed logarithmic amplification within 40 quantification cycles (Cq).

3. RESULTS

Trapping surveys resulted in the accrual of 4,500 total trap days. Survey efforts were constrained to some extent by habitat conditions and other factors. For instance, shallow, vegetated seasonal wetland where we intended to use drift fence were insufficiently wetted for trapping during the survey period. Also, diminished water levels made it difficult to place traps in close association with vegetation. Regardless, surveys were conducted at the intended intensity without interruption in all the wetland units. Sampling locations and results are depicted in Figure 1, below. Trap survey dates and trap locations (i.e., geocoordinates) are summarized in Appendix B, Table 1. Environmental DNA survey locations and results are summarized in Appendix B, Table 2.

No GGS were observed or captured during any VES or trapping survey. Although the fully-executed trapping surveys failed to detect individual GGS, GGS DNA was detected in seven unique locations on the southern half of the bank. While eDNA techniques do not allow us to determine demographic characteristics such as population size, size/age or sex distribution, they do provide information regarding spatial distribution. Results of these surveys confirmed GGS utilization on three of the four inundated aquatic GGS cells present on the bank at the time samples were collected. Environmental DNA sampling sites and results are depicted in Figure 1. Environmental DNA sampling locations and results are summarized in Appendix B, Table 2.

Water chemistry metric ranges were consistent with those supporting giant gartersnake at occupied sites throughout the species' range (Hansen et al. 2011). Water chemistry measurements obtained in 2017 are reported in Appendix B, Table 3. Composition of prey species collected in traps was comparable to that observed at other occupied sites (e.g., Hansen et al. 2011). Prey species observed included crayfish (*Procambarus clarkii*) and adult and larval bullfrogs (*Lithobates catesbeianas*). Other common species observed included black basses (*Micropterus* spp.) and sunfish (*Lepomis* spp.). Capture per unit effort (number of organisms per trap day; CPUE) of bycatch is provided in Appendix B, Table 4.

Figure 1. 2017 trap and eDNA sampling locations and results on the TNBC Cummings Tract

4. DISCUSSION

Environmental DNA methods provide a means of addressing limitations of visual and trapping surveys, because they 1) are cost-effective and feasible to deploy over a large survey area, 2) unambiguously identify target organisms and 3) are sensitive to trace amounts of DNA in sampled material (Jerde et al. 2011; Thomsen et al. 2010). Given that molecular diagnostic techniques may be more sensitive than traditional methods (Wilcox et al. 2016), information from eDNA can be used to obtain the critical presence/absence data that trapping surveys may fail to provide.

A clear progression in sampling technique and detection rates has occurred in areas where GGS are declining or persist at very low density. For example, from the 1940s through the 1970s and 1980s, GGS in the San Joaquin Valley occurred at densities facilitating routine observations through VES (G. Hansen 1988, 1996). When VES failed to produce detections, intensive trapping surveys confirmed presence at many sites (Dickert 2005), though detections have since diminished to the point that GGS now are presumed extirpated throughout much of the San Joaquin Valley (E. Hansen 2008, USFWS 2012). Techniques utilizing eDNA confirm that GGS are still present at some sites where intensive trapping has been unsuccessful (e.g., Hansen 2017). Techniques utilizing eDNA are now proving useful for elucidating patterns of occupancy with improved accuracy throughout the species' putative range.

The presence of cryptic species is ascertained by using molecular genetic assays to detect DNA that has been shed into the environment. The eDNA approach differs from traditional sampling in that a given survey does not capture the target organisms themselves, but the biological material those organisms leave in their vicinity that contain a "signal" of their genetic identity. Organisms liberate DNA into their surrounding environment by leaving behind indicators such as slime, scales, epidermal cells or feces (Janosik and Johnston 2015). Biological material containing DNA can be captured and isolated from water (or soil) samples, where purified total DNA can be interrogated for specific species of interest through use of molecular techniques (Jerde et al. 2011). While eDNA techniques do not allow us to determine demographic characteristics such as population size, size/age or sex distribution, they do provide information regarding spatial distribution. Such information is not only vital for evaluating occupancy, but can also be used to refine search areas and improve the efficiency of demography studies. The high detection probabilities of eDNA methods makes this approach suitable for monitoring the performance and compliance of species protection efforts.

GGS are a secretive and evasive species occurring at low density in some locations. Surveys addressing current distribution and occupancy must, therefore, include survey and analytical methods (e.g., Halstead et al. 2009, 2011) that account for low expected detection probabilities. However, detection probabilities associated with trapping surveys may be inadequate when population densities are exceptionally low. Trapping is also hindered by theft and tampering in areas of public access,

potentially impacting survey results and endangering the health of the animals present in the census population.

Although the fully-executed trapping surveys and VES surveys failed to detect GGS at the tract, GGS DNA was detected at five of the eleven locations sampled, confirming GGS utilization on at least three of the four inundated wetland units at the time samples were collected. Enhanced survey method sensitivity, such as that demonstrated here, will improve project and recovery planning for GGS. While these methods did not exist when the Natomas Basin Habitat Conservation Plan was developed, this work suggests they could be implemented as part of a comprehensive monitoring program improving survey sensitivity and interpretation of population status in the future.

Thank you for providing the opportunity to contribute to this important work. Please feel free to contact me if you have questions or require additional information.

Sincerely,

Eric C. Hansen
Consulting Environmental Biologist

5. REFERENCES

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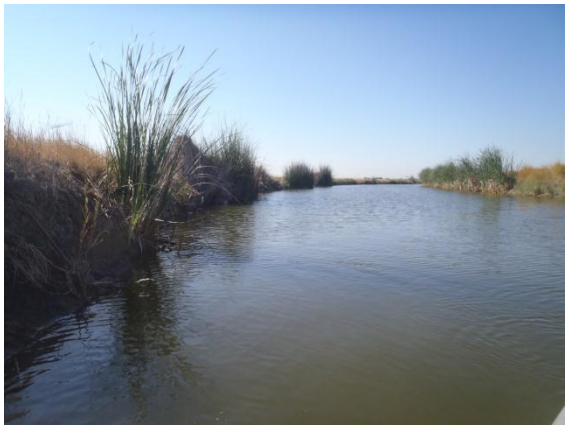
1. NA460 8/24/2017

2. NA460 9/8/2017



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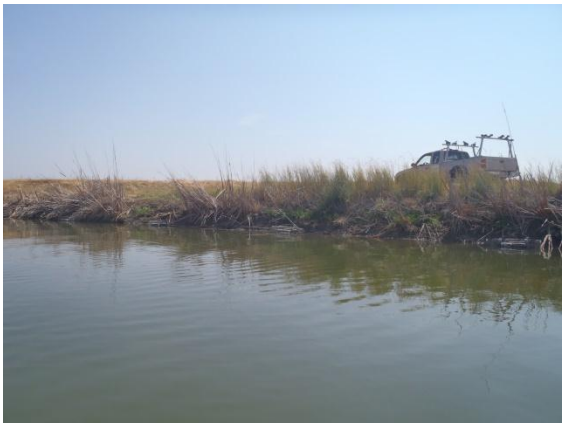

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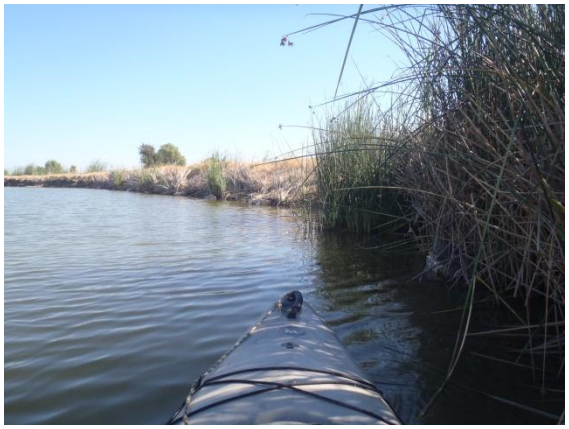

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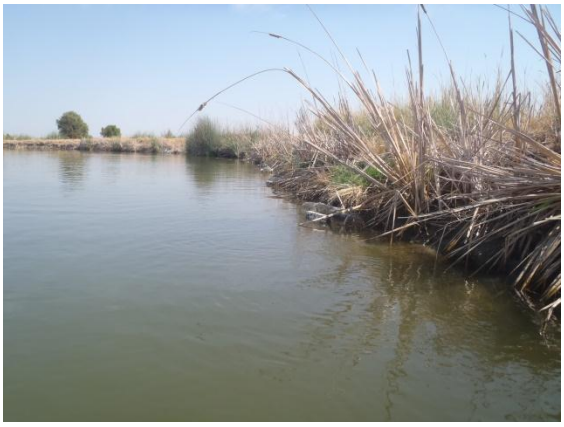

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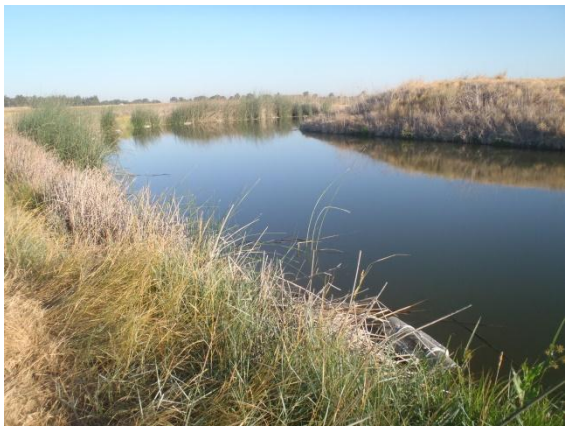

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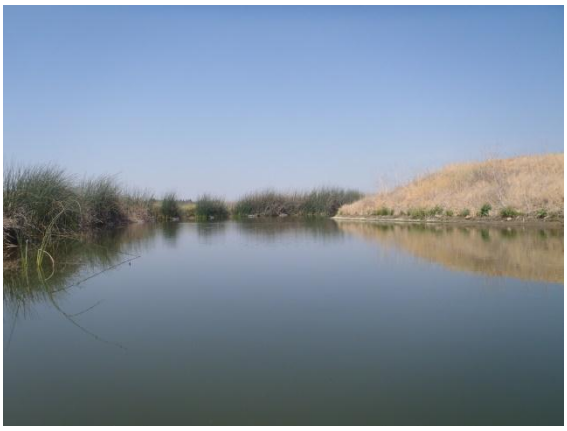

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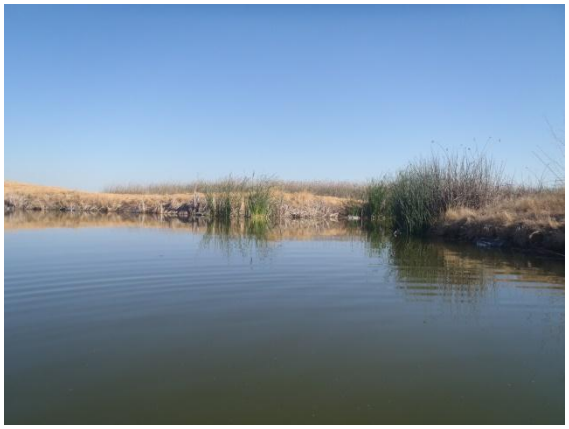

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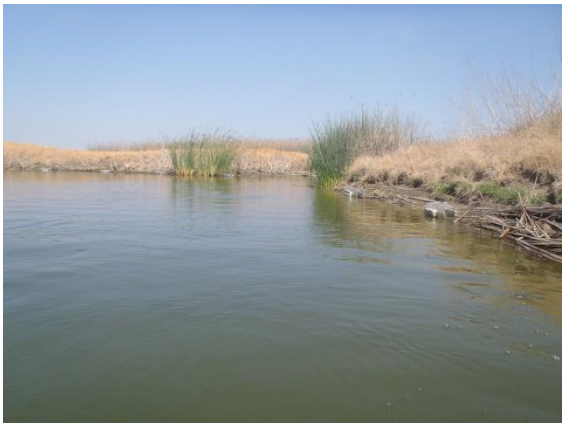

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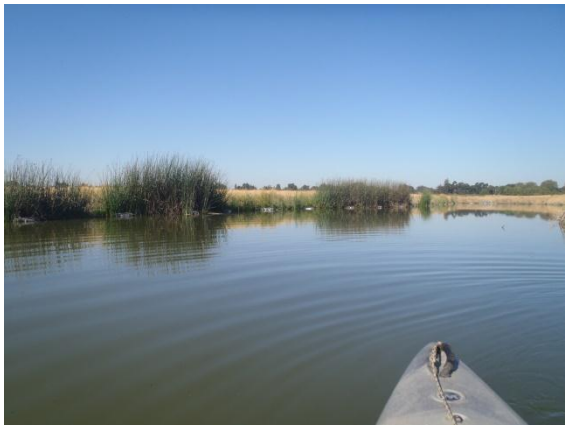

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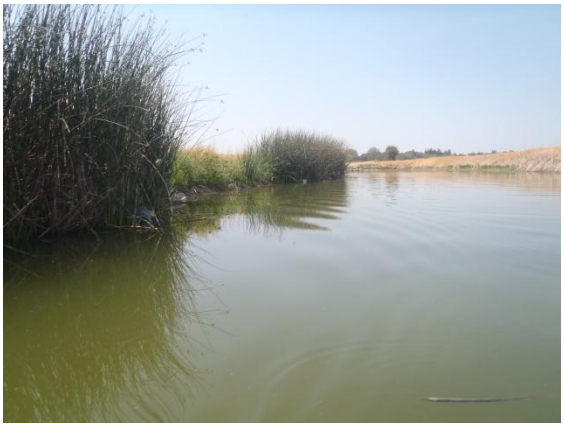

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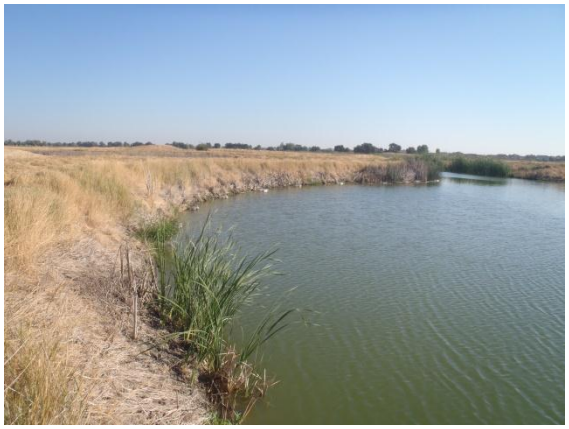

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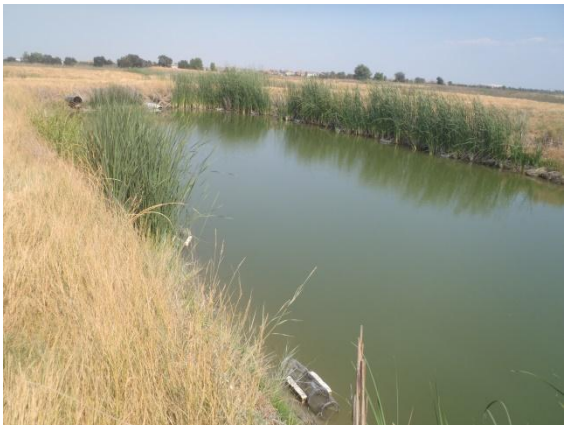

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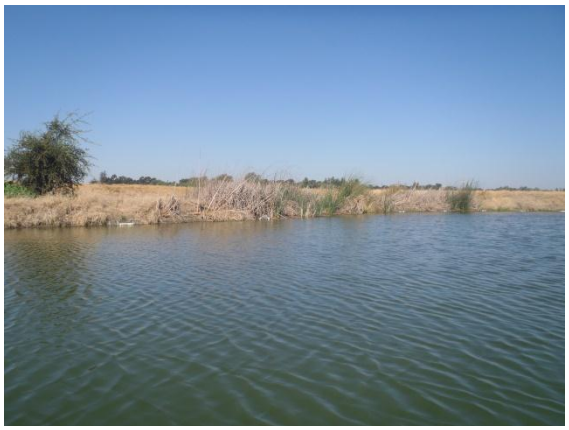

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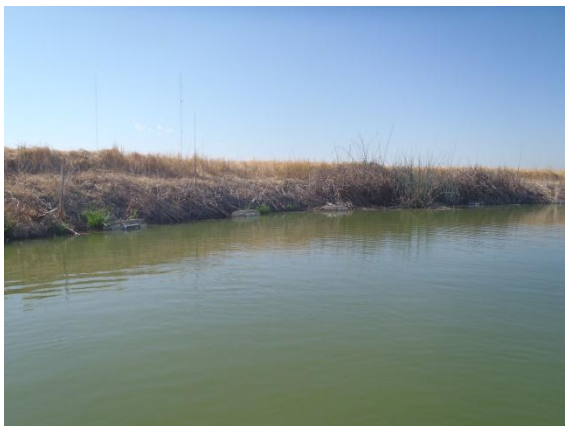

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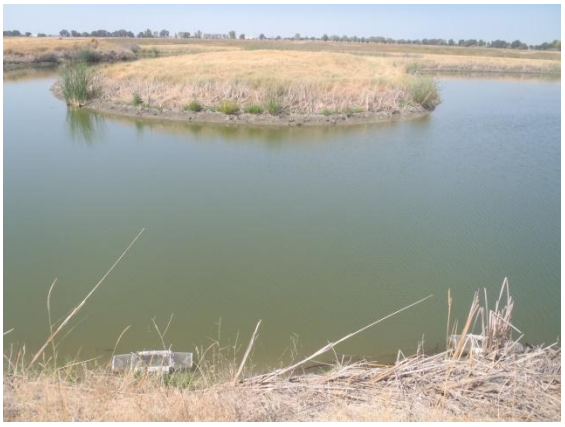
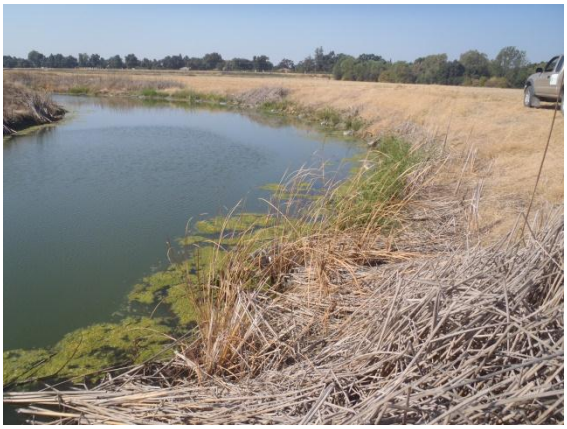


18. NA468 9/8/2017

19. NA469 8/25/2017



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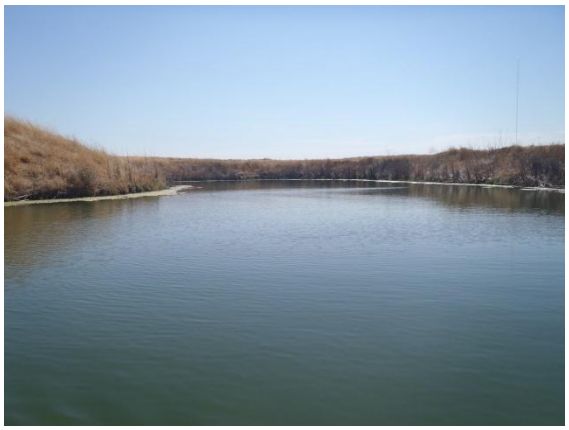

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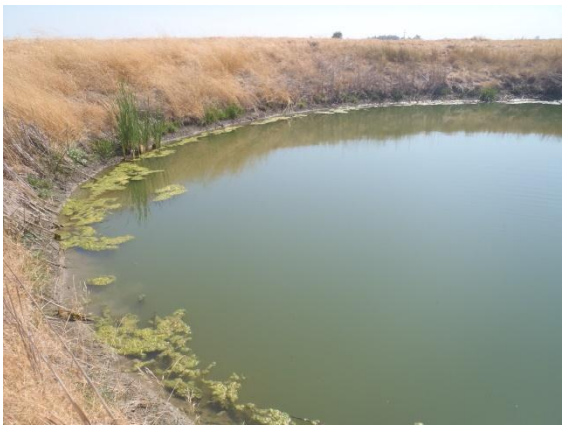

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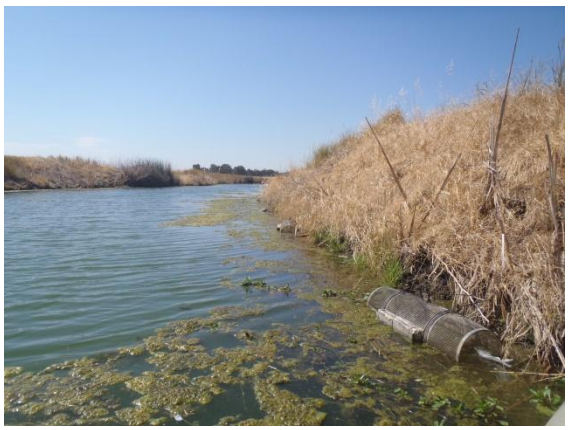

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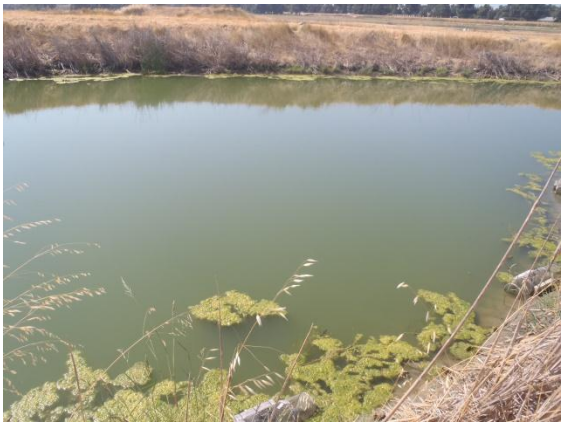

24. NA471 9/9/2017

Table 1: 2017 25-trap GGS trapping quadrat location descriptions and geo-coordinates[1] at TNBC's Cummings Tract

Quadrat ID	Location Description	Dates	Centroid Easting	Centroid Northing
NA460	Cell D	8/24/2017-9/8/2017	625973	4277994
NA461	Cell D	8/24/2017-9/8/2017	626008	4277895
NA462	Cell D	8/24/2017-9/8/2017	625965	4277876
NA463	Cell C	8/24/2017-9/8/2017	625778	4278006
NA464	Cell C	8/24/2017-9/8/2017	625867	4277913
NA465	Cell C	8/24/2017-9/8/2017	625890	4277795

[1] UTM NAD 83

NA466	Cell B	8/24/2017-9/8/2017	625703	4277865
NA467	Cell B	8/24/2017-9/8/2017	625680	4277965
NA468	Cell B	8/24/2017-9/8/2017	625638	4277960
NA469	Cell A	8/25/2017-9/9/2017	625508	4278013
NA470	Cell A	8/25/2017-9/9/2017	625513	4277874
NA471	Cell A	8/25/2017-9/9/2017	625600	4277891

Table 2: 2017 eDNA sampling site labels[2], results, and geo-coordinates[3] at TNBC's Cummings Tract

Feature_ID	Detection	Easting	Northing
Well Supply 1	Y	625348	4277785
Well Supply 2	Y	625446	4277786
Cell A1	N	625465	4277787
Cell A2	N	625593	4277847
Cell A3	Y	625555	4278032
Cell B1	Y	625753	4277941
Cell B2	N	625828	4277799
Cell C1	N	625834	4277914
Cell C2	N	625909	4277958
Cell D1	N	626007	4277828
Cell D2	Y	626010	4278023
Fisherman's Lake	N	625837	4278393

[2] Sites are represented from west to east on Figure 1
[3] UTM NAD 83

Table 3. 2017 Water Chemistry Metrics

Quadrat ID	Date	Time	H2O Temp (C)	Conductivity (mS/m)	Salinity (PPT)	DO % Saturation	pH
Cell A	9/6/2017	13:05	26.81	632	.30	142.2	8.34
Cell B	9/6/2017	13:07	27.70	489	.23	115.8	8.22
Cell C	9/6/2017	13:10	27.54	487	.23	105.6	8.37
Cell D	9/6/2017	13:12	27.67	555	.27	75.6	8.29

Table 4: 2017 prey catch per unit effort (CPUE) at TNBC's Cummings Tract

Quadrat ID	Trap Days	Ranid Adult		Ranid Larvae		Mosquitofish		Black Bass		Other Sunfish		Carp		Silverside		Black Bullhead		Crayfish		Combined Prey	
		Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE	Count	CPUE
NA460	375	0	0	0	0	30	.0800	8	.02133	0	0	0	0	3	.00800	0	0	31	.08267	72	.192
NA461	375	0	0	0	0	52	.13867	3	.00800	0	0	0	0	9	.02400	0	0	40	.10667	104	.277
NA462	375	0	0	0	0	31	.08267	8	.02133	0	0	0	0	7	.01867	0	0	23	.06133	69	.184
NA463	375	1	.00267	0	0	33	.08800	29	.07733	0	0	0	0	5	.01333	0	0	8	.02133	76	.203
NA464	375	2	.00533	0	0	9	.02400	25	.06667	0	0	0	0	0	0	0	0	5	.01333	41	.109
NA465	375	0	0	0	0	28	.07467	36	.09600	1	.00267	0	0	0	0	0	0	14	.03733	79	.211
NA466	375	0	0	0	0	101	.26933	16	.04267	51	.13600	0	0	1	.00267	0	0	29	.07733	198	.528
NA467	375	0	0	0	0	70	.18667	18	.04800	29	.07733	0	0	0	0	0	0	54	.14400	171	.456
NA468	375	0	0	0	0	17	.04533	20	.05333	22	.05867	0	0	2	.00533	0	0	46	.12267	107	.285
NA469	375	6	.01600	0	0	12	.03200	16	.04267	4	.01067	0	0	0	0	0	0	12	.03200	50	.133
NA470	375	4	.01067	0	0	8	.02133	22	.05867	10	.02667	0	0	0	0	0	0	13	.03467	57	.152
NA471	375	9	.02400	18	.04800	4	.01067	23	.06133	9	.02400	0	0	0	0	1	.00267	5	.01333	69	.184