

Section 6 (Texas Traditional) Report Review

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Project Title: Habitat expansion, genetic characterization, and population assessments of the highly endangered Leon Springs pupfish, *Cyprinodon bovinus*.

Final or Interim Report? Final

Grant #: TX E-150-R

Reviewer Station: Austin ESFO

Lead station concurs with the following comments: NA (reviewer from lead station)

Interim Report (check one):

- Acceptable (no comments)
 - Needs revision prior to final report (see comments below)
 - Incomplete (see comments below)
-

Final Report (check one):

- Acceptable (no comments)
 - Needs revision (see comments below)
 - Incomplete (see comments below)
-

Comments:

FINAL PERFORMANCE REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-150-R

F12AP01078

Endangered and Threatened Species Conservation

**Habitat expansion, genetic characterization, and population assessments of the highly endangered
Leon Springs pupfish, *Cyprinodon bovinus*.**

Prepared by:

Dr. Murray Itzkowitz



Carter Smith
Executive Director

Clayton Wolf
Director, Wildlife

26 October 2015

INTERIM REPORT

STATE: Texas GRANT NUMBER: TX E-150-R

GRANT TITLE: Habitat expansion, genetic characterization, and population assessments of the highly endangered Leon Springs pupfish, *Cyprinodon bovinus*.

REPORTING PERIOD: 1 September 2012 to 31 August 2015

OBJECTIVE(S): To mitigate the risk of extinction of the Leon Springs pupfish, *Cyprinodon bovinus*, by expanding its breeding habitat, monitoring subsequent reproductive behavior and success, and genetically testing for evidence of interspecific hybridization with other pupfish species in the resident populations.

Segment Objectives:

Task 1:

1. Develop an additional breeding site at Diamond Y Spring.
2. Expand the open water area at John's pool in the same manner as performed in the Monsanto area
3. Continue monitoring population sizes in both natural and artificial pools at Diamond Y, Monsanto Area, and begin monitoring John's Pool.
4. Continue monitoring of reproductive activities using videotapes to make quantitative accurate estimate of reproductive success and habitat use. Continue monitoring *G. nobilis* predation.
5. Develop four additional at the Monsanto Area approximately 100 m from those developed the previous year.

Task 2:

1. Develop an additional breeding site at Diamond Y Spring.
2. Continue monitoring population sizes in both the natural and artificial pools at Diamond Y, Monsanto Area, and John's Pool.
3. Continue monitoring of reproductive activities using videotapes to make quantitative accurate estimate of reproductive success and habitat use. Continue monitoring *G. nobilis* predation.
4. Develop four additional pools approximately 100 m from those developed the previous year, for a total of 12 new pools in 2012 and 2013.

Significant Deviations: None.

Summary Of Progress: Please see Attachment A.

Location: Diamond Y Draw, Pecos County, Texas.

Cost: Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.

Prepared by: Craig Farquhar

Date: 26 October 2015

Approved by: _____



Date: 26 October 2015

C. Craig Farquhar

ATTACHMENT A
FINAL REPORT: Habitat Expansion, Genetic Characterization, and
Population Assessments of the Highly Endangered Leon Springs Pupfish,
Cyprinodon bovinus.

Section 6 Grant (TX E-150-R)

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Abstract

This three year project, funded by the Texas Parks and Wildlife Department, was intended to reduce the risk of extinction of the Leon Springs pupfish, *Cyprinodon bovinus*, by expanding its breeding habitat, monitoring subsequent reproductive behavior and success, and genetically testing for evidence of interspecific hybridization with other pupfish species in the resident populations. The following abstracts the results of this project:

1. The current *Cyprinodon bovinus* at Diamond Y Spring shows no evidence of residual genetic material from past hybridizations with *C. variegatus*.
2. The population in the Lower Monsanto Area had gone extinct prior to the onset of this proposed project and thus we introduced 400 - 500 additional captive bred fish from the SNARRC in both 2013 and 2015.
3. In 2013 we increased the number of shallow pools in both Diamond Y Spring and in Lower Monsanto Area.
4. Using “Next Generation” genomic analyses (ddRAD), we uncovered significant genetic differences between the *C. bovinus* populations in Diamond Y Spring and at SNARRC.
5. The introduced individuals from the SNARRC exhibit similar territorial behavior to that of Diamond Y Spring fish, and males of both population sources are equally effective in excluding *Gambusia nobilis* from their territories.
6. The population of *C. bovinus* in Diamond Y Spring S has dramatically increased over the past 3 years and we have recently recorded the presence 78 territorial males, the highest number we have recorded in the past 15 years.

Introduction

Survival of the Leon Springs pupfish (*Cyprinodon bovinus*) has been of concern since 1965 when it was found to be not extinct (USFWS 1985; recovery plan). Hybridization of the only known natural *C. bovinus* population (Diamond Y Draw, Pecos Co., TX) with the foreign sheepshead minnow (*Cyprinodon variegatus*) was documented in the 1970s and again in the 1990s (Echelle and Echelle 1997), and efforts to eradicate the hybrids were conducted in 1976/77, 1998, and 2000 (Echelle et al. 2004). At Diamond Y Spring (see Fig. 1), an ichthyocide was used to eliminate all *Cyprinodon*. These were replaced with a pure stock of *C. bovinus* maintained at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC), Dexter, New Mexico. At other locations, known as Monsanto Area and John's Crossing (see Fig 1), several kilometers Northeast from Diamond Y Spring, in the "lower watercourse", pupfish were netted and removed over a two day period, followed by the introduction of large numbers of *C. bovinus* from SNARRC in an attempt to swamp out the any *variegatus* genes remaining in these pupfish populations. It is unclear if this technique was successful.

Independent of the restoration process, we have been observing the Diamond Y Spring population every summer since May 2000. By 2001 we verified that a large breeding population with well over 25 defended territories was present in habitat recently exposed in coordination with the population restoration efforts mentioned above, and another 25 territories occurred on a narrow natural shelf. This shelf area, which historically has been one of the most important pupfish breeding grounds, is located in the head pool spring outflow of the upper watercourse in Diamond Y Spring (Leiser & Itzkowitz 2003; Gumm et al. 2008; Gumm et al. 2011).

In spite of adding large numbers of captive fish from the SNARRC facility in 2000, only 10 pupfish were observed at Diamond Y Spring in 2006. This population contraction apparently resulted from a loss of spawning habitat by encroachment of bulrush (*Scirpus* sp.) and intense egg predation by the endangered fish, *Gambusia nobilis* (Gumm et al 2008). With financial support from the Section 6 program of the TPWD, we restored a section of the spawning habitat (see final reports, Itzkowitz 2007, Fig. 2), and with guidance and logistical support from the SNARRC, we verified that the 10 remaining pupfish at Diamond Y Spring were free of *variegatus* genes indicating that the previous restoration attempt by Echelle et al (2003) had been successful. Our expansion of the breeding habitat appeared to reduce gambusia predation, leading to an increase in the adult *C. bovinus* population to ~40-50 fish. This was a success, but the population remained small and at risk. For this reason, we proposed a further expansion of the shallow areas in this locality coupled with continued monitoring of the population. See Gumm et al. (2011) for complete review of this past project.

Relying on Diamond Y Spring as the primary locality to maintain this species posed risks inherent to any small body of water. Therefore, we proposed expanding the spawning areas in the Lower Monsanto Area (Fig. 1) several Km downstream from Diamond Y Spring. This distant locality has always supported a small pupfish population and in 2009 we began monitoring this community. We determined that approximately 100 pupfish were present in the Lower Monsanto Area, concentrated most heavily in a single deep pool. However, this pool was cold and we observed no breeding behavior there. We did observe breeding in a nearby shallow pool with about 20 reproductive males defending territories. The deep pool appeared to serve as a refugium, as pupfish were seen swimming back and forth across a shallow stream connecting the deep pool and scattered shallow pools.

In early 2010 the Nature Conservancy intentionally burned much of the grass surrounding Monsanto Area marsh habitat. This action perhaps stimulated bulrush growth, isolating the deep refugium pool from the surrounding shallow areas. The number of reproductive males in the shallow pool was much reduced from the previous summer and there was no evidence that water flowed above ground from the deeper pool to the shallow one, in spite of heavy rains that flooded other parts of the property. Also, while numerous pools with open water were apparent after the heavy rains in July 2010, they contained no pupfish. It was our aim to perform minor modifications that would connect these isolated shallow pools, which could then serve as ideal, diverse spawning habitat, with the deep pool serving as a refugium for the population in case of dropping water levels or during harsh winter conditions. In addition to expanding spawning habitat at Diamond Y and Lower Monsanto, it was deemed imperative to examine genetic diversity among the separate *C. bovinus* populations to determine if they are genetically distinct and whether there has been any loss of genetic diversity as a result of the population decline that was detected in 2006. Finally, because the manual methods used to remove hybrid fish in the 1990s could have missed some individuals, it was important to gauge whether the pupfish in Diamond Y Spring and Lower Monsanto were free of any introduced *C. variegatus* genes as a result of the previous documented or more recent but undocumented hybridization events.

Objective

To mitigate the risk of extinction of the Leon Springs pupfish, *Cyprinodon bovinus*, by expanding its breeding habitat, monitoring subsequent reproductive behavior and success, and genetically testing for evidence of interspecific hybridization with other pupfish species in the resident populations.

Location

Diamond Y Draw (Fig. 1), Pecos County, Texas, U.S.A.

Approaches, Methods, and Results

Approach 1. *Conduct molecular genetic analyses of pupfish from all ponds from Diamond Y Spring to John's Pool. To state an obvious point, very small populations can show genetic divergences quite quickly and if we are going to project this species, we should understand the initial population genetics of all local populations and insure uniformity among subpopulations.*

Methods:

The original proposal specified analysis of polymorphic microsatellite (short tandem-repeat) loci as the method for evaluating management-relevant genetic characteristics of *C. bovinus* for this project. Specifically, we proposed to 1) assess genetic diversity within and between the wild *C. bovinus* population at Diamond Y spring and the Lower Monsanto area, and 2) seek evidence of the presence of genetic material from a congener, *C. variegatus*, in the gene pool of *C. bovinus* at Diamond Y as a result of documented episodes of hybridization between *C. variegatus* and *C. bovinus* at that location in the 1970s and 1990s (the SNAARC population was founded from Diamond Y Spring fish prior to the detection of *C. variegatus* individuals or genetic material contamination in the upper section of the Diamond Y watercourse so considered uncontaminated by *C. variegatus*). While the objectives of these proposed genetic analysis did not change, there were two deviations from the plan. First, by the time the project began the population at Monsanto area had gone extinct and had to be replaced by new fish from SNARRC. Consequently we compared the genetic characteristics of these new fish to those of the native fish from Diamond Y Spring. Second, we chose to utilize a much more powerful and cost-effective genetic technology for achieving our genetic analysis objectives.

In place of microsatellite analysis, which would have provided data for ~20 genetic loci scattered across the *Cyprinodon* genome, we utilized ddRAD-seq (double-digest, restriction-site associated DNA sequencing) (Peterson et al. 2012; Kai et al, 2014), a “Next-Generation” sequencing approach that enabled us to simultaneously assess sequences at thousands of loci in the *C. bovinus* and *C. variegatus* genomes. The method is based on the production of reduced-

representation DNA libraries of restriction-site associated DNA, which generates a highly replicable sample of the same several thousand genetic loci (RAD-tags) for each individual fish sample. Homologous loci in most or all individuals examined can be sequenced with moderate (10-20x) coverage, enabling both the *de novo* discovery and genotyping of hundreds or thousands of single nucleotide polymorphisms (SNPs) that can be utilized to identify genetic differences between distinct populations or across geographic distance in continuous populations, as well as between members of distinct but closely related species. Cost to conduct the ddRAD-seq analysis was similar to that for the proposed microsatellite analyses, but yielded reliable genotyping data at 2,295 genetic loci for the Diamond Y Spring vs. SNAARC intraspecific population comparison and 3,338 loci for the *C. bovinus*/*C. variegatus* introgression analysis.

The following methodologic synopsis is distilled from Black (2015): ddRAD genomic DNA libraries, consisting of many tens of thousands of RAD-sequence fragments were generated from individual *C. bovinus* fin clips obtained from fish captured (and released) at Diamond Y Spring and SNAARC and *C. variegatus* captured (and released) at Balmorhea State Park, TX, using modifications of the ddRAD-seq method described by Peterson et al. (2012). Briefly, genomic DNA was digested using a mixture of two restriction endonucleases (RE), resulting highly repeatable RE fragments. The fragments from an individual fish were ligated to appropriate adapter oligonucleotides containing amplification primer sites, one of 48 five-nucleotide ‘barcode’ sequences, and one of 6 six-nucleotide ‘index’ sequences to produce fish-specific libraries. The use of unique barcode/index combinations for each individual fish library permits multiple individuals to be sequenced (and identified) on a single lane of the sequencing platform, which reduces the sequencing cost by two orders of magnitude compared to sequencing individual libraries. Following adapter ligation, the sequences (individually barcoded and indexed) were pooled and then size-selected for fragments of ~375 base pairs (bp), which were subsequently used for the sequencing procedure. Polymerase chain-reaction (PCR) amplification of the size-selected fragments was performed to incorporate the adaptors necessary for annealing to an Illumina flow cell during a sequencing run. The pooled ddRAD library, consisting of RAD-sequence fragments from multiple individuals, was sequenced on a single lane of an Illumina HiSeq 2500 DNA sequencer, yielding ~150 million, 101-bp sequence reads. The reads

were quality filtered using advanced computational algorithms, sorted into unambiguous 96-bp sequence ‘loci’, examined for the presence of SNPs, and finally attributed to individual fish on the basis of the unique barcode/index combination incorporated into the sequencing fragment during construction of the individual libraries. Totals of 2,295 and 3,338 reliable polymorphic loci were identified for the intra-species and interspecies hybrid analyses, respectively. A variety of descriptive and comparative statistics was generated from the data to assess intraspecific population genetic diversity and structure (differentiation) as well as evidence that would reveal to the persistence of *C. variegatus* genetic material in the Diamond Y *C. bovinus* gene pool.

Results

Results from a linkage disequilibrium-based estimation method suggest that the current SNARRC *C. bovinus* population has current effective size (N_e) of ~200 individuals, whereas the Diamond Y Spring population, which has suffered repeated demographic bottlenecks is much smaller, with current N_e ~30. Despite this difference, both populations retain substantial and similar high levels of genetic diversity (as assessed from the expected heterozygosity, H_e , across the RAD-tag loci), indicating that the recent (2006) bottleneck followed by rapid demographic recovery did not seriously suppress overall genetic diversity in the Diamond Y Spring population ($H_e = 0.302$) relative to that at SNARRC ($H_e = 0.296$), which is not known to have experienced major demographic contractions since its inception in 1976. Similarly, analysis of observed genotype frequency distributions revealed that breeding is essentially random within each population ($F_{is} = -0.028$ and 0.034 at Diamond Y Spring and SNARRC, respectively).

Contrasting with these results, the two populations exhibited a surprising level of divergence in allele frequency distributions. The average F_{st} value for the Diamond Y Spring vs. SNAARC populations (indicator of population subdivision and divergence) was 0.055. While this value is not particularly high relative to those found in broadly distributed species wherein the populations have been separated by hundreds or thousands of generations, this level of genetic structuring between populations that have been separated for 15 years is remarkable and a cause for concern. The divergence captured in this F_{st} metric derives from nearly 2,300 loci, almost all of which have locus-specific F_{st} values within ranges expected in the absence of divergent selection at the individual loci in the Diamond Y Spring and SNARRC environments. This

suggests that stochastic processes (genetic drift) alone have driven considerable divergence in a short period of time. But in addition, seven loci (~0.3%) did exhibit strong signatures of selection as judged from single-locus F_{st} values that significantly and substantially exceeded levels expected as a result of random processes alone (Black et al. manuscript in preparation).

Importantly, of these 7 loci, the one with the largest F_{st} value shows strong homology to a region in other Cyprinodontiformes that is tightly linked to a highly conserved vertebrate solute carrier gene involved in osmotic balance in fishes. If this linkage is conserved in pupfish as well (likely), it suggests that divergence between the Diamond Y Spring and SNARRC populations at this locus could be related to the substantial differences in water quality and salinity that exist between the Diamond Y and SNARRC environments.

Comparison of the genetic characteristics of *C. bovinus* with those of *C. variegatus* revealed no evidence of residual *C. variegatus* genomic sequences in the Diamond Y *C. bovinus* gene pool. Based on 278 fixed allelic differences between *C. variegatus* (source species) and the *C. bovinus* population at SNAARC (established prior to documented hybridization events at Diamond Y and, thus, representative of the uncontaminated recipient species), there was no evidence of *C. variegatus* genetic material in the Diamond Y gene pool. In addition, principle component and discriminant function analyses using the full (> 3,000 locus) datasets from *C. variegatus* and both *C. bovinus* populations, clearly separated the two recognized species while clustering the two *C. bovinus* populations as a single group. For example, the first principal component axis for analysis of the *C. bovinus*, *C. variegatus* dataset captured 65% of the total variance in the dataset and placed both *C. bovinus* populations in a single, tight cluster that was strongly discriminated from the cluster encompassing the *C. variegatus* samples. There was total absence of intermediate types between the two species. We conclude that if residual contamination of the Diamond Y Spring *C. bovinus* gene pool exists, it is of a magnitude too small to be of consequence in management decisions.

Approach 2. Increase spawning habitat at Diamond Y Spring

Methods and Results:

In 2007, we developed the methodology for expanding breeding areas by removing the bulrush (including roots) and adding cement steps. We submerged the cement steps to 15 cm to replicate the conditions on the “natural shelf” where the remaining pupfish continued to hold territories and spawn. In January 2013, we further expanded the breeding habitat in Diamond Y Spring by removing bulrush from four adjacent areas, thereby creating “constructed pools”, and again added cement steps. These constructed pools were located immediately downstream of the Diamond Y Spring pool. Each constructed pool comprised an open area of 5 m x 2 m that had a water depth of 15 cm over most of the pool area. Figure 2 A - E are photographs documenting the construction of the Diamond Y pools.

The four constructed pools were fed by the single stream that exited the main spring pool. We presumed, at the time, that pupfish originating in the main spring pool would have had easy access to any of the four pools by following the stream. As predicted, both male and female pupfish did begin to use these pools. These fish were somewhat smaller than those using the natural shelf suggesting that they migrated to find open breeding habitat. As of June 2015, approximately 9 males held territories in these constructed pools. We anticipate that should the population in the main pool continue to increase, so too should the population density in these constructed pools.

Approach 3. Increase spawning habitat at Monsanto Area

Methods and Results:

In the proposal we warned that the remaining fish in Monsanto Area’s deep pool (Lower Monsanto) appeared trapped without access to a shallow spawning area and, indeed, by the time the proposal was funded, the Lower Monsanto Area pupfish population had gone extinct. Thus, the “Approach” that we would renovate the previous spawning pool and expect the population to recover (as was done in Diamond Y Spring beginning in 2007) was no longer tenable. However, in an effort to preserve any pupfish that might persist hidden in these pools, we decided to construct two shallow pools flanking the deep refugium pool in January 2013. We lined both shallow pools with cement steps. Figures 3 A – D are photographs documenting the construction of the two shallow spawning pools at Monsanto Area.

We again searched for pupfish at Lower Monsanto Area in the late Spring and early summer, but found none. We then contacted Mike Montagne (FWS) who secured a permit for us to bring 400 pupfish from the SNARRC. We released these fish in early June and began an immediate and intense monitoring program (see below, Approach 4). Later in the summer the water declined dramatically, considerably reducing the areas and depths of the two spawning pools. Further renovations were done in late July allowing more water to flow from the deep pool to the shallow pools. A year later, during the 2014 summer, we observed a small number of nonterritorial pupfish that we estimated to be about 12 individuals. In June 2015 the water levels again were quite high and the number of nonterritorial fish again were estimated to be about 12 individuals. For this reason, we introduced an additional 400 pupfish from the SNARRC. Again the fish seemed healthy and well adapted to these pools. The two renovated spawning pools soon teemed with a pupfish spawning population. Furthermore, the renovations that had been performed in late summer of 2013 summer could be seen to be successfully and broadly connect the two constructed pools to the deeper water of the deep “refugium” pool. At the end of the summer work period (August 2015), the pupfish population appeared healthy and vigorous.

Approach 4. Monitor population sizes and interspecific egg predation:

Methods and Results:

A. Pupfish Population Numbers at Diamond Y Spring

We have been studying the pupfish at Diamond Y Spring since May of 2000. At that time, we estimated that about 50 males held territories. In 2003, we began to observe an obvious decline, with males holding territories only on a small shallow rock shelf (perhaps 6 m²) near the head pool spring outflow. In 2006, only 10 fish were observed of which only 2 were territorial males. It was at this point that we expanded the shallow habitat, and in 2007 a small increase in the number of territorial males was observed. From 2007 to 2013, the territorial males increased to 20 – 25 males and their reproductive behavior remained restricted to the natural shelf area and the area expanded by us in 2007. In 2015, the number of territorial males reached 78, more than 50% greater than our initial census numbers from 2000. Territorial males covered the natural shelf and the expanded area, and are now seen in peripheral areas of the

entire pool for the first time. Nine males held territories in the constructed pools. The largest males held territories on the natural shelf suggesting this was the prime spawning habitat. We anticipate that as the number of males continues to increase, more will establish territories in the constructed pools.

B. Are there behavioral and ecological differences among the pupfish in the three localities?

The long-term conservation of the Leon Springs Pupfish will depend on the further expansion of the numbers of individuals both within and between pools. With likely expansion of pools in the area, it is critical that we understand how the pupfish use new or renovated habitat. For this reason, soon after introducing the pupfish into the constructed Monsanto pools (June 2013), we quantified the reproductive behavior and habitat use by the pupfish in this pool and on the natural shelf and in constructed pools in the Diamond Y system. Our intent was to examine whether the introduced fish at Monsanto differed behaviorally and ecologically from pupfish in these other areas. To document the habitat use in all three areas, we built a string grid on the surface of each pool (Fig. 4). Using the grid for noting precise locations, we quantified the type and quantity of habitat in each grid box and quantified the locations of males and females as well as spawning locations (Fig. 5). Essentially we wanted to know how the captive fish used their constructed pool at Lower Monsanto compared to the constructed pool at Diamond Y Spring and the natural shelf at Diamond Y Spring. This work is currently submitted for publication (Al-Shaer et al. submitted) and here we provide an abbreviated version of the abstract from that manuscript.

The central question we asked is “... is it better to renovate an unoccupied site and introduce captive-bred individuals, or to expand an occupied site that would allow the current population to grow?” This is of critical importance to the conservation of *C. bovinus* because there are plans to increase the population size by creating new pools and stocking them with captive bred fish. We examined whether *C. bovinus* location preferences coincided with the presence of specific ecological factors. Wild *C. bovinus* in the natural breeding habitat (natural shelf at Diamond Y Spring) spawned more, had greater spawns per individual male, and had greater territorial stability compared to captive-bred *C. bovinus* in Monsanto or in constructed breeding habitats at Diamond Y Spring. Differences in social system stability and reproductive

success between sites may be due to variation in their ability to adapt to a renovated site as well as the ecological makeup of the habitat. We do not know if these differences have a genetic basis.

C. Are the Pupfish Producing Fry?

While our behavioral/ecological analyses do show social-behavioral differences between the pupfish in the three localities (constructed Monsanto, constructed Diamond Y, and natural shelf in Diamond Y Spring) these data do not provide details of fertility and offspring production. We developed a relatively simple measure of fertility by placing pupfish from SNARRC in small pens in a constructed pool at Monsanto and a constructed pool at Diamond Y (Fig 6). After 4 weeks, in both locations, we found fry in the pens. Thus we are confident that the breeding behavior we have seen in Monsanto did result in mating and the production of fry. It also is an indicator that the water quality in both areas allowed for embryological development of fertilized eggs. The data from these pens will be published in a subsequent paper (Black, in prep).

D. The Decline and Subsequent Reintroduction of Pupfish at the Lower Monsanto Area

Unfortunately, the water level at the Monsanto Area declined dramatically by August 2013, leading us to conduct immediate additional renovations. We do not know if the drop in water level influenced the survival of the pupfish, but a year later, in July 2014, we observed an estimated 12 adults in the shallow pools. While the presence of these few fish did verify that the water was of sufficient quality to maintain a viable pupfish population, the dramatic decline in the number of introduced fish remains largely unexplained (but see Discussion).

In June 2015, we again observed an estimated 12 adult pupfish in the constructed Lower Monsanto pools; these may have been the same individuals we observed in 2014. We then secured an additional 400 pupfish from SNARRC and added them to the renovated pools. Similar to the 2013 introduction, the pupfish appeared to acclimate quickly to their new environment, set up territories, and began reproducing. We have taken considerable data over the summer on the reproductive behavior of this new population with an emphasis on the ontogeny of their social behavior. In August 2015, with the water level remaining high, the

pupfish appeared healthy and reproductively active. At this point the Section 6 TPWD grant ended.

E. *Gambusia nobilis*, the Egg Predator of *C. bovinus*

We previously suggested that *Gambusia nobilis* was a contributing factor to the decline of the pupfish in Diamond Y Spring as they appear to be eating the pupfishes' freshly laid fertilized eggs (Gumm et al 2008). The gambusia clustered around spawning pupfish pairs and waited for the eggs to be deposited. This means that the gambusia had to remain in the male's territory, endure the aggression directed against them by the territorial male pupfish, and wait for females to enter the territory and spawn. In 2006, we hypothesized that increasing the available space for the pupfish would also reduce the density of the gambusia within territories and, in this way, reduce egg predation. Our previous study (Gumm et al 2008, 2011) showed that increased spawning space decreased gambusia density and that this decrease correlated with an increase in the subsequent population size of the pupfish.

The ability of the male to reduce gambusia numbers in his territory is a critical factor to his ability to reproduce. Thus, the behavior and ecology of the gambusia are not separate issues from the conservation of the pupfish. We were especially concerned that pupfish taken from the SNARRC may not be effective at removing gambusia from their territories because they have been bred for many generations in artificial pools without gambusia. We compared the ability of pupfish to exclude gambusia at Monsanto with the pupfish at Diamond Y Spring. This study (Paciorek et al., 2014) revealed that in spite of the pupfish's long history at SNARRC, they excluded gambusia as effectively as did the native pupfish at Diamond Y Spring. Here are two direct quotes from Paciorek et al (2014): "Regardless of habitat location or prior captivity, territorial *C. bovinus* significantly excluded *G. nobilis* within their direct vicinity (5 cm), but not from their entire territory." "This study provides empirical evidence of captive raised individuals behaving similarly to wild individuals upon reintroduction to their natural habitat..."

Discussion

Based on this project, three important results should be considered with regard to management planning. First, using Next-Generation genomics techniques, we have reaffirmed that the current *C. bovinus* population at Diamond Y Spring has not retained significant residual genetic material

from the documented hybridizations with *C. variegatus*, nor undergone more recent undetected hybridization events with this foreign congener. We have, however, detected significant genetic differences between the pupfish maintained at SNARRC and those in Diamond Y Spring. Given that these two populations have been separated by only 15 years (see Echelle et al., 2004), we expect that these differences will increase over time. We can only speculate about the adaptive implications of these genetic differences, but it is possible that one of the genetic differences relates to the fish's ability to osmoregulate. If this is the case, some of the genetic differences could be related to the high fluctuating salinity in the Diamond Y Spring system, which includes the Lower Monsanto Area (Goghici, 1997; George Veni & Assoc. 1991) in contrast to the relatively low salinity at SNARRC (based on a water chemistry report in 2013). We are concerned that only 10 individuals were alive in 2006 at Diamond Y Spring after seeding the system with many thousands of pupfish after previous hybrid removal efforts. Similarly, we found only a small number of fish remaining in Lower Monsanto after we added 500 individuals in 2013. We do not know why so few individuals survived after both introductions but it is possible that these individuals were part of a minority in the SNARRC stock that still possessed rare genotypes that enabled them to thrive in a higher salinity environment. It is our hope that with the addition of more SNARRC fish in 2015, additional individuals will survive. In any event, we recommend that any further use of the SNARRC population to start new communities in the Diamond Y system should be made quickly before further genetic divergences occur.

Another important result is that the population of pupfish at Diamond Y Spring has increased over the course of this 3 year project. We believe it is because we have increased the size of the shallow spawning habitat and reduced the impact of gambusia predation. Another possibility is that further genetic changes have occurred that made this population better adapted for survival in Diamond Y Spring.

Third, while we did observe differences in the spawning behavior of the recently introduced pupfish from the SNARRC relative to the population at Diamond Y Spring, we have no evidence that addresses whether these differences are genetically based or environmentally induced. However, the SNARRC and the current Diamond Y Spring pupfish are equally capable of defending their territories against gambusia; thus the genetic changes that have occurred in the

SNARRC population do not seem to have any impact on defense behavior against an important egg predator.

II. Significant Deviations: We decided against using the originally proposed microsatellite approach for genetic comparisons and instead used a more efficient and cost effective “Next Generation” genome wide-approach (ddRAD) for this purpose. With the extinction of *C. bovinus* from the Monsanto Area and John’s Pool, we were unable to perform the genetic comparisons between the two natural subdivisions of the *C. bovinus* population. Instead, we introduced new *C. bovinus* from the captive SNARRC population to the Monsanto Area habitat in 2013, and then compared these fish genetically with those still residing in Diamond Y Spring. With the dramatic reduction in the numbers of introduced fish at Monsanto, we again added more fish from SNARRC in 2015.

Literature Cited.

- Al-Shaer, L., Bloch, A., Paciorek, T., Carroll, Z., Black, A., & Itzkowitz, M. Submitted. Comparison of natural and renovated breeding habitat use in wild & captive bred populations of an endangered desert pupfish.
- Black, A. 2015. An integrative assessment of the ex situ conservation and reintroduction of an endangered species of pupfish (*Cyprinodon bovinus*): Investigation of genetic, morphological and behavioral variation. Ph.D. Dissertation. Lehigh University.
- Boghici, R. 1997. Hydrological investigation at Diamond Y Spring and surrounding area. M.S. thesis. Univ. Texas, Austin.
- Echelle, A. A., & A. F. Echelle. 1997. Genetic introgression of endemic taxa by nonnatives: a case study with Leon Spring pupfish and sheepshead minnow. *Conserv. Biol.* 11:153-161.
- Echelle, A. A., Echelle, A. F., Kiner, L., Garrett, G. P., Karges, J., Fishers, W. L. 2001. Final Report: Monitoring effects of a renovation project on endangered fish and invertebrates in Diamond Y Draw. Contract #388-0622, Endangered Resources Branch, Texas Parks and Wildlife Department.
- Echelle, A. F., Echelle, A. A., Bonnel, L. K., Allan, N. L., Brooks, J. E., & J. Karges. 2004. Effects of a restoration effort on an endangered pupfish (*Cyprinodon bovinus*) after genetic introgression by a non-native species. IN: Homenaje al Doctor Andres Resendez Medina. Ma. De L. Lozano-V. and A. J. Contreras- B. (eds). Universidad Autonoma de Neuvo Leon,

Monterrey, Mexico. 129-139

George Veni and Associates. 1991. Delineation and preliminary hydrologic investigation of the Diamond Y Spring, Pecos County, Texas. Prepared for the Texas Nature Conservancy. San Antonio, TX.

Gumm, J., Snekser, J. & Itzkowitz, M. 2008. Conservation and conflict between endangered desert fishes. *Biology Letters of the Royal Society*. 4:655-658.

Gumm, J., Snekser, J., Little, K., Leese, J., Imhoff, V., Westrick, B. & Itzkowitz, M. 2011. Management of interactions between endangered species using habitat restoration. *Biological Conservation*. 144: 2171 – 2176.

Itzkowitz, M. 2006. Leon Spring Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement. ESA Section 6 Grant Proposal, Texas Parks and Wildlife Department

Itzkowitz, M. 2007 Leon Spring Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement. Final Report Section 6 Grant, Texas Parks and Wildlife Department.

Kai W, Nomura K, Fujiwara A, Nakamura Y, Yasuike M, Ojima N, et al. (2014) A ddRAD based genetic map and its integration with the genome assembly of Japanese eel (*Anguilla japonica*) provides insights into genome evolution after the teleost-specific genome duplication. *BMC genomics*, 15, 233.

Leiser, J. K. & M. Itzkowitz. 2003. The costs and benefits of territorial neighbors in a Texas pupfish (*Cyprinodon bovinus*). *Behaviour*. 140: 97-112.

Leiser, J. K., Bryan, C. M., & M. Itzkowitz. 2006. Disruption of dear enemy recognition among neighboring males by female Leon Spring pupfish, *Cyprinodon bovinus*. *Ethology*. 112: 1-7.

Paciorek, T., Al-Shaer, L. & Itzkowitz, M. 2014. How territoriality affects the density of an egg predator: habitat renovations and reintroduction as a method of conserving two endangered desert spring fish. *Current Biology*. 60: 527-533.

Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE (2012) Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS one*, 7, e37135.

United States Fish and Wildlife Service. 1985. Leon Spring pupfish recovery plan.

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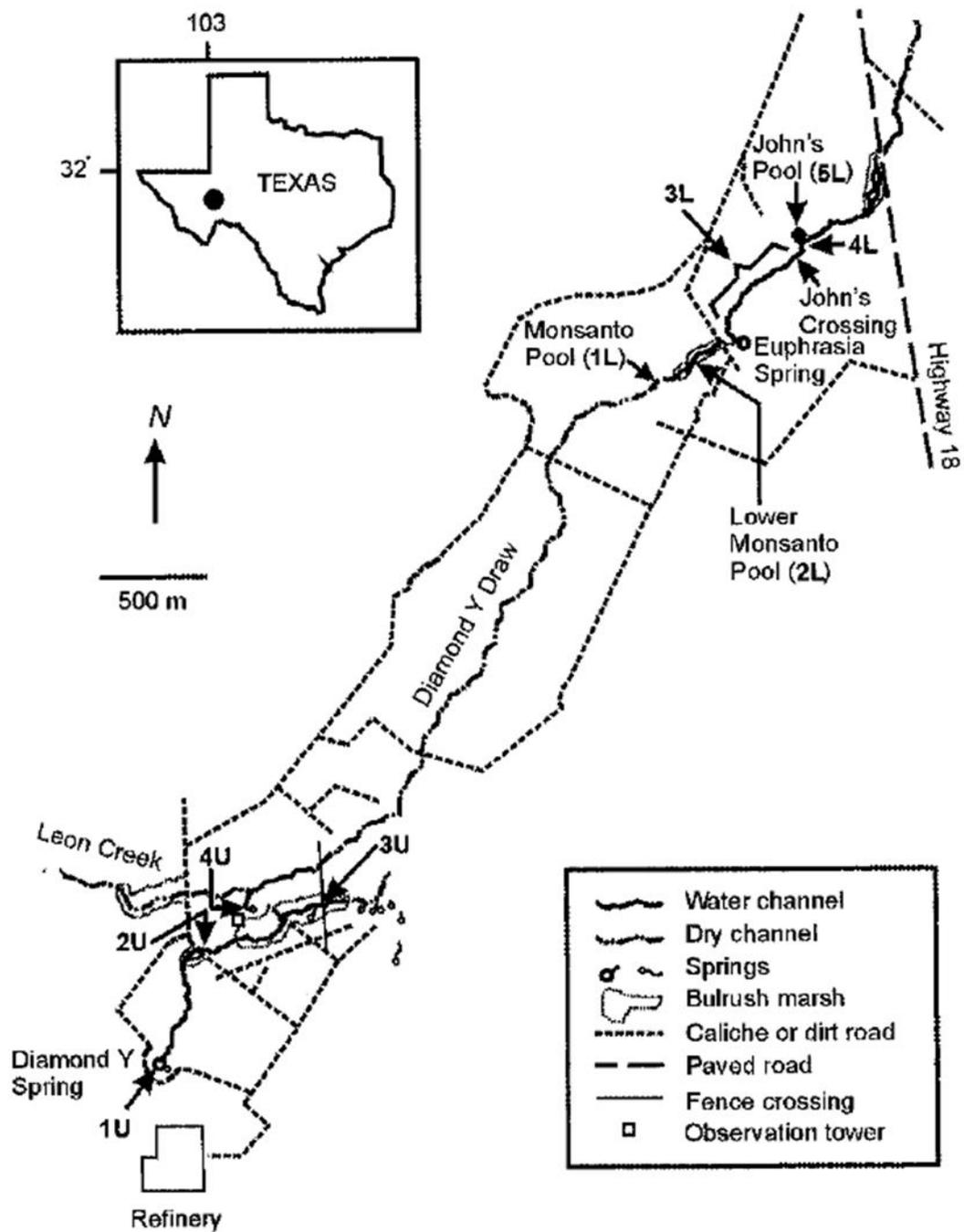


Figure 1: Sketch of Diamond Y Draw. The lower left is Diamond Y Spring. The Lower Monsanto on this sketch is the location for the Refugium Deep Pool which, in the text, also is term the Monsanto Area.

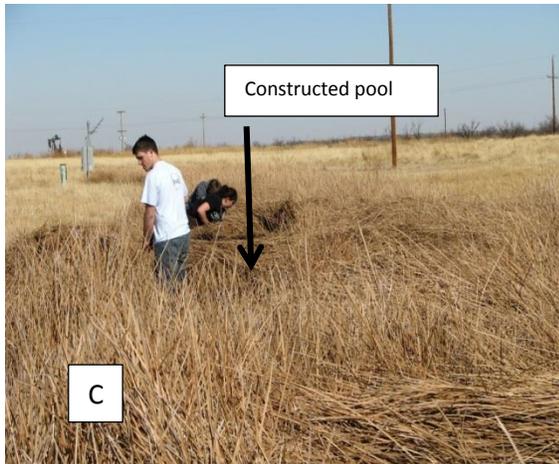
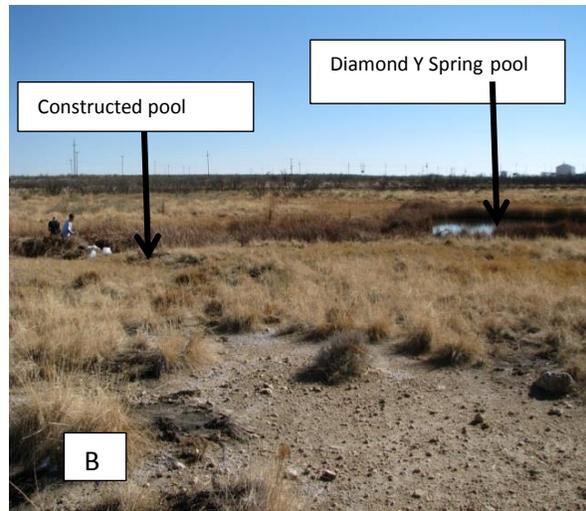
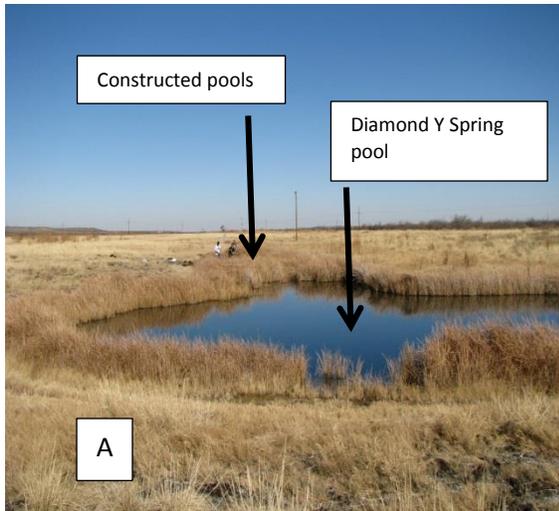


Figure 2 A, B, C, D, E : Photographs of the construction of pools at Diamond Y Spring

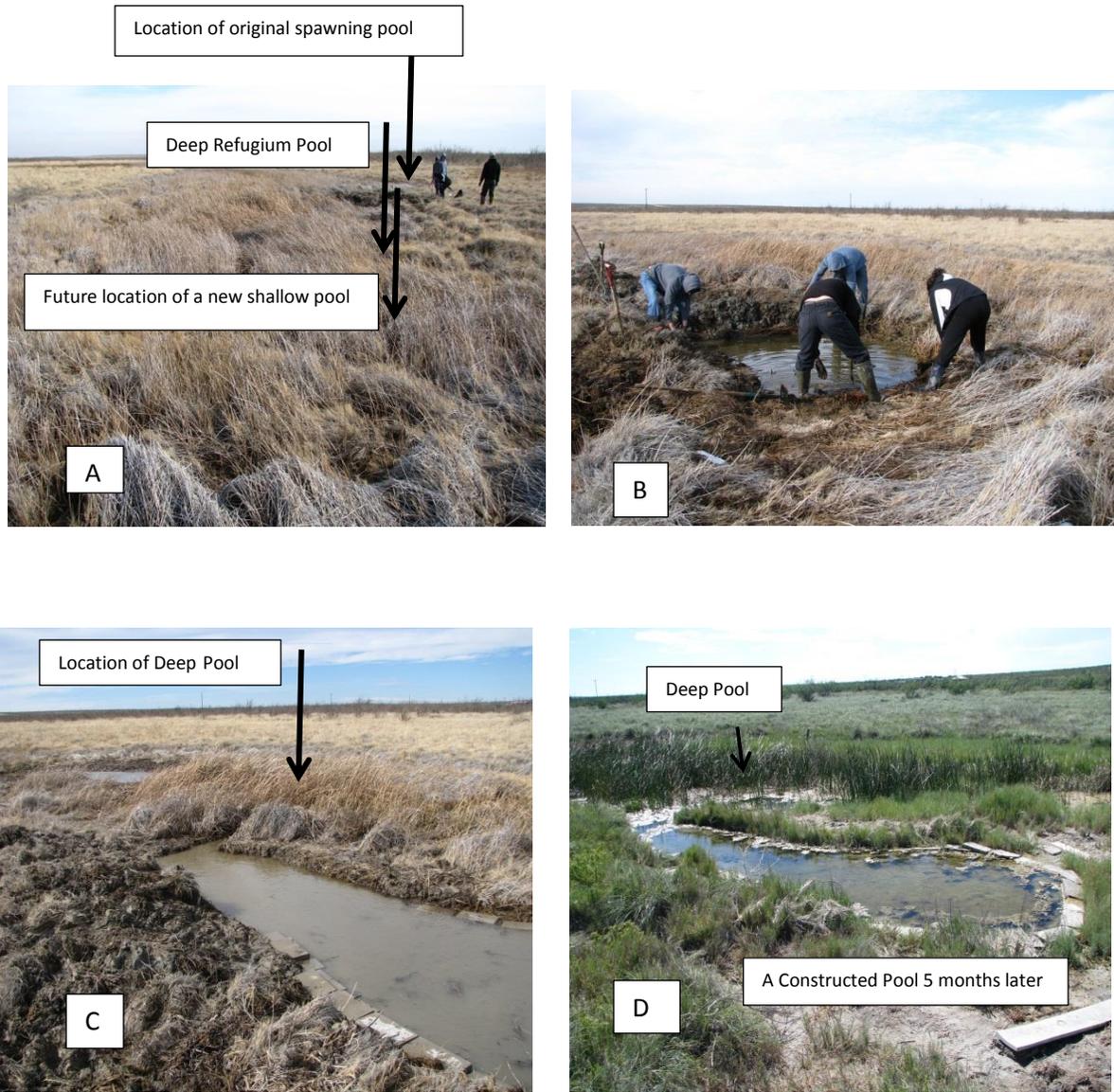


Figure 3 A, B, C, D. Photographs of the construction of a pool at the Monsanto Area.



Figure 4: The grid system used to document the spawning behavior and habitat use in a Diamond Y constructed pool.



Figure 5 : Data collection at a Monsanto constructed pool using the grid network. On the right Side are the pens used to test the fertility of the newly introduced pupfish.



Figure 6 : The pens placed in one Diamond Y constructed pool. The pens were Used to test the survival of offspring produced by the fish in Diamond Y Spring.