United States Department of the Interior

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JUN 2 7 2008

Dr. Craig Farquhar
Federal Aid Coordinator
Texas Parks and Wildlife Department
4200 Smith School Road
Austin, Texas 78744-3291

Dear Dr. Farquhar:

Enclosed is our review of the final section 6 report: E-83-R Leon Springs Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement.

Thank you for your continued efforts with this program. If you have any questions, please contact Christina Williams at 512 490-0057, extension 235.

Sincerely,

Adam Zerrenner
Field Supervisor

Enclosures

cc: Susan MacMullin, Region 2, Federal Aid, Albuquerque, New Mexico

Section 6 (Texas Traditional) Report Review

Attachment to letter dated JUN 2 7 2008

TPWD signature date on report 19 Dec 2007

Project Title: Leon Springs Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement

Final or Interim Report? Final

Grant #: E-83

Reviewer Station: Austin ESFO

Lead station was contacted and concurs with the following comments:
☐ Yes ☐ No ☒ Not applicable (reviewer is from lead station)

Interim Report (check one):

☐ is acceptable as is

☐ is acceptable as is, but comments below need to be addressed in the next report

☐ needs revision (see comments below)

Final Report (check one):

☐ is acceptable as is

☒ is acceptable, but needs minor revision (see comments below)

☐ needs major revision (see comments below)

Comments:

1. Please address Segment Objective, Task 4 regarding determining effective population size (in draft report on Page 5 as part of the Approach, c) – this objective does not appear to have been met.

2. Please edit for clarity on Page 12, second full paragraph, third sentence: "In 2006 the single territorial male had dozens of gambusia residing in territories and these gambusia groups seemed to become even larger when the male ~~when~~ began to spawned (pers. obs.)."

3. Please proof read the Addendum; it contains several typographical errors (suggested corrections are attached).

Addendum to:
Final Report of the
ESA Section 6 Grant Proposal
To Texas Parks and Wildlife Department

Leon Springs Pupfish Recovery and Genetic Diversity:
Spawning Habitat Restoration and Enhancement

Murray Itzkowitz, Ph.D.
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Lehigh University,
111 Research Dr.,
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On 7 October 2007 I visited Diamond Y Spring to evaluate the population size and breeding activity of the Leon Springs pupfish (*Cyprinodon bovinus*). To review, the Final Report states that during the 2008 summer we observed about 4 – 5 territorial males (approximately 4.5 cm total length) and a host of females. Within a 15 period, our videotapes recorded about 7 spawns per 15 min period. During this most recent visit, I positioned myself near the renovated area and the traditional spawning shelf and counted the numbers individuals and estimated their sizes. Because my presence may have caused some fish to flee, I also made several 20 min video taping of these localities. I also walked the along the edge of the pond searching for additional pupfish.

I neither directly observed nor video taped territorial behavior. The 11 pupfish that were observed were dispersed largely within the renovated area and consisted of: 1 female 5.5 cm, 2 males 4 - 4.5 cm, 2 females 4.0 cm, and 6 others 2.5 – 3.0 cm. One unsuccessful spawning attempt was observed between 4.0 cm male and a 4 cm female. This spawning attempt was interrupted by a 3 cm male. The 4.5 cm males were similar

in size to the territorial males I observed in July and August 2007. The 4 – 4.5 males followed females and courting them whenever the female the stopped swimming.

The reproductive season for this species is nearing the end and thus the lack of territorial behavior and low levels of reproductive activity were not surprising. Regarding the current population estimate, it is not a good sign that the population seems lower now than in August. It is possible that the ending of the breeding season coincides with individuals moving away from the breeding areas and into unobservable areas (e.g., deeper water). Alternatively, in spite of the reasonable levels of reproductive activity during the 2007 summer, the levels of egg predation by *Gambusia nobilis* prevented population growth. The number of small individuals (below 3 cm) indicates that some recruitment has occurred and I remain hopeful that my observations underestimated the actual population size. In March 2009, I again will return to Diamond Y Spring to again estimate the health of the pupfish population.

FINAL REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-83-R

Endangered and Threatened Species Conservation

Leon Springs Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement

Prepared by:

Murray Itzkowitz

Robert Cook
Executive Director

Mike Berger
Division Director, Wildlife

19 December 2007

FINAL REPORT

STATE: Texas **GRANT NUMBER:** TX E-83-R

GRANT TITLE: Endangered and Threatened Species Conservation

REPORTING PERIOD: 1/1/07 to 9/30/07

PROJECT TITLE: Leon Springs Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement.

OBJECTIVE(S):

To maintain and enhance a sustainable wild population of Leon Springs pupfish, *Cyprinodon bovinus*, at Diamond Y Spring, Texas.

Segment Objectives:

Task 1. Preliminary survey of population ("wild") size at Diamond Y Spring.
Task 2. Expand and enhance breeding habitat Diamond Y Spring.
Task 3. Collect DNA samples from Diamond Y Spring pupfish population and from populations as Dexter National Fish Hatchery and Technology Center.
Task 4. Determine amount of heterozygosity, inbreeding coefficients and effective population size for the population at Diamond Y Spring.
Task 5. Based upon results from genetics of the Diamond Y Spring population (Task 4), we will either (A) begin detailed monitoring of the breeding population until June 1, 2007, or (B) propose adding 500 -1000 individuals from the DNFHTC captive population to Diamond Y Spring (acquiring permits and documentation for this process will commence after the genetic work has been completed).
Task 6. Survey remainder of the upper and lower watercourses of Diamond Y Spring to estimate status of Leon Springs pupfish throughout the spring system.
Task 7. Intensive behavioral monitoring of the population at Diamond Y Spring through 15 Aug 2007.
Task 8. If current wild population is genetically distinct, or if we detect no increase in the population by June 1, 2007, we will recommend adding between 500 – 1000 adults.

Summary Of Progress:

Please see Attachment A.

Significant Deviations:

None.

Location: Pecos County, Texas.

Cost: **available upon completion of grant.**

Prepared by: Craig Farquhar **Date:** 19 December 2007

Approved by: _____ **Date:** 19 Dec 07
C. Craig Farquhar

ATTACHMENT A

Final Report of the

ESA Section 6 Grant Proposal

To Texas Parks and Wildlife Department

Leon Springs Pupfish Recovery and Genetic Diversity: Spawning Habitat Restoration and Enhancement

Principle Investigator:

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Abstract

The intent of the work was to rejuvenate the near extinct population of *Cyprinodon bovinus* (the Leon Springs Pupfish) in Diamond Y Spring, Ft. Stockton, Texas. We have closely followed the proposed objectives. We have increased the breeding habitat. There was a slight increase in the number of breeding males. Many spawning events were recorded. However, large numbers of *Gambusia nobilis*, an egg predator of the pupfish, were found in the territories. Using microsatellite analyses, we conclude that genetic variability is similar to the captive population at the Dexter National Fish Hatchery and Technology Center (DNFHTC). We offer a number of recommendations regarding the possibility of adding more fish to the Diamond Y Spring population.

Introduction

The federally endangered Leon Spring pupfish, *Cyprinodon bovinus*, is nearing extinction in its natural habitat. A general decline in the adult breeding population had been noted for several years (Itzkowitz, per obs.) but it was not until May 2006 that we recorded only about 10 adults (1 territorial male, 2 nonterritorial males, and about 7 females) and no juveniles at Diamond Y Spring. We believed that unless remediation occurred quickly, there was little hope for this species' survival at Diamond Y Spring.

The survival of this pupfish has been of concern since 1965 when it was found to be not extinct (USFWS 1985). In the 1970s and 1990s, hybridization of this small population with the sheepshead minnow (*Cyprinodon variegatus*) was documented (Echelle and Echelle 1997) and efforts were made to eradicate the hybrids between 1998 and 2000 (Echelle et al. 2004). Fish from a pure stock of *C. bovinus* maintained at the Dexter National Fish Hatchery and Technology Center (DNFHTC) were introduced and attempts to increase the breeding habitat were made by removing bulrush from the shallow areas (for summary of the renovations see Echelle et al. 2004). Independently of the restoration process, we have been observing this population since May 2000, and every summer thereafter. Leiser & Itzkowitz (2003) verified that by 2001, a large breeding population occurred with well over 25 territories established in the newly

exposed habitat coupled with another 25 territories on a narrow shelf. The shelf area has been one of the historical breeding grounds for this species, located in the head pool spring outflow of the upper watercourse in Diamond Y Spring. Besides these territories, there was an abundance of smaller reproductively active males (called "satellites" and "sneakers") that do not maintain territories but breed within the territories of the larger males. The non-territorial males may be important for maintenance of genetic diversity in the population.

Objective:

To maintain and enhance a sustainable wild population of Leon Springs pupfish, *Cyprinodon bovinus*, at Diamond Y Spring, Texas.

Expected Results or Benefits:

A. Provide an analysis of the genetic diversity of the remaining wild population of the Leon Springs pupfish and compare it to the captive population maintained at the DNFHTC.

B. Expand the breeding habitat of the Leon Spring pupfish at Diamond Y Spring.

C. Monitor the pupfish's population size, habitat use, and reproduction throughout Diamond Y Spring.

D. Introduce pupfish from DNFHTC if the population at Diamond Y Spring does not positively respond to habitat modifications.

Location: Field work: Latitude and longitude: 30degrees 00'30 N 102 degree 55'00 W. The study site is about 8 miles NNE of Fort Stockton, Pecos County, Texas, West of State Highway 18 crossing of Diamond Y Draw. The Diamond Y Draw consists of two

watercourses, each about 1 km in length, separated by about 2 km of dry spring bed. This occurs as part of the Diamond Y Draw/ Leon Creek drainage (Fig 1).
Laboratory work: Dexter National fish Hatchery and Technology Center. Dexter, New Mexico.

Methods and Results

Text in quotes and *in italics* are taken from the initial ESA Section 6 Grant proposal to the Texas Parks and Wildlife Department. The text in **bold** indicate the results of this work.

Approach: "*This proposal focuses on the population of Leon Springs pupfish found in the upper watercourse of Diamond Y Spring, Texas. This site contains the historically best spawning areas for pupfish and therefore is indicative of potential problems throughout the system. Recent observation (June 2006) of the lower watercourse of Diamond Y Draw revealed no ideal spawning habitat for pupfish and suggested numbers of pupfish (< 5) are too low for either genetic analysis or behavioral observation. If we are successful in restoration of the population at Diamond Y Spring, further restoration will be proposed for the lower watercourse of Diamond Y Draw."*

"January 1, 2007 – February 15, 2007:

a) *Take preliminary data on numbers of fish in the population and habitat usage for breeding."*
b) *Expansion the breeding habitat, similar to that done in 2000, by removing of a portion of the bulrushes approximately 10 m x 1.5 m^2 beginning immediately downstream of the breeding shelf in the head pool.*
c) *Roughened ceramic tiles and limestone rock will be inserted on the newly exposed area. This hard covering will inhibit the return of the bulrush and provide suitable spawning surface for the pupfish."*

Results:

We observed several adult pupfish (perhaps less than 5 individuals) in January 2007. Parts b and c were completed. We first removed the bulrush grass near the substrata and then, with pick and shovel, removed the roots down to approximately 15 cm. We then added cement steps that were 18" long, 8" wide, and 2" thick (Fig. 2, 3, 4). The muddy water cleared quickly and we observed gambusia swimming over the newly exposed shelf within 24 hours. (Please note: Although this work was proposed and completed in January, the funds did not arrive till late March 2007).

Approach Continued:

"February 15, 2007 – March 15, 2007:

a) *Collection of samples of DNA from the pupfish population. Similar DNA samples will be collected from the individuals at DNFHTC.*
b) *Sampling methodology: Specimens will be collected from the upper watercourse of Diamond Y Draw with dip nets, minnow traps and seines. Fish will be treated with MS-222, a mild anesthetic. On a clean, flat plastic surface a 1 x 2 mm cut will be made into the bottom posterior of the caudal fin with a sterile scalpel. Fin clips are commonly used as a technique in order to obtain non-lethal tissue samples for genetic analyses (Wilson & Donaldson 1998; Tyus et al. 1999). Taking fin clips has not been shown to significantly affect growth or mortality in many species of fishes (Armstrong 1947; Coble 1967; Tyus et al. 1999). No injury or mortality is expected from this sampling. Fish will be measured and observed until recovery from anesthesia. They will then be immediately replaced into the pond while the tissue sample will immediately be placed in a 1.5 ml Eppendorf tube with 100% Ethanol. The ethanol will be changed within 4 hours of tissue collection to ensure that the DNA in the tissue samples does not degrade.*
c) *Using the molecular laboratory facilities at DNFHTC, we will determine the amount of heterozygosity, inbreeding coefficients and effective population size for the population at Diamond Y Spring.*

d) *Molecular techniques: Genomic DNA will be extracted using an extraction kit. Primers have been characterized for 13 microsatellite loci in the closely related Cyprinodon variegatus and 9 microsatellite loci in another closely related species, C. pecoensis (Burg et al. 2002). Microsatellite markers will be used to determine population genetic variables. Specifically, these methods will be used to determine the amount of heterozygosity, inbreeding coefficients and effective population size for each population. The Diamond Y Draw population will be compared to samples from the pure population at the DNFHTC. In addition, levels of heterozygosity will be compared to those from other genetic work on C. bovinus (Echelle and Echelle 1997; Echelle et al. 2004)."*

Results:

Very few pupfish were observed during this period of time. Using dip nets and umbrella nets, we collected two pupfish from Diamond Y Spring in efforts throughout the week of March 5th, 2007. These two fish were males and were showing some signs of territoriality (restricting their movements). Funnel trapping in the upper and lower watercourses yielded no pupfish. Additionally, cast-nets proved to be unsuccessful in both the upper and lower watercourses. Seining was abandoned as a strategy to collect pupfish due to practical concerns (e.g. water too deep) and concerns about its destructive potential to any developing pupfish eggs. We decided to wait until June when we hoped the population would be larger and the pupfish would be restricting much of their movements to the shallow areas.

Approach continued:

"March 16, 2007- June 1, 2007:

a) *Once a determination has been made regarding the genetics of the Diamond Y Spring population, we will either (A) begin detailed monitoring of the breeding population until June 1, 2007, or (B) propose adding 500 -1000 individuals from the DNFHTC captive population to Diamond Y Spring (acquiring permits and*

documentation for this process will commence after the genetic work has been completed).

b) *Sample the remainder of the upper and lower watercourses of Diamond Y Spring to estimate the status of Leon Springs pupfish throughout the spring system.*

c) *Intense behavioral monitoring of the population at Diamond Y Spring. This will be done bi-weekly and will be done similarly to my monitoring in the past 6 years (Leiser & Itzkowitz 2003; Leiser et al. 2006). Video recordings will be taken of sections of the historical and newly restored breeding areas and will be analyzed for numbers and aggressive behaviors of males, numbers and locations of spawnings, and interactions in all areas with Gambusia nobilis. At the present time we have strong evidence that predation by Gambusia may be severely impacting pupfish populations via egg predation. It is possible that simply expanding the area of suitable breeding habitat will assist the current population in overcoming this intense egg predation. For this reason, we propose examining the effects of the habitat modification on the current population before the reintroduction of new individuals from the DNFHTC. We also feel it is imperative to understand how the pupfish will utilize the new habitat and where they will locate their territories.*

June 2, 2007 – August 15, 2007:

a) *If the current population is genetically distinct (i.e., shows differing amounts of heterozygosity, inbreeding from the DNFHTC population) or if we detect no increase in the population by June 1, 2007, we will recommend adding between 500 – 1000 adults. This action will be taken in close coordination with, and approval by, the U.S. Fish and Wildlife Service and the Texas Parks and Wildife Department.*

b) *Behavioral monitoring will continue until August 15, 2007."*

Results:

Survey:

We surveyed the water course and observed 2 nonbreeding pupfish at "John's Pool" and up to several dozen more at Monsanto Pool. The structure of Monsanto Pool made it extremely difficult to move close to the open water and we were unable to collect any fish. We believe that any attempts to collect fish using throw nets, seining, or funnel traps will not provide a reasonable estimate of the population. We suggest that a platform is built; making is easier to gain access to the open water.

Genetic Analyses:

Methods:

Fin clips were taken from *C. bovinus* individuals (n = 48) maintained at the DNFHTC in January 2007. We also received DNA *C. bovinus* samples (N = 28) from Dr. Anthony Echelle at Oklahoma State University. These samples were collected from Diamond Y Spring in 2001, approximately 2 years after the restoration of this population using a *C. bovinus* stock maintained at DNFHTC (Echelle 2001). In addition, reference samples of DNA from *C. variegatus* (n = 48) also were obtained from the Dr. Anthony Echelle. These samples were collected in the late 1990s from Lake Balmorhea, Reeves Co., Texas. These samples represent the source of *C. variegatus* that was introduced into Diamond Y Draw (Echelle & Echelle 1997).

Collections of tissue samples were made at Diamond Y Spring in June, 2007. Fish (N = 20) were collected by umbrella nets on the breeding grounds and dip nets throughout the pool. These methods resulted in minimal disturbance to the habitat. After collection, fish were anesthetized with MS-222, measured, and a 1-2 mm^2 clip was taken from the lower portion of the caudal fin. No mortality occurred as a result of collection or tissue sampling. Tissue samples were preserved in 100% Ethanol and stored at the Dexter NFHTC until analysis.

All genetic analyses were performed at the DNFHTC and adhered to the standard operating protocols of the DNFHTC. DNA was isolated from DNFHTC and Diamond Y Spring samples using DNeasy single tube kits (Quigen). The quantity of DNA was determined with spectrophotometry (Nanodrop) and all

samples yielded between 7.2 – 63.1 µl/ng with a mean of 36.675 µl/ng. Seven microsatellites were used for genetic analyses and analyzed on an ABI 3130*xl* Genetic Analyzer after polymerase chain reaction (PCR) amplification in which forward primers were fluorescence labeled (Table 1). Each PCR reaction was performed in a 10 µl reaction volume using 3.5 µl ddsH20, 2 µl Buffer/MgCl, 1.5 µl dNTPs, 0.175 µl taq DNA polymerase, 0.5 µl forward and backward primers and 2 µl DNA template. Thermocycling parameters were 9 min at 95 ^{o} C, 33 cycles of denaturation at 94^{o} C for 1 min, 56 ^{o} C for 45 s and extension at 72 ^{o} C for 1 min, followed by a final extension step at 72 ^{o} C for 7 min. After amplification, 1-2 µl PCR product mixed with 0.88 µl HiDye and 0.12 µl GeneScan- LIZ 600 Size Standard (Applied Biosystems) and run on an ABI *3130xl* Genetic Analyzer. Scoring was done with Genemapper Software version ?? and all data analysis was conducted using GenalEx 6.1 (citation for GenalEx).

Results of Genetic Analyses:

A principle component analysis revealed overlap in the Diamond Y Spring, Echelle and the DNFHTC populations, (Fig 5A, B, C, D). Comparing the three *C. bovinus* populations to the *C. variegatus* population (collected from Balmorhea, TX) shows that the samples of *C. variegatus* do not cluster tightly with any of the *C. bovinus* samples from any population (Fig 5A).

A population assignment test was conducted in which each *C. bovinus* sample was assigned to their own, or other populations based on analyses using the 7 microsatellite loci (Table 3). There are high levels of between population assignments for *C. bovinus*, with 50% of the Diamond Y Spring samples being assigned to the Echelle or DNFHTC populations. None of the samples of *C. bovinus* from any population were assigned to the population of *C. variegatus*. Further, all *C. variegatus* were assigned to their own population.

All three *C. bovinus* populations were polymorphic for all loci (see also Table 2). The lack of homozygosity at any locus is suggestive that inbreeding and/or genetic drift, if they occurred, were relatively weak processes. All populations had mean numbers of alleles between 10 - 13 (Table 4); and more than half of these

alleles were at frequencies higher than 0.05 (Table 4). High levels of heterozygosity were seen across all populations and at most loci (see table 2 for summary of Diamond Y Spring population). Low Fis values indicate that there is little inbreeding in any of the populations. Further, low mean Fst values indicate that there is little divergence among the populations (Table 5).

There were 5 private alleles (found in 4 individuals out of 20 sampled) in the Diamond Y Spring population, 8 private alleles found in the current DNFHTC population and one private allele found in the Echelle population (Table 6). This variation between populations may be due to sampling errors or small numbers of samples taken. When considering all 3 *C. bovinus* samples, 14 different private alleles have appeared in about 6 years. The population of *C. variegatus* also had many private alleles. This further indicates that there is no gene flow between the species and that the Diamond Y Spring population has not introgressed a third time.

Reproductive Behavior and Influence of Gambusia:

Five males were observed to be territorial during July-August and only 3 of these were consistently territorial over multiple days. These three males were observed defending territories over the newly cleared areas while the remaining two males had used the old spawning shelf and maintained poorly defined territorial boundaries. All five males were observed to spawn at least once during our observations. Many females and juveniles were observed. Although nonterritorial satellite and sneaker males mimic the coloration of females and thus could be confused with females, we observed no female-like fish that attempted to spawn with females. Thus, if small nonterritorial males did exist, they were not spawning.

The 4 territorial males were videotaped on multiple days for 15 minutes each (see Figure 3 for quantitative details). Of particular importance is that a mean of 68 chases were directed at gambusia intruders and the mean number of spawns was 7. In the Discussion, these numbers will be compared to a previous study at Diamond Y Spring.

Adding Additional Fish to Diamond Y Spring:

Although the proposal stated that we may or may not propose adding additional fish during the 2007 summer, we decided against making this decision at that time, largely because we were unable to complete the genetic analysis until mid-September 2007. In the Discussion (see below) we now consider this possibility.

Discussion

The genetic analyses indicate that the small population at Diamond Y Spring remains similar to the current population maintained at DNFHTC and remains highly dissimilar to the reference population of *C. variegatus*. Thus, introgression with *C. variegatus* (Echelle et al. 2004) has not re-occurred. Furthermore, there is little evidence of genetic divergence between the current diamond Y Spring population and the Echelle samples taken in 2001. However, we did find 20% (4/20) of the fish carrying private alleles. Interestingly, the Echelle samples had one private allele (i.e., no longer represented in either the DNFHTC or the Diamond Y Spring samples). At the present time, we are unable to account for the appearance and disappearance of these private alleles but may be the result of sampling errors. In any case, they may have an impact on future conservation measures (see below).

Leiser & Itzkowitz (2003) described the reproductive behavior of the Leon Springs Pupfish in Diamond Y Spring during the 2001 summer. They divided territories into two categories: clustered and dispersed. Clustered territories were those that shared boundaries with other territorial males while dispersed were territories without neighbors. Clustered males chased significantly fewer heterospecific intruders than males with dispersed territories (Fig. 7). These heterospecific intruders were mainly *Gambusia nobilis* and our observations indicate that they may be important predators of pupfish eggs. The differences

between clustered and dispersed territories suggested that male *C. bovinus* were best able to repel egg predators because several neighboring males simultaneous attacked fleeing gambusia. This technique seemed highly effective in dispersing gambusia away from territorial areas.

The territorial males observed in 2007 more closely resembled the dispersed males observed in 2001 but had more gambusia within their territories. The larger breeding area and high numbers of dispersed territories in 2001 may have dispersed the gambusia population and thus, provided males defending these territories some protection from egg predators. In contrast, higher numbers of gambusia per territory in 2007 indicates that the few territorial males we observed in this year were likely experiencing high levels of egg-predation. In addition to lower numbers of gambusia egg predators per territory in 2001, more females entered territories, more females spawned, and more eggs were deposited than in 2007 (Fig. 8 & 9). Thus, the intense egg predation coupled with the low levels of reproduction in 2007 may explain the continued low population numbers.

We hypothesized that increasing the shallow areas of the Spring would sufficiently disperse the gambusia so they would no longer accumulate in the remaining pupfish territories. In fact, this did occur. In 2006 the single territorial male had dozens of gambusia residing in territories and these gambusia groups seemed to become even larger when the male when began to spawned (pers. obs.). This accumulation behavior of gambusia continued to occur in 2007, however, there were typically less than 5 gambusia around spawning males. Reduced densities of gambusia around spawning pairs coupled with an increase in the number of territorial males (from 1 to 5) lead us to remain optimistic that Diamond Y Spring can now support a viable pupfish population. It remains unclear whether the current pupfish population can generate self sustaining numbers as we had seen in previous years and perhaps may require the addition of pupfish from DNFHTC.

Based on the reproductive data and the genetic analyses, we offer three alternative recommendations regarding the adding of pupfish from DNFHTC.

A. *Argument for adding additional fish*: The small number of breeding individuals along with the high numbers of gambusia egg predators suggest that the Diamond Y Spring community remains highly susceptible to inbreeding and/or extinction. The lack of divergence from the DNFHTC populations indicates that adding additional fish will have (1) no profound effect on the current genetic composition of the population and (2) prevent the current population from a future genetic divergence. Much like the exchanging of individuals between fish hatcheries to maintain genetic similarity, it may be beneficial to prevent any future divergence of the Diamond Y Spring population from the DNFHTC population. If divergence does occur, it may be necessary to maintain a refugium for this new *C. bovinus* "type" and may result in the loss of a natural 'home' locality for the current population at DNFHTC.

B. *Argument for not adding additional fish*: The marginal increase in the number of breeding males has occurred and this might be an indicator of an expanding population. Pupfish, in general, have a high reproductive potential which allows them to expand rapidly into newly exposed habitat. The expansion of the breeding habitat in Diamond Y Spring may be responsible for this modest increase and more time may be needed to develop a larger population. A strong case for not adding new fish would be seeing (1) a minimum of 15 to 20 males defending territories, (2) a similar number of nonterritorial satellite males, and (3) a breeding rate similar to that observed in 2001 (see Leiser & Itzkowitz, 2003). Finally, we do not yet fully understand the significance of the private alleles in Diamond Y Spring and in the DNFHTC populations. Perhaps we should continue to perform genetic analyses on a timely basis before we perturb the system with additional fish.

C. *Compromise Alternative*: If more fish are to be added, we suggest it be delayed for two reasons. First, if the current small population expands, it may be unnecessary to add more fish, at least in the near future. Such an expansion of the current population will provide important evidence, with implications for many endangered species of pupfish, that supplementing the habitat with additional

spawning grounds has been a successful remedy. Given the high heterozygosity in the current small population, a drastic decline in the genetic health of the population in the immediate future seems unlikely. Thus, waiting before making a final decision seems relatively risk-free.

One of us (M. Itzkowitz) will visit to Diamond Y Spring in October 2007, March 2008, and May 2008 (and through much of the 2008 summer) to further monitor this population. If the population remains low and more fish are added, we will be in an excellent position to monitor these fish over the 2008 summer.

Acknowledgments

This research was funded by a Section 6 award from the TPWD. The DNFHTC provided housing for Jennifer Gumm and provided all materials necessary for running of the genetic analyses. We are grateful to Dr. Connie Keeler-Foster of the DNFHTC and the staff of the molecular ecology laboratory for their continuous advice and support for this project. Nathan Allan of the FWS (Austin) was essential in securing the funding for this project. He has also provided continuous and enthusiastic support for our research on this pupfish over the past several years. The Nature Conservancy in Texas owns the Diamond Y Springs property and we are impressed with their leadership and vigilance in protecting the pupfish and the pupfish's habitat.

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Table 1. Microsatellite markers used to assess population genetics in *C. bovinus*. Annealing temperatures (T_A) are shown for each locus. Dye = Name of the florescent-labeled dye for that marker.

Locus	Primer 5' -> 3'	GenBank	T_A	Dye	Repeat motif
GATA2	A: TCGGATGCTCAGTCAGTACG B: ATGAACAACGAGTCACACGC	AF398010	45/48	NED	$(GATA)_{30}$
GATA9	A: TCTTGGTGAAAAGGGACTATACG B: GCGTTCTCGAGCTTGTTTAG	AF398012	50/53	FAM	$(GATA)_{29}$
GATA10	A:TTTAAGGCTGTGGTCCAACTG B: AGGTGAGAGACAGCGACTGG	AF398013	50/53	VIC	$(GATA)_{33}$
GATA26	A: ACCTCTCAAGGCAAACAACG B: TCCCACGATAGCTCAGACG	AF398018	50/53	FAM	$(GATA)_{39}$ $(GACA)_3$
GATA39	A: CCTTAGGTGCCTGTGTGAGC B: TGGGAGGTGAACTAAAGATGC	AF398019	50/53	NED	$(GATA)_{28}$
GATA73	A: GGAGACGGTAATCTAGCCAGG B: TCCCCTACCACATAGAGAGGG	AF398020	40/43	PET	$(GATA)_{47}$
CmD16	A: CGGAAATGATATGAGCAGCCC B: GGTCCCATGTTTACCCTC	AF398025	58	VIC	$(GATA)_{27}$

Table 2. Characteristics of microsatellites in the Diamond Y Spring samples. Ho = observed heterozygosity, He = expected heterozygosity, Fis = inbreeding coefficient.

Locus	Fragment Size Range	Number of Alleles	Ho	He	Fis
GATA2	210- 270	13	0.950	0.865	-0.054
GATA9	238- 330	13	0.750	0.899	0.149
GATA10	138-146	2	0.200	0.180	-0.087
GATA26	355- 415	10	0.800	0.808	-0.031
GATA39	324- 404	17	0.900	0.898	-0.018
GATA73	309- 433	11	0.900	0.859	-0.072
CmD16	386- 466	8	0.800	0.734	0.004

Table 3. Population assignment data

Population	Self Pop.	Other Pop.
DNFHTC	28	20
Diamond Y	10	10
Echelle	17	11
Balmorhea	39	
Total	94	41
Percent	70%	30%

The number of samples that are assigned to their own vs. other populations based on analysis of 7 microsatellite loci. While there are high levels of between population assignments for *C. bovinus*, none of these samples were assigned to the population of *C. variegatus*. Further, all *C. variegatus* were assigned to their own population.

Table 4. Allelic patterns across populations

Mean values			
Population	**DNFHTC**	**Diamond Y**	**Echelle**
Na	13.143	10.571	10.286
Na Freq. >= 5%	7.286	6.857	6.857
No. Private Alleles	1.571	0.714	0.286
He	0.762	0.749	0.747
UHe	0.771	0.768	0.761

Na = No. of Different Alleles
Na (Freq >= 5%) = No. of Different Alleles with a Frequency >= 5%
Ne = No. of Effective Alleles = 1 / (Sum pi^2)
No. Private Alleles = No. of Alleles Unique to a Single Population
He = Expected Heterozygosity = 1 - Sum pi^2
UHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He

Table 5 Summary of F statistics. Fis = inbreeding coefficient, Fst = genetic distance, Nm =

All Pops.	Locus	Fis	Fst	Nm
	Gata26	-0.031	0.012	19.778
	Gata 10	-0.087	0.006	44.248
	Gata39	-0.018	0.019	13.161
	Gata9	0.149	0.021	11.837
	CmD16	0.004	0.017	14.455
	Gata2	-0.054	0.027	9.003
	Gata73	-0.072	0.018	13.818
	Mean	-0.015	0.017	18.043
	SE	0.030	0.003	4.537

Table 6. Summary of Private Alleles for *C. bovinus* populations

Pop	Locus	Allele	Freq
DNFHTC	Gata26	359	0.010
DNFHTC	Gata9	262	0.011
DNFHTC	Gata9	274	0.021
DNFHTC	Gata9	318	0.011
DNFHTC	Gata9	334	0.021
DNFHTC	Gata2	278	0.021
DNFHTC	Gata73	301	0.010
DNFHTC	Gata73	353	0.031
Diamond Y Spring	Gata39	376	0.025
Diamond Y Spring	Gata39	400	0.025
Diamond Y Spring	Gata39	404	0.025
Diamond Y Spring	CmD16	414	0.025
Diamond Y Spring	Gata73	425	0.025
Echelle	Gata9	246	0.056

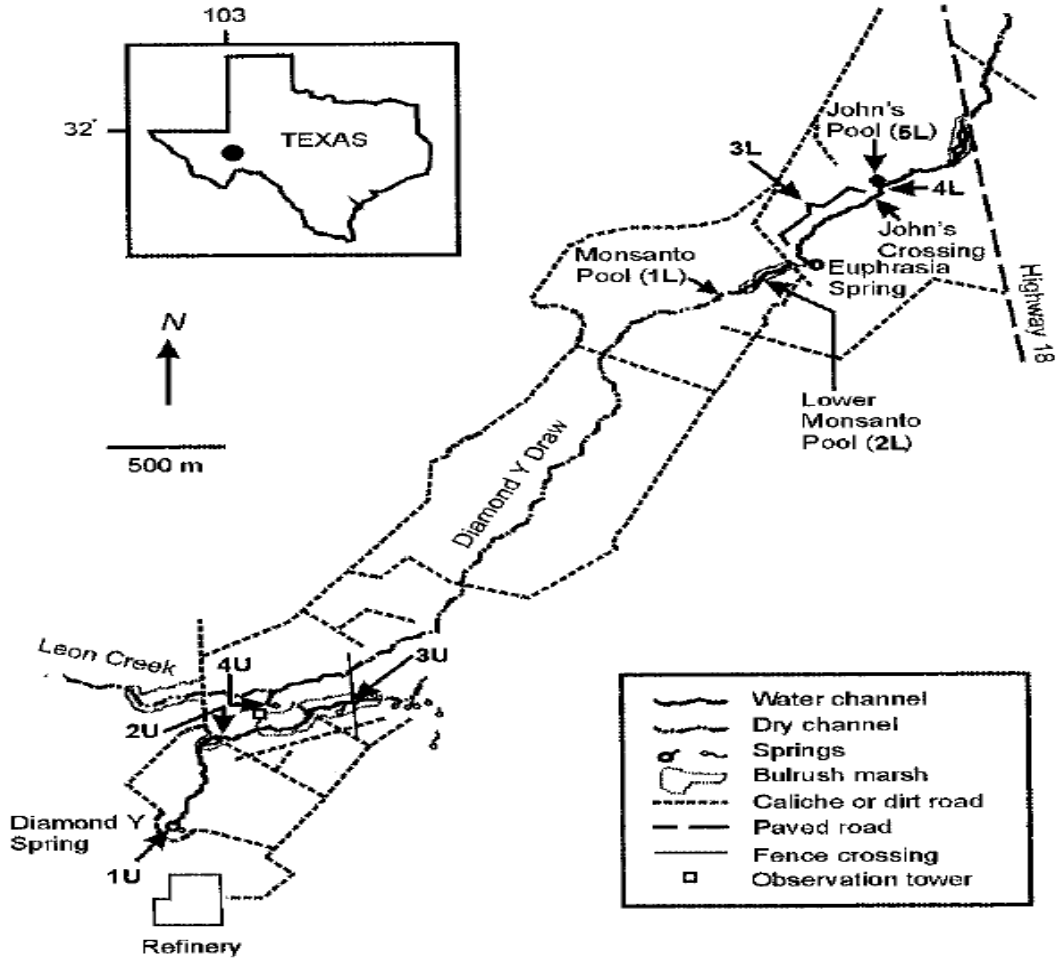

Fig. 1. Diamond Y Draw, Pecos County, Texas. Inset with dot shows location of Diamond Y Draw. Sampling sites for pupfish are indicated by 1-4U in the upper watercourse and 1-5L in the lower watercourse. Other labels refer to landmarks mentioned in the text.

Figure 1. From Echelle et al. 2004.

Figure 2. Diamond Y Spring showing the location of historic breeding site (oval blue area) that previously had maintained about 25 territories. The location of the future new spawning site is a 1 m strip of bulrush that runs 90 degrees from the historic shelf for about 4 meters.

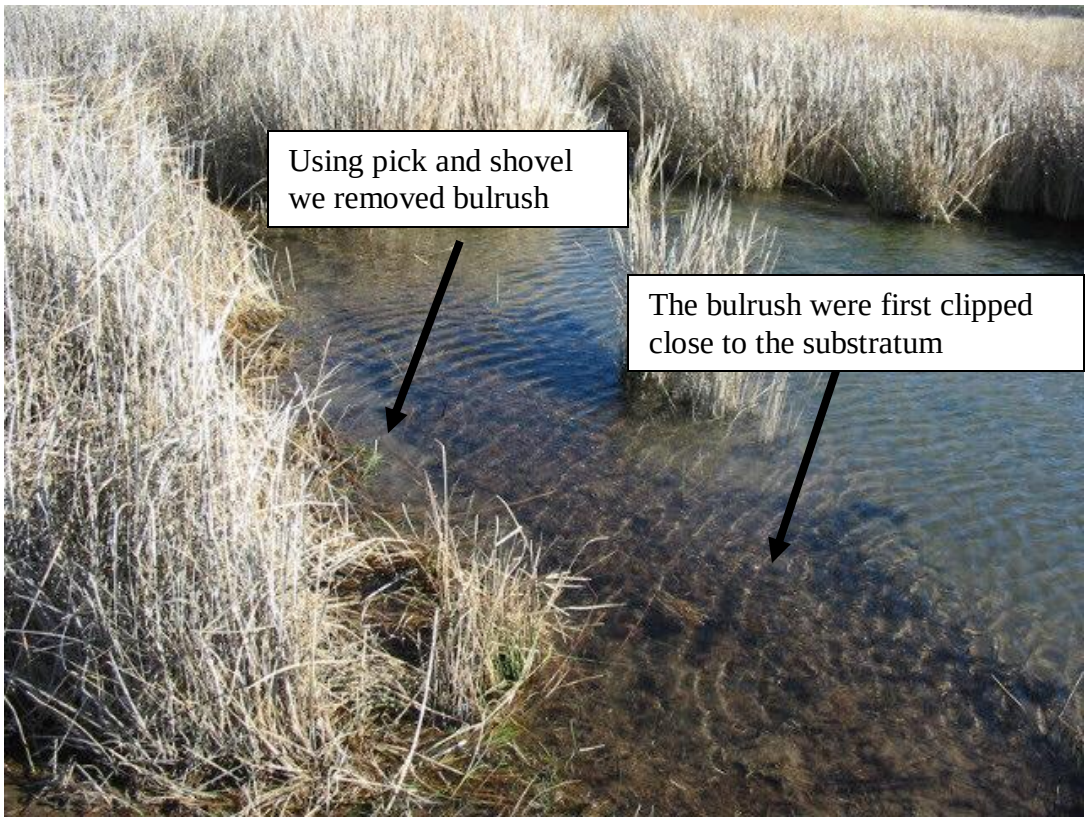

Figure 3. The bulrush have been cleared and we are beginning to dig down about 15 cm to remove the roots.

Figure 4. Cement steps are used to ‘tile’ the cleared area.

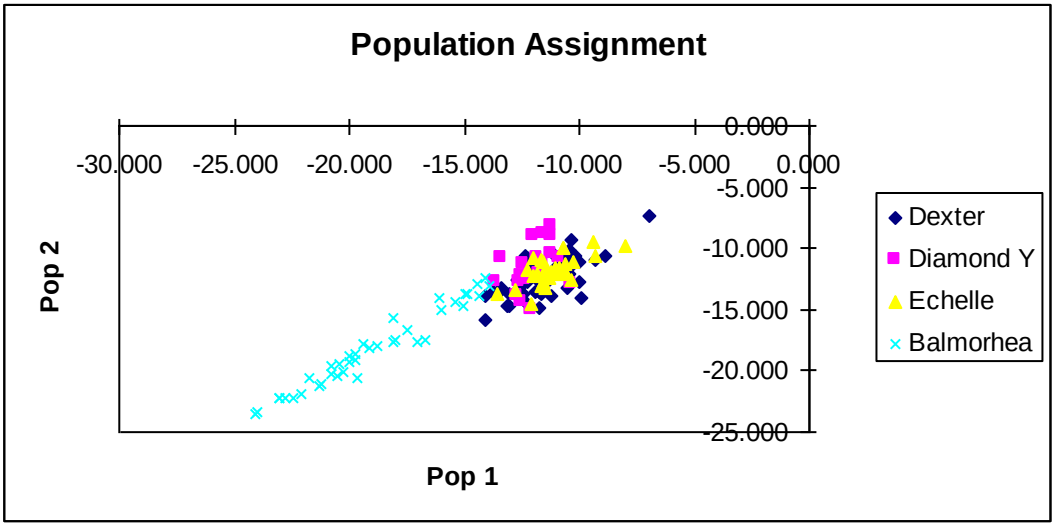

Figure 5A. This population assignment shows that the samples of *Cyprinodon variegatus* from Lake Balmorhea do not cluster with any of the *C. bovinus* samples from any population.

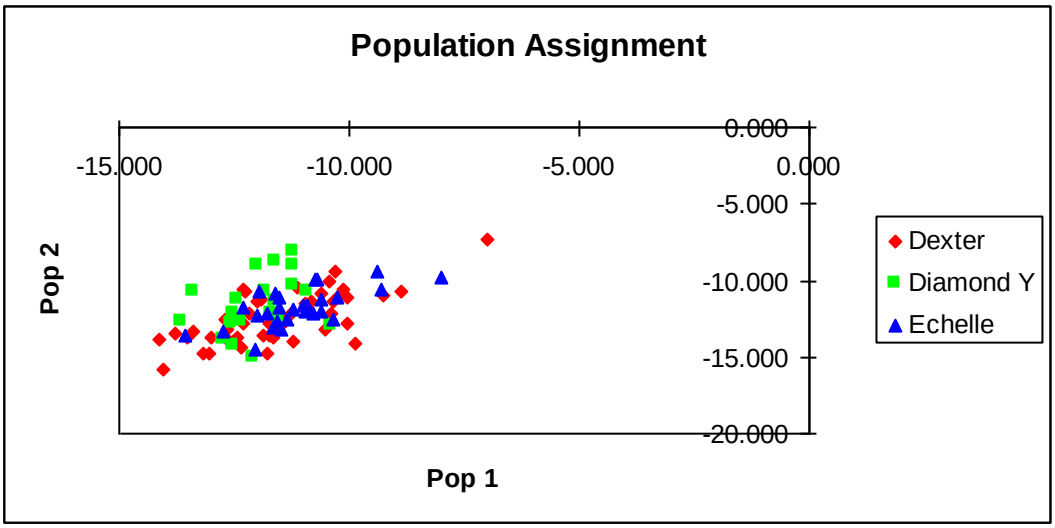

Figure 5B. This population assignment shows the distribution of C. bovinus across the Dexter, Diamond Y and historical Echelle populations.

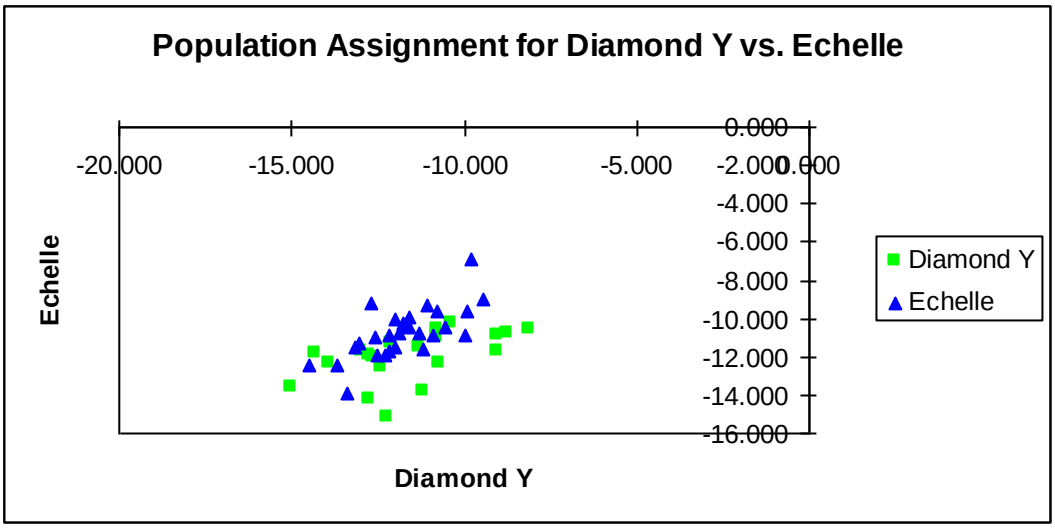

Figure 5C. Pairwise population assignments of Diamond Y Spring and the Echelle samples from Diamond Y Spring.

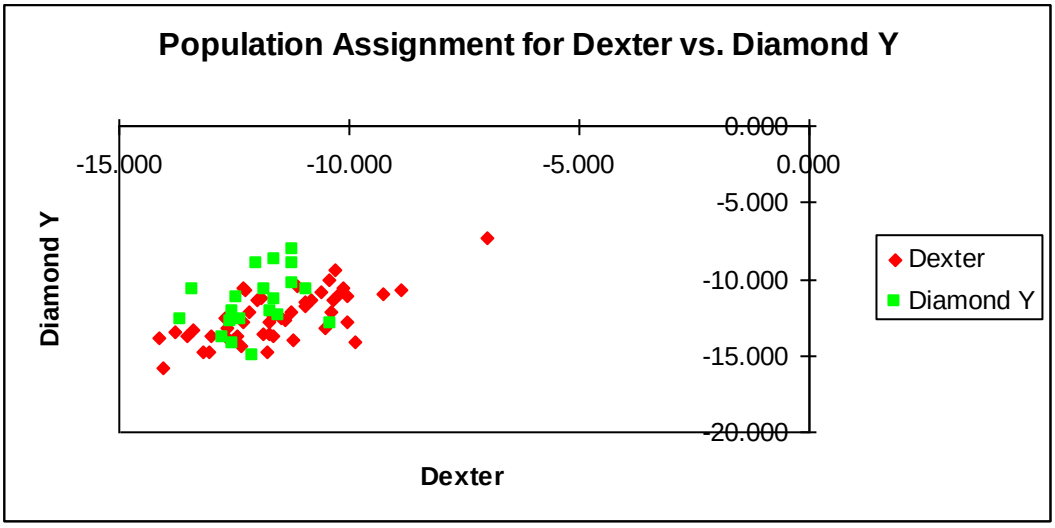

Figure 5D. Pairwise population assignments of DNFHTC samples and Diamond Y Spring samples.

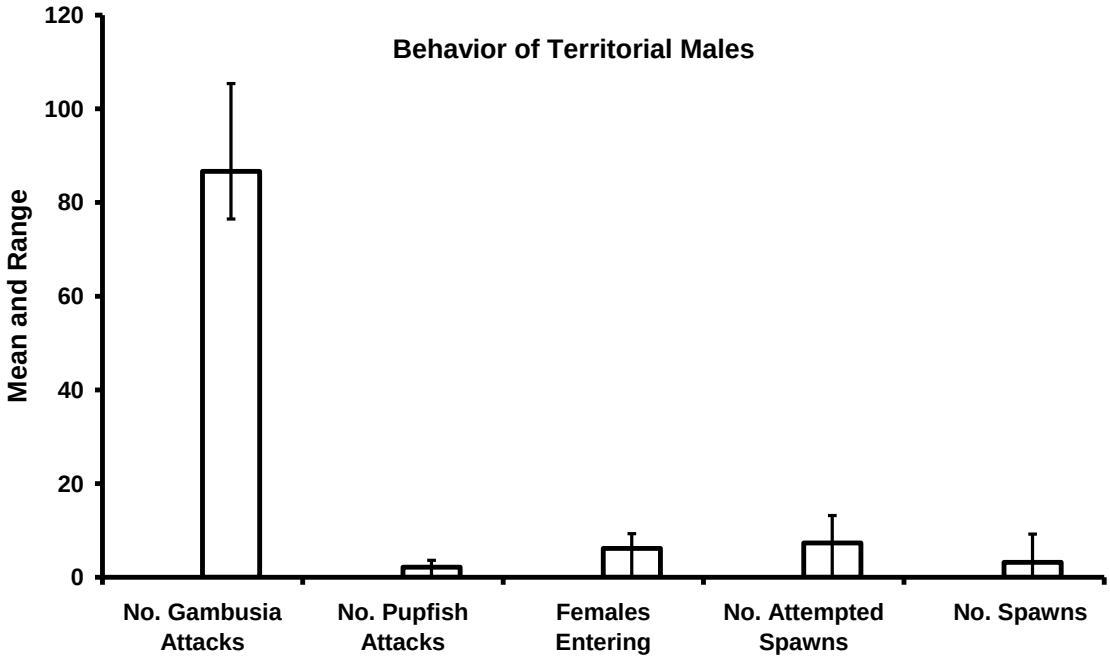

Figure 6. Mean and range for the number of attacks the 5 territorial males directed at Gambusia, attacks against other pupfish, number of females that entered, number of spawns that were attempted, and the number of spawns that were completed.

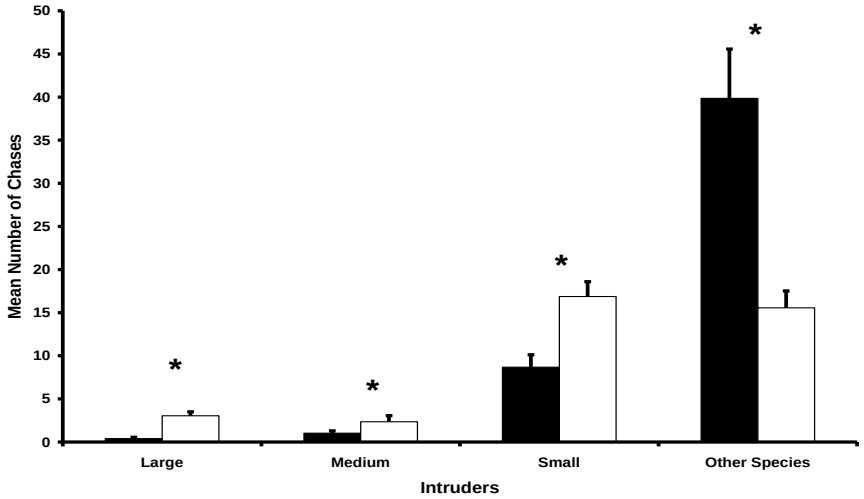

Figure 7. Mean (+ SE) numbers of times that residents defending clustered (light bars) and dispersed territories (dark bars) chased large, medium, and small conspecific intruders and heterospecific intruders. Males on clustered territories chased conspecific intruders more than dispersed males, while dispersed males chased heterospecific opponents more than clustered males did. (Taken from Leiser & Itzkowitz 2003)

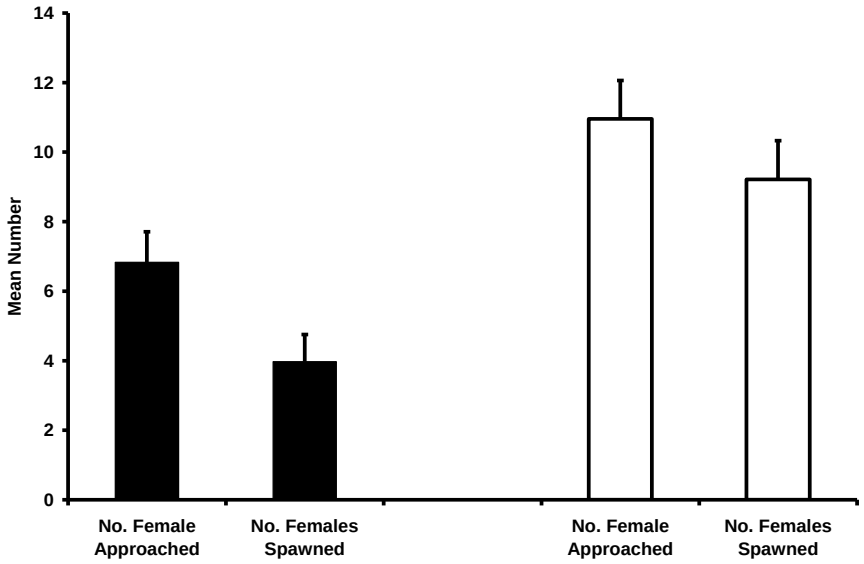

Fig. 8. The number of females that spawned with residents in clustered (open boxes; dashed line) and dispersed territories (black diamonds; solid line) as a function of the number of females approached by residents. (Taken from Leiser & Itzkowitz 2003)

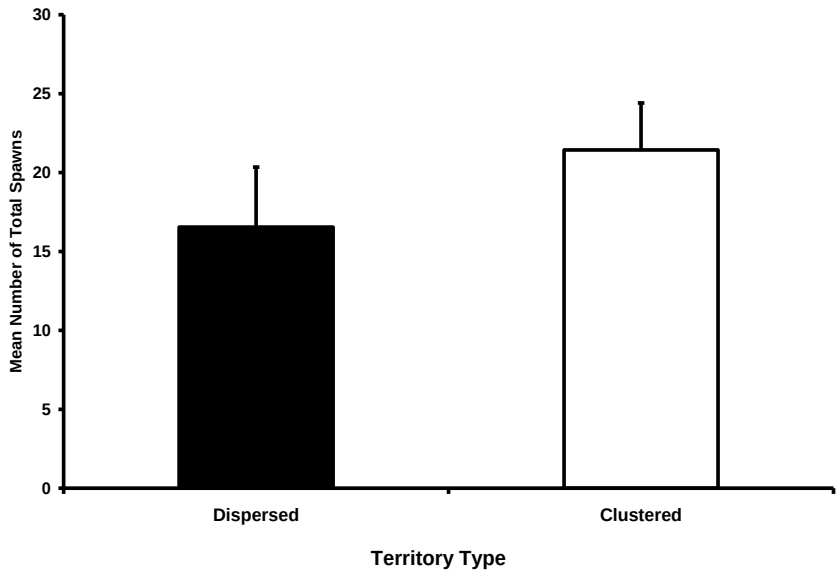

Figure 9. Mean (+ SE) total numbers of spawns received by clustered compared to dispersed territory residents. There was no significant difference in the total number of spawns between the two types of territories. (Taken from Leiser & Itzkowitz 2003)

Addendum to:
Final Report of the
ESA Section 6 Grant Proposal
To Texas Parks and Wildlife Department

Leon Springs Pupfish Recovery and Genetic Diversity:
Spawning Habitat Restoration and Enhancement

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On 7 October 2007 I visited Diamond Y Spring to evaluate the population size and breeding activity of the Leon Springs pupfish (*Cyprinodon bovinus*). To review, the Final Report states that during the 2008 summer we observed about 4 – 5 territorial males (approximately 4.5 cm total length) and a host of females. Within a 15 period, our videotapes recorded about 7 spawns per 15 min period. During this most recent visit, I positioned myself near the renovated area and the traditional spawning shelf and counted the numbers individuals and estimated their sizes. Because my presence may have caused some fish to flee, I also made several 20 min video taping of these localities. I also walked the along the edge of the pond searching for additional pupfish.

I neither directly observed nor video taped territorial behavior. The 11 pupfish that were observed were dispersed largely within the renovated area and consisted of: 1 female 5.5 cm, 2 males 4 - 4.5 cm, 2 females 4.0 cm, and 6 others 2.5 – 3.0 cm. One unsuccessful spawning attempt was observed between 4.0 cm male and a 4 cm female. This spawning attempt was interrupted by a 3 cm male. The 4.5 cm males were similar

in size to the territorial males I observed in July and August 2007. The 4 – 4.5 males followed females and courting them whenever the female the stopped swimming.

The reproductive season for this species is nearing the end and thus the lack of territorial behavior and low levels of reproductive activity were not surprising. Regarding the current population estimate, it is not a good sign that the population seems lower now than in August. It is possible that the ending of the breeding season coincides with individuals moving away from the breeding areas and into unobservable areas (e.g., deeper water). Alternatively, in spite of the reasonable levels of reproductive activity during the 2007 summer, the levels of egg predation by *Gambusia nobilis* prevented population growth. The number of small individuals (below 3 cm) indicates that some recruitment has occurred and I remain hopeful that my observations underestimated the actual population size. In March 2009, I again will return to Diamond Y Spring to again estimate the health of the pupfish population.