Section 6 (Texas Traditional) Report Review

Form emailed to FWS S6 coordinator (mm/dd/yyyy): 12/30/2010

TPWD signature date on report: 12/8/2010

Project Title: The Leon Springs Pupfish Recovery Project at Diamond Y Draw: Further Monitoring of the Population and the Breeding Habitat

Final or Interim Report? Final

Grant #: ~~E-103-1~~ E-106-1

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

ESA Section 6 Grant Program

Texas

USFWS Federal Assistance Grant E-106-1

THE LEON SPRINGS PUPFISH RECOVERY PROJECT AT DIAMOND Y DRAW: FURTHER MONITORING OF THE POPULATION AND THE BREEDING HABITAT

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Date: December 1, 2010

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Reporting Period: October 1, 2009 to September 30, 2010

Annual Report of the

ESA Section 6 Grant Proposal

To Texas Parks and Wildlife Department

December 2010

The Leon Springs Pupfish Recovery Project at Diamond Y Draw:

Further Monitoring of the Population and the Breeding Habitat

Grant E106

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Abstract

Here I report on the results of a two year project designed to monitor the near extinct population *of Cyprinodon bovinus* (the Leon Springs Pupfish) in Diamond Y Spring, Ft. Stockton, Texas. The diminished pupfish population size was thought caused by both the reduction in the availability of shallow areas by the pupfish to spawn and the egg predation by the highly endangered Pecos gambusia (*Gambusia nobilis*). In January 2007 we had removed emergent vegetation from several square meters of shoreline and placed cement steps on the substratum. The intent was to increase the spawning habitat for the pupfish. Also, increasing the shallow spawning habitat was predicted to spread the gambusia population and thus reduce their egg predation on the spawning pupfish. In 2008, a final report at the end of an initial one year project, I reported that the *C. bovinus* had a "modest" increase in number of territorial males and a reduction in the number of *G. nobilis* preying on freshly laid eggs. In effect, the expansion of the breeding habitat did appear to have the desired effect. Here I report on the results from the last two years. Currently, the pupfish population size is nearly identical to that found in the 2008 and the gambusia predation remained low. My estimate of fecundity is also similar to 2008. I have added Lower Monsanto Pool, located several km north of Diamond Y Springs, to my monitoring schedule. This population is larger but it remains unclear if they retain genes from the introduced variegated pupfish, *C. variegates*, as reported previously. I now report that Lower Monsanto Area may be in jeopardy because of an aggressive increase in the bulrush (*Scirpus sp.)* weeds causing a choking of the shallow streams that connected the Monsanto Deep Pool (a refugium) to the shallow nearby spawning pools.

Introduction

The federally-endangered Leon Springs pupfish, *Cyprinodon bovinus*, is on the verge of extinction in its natural habitat in Diamond Y Draw (Fig 1). In May 2006 at Diamond Y Spring, we recorded approximately 10 adults (males and females) and no juveniles. In January-August 2007, with the support of an ESA Section 6 Grant from the Texas Parks and Wildlife Department (Itzkowitz 2006), we physically restored a small segment of the disappearing breeding habitat in Diamond Y Spring (see Fig 2). This is not the first restoration effort designed to protect the Leon Springs pupfish at Diamond Y Spring (see USFWS 1985). Echelle et al. (2001) replaced the resident hybridized pupfish population (see Echelle & Echelle 1997) with a pure strain from Dexter National Fish Hatchery and Technology Center (DNFHTC), removed exotic species, and did a minor habitat restoration by removing some bulrush (*Scirpus sp.)* plants to increase the pupfish spawning area (see also Echelle et al 2004).

I have been observing this population every summer since May 2000, independent of the Echelle et al. restoration. We have verified (Leiser & Itzkowitz 2003; Leiser et al. 2006) that in May 2001, a vibrant breeding population existed, with well over 25 territories established in the then newly-exposed habitat, and another 25 territories on a narrow shelf (note: The shelf area has been one of the historical breeding localities for this species in Diamond Y Spring). Numerous adult females and non-territorial males were also recorded. The success of the 2001 Echelle restoration was short-lived, perhaps

because some of the recommendations offered at the conclusion of the Final Report (Echelle et al. 2001) (e.g., long-term monitoring of the population and continued control of the bulrush) were not followed.

This project was designed to ensure our restoration effort would be successful on a longer-term basis. This final report provides the results of the two year monitoring effort that was designed to investigate four important questions: (1) Are the historical and restored breeding sites at Diamond Y Spring being used at a maximal level by the pupfish? (2) Is the population sustaining itself and perhaps increasing? (3) Is there a migration of individuals from Diamond Y Spring to other parts of the Diamond Y Draw water system? (Note: Monitoring the effects of existing populations and habitats was suggested in the 1985 recovery plan; USFWS 1985, 1.38) (4) If the restored breeding site is not functioning as proposed, will further modifications be helpful?

Objective:

To assess the status and to contribute to the recovery of the Leon Springs pupfish at Diamond Y Draw, Pecos County, Texas

Expected Results or Benefits

This proposed project is designed to assess the effects of a habitat restoration at Diamond Y Springs. If the restoration is successful, the benefit will be the development of a sustainable population of the Leon Springs pupfish. The extent to which this restoration is successful will be easily quantified by: (1) observable crowding of the historical breeding shelf in Diamond Y Spring; i.e., about 30 large males defending small

territories (approximately 30 cm in diameter), a similar number of smaller "satellite" males that are also reproductively active, at least 60 breeding females, and a host of juveniles; (2) a similar number of territorial males, satellite males, females, and juveniles on the newly developed 30 sq. ft. of breeding habitat that will be near to the shelf; (3) an increasing total number of non-breeding fish in the Diamond Y Spring, as observed over the next two summers, (4) no evidence of hybridization with other pupfish species; (5) the addition of two restored breeding habitat in other parts of Diamond Y Draw; (6) the utilization of these two restored breeding areas by breeding pupfish; and (7) increasing numbers of pupfish throughout the water system.

At the present time, I agree with Echelle et al. (2001) that the decline in the pupfish population was caused by the invasion of bulrush into the pupfish's shallow breeding areas (perhaps due to a minimal lowering of the water level). Echelle et al. (2001) strongly recommended some type of bulrush control system be developed in the future. Our use of submerged cement tiles was predicted to be highly effective in this regard. A secondary contributor to this decline in pupfish numbers was the egg predation by the Pecos gambusia. This was observed clearly when pairs of pupfish that spawned attracted swarms of Pecos gambusia that appeared to eat the newly deposited eggs (Gumm et al. 2008). I expect that a critical benefit of this project will be to determine whether the increased number of territorial clusters will deter the Pecos gambusia from swarming near to breeding pairs of pupfish. Given the different ecological requirements of the pupfish and the Pecos gambusia, I believe that expanding the breeding habitat of the pupfish will have no detrimental influence on the numbers of Pecos gambusia. Thus,

besides increasing the number of pupfish territorial clusters to deter the Pecos gambusia, an additional benefit from this project will be to spread out the pupfish breeding population and thereby dilute the effect of the predatory Pecos gambusia.

Location:

Diamond Y is located 30'30 N 102 degree 55'00. The study site is about 8 NNE of Fort Stockton, Pecos County, Texas, and West of State Highway 18 crossing Diamond Y Draw.

Methods and Results

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

Approach: "*The over-arching objective is straightforward: to support a naturally reproducing population of the Leon Springs pupfish in its natural habitat. The three underlying objectives for this proposed project are: (1) to determine whether the habitat restoration (and possible restocking program) performed during Spring '07 are successful at producing a sustainable population; (2) to increase the number of restored breeding sites in other parts of Diamond Y Draw; and (3) to increase the number of pupfish throughout the Diamond Y Draw water system."*

Tasks Overview:

"There are two separate components to this proposed project: First, an assessment of the restorations…" "Based on this assessment… we may increase the number of breeding sites to other parts of this water system. Second, whether or not we increase the number of breeding sites, we will monitor the breeding success and population size of the pupfish…"

***Task 1**: "Monitor breeding. On a biweekly basis, we will videotape all territories on the breeding shelf and the restored breeding area. Typically I can record approximately 3 to 5 neighboring territorial males at the same time. Each segment will be recorded for 15 minutes. Over the 12-week period, we will have many records of the same locality and the same males."*

***Task 2**: "Analyze the videotapes. I have used such recordings with considerable success (e.g., Leiser & Itzkowitz 2003, Leiser et al. 2006) to get an estimate of breeding activities on both the micro (i.e., each male) and macro levels (i.e., community of males). Over the course of several days, nearly all breeding males will be recorded. At a later time, the following quantitative data will be taken from the video records: (a) the number of territorial males; (b) the size of their territories; (c) the number of times a female entered a territory and either spawned or did not spawn (if the female did spawn, where in the territory were the eggs deposited?); (d) the number of reproductively active non-territorial males within the territory (note: these are called sneaker or satellite males and they are a critical component in the maintenance of genetic diversity); (e) the number of*

successful and unsuccessful spawnings of these non-territorial males; (f) the egg predation by the Pecos gambusia."

Results (Tasks 1 & 2)

Diamond Y Spring

Besides monitoring the Diamond Y Spring community during the 2009 and 2010 summers, I also made an initial visit during the first and second week of April 2009 and early April 2010. Approximately 10 adult males were present. In May 2009 and 2010, I counted 11 territorial males.

Although this final report covers the years 2009 and 2010, I placed these years into a larger context by considering the population from 2000 – 2005, the rapid decline in numbers (2006), the restoration of the habitat (2007), and the subsequent 3 years of monitoring of the population (2008 – 2010). From 2001 to 2007 (i.e., including 2006), all territories were found only on the "natural shelf." In January 2007, vegetation was removed from a portion of the shoreline area that had previously supported territories; cement steps lined the newly exposed open shallow area. Beginning in the summer of 2007, more territories were established in the exposed area than on the natural shelf. Essentially the territories were more dispersed than prior to 2006. Similar to the monitoring in 2008, in 2009 - 2010 we made extensive repetitive observations of the reproductive activity in Diamond Y Spring both in the natural undisturbed shelf (termed "natural shelf") and the shallow area where the bulrush were moved ("exposed area').

In each of the 4 summers (2007 – 2010) after the emergent plants were removed, the total number of territories did return to the numbers seen immediately prior to the massive decline in 2006 but not to the levels reached in 2001 (Fig 2). It is unclear if the large increase in the number of territories in 2001 was an aberration; my recollections are that these numbers are more similar to the numbers prior to 2000. Prior to 2000 many more territories existed in shallow areas that eventually became overgrown with bulrush (e.g., see Leiser & Itzkowitz 2003, Leiser et al. 2006). Thus the surge in the number of territories in 2001 on the natural shelf may have resulted from the inability of adult males to find other shallow areas to establish their territories. However, in spite of the larger numbers of pupfish prior to 2000, it remains possible that the current numbers of territories in 2009 – 2010, in both the natural and exposed areas, have reached the levels that we should now expect for Diamond Y Spring. I urge that more effort be expended to increase the population size. There is no doubt that the total number of fish in this pool is less than 50 individuals (including territorial males, nonterritorial adult males, adult females, and sub adults) and I believe this number is much too low to (1) sustain the population after a minor habitat perturbation (e.g., predation by shore birds and snakes) and (2) prevent inbreeding.

The effect of increasing the shallow habitat available for territories had the predicted effect on the *Gambusia nobilis* community. That is, the gambusia were predicted to become more dispersed and be less likely to prey on pupfish eggs. A thorough documentation of the influence of the habitat renovations at Diamond Y Spring are currently in review (Gumm et al. in review). This manuscript

summarizes the effects of the renovations on the gambusia's influence on the spawning pupfish. Using the data collected through 2009, I summarized that paper in last year's annual report to the TPWD (Itzkowitz, 2009). I repeat that summary here: "Using *an ANOVA, we found there was an overall significant difference in the number of gambusia found near tags (i.e., arbitrarily chosen areas of the shelf in which spawning was not occurring) and spawning pairs for 2008 and 2009 (ANOVA, (F (3,290) = 32.15, p < 0.0001; Fig 3, 4). In both years, the number of gambusia was significantly higher near spawning pairs (Tukey HDS Tests; 2008: p < 0.0001, 2009: p < 0.0001). There was no significant difference for gambusia near tags between the two years (Tukey HDS, p = 0.32) but there was a significantly lower number of gambusia near spawning pairs in 2009 compared to 2008 (Tukey HDS Test; p = 0.0015). Thus, both years were consistent in showing a significant difference in the number of gambusia around spawning pairs than those around white tags.*"

With the addition of data from 2010, I have modified the figure in Gumm et al. (in review) (see Fig. 3). The estimated number of gambusia found within the territory and the number found within 5 cm of a spawning pair are consistent across all three years (2008 - 2010) and all are considerably different from 2006. Thus, the habitat manipulations performed in 2007 appears to have caused the desired effect of reducing the density of gambusia in territories and around spawning pairs. Essentially, creating more open spawning habitat for the pupfish did not lead to a concomitant increase in the gambusia population.

In 2009 and 2010, there was also a significant positive correlation between the mean number of gambusia around a tag within the territory of a male and the

mean number of gambusia around the same male when he spawned (Fig. 5: 2009: r = 0.94, N = 9, P < 0.0001; 2010: r = 0.75, N = 10, P < 0.05). These significant correlations indicate that the gambusia within the territories are the ones that most likely prey on the eggs of spawning females. Thus, reducing the number of gambusia "waiting" in a territory is an effective way to reduce this egg predation. In effect, further increasing the available shallow habitat will correspondingly decrease the gambusia density, even if the number of territories do not increase.

Lower Monsanto Area

In 2009 I began a survey of the pupfish population size at the Lower Monsanto Pool (Fig 6). This pool has a long history of maintaining pupfish but the geographic nature of the pool (steep unstable shoreline with extremely dense emergent vegetation) made it hazardous and destructive to approach the pool's edge to observe the fish. Furthermore, previous attempts to sample the population using seines and throw-nets have been unsuccessful. Beginning in 2009, I was able to visually assess the fish population using an underwater television camera (*Aqua Vu*) attached to a 4.5 m pole. Images from the *Aqua Vu* camera were recorded with a laptop computer. The intent was to generate an estimate of the number of pupfish found in the pool. Although the pool was quite deep, I observed no pupfish below 50 cm and the vast majority were within 15 cm of the surface. In an attempt to quantify the population size, I performed multiple visual transects across the pool with the *Aqua vu* camera. This revealed that much of the pool had no fish in both 2009 and 2010 and the fish were largely concentrated in an aggregation at the

southern edge of the pool, near the surface. I decided that an estimate of the number of fish within the aggregation would provide an estimate of the total number of fish in the pool. For this reason, I placed the camera within 15 cm of the surface, in the center of the aggregation, and made a 180 sec recording. I found no indication that the fish avoided or were attracted to the camera. My intent was to estimate how many pupfish swam across the screen, at a distance of 20 cm or less. All pupfish were between 3 – 5 cm. Surprisingly, I saw no immature individuals and this coincides with my consistent observation that I have never observed any reproduction in this pool. All fish seemed extremely healthy. In July 2009, I counted 82 adults with 45 males and 37 females. In 2010, I recorded 80 fish, 36 males and 54 females. Figure 7 illustrates a "snapshot" taken off of the 180 sec period; 5 adult pupfish are seen. Thus, the estimated number of individuals was similar for both years suggesting that the population size is relatively stable.

My several previous visits to the Lower Monsanto Area (see Fig. 1) prior to 2009 revealed several shallow pools near the main pools and these often supported Pecos gambusia but no pupfish. A startling change was observed first in May 2009 with the appearance of some pupfish in what appeared to be a runoff shallow pool next to the Lower Monsanto Pool. A narrow, very shallow, water "bridge" still existed connecting the Lower Monsanto Pool and the runoff pool. This shallow pool was approximately 25 m^2 with an average depth of approximately 20 cm (Fig. 8). There was little hard bottom; the substratum consisted of a soft easy-to-disturbed flocculent silt with clumps of filamentous algae. Given that pupfish always prefer hard-bottom substratum for spawning, this habitat seemed poor quality.

In spite of the substratum quality, we did observe reproductive activity in this runoff pool in July 2009. Please note, there was no reproduction observed in the main deeper pool (Deep Pool). We counted 25 adult male pupfish and 19 female pupfish in this runoff pool. We then videotaped individual males to judge their reproductive success. After several days of observation and videotaping, we found that, on average, about 3 females entered the males' territories during a 20 min period and of these females averaged only one spawn. Territorial males made only sporadic and infrequent chases at gambusia. This was not surprising, given the large area of the pool coupled with the low numbers of Pecos gambusia.

In April 2010, the Lower Monsanto Area had changed dramatically. The Nature Conservancy had burned a significant amount of the terrestrial vegetation surrounding the ponds in the late winter of 2010. In April the vegetation surrounding the pools was completely burned and the bulrush in the shallow water was trimmed by fire (Fig. 9, 10 , 11). We recorded many large, seemingly healthy, non-spawning adults in the Deep Pool. However, the fish community in the shallow neighboring shallow pool had all but disappeared. It is unclear what had happened to the pupfish we had observed in this pool during the previous summer but it is possible that they migrated back to the Deep Pool or they simply did not survive the winter.

In May 2010 the grass and the bulrush were recovering from the fire-burn and we noticed that the shallow pool was now smaller with the encroachment of new bulrush. There was no obvious water flow between the Deep Pool and the shallow neighboring pool; we observed a few nonbreeding pupfish in the shallow pool. In

July, the bulrush were extremely high, very dense, and the water bridge observed in 2009 was choked with the bulrush (Fig. 12, 13). The shallow pool was approximately 1/3 smaller caused by the encroaching of the bulrush (Fig. 14).

There were only 4 territorial males in the shallow pool. We were unable to count the females because they tended to remain hear the bulrush. All males were breeding and each male had a higher number of successful spawns per 20 min observations (Approx. 5) than those observed in 2009 (Approx. 3 spawns) and those concurrently observed in Diamond Y Spring (Approx. 3). I see this low number of breeding males as a signal for a potential disaster!

I believe the numerous shallow pools in the Lower Monsanto Area cannot maintain year-round populations of pupfish. Interestingly, these pools usually contain gambusia. Thus, they can maintain fish, just not pupfish. I believe that the common ingredient in the survival of pupfish in Diamond Y Spring and the Lower Monsanto Area Deep Pool is that deep water must be available to the pupfish. I believe this deep water protects the fish from the low winter temperatures or some other abiotic and/or biotic factors or that co-occur with shallow water. In effect, I believe these deep water areas serve as refugia for nonbreeding pupfish. When the temperature warms, the pupfish move out of the deeper area and breed in the shallow areas. Unlike Diamond Y Spring where the deep and shallow areas are contiguous, in Lower Monsanto, the shallow pools are reliant on overflows from the deep pool. When the overflows (i.e., "water bridges") stop, the pupfish are unable to leave to breed in the shallow neighboring pools or return from the shallow pools for the winter months.

"*Task 3*: *Estimate population sizes. There are many ways to assess population sizes but often they involve manipulations of the fish (e.g., fin clips, tags) that are probably inappropriate for such an endangered species. Instead, I propose placing funnel fish traps in the water for 24 hours, counting and measuring all trapped fish, and then releasing them. This will be done once per month in each of the three summer months. Over a 3-month period, I should be able to assess the status of the population adequately. I propose using 12 such traps in Diamond Y Spring. I also propose that such a sampling be done throughout the Diamond Y Draw watercourse. The intent is to determine whether an increasing Diamond Y Spring pupfish population will migrate into new areas of the Diamond Y Draw water system."*

Results (Task 3)

We made frequent observations around the Diamond Y Spring and found no other pupfish either swimming in the open water or near the pond edge nor did we see any other territorial male. A thorough examination of the marsh area adjacent to the Spring resulted in no visual sightings of pupfish. Our made preliminary attempts to capture pupfish using funnel traps and these attempts were all unsuccessful. Because of these observations, we believe nearly all pupfish in Diamond Y Spring occurred on or near the natural and exposed shelf. See the previous section for the quantitative details of the pupfish population in Diamond Y Spring from 2000 to 2010.

In 2009 and 2010, we made extensive visual surveys in the Lower Monsanto Area. There are a surprisingly large number of shallow pools and many of them contain gambusia. Only 2 ponds supported pupfish, the Deep Pool and the neighboring shallow pool. These two pools had been connected in the recent past.

***"Task 4**: Additional Restoration. If the restorations at Diamond y Spring are successful, I propose establishing two additional breeding sites in other parts of the water course. Their design will be similar to that used in Diamond Y Spring, e.g., shoreline areas will receive 30 sq. ft. of roughened ceramic tiles approximately 15 cm below the water line (Itzkowitz 2006). These tiles will prevent the regrowth of bulrushes and will provide the hard substrata that are required for pupfish spawning. Specific locations in downstream areas will be chosen based on suitable habitat conditions (e.g., water depth, flow, and substrate). Along with these habitat modifications, I also propose adding between 200 – 400 adult pupfish from the stock held at DNFHTC, if earlier work shows this augmentation is warranted. This effort will be closely coordinated with the U.S. Fish and Wildlife Service and Texas Parks and Wildlife Department."*

Results (Task 4)

I did not remove any additional vegetation because considerable space was still available for territories on both the natural shelf and the exposed areas. The numbers of males for the past three years are similar and represent a large increase over the numbers observed in 2006 (Fig 3). Also the number of spawnings per male per 20 min are similar over the past several years but have yet to reach the much

higher levels seen in 2001 (Leiser & Itzkowitz 2003). While the increase in the number of territories is gratifying, I consider this population to be at risk. I did not see the large shoals of immature individuals that I would expect in an expanding population. I see the population as simply maintaining itself, in spite of more than doubling the available spawning area. Thus, any minor perturbation, such as bird predation, could cause the population to go extinct.

I believe the population in the Lower Monsanto Deep Pool is large enough to sustain small perturbations without going extinct. However, while the fish within the Lower Monsanto Pool are a variety of sizes (i.e., ages), I have not seen any individual less than 2 cm. This observation suggest that there is no reproduction occurring in the pool but must be occurring elsewhere, perhaps in the runoff pool. If this is the case, there must be migration of young adult individuals (> 3 cm) into the Pool.

In spite of finding a relatively robust population of pupfish at the Lower Monsanto Pool, I am concerned for two reasons: First, there has been no genetic analyses of these fish and thus they may or may not retain the introgressed *C. variegates* genes that were discovered previously (Echelle & Echelle 1997). Second, except when runoff occurs, the pupfish appear restricted to the Lower Monsanto Deep Pool. If the water level is reduced, the pupfish will be confined to the Deep Pool refugium where reproductive behavior has yet to be documented.

***"Task 5:** Gambusia Egg Predation**.** Evaluate the extent to which Pecos gambusia preys on pupfish eggs. The videotape record of pupfish territorial males will indicate the*

density of Pecos gambusia within the territories and the swarming of the Pecos gambusia around spawning pupfish pairs is an obvious indicator of their egg predation."

Results (Task 5)

In 2006, Gumm et al (2008) observed that Pecos gambusia swarmed around spawning pairs of pupfish at Diamond Y Spring (Fig 5). It appeared that the gambusia responded to female Leon Spring pupfish that approached the substrate in order to spawn. On average, 19 ± 1 gambusia were present within one-fish body length of each Leon Spring pupfish spawning. Significantly fewer gambusia were found randomly on the shelf at numbered tags (only 3 ± 0.2). The number of gambusia present around spawning pairs was significantly lower after the restoration in 2008, 2009 and 2010 (Fig 4). The numbers of gambusia recorded near the numbered tags averaged close to 2 individuals, while at spawning pairs the numbers were between 4 and 5. Comparing the number of gambusia near pupfish spawning and at numbered tags, both pre- and post–restoration, reveals significantly more gambusia near spawning pairs in 2006 as compared to tags in either year or at spawning pairs in 2008 - 2010 (see Results in Task 4). For both 2008 - 2010, territorial males averaged about 3 spawning per 20 min period.

Discussion

Diamond Y Spring:

It is clear that the pupfish populations during the 2008, 2009, and 2010 summers were larger than during 2006 and nearly double since 2007 (5 territorial males; see Itzkowitz 2007). While the number of territorial males increased over the past two years, it still represents a fraction of the numbers observed in 2001. Irrespective of which numbers are considered, they are simply all TOO SMALL! No reasonable conservation biologist would believe that a population of less than 100 individuals is sufficient to maintain the species. Right now, I consider the Diamond Y Springs population be about 50 individuals. Thus, while I am exceedingly encouraged that the renovations have helped maintain the species, more work must be done. It is possible that the reduction in gambusia caused by our renovations around spawning pairs was insufficient to reduce their influence on offspring survival. However, the number of pupfish present is certainly enough to 'seed' the next reproductive event in the Spring 2011. For this reason I do not recommend any restocking with additional pupfish fish from the Dexter National Fish Hatchery and Technology Center at this time.

A critical difference between the territories observed in 2001 and those observed in 2008 - 2010 is that lower numbers of eggs were deposited in the latter years. Thus the territories in 2001 received far more spawns per female than those observed for 2008 - 2010. I cannot account for this decline in fecundity per female but this reduction could account for the continued small number of territorial males observed in 2009 – 2010 in Diamond Y. I am concerned by this fecundity level as it may limit the pupfish's ability to outpace the gambusia predation. It is possible is that these pupfish have lost critical adaptations during their many generations of

captivity in the DNFHTC. Previous studies have observed that captive pupfish stocks do change both genetically and phenotypically (Wilcox & Martin 2006). In the future, if the Monsanto population is free of *C. variegatus* genes, I suggest that we should transplant some of these individuals into Diamond Y Spring.

The pupfish did use the cement tiles in the restored habitat (Fig 4). However, while this artificial substratum was sometimes used by females for spawning, the natural substratum was clearly more desirable to territorial males. We believe that as the population size increases, competition for space will cause males to defend the cement tile areas as well. The current value of the cement tiles is that they do act as a deterrent to bulrush expansion and thus should be used for any future renovations that increase the shallow spawning areas.

In 2006, gambusia egg predation on spawning pupfish was obvious (see Gumm et al. 2008). This predation was problematic because both the gambusia and the pupfish are endangered; we cannot simply reduce the gambusia population. Our hypothesis that increasing the shallow areas would disperse the gambusia population and thus reduce their egg predation on pupfish was supported. With the dispersing of the gambusia population, territorial male pupfish seemed be more effective at chasing them from their territories and, this also, should have reduced egg predation. It is my hope that a further increase in the numbers of side-by-side territorial males in the succeeding years would further increase their effectiveness at repelling gambusia.

In summary, our observations indicate that the restoration of some breeding habitat using cement tiles was successful as indicated by the reduction in the

numbers of gambusia near spawning pairs and the increase in the number pupfish. However, in spite of the reduction in egg predation, the population of pupfish at Diamond Y Spring remains much lower than predicted, the fecundity appeared much lower than previously observed, and recruitment appears to only replace the current numbers of pupfish. I strongly suggest that (1) more shallow areas be developed in Diamond Y to further reduce gambusia predation and (2) individuals that have not spent many generations in captivity (i.e., those from the Lower Monsanto population if proven to be free of C. variegatus genes) be transplanted into Diamond Y Spring.

Lower Monsanto Area:

I strongly believe that this area provides the most promise for preventing the extinction of the Leon Springs Pupfish. There are numerous permanent shallow ponds that could provide a series of semi-isolated spawning localities. It has a refugium that maintains seemingly healthy adults. However, it is unclear if the fish in this area are free of the *C. variegatus* genes (Echelle & Echelle 1997). Without that critical information, further efforts to restore and improve the habitat are unwarranted. Currently, the Lower Monsanto Area has a refugium (the Deep Pool) and a single spawning area (shallow neighboring pool). The "water bridge" that connects the two areas is essential because it allows the pupfish that over-wintered in the Deep Pool to migrate into the spawning area in the Spring. The recent fire burn appears to have stimulated bulrush growth causing these weeds to choke off

the water flow. The spawning pool is now isolated from the refugium. However, the overgrowth of the bulrush is easily remedied.

Recommendations:

The renovation that increased the spawning area and reduced the gambusia egg predation at Diamond Y Spring is successful. As far as I know, this has been the one of most successful conservation efforts for any endangered pupfish species. However, the species is still at considerable risk. I recommend increasing the spawning areas in Diamond Y Spring, downstream from the current renovated area. It is important to spatially separate the spawning areas to mediate against local perturbations. I am concerned that this population may have lost critical adaptations from its long captivity and I suggest that individuals from the Lower Monsanto Area be transplanted directly into this Spring. In my opinion, the Lower Monsanto Area provides the best opportunity to maintain a sufficiently large population of pupfish in semi-isolated pools. However, before any renovations or transplantations occur, a thorough genetic analysis must be made of the current population to judge if previous attempts to remove the *C. variegatus* genes has been successful (see Echelle & Echelle 1997). If this population is free of introduced variegatus genes, further habitat renovations are warranted (i.e., using the Deep Pool as the winter refugium and creating permanently open water bridges to many interconnected shallow spawning pools).

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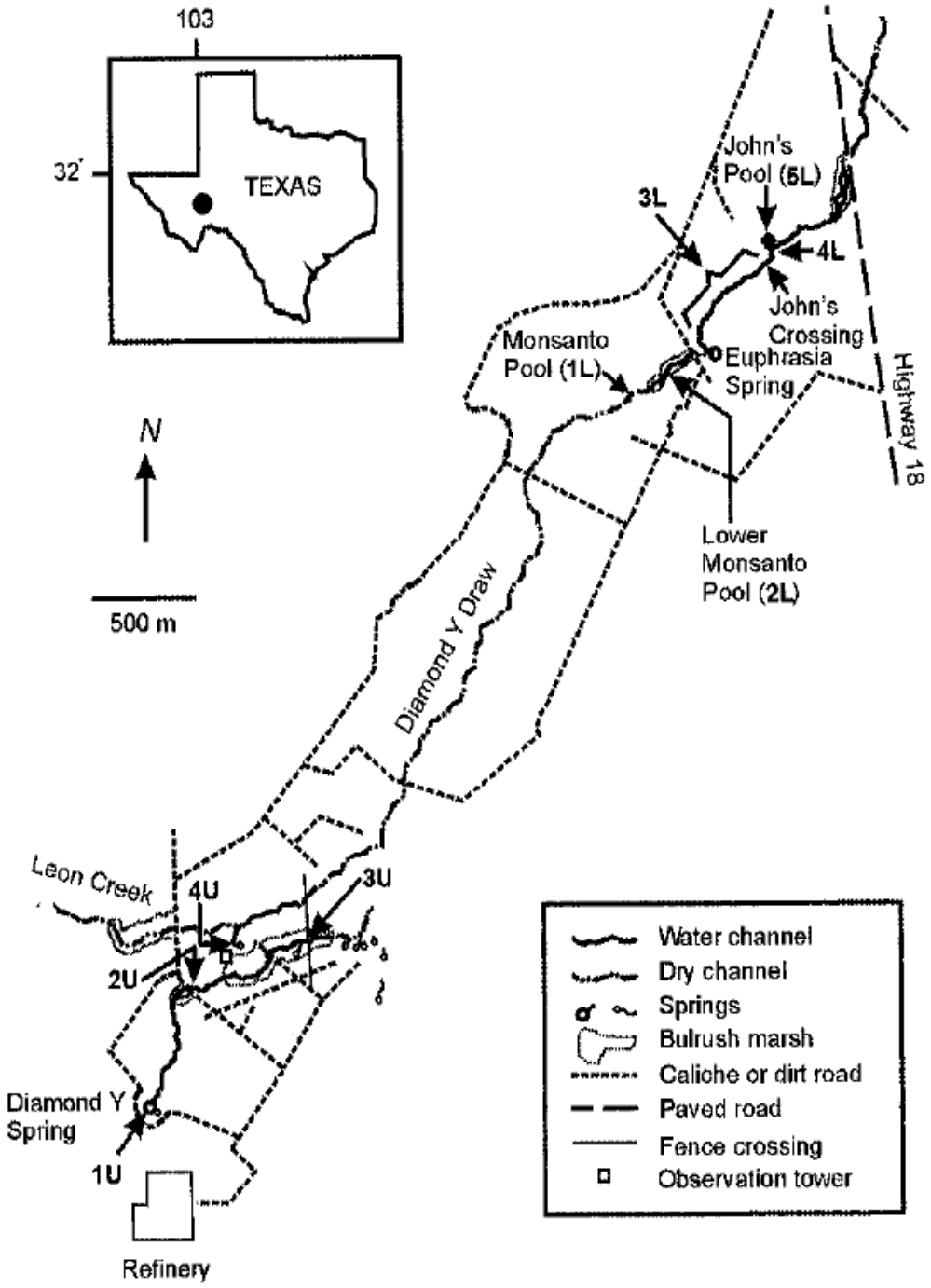

Figure 1. Map of the proposed study sites. Modified from Echelle et al (2001). 1U – 4U and 1L – 4L refer to previous collecting sites. Lower Monsanto Pool is renamed "Monsanto Pool" also termed the "refugium pool" in this proposal. In this final report, the "Monsanto Area" refers to all areas from Monsanto Pool up to John's Pool.

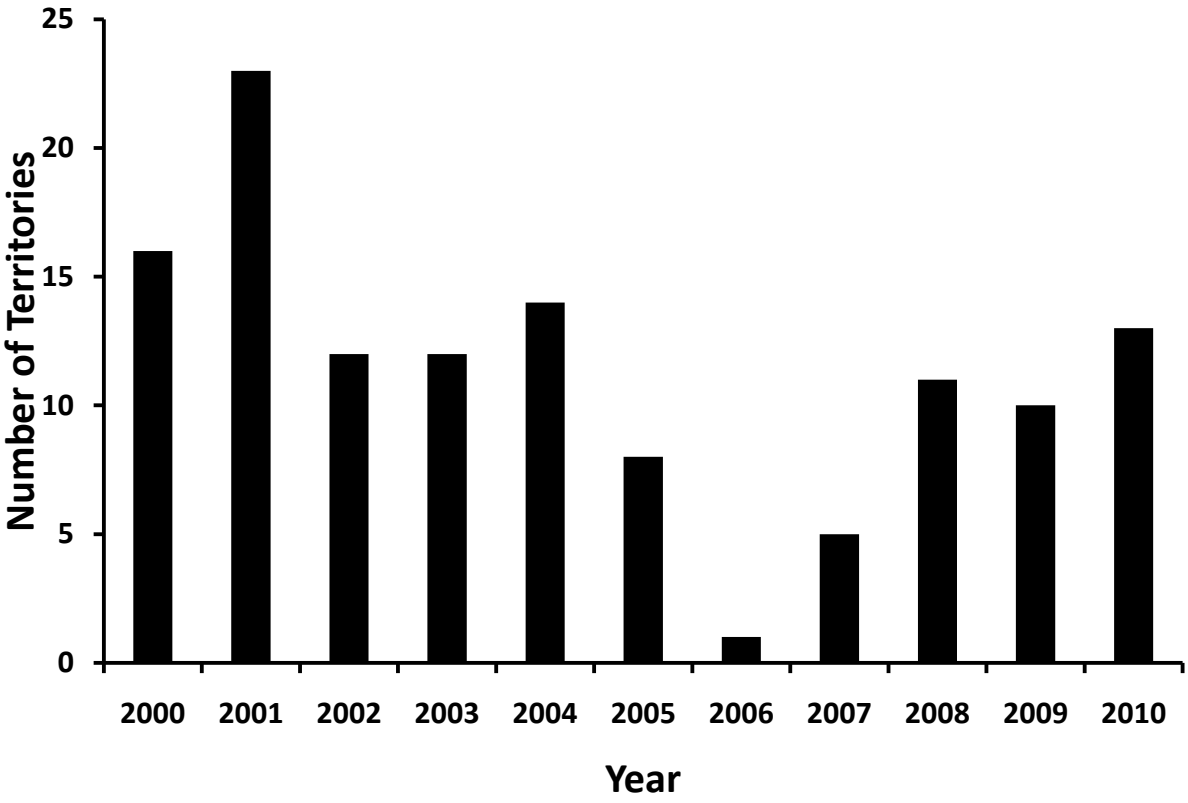

Figure 2. The number of territories observed in Diamond Y Spring, Texas. From 2001 – 2006, territories occurred only on the "natural shelf." In subsequent years, territories were observed on both the natural shelf and the newly exposed area.

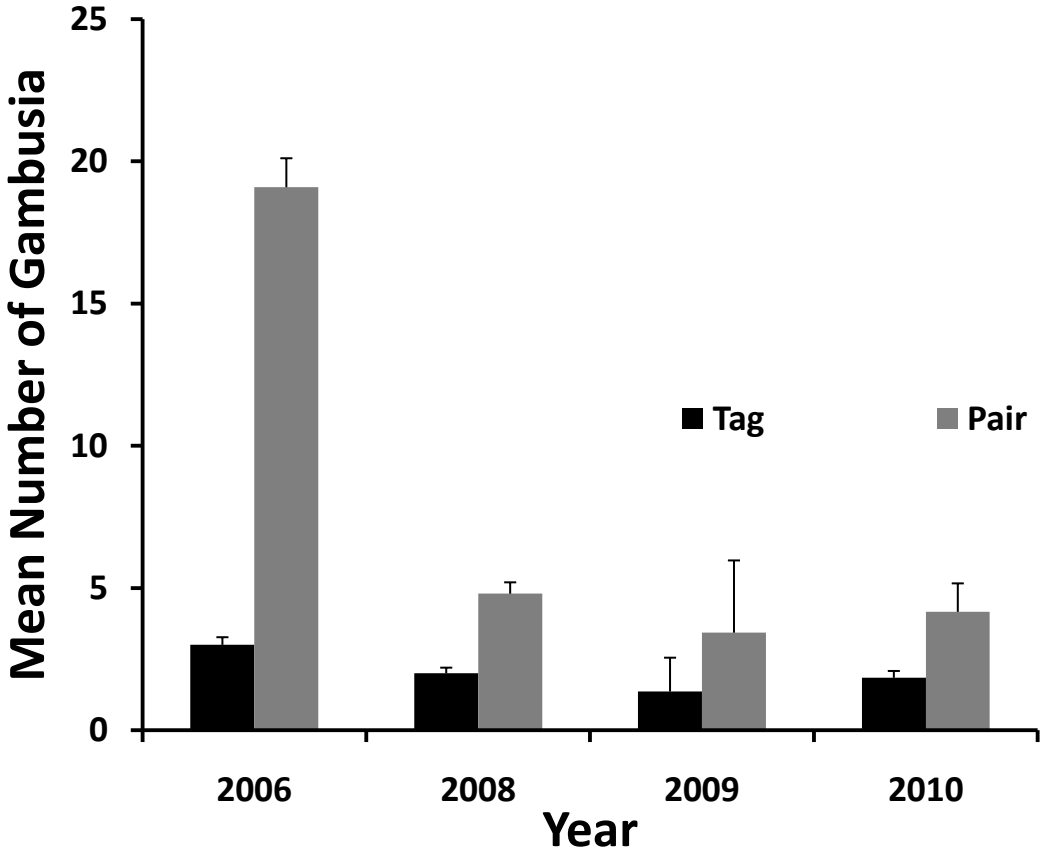

Figure 3. The mean number (± S.E.) of gambusia within one body length (5 cm) for the tag (black bar) or the spawning pairs (gray bar). The data for 2006 was modified from Gumm et al (2008).

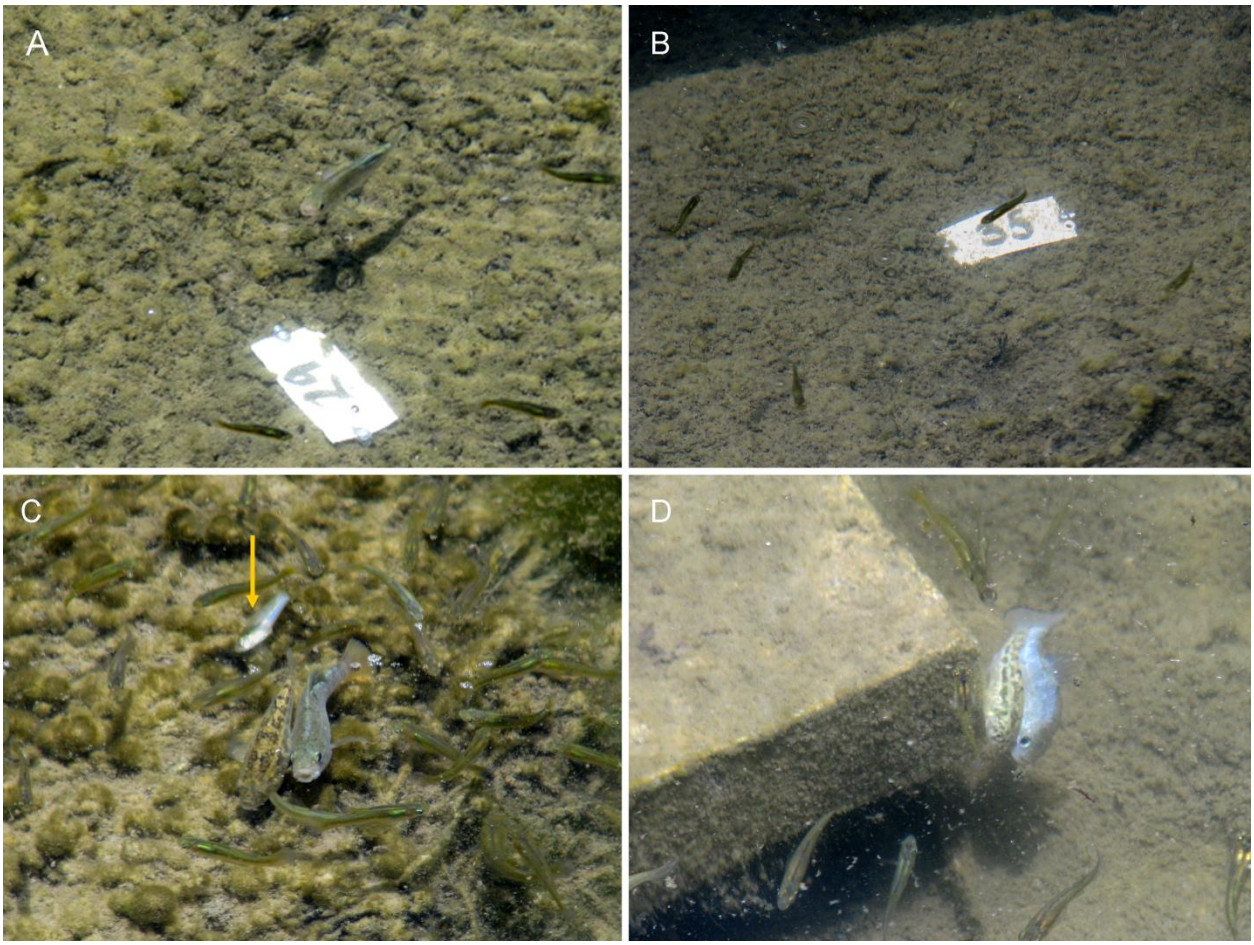

Figure 4. Leon Spring Pupfish at Diamond Y Spring 2008. (A) A large male Leon Spring Pupfish defends his territory. (B) Numbered tag used to delineate breeding shelf areas and estimate the relative distribution of *G. nobilis*. (C) Spawning of Leon Spring Pupfish surrounded by *G. nobilis*. A small sneaker or satellite male Leon Spring Pupfish approaches the spawning to attempt fertilization (yellow arrow). (D) Territorial male Leon Spring Pupfish spawning on the edge of one of the cement tiles in the restoration area.

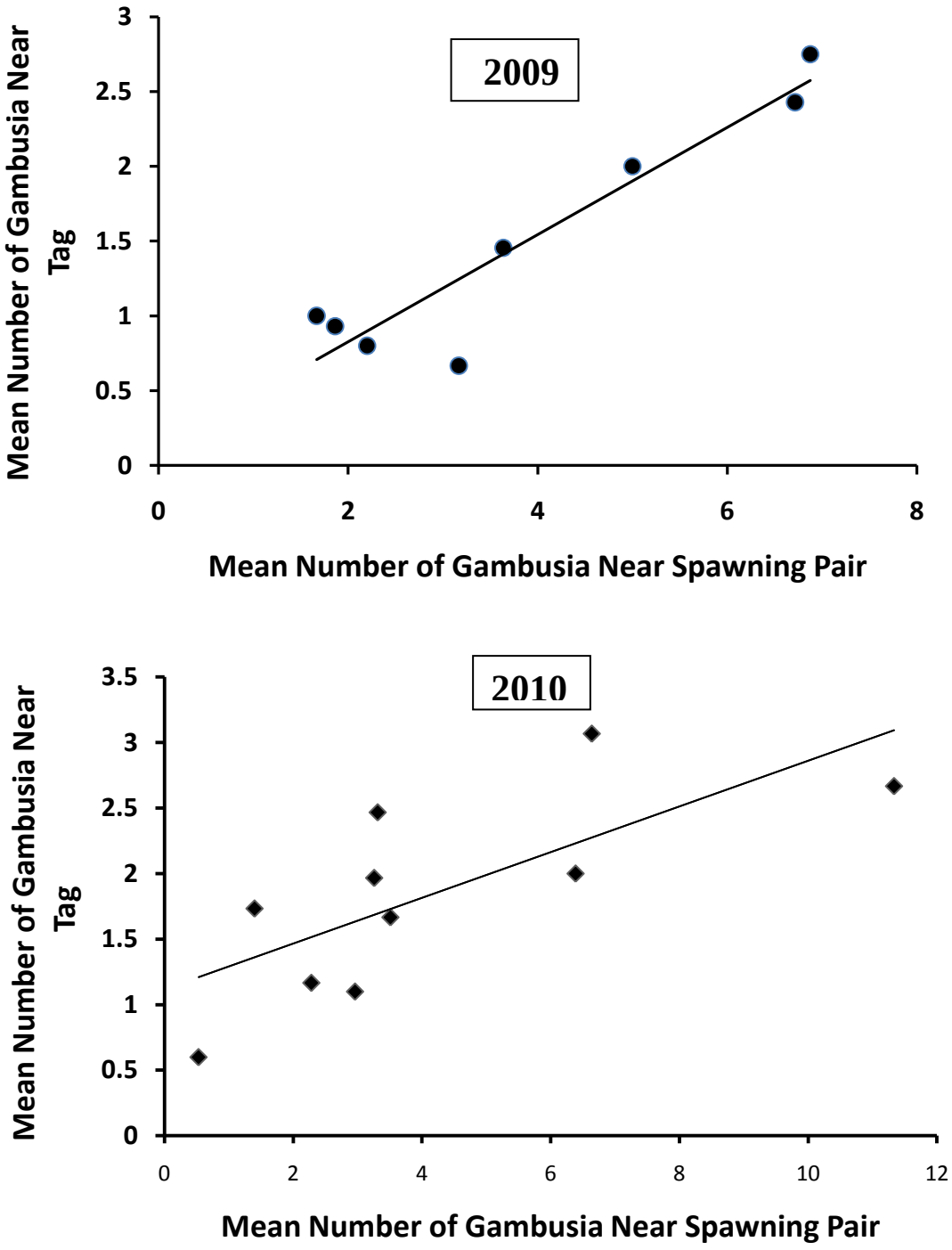

Figure 5. In 2009 (upper panel) and 2010 (lower panel), the mean number of gambusia within 5 cm of the tag located in a territory compared to the mean number of gambusia within 5 cm of the spawning pair in the same territory at Diamond Y Spring.

Figure 6. Photograph of the Monsanto Area refugium pool in July 2009. The size of the pool is about 3 x 2 m with a depth of about 1.5 m. Notice the large amount of seemingly dead bulrush. The white arrow indicates the beginning of the water bridge to the adjacent spawning pool.

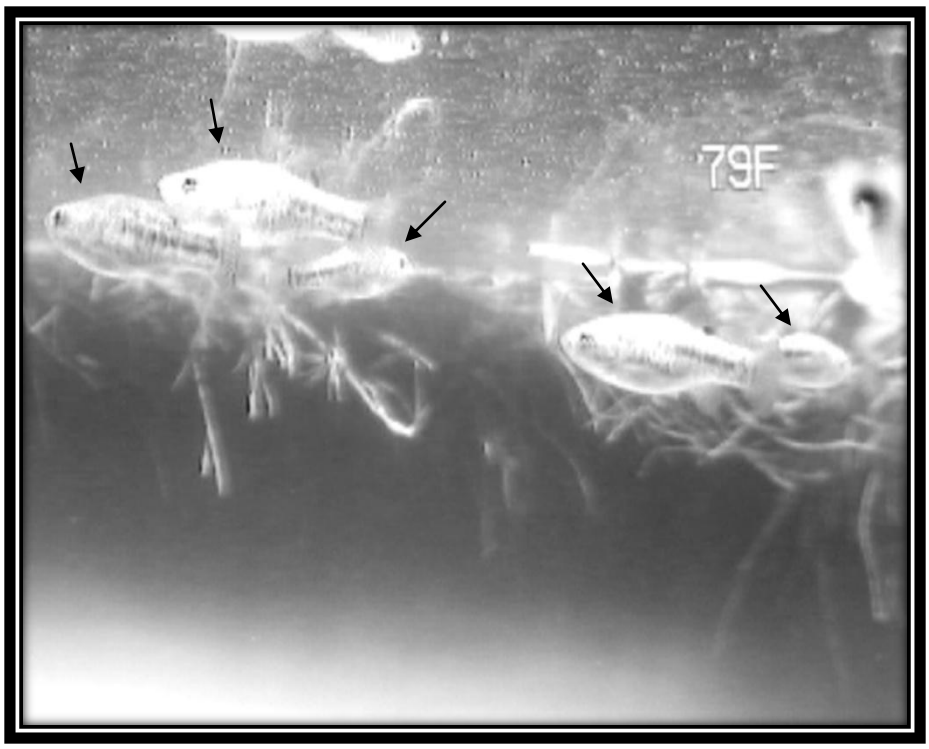

Figure 7. An image of 5 pupfish (see arrow) taken in the Lower Monsanto Refugium in July 2009 using the Aqua Vu television attached to a computer. The temperature was recorded automatically at 79F (26.1C). These fish are within 5 cm of the surface.

Figure 8. Photograph of the water bridge (left arrow) connecting the Deep Pool and the shallow runoff pool (spawning pool)(right arrow). Notice relatively short bulrush at top of spawning pool and the absence of bulrush at the lower portions of the water bridge and the spawning pool.

Figure 9. Photograph of the Lower Monsanto Area April 2010. Arrow indicates Deep Pool (refugium)

Figure 10. Photograph of the Monsanto Deep Pool (refugium) in April 2010. Notice the burned brush, grass, and trimmed bulrush.

Figure 11. Photograph of the shallow runoff pool (spawning pool) neighboring the Deep Pool (on left out of view)(April 2010)

Figure 12. Photograph of refugium pool at Monsanto Area in July 2010. Indicates a dramatic increase in bulrush. Arrow indicates the pool.

Figure 13. Photograph of the edge of the spawning pool at Monsanto Area in July 2010. The water bridge had connected this pool with the refugium pool (lower arrow) no longer exisits. The upper arrow indicates the location of the Deep Pool (refugium pool).

Figure 14. Photograph of the spawning pool at Monsanto Area in July 2010. Notice that the bulrush completely ring what is now a much smaller pool than observed in 2009 (approx. 1/3 the area of the previous year; see Fig. 9). The bulrush at the lower edge of the pool (did not exist in 2009) had been trimmed by hand to gain a better view of the pupfish and the bulrush at the upper side of the pool has been cut and trampled for the same reason.