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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.

By,

Andrew N. Black

A Thesis

Presented to the Graduate and Research Committee

of Lehigh University

in Candidacy for the Degree of

Doctorate of Philosophy

In

Integrative Biology

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This thesis is accepted and approved in partial fulfillment of the requirements for the
Doctorate of Philosophy.

Andrew N. Black

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Endangered Species Of Pupfish (*Cyprinodon bovinus*): Investigation Of Genetic,
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I would like to dedicate this dissertation to my son Cyrus V. Black; perseverance and tenacity can get you anywhere you desire to go in this world.

TABLE OF CONTENTS

Acknowledgments.....	iv
Dedications.....	vi
Table of Contents.....	vii
List of Figures.....	x
List of Tables.....	xii
General Abstract.....	001
General Introduction.....	003
I. The History and Conservation of The Leon Spring pupfish.....	007
I.1 Introduction.....	007
I.2 Species description.....	008
I.3 Conservation history.....	010
I.4 Recent management.....	013
I.5 Discussion.....	016
I.6 Further considerations and conclusions.....	017
II. The <i>ex situ</i> conservation of <i>Cyprinodon bovinus</i>	025
II.1 Introduction.....	025
II.2 Materials and methods.....	028
II. 2.1 Subject species.....	028
II. 2.2 Sample collection.....	030
II. 2.3 Morphological variation	030
II. 2.4 Molecular techniques and bioinformatics	032
II. 2.5 Neutral and adaptive variation.....	036

II.3 Results.....	038
II. 3.1 Morphological variation.....	038
II. 3.2 SNP discovery and filtering.....	039
II. 3.3 Neutral and adaptive variation.....	040
II.4 Discussion.....	042
II. 4.1 Morphological variation.....	043
II. 4.2 Neutral and adaptive variation	044
II. 4.3 Implications for future conservation.....	047
III. Assessment of introgressive hybridization.....	057
III.1 Introduction.....	057
III.2 Materials and methods.....	060
III. 2.1 Sample collection.....	060
III. 2.2 Morphology.....	060
III. 2.3 SNP discovery and filtering.....	062
III. 2.4 Genetics.....	063
III. 2.5 Extension of previous chapters results.....	064
III.3 Results.....	065
III. 3.1 Morphology.....	065
III. 3.2 SNP discovery and filtering.....	066
III. 3.2 Genetics.....	068
III.4 Discussion.....	067
III. 4.1 Morphology.....	068
III. 4.2 Genetics.....	069

IV. The reintroduction of <i>Cyprinodon bovinus</i>	079
IV.1 Introduction.....	079
IV.2 Materials and methods.....	082
IV. 2.1 Study location and species.....	082
IV. 2.2 Experimental design.....	083
IV. 2.3 Statistical analysis.....	086
IV.3 Results.....	087
IV.4 Discussion.....	087
IV. 4.1 Concluding note.....	090
V. Density-dependent effects of a putative egg predator.....	096
V.1 Introduction.....	096
V.2 Materials and methods.....	097
V. 2.1 Study location and species.....	097
V. 2.2 Density dependent behavior and fecundity.....	098
V.3 Results.....	100
V.4 Discussion.....	100
General Discussion.....	103
Literature Cited.....	108
Appendices.....	121
Appendix A: Tables A1.01 – A5.02.....	121
Appendix B: Figures A1.01 – A3.01.....	126
Glossary.....	135
Vitae.....	139

LIST OF FIGURES

Figure I.1 Male and female <i>C. bovinus</i> , study locations, and habitat restoration.....	019
Figure I.2 Geographic range of <i>C. bovinus</i>	020
Figure I.3. Population estimates of <i>C. bovinus</i>	021
Figure I.4 Reproductive behavior and opportunity.....	022
Figure I.5 Mean annual reproduction vs. territorial male estimates.....	023
Figure II.1 Map of DY and SNARRC.....	050
Figure II.2 Procrustes superimposition and landmarks used.....	051
Figure II.3 Canonical Variate Analysis (CVA) of landmarks.....	052
Figure II.4 Bayescan results for outlier detection.....	053
Figure II.5 Pairwise F_{ST} distribution.....	054
Figure II.6 Principle Component Analysis (PCA).....	055
Figure III.1 Map of the three collection sites.....	071
Figure III.2 External characteristics of the three <i>Cyprinodon</i> populations.....	072
Figure III.3 Principle Component Analysis (PC1/PC2).....	073
Figure III.4 Principle Component Analysis (PC2-PC4).....	074
Figure III.5 Histogram of cross-validation scores.....	075
Figure III.6 Discriminant Analysis of Principle Components (DAPC).....	076
Figure III.7 <i>Structure</i> results.....	077
Figure IV.1 Map of study locations for the wild and reintroduced population.....	091
Figure IV.2 Reproductive behavior for the wild and reintroduced population.....	092
Figure IV.3 Foraging behavior for the wild and reintroduced population.....	093
Figure IV.4 Aggressive behavior for the wild and reintroduced population.....	094

Figure IV.5 Territory size for the wild and reintroduced population.....095
Figure V.1 Reproductive behavior and fecundity.....102

LIST OF TABLES

Table I.1 Summary of recovery plan goals.....	024
Table II.1 Procrustes distances between captive and wild <i>C. bovinus</i>	056
Table II.2 Water quality contrast between DY and SNARRC.....	056
Table III.1 Pairwise F_{ST} values.....	078
Table III.2 Classification / misclassification table.....	078

GENERAL ABSTRACT

The overarching objective of this thesis was to: (I) synthesize and review the conservation history of an endangered species of pupfish (*Cyprinodon bovinus*), (II) test for genetic and phenotypic divergence between a captive and wild population, (III) examine the wild population for signs of introgression with its congener, *C. variegatus*, (IV) evaluate evidence for the exhibition of maladaptive behaviors following the release of captive animals into the wild, and (V) examine how the presence of a putative egg predator (*Gambusia nobilis*) may affect density-dependent behavior and the future persistence of *C. bovinus* in the wild. The first chapter illustrated that while there has been a contemporary increase in the number of territorial *C. bovinus*, there may be unintended consequences of habitat restoration projects on reproductive success. In the second chapter, landmark-based geometric morphometrics revealed considerable morphological divergence in body shape and examination of both neutral and adaptive variation revealed significant levels of genomic divergence as well as evidence for local adaptation, possibly relating to differences in salinity between environments. While the captive population showed higher levels of genetic diversity, the wild population has maintained substantial genetic variation despite its small estimated effective population size. The third chapter revealed that the wild population failed to show substantial evidence of introgression or contemporary hybridization with the congener, *C. variegatus*, which was demonstrated by a lack of morphological overlap with *C. variegatus*, distinct genotypic clustering, and high levels of genetic divergence with the congener. The fourth chapter illustrated that following the release of captive *C. bovinus* into

their ancestral habitat, the behavior of the reintroduced population was both quantitatively and qualitatively similar to that of the wild population; the reintroduced captive and wild populations exhibited comparable levels of reproduction, foraging and agnostic behavior. The fifth chapter demonstrated that *G. nobilis* failed to exert a negative density-dependent effect on *C. bovinus* reproductive behavior or fecundity.

GENERAL INTRODUCTION

Imperiled species are reared in captivity for the purpose of reintroduction, to supplement declining wild populations or to maintain a continued representation of the population (Kleiman 1989; Brown and Day 2002). Support of this conservation practice can be clearly demonstrated by high profile cases such as the black-footed ferret [*Mustela nigripes*] (e.g. Jachowski and Lockhart 2009), the California condor [*Gymnogyps californianus*] (e.g. Meretsky *et al.* 2000), the Owens pupfish [*Cyprinodon radiosus*] (e.g. Miller and Pister 1971) and the Arabian Oryx [*Oryx leucoryx*] (e.g. Spalton *et al.* 1999). By preventing the extinction of these species in the wild, captive breeding (or *ex situ* conservation) can be viewed as a viable option for combating biodiversity decline (Ebenhard 1995).

While captive breeding was first initiated and reserved for the most critically endangered species it has now increased in frequency for use with both threatened (Araki *et al.* 2007) as well as endangered populations (Snyder *et al.* 1996). For example, it has been estimated that 2000 - 3000 terrestrial vertebrates will be recommended for captive breeding management over the next several centuries (Soulé *et al.* 1986). However, with a rising number of *ex situ* populations under management, concerns associated with captive breeding have begun to surface (Snyder *et al.* 1996).

By raising animals in an environment very different from their natural one, species are exposed to varying sources of both natural and sexual selection in captivity (Snyder *et al.* 1996). This can cause genetic adaptation, or domestication, to the captive environment and has been documented across a wide range of taxa (Heath *et al.* 2003;

Frankham 2008). Domestication, arising from a combination of environmental change as well as genetic adaptation has been a major cause of divergence between captive and wild populations (Price 1999). Compared to their wild counterparts, species maintained in captivity have revealed differences in genetic structure as well as a divergence in morphology and behavior (Wilcox and Martin 2006; Blanchet *et al.* 2008). The alteration in these traits is considered to arise in part due to adaptation to a benign environment, and can have a drastic effect on the establishment and persistence of captive animals when they are reintroduced into their former habitat (Woodworth *et al.* 2002; Frankham 2008; Christie *et al.* 2012).

A reintroduction is defined as "an attempt to establish a species into an area which was once part of its historical range, but from which it has been extirpated or become extinct" (IUCN 1998). While there are over 489 species in focus of recent, current or planned reintroduction efforts for conservation purposes (Seddon *et al.* 2007), success rates are typically low with a majority failing due to a high post-release mortality (Teixeira *et al.* 2007). In fact, Fischer and Lindenmayer (2000) estimated the success rate of reintroductions at 23%. Possible causes could stem from domestication, the decreased ability to adapt to a novel habitat, obtain adequate resources, reproduce or avoid predation (Teixeira *et al.* 2007).

Animal behavior is rarely incorporated into conservation management, but by assessing possible maladaptive behavior it is feasible to improve overall reintroduction success, especially in regard to predation, competition and social behavior (Sutherland 1998). Despite the challenges facing many reintroduction programs, studies are lacking in

assessment of the causation contributing to low reintroduction success in the wild (Seddon *et al.* 2007).

Some of the most extensive research that has been conducted in understanding the costs and benefits of captive breeding has focused on fish, which have received centralized attention due to their high socio-economic value in aquaculture. However, comparative analysis between captive and wild populations has demonstrated similar captivity induced changes. For example, captive animals have shown deviation in morphology (Hard *et al.* 2000; Blanchet *et al.* 2008; Collyer *et al.* 2011), growth (Johnsson *et al.* 1996), population genetic structure (Wilcox and Martin 2006), aggression (Wilcox and Martin 2006; Blanchet *et al.* 2008), predator avoidance (Johnsson *et al.* 1996), reproductive behavior (Fleming *et al.* 1996; Araki *et al.* 2007) as well as egg size (Heath *et al.* 2003) relative to their wild counterparts.

The pupfishes (family Cyprinodontidae) of southwestern North America are commonly maintained in captivity, as the sustainability of these wild populations is frequently contingent on external support (Miller and Pister 1971; Koike *et al.* 2008). Maintaining these species in captivity, or in artificial environments, has resulted in genetic divergence among sub populations (Wilcox and Martin 2006). Captive pupfish populations have also shown a high degree of non-genetic morphological plasticity in addition to rapid genetically based divergence in body shape (Collyer *et al.* 2005, 2007, 2011) as well as changes in head and mouth morphology due to diet specialization (Martin and Wainwright 2011). This phenotypically plastic predisposition may underlie the remarkable ability of pupfish to survive under extremely adverse and variable environmental conditions (Bennett and Beitinger 1997; Feldmeth and Brown 1971) and

has been suggested to facilitate the directional evolution of body shape characteristics (Collyer *et al.* 2007). As the reestablishment of captive animals in wild habitats may be affected by the decreased ability to adapt to local, natural selection pressures, shifts in both genetic and phenotypic characteristics are of concern, especially in view of the fact that a majority of desert fish reintroductions fail to establish (Henderickson and Brooks 1991).

As it is difficult to predict the adaptive importance of specific genetic characteristics for the future persistence and adaptation of a population, the preservation of extant, natural genetic and phenotypic characteristics is believed to be an effective strategy to increase the ability of future reintroduced individuals to adapt to ancestral habitats (Frankham 1986). However, because selection and genetic drift can shape the genetic architecture of subdivided populations in different ways, genetic cohesion between captive and wild populations can be difficult to preserve. It is therefore important to assess how a founding captive population and a wild population compare with regard to the preservation of genetic variation and whether they have, despite their isolation, maintained genetic and morphological cohesion.

Therefore, the objective of this thesis is to use a comparative approach (between a wild and captive population) to evaluate the potential effects of *ex situ* conservation on the preservation of genetic and phenotypic characteristics, and how deviation in these features may then affect the ability of reintroduced animals to become established in their ancestral habitat.

I. THE HISTORY AND CONSERVATION OF THE LEON SPRINGS PUPFISH

I.1 Introduction

The increased threat of extinction for many endangered species has led to the implementation of a variety of recovery plans, ranging from single-species efforts to whole-habitat restorations (Lundquist *et al.* 2002; Sodhi *et al.* 2011). While extensive effort is often invested into the preservation of endangered species and their ecosystems (Hoekstra *et al.* 2002), many conservation efforts are often ineffective (Butchart *et al.* 2010). However, examination of implemented methodology, both successful and unsuccessful, can be instructive and directly or indirectly contribute to the sustainability of the target species as well as inform those with similar recovery plans (Clark *et al.* 2002; Lundquist *et al.* 2002; Bottrill *et al.* 2011; Sodhi *et al.* 2011; Bottrill and Pressey 2012; Masica *et al.* 2014).

The endangered Leon Springs pupfish (*Cyprinodon bovinus*) can help provide insight into the success and failures of other equivalent recovery plans. To date, a single wild population of *C. bovinus* occurs in an isolated desert spring in southwestern Texas (Gumm *et al.* 2011). Similar to many other desert pupfish species, habitat loss has contributed to their decline in abundance and distribution (Williams *et al.* 1985; Hendrickson and Romero 1989). Within the last 30 years this species of pupfish has also endured hybridization and introgression with non-natives, egg predation by the sympatric Pecos gambusia (*Gambusia nobilis*), and pollution (Echelle and Miller 1974; Kennedy 1977; Hubbs 1980; Echelle and Echelle 1997; Echelle *et al.* 2004), resulting in federally listing *C. bovinus* as an endangered species (US Federal Register 2008). Because of these

extrinsic factors, the sustainability of endangered pupfish populations is often highly contingent on careful surveillance and the protection of the remaining habitat (Williams *et al.* 1985).

Therefore, the overarching goals of this chapter are to survey the history and examine the progress of a multi-decade recovery plan focused on *C. bovinus* (Table I.1). Assessing these conservation efforts will allow management to identify the strengths and weaknesses of these strategies, determine the direction of future conservation efforts for *C. bovinus*, and provide others with a framework for the implementation, assessment, and re-examination of equivalent recovery plans. To this end, the following work has a three-fold purpose: (i) to provide a description of the behavioral ecology of *C. bovinus*, (ii) synthesize the conservation literature on this species over the last 50 years and, (iii) review and expand on the results of over a decade of work maintaining the sole remaining wild *C. bovinus* population. Following this, conclusions are drawn by discussing some of the critical issues that need to be addressed for the continued sustainability of *C. bovinus*.

I.2 Species description

The Leon Springs pupfish are a small (≤ 7 cm) sexually dimorphic freshwater fish that have stout bodies and short fins (Echelle and Miller 1974; Kennedy 1977). Males have a prominent predorsal ridge, a conspicuous terminal black stripe on their caudal fin, and yellow fin pigmentation, while females typically have a narrow body with discontinuous lateral bars, grey fins, and bear a resemblance to immature / juvenile males (Figure I.1A & Figure I.1B; for additional information see: Echelle and Miller 1974). *C.*

bovinus are estimated to live 20 - 23 months and reach maturity within just a few months (Kennedy 1977).

Similar to other pupfish species (Itzkowitz 1974; Kodric-Brown 1986), the breeding season for *C. bovinus* begins mid-April and concludes mid-September, with a peak in spawning activity mid-July (Kennedy 1977). At the onset of the breeding season, large male *C. bovinus* emerge from deeper water to secure open breeding territories (44 cm \pm 10 SD), differing slightly from several other *Cyprinodon* sp. that utilize rock formations and sunken debris as territorial centerpieces (Kodric-Brown 1977, Ludlow *et al.* 2001). Pupfish compete intensely to claim these limited breeding sites, resulting in conditional breeding behavior, which can be observed by the occurrence of both territorial and non-territorial males (Kodric-Brown 1986).

Territorial *C. bovinus* are generally larger than non-territorial males and adopt a blue nuptial coloration during the summer breeding season (Echelle and Miller 1974). Territorial males will actively defend small heterogeneous sites and attempt to exclude all conspecific and heterospecific intruders from their territories, with the exception of receptive females (Leiser and Itzkowitz 2003a). These aggressive behaviors can include direct contact, such as chasing, fighting and/or biting intruders; as well as the non-contact signal of lateral displays (Leiser and Itzkowitz 2003a). Territorial males will exhibit elevated levels of aggressive behavior toward heterospecifics and non-territorial male conspecifics, while non-territorial males are generally smaller (i.e. satellite males), and may bear a cryptic female coloration (i.e. sneaker males; Echelle and Miller 1974; Leiser and Itzkowitz 2003a). Accordingly, these non-territorial males will usually exhibit

reduced levels of agonistic behavior and will steal copulations with females within the boundaries of other males' territories (Leiser and Itzkowitz 2003a).

The courtship and mating behaviors of *C. bovinus* also show similarities with other *Cyprinodon* species (for a review see: Kodric-Brown 1981). While *C. bovinus* females are promiscuous, they are also highly discriminatory of potential mates, visiting all available territorial males before selecting a partner for spawning (Leiser *et al.* 2015). Once an acceptable male is identified, a female will descend to the substrate, the pair will align and form a sigmoidal shape, which is followed by a rapid jerking movement during egg deposition (Figure I.1C; Gumm *et al.* 2008). Females will typically deposit one to several demersal eggs (≤ 0.4 mm in diameter) on the substrate during a spawning event (Kennedy 1977; Leiser and Itzkowitz 2003a). Similar to other species of pupfish, males provide no parental care to these deposited eggs (Kodric-Brown 1977), with the exception of inadvertently driving away male intruders from his territory, which can help reduce filial egg cannibalism (Loiselle 1983). This is particularly relevant because *C. bovinus* lives sympatrically with a suspected egg predator, the endangered *Gambusia nobilis* (for details see Gumm *et al.* 2008, 2011; Figure I.1C).

I.3 Conservation history

C. bovinus have historically been distributed throughout Leon Creek, both upstream and downstream to highway 18 north of Fort Stockton, Texas (Figure I.2A; Kennedy 1977; Hubbs *et al.* 1978). Recently however, the range of *C. bovinus* has become restricted within Diamond Y Draw, a historical tributary of the Pecos River (Veni 1991), to two discrete locations: Diamond Y Spring (31° 0'4.75" N, 102°55'27.09"

W) and Monsanto Pool (31°1'51.60" N, 102°53'49.73" W; Figure I.2B). Separated by 4.2 km of predominantly dry land, the draw flows from Diamond Y Spring NNE to Monsanto Pool, predominantly fed by the Rustler aquifer (Boghici 1997). Water in this area is low in dissolved oxygen and high in chloride, sodium, and sulfate ions (Brune 1975; Veni 1991).

Diamond Y Spring is located 8 km west of highway 18 and is comprised of a circular head pool ~ 419 m² and 3.8 m in depth with steep undercut banks and a shallow 8 m² natural breeding shelf (Veni 1991; Gumm *et al.* 2011). From the head pool, water flows down a long stretch of land choked with bulrush (*Scirpus americanus*; ~ 2 m in width and 5 - 15 cm in depth) before terminating into the ground 1 - 2 km to the NNE (Echelle and Miller 1974). Monsanto Pool, located downstream from Diamond Y Spring, is primarily fed by groundwater seepage and potentially by a nearby Euphrasia Spring (31°01'56.1" N, 102°53'39.2" W; Veni 1991; Figure I.2B). Monsanto pool has maintained a small population of *C. bovinus*, which overwinter in a deep ~ 1 m² refugium, exiting to spawn in the surrounding shallow ponds (M. Itzkowitz, personal observation). Much of the area surrounding the refugium contains dense emergent vegetation (~ 10 m²), with an estimated water depth of 5 - 10 cm, which can impede spawning by overtaking the shallow breeding areas. Unlike Diamond Y Spring, there is an absence of compact substrate at this locale, and the majority of the shallow areas are muddy and full of flocculent silt and algal material (A. Black, personal observation).

The first documented collection of *C. bovinus* occurred in 1851 (16 specimens collected by John H. Clark) from Leon Springs (or Lake Leon; 30°53'N, 103°01'W; Brune 1975) during a U.S. and Mexican boundary survey (Girard 1959; Echelle and

Miller 1974; Kennedy 1977; Hubbs 1980), yet subsequent surveys of the area surrounding the initial collection site were fruitless and *C. bovinus* were declared extinct (Kennedy 1977; Hubbs 1980). However, conservation efforts were renewed in December of 1965 when Minckley and Barber collected a putative *C. bovinus* sample from a desert spring north of Fort Stockton, TX (Echelle and Miller 1974; Kennedy 1977; Hubbs 1980). The collected species was later verified as *C. bovinus* during future surveys in the downstream watercourse of Diamond Y Draw (Echelle and Miller 1974).

The population persevered uninterrupted until the release of a baitfish (the sheepshead minnow; *Cyprinodon variegatus*) precipitated hybridization and genetic introgression with *C. bovinus* during the 1970s (Hubbs 1980) and then again in the 1990s (Echelle and Echelle 1997; Echelle *et al.* 2004). Balmorhea Lake, (30°57'46.46" N, 103°43'12.82" W) in Reeves County, Texas (76 km west of Diamond Y Spring), was the apparent source of the introduced non-natives (Echelle and Echelle 1997). Following the introduction of *C. variegatus* into Balmorhea Lake during the 1960s, there have been documented hybridization events with other Cyprinodontidae, such as *C. elegans* (Commanche Spring pupfish; Echelle and Echelle 1994) and *C. pecosensis* (Pecos pupfish; Echelle and Connor 1989; Wilde and Echelle 1992; Childs *et al.* 1996; Rosenfield and Kodric-Brown 2003).

C. variegatus were first identified in Diamond Y Draw in 1974 near the highway 18 bridge (Kennedy 1977) and the first documented *C. bovinus* x *C. variegatus* hybrids occurred in 1975 within the downstream watercourse (Echelle and Echelle 1997). From 1976 - 1978, conservation efforts focused on eradicating hybrids through intensive seining and the use of ichthyotoxins (for additional details see: Hubbs 1980). Both

morphological characteristics and allozyme electrophoresis verified that the renovation had been successful; no morphological or genetic signatures of introgression were observed or detected in any of the specimens collected from the downstream watercourse (Hubbs 1980; Echelle and Echelle 1997). As a safeguard, 80 genetically uncompromised *C. bovinus* were translocated from the upstream watercourse to the Southwestern Native Aquatic Resources and Recovery Center (SNARRC; formerly Dexter National Fish Hatchery and Technology Center) as an assurance colony (Edds and Echelle 1989).

A second, more pervasive, invasion event occurred again during the early 1990s when diagnostic allozyme and mtDNA markers were detected in individuals from both the upper and lower section of the watercourse (Echelle and Echelle 1997; Echelle *et al.* 2004). The severity of the invasion was substantial enough to result in a second project to eradicate the genetically compromised *C. bovinus* (Echelle *et al.* 2004). From 1998 – 2001, extensive seining and ichthyotoxins were once again employed to remove putative hybrids throughout the watercourse (Echelle *et al.* 2004). Following completion of the renovation, between 5 000 and 10 000 captive *C. bovinus* were subsequently reintroduced from SNARRC to restock the decimated *C. bovinus* population as well as swamp out any potential exotic alleles that may have remained (Echelle and Echelle 1997; Echelle *et al.* 2004). Few non-native allozyme markers (ranging in frequency from 0.0 – 4.2 %) were found in any of the post-renovation *C. bovinus* samples (Echelle and Echelle 1997; Echelle *et al.* 2004).

I.4 Recent management

Since the large-scale release of captive *C. bovinus* into the wild in the early 2000s, the population size rapidly declined until in 2006, only one territorial male was reported (Gumm *et al.* 2008). This precipitated an extensive habitat restoration project that entailed clearing an area of *S. americanus* vegetation ~ 10 x 1.5 x 0.2 m immediately downstream of the natural breeding shelf at Diamond Y Spring in 2007. Cement tiles (30 x 10 x 5 cm) were placed in the cleared area to prevent regrowth of *S. americanus* and to provide suitable breeding grounds for pupfish (Figure I.1D; Kodric-Brown 1978, 1981). Habitat restoration methods were repeated prior to the breeding season in January 2013 and 2014 to create four additional breeding pools (7 x 2 x 0.2 m each) further downstream of the natural breeding shelf. The reiterated habitat expansions sought to decrease the extent of vegetation coverage and provide additional substrate availability for territorial males.

Leading up to and following these habitat restoration projects, annual surveys were conducted to evaluate fluctuations in the *C. bovinus* population size at Diamond Y Spring from 2001 - 2014 and at Monsanto Pool from 2009 - 2012. To estimate population size each year the number of territorial males was documented, which was used as a proxy for total population size. A population census was not possible due to the extent of vegetation coverage, but territorial male counts have been used previously to estimate population size in other pupfish species (Kodric-Brown 1978) as well as *C. bovinus* (Gumm *et al.* 2011).

Following the large-scale reintroduction of *C. bovinus* in 2000 (Echelle *et al.* 2004), there has been a decline in the estimated number of territorial males on the natural breeding shelf of Diamond Y Spring, with an all-time low of one territorial male

observed in 2006 (Figure I.3). However, following the extensive habitat restoration project that occurred at Diamond Y Spring in 2007 (Figure I.1D), the population has shown a rapid, and stepwise, increase in estimated population size (Figure I.3). In fact, the most recent population size estimate at this location (2014 = 20 territorial males) is close to the estimated population size that was observed following the reintroduction event in 2001 (Figure I.3).

Population size estimates at Monsanto Pool were less encouraging. While the 2009 survey revealed 82 adults (45 males; 37 females), and the 2010 survey showed 80 fish (36 males; 44 females), soon after the 2010 survey, the pool became severely choked with bulrush (*S. americanus*) and water levels receded (≤ 5 cm). An ancillary census following the 2010-breeding season revealed just 24 adult and 17 juvenile *C. bovinus*, and in 2012 only 4 adults remained. Since 2012, subsequent surveys of this location have been unsuccessful, leading to the conclusion that *C. bovinus* have become extirpated at this site (M. Itzkowitz, personal observation). The extirpation at this location was likely due to the observed increase in *S. americanus* coverage that had overrun the shallow breeding areas, which are essential for the persistence of pupfish populations (Kodric-Brown and Brown 2007).

Following the habitat restorations in 2011 and 2012, male *C. bovinus* were observed defending territories in the newly established pools both within, and downstream from the natural breeding shelf (Table I.1). Males exhibited characteristic behavior within these newly excavated sites, and were observed chasing away intruding conspecifics and heterospecifics in addition to spawning with arriving females. However, across all breeding territories, males exhibited a downward trend in the frequency of their

reproductive behavior (Figure I.4A), which was highly correlated with an increase in the number of territorial males (Figure I.5 & Table A1.01; Spearman's rank correlation, $\rho = -0.42$, $N = 78$, $P < 0.001$). With the exception of 2011, there was no noticeable pattern in the number of females entering male territories (Figure I.4B).

I.5 Discussion

The recovery plan of *C. bovinus* (Table I.1) appears to have been successful, yet may have also produced unintended consequences. Following the population decline at Diamond Y Spring in 2006, work began with the objective of maintaining and enhancing a sustainable wild population of *C. bovinus*. After the 2007 habitat restoration (Figure I.2D), when emergent vegetation was removed to expand the natural breeding area, the wild population began to increase in size (Figure I.3), presumably highlighting a positive effect of the restoration efforts. However, with an increase in the number of territorial males (Figure I.3), promiscuous females (which have been shown to be choosy in this species; Leiser *et al.* 2015) would have a larger group of potential mates to choose from, which may be decreasing individual territorial male reproductive success (Spence and Smith 2005; Figure I.5). This suggests that with an increase in territorial males, perhaps females are spending more time assessing males / habitat quality prior to spawning, which may be resulting in the observed decrease in reproductive success (Figure I.5).

It is also possible that the individual decrease in male reproductive success could be due to an unequal sex ratio (Clutton-Brock and Parker 1992). While the mechanism contributing to this reduce reproductive success are difficult to delineate, these results

may represent potential negative by-products of expanding the natural habitat of *C. bovinus*.

I.6 Further considerations and conclusions

In addition to focusing on *C. bovinus*, the environment itself must also be considered. Habitat destruction, such as groundwater pumping and drying of surface springs, represents a major challenge for maintaining small populations of desert fish (Williams *et al.* 1985). For example, groundwater pumping was identified as a primary reason for the extinction of the Commanche Springs pupfish (*C. elegans*) during the 1950s (Hubbs and Echelle 1972; Leiser and Itzkowitz 2003b). With local seasonal fluctuations in water flow common, even a small decrease in the water level could impact the amount of available breeding habitat and therefore persistence of *C. bovinus* (Brune 1975; Kennedy 1977). Therefore, in order to protect the habitat and mitigate further habitat loss, extensive aquatic reclamation needs to occur. At a minimum, the continued upkeep of *S. americanus* at Diamond Y Spring is essential for maintaining the available breeding habitat and preserving the current population size. However, the potential effects that altering habitat may have on the behavioral ecology of *C. bovinus* needs to be carefully monitored.

The massive reintroduction of captive *C. bovinus* released 5 000 - 10 000 pupfish into the Diamond Y Draw (Echelle *et al.* 2004). While a population census has not occurred (nor would it be feasible), anecdotal evidence places the population size at roughly 250 individuals within Diamond Y Spring, and surveys have failed to locate any other local populations. Therefore, survivorship of reintroduced *C. bovinus* was

exceedingly low. At this time, the reason for such low settlement success is unknown but is likely due to rapid habitat loss (Williams *et al.* 1985).

Based upon the historical incidence of introduced *C. variegatus* into Leon Creek, it is imperative to continuously monitor and protect Diamond Y Draw from future introductions of exotic species. While the elimination of genetically compromised *C. bovinus* and subsequent release of pure *C. bovinus* from SNARRC is a viable solution, it should be done as a last resort in order to prevent the loss of any private alleles that may only be occurring in the wild population (Echelle and Echelle 1997; Echelle *et al.* 2004).

In order to preserve the remaining wild *C. bovinus* population and continue to develop the recovery plan (Table I.1), the following conservation strategies are emphasized: (1) continued habitat restoration to increase (or maintain) the amount of available breeding habitat, (2) careful monitoring of Diamond Y Draw for introduced non-native species and putative *Cyprinodon* hybrids, (3) thorough and sustained assessment of the hydrogeology of Diamond Y Draw and the aquifer system that supports it, and (4) further examination of possible effects inadvertently introduced by increasing breeding habitat.

Figures I:

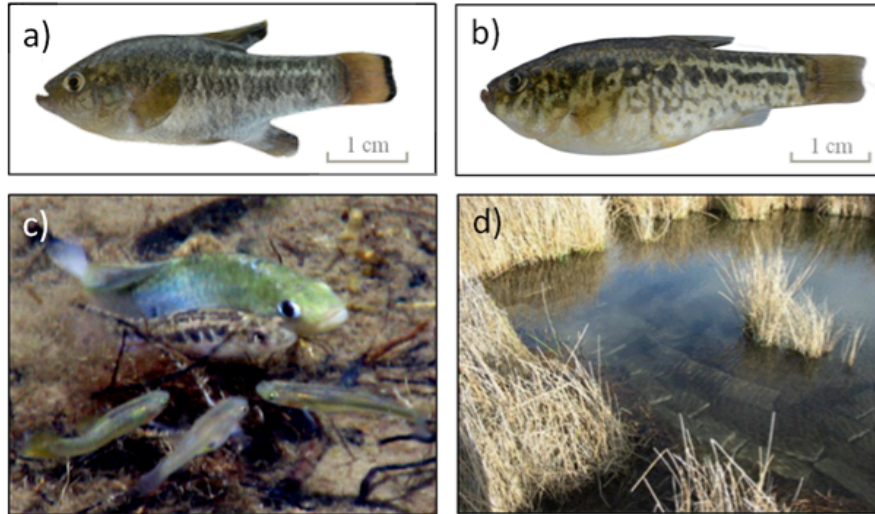


Figure I.1 Male and female *Cyprinodon bovinus*, spawning behavior, and habitat restoration: a) male, and b) female *C. bovinus*; c) spawning between a territorial male (top) and female (bottom) *C. bovinus* and Pecos Gambusia (*Gambusia nobilis*; pictured below the spawning) the putative egg predators; d) habitat restoration project at Diamond Y Spring conducted in 2007 entailed the manual excavation of bulrush (*Scirpus americanus*) vegetation to provide an additional open, shallow, breeding territory habitat. Cement tiles were used to prevent *S. americanus* regrowth and to provide additional oviposition substrate for *C. bovinus*.

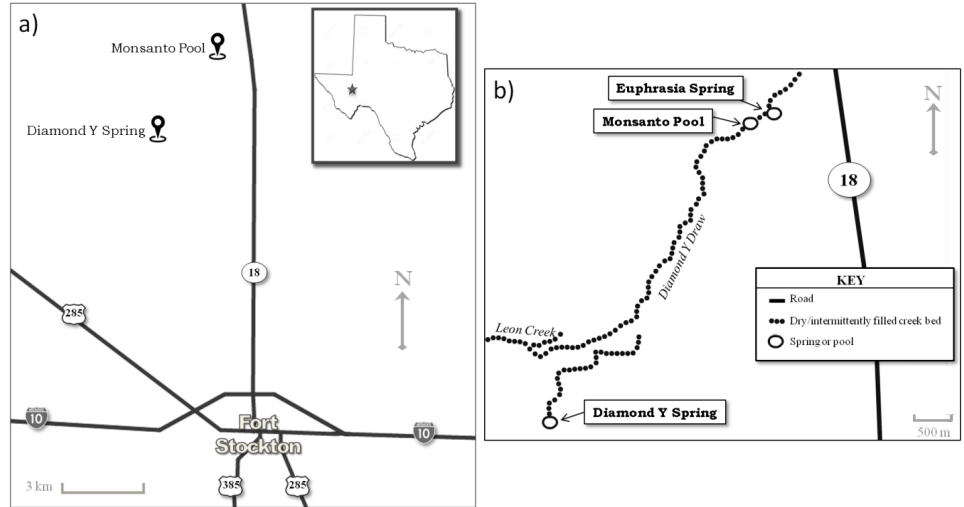


Figure I.2 The geographic range of *Cyprinodon bovinus*: a) the two study locations are located ~ 18 km north of Fort Stockton TX and b) a close-up of the two locations within Diamond Y Draw, which are separated by ~ 4 km of predominantly dry land.

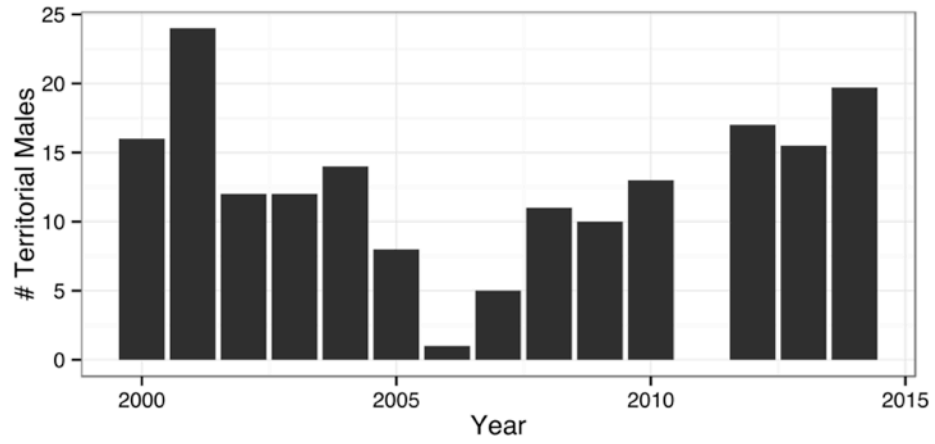


Figure I.3 Population estimates of *Cyprinodon bovinus* over 15 years based upon the number of territorial males observed at Diamond Y Spring. *Note: a population size estimate was not conducted in 2011.*

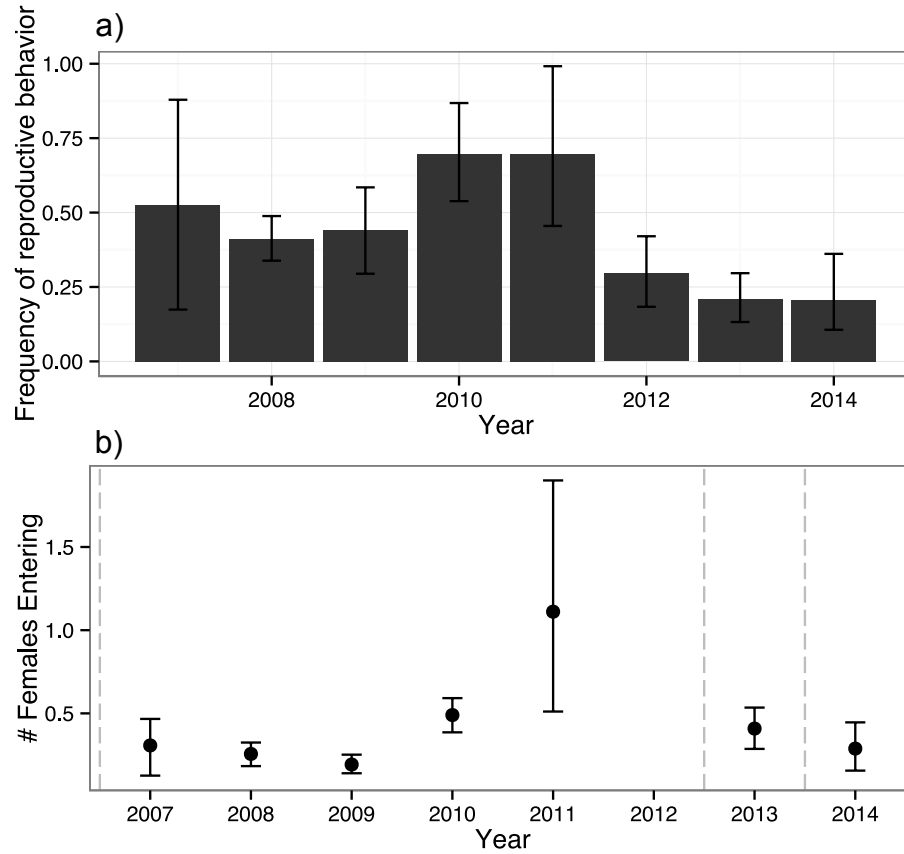


Figure I.4 Reproductive behaviors and females occurring within *C. bovinus* territories of territorial males across the 2007 - 2014 breeding seasons at Diamond Y Spring: a) Mean frequency (\pm SE) of reproductive behaviors (spawns and attempted spawns) by territorial males across the 2007 - 2014 breeding seasons; b) Mean number of females (\pm SE) observed entering male territories. A gray dashed line depicts years that habitat restoration projects occurred. *Note, data was not obtained for the number of females entering male territories in 2012*

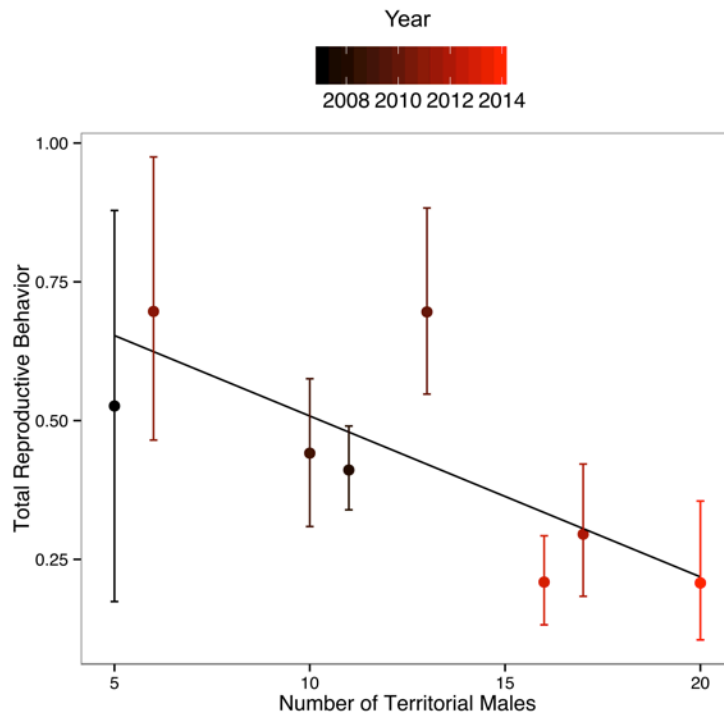


Figure I.5 Negative correlation between mean (\pm SE) annual reproductive behaviors (spawns & attempts) and the number of territorial males at Diamond Y Spring, factored by year (2007 – 2014; Spearman’s rank correlation, $\rho = -0.42$, $P < 0.001$, $n = 78$).

Table I.1 Summary of the three main goals of the *Cyprinodon bovinus* Recovery Plan and associated expected outcomes, actions, assessment tools, and findings.

EXPECTED OUTCOMES	ACTION	ASSESSMENT TOOL	FINDINGS
Goal 1: Habitat Restoration			
Entire habitat used for breeding, including newly renovated shelf	Increase the size of the breeding habitat through extensive restoration	Observations of reproduction and other behavior within the newly renovated area	Behavioral observations reveal breeding across the original natural shelf as well as the newly renovated area.
Pupfish observed throughout the spring system	Survey for evidence of population expansion throughout spring system	Observations of reproduction and other behavior within new areas of the spring system	No pupfish found in the exterior spring system after 2012. Absence likely due to poor quality habitat for breeding, ground water drainage, and bulrush encroachment.
Goal 2: Monitor population size			
A significant increase in overall population size	Monitor population size	Yearly (2000-2014) counts of territorial males	After all time low population in 2006, numbers have increased.
Goal 3: Monitor and evaluate habitat use, behavior, and reproduction			
Use of entire habitat for breeding, including newly renovated shelf	Monitor habitat use	Observations of use of natural habitat at the breeding shelf, throughout the spring system, and newly created habitat	Spawning occurred throughout the natural shelf and newly renovated areas.
Increase in overall reproductive success	Evaluate spawning success	Annual analysis of male reproductive success	Male spawning success has decreased, possibly due to increased habitat, which disbursed male territories.

II. THE EX SITU CONSERVATION OF CYPRINODON BOVINUS

II.1 Introduction

The integration of genetic monitoring and assessment into conservation biology allows managers to quantitatively gauge the efficacy of ongoing recovery plans through the evaluation of population structure and the evolutionary potential of endangered species (Hendrick 2001; Frankham 2005; Schwartz *et al.* 2007; Harrisson *et al.* 2014). Historically, conservation geneticists have relied on tools that provide data from only a limited number of anonymous, presumably neutral markers (e.g. short-tandem-repeat polymorphisms) to examine population structure (Wan *et al.* 2004). Recently however, Single Nucleotide Polymorphisms (SNPs) have gained popularity due to their abundance, high quality, and presence across both coding and non-coding regions of the genome (Vignal *et al.* 2002; Morin *et al.* 2004).

With declining costs of Next Generation Sequencing (NGS) technology and continuing improvement in bioinformatic tools (Shendure and Ji 2008), powerful techniques for assessing variation at thousands or even tens of thousands of genetic loci in multiple individuals are rapidly being adopted for ecological and conservation research applications (Kohn *et al.* 2006; Allendorf *et al.* 2010; Rice *et al.* 2011; Narum *et al.* 2013; Reitzel *et al.* 2013; Martin and Feinstein 2014). These emerging tools facilitate the increased resolution of neutral population structure and can simultaneously help to identify genomic regions that are evolving under novel selective pressures (Nielsen *et al.* 2009; Narum *et al.* 2010; Rice *et al.* 2011; Funk *et al.* 2012; Lemay and Russello 2015). By examining genetic markers on a genome-wide basis, NGS technologies can therefore

provide valuable insight into the genetic status and adaptive potential of managed and endangered populations (Kohn *et al.* 2006; Allendorf *et al.* 2010; Funk *et al.* 2012; Gruenthal *et al.* 2013; Ogden *et al.* 2013).

Endangered species are frequently reared in captivity to provide stock for reestablishing or augmenting declining wild populations, or as a hedge against the imminent extinction of species of particular public focus (Kleiman 1989; Seddon *et al.* 2007; Seddon 2010). While augmentation of imperiled populations can be requisite for the enhancement and survival of many natural populations (Kozfkay *et al.* 2008), the establishment and persistence of captive animals in natural habitats can be greatly affected by extrinsic factors (Williams *et al.* 1985; Holsman *et al.* 2012; Bennett *et al.* 2013) in addition to the intrinsic genetic characteristics of the source population (Woodworth *et al.* 2002; Frankham 2005; Araki *et al.* 2007; Carroll and Fox 2008; Milot *et al.* 2014).

Traits favored by selection in captivity may be maladaptive upon release into a natural environment, well within the time scale of most conservation management plans (Ford 2003; Heath *et al.* 2003; Frankham 2008; Williams and Hoffman 2009; Christie *et al.* 2012; Milot *et al.* 2014). This reduction in local adaptation coupled with concomitant habitat loss and fragmentation has the potential to produce a high initial mortality rate following the release of captive-bred animals into natural habitats (Brown and Day 2002; Laikre *et al.* 2010; Tracy *et al.* 2011; Holsman *et al.* 2012). Because only a small fraction of released individuals may survive, the resulting demographic and / or genetic bottlenecks can effectively reshape the genetic architecture of wild populations, despite the release of a large number of captive animals (McCullough *et al.* 1996; Friar *et al.*

2000; Ramey *et al.* 2000; Bristol *et al.* 2013).

Because of the important role genetic variation has with increasing a populations resiliency to environmental change (such as reintroducing into a novel habitat), it is therefore important to assess how captive and wild populations compare with regard to the preservation of genetic variation and whether they have, despite their isolation, maintained genetic and phenotypic similarity (Wisely *et al.* 2008). However, because selection and genetic drift can shape the genetic architecture of isolated populations in different ways, maintaining natural genetic and phenotypic characteristics can be difficult (Wilcox and Martin 2006; Blanchet *et al.* 2008). Comparing the genetic characteristics of an established captive population of known origin and demographic history to those of a wild population can enable strong inferences about the genetic health and status of wild populations based on levels of genetic variation and demographically important metrics such as inbreeding levels and effective population size (Witzenberger and Hochkirch 2011; Gonzalez *et al.* 2014). Additionally, by complementing a genetic assessment with phenotypic data, further inferences about spatial patterns of adaptation can be made (Kawecki and Ebert 2004; Funk *et al.* 2012).

With these concerns in mind, a NGS approach was employed to conduct a genome-wide assessment of neutral and adaptive population structure, which was accompanied with morphological data in order to gauge variation occurring within and between a captive and wild population of the endangered Leon Spring pupfish (*Cyprinodon bovinus*).

For assessment of both genomic and morphological patterns, the null hypothesis was posited to be an absence of divergence between the two populations, which would

provide conceptual support for preserving the natural genetic and phenotypic characteristics through the augmentation of the wild population from captive stock. Alternatively, rejection of the null hypothesis could signal the effects of environmental stochasticity and / or the occurrence of local adaptation, which could potentially compromise future supplementation efforts and therefore encourage reconsideration of the current *C. bovinus* management strategy.

II.2 Materials and methods

2.1 Subject species

The pupfishes (Cyprinodontidae) of southwestern North America are commonly maintained in captivity, as the sustainability of these wild populations is frequently contingent on external support (Miller and Pister 1971; Wilcox and Martin 2006; Koike *et al.* 2008; Finger *et al.* 2013). Between captive and wild populations, restricted gene flow has contributed to genetic divergence across populations (Wilcox and Martin 2006; Finger *et al.* 2013) with pupfish maintained in these environments showing a high degree of non-genetic morphological plasticity in addition to rapid genetically based divergence in body shape (Collyer *et al.* 2005, 2007, 2011). This phenotypically plastic predisposition may underlie the remarkable ability of pupfish to survive under extremely adverse and variable environmental conditions (Feldmeth and Brown, 1971; Bennett and Beitinger 1997) and may facilitate the directional evolution of body shape characteristics (Collyer *et al.* 2007). As the reestablishment of captive animals in wild habitats may be affected by the decreased ability to adapt to local, natural selection pressures, shifts in both genetic and phenotypic characteristics are of concern, especially in view of the fact

that a majority of desert fish reintroductions fail to establish (Hendrickson and Brooks 1991).

C. bovinus are an endangered desert pupfish restricted to an isolated spring system in SW Texas, USA (US Federal Register 2008). Following near extinction due to habitat loss and an episode of ‘genetic contamination’ from the non-native sheepshead minnow (*Cyprinodon variegatus*; Echelle and Echelle 1997), *C. bovinus* have been maintained in captivity at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC; Dexter, NM) as a “genetic replicate” since 1976 (Edds and Echelle 1989). During the mid 1990’s, a second hybridization event occurred with *C. variegatus*, and due to the rapid and ubiquitous spread of *C. variegatus* throughout the entire spring system, the wild population was largely eradicated to remove genetically compromised individuals (Echelle and Echelle 1997).

Following the apparently successful renovation, a large-scale release of captive pupfish (~ 10 000 individuals) occurred from 1998 - 2001 to replenish the culled *C. bovinus* population and to dilute the frequency of *C. variegatus* alleles in rare hybrids that might still be present in the watercourse; the number of remnant wild pupfish was never quantitated prior to this restocking (Echelle *et al.* 2004). Despite the large number of captive *C. bovinus* released into the wild, the population size rapidly declined, until in 2006, less than 5 individuals were observed. This precipitated multiple habitat restoration projects, which effectively counteracted the downward trend in population size (Gumm *et al.* 2008, 2011); anecdotal evidence places the current population size ~ 250 individuals (A. Black, personal observation).

2.2 Sample collection

Wild and captive *C. bovinus* were collected from Diamond Y Spring, TX (DY; 31°0'4.75"N, 102°55'27.09"W) and supplied by Southwestern Native Aquatic Resources and Recovery Center (SNARRC; Dexter, NM), respectively (Figure II.1). Fish were captured using minnow traps and sedated with ~ 50 ppm Tricaine methanesulfonate (MS-222) in natural spring water. Each individual was then measured (with electronic calipers) and photographed prior to the removal of a small tissue segment from the medial caudal fin. Photographs were taken using a Nikon D5100 digital SLR camera (16.2 megapixels) with an 85 mm lens mounted to a copy stand. Prior to each photograph, individuals were patted dry with kimwipes and carefully oriented left side up over a 2 mm reference grid ~ 0.25 m below the camera lens. Digital images of the left lateral surface of each individual fish were captured under natural lighting conditions and fish were subsequently placed in holding aquaria to await recovery from anesthesia. After fish resumed normal swimming behavior, all individuals were successfully released back into their respective habitats with no observed losses.

2.3 Morphological variation

To quantitate variation in body shape between populations, landmark-based geometric morphometrics, a commonly used technique for ecological, evolutionary and developmental inquiry (Bookstein 1991; Rohlf and Marcus 1993; Marcus 1996), was employed. By quantifying relative distances between anatomically homologous points (i.e. landmarks), shape complexity was extracted in the form of Cartesian Coordinates, which were subsequently used as ordination variables in the analysis of body shape.

Following digital image acquisition, photographs were uploaded to a computer and 12 landmarks were assigned to each individual image based upon anatomical reliability and prior success in mapping body shape variation in other *Cyprinodon* species (Collyer *et al.* 2005; Figure II.2A). A single investigator (A. Black) digitized all 12 landmarks for each specimen using *tpsDig2* (Rohlf 2013), and the Cartesian coordinates (x, y) of each landmark for all individuals were imported into *MorphoJ* (Klingenberg 2011), where geometric morphometric methodology largely followed Klingenberg *et al.* (2003).

Centroid size, defined as the square root of the sum of squared distance from each landmark to their configurations centroid, was used to quantitate body size (Bookstein 1991). To determine whether sexual dimorphism and population source had effects on body size, a two-way analysis of variance (ANOVA, Type II) was implemented using the *Mass* package (Venables and Ripley 2002) in the R environment (R Development Core Team 2014). Non-shape variation was held constant by Procrustes superimposition (Figure II.2; Dryden and Mardia 1998; Klingenberg 2011), which implements an iterative least squares superimposition of landmark coordinates by first scaling individuals to unit size and then aligning the centroid to the origin of a shared coordinate map; this superimposition effectively removes minor differences in the size and orientation of individuals (Rohlf and Slice 1990). However, as Procrustes superimposition does not correct for allometric variance, which can account for a large component of shape variation in fishes (e.g. Reis *et al.* 1998), a multivariate regression of shape onto size was performed and tested against the null hypothesis of independence (Monteiro 1998).

To reduce the dimensionality of the data, as well as to visualize the main axes of variation, a Canonical Variate Analysis (CVA) was performed using ‘sex’ and ‘population source’ as classification variables (Klingenberg 2011). CVA is an ordination method that emphasizes the differences that vary most between groups by maximizing between-group variation relative to within-group variation (Legendre and Legendre 1998). To visualize group shape transformations, a Thin-Plate-Spline method was used to generate transformation grids along the CV axes; these grids represent shape deformation from the mean landmark configuration along the major axes of variation. Procrustes distance, the square root of the sum of squared distance between corresponding landmarks, was used for pairwise comparisons as an absolute metric to distinguish groups through use of permutation rounds (10 000 iterations). Classification rates were calculated using cross-validated discriminant functions, which use a ‘leave-one-out’ approach to generate an estimate of membership assignment probability *in a priori* defined groups (Lachenbruch 1967).

2.4. Molecular techniques and bioinformatics

To analyze neutral population structure and examine genomic regions for signatures of selection, a genome-wide strategy was employed based on restriction Associated DNA sequencing (RAD-seq) technology (Baird *et al.* 2008). RAD-seq utilizes restriction endonucleases to create a reduced-representation sequence library that enables the identification of thousands of SNPs spanning the genome, simultaneously yielding SNP genotypes for all individuals analyzed (Davey *et al.* 2013), even in non-model organisms for which no reference genome assembly exists (Ekblom and Galindo 2010;

Davey *et al.* 2011). However when used without a reference genome, this technology can require excessive amounts of sequence data to enable the adequate read depth required to achieve high confidence in SNP calls (Peterson *et al.* 2012; Kai *et al.* 2014).

To reduce the amount of genotyping error by optimizing the number of reads sequenced in a non-model organism, an extension of traditional RAD-seq was employed, based upon double digest restriction Associated DNA sequencing (ddRAD-seq) technology (Peterson *et al.* 2012). This method employs a cocktail of a rare-cutting enzyme and a frequent-cutting enzyme to digest the genomic DNA, followed by a precise size selection step (opposed to the random shearing conducted with RAD-seq) to create a highly reproducible, reduced representation genomic library (Peterson *et al.* 2012).

Tissue samples (n = 48; 24 from each population) were individually stored in 500 μ L of RNAlater and maintained at 4°C pending digestion with Proteinase K (Qiagen; Hilden, Germany). Genomic DNA was extracted using a standard phenol-chloroform protocol, and DNA concentrations were quantified for each sample with a Qubit®2.0 Fluorometer (Life Technologies; Carlsbad, CA). Genomic DNA quality was assessed using a 260 / 280 absorbance ratio on a NanoDrop 1000 Spectrophotometer (Thermo Scientific; Waltham, USA) and by gel electrophoresis (1% agarose gel).

ddRAD library preparations followed the methodology originally described in Peterson *et al.* (2012) with minimal modification. For each sample, one μ g of genomic DNA was digested overnight at 37°C in 50 μ L reactions containing 20 units each of High Fidelity *Eco*RI and *Msp*I restriction enzymes, NEBuffer 4, and water (New England Biolabs; Beverly, MA). Following digestion, samples were purified with 1.5x volume of Ampure XP beads (Beckman Coulter; Miami, FL) on a 96-well magnetic plate. For each

sample, 100 ng of fragmented DNA was ligated to a universal P2 and P1 adapter (which contained 48 unique 5-base barcodes adjacent to genomic DNA fragments). All barcodes differed by at least 2-base positions, enabling an accurate read assignment (95 – 99%) during the sample demultiplexing procedure (Peterson *et al.* 2012). Following ligation and subsequent Ampure XP bead cleanup, samples were pooled and size selected for a range of 375 ± 38-bp (BluePippin, Sage Science; Beverly, MA), and individual libraries were PCR amplified with uniquely indexed primers using a Phusion Polymerase kit (New England Biolabs) following the manufacturers default guidelines. Reactions were transferred to a preheated Thermocycler (Eppendorf; mastercycler®pro) and amplified using the following conditions: 1 cycle of 98°C for 30 s, 12 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 30 s, and a final cycle at 72°C for 10 min. Following amplification, both libraries were again cleaned with Ampure XP beads, assessed for quality using a bioanalyzer, and pooled together at equimolar concentrations. The final pooled library was then run on a single Illumina Hiseq 2500 lane (Institute of Biotechnology, Cornell University), using 2 x 101-bp sequence chemistry. Only single-end read data were used in the current study.

Raw Illumina reads were assessed for quality using *FastQC* (Babraham Bioinformatics, Babraham Institute; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and subsequently assembled into putative genetic loci using the open source pipeline *Stacks* (v.1.9; Catchen *et al.* 2011, 2013). The ‘process radtags’ script in the *Stacks* pipeline was first used to demultiplex raw sequences by assigning individual reads to corresponding samples through the unique combination of ligated in-line barcodes and standard Illumina read

indices (Peterson *et al.* 2012). After trimming off the verified barcodes, single end reads were checked for the presence of the *EcoRI* restriction cut-site sequence (GAATTC), and those with base-call errors were discarded. Using a sliding window (15 % of read length), reads that showed a continual decrease in quality score (Q score < 10 [90 % base call accuracy]) were also removed.

Demultiplexed and filtered reads were uploaded onto a computing cluster composed of 32 processor cores and 128 GB of memory (High Performance Computing; Lehigh University) where the reads were assembled using *Stacks* ‘denovo_map.pl’ wrapper program (which sequentially executes each core component of the pipeline; Figure A2.01). All ‘denovo_map.pl’ parameters were set to default (parameters in italics: *-m 3, -n 0, -t, -M 2, -N 4*) and stacks with an excessive number of reads (> 2 SD above the mean depth) were filtered to remove stacks that potentially contained more than one merged locus (for additional details see: Catchen *et al.* 2011, 2013).

Missing data in RAD-seq can arise due to variation in recognition sites, errors introduced during library preparation, or sequencing base miscalls (Arnold *et al.* 2013; Davey *et al.* 2013; Gautier *et al.* 2013). Using a conservative approach to reduce ascertainment bias, loci were required to be present in $\geq 80\%$ of individuals within each population, with a minimum stack depth of 5x and a minor allele frequency > 0.02. Additionally, to maintain independence of loci with multiple polymorphisms, only the first SNP from each locus was retained. Using a False Discovery Rate (FDR) of $\alpha = 0.05$ (implemented in R’s *Stats* package), loci were also excluded if their genotype frequency distribution deviated significantly (within both populations) from Hardy-Weinberg Equilibrium expectation (Wigginton *et al.* 2005). The ‘populations’ module of *Stacks* was

used to calculate the number of private variants, F_{IS} , Nucleotide Diversity (Π), Observed (H_{OBS}), and Expected heterozygosity (H_{EXP}) for every filtered SNP in the dataset. SNPs were exported from *Stacks* in Variant Call Format (Danecek *et al.* 2011) and converted to Plink (Purcell *et al.* 2007) for additional data management. Filtered loci were then reformatted using *PGDspider* (v.2.0.5.2; Lischer and Excoffier 2012) for future software import.

2.5 Neutral and adaptive variation

Delineation of both neutral and adaptive genetic variation can be important for evaluating discreteness among endangered populations, especially in the face of low gene flow and environmental differences (Funk *et al.* 2012). Assessment of neutral variation can provide insight into stochastic processes as well as the demographic history of populations (Wright 1931). Evaluation of adaptive variation, on the other hand, can help identify and potentially provide an understanding of local adaptation occurring within populations; this can help inform managers of divergent selection patterns that could negatively affect future supplementation, reintroduction, or translocation efforts. Therefore, following the workflow of Funk *et al.* (2012), two different datasets were created: 1) a ‘neutral’ dataset, and 2) an ‘adaptive’ dataset. These datasets were then used to independently examine the relative contribution that these two processes have on the genetic distinctiveness of the two *C. bovinus* populations.

First, to examine the likelihood that divergent selection regimes could be promoting genetic adaptation to specific local environments (e.g. captive habitat), selectively neutral loci were distinguished from those potentially under selection

(genomic regions more divergent between populations than that expected by chance, or ‘outlier’ loci), using *Bayescan* (v2.1; Foll and Gaggiotti 2008; Foll *et al.* 2010; Fischer *et al.* 2011). This implements an F_{ST} -based approach to estimate the probability that a locus is under selection by comparing two models (in the presence / absence of selection) using a Markov Chain Monte Carlo procedure. Individuals were grouped according to population and a prior odds of 10 was used (implying the prior belief that the neutral model is 10x more probable than the model with locus-specific selection) to obtain the posterior probability of a neutral model using default settings and an FDR of 0.05. Putative outliers were then coded according to their number in the *Stacks* catalogue, and were separated by supplying a “whitelist” (which will include specific SNPs) to the ‘population’ module in *Stacks* (Catchen *et al.* 2013). The consensus sequences of putative outliers were then subjected to sequence similarity searches with known NCBI sequences using BLAST (Altschul *et al.* 1997).

To generate the ‘neutral’ dataset, outliers that were putatively under divergent selection were removed from the dataset by supplying a “blacklist” (which will exclude specific SNPs) to the ‘population’ program. Contemporary effective population size (N_e) was then estimated from this dataset by using the linkage disequilibrium method implemented in the software package *NeEstimator* (v.2.01; Do *et al.* 2014). *GenoDive* (v.2.0; Meirmans and Van Tienderen 2004) and the *Pegas* package in R (Paradis 2010) were used to assess population differentiation through mean and pairwise F_{ST} values (Weir and Cockerham 1984) for both the ‘neutral’ and ‘adaptive’ dataset independently; significance was assessed through use of 999 permutations to test if F_{ST} differed significantly from zero. Finally, clusters of genetically similar individuals were visualized

with an individual principal component analysis (PCA) for both datasets using the package *Adegenet* in R (Jombart 2010). For the PCA, data were scaled and centered and missing values were replaced with mean values for each locus.

II.3 Results

3.1 Morphological variation

Landmark coordinates were assigned to 66 wild (DY; male / female = 35 / 31) and 72 captive (SNARRC; 40 / 32) adult *C. bovinus* (Figure II.2B). Centroid size (CS) was calculated and log transformed for normality for both DY (3.898 mm \pm 0.131 SD) and SNARRC (3.851 mm \pm 0.069 SD) populations (Figure A2.02). There was a strong association between caliper measured length (log total length [mm]; measured from snout to terminal caudal fin) and logCS (Pearson's correlation = 0.98, N = 138, P < 0.001, Figure A2.03 & Table A2.01). A two-way ANOVA revealed no significant main effect of 'population source' ($F_{1,134} = 1.55$, P = 0.215), 'sex' ($F_{1,134} = 0.357$, P = 0.551) or the 'population source' * 'sex' interaction ($F_{1,134} = 0.192$, P = 0.662; Table A2.02 – A2.04) on 'logCS'. A pooled within-group regression (population source, sex) of shape (i.e. Procrustes coordinates) onto size (i.e. logCS) predicted 4.7 % of shape variation (10 000 iterations; P = 0.001), so to account for allometric variance, residuals were used for all subsequent morphometric analyses (Figure A2.04 & Table A2.05).

Matrices derived from the residuals of pooled within-group data were used for a CVA and the results were visualized using the first two canonical axes (Figure II.3). Permutation tests revealed significant differences between all mean treatment groups (Procrustes Distance; P = 0.001; 10 000 Iterations [Table II.1]). Canonical Variate 1

(CV1, 74.39 % of explained variance) clearly illustrated variation occurring between the two sexes, which was demonstrated predominantly by difference in body depth. Transformation grids associated with CV1 illustrated that males exhibited greater body depth, while females were more streamlined (Figure II.3D & E). Canonical Variate 2 (CV2, 22.01 % of explained variance) characterized shape variation occurring between the two populations. Transformation grids associated with CV2 illustrated that group separation was most apparent in the anterior tip of the snout (Landmark 1), ventral opercular slit (Landmark 12) and the pectoral fin attachment points (Landmarks 10 + 11). Compared to the consensus shape, the SNARRC population exhibited a downward sloping head while the DY population displayed a more upward head slope and dorsal shift in pectoral fin attachment points (Figure II.3B & C). Cross-validation of discriminant functions showed clear separation between populations ($P = 0.001$; 10 000 iterations) and correctly classified individuals to their corresponding group at 91% and 88 % accuracy for the DY and SNARRC population, respectively (Figure A2.05 & Table A2.06).

3.2 SNP discovery and filtering

One Illumina HiSeq 2500 lane yielded 141 551 650 single-end 101-bp reads. Raw sequences had a GC content of 38 % and base calls had a mean *phred* quality score of 34, with 85% having $Q \geq 30$ (99.9 % base call accuracy). A visual examination of terminal read quality showed no substantial decrease in base call accuracy, so the entire read length was retained for all reads (after truncating the 5-base barcodes). After demultiplexing the first index ($n = 48$ individuals [a second index was used for a separate

study]), reads were filtered based on: (i) indeterminate barcodes (309 526 [0.4 %]); (ii) sub threshold quality (4 193 636 [5.8 %]) and; (iii) ambiguous RADtags (501 835 [0.7 %]). Out of the total sequence reads generated from a single index, 93 % (66 542 557) remained with 1 444 182 mean reads per individual.

Filtered and trimmed 96-base reads were then aligned *de novo* into stacks of homologous reads using *Stacks* core pipeline, 'denovo_map.pl'. Following completion of the 'denovo_map.pl' script, 339 126 consensus stacks within each individual were uploaded into the *Stacks* catalogue. After removing two individuals from SNARRC due to low genotyping rate (> 90 % missing data), 23 692 stacks (38 442 SNPs) across all 46 individuals remained with a mean site depth of 11x (± 4 SD); mean sequencing coverage was similar between DY (12 ± 4 SD) and SNARRC (9 ± 3 SD). The requirement for loci to be present in ≥ 80 % of individuals ($-r 0.80$) from both populations ($-p 2$) with a minimum stack depth of 5X ($-m 5$) resulted in the retention of 4 328-biallelic SNPs in 3 241 loci (mean = 1.34 SNPs per locus) following implementation of *Stacks* 'Populations' module. Both the total number of private variants and minor allele frequencies were higher in the SNARRC population (1215 and 0.1447, respectively) relative to the DY population (457 and 0.1334, respectively; Figure A2.06). Descriptive statistics illustrated that overall DY had lower diversity values ($H_{OBS} = 0.2053$, $H_{EXP} = 0.1864$, $\Pi = 0.1906$) compared to SNARRC ($H_{OBS} = 0.2145$, $H_{EXP} = 0.2060$, $\Pi = 0.2113$; Figure A2.07), with DY exhibiting a slightly lower inbreeding coefficient ($F_{IS} = -0.0326$ [DY]; $F_{IS} = 0.0008$ [SNARRC]).

3.3 Neutral and adaptive variation

After filtering SNPs with *Vcftools*, inclusion thresholds removed an additional 167 SNPs with a minor allele frequency ≤ 0.02 and 51 SNPs due to deviation from the expectations of Hardy-Weinberg equilibrium ($\alpha = 0.05$ following FDR correction), resulting in a matrix of 2 023 SNPs which was subsequently used to identify regions of the genome that were putatively under divergent selection. Using a prior odds of 10, *BayeScan* identified eight loci ($\sim 0.4\%$ of SNPs) that showed significant deviation from a neutral background, with high estimated F_{ST} values (0.1478 – 0.1948; Table A2.07) relative to the background F_{ST} (Figure II.4). Out of these eight outliers (Table A2.08), only one, pupfish ddRAD sequence 1363, showed moderately strong matches to known sequences in the NCBI nr database, as detected by several different BLAST strategies. Interestingly, 1363 exhibited the highest F_{ST} value in the dataset (0.587; Figure II.5) and was aligned most strongly to a predicted gene (sodium-driven chloride bicarbonate exchanger-like transcript; LOC102232885) associated with a sodium-driven chloride bicarbonate exchanger function in *Xiphophorus maculatus* (Genbank: XM_005805324, LOC102232885) with an expected value of $2e-08$ and a max score of 68 (Table A2.09). This gene is homologous to the large *SLC4a10* family of vertebrate solute carrier family 4, sodium bicarbonate transporter genes.

Using all eight outlier loci in a principle component analysis illustrated that the first two principle components explained the majority of variation (PC1 = 68%, PC2 = 19%) and clearly clustered the two groups based upon population origin (Figure II.6A). Examination of a loading plot calculated using all 2023 SNPs in an individual PCA revealed that five of the eight outliers identified by *Bayescan* composed the majority of the variation in the PCA (Figure A2.08 & Table A2.11). When evaluating the partitioning

of genetic variance, the two populations revealed a substantial level of genetic differentiation in the ‘outlier’ dataset (mean $F_{ST} = 0.540$; $P = 0.001$) and examination of genetic structure between populations revealed high pairwise F_{ST} values for all eight loci (min / max = 0.418 / 0.587; Figure II.5).

The removal of the eight outliers resulted in a ‘neutral’ dataset of 2 015 SNPs. Within this dataset significant genetic differentiation was also observed (mean $F_{ST} = 0.049$; $P = 0.001$). Based upon the minor allele frequency cutoff, contemporary N_e estimates for wild *C. bovinus* ranged from 28 (95 % CI = 27.6 – 28.5, minor allele frequency = 0.05) to 32.9 (95 % CI = 32.4 – 33.4, minor allele frequency = 0.02), while estimates for captive *C. bovinus* ranged from 221.5 (95 % CI = 200.9 – 246.8, minor allele frequency = 0.05) to 175.3 (95 % CI = 164.0 – 188.1, minor allele frequency = 0.02, Table A2.10). Using allele frequencies derived from the neutral dataset illustrated that the eigenvalues associated with the first and second principle components composed a minor amount of the total variation (8% and 4% of variance explained, respectively) but clearly clustered the two groups by population origin (Figure II.6B).

II.4 Discussion

To assess the effectiveness of the current *C. bovinus* captive breeding program and historical release of captive stock into the natural Diamond Y habitat, a genome-wide approach was used to measure levels of genetic variation within, and subdivision between, contemporaneous samples of individuals from the captive and wild population. Results revealed that the large-scale release of thousands of captive animals failed to prevent a severe genetic bottleneck that appears to have contributed to loss of genetic

variation and substantial neutral genetic divergence between these two “subpopulations” (defined as such based upon two episodes of unilateral gene flow between SNARRC and DY). Divergence also extended beyond these stochastic processes, which was evident by the presence of multiple genetic outliers and significant morphological divergence between the two populations, suggesting that selection may be promoting local adaptation, which may also be shaping population specific body shape characteristics. As the preservation of natural qualities is of utmost importance in conservation breeding programs, evidence for such substantial levels of divergence holds serious implications with regard to the preservation of natural genetic and phenotypic characteristics.

4.1 Morphological variation

The two populations of *C. bovinus* exhibited significant body shape differentiation. Clearly discernible was variation in pectoral fin attachment points and in the slope of the snout (Figure II.3B & C), a modification that could have a direct effect on foraging behavior. Of note is that this difference in head orientation results in a distinct upward repositioning of the head in fish from the captive population (relative to DY), a finding that was also reported by Wilcox and Martin (2006) who illustrated that *C. diabolis* maintained in artificial refuges showed an upward slope to the head relative to the natural Devils Hole population.

The captive *C. bovinus* population is maintained in a 0.10-acre earthen pond at SNARRC, which is enhanced with organic fertilizers; approximately 30% of the pond possesses vegetation covering (personal communication, M. Ulibarri). While speculative, it is therefore possible that reduced substrate heterogeneity or increased nutrient

availability may have relaxed selection within captivity, resulting in modification of foraging behavior (relative to the wild) and consequently the observed morphological shift in head position. While the functional significance of this morphological change is unknown, future evaluation of variation in feeding mechanics or trophic structure between populations may help elucidate this difference (McGee *et al.* 2013).

Body shape also significantly differed between sexes, which were most evident in body depth (Figure II.3D & E). Relative to females, larger size and an exaggerated body depth in males has also been reported in other studies examining pupfish morphology (Collyer *et al.* 2005), and may play a key role in agonistic displays and territory defense (Kodric-Brown 1978). While there were significant differences in shape between the two sexes from both populations (Table II.1), it remains unknown if local variation in social behavior may be further driving this sexual dimorphism.

The population-specific shape characteristics that were detected could be a function of phenotypic plasticity independent of genetic differences between the populations, or could be genetically based as a result of rapid evolution (Collyer *et al.* 2011). Based on the employed methods, the discrimination between these two, non-mutually exclusive, possibilities was not possible.

4.2 Neutral and adaptive variation

Genetic drift can rapidly erode genetic diversity in small populations over a contemporary time scale and promote interpopulation divergence through stochastic changes in allele frequencies (Briscoe *et al.* 1992; Hartl and Pucek 1994; Jezkova *et al.* 2014). This was evident in the asymmetrical distribution of private alleles and substantial

differences in allele frequencies, which were clearly discriminated on the PC1 and PC2 axes (Figure II.6B). Examination of the genetic composition of both captive and wild populations revealed that the captive population retained higher levels of genetic variation relative to the wild population, a situation that opposes that typically observed in conservation breeding. While loss of heterozygosity can occur rapidly in managed populations (Briscoe *et al.* 1992; Hartl and Pucek 1994; Jezkova *et al.* 2014), it appears SNARRC has efficiently maintained levels of heterozygosity in the captive population (relative to the wild population) following the founding event in 1976. However, genetic bottlenecks in the wild can precipitate genetic changes similar to those associated with the founding of captive populations, such as rapid reductions in genetic diversity (Nei 1975; Leberg 1992; Robichaux *et al.* 1997; Taylor *et al.* 2007) and effective population size (Ryman and Laikre 1991). This appears to have been the case with the DY *C. bovinus*. Since 2006, extensive habitat restoration projects have led to a rapid increase in the estimated DY population size (Gumm *et al.* 2008, 2011), yet based on diminished genetic diversity and low estimated effective population size of this population ($N_e = 28$), it is apparent that genetic drift has exerted a significant effect on allele frequencies, including both frequency shifts and allelic loss. This is of concern because the fixation of mildly deleterious alleles at multiple loci can contribute to the extinction of small populations (e.g. with $N_e < 50$; Higgin and Lynch 2001; Rowe and Beebee 2003). While the census population size (N) of DY is unknown, it is suspected to be ~ 250 , which would result in N_e / N ratio around 0.112, consistent with the median N_e / N ratio of 0.11 observed across the 102 endangered wildlife species reported by Frankham (1995).

The captive SNARCC population was founded ~40 years ago from wild DY stock and ~ 10K captive bred SNARCC animals were released into the wild 14 years ago. It is therefore difficult to define a precise temporal separation metric between these two populations; however, it is certainly less than 40 years (~ 80 generations). Regardless of the time frame, the mean F_{ST} value for ‘neutral’ SNPs was considerable (0.049), with high variation in pairwise F_{ST} values (min / max = -0.0396 / 0.4216; Figure II.5), suggesting that the founding event coupled with the two documented population bottlenecks have exerted a large effect on population structure. This amount of genetic differentiation is close to an order of magnitude less than that observed in the ‘adaptive’ dataset, but nevertheless represents considerable genetic divergence over a relatively brief period of separation.

Because the sustainability of endangered populations can be contingent on the maintenance of genetic diversity, preservation of standing variation is a primary goal in conservation breeding programs (Pelletier *et al.* 2009; Engelhardt *et al.* 2014). Unfortunately, efforts failed to determine homologous relationships or likely conserved syntenic genomic positions for any of the eight-outlier loci that appeared to be under positive selection in the pupfish ddRAD dataset. Only one of these ddRAD fragments, sequence 1363, showed strong alignment to an annotated gene in highly inclusive BLAST strategies. This alignment, to a predicted sodium-driven chloride bicarbonate exchanger-like (LOC102232885) gene of *Xiphophorus maculatus* (Southern platyfish), was initially intriguing due the fact that *X. maculatus* and *C. bovinus* are members of the same family (Poeciliidae) and because this gene is homologous to members of the highly conserved vertebrate *Slc4a10* gene family, which are important in regulating bicarbonate

secretion and cellular pH. These functions are likely to be affected by environmental inorganic solute concentrations in aquatic organisms. Since DY and SNARRC differ greatly in water chemistry (Table II.2), a relationship between variation in a *C. bovinus* *Slc4a10* homologue and water conditions could be a driver of divergent selection on sequence 1363. However, inspection of the *X. maculatus* LOC102232885 gene model shows that the region to which the *C. bovinus* 1363 ddRAD sequence aligns is not within the protein coding region of this predicted gene, but lies instead in a 1167 nt region that is 3' to the canonical SLC4A10 protein-coding region of other vertebrates.

Nevertheless, the *C. bovinus* 1363 ddRAD sequence does appear to represent a conserved sequence in vertebrate genomes. For example it aligns with substantial agreement (e.g. E values of 1e-10 to 8e-07; identities of 76 - 84%) to sequences in unannotated sequences in several linkage groups in Zebrafish (*Danio rerio*), several other fish species predicted collagen alpha-2 (VI) chain-like sequences, and many unanchored scaffolds in other fishes and non-fish vertebrates. It also shows good alignment (8e-07; 76 % identity) to sequence within the *mesp* gene homologue in Medaka (*Oryzias latipes*). Further characterization of this fragment is, however, beyond the intended scope of this study.

4.3 Implications for future conservation

The evaluation of genetic structure revealed that genetic differentiation was present in regions of the genome subject to non-adaptive forces (e.g. genetic drift), as well as regions likely under the influence of divergent selection (Figure II.6). Because it is difficult to predict the adaptive importance of specific genetic characteristics for the

future persistence and adaptation of a population, the preservation of extant, natural genetic and phenotypic characteristics is believed to be an effective strategy to increase the ability of captive populations to produce individuals that can survive and reproduce under ancestral conditions (Frankham 1986). Despite the fact that genetic cohesion can be maintained by periodic artificial gene flow between captive and the wild populations, no such transfer has been conducted since the founding of the captive SNARRC population (Echelle and Echelle 1997). Enabling immigration between populations could slow the rate of genetic adaptation to the artificial environment and balance levels of heterozygosity between the two groups (Frankham and Loebel 1992; Araki *et al.* 2007). Therefore, in light of the high levels of neutral genetic divergence, the putative evidence of local adaptation, and the asymmetrical partitioning of genetic variation documented, the periodic reciprocal transfer of genetic material between the DY and SNARRC populations is strongly recommended.

Despite failure to identify environmental factors that might drive divergent selection for any of the eight-outlier loci, the fact that water chemistries at DY and SNARRC are so different (Table II.2), suggests that water quality could play a role in selective divergence of these populations. Additionally, while the underlying developmental cause for divergence in body shape is unknown, pupfish body shape characteristics can be influenced by salinity differences between environments (Collyer *et al.* 2005). If local adaptation is in fact occurring based on spatial differences in water conditions, adjusting the salinity of the captive population to an intermediate value may be an effective initial strategy to help captive animals survive and breed in their natural habitat. In summary, reciprocal gene flow, the evaluation of water chemistry tolerance,

feeding mechanics, variation in trophic structure between environments, and understanding the genomic contexts of loci that show documented outlier status with regard to neutral evolutionary models are areas that merit attention in connection with the future release of captive *C. bovinus* to help ensure successful future supplementation efforts.

Figures II:

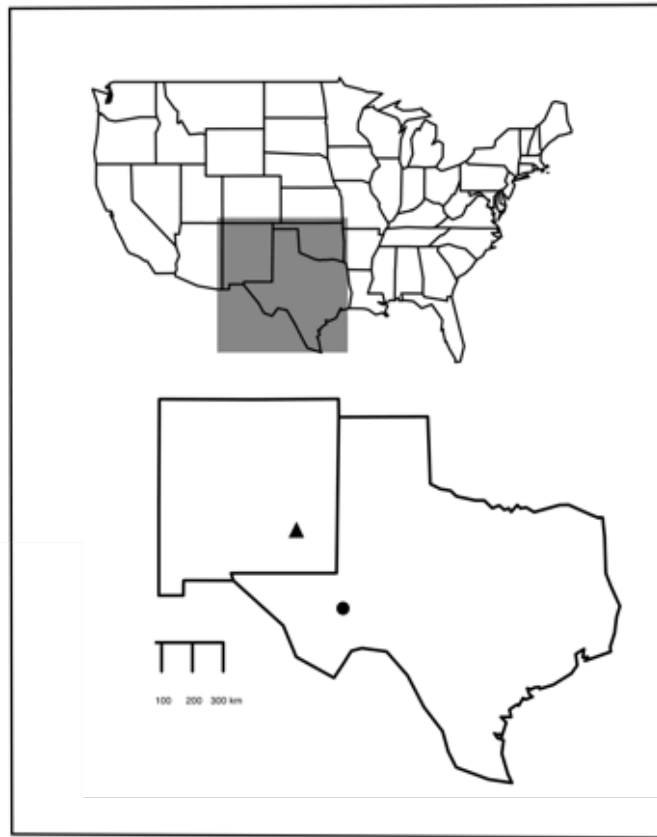


Figure II.1 Map illustrating the location of the wild *Cyprinodon bovinus* population (DY; circle) in SW Texas and the captive population (SNARRC; triangle) in SE New Mexico.

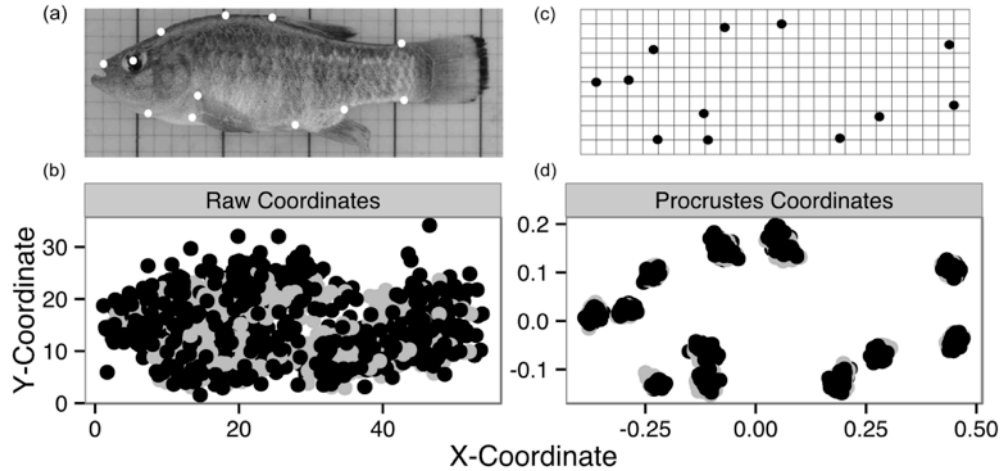


Figure II.2 12 landmarks used to quantitate shape variation in *Cyprinodon bovinus* and Procrustes superimposition. (a) Landmarks assigned clockwise from the left to: 1) tip of the snout, 2) eye center, 3) predorsal elevation, 4) dorsal elevation, 5) anterior base of dorsal fin, 6) upper margin of caudal peduncle, 7) lower margin of caudal peduncle, 8) posterior insertion of anal fin, 9) anterior insertion of anal fin, 10) dorsal base of pectoral fin, 11) ventral base of pectoral fin, and 12) ventral opercular slit. (b) Illustrates the raw coordinates of wild (black) and captive (grey) *C. bovinus* prior to Procrustes superimposition, (c) the resulting consensus shape and (d) illustrates the coordinate shift following Procrustes superimposition of raw coordinates of wild (black) and captive (grey) *C. bovinus*.

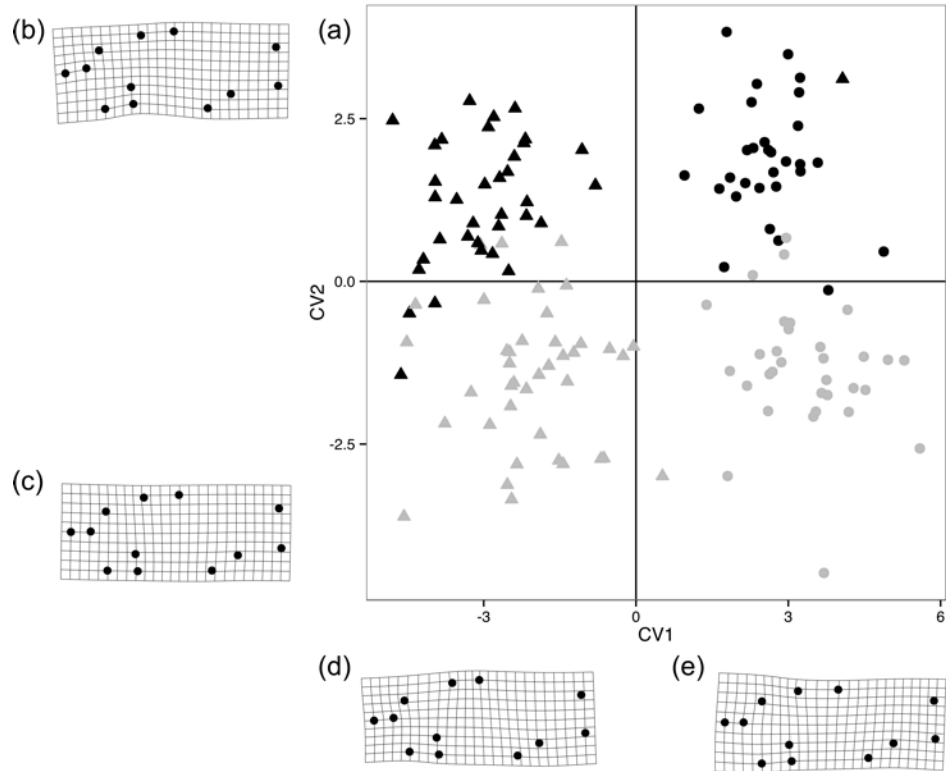


Figure II.3 The ordination of body shape from a pooled Canonical Variate Analysis (CVA) using corrected data. (a) Biplot of the first two CVs with symbols representing individuals. Shape information derived from 12 landmarks assigned to the wild (DY; black) and captive (SNARRC; grey) populations with male (triangles) and females (circles) factored into the model. Transformation grids are associated with CV1 (-6, +6) and CV2 (-3, +5) relative to the mean configuration shape (0, 0). CV2 is associated with shape deformation in the wild (b) and captive (c) populations while CV1 is associated with shape deformation in males (d) and females (e).

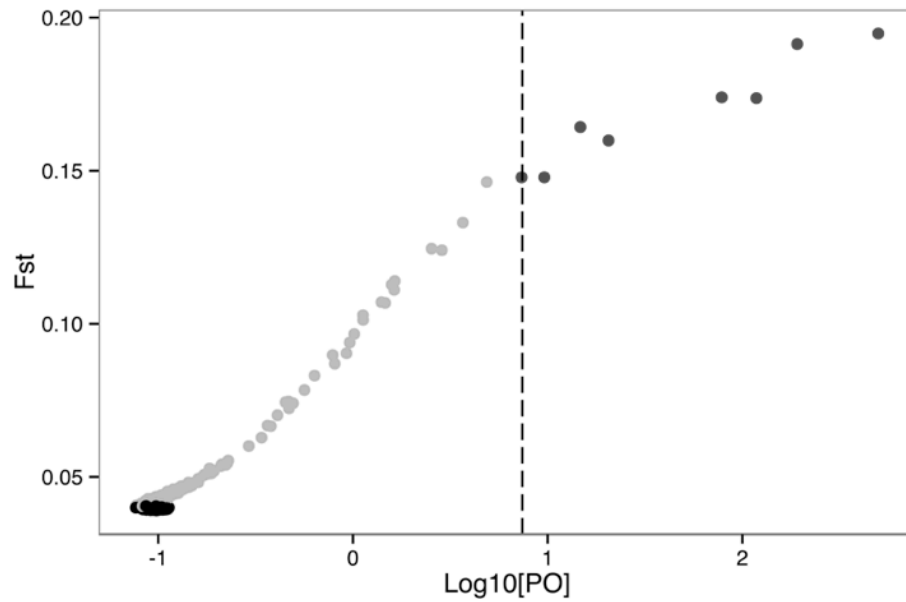


Figure II.4 BayeScan results for outlier tests of polymorphic loci ($n = 2\,023$). Y-axis, genetic divergence (F_{ST}) in *Cyprinodon bovinus*. X-axis, logarithm to base 10 of the posterior odds. Black data points reflecting balancing selection, light grey reflecting neutral selection, and dark grey representing divergent selection. The long dashed line represents a False Discovery Rate of 0.05, which represents the minimum value at which a locus may be considered to deviate significantly from a neutral model.

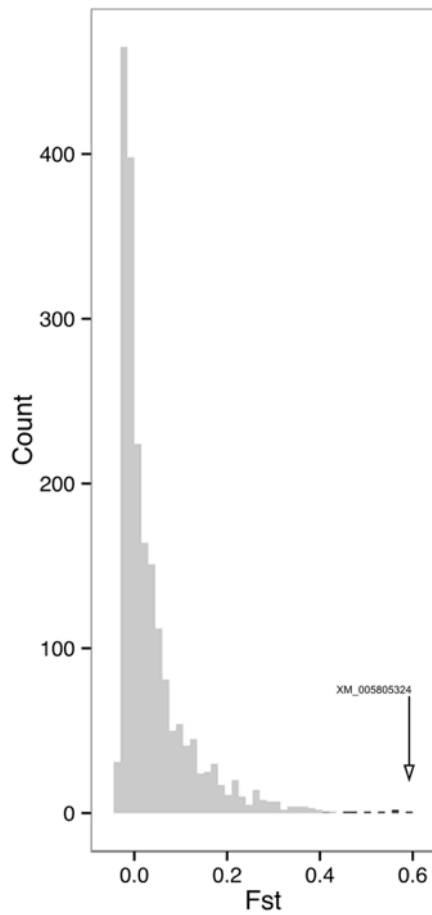


Figure II.5 Pairwise frequency distribution of F_{ST} values from 2 023 SNPs typed in 46 individuals across both populations. F_{ST} values were calculated as in Weir and Cockerham (1984). Grey bars represent SNPs from the ‘neutral’ dataset ($n = 2\ 015$) and black bars represent SNPs from the ‘adaptive’ dataset ($n = 8$). Arrow represents outlier 1363 that matched a predicted gene sequence (with accession number) in *Xiphophorus maculatus*.

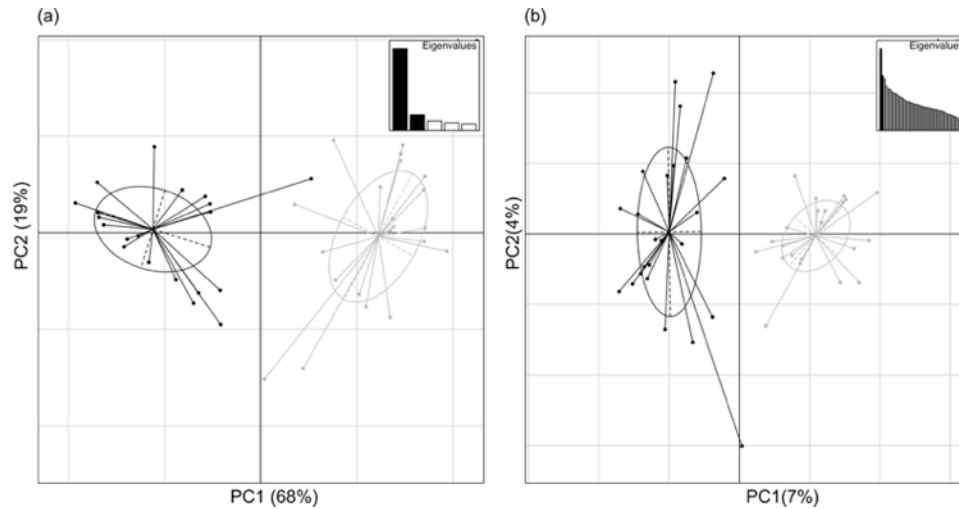


Figure II.6 First two axes from a centered and scaled principle component analysis of allele frequencies from the (a) ‘adaptive’ dataset (8 SNPs) and (b) ‘neutral’ dataset (2 015 SNPs). Dots represent individuals for the wild (DY; black) and captive (SNARRC; grey) populations. Scree plots illustrate the eigenvalues with the relative contribution labeled on each axis for each dataset.

Tables II:

Table II.1 Pairwise Procrustes distance measures using corrected data. Calculated from a pooled within group Canonical Variate Analysis.

	Wild Females	Wild Males	Captive Females
Wild Males	*0.0634		
Captive Females	*0.0210	*0.0670	
Captive Males	*0.0548	*0.0263	*0.0533

* Indicates significance at $P = 0.001$ using 999 permutations

Table II.2 Readings at DY were taken in the field from 06 – 07 / 2013 using a LaMotte Water Test Kit (#3633-04) following the manufacturers instructions. Readings from SNARRC were obtained from analytical reports completed on pond surface water or from weekly logs from 07 - 08 / 2013.

	DY	SNARRC
Chloride	3079	630
pH	7.0	7.5
Alkalinity	364	82
Dissolved O ₂	11	7
Hardness	2776	3000

*All concentrations are in mg / L

III. ASSESSMENT OF INTROGRESSIVE HYBRIDIZATION

III.1 Introduction

Interspecific hybridization, crossbreeding between two different species, can effectively alter the genetic structuring of populations and introduce variation at a much faster rate than mutation alone (Allendorf and Leary 1988; Martinsen *et al.* 2001; Laikre *et al.* 2010; Brennan *et al.* 2012). Interspecific hybridization can also disrupt local adaptation (Allendorf *et al.* 2001), which can effectively reduce fitness (Muhlfeld *et al.* 2009). In the most extreme cases, hybridization can effectively eliminate native genomes, even in the absence of introgression (Rhymer and Simberloff 1996). For rare or endangered species, introgressive hybridization can be particularly troublesome following the introduction or contact with a more abundant species (Rhymer and Simberloff 1996; Allendorf *et al.* 2001), especially if hybrid swarms are created between endemic species and non-natives (Echelle and Connor 1989). Because of these factors, introgressive hybridization can pose a significant threat to the effective management of imperiled populations.

One management solution in conservation is to simply eradicate genetically compromised individuals (Hubbs 1980; Rhymer and Simberloff 1996; Echelle *et al.* 2004), yet as evolutionary biologists are discovering, hybridization is a continuously occurring process, which has contributed greatly to shaping the evolutionary trajectories of both plants (Stebbins 1950; Arnold *et al.* 1999) and animals (Dowling and Secor 1997; Arnold 1997). While this is a distinction that has fueled considerable debate (Allendorf *et al.* 2001; Edmands 2007), it can generally be classified as detrimental when endangered

species undergo introgressive hybridization due to anthropogenic events (Allendorf *et al.* 2001). Because many fish endemic to the southwestern Americas have shown declines in population size due to anthropogenic factors, such as human-mediated dispersal of non-native species and habitat degradation (Miller *et al.* 1989; Minckley and Deacon 1991; Smith 1992), the sustainability of many endangered fish species can be difficult.

Frequently, exotic species are inadvertently, or purposely, introduced into waterways by anthropogenic means, such as from baitfish or for biological control (Hubbs 1980). For example, following their introduction during the 1980s, *Cyprinodon variegatus* led to the rapid formation of a hybrid swarm that contributed to the eradication of pure *C. pecosensis* throughout a majority of their habitat range (Echelle and Connor 1989). Following their introduction into Balmorhea Lake (Reeves CO., Texas) during the 1960s, *C. variegatus* has hybridized with multiple other pupfish species such as the Comanche Spring pupfish (*C. elegans*; Echelle and Echelle 1994), the Pecos pupfish (*C. pecosensis*; Wilde and Echelle 1992; Childs *et al.* 1996), and the Leon Springs pupfish (*C. bovinus*; Hubbs 1980; Echelle and Echelle 1997).

The Leon Springs pupfish (*Cyprinodon bovinus*) are a federally listed endangered desert pupfish isolated to a single spring in southwestern Texas, Diamond Y Spring (DY; 31° 0'4.75" N, 102°55'27.09" W; Figure III.1), where they are currently protected within the Diamond Y Preserve and under management of the Texas Nature Conservancy.

Facilitated by anthropogenic measures, the inadvertent or intentional release of *C. variegatus* in the natural habitat of *C. bovinus* was first documented in the 1970s, which precipitated the selective eradication of genetically compromised individuals (Echelle and Echelle 1997). As an assurance colony, 80 genetically uncompromised *C. bovinus*

were translocated from the upstream watercourse to the Southwestern Native Aquatic Resources and Recovery Center (SNARRC; formerly Dexter National Fish Hatchery and Technology Center; Figure III.1) in 1976 (Edds and Echelle 1989). Both morphological characteristics and allozyme electrophoresis (Hubbs 1980) verified that the renovation had been successful; no morphological or genetic signatures of introgression were observed or detected in any of the specimens collected from the downstream watercourse or brought into captivity (Echelle and Echelle 1997; Hubbs 1980). However, in the mid - late 1990s *C. variegatus* was introduced again into the Diamond Y Draw, which prompted a management decision to cull the native *C. bovinus* population and restore it with a large-scale (~ 5 – 10 000) release of captive fish into Diamond Y Draw (Echelle *et al.* 2004). Following the release and attempted establishment of the captive stock, few non-native allozyme markers were found in the wild (frequency 0 – 4.2 %; Echelle and Echelle 1997; Echelle *et al.* 2004), yet genetic monitoring for hybridization in the wild has not transpired since.

In the previous chapter, both divergent selection and genetic drift were identified as probable drivers for the substantial levels of genetic (and potentially morphological) divergence observed between the wild (DY) and captive (SNARRC) *C. bovinus* population. However, as hybridization can enhance both genetic and phenotypic variation (Brennan *et al.* 2012), it leaves the untested hypothesis that introgressive hybridization may be responsible for the documented genetic and possible phenotypic divergence. To address this, the previous chapter's results were extended to examine the wild *C. bovinus* population for associations with the genetic and morphological characteristics of *C. variegatus*. The prediction was that the presence of introgressive hybridization would

leave a genomic signature in the wild population and reveal morphological overlap or an intermediate body shape between the wild *C. bovinus* population and the congeneric *C. variegatus*.

III.2 Materials and methods

2.1 Sample collection

On 08 - 23 - 2013, forty (20 male / 20 female) adult *C. variegatus* samples were caught using minnow nets from Balmorhea Lake (BL; 30°57'46.46"N, 103°43'12.82"W) Reeves County, the suspected source population of the *C. variegatus* historically introduced into Diamond Y Draw (Echelle and Echelle 1997; Figure III.1). Tissue samples and images were acquired from these samples following the methods listed in the previous chapter and in accordance with stipulations defined in TX Parks and Wildlife permit No. SPR-0812 - 967.

2.2 Morphology

Historically, *C. bovinus* have provided visual evidence for introgressive hybridization by exhibiting morphological traits that were characteristic of *C. variegatus* (Echelle and Echelle 1997). To evaluate contemporary evidence for morphological introgression in DY, photographs of the left lateral surface of each individual fish were taken using a Nikon D5100 digital SLR camera (16.2 megapixels) with an 85 mm lens mounted to a copy stand.

To look for an association in morphological characteristics among populations, landmark-based geometric morphometrics (Bookstein 1991; Rohlf and Marcus 1993;

Marcus *et al.* 1996) was employed. A single investigator (A. Black) digitized 12 landmarks for each specimen using *tpsdig2* (v2.16, Rohlf 2013) and non-shape variation was held constant through Procrustes superimposition (Rohlf and Slice 1990). Procrustes superimposition effectively eliminates non-shape variation by translating all specimen to the origin, scaling them to unit centroid size, and optimally rotating them until corresponding landmarks across all specimen are aligned as closely as possible (Rohlf and Slice 1990). For additional information about image acquisition and digitization methodologies, see chapter II.

The Cartesian coordinates (x, y) of each landmark for all individuals were imported for use with the *geomorph* (Adams and Otárola-Castillo 2013) package in the R environment (R Development Core Team 2014), where a Principle Component Analysis (PCA) was then performed to reduce the dimensionality of the data and to visualize the Procrustes aligned specimen. This ordination method offers the advantage of requiring no *a priori* defined groups, which allows the visual discrimination of spatial clustering purely based upon morphological characteristics (e.g. putative morphological introgression). The contribution of each Principle Component (PC) was calculated and displayed in a scree plot, with deformation grids illustrated at the ends of the axes showing maximal variation (PC1 – PC3). Coordinates were then imported into *morphoJ* (Klingenberg 2011) for discriminant function analysis where classification rates were calculated using cross-validated discriminant functions, which use a ‘leave-one-out’ approach to generate an estimate of membership assignment probability in predefined groups (Lachenbruch 1967).

2.3 SNP discovery and filtering

To evaluate the wild population for signatures of introgression, a genome-wide strategy was employed based on double digest restriction Associated DNA sequencing (ddRAD-seq) technology (Peterson *et al.* 2012) following the methods described in chapter II. Briefly, genomic DNA from each individual (from both species) passing quality control thresholds ($n = 24$; 16 samples were removed due to low [DNA]) was digested with two restriction enzymes (High Fidelity *EcoRI* and *MspI*), ligated with barcoded adapters (1 - 48), PCR amplified with indexed flow cell adapters, size selected (375 ± 38 -bp), and sequenced on a single Illumina HiSeq 2500 lane (Institute of Biotechnology, Cornell University) using 2 x 101-bp sequence chemistry.

Demultiplexed and filtered reads were uploaded onto a computing cluster composed of 32 processor cores and 128 GB of memory (High Performance Computing; Lehigh University) where the reads were assembled using *stacks* 'denovo_map.pl' wrapper program (which sequentially executes each core component of the pipeline) using the following parameters: *-m 3, -n 2, -t, -M 2, -N 4*. Briefly, 'ustacks' combined reads of identical sequences (i.e. stacks) within each individual and merged these stacks based upon the number of nucleotide differences to accommodate individual polymorphisms, 'cstacks' created a catalog of all stacks across all individuals based upon the number of nucleotide differences, and 'sstacks' then matched each individual from each population against the catalog to determine the allelic state using a maximum likelihood framework. Stacks with an excessive number of reads (> 2 SD above the mean depth) were removed to screen out stacks potentially containing more than one merged locus (for additional details see: Catchen *et al.* 2011, 2013).

To avoid the loss of data and prevent a potential biased screening process, tolerance levels for missing data were relaxed to 50% (Huang and Knowles 2014); loci were retained if they were present in $\geq 50\%$ individuals examined, with a minimum stack depth of 10x, and a minor allele frequency (MAF) > 0.05 . Using a False Discovery Rate of $\alpha = 0.05$ (implemented in R's *stats* package) loci were excluded if their genotype frequencies deviated significantly ($P < 0.05$) from Hardy-Weinberg Equilibrium expectations for two or more populations (Wigginton *et al.* 2005). Additionally, to maintain independence of loci with multiple SNP sites, only the first SNP from each locus was retained.

2.4 Genetics

To examine genotypic clustering, all filtered SNPs were grouped by population and uploaded for analysis with a Discriminate Analysis of Principle Components (DAPC; Jombart 2008) in R's *Adegenet* package. This test first transforms the multilocus genotype of all individuals with a principle component analysis prior to running a discriminant function analysis to provide a spatial clustering of individuals. To prevent over fitting of discriminant functions by using an excessive amount of variation, 1/3 of the total principle components (23 PCs) were initially retained and optimized by using the 'optim.a.score' function (Figure A3.01). *Genodive* (v2.0; Meirmans and Van Tienderen 2004) and the *pegas* package in R (Paradis 2010) were used to assess population differentiation through mean and pairwise F_{ST} values (Weir and Cockerham 1984) and significance was assessed through use of 999 permutations to test if F_{ST} differed significantly from zero. *Genodive* was additionally used to calculate a Hybrid

Index, which is a maximum-likelihood estimate of the proportion of alleles a putative hybrid individual obtained from either parental genotype (Buerkle 2005). A hybrid index of 0.50 would indicate an equal contribution of parental alleles in a hypothetical hybrid.

Introgressive hybridization was further evaluated by filtering loci that were fixed between reference and alternative populations ($F_{ST} = 1$; SNARRC * BL), which were then imported into *structure* (Pritchard *et al.* 2000) to identify similar clusters of individuals based upon allele frequencies. This model estimates the group membership of each individual, assuming Hardy-Weinberg and linkage equilibrium within groups (Pritchard *et al.* 2000). Parameters were run using correlated allele frequencies, an admixture model, a burn in period of 10 000, 100 000 repetitions, and k was run for 1 - 5 possible genotypic clusters with 10 iterations for each k value. Results were uploaded to *structure harvester* (Earl *et al.* 2012) where the optimal k was identified using Evanno *et al.* (2005) delta k formulation method. Files were then imported into *clumpp* (Jakobsson and Rosenberg 2007) for aggregation, and Q-matrices were visualized with *distruct* (Rosenberg 2004).

2.4 Extension of previous chapters results

Raw demultiplexed sequence reads and raw coordinates obtained from digitized photographs of *C. bovinus* from chapter II were processed along with original sequence data and digitized photographs of *C. variegatus*. The barcoded index containing pooled *C. variegatus* samples were sequenced in the same final library as the wild and captive populations of *C. bovinus*. Pooled genomic and morphometric datasets were then used to examine the wild *C. bovinus* population for evidence of introgressive hybridization by

using the *C. variegatus* and the captive *C. bovinus* population as reference genotypes/phenotypes.

III.3 Results

3.1 Morphology

Figure III.2 illustrates the external characteristics exhibited by the two *C. bovinus* populations for contrast with *C. variegatus*. Representative of *C. bovinus*, males from both populations showed a slightly convex terminal caudal fin (Figure III.2A & Fig2E), opposed to the slightly concave fin of *C. variegatus* males (Figure III.2C; Echelle and Echelle 1997). Multiple female *C. bovinus* from SNARRC (Figure III.2F) showed similar pigmentation patterns to female *C. variegatus* (Figure III.2D), which appeared absent or reduced in the wild *C. bovinus* population (Figure III.2B). Specifically, several females from SNARRC showed conspicuous vertical bars, opposed to the characteristic intermittent vertical bars characteristic of female *C. bovinus*, which were observed in DY.

After importing the Cartesian Coordinates of all specimens into R (n = 178), a PCA was conducted on the superimposed specimen from all three populations. The range of variation in PC1 (40.5% variation explained) showed little apparent group discrimination when individuals were labeled by population source (Figure III.3A). However group division became clearly evident when individuals were labeled according to sex (Figure III.3B). Deformation grids along PC1 illustrated difference occurring in body depth (Figure III.3). The range of variation of PC2 (12.1% variance explained) was illustrated by shape change between the two *C. bovinus* populations (Figure III.4A), with *C. variegatus* grouping between the two *C. bovinus* populations with minor overlap with

individuals from DY (Figure III.4A). Deformation grids along PC2 illustrated the same difference in head slope orientation previously outlined in chapter II (Figure III.4A). The range of variation of PC3 (9.8% of variation explained) grouped the two *C. bovinus* populations discretely from *C. variegatus* (Fig4B). Deformation grids along PC3 illustrated differences occurring in the predorsal elevation, body depth, and dorsal fin attachment point (Figure III.4B).

To examine the wild population for the presence of morphological intermediates, a discriminant function analysis was conducted on all individuals from all three populations. Discriminant function analysis provided strong support for separating the morphological characteristics of the two *C. bovinus* populations from *C. variegatus*, with little evidence for overlap (Figure III.5). Discriminant function with cross-validation correctly assigned *a priori* defined groups with a high accuracy for both captive ($T^2 = 1092.4$, P-value < 0.001) and wild populations ($T^2 = 832.9$, P-value < 0.001) when a pairwise comparison was conducted with *C. variegatus* (Table III.2).

3.2 SNP discovery and filtering

After removing two individuals from SNARRC due to low genotyping rate (< 90 %), a total of 40 498 loci (75 427 SNPs) across all 70 individuals were retained with an average of 31 302 loci per individual and a mean merged depth of 12x (± 4 SD). Mean depth was well balanced between DY (15 ± 4 SD), SNARRC (11 ± 3 SD), and BL (10 ± 3 SD). By requiring loci to be present in $\geq 50\%$ of individuals within each population with a minimum read depth of 10x, this was reduced down to 4 174-biallelic loci (9 686 SNPs). Using only the first SNP of each locus, 18 SNPs were removed due to deviation

from Hardy-Weinberg Equilibrium (following FDR correction, $\alpha = 0.05$), and 818 SNPs were removed with a MAF < 0.05 . This resulted in a final filtered dataset of 3 338 SNPs which were used to evaluate the wild *C. bovinus* population for evidence of introgressive hybridization.

3.3 Genetics

A DAPC clearly differentiated the two *C. bovinus* populations from *C. variegatus* and showed a clear membership assignment probability for the two *Cyprinodon* species (Figure III.6); both *C. bovinus* populations grouped together with the absence of intermediate genotypes relative to *C. variegatus*. When evaluating the partitioning of genetic variation, DY revealed significant levels of genetic differentiation with BL ($F_{ST} = 0.837$; $P < 0.001$), which was also observed when comparing SNARRC and BL ($F_{ST} = 0.829$; $P < 0.001$; Table III.2). Using SNARRC as the reference population and BL as the alternative population showed that the DY population provided little to no support as putative hybrids ($h = 0.9965$, \ln (likelihood) = - 0.567.19, lower = 0.994, upper = 0.998).

Population structuring was further evaluated using 278 diagnostic SNPs that were fixed between the SNARRC and BL population. Results showed an optimal genotypic clustering at $k = 2$, which clearly separated the two species (Figure III.7A). Increasing the number of clusters to $k = 3$ illustrated population structuring occurring between the two *C. bovinus* populations with little evidence of genotypic influence of *C. variegatus* in the wild *C. bovinus* population (Figure III.7B).

III.4 Discussion

While assessment of both genetic and morphological characteristics can help detect cryptic cases of hybridization (Gaubert *et al.* 2005), results illustrated a lack of genetic or phenotypic evidence to indicate influence from *C. variegatus*, which suggests that hybridization has likely not occurred since the previous identification of hybrids in the Diamond Y Draw during the 1990's. This was apparent by both *C. bovinus* populations exhibiting comparable disparity with the morphological characteristics of *C. variegatus* and similar levels of genetic differentiation with *C. variegatus*. Additionally, using SNPs that were fixed between the captive and *C. variegatus* population failed to detect any patterns of genetic influence from *C. variegatus*.

4.1 Morphology

While the majority of shape variation was likely attributed to differences occurring between the sexes (Figure III.3B), the three populations showed considerable, and significant, separation (Figure III.3 – Figure III.5, Table III.1). While there was some ambiguity in the evaluation of the wild populations external characteristics (i.e. pigmentation patterns) relative to *C. variegatus* (Figure III.2), the absence of geometric intermediates or overlap implies the absence of recent morphological introgression with the wild *C. bovinus* population. Additionally, cross-validation of discriminant functions showed a high classification rate for *a priori* defined groups and with one exception showed a perfect assignment rate (Table III.2). As there were no misclassifications of any of the wild individuals with *C. variegatus*, based upon morphological characteristics, it appears that hybridization has not occurred recently in the Diamond Y Draw.

However, the occurrence of introgression is not always reflected in morphological

variation, as individuals that show a “parental” phenotype may in fact show molecular evidence for introgression (Rhymer and Simberloff 1996). While using morphological characteristics to identify hybrids is often generally quite feasible, similar to molecular markers, the difficulty increases with the number of back-crosses, or if weighted towards one parental species or sex (Boecklen and Howard 1997). So while morphological characteristics provided insufficient evidence for the presence of introgressive hybridization, it does not fully eliminate the possibility.

4.2 Genetics

Insubstantial evidence was also found in the wild *C. bovinus* population to indicate the presence of genetic introgression with *C. variegatus*; both *C. bovinus* populations were genetically distinct from *C. variegatus*. Mean F_{ST} values showed that both DY (0.837) and SNARCC (0.829) displayed similar, and significantly elevated, levels of genetic differentiation with *C. variegatus* (Table III.1). Group separation was additionally confirmed in a DAPC, which clearly illustrated that both *C. bovinus* populations clustered together, and substantially apart from *C. variegatus* (Figure III.6). When using diagnostic markers fixed between SNARCC * BL in a *structure* analysis, results also confirmed that the two *C. bovinus* populations grouped together, with no evidence of genotypic clustering with BL.

Results revealed a promising lack of evidence for introgressive hybridization in the wild *C. bovinus* population, as illustrated by the absence of genomic and morphological *C. variegatus* characteristics. Yet, it is possible that the *C. bovinus* population maintained in captivity (which were then released back into the wild) had

considerable numbers of *C. variegatus* alleles integrated into their genomes, which were undetected by the initial screening process in the mid 1970s. Therefore, using the genotype of the captive *C. bovinus* population as a reference may have introduced a substantial bias into the genetic assessment for introgression. While it is possible that the low-resolution markers used to evaluate the eradication of non-native genes in the late 1990s (i.e. allozyme markers) may have been insufficient to detect the cryptic signature of hybridization, it is a rather moot point now, as there remain no other source of *C. bovinus*.

The lack of genetic/phenotypic evidence suggests that recent hybridization or historical introgression was not a principal mechanism for the documented population divergence previously reported between the captive and wild *C. bovinus* populations. This provides further support for conducting an immediate reciprocal genetic inoculation between captive and wild *C. bovinus* populations, to help mitigate population divergence, while minimizing the threat of introducing non-native genetic material. In the mean time, it is imperative to continually monitor the genetic and phenotypic characteristics of the wild *C. bovinus* population to help minimize the negative effects of future introduction events and ensure that genetic integrity is maintained in the sole remaining wild *C. bovinus* population.

Figures III:

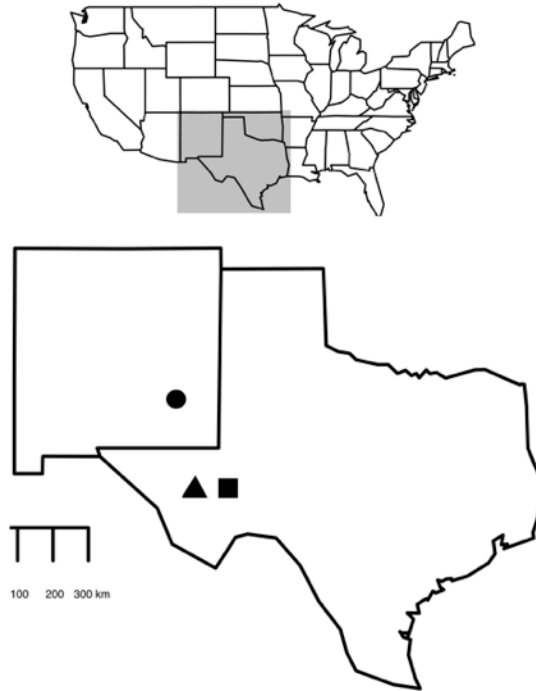


Figure III.1 Map illustrating the location of the wild *C. bovinus* population (DY; square), the *C. variegatus* population from Balmorhea Lake (BL; triangle), and the captive *C. bovinus* population (SNARRC; circle).

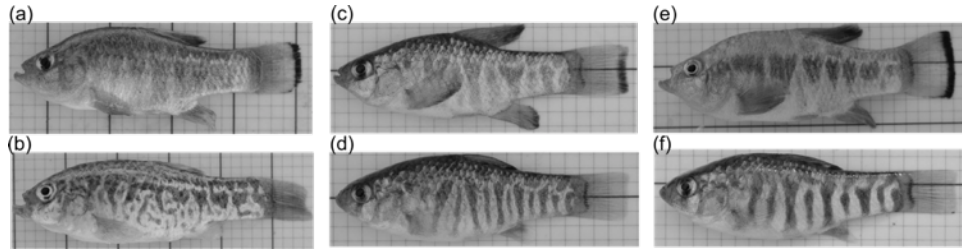


Figure III.2 Representative images of (a) male and (b) female *C. bovinus* from DY, (c) male and (d) female *C. variegatus* from BL, and (e) male and (f) female *C. bovinus* from SNARRC.

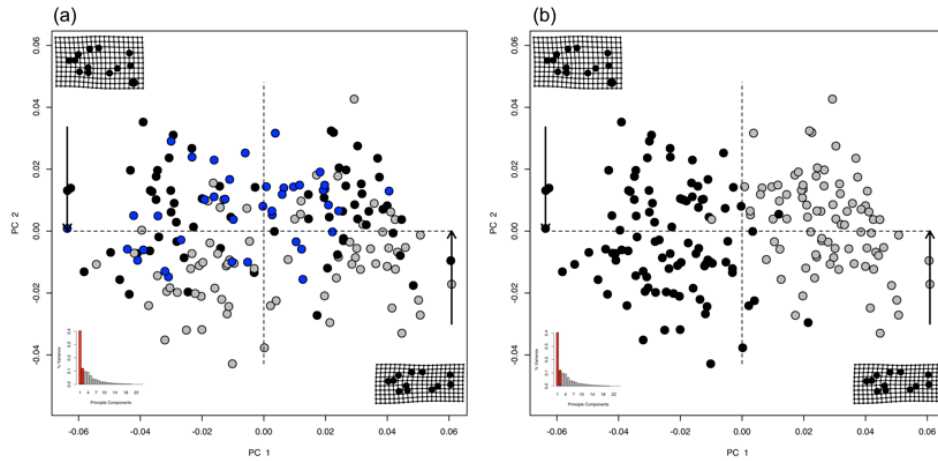


Figure III.3 Biplot of PC1 (40.5% variation explained) and PC2 (12.1% variation explained) from a Principle Component analysis of body shape variables factored for illustration purposes by (a) population (DY, black; SNARRC, grey; BL, blue) and (b) sex (male, black; female, grey). Deformation grids represent variation in body shape occurring at the ends of each axis.

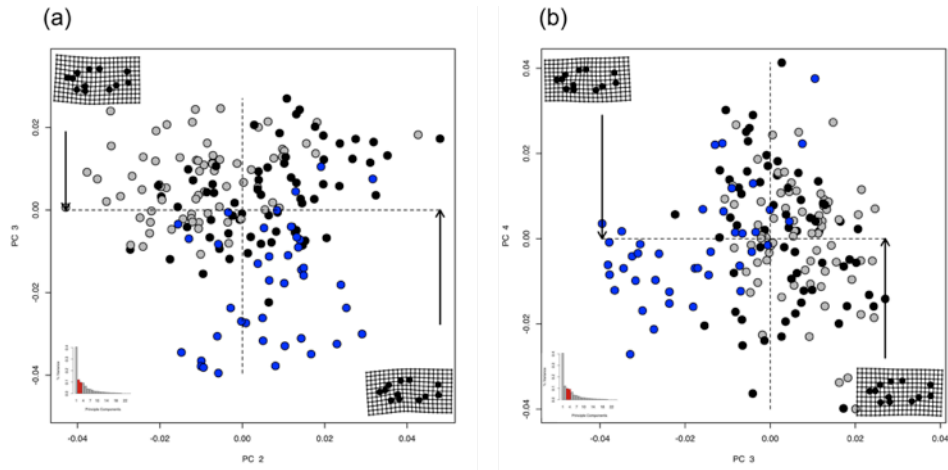


Figure III.4 Biplot of (a) PC2 (12.1% variation explained) and PC3 (9.8 % variation explained) and (b) PC3 and PC4 (9.2 % variation explained) from a Principle Component analysis of body shape variables. Individuals were factored by population (DY, black; SNARRC, grey; BL, blue). Scree plot in lower left hand corners illustrates the relative contribution of each principle component (respective PCs are highlighted in red). Deformation grids represent variation in body shape occurring at the ends of each axis.

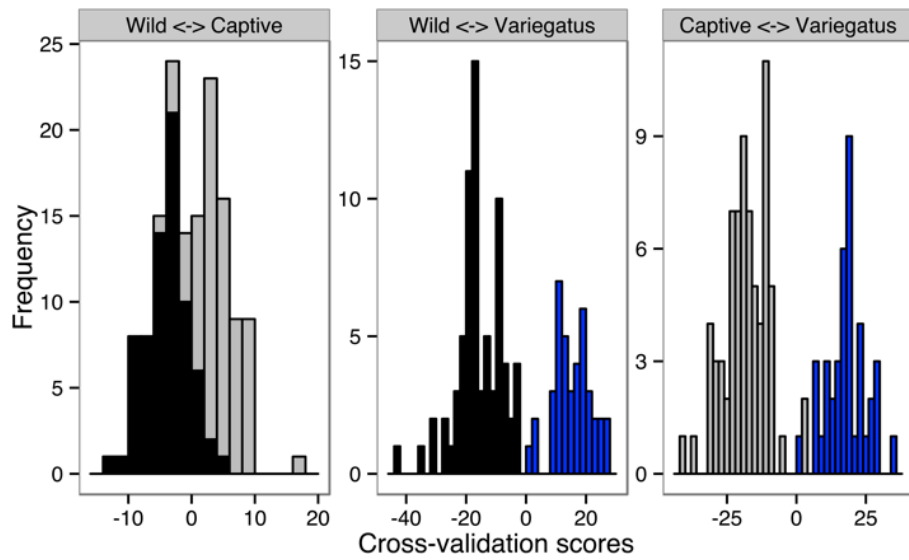


Figure III.5 Histogram of cross-validation scores from a pairwise discriminant function analysis used to examine differences in body shape between the wild (DY; black), captive (SNARRC; grey) *C. bovinus* populations, and *C. variegatus* (BL; blue).

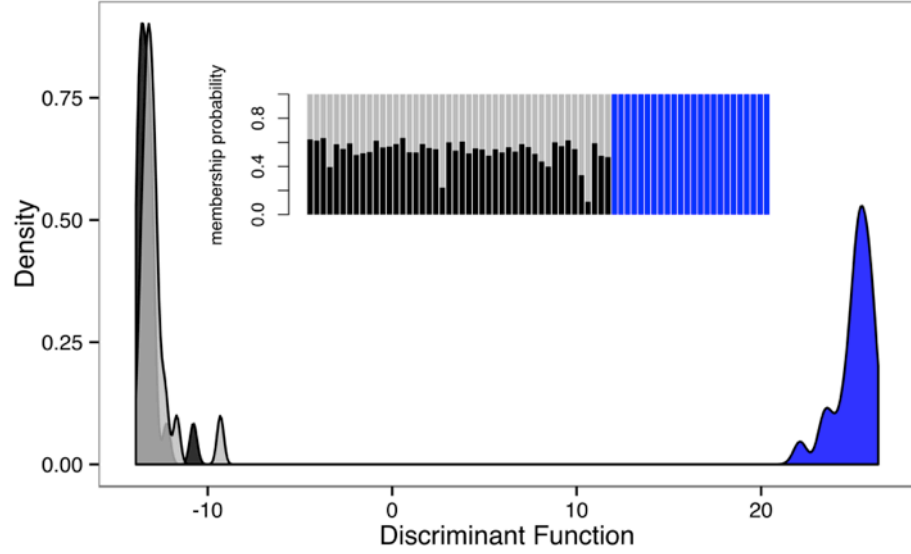


Figure III.6 Discriminant Analysis of Principle Components using 3 338 SNPs after retaining 1 principle component and the associated membership probability for individuals from the wild (DY; black) and captive (SNARRC; grey) *C. bovinus* population, and individuals from the *C. variegatus* population (BL; blue).

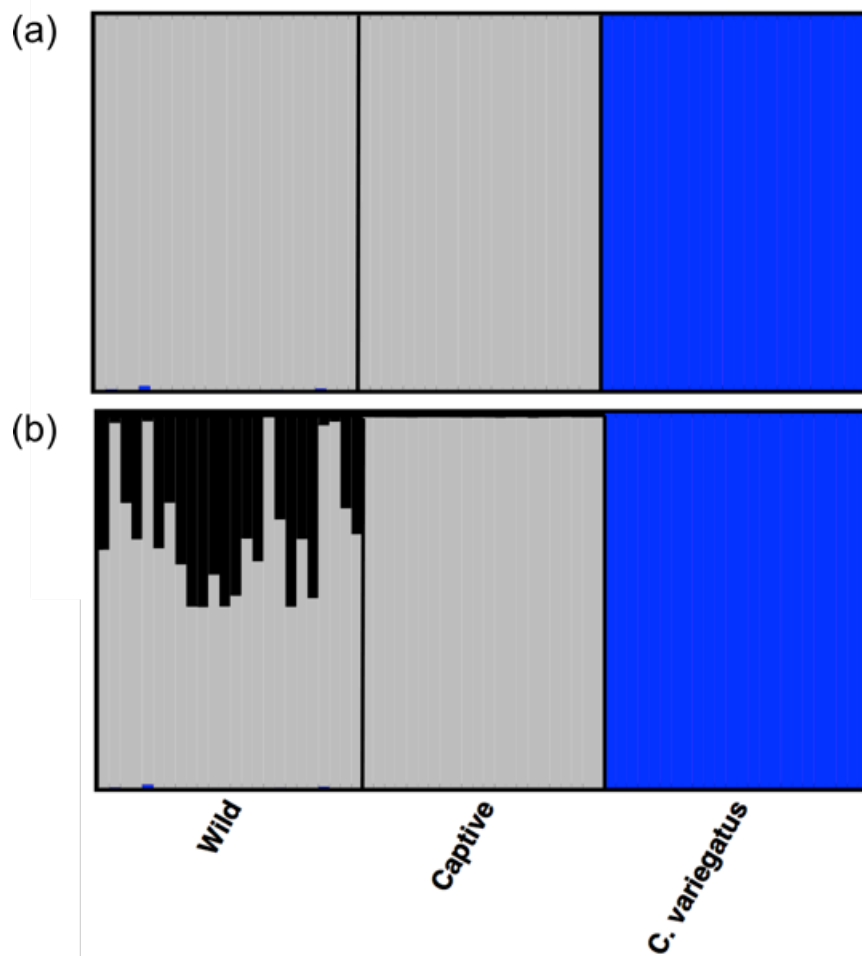


Figure III.7 Structure run for $k = 2$ (a) and $k = 3$ (b) from all three *Cyprinodon* populations using 278 SNPs fixed between the captive *C. bovinus* and *C. variegatus* population. Optimal k (2) was identified by Evanno's method.

Tables III:**Table III.1** Mean pairwise F_{ST} values from 3 338 SNPs typed in 70 individuals across all three populations. F_{ST} values were calculated as in Weir and Cockerham (1984) and significance was assessed through 999 permutations.

Population	Wild	Captive	<i>C. variegatus</i>
Wild		0.061	0.837
Captive	$P < 0.001$		0.829
<i>C. variegatus</i>	$P < 0.001$	$P < 0.001$	

Table III.2 Classification / misclassification rates from cross-validation of pairwise discriminant function analysis using morphological characteristics.

	Wild	Captive	Total	% Correct
Wild	60	6	66	91
Captive	5	67	72	93
	Wild	Variegatus	Total	% Correct
Wild	66	0	66	100
Variegatus	0	40	40	100
	Captive	Variegatus	Total	% Correct
Captive	71	1	72	99
Variegatus	0	40	40	100

IV. THE REINTRODUCTION OF CYPRINODON BOVINUS

IV.1 Introduction

Endangered species are often reared in captivity to augment declining wild populations, or to reintroduce them into their historical range from which they have become extirpated (Seddon *et al.* 2007; Seddon 2010). While the reintroduction of captive animals is a commonly employed method used to restore native species into their former range, success rates of reintroductions are generally low, in part due to the impaired ability of captive animals to become established or persist in their ancestral habitat (Kleiman 1989; Fischer *et al.* 2000; Seddon *et al.* 2007; Teixeira *et al.* 2007).

Establishment in ancestral habitats can be contingent on adaptive potential, local habitat quality and the resulting reproductive and survivorship rates of reintroduced populations (Hendrick 2001; Armstrong and Seddon 2008). However, by raising animals in an environment very different from their natural one, species in captivity can be exposed to different selection regimes than those present in the wild (Heath *et al.* 2003; Christie *et al.* 2012), which can shape both genetic and phenotypic characteristics of captive animals through domestication selection (Snyder *et al.* 1996; Blanchet *et al.* 2008; Frankham 2008). Therefore, unfavorable consequences associated with conservation breeding have the potential to negatively impact the ability of captive animals to reproduce or survive when released back into the wild (Snyder *et al.* 1996; Woodworth *et al.* 2002).

Some of the most extensive research that has been conducted into understanding the association between conservation breeding and reintroduction biology has focused on

teleosts, which have received centralized attention due to their high socio-economic value in the aquaculture field (e.g. Araki *et al.* 2007; Milot *et al.* 2012). Comparative analyses between source and recipient populations have shown how raising fish in a captive environment can affect local fitness, foraging ability and aggression levels (Kelley *et al.* 2006; Araki *et al.* 2007; Milot *et al.* 2012). These effects can be readily apparent in fish, likely due to their short generation times and high population density coupled with an overabundance of a predictable and reliable food source in captivity.

The pupfishes (family Cyprinodontidae) of southwestern North America have received extensive conservation focus as they are commonly maintained in captivity or artificial refugia (Echelle and Echelle 1993; Duvernell and Turner 1998; Koike *et al.* 2008). Within these artificial environments, changes in both genetic and phenotypic characteristics, relative to those exhibited in the wild, have been documented (Wilcox and Martin 2006; Collyer *et al.* 2011). While multiple studies have predicted, or implied, that genetic or phenotypic alterations in captivity may have an effect on reintroduction success (e.g. Lema and Nevitt 2006; Wilcox and Martin 2006; Collyer *et al.* 2005, 2011), this has not been explicitly tested under natural and semi-controlled environments using any endangered *Cyprinodon* species.

The Leon Spring pupfish (*Cyprinodon bovinus*; Baird and Girard [1851]) are an endangered species isolated to a single desert spring in southwestern Texas (US Federal Register 2008). Since reaching near extinction in the mid 1900s, a founding population of *C. bovinus* has been maintained since 1976 at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC [formerly Dexter National Fish Hatchery and Technology Center] Edds and Echelle 1989), which has been critical for the continuation

of this species (Echelle *et al.* 2004). As a result of the introduction and subsequent interspecific hybridization with the congeneric sheepshead minnow (*Cyprinodon variegatus*), a majority of the wild population was culled in the early 2000s, which led to the large-scale release of ~ 10 000 pupfish to replenish the wild population (Echelle and Echelle 1997; Echelle *et al.* 2004). Since the release of captive *C. bovinus* into the wild, the population size has rapidly declined until 2006, when less than five individuals were reported (Gumm *et al.* 2008).

The proximate reasons for the low observed establishment of captive fish are unknown, yet by employing a comparative approach with an established wild population of *C. bovinus*, it is possible to simultaneously identify and potentially assess the functional significance of principal behavioral disparities of captive animals in their ancestral habitat. That is, maladaptive behavior can be identified in reintroduced captive animals by looking for deviation in behavioral patterns exhibited by wild animals (Mathews *et al.* 2005).

In the current study, a habitat restoration project was employed to accommodate an experimental approach, followed by a reintroduction of captive *C. bovinus* into an isolated habitat, located 2 km NNE of the sole remaining wild population. To evaluate the behavioral patterns of captive animals, the reintroduced population was monitored throughout the summer of 2013. A comparative approach was used (wild vs. reintroduced population) to identify the presence of maladaptive behavior, which may be a result of conservation breeding and the associated divergent selection regimes. The null hypothesis was posited to be an absence of maladaptive behavior exhibited by the reintroduced captive population, which would indicate the preservation of natural

characteristics. Alternatively, rejection of the null hypothesis could signal the occurrence of novel adaptations in either or both populations, which could potentially compromise the sustainability of this endangered species and therefore encourage reconsideration of the current *C. bovinus* management strategy.

IV.2 Materials and methods

The behavior of wild *C. bovinus* was observed at Diamond Y Spring, TX (DY; 31°0'4.75"N, 102°55'27.09"W) from June – July 2013 (Figure IV.1). Four hundred adult captive *C. bovinus* were supplied by Southwestern Native Aquatic Resources and Recovery Center (SNARRC; Dexter, NM) and were transported and released into Monsanto Pool, TX (MP; 31°1'51.60"N, 102°53'49.73"W) where their behavior was also observed from June - July 2013 (Figure IV.1). All fishes were handled in accordance with stipulations defined in TX Parks and Wildlife permit # SPR – 0812 - 967.

2.1 Study location and species

DY is comprised of a circular head pool ~ 419 m² and 3.8 m in depth with steep undercut banks and a 8 m² breeding shelf (Veni 1991; Gumm *et al.* 2011). From the head pool, water flows down a long stretch of land choked with *Scirpus americanus* (~ 2 m in width and 5 - 10 cm in depth) before terminating into the ground 1 - 2 km to the NNE (Echelle and Miller 1974). MP, located 2 km downstream from DY, contains dense emergent vegetation (~ 1 -2 km stretch) and maintains a deep 1 m² refugium with a small peripheral clearing (~ 3 m²; A. Black, personal observation). Historically, MP supported a

small population of *C. bovinus*, which became extirpated in 2012 (M. Itzkowitz, personal observation).

Cyprinodon bovinus are a small (≤ 7 cm) promiscuous annual breeder that maintains small territories on a shallow (5 – 10 cm) breeding shelf throughout the summer. During the breeding season (May ~ Oct), territorial male *C. bovinus* adopt a blue nuptial coloration and actively defend small heterogeneous sites (Echelle and Miller 1974). Females will enter a male's territory in the upper water column, and if the female is interested she will descend to the substrate, allowing the male to align himself parallel to her. The pair will then form a sigmoidal shape, followed by a rapid jerking movement during egg deposition (Leiser and Itzkowitz 2003a). Similar to other species of pupfish, males provide no parental care of these deposited eggs, with the exception of inadvertently driving intruders from his territory (Leiser and Itzkowitz 2003a). Territorial males are commonly observed chasing, fighting and / or biting intruding conspecific males in addition to *Gambusia nobilis*, a sexually dimorphic small live bearing member of the Poeciliidae family (for additional details see: Gumm *et al.* 2008, 2011). *Cyprinodon bovinus* are opportunistic generalists which feed on diatoms, amphipoda, algae, gastropoda, as well as seeds and have been described displaying "pit digging", a behavior used to churn up the substrate in an attempt to excavate any buried food (Kennedy 1977).

2.2 Experimental design

To identify the presence of maladaptive behavior in reintroduced captive fish, the behavior of adult territorial male *C. bovinus* was observed under natural and semi-controlled conditions for both wild and reintroduced captive *C. bovinus* populations.

Natural behavior was observed June - July 2013, which entailed the passive observation of ten territorial males in the wild population (DY) as well as the recently reintroduced captive population (MP). At both DY and MP, ten numbered 2 x 5 cm, weighted plastic tags were placed within each focal male territory. These tags facilitated the analysis of the same male's behavior across the breeding season and also provided scale for estimating territory size (see below).

Once a day at both locations, video cameras (JVC GZ-R10B) equipped with polarized lenses were placed on tripods on the shore directly above each tagged territory ($n = 10$ males / location) and 15 min recordings were taken between 10 am and 4 pm. For evaluation of recorded data, behavior was undocumented for the first 5 minutes to allow males to acclimate to the video camera, and a total of 10 min was analyzed from each recording. Due to distorted recordings caused by wind or excessive sun glare, the number of recordings varied by territorial male (mean = 7.4; min / max = 5 / 12) representing ~ 25 hours of total video footage [12.5 hrs (DY); 12.2 hrs (MP)].

From each video recording, the following behaviors / parameters were quantitated: 1) *total aggressive behavior*, 2) *foraging behavior*, 3) *total reproductive behavior*, and 4) *territory size*. For quantification purposes, *total aggressive behavior* was comprised of fighting (direct, violent contact between conspecific males), male conspecific and *G. nobilis* chases (mutual acceleration), and lateral displays (extension of dorsal / pectoral fins). *Total reproductive behavior* was comprised of the number of

spawning events (sigmoidal orientation with subsequent pause) in addition to the number of spawning attempts (male parallel to female but no sigmoidal orientation). *Foraging behavior* was recorded as a discrete event whenever the male struck / dislodged the substrate. *Territory size* was estimated using *imagej* (Abramoff *et al.* 2004) by calculating the video area of each recording (using the plastic tag as scale), regressing the percent of time the male spent within the video frame (y) on territory diameter ($\sqrt{\text{area}} = x$), and using the slope to predict the territory size (cm) at $y = 0.75$. All four measures were individually averaged to obtain a mean estimate for each territorial male and an average was obtained for each location (DY & MP). All behaviors were then converted to frequency measures (min).

Scirpus americanus encroachment is a predominant cause of habitat loss at both locations (MP & DY), which severely limits availability of experimental substrate. Therefore, to provide accessible habitat for the semi-controlled experiments, two pools were manually excavated immediately downstream from the breeding shelf at DY and upstream from the refugium at MP during January 2012. Following the construction of all four pools (700 x 200 x 20 cm each), cement tiles (30 x 10 x 5 cm) were submerged in cleared areas to prevent the regrowth of *S. americanus*. To compensate for density dependent behavior, six mesocosms (three at each location) were placed in newly reclaimed habitats and were provided with small diameter river rocks (≤ 1.25 cm) and 2 L of substrate / mud from the surrounding area for food (e.g. amphipods); the six m^3 mesocosms were manufactured using 1.90 cm pvc pipe, 0.025 mm grey fiberglass screening, and zip ties.

Cyprinodon bovinus and *G. nobilis* were caught with minnow traps, weighed, measured and separated into mesocosms based upon similar size. Each mesocosm contained 3 male *C. bovinus* (mean TL = 5.5 cm \pm 1.4 SD), 3 female *C. bovinus* (mean TL = 4.9 cm \pm 0.5 SD) and 6 female *G. nobilis* (mean TL = 4.1 cm \pm 0.2 SD) to standardize size and species composition. In each mesocosm, three 10 x 20 cm weighted scouring pads (Scotch-Brite) were added (prior to the addition of residents) for spawning substrate.

Using a mounted video camera, each mesocosm was recorded daily for a period of 35 min between 10 and 3 pm (06/09/2013 – 06/14/2013). From these recordings, following a 5 min acclimation time, the behavior of all males in each mesocosm were analyzed for 30 min each for a total of 18 hrs of documented video footage [9 hrs (DY); 9 hrs (MP)]. Using the same definitions as in the previous experiment, the following behaviors were documented: 1) *total reproductive behavior*, 2) *total aggressive behavior*, and (3) *foraging behavior*. Each mesocosm was averaged by male and by behavior to obtain a mean estimate for each mesocosm and an average was then obtained for each location (DY & MP). All behaviors were then converted to frequency measures (min).

2.3 Statistical analysis

Both populations were tested for multivariate normality (Shapiro's test [W ; $\alpha < 0.05$]) and the effect of population source on behavior was assessed with a multivariate analysis of variance (MANOVA) using the packages *WRS* (Wilcox and Schonbrodt 2015), *reshape* (Wickham 2007), *mvnrmtest* (Slawomir 2012) and their associated dependencies in the R environment (R Development Core Team 2014). Using $\alpha = 0.05$, a

power analysis of the semi-controlled data revealed a low probability of discovering a main effect for each behavioral variable (unpaired t-test; $d = 0.03 - 0.25$), so results from the mesocosms were used for descriptive purposes only and figures from both experiments were combined to provide a visual comparison using the package *ggplot2* (Wickham 2009) in R.

IV.3 Results

Due to violations in multivariate normality for both wild ($W = 0.565, P < 0.0001$) and reintroduced ($W = 0.839, P < 0.05$) populations, a robust MANOVA was implemented using ranked data as in Choi and Marden (1997). For the natural behavior, there was no significant main effect of population (DY, MP) on any of the outcome variables ($H_4 = 2.66, P = 0.616$).

IV.4 Discussion

While traits selected for in captivity may no longer be adaptive when animals are released back into the wild, insufficient evidence was found to indicate that captive *C. bovinus* exhibited maladaptive behavior upon release into their ancestral habitat. By evaluating the territory size, foraging behavior, reproductive behavior, and agonistic behavior of the reintroduced population, relative to an established (and theoretically adapted) *C. bovinus* population, no significant deviations in any of the documented behavioral metrics were found. Similar patterns were additionally observed after controlling for the presence of density dependent behavior. These results provide an optimistic perspective on the role that conservation breeding may play in regard to the

reintroduction of endangered desert fish and offers an experimental approach to help evaluate the current or future adaptive ability of reintroduced animals under standardized local conditions.

The initial survivorship (while not quantitated) of *C. bovinus* appeared very high, signifying that the ensuing shift in abiotic conditions appeared to play a minor immediate role. Additionally, the high survivorship also implies that minor stress was imposed during transport from SNARRC, which can negatively impact the establishment of reintroduced animals (Hartup *et al.* 2005; Teixeira *et al.* 2007). Upon release into their ancestral habitat, the reintroduced captive fish showed characteristic levels of reproductive behavior. In fact, under natural conditions the reintroduced population actually exhibited slightly elevated levels of reproductive behavior (relative to the wild population; Figure VI.2). However, as this pattern was absent in the semi-controlled mesocosms, this could be attributed to differences in density between the two locations (Figure VI.2).

For both natural and semi-controlled conditions, the wild population exhibited slightly elevated levels of foraging behavior compared to the reintroduced population (Figure VI.3). While non-significant, this may be a result of inherent habitat differences found in captivity. For example, the captive population is maintained at SNARRC in a 0.10 acre pond with rooted vegetation covering ~ 30 % of the pond (personal communication, M. Ulibarri). If the predominant food source in these ponds is primarily obtained from / within vegetation (opposed to scavenging in the substrate) it may have an effect on foraging strategy in natural habitats. Unfortunately, an absence of aquatic plants

near the observed territorial fish at MP prevented testing this hypothesis in regard to foraging strategies between locations. Future examination of stomach contents or behavior in captivity may help elucidate any variation occurring between locations / populations.

While there are currently roughly 6000 *C. bovinus* maintained in the pond at SNARRC (personal communication, M. Ulibarri), no significant differences in aggression levels (Figure VI.4) or territory size (Figure VI.5) were discovered between the wild and reintroduced populations; males at both locations exhibited comparable levels of agonistic behavior and defended similar sized areas. This is surprising, as high density has been shown to have an effect on both aggression levels and territory size and (Kodric-Brown & Mazzolini 1992; Price, 1999; Kelley *et al.* 2006; Blanchet *et al.* 2008). However, while absent under natural conditions, slightly elevated levels of aggression were observed in captive fish maintained in mesocosms (Figure VI.4), which would also be an expected carryover effect of being maintained at high density in captivity. Additionally, reintroduced captive fish did show a trend for reduced territory size (relative to wild individuals), which may also be a result of being maintained at higher density in captivity; Figure VI.5). Because sample sizes were constrained by the number of mesocosms that could be established in the renovated habitat and the number of territorial males, it is difficult to discount the observed trend in aggression levels or territory size between locations.

In summary, analysis failed to isolate any maladaptive behavior in a recently reintroduced population of *C. bovinus*. However, results of this study faced the small

sample sizes that characterize many reintroduction studies, and should therefore be interpreted accordingly. Additionally, due to limitations in the number of behavioral measures that could be documented, it remains a possibility that other behavioral measures may in fact have shown differences between populations. Nevertheless, because of the lack of established differences in behavioral patterns between wild and reintroduced *C. bovinus*, it seems probable that other factors, such as habitat loss and fragmentation, were the predominant issues that contributed to the historically poor establishment and persistence of captive fish in the wild.

4.1 Concluding note

In August 2013 the water column dropped drastically at MP and as a result the population appears to have mostly perished; a recent census revealed that only twelve *C. bovinus* remained. A primary factor in the historical, and apparently current, persistence of this population is due to the presence of a relatively deep refugium, which helps buffer against seasonal fluctuations in local water flow and depth (Brune 1975; Hubbs *et al.* 1978). However, it appears that the refugium may be insufficient for the maintenance and sustainability of a stable population at this location. The drastic (and rapid) erosion of this population provides additional evidence that assessment of groundwater pumping or habitat loss merit further attention prior to the future reintroduction of this imperiled species.

Figures IV:

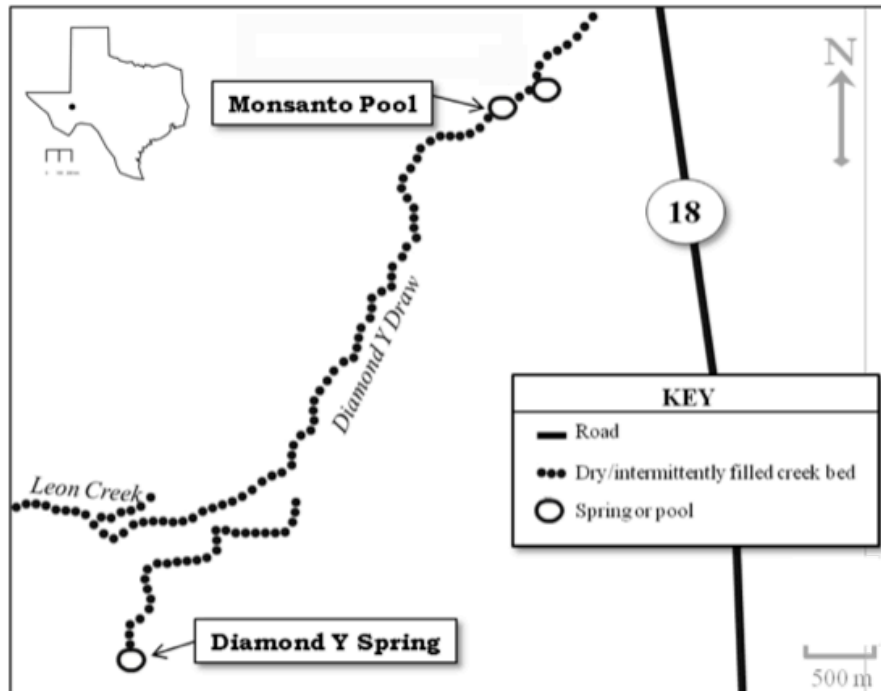


Figure IV.1 The geographic range of *Cyprinodon bovinus* in SW Texas. The sole remaining wild population of *C. bovinus* occurs in Diamond Y Spring (DY) with the reintroduction site (MP) ~ 4.2 km downstream.

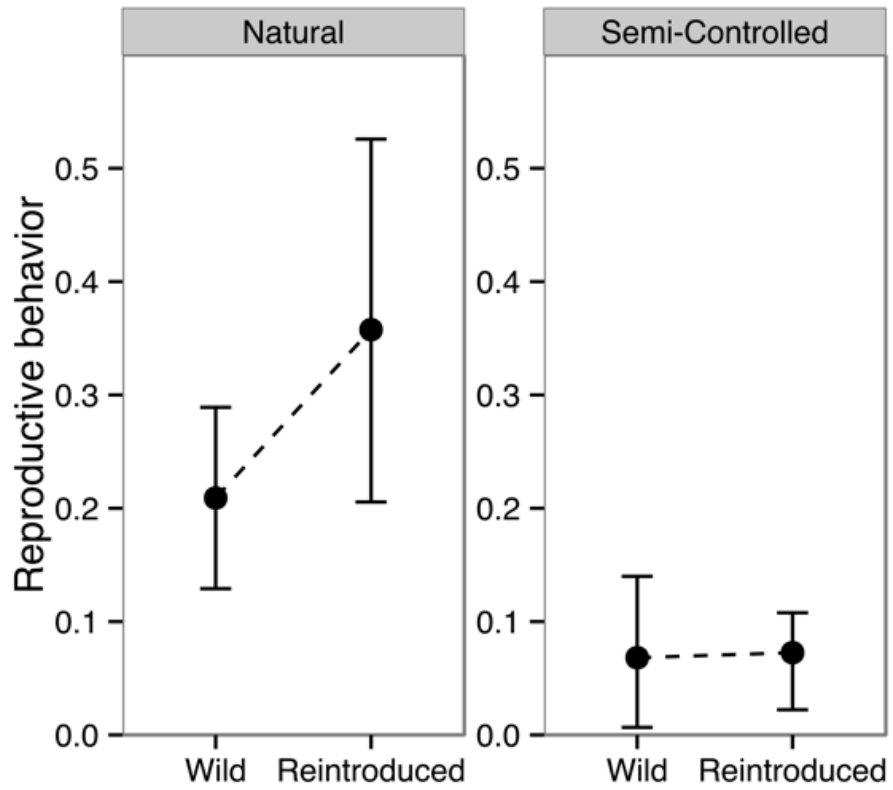


Figure IV.2 Mean reproductive behavior / min \pm SE for the wild (DY) and reintroduced captive population (MP) under natural and semi-controlled conditions. Dashed line for inter-population illustration only.

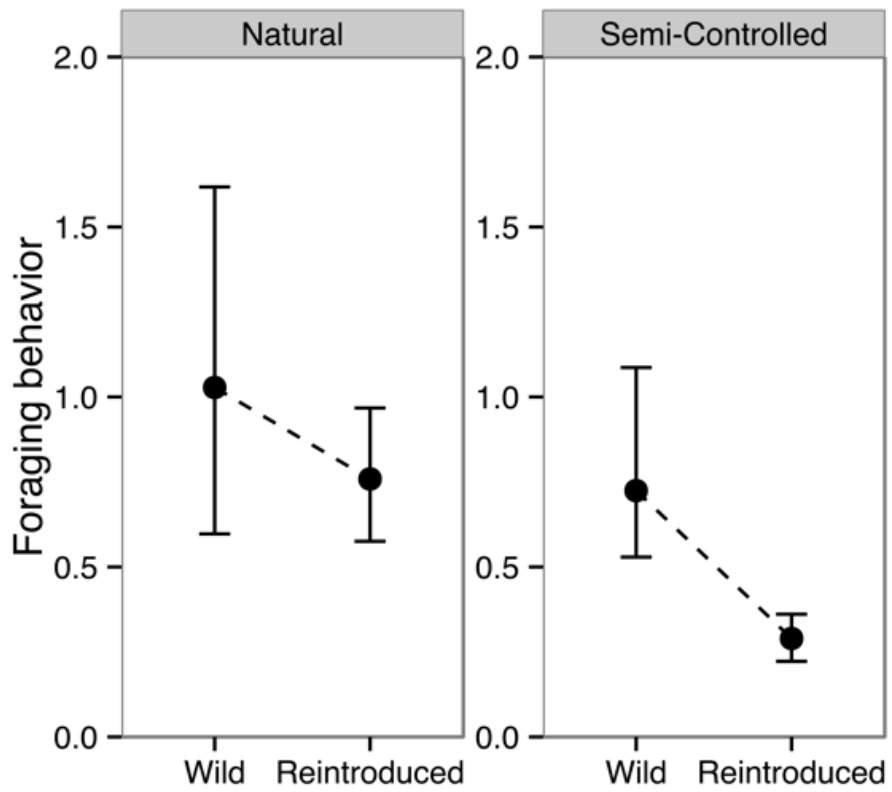


Figure IV.3 Mean foraging behavior / min \pm SE for the wild (DY) and reintroduced captive population (MP) under natural and semi-controlled conditions. Dashed line for inter-population illustration only.

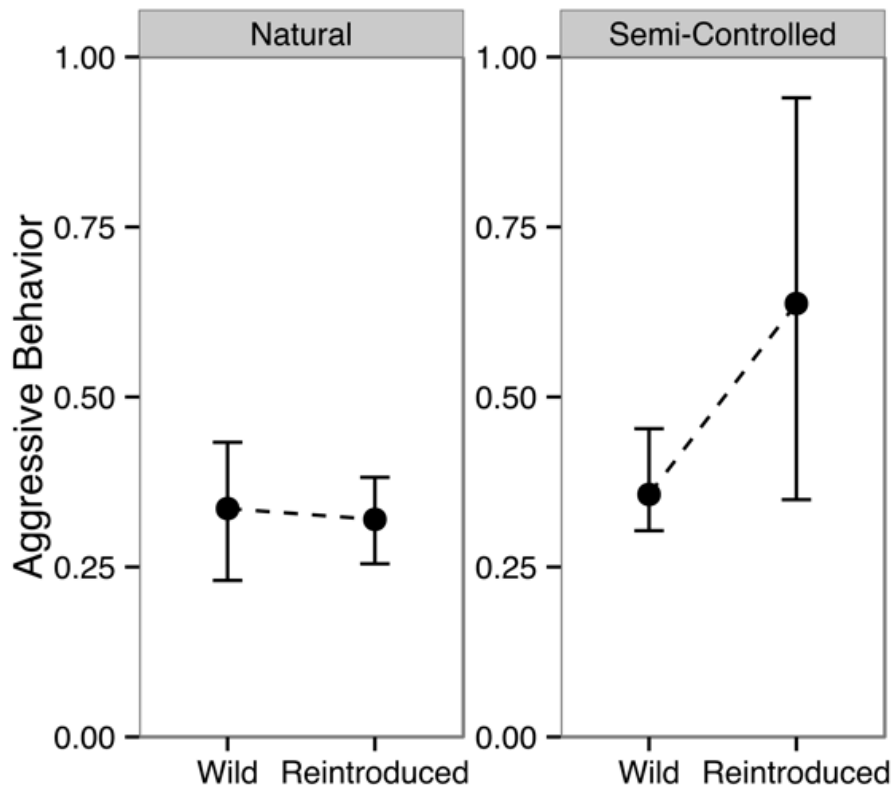


Figure IV.4 Mean aggressive behavior / min \pm SE for the wild (DY) and reintroduced captive population (MP) under natural and semi-controlled conditions. Dashed line for inter-population illustration only.

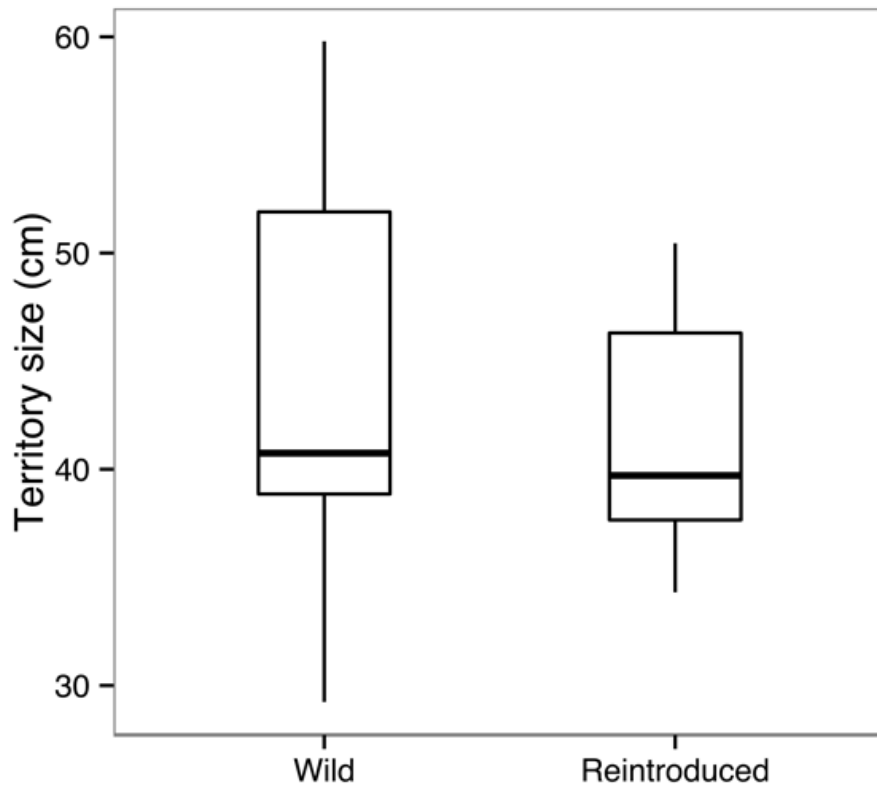


Figure IV.5 Boxplot illustrating estimated male territory size (diameter) for the wild (DY) and reintroduced captive population (MP) under natural conditions. Boxplots represent the median with hinges representing the first and third quartiles. Whiskers represent $1.5 \times$ Interquartile range.

V. DENSITY-DEPENDENT EFFECTS OF A PUTATIVE EGG PREDATOR

V.1 Introduction

Because of natural or anthropogenic mediated habitat loss, maintaining the sustainability of an endangered species is a difficult endeavor, even with an effective recovery plan (Hoekstra *et al.* 2002; Butchart *et al.* 2010). These challenges can be further compounded when habitat loss is accompanied by potentially deleterious interspecific interactions, which can require active intervention to manage complex interactions occurring between sympatric species (Soule *et al.* 2003; Tilman 2007). As in the case of introduced species, a common solution is to typically eradicate, or reduce, the exotic species in order to alleviate negative impacts (Westman *et al.* 2002; Lessard *et al.* 2005).

However, when addressing negative interactions occurring between two endangered species, simply eradicating or reducing the abundance of one of the species in question is not a viable option. This challenging dynamic can occur when sympatric species compete for similar resources and can require the careful management of ecological parameters to help maintain and preserve the fragile status and co-existence of both species in question (Soule *et al.* 2003; Oro *et al.* 2009). Successfully managing these parameters can be particularly challenging however, when an endangered species is suspected of directly reducing the fecundity of another sympatric endangered species (Gumm *et al.* 2008, 2011).

The Leon Spring pupfish (*Cyprinodon bovinus*) is an endangered species that occurs sympatrically with *Gambusia nobilis*, a small live-bearing member of the

Poeciliidae family; both species are the focus of ongoing recovery plans (Federal Register 2008). *G. nobilis* occur at very high densities in their native habitat, and commonly exhibit aggregation behavior around spawning pupfish pairs, suggesting that they may be preying upon newly deposited *C. bovinus* eggs (Gumm *et al.* 2008, 2011). Because Poeciliidae have been documented to outcompete native species for resources (Courtenay and Meffe 1989; Mills *et al.* 2004; Rehage *et al.* 2005) and prey on native species (Meffe 1985), concerns have been raised about the threat this species poses to the continued sustainability of *C. bovinus* (Gumm *et al.* 2008, 2011).

At a contemporary time scale, the primary conservation action has been to reduce the putatively negative interspecific impacts with *C. bovinus* by diluting their spatial contact with *G. nobilis* through habitat expansions. These habitat expansions facilitate the increased dispersal of both species, subsequently reducing the magnitude of any potential deleterious interactions. However, it is unknown how these habitat expansions, and subsequent shifts in *G. nobilis* density, affect the reproduction of *C. bovinus*. Therefore, the current study sought to experimentally test the behavior and fecundity of *C. bovinus* at varying densities of *G. nobilis*.

V.2 Materials and methods

2.1 Study location and species

Both *C. bovinus* and *G. nobilis* (Echelle and Echelle 1980; Hubbs *et al.* 2002) occur within Diamond Y Spring, a historical tributary of the Pecos River (Veni 1991), and are commonly observed in conjunction on a shallow breeding shelf (8m²) where *C. bovinus* males defend small territories throughout their breeding season (~ May – Oct).

Female *C. bovinus* will enter these territories in the upper water column, and if the female is interested, will descend to the substrate and form a sigmoidal shape with the male, which is followed by a rapid jerking movement during egg deposition (Leiser and Itzkowitz 2003a). Similar to other species of pupfish, males provide no parental care of these deposited eggs (Leiser and Itzkowitz 2003a).

2.2 Density dependent behavior and fecundity

Eight 1-m³ mesocosms were manufactured using 1.90 cm pvc pipe, 0.025 mm grey fiberglass screening, and zip ties. Mesocosms were placed in shallow water (10 - 15 cm), where small diameter river rocks (≤ 1.25 cm) were added to weigh down the fiberglass screening and ~ 2 L of substrate / mud was provided from the surrounding area for food (e.g., amphipod source). All mesocosms were placed in newly renovated ponds downstream from the natural breeding shelf to minimize disturbance.

To examine potential density dependent effects imposed by *G. nobilis*, two male (mean TL = 4.34 cm \pm 2.7 SD), and two female (mean TL = 4.2 cm \pm 2.9 SD) *C. bovinus* were assigned to each mesocosm based upon comparable size and were exposed to varying numbers of *G. nobilis* over three weeks in June 2014. A repeated measure design was implemented for each mesocosm (n = 8), which were each exposed to three treatment conditions for a period of seven days each: (i) zero *G. nobilis*, (ii) thirty *G. nobilis*, and (iii) sixty *G. nobilis*. These densities were chosen to cover the range of observed *G. nobilis* densities that have been documented to occur on the natural shelf at DY (unpublished data; A. Black). The order of treatment conditions was randomized for each mesocosms and commotions were created in the absence of *G. nobilis* to model

disruptions that occurred while adding *G. nobilis*. In each mesocosm, a single 10 x 20 cm weighted scouring pad was added (prior to the addition of residents) to provide a spawning substrate.

Twice a week, for three weeks, all eight mesocosms were filmed with a mounted video camera (JVC GZ-R10B) between 10:00 and 15:00 for a period of 25 min on the 5th and 7th day of each treatment week. Behavior was undocumented for the first five minutes to allow the fish to acclimate to the video camera, resulting in ~ 20 min of documented behavior from each recording; due to variation in recording times (mean = 18.4 min \pm 2.5 SD) behavior was converted to a frequency measure (per min). From each recording, the number of spawning events (number of spawns and spawning attempts) that occurred within each mesocosm was documented. Following the conclusion of filming on the 7th day, all spawning pads were carefully transferred (under water) into separately labeled containers filled with natural spring water to obtain a visual count of the number of deposited eggs. Each spawning pad was evaluated multiple times to obtain the maximum number of eggs found across the entire area of each spawning pad. New spawning pads were then added to each mesocosm and the ensuing treatment was started following the removal, and (if required) subsequent addition of *G. nobilis*.

A repeated-measures ANOVA design was implemented separately for the two dependent variables (total reproductive behavior and number of eggs), which were each tested for normality (Shapiro-Wilks; $P > 0.05$ = normality) and for violations in sphericity (Mauchly's test; $P > 0.05$ = sphericity). Statistical analysis utilized the *stats* and *ez* packages (Lawrence 2013), and figure generation used the *ggplot2* package (Wickham 2009) in the R environment (R Development Core Team 2014).

V. 3 Results

Both dependent variables were rank transformed due to a violation in normality ($P < 0.05$). A repeated-measures ANOVA illustrated that *G. nobilis* density was not a significant main effect on either *total reproduction* ($F_{2,14} = 1.38$, $P = 0.28$; Figure V.1A) or *egg number* ($F_{2,14} = 1.34$, $P = 0.29$; Figure V.1B).

V.4 Discussion

Insufficient evidence was found to suggest that *G. nobilis* exerted a distinct negative effect on either of the documented measures examined in a series of mesocosm experiments. Results demonstrated that the three treatment conditions failed to show an effect on the number of eggs laid or the frequency of reproductive behavior. However, it is possible that the abrasive surface of the spawning pads effectively trapped the deposited eggs, and therefore reduced the ability of *G. nobilis* to extract the eggs in the mesocosm experiments.

Another possible explanation for the similar levels of deposited eggs across treatments may be due to the fact that pupfish have been documented to engage in filial cannibalism, preying on eggs fertilized by other males (Loiselle 1983). If this were occurring within mesocosms, it is possible that the absence of *G. nobilis* may have allowed male *C. bovinus* to partition more time scavenging for eggs. Alternatively, in mesocosms containing *G. nobilis*, males may have devoted more time driving *G. nobilis* away from the spawning pad, which reduced the magnitude of filial cannibalism. Yet this does not appear to be the case, as comparable patterns were observed for the number of

deposited eggs and the frequency of reproductive behavior, which would have shown an elevated number of spawns (relative to the number of eggs) in the absence of *G. nobilis*.

In conclusion, an experimental approach was used to evaluate the density dependent effects of a putative egg predator on the reproductive behavior and fecundity of *C. bovinus*. Insufficient evidence of a clear negative density dependent effect implies that the proposed deleterious interaction occurring between *C. bovinus* and *G. nobilis* may be of lower severity than initially anticipated. While the results of the study were surprising based upon the supposed deleterious interaction occurring between these two species (Gumm *et al.* 2008, 2011), it suggests that experimental approaches may be more discriminating of complex relationships occurring within / between sympatric species. The lack of evidence suggesting a clear negative interaction between these two endangered species implies that perhaps conservation effort should shift away from reducing the interaction between these two species towards placing emphasis on the sustainability of both species.

Figures V:

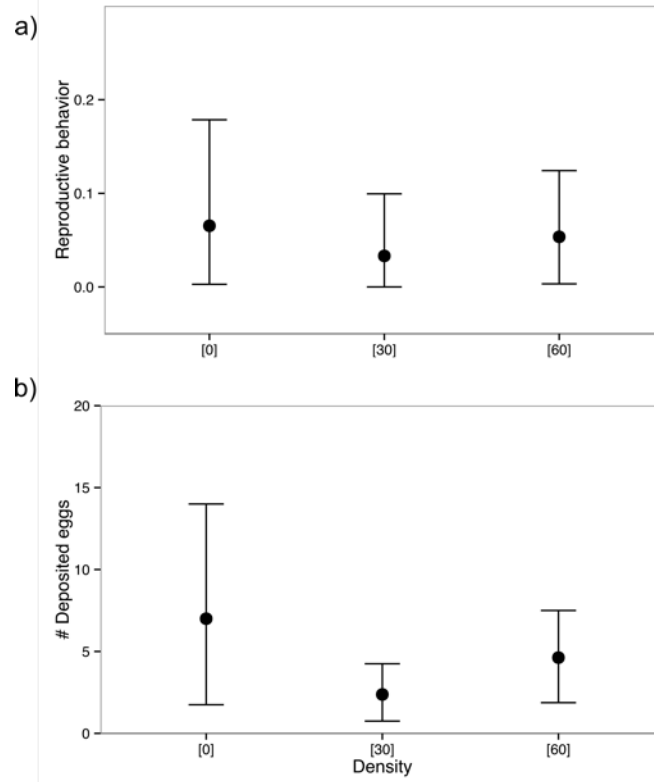


Figure V.1 Illustrates (a) mean \pm SE reproductive behaviors / min exhibited by territorial males and (b) mean \pm SE number of eggs deposited on spawning pads in mesocosms following exposure to 0, 30, or 60 *G. nobilis*.

GENERAL DISCUSSION

The overarching objective of this thesis was to evaluate the recovery plan of *Cyprinodon bovinus* and to examine how potential consequences of *ex situ* conservation may shape population distinctiveness and ultimately affect the ability of reintroduced captive animals to become established in their ancestral habitat. To provide an adequate foundation, I first conducted a thorough literature search over a 50-year period and examined the effects of the contemporary conservation of this endangered species. I then evaluated the potential consequences of *ex situ* conservation by examining genetic and phenotypic characteristics that may be divergent between the sole remaining wild population and a population maintained in captivity for the last ~ 40 years. Due to the past incidences of documented hybridization events with a non-native, I also examined the wild population for genetic and phenotypic evidence of introgression, which would bias the comparison of captive and wild characteristics. Following this evaluation, I coordinated and monitored a reintroduction attempt to evaluate whether captive animals exhibited any maladaptive behaviors upon release into their ancestral habitat. Finally, I used a series of field experiments to examine if the wild *C. bovinus* population showed any negative density-dependent effects from a putative endangered egg predator (*Gambusia nobilis*) in order to predict the long term impact they may be exerting on the persistence of this imperiled species of pupfish.

The execution of the recovery plan for *C. bovinus* appears to have been successful, as the removal of emergent vegetation has apparently assisted with increasing the estimated size of the wild population. However, by increasing habitat area it may

have also dispersed *C. bovinus* territories, effectively reducing their density, and in turn negatively affecting their overall reproductive success. This may be a result of females spending more time assessing an increased number of territorial males.

To assess the effectiveness of the current *C. bovinus* captive breeding program, I used a genome-wide approach to measure levels of neutral and adaptive genetic variation within, and subdivision between, contemporaneous samples of individuals from the captive and wild populations. Results revealed that despite relatively high levels of genetic diversity in the captive population, the reintroduction of thousands of captive animals failed to prevent a severe genetic bottleneck, which appears to have reduced the genetic diversity of the wild population and possibly contributed to the substantial genetic divergence between these two populations. In addition to the effects of genetic drift, I identified multiple outlier SNPs that showed signs of divergent selection, one of which matched to a predicted gene likely involved with osmoregulation. I also detected significant morphological divergence between the two populations, which may also be shaped by differences in local habitat conditions (e.g. salinity). Results illustrated that contemporary hybridization or historical introgression was not a principal mechanism for the documented population divergence between the captive and wild *C. bovinus* populations; this was evident by both *C. bovinus* populations showing an absence of morphological overlap with *C. variegatus*, high levels of genetic differentiation with *C. variegatus*, and the limited allocation of shared alternative alleles.

To evaluate if traits potentially selected for in captivity may no longer be adaptive when animals are released back into the wild, I monitored the reintroduction of captive *C. bovinus* into an extant habitat. I found insufficient evidence to indicate that captive *C.*

bovinus exhibited maladaptive behavior upon release into their ancestral habitat. Similar responses were additionally confirmed after controlling for the presence of density dependent behavior.

To evaluate the potential long-term effect that a putative egg predator (*G. nobilis*) may have on the sustainability of *C. bovinus*, I observed their behavior and fecundity under varying densities of *G. nobilis* in controlled field experiments. Based upon observed similarities in the number of deposited eggs and the frequency of exhibited reproductive behavior across *G. nobilis* densities, I found insufficient evidence to conclude that *G. nobilis*, a putative egg predator, exerted a distinct negative density-dependent effect on either the reproductive behavior or fecundity of *C. bovinus*.

To summarize, I will briefly outline the implications that the recovery plan, *ex situ* conservation, reintroduction attempt, and *G. nobilis* densities may have on the future sustainability of *C. bovinus*. While the recovery plan appears to have been successful, based upon an increase in the number of territorial males, it appears that additional releases of captive fish may need to occur alongside habitat expansions to offset a decrease in individual male reproductive success. This should assist with maintaining an effective ecological density and preserve levels of male reproductive success. For the evaluation of *ex situ* conservation, the documented evidence for such substantial levels of divergence between captive and wild populations hold serious implications with regard to the preservation of natural genetic and phenotypic species characteristics. Based upon the evidence for local selection and the asymmetrical distribution of genetic diversity between populations, I conclude that reciprocal genetic inoculation would greatly assist

in re-establishing genetic cohesion between these two populations. While the foundation for the documented phenotypic divergence is unknown, altering abiotic conditions in captivity (i.e. salinity) may help equalize body shape characteristics that were documented occurring between these two populations. Additionally, because I found no evidence for introgression with the non-native sheepshead minnow (*C. variegatus*) (based upon the evaluated morphological or genetic characteristics) it appears that the reason for the documented divergence between these two populations was not due to introgressive hybridization. Therefore, this represents a critical time period, where a translocation of wild individuals into captivity could transpire with minimal concerns of facilitating the inadvertent introduction of non-native, introgressed genetic material.

Taken together, the reason for the historically limited establishment ability of captive *C. bovinus* in their ancestral habitat appears not to have been due to maladaptive behavioral in the released captive fish. However, because of the evidence for local adaptation, future work is required to investigate the possibility that abiotic conditions may have led to the poor establishment and persistence of the historically released fish. In conclusion, I stress the importance of prioritizing the following goals to aid in the sustainability of this endangered species: (1) the reciprocal genetic inoculation between captive and wild populations to help reestablish population cohesion, (2) the continued monitoring of Diamond Y Draw for introduced non-native species and putative *Cyprinodon* hybrids, (3) continued habitat restoration and simultaneous release of captive fish to increase the amount of available breeding habitat and maintain high reproductive success, and (4) the thorough assessment of salinity tolerance between environments and

its effects on survivorship and development.

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APPENDICES

Appendix A

Table A1.01 Bivariate Shapiro-Wilk's Normality Test prior to conducting a Spearman's correlation between reproductive behavior and the number of territorial males.

	P-value	W
Reproduction	0.0003769	0.9306
# Males	0.0001096	0.9195

Table A2.01 Shapiro-Wilk's Normality Test prior to conducting Pearson's correlation between Log total size and Log Centroid Size.

	W	P-value
Wild	0.9654	0.0624
Captive	0.9774	0.2202
Male	0.9781	0.2202
Female	0.9823	0.4975

Table A2.02 Shapiro-Wilk's normality test for Log Centroid Size prior to conducting a two way ANOVA.

Log.CS	W	P-value
Wild	0.9671	0.0771
Captive	0.9774	0.2202
Male	0.9752	0.1474
Female	0.9879	0.7943

Table A2.03 Levene's Test for Homogeneity of variance (center = median) prior to conducting a two way ANOVA on Log Centroid Size.

	df	F-value	Pr(>F)
Population	1,136	11.829	0.09
Sex	1,136	1.2018	0.2749

Table A2.04 Type 2 sequential sum of squares ANOVA results for the effect of Population source and Sex on Log Centroid size when comparing the wild and captive *C. bovinus* populations.

	Df	Sum Sq	F-value	Pr(>F)
Population	1	0.01671	1.5550	0.2146
Sex	1	0.00383	0.3568	0.5512
Population*sex	1	0.00207	0.192	0.6617

Residuals	134
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Table A2.05 Within group sum of squares for multiple regression, pooled within subgroups (Population & Sex).

Total SS:	0.17483497
Predicted SS:	0.00826233
Residual SS:	0.16657264

Table A2.06 Classification / misclassification tables from cross-validated discriminant functions.

	DY	SNARRC	Total	%Correct
DY	60	6	66	90.9
SNARRC	9	63	72	87.5

Table A2.07 Bayescan results for the outlier analysis. Probabilities and F_{ST} values for the 8 SNPs that showed significant evidence of divergent selection.

SNP	Probability	log10(PO)	Q-value	Alpha	F_{ST}
1363	0.998	2.698	0.0020004	1.6896	0.19482
15156	0.9948	2.2816	0.0036007	1.6652	0.19138
11917	0.9916	2.072	0.005201	1.5392	0.1737
24187	0.9874	1.894	0.0070514	1.5408	0.17397
284	0.95359	1.3128	0.014923	1.4157	0.15988
17015	0.93639	1.1679	0.023038	1.4346	0.16425
18316	0.90578	0.98289	0.033207	1.302	0.14785
9507	0.88018	0.86603	0.044034	1.2885	0.14784

Table A2.08 Consensus read fragments for outlier SNPs.

snp	Read Sequence
284	AATTCTCCACTATACCTTTAATATCCAGTTTTTGATCTTTGGATGTTTTATTCAAGAAGTGTGTAACCTCTGATGTCTCTACCAGTTCTTATTGGT
1363	AATTCTAGTGTGATACTTTGAGAGGATGCCACCTGTGCAATACCTCCAGTTTTGAAATTCCTCACCCTAAATATATCTCTAAGGTATTATTGGT
9507	AATTCCTATTAGTTAAAAAACAATAATTACCATTGCTCACATTGTCATTGATTCTTTATAGTCTTTTCCATTTAGATTGTTAAT
11917	AATTCTTCTTCAGGTGAAAATTCAGTCTCATCTTCTCTGGACCATCTAATTGTATAGGAAACAGAAAGTAAGGTTAGTTTCTTGAGTTATTGCAA
15156	AATCCGTTATTAACAGCAGGCAGAACATTTCGTAATCTCTGCCTGTGAGAATATGAAACAGTTGGAAGGCTGGGAAATGCTACATTTTAAT
17015	AATTCTAACTTTATCCAGTAGAATGTAACACCATGACCCCTCCACCATTAGTTTCCCTTCTCATTATTTCTGTTTATAACCTACCACCTGTA

	AATTCATTGATCAGTTTATTTTCAGTAGCACCAATTAGCAACACGCATCATCTCAGGGAACTTTATAGAGAT
18316	AATTCAGCGCAAGATAGTCTAAC
	AATTCAGCGGAGACAGACAGTACTTAAAGGTTTTTATTGACACCAATGCAGAGCTGTGGATCTGCCGC
24187	AGCTCGAGGAGGGCAGGGCACTG

Table A2.09 BLAST results for outlier sequence 1363

SNP	Sequence	E-Value	Query Cover	Max Score	Iden	Accession
1363_A	AATTCATTGATCAGTTTATTTTCAGTAGCACCAATTAGCAACACGCATCATCTCAGGGAACTTTATAGAGAT	2.00E-08	78	68	80	XM_00580 5324.1
	ATGCCACCTGTGCAATACCTCCAGTT					
	TTGAAATTCCTCACCCTAAATATA					
	TCTCTAAGGTATTATTGGT					
1363_G	AATTCATTGATCAGTTTATTTTCAGTAGCACCAATTAGCAACACGCATCATCTCAGGGAACTTTATAGAGAT	2.00E-08	78	68	80	XM_00580 5324.1
	ATGCCACCTGTGCAATACCTCCAGTT					
	TTGAAATTCCTCACCCTAAATATA					
	TCTCTGAGGTATTATTGGT					

Table A2.10 Estimated contemporary effective population size (N_e) and confidence intervals calculated at various minor allele frequencies for both wild (DY) and captive (SNARRC) populations under random mating using the linkage disequilibrium method implemented in *NeEstimator*.

Population	$N_{E-0.05}$	$N_{E-0.02}$	$N_{E-0.01}$	N_{E-0+}
	5 % CI	200.9	164.0	164.0
Captive		221.5	175.3	175.3
	95 % CI	246.8	188.1	188.1
Wild	5 % CI	27.6	32.4	32.4
		28.0	32.9	32.9
	95 % CI	28.5	33.4	33.4

Table A2.11 Relative loading values reported from a Principle Component Analysis using all 2023 SNPs, with the top 16 alleles listed above a defined .00205 cutoff threshold. The five SNPs identified by *BayeScan* potentially under divergent selection are **Bolded**.

SNP	Var.contribution
24187.110	0.003196072
24187.130	0.003196072
11917.120	0.003196072
11917.130	0.00319607
13219.110	0.002467594
13219.130	0.002467594
5571.100	0.002429089

5571.120	0.002429000
1363.100	0.002402284
1363.120	0.002402280
284.110	0.002363082
284.130	0.002363082
17015.100	0.002314515
17015.120	0.002314515
5035.110	0.002066608
5035.130	0.002066608

Table A3.01 Structure Harvester Values. Optimal k (2) was identified by Evanno's method.

K	Reps	Mean LnP(K)	Stdv LnP(K)	Ln'(K)	[Ln''(K)]	Delta K
1	10	-25254.51	0.7047	NA	NA	NA
2	10	-338.72	0.2658	24915.79	24922.81	93753.9779
3	10	-345.74	113.9644	-7.02	6.02	0.052824
4	10	-358.78	107.3136	-13.04	8.9	0.082935
5	10	-362.92	150.0579	-4.14	NA	NA

Table A4.01 Test for multivariate normality for both wild and reintroduced populations for all four behavioral variables documented for the 'natural' condition.

	W	P-value
Wild	0.5648	0.00002113
Reintroduced	0.8399	0.04397

Table A5.01 Tests for normality (Shapiro-Wilk) and Sphericity (Mauchly's test) prior to running a repeated measures ANOVA.

	Shapiro-Wilk		Mauchly's	
	W	P-value	W	P-value
<i>Egg number</i>				
[0]	0.722	0.0145	0.9406	0.8321
[30]	0.837	0.07011	NA	NA
[60]	0.9017	0.299	NA	NA
<i>Total reproduction</i>				
[0]	0.5268	0.00002168	0.8647	0.646
[30]	0.4184	0.00000105	NA	NA
[60]	0.6547	0.000686	NA	NA

Table A5.02 Repeated-Measures ANOVA for the main effect of *Gambusia nobilis* density on *egg number* and *total reproduction*.

	DFn	Dfd	F	P-value
Eggs	2	14	1.345	0.2922
Total Reproduction	2	14	1.38	0.2836

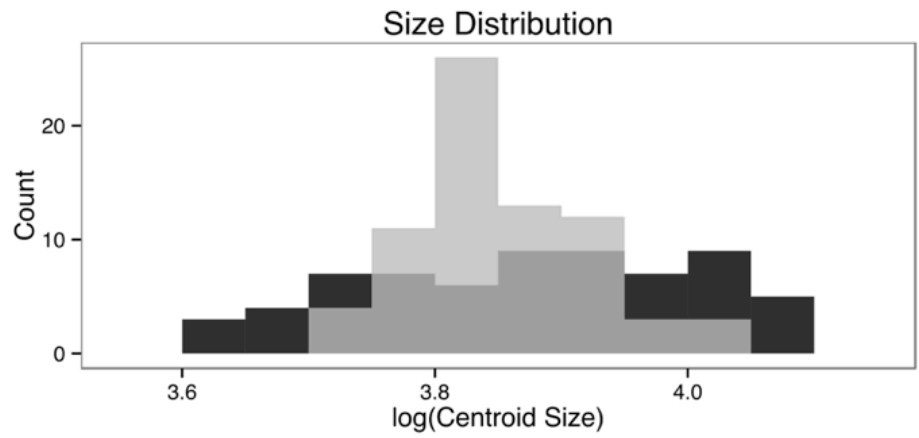


Figure A2.02 Histogram distribution of Log Centroid Size (mm) for the wild (black; DY) and captive (grey; SNARRC) *C. bovinus* populations.

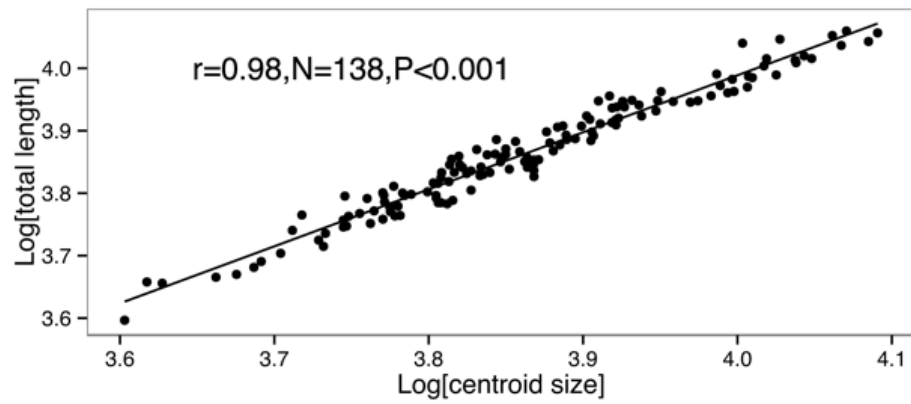


Figure A2.03 Pearson's correlation between Log Total length (mm) and Log Centroid Size (mm) for all *C. bovinus* samples (N= 138).

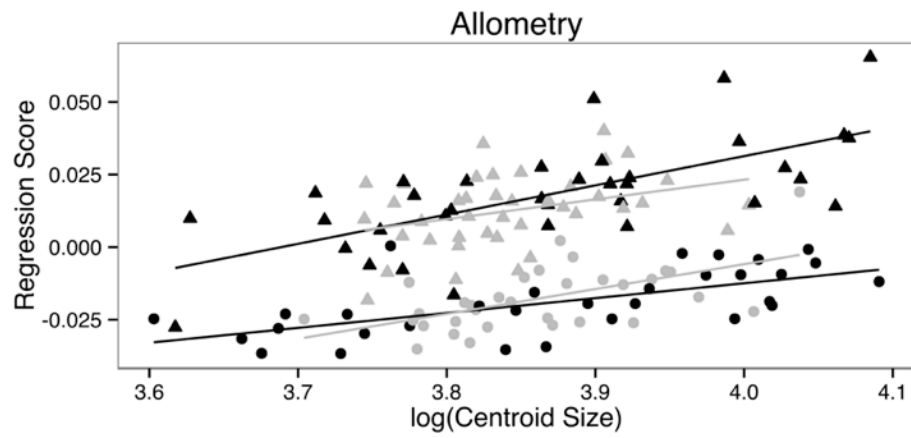


Figure A2.04 Allometric variation demonstrated by a regression of size (Log Centroid Size; mm) on shape (Procrustes Coordinates) for the wild (DY; black) and captive (SNARRC; grey) populations, with males (triangles) and females (circles) factored into the model.

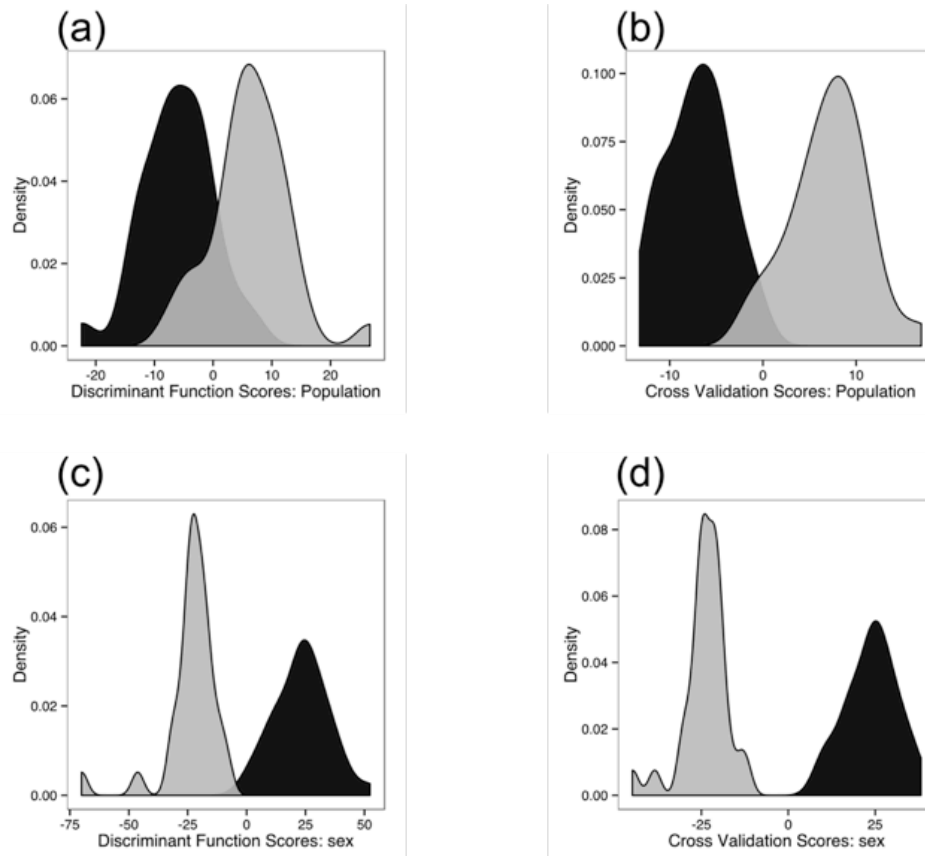


Figure A2.05 Density histogram of discriminant function and cross validation scores from Discrimination Function Analysis of population (a & b; DY = black, SNARRC = grey) and sex (c & d; male = black, female = grey).

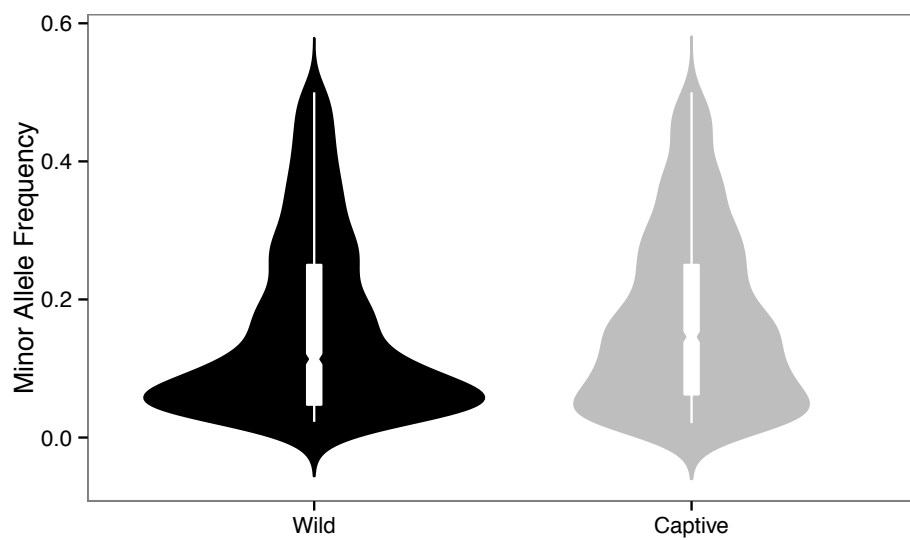


Figure A2.06 Untrimmed violin plot of 2 015 loci outlining the distribution of the Minor Allele Frequency for the wild (DY; black) and captive (SNARRC; grey) populations.

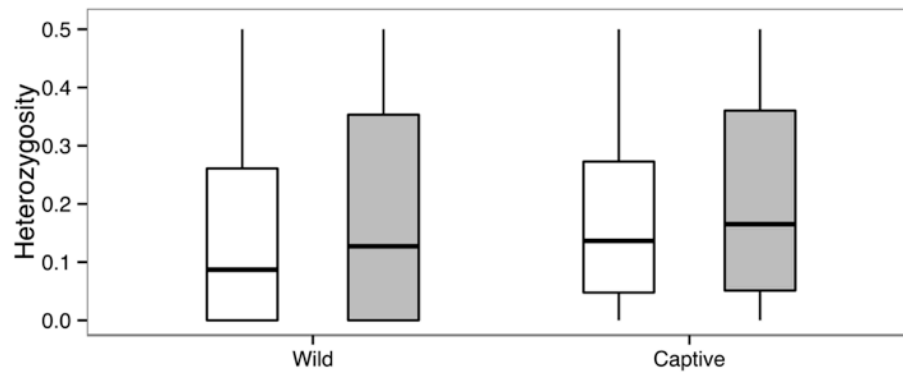


Figure A2.07 Boxplot of all SNPs, outlining the distribution of Observed (white) and Expected (grey) Heterozygosity for the wild (DY) and captive (SNARRC) populations. Boxplots represent the median with hinges representing the first and third quartiles. Whiskers represent $1.5 * \text{Interquartile range}$.

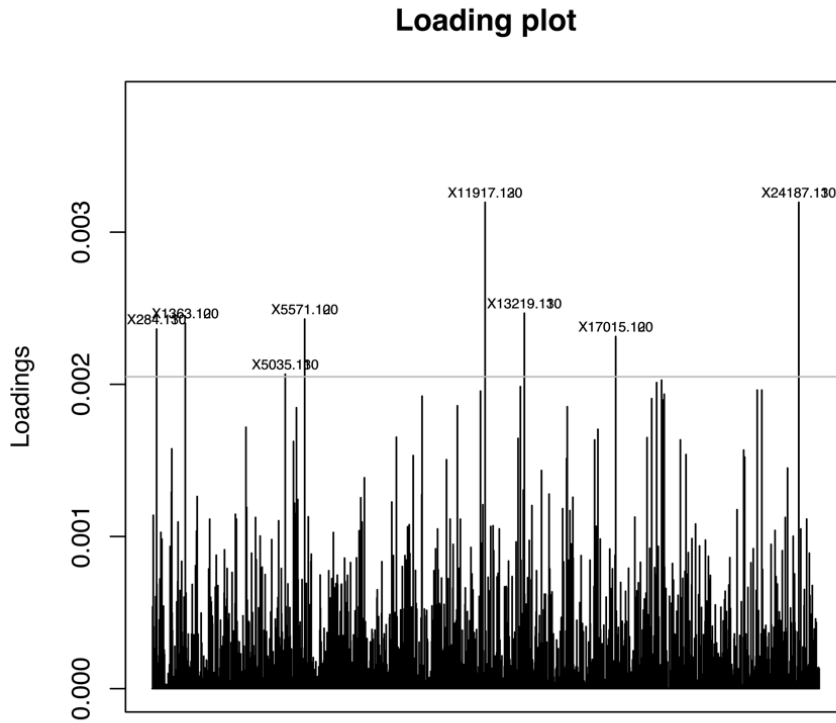


Figure A2.08 Loading plot generated from using all 2023 SNPs, with the horizontal line representing an arbitrary threshold value of .00205. Illustrates which SNPs contributed most to the individual principle component analysis.

a-score optimisation - spline interpolation

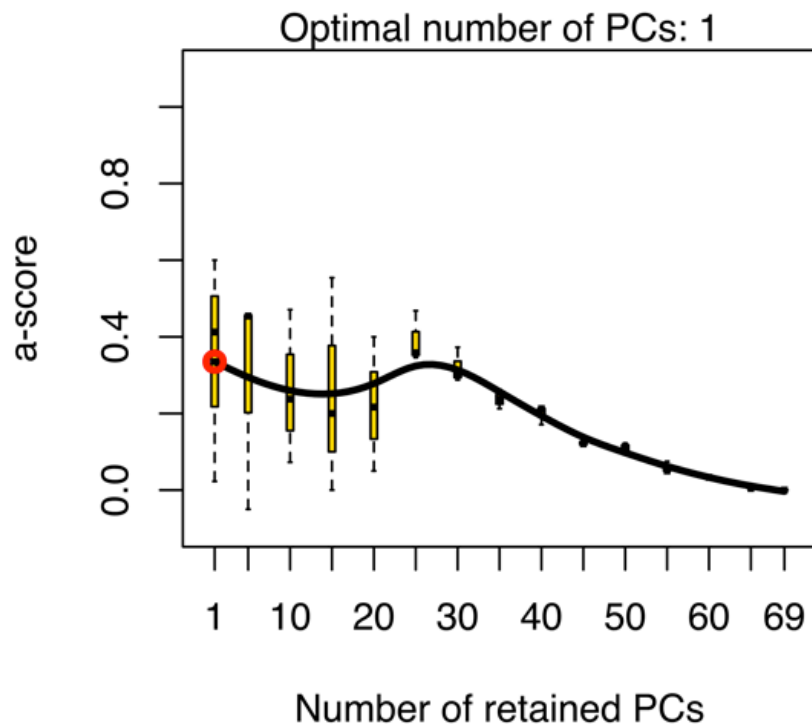


Figure A3.01 To avoid over fitting, the a-score function was implemented to select the optimal number of principle components for the Discriminant Analysis of Principle Components.

GLOSSARY:

- Adaptive Genetic Variation:** Refers to genetic variation that affects fitness. The variant can either be fixed (as it has been present in the population for a long time) or be polymorphic and rising in frequency.
- Allometry:** Changes in body shape variation that accompany or is caused by alteration in size.
- Allozyme electrophoresis:** Historically used dominant protein marker whose application is becoming rare.
- Ampure XP beads:** Binds to contaminants such as salts, primers, and dNTPs. A magnet is then used to remove the beads and bound contaminants to clean samples and keep amplicons.
- Ascertainment Bias:** Refers to discovering loci prior to genotyping individuals. This biases methods by yielding an unrepresentative selection of loci that does not accurately represent the spectrum of allele frequencies in a population(s).
- Bioanalyzer:** Used to assess the success of the size selection step and subsequent PCR amplification prior to pooling and sequencing.
- Bioinformatics:** Incorporation of computer programming into biological methodologies.
- Cartesian Coordinates:** A pair of numerical coordinates (x, y) for designating a specific location (e.g. landmark).
- Centroid Size:** The centroid size of a landmark configuration is defined as the square root of the sum of the squared distances of all landmarks. It effectively encompasses the “spread” of all landmarks around the centroid (center) of the configuration. Centroid size will have the same units as that which was originally measured (e.g. mm).
- Centroid:** The center of gravity, the x and y coordinates are averaged separately.
- Congeneric:** Belonging to the same genus.
- Conspecific:** Belonging to the same species.
- ddRAD-seq:** Genomic DNA is digested using two restriction enzymes, and the resulting fragments are ligated with flanking adapters. These fragments then go through a precise size selection step to select a certain size range of fragments (e.g. 300 bases). These selected fragments are then PCR amplified and sequenced. By comparing the same loci across individuals, SNPs can be identified.
- Demographic History:** The historical reconstruction of metrics such as fluctuation population size, sex ratios, growth rates etc.
- Demographic Stochasticity:** Refers to random processes within a population, which will generally be more pronounced with smaller populations. For example, the number of offspring surviving each season will be variable (across seasons) but can have a drastic, and unpredictable, effect on population size.
- Digitization:** Simply refers to digitally assigning landmarks to an image, may occur following image enhancement (e.g. Photoshop) to assist with identifying anatomical structures.
- Divergent Selection:** Different sources of selection favor different alleles in different populations, resulting in divergence in allele frequencies between populations.
- EcoRI and MspI:** Were the two restriction enzymes used in the study, a 6 and 3 bp cutter respectively.
- Effective population size (N_e):** Reflects the rate at which genetic diversity will be lost following genetic drift. The ideal population size that would have the same rate of change as the population under analysis.
- Environmental Stochasticity:** Similar to Demographic stochasticity, it refers to variability in birth and death rates, but is a direct consequence of extrinsic factors such as predation, weather, competition, or disease.
- Estimating effective population size:** For a single-sample estimator, the linkage disequilibrium (LD) method can be used. This is based on the principle that in small populations, genetic drift increases the likelihood of non-random associations to occur among alleles from different loci, since only few parents will contribute their alleles to the next generation.
- Evolutionary Potential:** Relates directly to the amount of genetic diversity within an individual/population, which can affect a species ability to adapt to environmental change. Populations exhibiting low diversity, or potential, have limited resilience and adaptive potential when faced with environmental change.

Exact Test: Deviations from HWE can indicate one of the paramount assumptions was not met or that there was genotype error. The exact test is based upon contingency tables and involves calculating the theoretical probability and statistically comparing it for deviation from observed.

Expected Heterozygosity: The frequency of heterozygotes that would be expected in the population is in HWE.

FastQC: Provides a quality control check of raw sequence data after you get the sequence data back. Will provide an overview of phred scores, GC content, etc.

Fixed markers: Loci that are diagnostic for a species or population because they are fixed for different alleles between the two populations (e.g. A vs C)

Fst: Fixation index is a standardized index that illustrates the amount of genetic variation occurring between populations relative to the total variation. $F_{ST} = (Het_{Total} - Het_{Sub}) / Het_{Total}$. A F_{ST} of 0.05 means an overall reduction in average heterozygosity is close to 5% of the total heterozygosity. Another way to say it is that 95% of genetic variation can be found within populations.

Gel electrophoresis: Can be used to ensure high molecular weight genomic DNA. Severely degraded DNA would show up a complete smear, with a lack of high molecular weight DNA.

Gene flow: The exchange of genetic material between two (or more) populations through migration (assisted or passive).

Genetic cohesion: This is not referring to the species concept, but relates to preserving species integrity by maintaining similar characteristics between populations, whether it be morphological or genetic in nature.

Genetic Differentiation: Populations differ in their allele frequencies

Genetic Divergence: A process in which two populations independently accumulate genetic changes through time.

Genetic Monitoring / Assessment: Refers to the quantification of temporal changes in a natural population. This usually entails using molecular techniques to evaluate population genetic metrics or to study demography. This differs from “genetic assessment”, which entails a single snapshot in time, but usually looks at similar parameters.

Genetic Stochasticity: This refers to changes in the genetic structure of populations, unrelated to systematic processes (selection, inbreeding, or migration). An example is genetic drift.

Genetic Variation: Broadly refers to variation at the genetic level. It can occur at multiple levels, such as at the chromosomal level, in genes, DNA, proteins, or in the functional differences of proteins.

Genetic/demographic Bottleneck: Refers to the loss of genes at the level of individuals within a population following a reduction in population size.

Genotype Frequency: The frequency at which a given locus exists (e.g. AG) in a population.

Heterospecific: Belonging to different species

Hybrid Swarm: A population of individuals that are all hybrids due to varying numbers of backcrosses with parental genotypes.

Hybridization: Interbreeding between individuals from different populations or species

Ichthyotoxins: Compounds that are toxic to fish

In silico: Sequenced Genomic data is screened for the identification of putative polymorphisms.

Inbreeding levels: Breeding or mating between related individuals. This can change genotype frequencies, leading to elevated levels of homozygotes than would be expected under HWE. The inbreeding coefficient (F) quantitates this measure, by representing the difference between expected and observed levels.

Introgression: Gene flow between populations whose individuals have hybridized

Introgressive Hybridization: Encompasses both hybridization and introgression, referring to the production of viable/ fertile offspring of mixed ancestry.

Landmark Based Geometric Morphometrics: In brief, this entails taking high resolution images of specimen, assigning landmarks (coordinates) to specific areas of the body, and using these landmark coordinates to compare variation in body shape across individuals / populations.

Linkage Disequilibrium: The non-random association of alleles at different loci within a given population.

Minor Allele Frequency: The frequency at which the least common allele (q) occurs within a single population.

Morphological Introgression: Refers to a differing proportion of morphological characteristics observed in an individual due to influence from either “parental” phenotype. Sharing a range of morphological characteristics from two different populations / species.

NanoDrop 1000 Spectrophotometer: Nucleic acids have a specific absorbance at 260 nm and proteins have one at 280 nm. Thus by assessing the relative ratio between these two absorbance’s, it can give an indication of the purity of the sample. A ~ 1.8 ratio would be considered “pure” DNA.

Neutral Genetic Variation: Refers to variation in populations that is governed by stochastic processes such as drift, recombination and migration.

Next Generation Sequencing (NGS) technology: A broad category that includes multiple sequencing platforms such as: Sanger, 454 pyrosequencing, Illumina Genome Analyzer, AB Solid, and HeliScope. My research trajectory employed Illumina (or Solexa), which involves using bridge amplified PCR to sequence adapter-flanked fragments up to several hundred base-pairs in length.

Observed Heterozygosity: The number of heterozygotes at a particular locus divided by the number of individuals in a population.

Ordination: In statistics, this entails taking a high dimension data set (large number of variables) and distilling it down to 2 - 3 dimensions.

Outbreeding depression: Reduction in fitness in hybrid individuals (from intra- or interspecific hybridization) relative to parental genotypes.

Outlier Analysis: To identify regions of the genome that are showing signs of positive or balancing selection, outlier tests can be employed to assess individual locus for deviation from a model of neutrality (which is determined primarily by drift and gene flow). Levels of differentiation at a given locus are compared to levels of differentiation across the genome to determine the presence of selection.

P2 and P1 adapters: Ligated to the flanking regions of fragments after digestion. Permits barcoding each fragment and amplifying it in the future.

Phenol-chloroform Extraction: A liquid-liquid extraction molecular technique used to separate proteins from nucleic acids based upon differences in acidity as well as density.

Phenotypic plasticity: The capability to develop into multiple alternative phenotypes under different environmental conditions.

Population Assignment Tests: Populations assignment tests employ a Bayesian analyses to estimate the number of clusters (populations; K) by maximizing HWE and linkage equilibrium.

Population Genetic Structure: Refers to the partitioning of genetic variation both between and within populations. This allows quantifying how genetic diversity contributes to the genetic architecture of populations or metapopulations.

Principle Component Analysis: A powerful tool that enables one to reduce the number dimensions in a massive multivariate dataset into a few synthetic variables (PCs). This uses allele frequencies to obtain a summary of the genetic variability among individuals and populations without using any group assignment.

Private Variant: The presence of a variant within a population, that is not found in other population(s), such as an allele / SNP.

Procrustes distance: The differences left between landmarks after superimposition can provide a measure of shape difference. The square root of the sum of squared distances between homologous landmarks between individuals. That is, the square root of the sum of the areas of the circles (between two configurations/means).

Procrustes Superimposition: A necessary step in the analysis of shape, involves removing non-shape variation that is associated with differences in size, position, and orientation of individual specimen. Individuals are scaled to a standard size, standard position, and standard orientation.

Proteinase K: Enzyme that digests protein and nucleases.

Qubit®2.0 Fluorometer: Used to calculate [DNA] by using a florescent dye that binds to the target and the degree of fluorescence is used as an index of concentration.

Reduced Representative Library: Refers to the reproducible sequencing of segments, to obtain a “sample” of sequences densely spread across the genome. Is very similar to obtaining a sample from a population to make estimates about various measures.

Refugium: Isolated body of water

- RNAlater:** An aqueous reagent that permeates tissue and protects cellular DNA and RNA, effectively preventing degradation prior to DNA extraction.
- Selection:** In general the effects of natural selection compete with the effects of drift, and are therefore dependent partly on the effective size of a population. In populations with small N_e , drift will out swamp any effect selection may be having and the opposite is true for large populations.
- Size selection:** To standardize the size of the fragments, a precise size selection step occurs prior to PCR. Otherwise, each library would have a substantial range of fragment sizes, which would reduce the amount of useable data following sequencing.
- SNPs:** Single Nucleotide Polymorphisms, as the name implies are single nucleotide change in a DNA sequence. These are bi-allelic co-dominant markers that can be found in either coding or non-coding regions of the genome.
- Stacks:** A pipeline used to align raw reads *de novo*, form Loci, call SNPs within these loci, and calculate multiple population genetic parameters.
- Sustainability:** Maintaining diversity and viability
- Sympatric:** Occur in the same geographic area.
- Thin-Plate-Spline:** An interpolation technique used to visualize shape variation. This is done by using transformation grids that fit perfectly at all landmarks in the analysis. Therefore, by using the consensus shape, you can visualize how a given group / mean shape differs by the direction that the grid is stretched. This will illustrate areas that differ more than others.
- Tricaine methanesulfonate:** Is a white powder commonly used for the sedation, anesthesia or euthanasia of fish.
- Variation in the Number of Tandem Repeats:** Microsatellites, also known as simple sequence repeats, are stretches of DNA that consist of tandem repeats of 1-6 base pairs. VNTRs are markers that are repeating areas of DNA, which is used to quantitate variation in the number of single repeats (SSR) or tandem repeats (STR), such as microsatellites.

VITAE

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Education

Lehigh University (2010 -2015)
PhD
Integrative Biology

MSU Denver (2004-2008)
BSc
Major: Biology
Minor: Chemistry

Professional Experience

Teaching Assistantships

- > BIOS 120 Biology Core III: Integrative and Comparative
(Spring 2013)
Experimental and historical approaches to the analysis of structural and functional properties in organisms. Use of scientific method to study species diversity. Introduction to the analysis of organismal attributes that explain behavioral repertoire and ecological relationships.
- > BIOS 277 Experimental Neuroscience Laboratory
(Fall 2010, 2013, 2014)
Structure and function of the mammalian brain with special attention to cellular morphology and organization. Standard, cutting edge techniques to determine how the shape and function of the nervous system regulates behavior. Experimental design, hypothesis testing, statistical analysis, reading and writing of scientific papers, basic histology and imaging.
- > BIOS 336 Animal Behavior Laboratory
(Spring 2012)
Emphasis on observing animals, performing experiments, collecting and analyzing data, and individual research.

Research Assistantships

- > Thorne Fellowship (Spring 2015)

- > Lab Manager (Spring 2010, 2011, Fall 2012)
- > BDSI Team Leader (Summer 2011)
- > Research Assistant MSCD (Fall 2008)

Research Interests

- > Conservation Biology, Ecology, Evolution, Conservation Genomics, Animal Behavior.

*I am interested in evolutionary ecology, with research experience in both freshwater and marine systems. I have used field-based approaches to independently examine genetic and phenotypic divergence in an endangered species of pupfish (*Cyprinodon bovinus*). My research utilizes bioinformatics coupled with Next-Generation Sequencing (ddRAD), landmark-based geometric morphometrics and ecological assays to critically examine how stochastic processes and divergent selection may contribute to the population discreteness of an endangered desert spring pupfish. In addition to this multidisciplinary research, I have also focused on habitat restoration, biological invasions, sexually selected traits, mate choice and game theory in a diverse range of teleost species.*

Publications

- > Black A, Weimann S, Imhoff V, Richter M, Itzkowitz M (2014) A differential prey response to invasive lionfish, *Pterois volitans*: Prey naiveté and risk-sensitive courtship. *Journal of Experimental Marine Biology and Ecology* 460, 1-7.
- > Black A, Imhoff V, Leese J, Weimann S, Gumm J, Richter M, Itzkowitz M (2013) Attack Intensity by two species of territorial damselfish (Pomacentridae) as estimates of competitive overlap with two species of wrasse (Labridae)? *Journal of Ethology* 32, 63-68.
- > Black A, Draud M, Richter M, Itzkowitz M (2014) Are conspecific and heterospecific opponents assessed similarly? A test in two species of territorial damselfish (Pomacentridae). *Behavioural Processes* 106, 107-110

In Review:

- > Black A, Hollenbeck C, Samollow P (2015) Genetic and phenotypic divergence between captive and wild populations of the endangered Leon Springs pupfish (*Cyprinodon bovinus*). *Molecular Ecology*
- > Black A, Snekser J, Al-Shaer L, Paciorek T, Bloch A, Little K, Itzkowitz M (2015) An assessment of a multi-decade conservation effort: the past, present, and future of the endangered Leon Springs pupfish (*Cyprinodon bovinus*). *Aquatic Conservation*

- > Black A, Itzkowitz M (2015) Implications for conservation breeding: The reintroduction of the endangered Leon Spring pupfish. *Animal Conservation*
- > Black A, Paciorek T, Itzkowitz M (2015) Density dependent effects of a putative egg predator on the behavior and fecundity of an endangered species of pupfish. *Southwestern Naturalist*

In Preparation:

- > Black A, Samollow P (2015) Examination of introgression as a cause for divergence between an endangered captive and wild population of the Leon Springs pupfish (*Cyprinodon bovinus*)
Target Journal: *Biological Conservation*
- > Weimann S, Black A, Leese J, Burger R, Richter M, Itzkowitz M (2015) Sound Production In Sympatric Damselishes: Analysis Of Signal Properties And Vocalization Behavior In Two Closely Related Species. Target Journal: To Be Decided

Academic Presentations

- > Andrew Black (2013) “An integrative assessment of the Ex Situ conservation and reintroduction of a critically endangered species (*Cyprinodon bovinus*): Investigation of genetic, morphological and behavioral variance”. Graduate student symposium. Lehigh University, Bethlehem, PA.
- > Andrew Black (2012) “An integrative assessment of the Ex Situ conservation and reintroduction of a critically endangered species (*Cyprinodon bovinus*): Investigation of genetic, morphological and behavioral variance”. Graduate student symposium. Lehigh University, Bethlehem, PA.
- > Andrew Black (2012) An examination of prey naiveté in response to the Indo-Pacific lionfish [*Pterois volitans*]. Lehigh Valley Evolution and Ecology Symposium, Allentown, PA.
- > Andrew Black (2011) An examination of prey naiveté in response to the Indo-Pacific lionfish [*Pterois volitans*]. Lehigh Graduate Student Presentation, Bethlehem, PA
- > Black A, Draud M, Itzkowitz M (2010) “Intra and interspecific attack assessment in two closely related Caribbean damselfish”. Lehigh Valley Evolution and Ecology Symposium, Allentown, PA.
- > Black A, Draud M, Itzkowitz M (2009). “Intra and interspecific attack assessment in two closely related Caribbean damselfish”. Graduate student symposium. Lehigh University, Bethlehem, PA.

Work History

2004-2010

NMFS Fishery Biologist

- > Observation and evaluation of commercial fishing regulations and by-catch. Involved otolith extraction, species identification, documentation of travel coordinates and assessment of crew safety and fishing practices.

Tsar nicoulai: Sturgeon aquaculture

- > Water quality readings, microchip injections, overall system maintenance, caviar sampling, sorting and census, sturgeon fry nursery establishment, shipping and monitoring of live fish, sex biopsy of fish, tank establishment and maintenance.

Volunteer Experience:

Denver Aquarium: Feeding and tank maintenance

Denver Water: Water sampling and analysis

Computational Program Skills:

- > R, Linux, GenePop, Arlequin, Structure, BayeScan, MorphoJ, tpsdig, tpsrsw, Univariate & Multivariate statistics, FastQC, Stacks, Vcftools, Plink, PgdSpider

Laboratory Skills:

- > ddRAD-seq library preparation, PCR, Landmark-based geometric morphometrics, Phenol Chloroform DNA extraction, Electrophysiology equipment, Specimen dissection, Species ID, NanoDrop, Qubit, Ampure XP

Miscellaneous Skills:

- > Habitat Restoration, Experience working with Endangered Species, Water quality experience, Diesel engine maintenance, Open Water Scuba Certified, Small boat handling (<58ft), Species identification, handling, and trapping, Navigational proficiency (GPS, LOREN, Charts)

References:

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