5-1-2011

Divergence in the ecology of two species of Gambusia in secondary contact

Daniella M. Swenton

Daniella Marilyn Swenton
Candidate

Biology
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

, Chairperson

DIVERGENCE IN THE ECOLOGY OF TWO SPECIES OF *GAMBUSIA* IN SECONDARY CONTACT

BY

Daniella M. Swenton

B.S., Environmental Science, University of Vermont, 2003

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

May, 2011

Dedication

This dissertation is dedicated to my loved ones. My dad adores exploring nature and inspired that passion in me. Deb is a well of unconditional love & support. My sister, Deborah, & Hank, Bean, Jas, & Mer are the calm anchor to my whipping kite. My mom always encourages me to reach higher. My brothers keep me on my toes and in smiles. Erin, Cyndi, Cara Lea, Sarah, Tansey, Lis, Yadeeh, Maria, Brit, 'Rie, Apple, Fred, Andee, & Shannon are my spiritual and girl-power strength. And finally to Travis, for your one shine.

Acknowledgements

I would like to thank my dissertation committee, Astrid Kodric-Brown, Tom Turner, Blair Wolf, & Joel Trexler. I would also like to acknowledge the support from members of the UNM Biology Department including my friends and colleagues in the Kodric-Brown, Turner, & Wolf labs, especially Lisa Schwanz, Pam Keil, Tom Kennedy, Rick Winslow, and Robin Warne. This dissertation would not have been possible without the support of the stickleback crew, especially John Baker & Susan Foster. Finally, a very special thanks to my dear friend and colleague, Ric Charnov, to whom I owe my fascination with life history theory.

DIVERGENCE IN THE ECOLOGY OF TWO SPECIES OF *GAMBUSIA* IN SECONDARY CONTACT

BY

DANIELLA M. SWENTON

ABSTRACT OF DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

May, 2011

DIVERGENCE IN THE ECOLOGY OF TWO SPECIES OF *GAMBUSIA* IN SECONDARY CONTACT

BY

DANIELLA M. SWENTON

B.S., Environmental Science, University of Vermont, 2003
Ph.D., Biology, The University of New Mexico, 2011

ABSTRACT

Gambusia nobilis, a federally endangered species, and *G. affinis* (Poeciliidae) are small, livebearing fishes found in the southwestern U.S. The invasive *G. affinis* has been introduced globally to control mosquito populations. It is found in some populations of *G. nobilis* on Bitter Lake National Wildlife Refuge (BLNWR), the field site for this study. It is unclear to what extent the two species have diverged in behavior and ecology and how extensively they have hybridized on BLNWR, thereby threatening the endangered *G. nobilis* via genetic introgression. In this study I examined divergence in behavior and ecology between two species with four main objectives: (1) To determine if there is assortative mating between the two species; (2) To determine if there is divergence in life history characteristics between the two species; (3) To assess habitat and dietary differences between extant populations of the two species; and (4) To determine the degree to which the two species have genetically introgressed on BLNWR. I found these two fishes are markedly different in behavior and ecology. Males and females of both species show assortative preference in visual/olfactory tests. This

assortative preference pattern was held during copulation, probably as a function of female choice. Data from field caught and lab breeding individuals show differences in key life history traits that reflect the trade-offs in their current environments. To assess ecological divergence I characterized the habitats of the two species. *G. affinis* persists in ephemeral environments and *G. nobilis* is restricted to spring-fed, stenohaline and stenothermal habitats. The two species also show differences in ecological niche as *G. nobilis* appears to feed at a higher trophic level. Finally, I characterized genetic patterns of hybridization. I found low genetic diversity for G. *nobilis,* probably a result of range contraction. Gene flow and rates of hybridization are low providing further evidence for divergence and reproductive isolation between these two species. The results presented here include characterization of the habitat requirements, heterospecific interactions, and population genetics of these two species on BLNWR and may be helpful to management of a sensitive species such as *G. nobilis*.

TABLE OF CONTENTS

List of Figures ..... **xii**

List of Tables ..... **xv**

Chapter 1: Sex Differences in Mate Preference between Two Hybridizing Species of Poeciliid Fish **1**

Abstract 1

Introduction 1

Methods 5

Fish collection and maintenance. 5

Trial methods. 5

Visual/Olfactory tests. 6

Open mating tests. 7

Data analysis. 8

Results 9

Visual/Olfactory tests. 9

Open mating tests. 10

Discussion 12

Acknowledgements 14

References 16

Tables and Figures 22

Chapter 2: Habitat and Life History Differences between Two Species of *Gambusia* **26**

Abstract 26

Introduction 27

Methods 30

Field study. 30

Laboratory breeding. 31

Dissections of fish from museum collections. 32

Data analysis. 33

Results 34

Habitat characteristics. 34

Demographic patterns during the breeding season. 34

Maternal investment. 35

Discussion 36

Acknowledgements 41

References 43

Tables and Figures 48

Chapter 3: The Use of $\delta^{15}N$ and $\delta^{13}C$ to Assess Feeding Niche Space of Fishes in a Desert Spring System 56

Abstract 56

Introduction 57

Methods 60

Study site. 60

Sample collection. 61

Stable isotope analysis. 62

Data analysis. 63

Results 64
Habitats. 64
Primary producers. 64
Fishes. 65
Community level patterns. 67
Discussion 68
Habitat variation in water quality and primary producers. 70
Variation in feeding niches of fishes as revealed by stable isotope analyses. 72
Acknowledgements 76
Literature Cited 77
Tables, Appendices, and Figures 84
Chapter 4: Genetic Characterization of the Endangered *Gambusia nobilis*, the Invasive *G. affinis* and Their Hybrid Zone on Bitter Lake National Wildlife Refuge 103
Abstract 103
Introduction 104
Methods 108
Study site and fish collections. 108
Molecular methods. 110
Analysis of genetic variation and structure. 111
Population assignments. 112
Results 113
Patterns of genetic diversity & variation. 113

Genetic population differentiation. 114

Species assignment. 116

Discussion 117

Acknowledgements 123

Literature Cited 124

Tables, Appendices, and Figures 130

List of Figures

Chapter 1:

Figure 1. Association time in seconds with standard error bars during visual/olfactory preference tests with females of both species for Gambusia affinis males and G. nobilis males 23

Figure 2. Association time in seconds with standard error bars during visual/olfactory preference tests with males of both species for Gambusia affinis females and G. nobilis females 24

Figure 3. Copulation Success Rate open in mating trials with male-male competition for all female x male interactions (G.a. = G. affinis individual, G.n. = G. nobilis individual 25

Chapter 2:

Figure 1. Schematic diagram depicting the evolution of an optimal life history strategy. Environmental characteristics are considered in shaping individual life history phenotypes on which natural selection acts. 50

Figure 2. Abiotic habitat characteristics for both species during 2008..... 51

Figure 3. (a) Standard length (mm) distribution of females over the breeding season from museum specimens of Gambusia affinis. (b) Standard length distribution of females over the breeding season from museum specimens of G. nobilis. 52

Figure 4. Plot of log female somatic mass (g) by log average embryo mass in her brood (g). 53

Figure 5. (a) Box plot of brood size from each brood from Gambusia affinis females over the breeding season. (b) Box plot of brood size from each brood from Gambusia nobilis females over the breeding season..... 54

Figure 6. An individual's life history by species. 55

Chapter 3:

Figure 1. Sampling localities on Bitter Lake National Wildlife Refuge..... 97

Figure 2. Measurements of habitat variables taken in 2008 across field sampling localities at Bitter Lake NWR in the months of May, June and July: (a) dissolved oxygen (mg/L), (b) salinity (ppt), (c) temperature (°C), and (d) conductivity (S/cm). 98

Figure 3. Adjusted $\delta^{15}N$ values for Cyprinodon pecosensis across sampling localities.. 99

Figure 4. (A) Mean $\delta^{15}N_{base}$ values in ‰ AIR for each fish species surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge with standard error bars. 100

Figure 5. $\delta^{13}C$ and $\delta^{15}N$ biplot showing mean stable isotope values of all fish (diamonds), macroinvertebrate (squares) and plant (triangles) species in all sinkhole sampling localities surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge for (a) sinkholes with only C. pecosensis, (b) SH27S with only C. pecosensis and G. nobilis, and (c) sinkholes with three or more species.. 101

Figure 6. $\delta^{13}C$ and $\delta^{15}N$ biplot showing mean stable isotope values of all fish (diamonds), macroinvertebrate (squares) and plant (triangles) species in all sinkhole sampling localities surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge for (a) sinkholes with three or more fish species present, (b) stream habitats with three or more species present. 102

Chapter 4:

Figure 1. Map of populations sampled on Bitter Lake National Wildlife Refuge..... 140

Figure 2. Plot scores of PCI and PCII from the principle components analysis of the multilocus, microsatellite genotype for the 16 populations of G. affinis, G. nobilis and putative hybrid zones on Bitter Lake National Wildlife Refuge..... 141

List of Tables

Chapter 1:

Table 1. Summary of preference times in seconds and male behavior (Lead chase time (in seconds), copulation attempts and Copulation Success Rate) across all trials by experiment type. 22

Chapter 2:

Table 1. Fish community assembly data in habitats of Gambusia nobilis and G. affinis during the 2008 breeding season. 48

Table 2. Percentage of males, females and juveniles in populations of *Gambusia nobilis* and *G. affinis* in allopatric habitats over the 2008 breeding season at Bitter Lake National Wildlife Refuge. 49

Table 3. Brood characteristics for *G. affinis* and *G. nobilis* from dissected museum specimens. 49

Chapter 3:

Table 1. Fish assemblages of the 12 sampling localities at BLNWR in 2008 with six most abundant species. 84

Table 2. Summary of sampled habitat characteristics. 85

Table 3. Results of ANOVA on variation in water quality measurements on Bitter Lake National Wildlife Refuge in 2008. 85

Table 4. Results of stable isotope analysis on chara and algae in all sampling localities at Bitter Lake National Wildlife Refuge in 2008. 86

Table 5. Variation in isotope values over time for algae and chara. 87

Table 6. Variation in isotopic values over time for each species collected. 88

Table 7. Euclidean distances (ED) of *C. pecosensis* centroid of $\delta^{13}C$ x $\delta^{15}N$ by site from the $\delta^{13}C$ x $\delta^{15}N$ centroid of each site for all taxa, the $\delta^{13}C$ x $\delta^{15}N$ centroid of *C. pecosensis* across all sampling localities, and the $\delta^{13}C$ x $\delta^{15}N$ centroid of all taxa across all sampling localities. 89

Chapter 4:

Table 1. F_{IS} values calculated from six microsatellites of each population on Bitter Lake National Wildlife Refuge. 130

Table 2. Number of alleles and allelic richness of the six microsatellite loci used in this study by pooled groups of individuals. 131

Table 3. Summary of the analysis of molecular variance (AMOVA) within and among populations of *Gambusia* and by species at Bitter Lake National Wildlife Refuge. 132

Table 4. Pairwise F_{ST} values (below diagonal, calculated from microsatellite data) among populations on Bitter Lake National Wildlife Refuge. 133

Table 5. Summary of the analysis of molecular variance (AMOVA) among populations and among and within individuals of *Gambusia* on Bitter Lake National Wildlife Refuge. 134

Table 6. Pairwise F_{ST} values (below diagonal, calculated from microsatellite data) among pooled populations and type on Bitter Lake National Wildlife Refuge..... 135

Table 7. Proportion of membership of each population in each of the 2 clusters from STRUCTURE analysis 136

Table 8. Summary of species assignment of individuals by population using STRUCTURE analysis. 137

Chapter 1:

Sex Differences in Mate Preference between Two Hybridizing Species of Poeciliid Fish

Abstract

When hybridization between two species in secondary contact is costly, natural selection should favor premating isolation barriers. The invasive *Gambusia affinis* has been introduced to habitats of a closely related species, the endangered *G. nobilis*. Although other *Gambusia* species readily hybridize in secondary contact, previous studies in this system found low abundance of hybrids in sympatry. To examine if hybridization is limited by behavioral premating isolation that may have evolved in allopatry, I examined each species' mating preferences using individuals from allopatric populations in male and female visual/olfactory association preference tests as well as open mating tests with and without male-male competition. *Gambusia affinis* and *G. nobilis* males had significant association preference for conspecific females in visual/olfactory tests. Only *G. nobilis* females had statistically significant preference for conspecific males. In open mating tests males of both species had lower chase times overall when in competition but there was no difference in number of copulation attempts. Males of both species had higher copulatory success rates with conspecific females when in competition, suggesting females may exert some control over copulation success of males. These results suggest that there are differences in mating preferences between these species. This mate choice may act as a premating isolating barrier to reduce hybridization in sympatry, a proposed threat to the endangered *G. nobilis*.

Introduction

In allopatric speciation, geographic barriers isolate two populations. Over time traits may change in response to local environment conditions to the point at which the two

populations are considered different species (Dobzhansky 1937; Mayr 1963; Servedio 2001). A by-product of this allopatric ecological divergence may be the evolution of traits that lead to changes in mate preference (Rundle & Nosil 2005). These novel traits may provide sufficient reproductive isolation in secondary contact to maintain unique species' identities (Rundle 2002; Geyer & Palumbi 2003; Schluter 2003) or fail to do so and lead to unchecked hybridization of the two species (Arnold & Hodges 1995; Rosenfield et al. 2004). Reproductive isolation barriers can be premating or postmating in nature. With premating isolation, matings do not occur because of ecological or behavioral/morphological barriers. In many species, premating barriers are stronger than postmating barriers. This may occur for many reasons including strong sexual selection leading to rapid allopatric divergence of traits associated with mating or natural selection in sympatry to avoid potentially costly hybridization (Dobzhansky 1940; Miyatake & Shimizu 1999; Hurt *et al*. 2005).

Sexual Isolation (SI) may be a mechanism of reproductive isolation (Coyne & Orr 2004). SI, also called behavioral isolation, predicts that reproductive isolation is driven primarily by differences in traits related to sexual behavior including mating signals and preferences. It is usually thought to be indirectly selected in allopatry but can evolve rapidly in strongly sexually selected species (Fisher 1930; Andersson 1994). A by-product of divergence in allopatry may be changes in mate preference or other characteristics upon which sexual selection can act (Muller 1942; Nosil et al. 2007). Traits important in vertebrate species recognition as well as intraspecific mate preference quality include coloration (fishes: Seehausen & van Alphen 1998; Strecker & Kodric-Brown 1999, birds: Andersson 1994), secondary sexual ornaments (fishes: Basolo 1990, reptiles: Schwartz & Henderson 1985, birds: Young et al. 1994), chemical cues (fishes: McLennan & Ryan 1997, salamanders:

Arnold & Houck 1982, mammals: Laukaitis 1997), vocal calls (amphibians: Wells 1977; Gerhardt & Doherty 1988, reptiles: Stamps 1977, birds: Irwin & Price 1999), courtship displays (fishes: Shaw et al. 2007, salamanders: Halliday 1990, reptiles: Stamps & Barlow 1973) and size (fishes: McKinnon et al. 2004, reptiles: Tokarz 1995). These traits are rarely mutually exclusive. Individuals may use several signals to discriminate between conspecifics and heterospecifics (Ptacek 2000). I investigated the degree of premating isolation via SI in a model system of fishes. The two species under study hybridize at low levels in sympatry suggesting that speciation may not be complete but that a certain degree of premating isolation may have evolved, possibly in allopatry.

Gambusia nobilis and *G. affinis* (Poeciliidae) are small, short-lived livebearing fishes found in the southwestern U.S. that speciated in allopatry (Hubbs 2001; Echelle & Echelle 1980). *Gambusia affinis* is native to warmer American waters but has been introduced globally to control mosquito populations. It has invaded three of four remaining populations of *G. nobilis* and threatens persistence of this endangered species via competition and hybridization (Courtenay Jr. & Meffe 1989; Echelle & Echelle 1980). *Gambusia* males are typically smaller than females and have a modified anal fin, gonopodium, for spermatophore transfer. *Gambusia* males often do not court females and use force copulation instead (Constanz 1989). In order to fertilize a female, a male will chase, jockey underneath her, swing his gonopodium forward, and transfer spermatophores into or near her genital pore. With no paternal care males will attempt to maximize copulations by mating with many females or maximize paternity certainty by mating multiple times with highly fecund females (Plath *et al*. 2007). Due to the nature of the force copulation system, female strategy may by

cryptic in nature (Bisazza *et al.* 2001). Each live brood is a costly investment for females thus selection should favor those that exert control over paternity (Arnqvist & Rowe 2005).

At Bitter Lake National Wildlife Refuge (BLNWR) outside of Roswell, New Mexico *G. affinis* and *G. nobilis* are allopatric throughout as well as sympatric in some sites. The first documentation of co-occurrence at BLNWR was made in surveys after 1938 (Koster personal journals, unpublished; BLNWR survey records, G. Warrick, pers. comm.). Although *G. affinis* readily hybridize with many other *Gambusia* species (e.g. Meffe & Snelson Jr. 1989; Scribner & Avise 1994) they are known to hybridize with *G. nobilis* at a relatively low frequency (<10%) in one area of sympatry, Blue Spring near Carlsbad, NM (Echelle & Echelle 1980). The low occurrence of hybridization suggests reproductive isolation. There is evidence for strong postzygotic barriers between these two species. The hybrid and backcross offspring are often malformed, with abnormally large, deep-bodied males as well as delayed maturation or androgynization and possible sterilization of individuals (Hubbs 1959; Echelle & Echelle 1980; Swenton & Kodric-Brown *in prep*). In this study I determined if 1) in addition to postzygotic barriers, there were also prezygotic barriers and 2) if the premating isolation via SI between these two species evolved in allopatry.

In this study, I investigated the role of SI in premating isolation between the two species from allopatric populations. If sexual selection for traits that evolved in allopatry is the mechanism underlying species isolation, then I expect assortative mating (i.e. preferential mating with conspecifics over heterospecifics) based on secondary sexual traits, morphology and behavior. I tested the strength of assortative preference in two experiments: (1) male association preference in visual/olfactory laboratory tests and (2) female association

preference in the same manner. I also tested the strength of assortative mating in an open mating experiment with and without male competition to determine if the visual/olfactory trials accurately predicted mating behavior. By examining female and male preference during actual breeding we may be able to tease apart subtle female preference (Parker 1983; Servedio 2007).

Methods

Fish collection and maintenance. *Gambusia nobilis* and *G. affinis* were collected from allopatric populations in 2006-2008 from BLNWR (N 35° 02.023 W 106° 56.474). *Gambusia nobilis* were collected from Sinkhole 37. *Gambusia affinis* were collected in a ditch in unit 16 near the southern end of the refuge. Fish were maintained in 38L or 76L aquaria with undergravel-filtration. Species and sexes were kept separate. Water was maintained at 8 ppt salinity using CoraLife$_{TM}$ (IL, USA) Marine Salt. Aquaria were kept under natural light as well as full-spectrum UV lamps that were set to the natural light cycle. Fish were fed once per day with a 70:30 mixture of Tetramin (Tetra, Blacksburg, VA, USA) flake food and Freeze-Dried (UT, USA) brine shrimp and were provided with as much food as they would consume in 5 minutes. Experiments were conducted in accordance with UNM IACUC Protocol number 04MCC006. *Gambusia nobilis*, a federally endangered species, were acquired, maintained and tested in accordance with NM state permit 2968 and federal permit TE676811-0.

Trial methods. All trials were conducted in 76L aquaria under full-spectrum UV lighting or natural lighting in a greenhouse in March-October of 2006, 2008 and 2009 between 10:00-16:00 hours. Each trial consisted of four tests in the following order: (1) male visual/olfactory preference test (n = 40), (2) female visual/olfactory test (n = 35), (3) single

male open mating test (n = 10), and (4) male-male competition test (n = 23). For each trial I used four individuals, a male and female of each species. Males and females were reused across tests within but not across trials. A subset of fish from the visual/olfactory tests were used in the open mating tests so sample sizes vary between tests. Males were size matched within 3mm of one another and females were size matched with 5mm of one another. Fishes were size matched to avoid the confounding factor of female size (e.g. fecundity) in male choice and males were size matched to avoid any confounding competitive effects in open mating trials (e.g. faster swimming due to larger size).

Males ranged in size from 16.3 mm to 26.88 mm (X= 21.45 $\pm$ 0.37, n = 80) and females from 21.00 mm to 38.96 mm (X= 30.73 $\pm$ 0.59, n = 80). Between each trial I changed tank water. All females used in trials were either virgins or 2nd year females. Second year females had not been in contact with males for at least three months. Female species within trials were matched in age.

Visual/Olfactory tests. A binary preference experimental setup was used for visual/olfactory association preference tests. The test aquarium was covered on three sides and divided into three sections via two perforated, clear partitions to allow visual/olfactory communication. The same setup was used to measure male and female preference. In each test order of presentation was randomized.

In male preference tests I placed one female of each species in opposite side partitions. Side placement was randomized throughout all trials. After one hour, a male of each species was placed one at a time, in the center partition and allowed to investigate females. Each test lasted 10 minutes. Male preference was determined as number of seconds spent within 5cm of each female's partition. Female association preference tests were

conducted at least two hours after females were introduced to the tank and were identical in method to male preference tests, except sexes were reversed in the experimental setup. Side placement and introduction order was randomized.

Open mating tests. Open mating trials were conducted to determine if visual/olfactory trials were an accurate representation of actual mate preference as well as to quantify mating behaviors. The same sets of males and females were used from visual/olfactory tests. This experiment consisted of two parts: a single male test and a male-male competition test in order to distinguish between effects of male and female preference and male-male competition. In single male tests, conducted in only 2009, males were placed singly in the tank and acclimatized for 30 minutes before the introduction of a female singly so there was one male and one female in a tank at a time. Each male was tested for 10 minutes once he began following the female. After the male was tested with a female of one species she was removed and the female of the other species was introduced for the second half of the test. The order of tests was randomized.

In male-male competition test males were placed together in the aquarium and acclimatized for an hour. Females, in random order, were introduced to the aquarium. The 10 minute test time started once a male began following the female. After the 10 minutes the first female was removed from the tank. The second female was introduced and tested in the same manner. In single male and male-male competition tests I recorded number of copulation attempts (defined as when a male is under a female and thrusts his gonopodium towards the female genital pore), copulation successes (defined as when a male thrusts his gonopodium at the female followed by the female twisting her body for removal), and time each male spent in the lead chasing females.

Data analysis. I conducted statistical analyses using JMP Version 7 (SAS Institute Inc. 2007, South Cary, NC, U.S.A.) and VassarStats© (Richard Lowry, Vassar College). To determine whether male *G. affinis* and *G. nobilis* respond differently to conspecific and heterospecific females, responses of 20 species pairs were examined. Because males and females were not reused across trials, only within trials, each individual represented an independent data point across trials. When comparisons were made between different experimental tests (e.g. male and female visual/olfactory, single male and male-male competition) within trials, data points were considered correlated and appropriate statistical tests were used as the same set of males and females were used across experiment tests within one trial. Association time data were transformed by raising time to (1/2) power to normalize distribution. All transformed association preference data were tested for goodness of fit and were normally distributed. I tested for differences in preference by *G. nobilis* and *G. affinis* males and females for heterospecific and conspecific individuals with a two-way paired *t* test.

Copulation success rate in open mating trials was measured as the inverse of the number of copulation attempts divided by number of copulation successes (1/(copulation attempts/successes)). If an individual had a score of 0 for either copulation attempts or successes I replaced it with .001 to prevent division by 0. As I only conducted single male trials in 2009, when comparing single male trials to male-male competition trials I only included 10 correlated trials from 2009. Copulation success rate and lead chase time data were transformed by raising to (1/2) power to normalize the distribution. All transformed data were tested for goodness of fit and were normally distributed. When examining male-male competition trials I used a paired two-tailed *t*-test to determine if there were significant

differences between males in copulation success rate by female species. I also used this to determine if males differed in degree of female chasing during attempted copulation. Due to low sample size, in any comparisons including single-male trials I used non-parametric two-tailed Wilcoxon Signed-Rank test. I used a three-way ANOVA with the factors of test type (male-male competition or single male test), male species, and female species to determine which factors significantly affected copulation success rate.

Results

Visual/Olfactory tests. Mean association preference time between conspecific and heterospecific females was different for *G. affinis* and *G. nobilis* males. Males of both species showed an association preference for conspecific females (*Gambusia affinis* males: Paired *t* test: $t_{39} = -1.95$, $P = 0.003$; *Gambusia nobilis* males: Paired *t* test: $t_{39} = -3.02$, $P = 0.004$; Table 1; Fig. 1).

Gambusia nobilis females spent significantly more time investigating *G. nobilis* males than *G. affinis* males (Paired *t* test: $t_{34} = -3.29$, $P = 0.002$; Table 1; Fig. 2) In contrast, *G. affinis* females did not have a significant association preference for conspecific males over heterospecific males (Paired *t* test: $t_{34} = 0.99$, $P = 0.229$; Table 1; Fig. 2). Due to a reasonably low sample size, I subsequently performed a power analysis on the *t* test for *G. affinis* female association time to determine the power with which I could ascertain if, in fact, there is no difference in association between male types. At the $\alpha = 0.05$ level I obtained a power of 0.224, which is relatively low. This combined with the non-significant *t* test suggests that I cannot determine with any real power if, in fact, there are no differences in association times of *G. affinis* females with conspecific and heterospecific males.

Open mating tests. ***Single male test.*** There was no difference in lead chase time between *G. affinis* and *G. nobilis* males across all females and all trials (Wilcoxon Signed-Rank test: $W = -1.07$, $P = 0.285$; $n = 10$ trials). *Gambusia affinis* males did not differ significantly in lead chase time between conspecific and heterospecific females (Wilcoxon Signed-Rank test: $W = 0.43$, $P = 0.667$; $n = 10$ trials; Table 1) nor did *G. nobilis* males (Wilcoxon Signed-Rank test: $W = -0.43$, $P = 0.667$; $n = 10$ trials; Table 1). All *G. affinis* males ($n = 10$) attempted to copulate at least once with conspecific females. Of 10 males, 8 attempted to copulate with heterospecific females. All *G. nobilis* males ($n = 10$) attempted to copulate with conspecific females and 8 attempted to copulate with heterospecific females. *Gambusia affinis* males did not differ in copulation success rate between conspecific and heterospecific females (Wilcoxon Signed-Rank test: $W = 2.12$, $P = 0.340$; $n = 10$ trials; Table 1). *Gambusia nobilis* males also did not differ significantly in copulation success rate between conspecific and heterospecific females (Wilcoxon Signed-Rank test: $W = -1.25$, $P = 0.10$; $n = 10$ trials; Table 1).

Male-male competition test. Male lead chase times of females were not different between male types (species) over all tests with conspecific and heterospecific females (Paired *t*-test: $t_{22} = 0.7$, $P = 0.488$). *Gambusia affinis* males did not have a greater chase time with conspecific females over heterospecific females (Paired *t*-test: $t_{22} = -0.35$, $P = 0.730$; Table 1) nor did *G. nobilis* males (Paired *t*-test: $t_{22} = -0.18$, $P = 0.859$; Table 1). Nineteen of all *G. affinis* males ($n = 23$) attempted to copulate at least once with conspecific female. Of 23 males, 18 attempted to copulate with heterospecific females. Twenty-one of all *G. nobilis* males ($n = 23$) attempted to copulate with conspecific females and 17 attempted to copulate with heterospecific females. There was no difference in number of copulation attempts

between *G. affinis* and *G. nobilis* males across all trials (*G. affinis* males: 7.35 $\pm$ 1.24; $n = 23$ males; *G. nobilis* males: 6.98 $\pm$ 1.43; $n = 23$ males; Paired *t*-test: $t_{45} = 0.22$, $P = 0.827$). There was no difference in number of copulation attempts for males of both species between interactions with conspecific and heterospecific females (*G. affinis* males: Paired *t*-test: $t_{22} = 0.66$, $P = 0.516$; *G. nobilis* males: Paired *t*-test: $t_{22} = 1.29$, $P = 0.210$; Table 1). Males of both species had higher copulation success rates with conspecific females over heterospecific females (*G. affinis* males: Paired *t*-test: $t_{22} = 2.33$, $P = 0.029$; *G. nobilis* males: Paired *t*-test: $t_{22} = -6.99$, $P < 0.0001$; Table 1; Fig. 3).

Single male vs. male-male competition tests. *Gambusia affinis* males had greater lead chase times in single male trials over male-male competition trials (Wilcoxon Signed-Rank test (two-tailed): $W = 3.24$, $P = 0.001$; $n = 10$ trials). *Gambusia nobilis* males showed the same pattern (Wilcoxon Signed-Rank test (two-tailed): $W = 2.04$, $P = 0.041$; $n = 10$ trials). There was no difference in number of copulation attempts for *G. affinis* males between single male and male-male competition trials (Wilcoxon Signed-Rank test (two-tailed): $W = 0.5$, $P = 0.617$; $n = 10$ trials) and *G. nobilis* males showed the same pattern (Wilcoxon Signed-Rank test (two-tailed): $W = 1.87$, $P = 0.062$; $n = 10$ trials). *Gambusia affinis* and *G. nobilis* males had greater copulation success rates in conspecific pairings in male-male competition trials but not single male trials. (*G. affinis* males: Wilcoxon Signed-Rank test: $W = -2.78$, $P = 0.005$; $n = 10$ trials; *G. nobilis* males: Wilcoxon Signed-Rank test: $W = -1.13$, $P = 0.010$; $n = 10$ trials). The results of the three-way ANOVA showed a significant effect of the interaction between the factors of male species and female species on copulation success rate ($F_{1, 124} = 22.71$, $P < 0.0001$). There were no significant effects of the

factors of test type, female species, male species or other interactions between factors on copulation success rate.

Discussion

The results presented here suggest that mating behaviors differ between *Gambusia affinis* and *G. nobilis*, Furthermore, there is evidence that these species may have assortative preferences for conspecifics, at least partially mediated by female choice. Mate choice may act as a premating isolating barrier to reduce hybridization in sympatry, as males of both species had greater association preference times for conspecific over heterospecific females in visual/olfactory tests. Female *G. affinis* did not differ in association preference between conspecific and heterospecific males. Female *G. nobilis*, however, spent more time with conspecific males. In open mating tests most males would attempt mating with heterospecific females. In single male tests there was no difference in copulation success rate between conspecific and heterospecific pairings. The opposite trend was found in male-male competition tests where copulation success rate was higher in conspecific versus heterospecific pairings. Males of both species had greater lead chase times when not in competition but there was no difference in copulation attempts between single male and male/male competition tests, suggesting that perhaps it is female choice that is mediating higher copulation success rate when males are competing. As males do not engage in courtship female preference is likely subtle or cryptic.

Hybridization between *G. affinis* and *G. nobilis* is at low levels in the wild, suggesting strong reproductive isolation between the two species (Echelle & Echelle 1980). Previous studies have indicated that postzygotic barriers exist between these species; this study indicates that prezygotic barriers may also be present (Echelle & Echelle 1980; Hubbs

2001). As fishes used in this study came from allopatric populations with no history of contact, conspecific mate preferences demonstrated here must have evolved in allopatry, possibly as a byproduct of ecological divergence (Nosil *et al.* 2007). Males attempting to mate with any female they were presented with suggests visual/olfactory preferences for conspecifics is not a sufficient barrier to hybridization. I also tested for the effect of intraspecific sexual selection on male choosiness. Plath et al. (2008) indicates that male choosiness in some species of Poeciliid may increase in competition explaining differences in copulation success between tests. Copulation success rate, however, did not differ between tests where males had access to females in competition or singly. This lack of difference in male behavior suggests that varying copulation success may be mediated by subtle female preference. This may result from experimental design. Females in single male preference trials have no choice. When presented with choice, however, they may influence male copulatory success by choosing conspecifics, suggesting that female preference is important in this system. Thus, although male promiscuity would tend to facilitate hybridization, subtle female preference will limit hybridization and act as a mechanism of premating isolation (Fedina & Lewis 2008).

These results provide insight into the role of sexual selection in premating isolation when male and female preference is considered. Poeciliid females are livebearers. Selection on females to choose a good mate should be high because of their relatively heavy investment in offspring. Differential success of conspecific matings in the open mating trials suggests that females may exercise some degree of control over actual copulation success by males (McPeek 1992, Gould *et al.* 1999, Bisazza *et al.* 2001). Females may mediate higher copulation success rate between conspecifics when they are given a choice. Evidence for

subtle or cryptic female choice in poeciliid mating systems without courtship is not strong (Bisazza & Marin 1995) since there are few studies that have examined female preference in a force copulation system (Wilson *et al.* 2007). Livebearing fishes, such as guppies and *Gambusia* can store sperm, and there is evidence for sperm competition in the latter (Evans & Rutstein 2008). In this way, females can control paternity of their offspring. A thorough examination of sperm competition in *Gambusia* may provide insights into mechanisms by which females may further manipulate paternity of their offspring.

What remains puzzling is the behavior by which these females may selectively copulate at a higher frequency with conspecific males. One possibility is that females may use an increase in burst swimming speed to avoid heterospecific males. Despite higher copulation success rate of conspecific males, however, heterospecific males still managed to force copulate, albeit at lower frequencies. Given the low rate of wild hybridization females may have further, prezygotic means to cryptically select paternity of their offspring such as preferential sperm storage or fertilization. Empirical studies on such force-copulation systems are interesting and important to understand how female and male preference can collectively influence maintenance of species identity, particularly in secondary contact.

Acknowledgements

I wish to thank A. Kodric-Brown for providing advice on design, analysis, and presentation of this work. A. Olson assisted with all experiments. I also wish to thank J. A. Baker, C. Tech, D. L. Swenton, A. Edelman, J. McIntyre, L. Schwanz, A. Barlow, and R. West for providing helpful comments on this manuscript. B. Belding assisted with fish maintenance. This work was funded by a grant through NM Department of Game and Fish Share with Wildlife Program to A. Kodric-Brown and D. Swenton, and a NSF Doctoral

Dissertation Improvement Grant, an Alvin and Caroline Groves Research, Crawford Scholarship & GRAC grant (UNM Biology Department), & SRAC grant (UNM Graduate and Professional Student Association, Student Resource Allocations Committee) to D. Swenton.

References

Andersson, M. 1994: Sexual selection. Princeton University Press, Princeton.

Arnold, M. L. & Hodges, S. A. 1995: Are natural hybrids fit or unfit relative to their parents? Trends in Ecology and Evolution **10**, 67-71.

Arnold, S. J., Houck, L. D. 1982: Courtship pheromones: evolution by natural and sexual selection. In: Biochemical Aspects of Evolutionary Biology (Nitecki, M. H., ed). University of Chicago Press, Chicago, pp. 173-211.

Arnqvist, G. & Rowe, L. 2005: Sexual Conflict. Princeton University Press, Princeton.

Basolo, A. L. 1990: Female preference predates the evolution of the sword in swordtail fish. Science **250**, 808-810.

Bisazza, A. & Marin, G. 1991: Male size and female mate preference in the eastern mosquitofish (*Gambusia holbrooki)*. Copeia **1991**, 730-735.

Bisazza, A. & Pilastro, A. 1997: Small male mating advantage and reversed size dimorphism in poeciliid fishes. Journal of Fish Biology **50**, 3977-4006.

Bisazza, A., Manfredi, S., & Pilastro, A. 2000: Sexual competition, coercive mating and mate assessment in the one-sided livebearer, *Jenynsia multidentata*: are they predictive of sexual dimorphism? Ethology **106**, 961-978.

Bisazza, A., Vaccari, G., & Pilastro, A. 2001: Female mate choice in a mating system dominated by male sexual coercion. Behavioral Ecology **12**, 59-64.

Constanz, G. D. 1989: Reproductive biology of poeciliid fishes. In: Ecology and Evolution of Livebearing Fishes (Poeciliidae) (Meffe, G. K. & Snelson Jr., F. F., eds). Prentice Hall, New Jersey, pp. 33-50.

Courtenay Jr., W. R. & Meffe, G. K. 1989: Small fishes in strange places: A review of introduced poeciliids. In: Ecology and Evolution of Livebearing Fishes (Poeciliidae) (Meffe GK, Snelson Jr. F. F., eds). Prentice Hall, New Jersey, pp. 319-331.

Coyne J. A. & Orr, H. A. 2004: Speciation. Sinauer Associates, Sunderland.

Deaton, R. 2008: Use of microsatellite paternity analysis to determine male mating success in the western mosquitofish, *Gambusia affinis*. Behaviour **145**, 795-814.

Dobzhansky, T. 1937: Genetics and the Origin of Species. Columbia University Press, New York.

Dobzhansky, T. 1940: Speciation as a stage in evolutionary divergence. American Naturalist **74**, 312-321.

Echelle, A. A. & Echelle, A. F. 1980: Status of the Pecos Gambusia, *G. nobilis*. Endangered Species Report No. 10:1-73, U.S. Fish and Wildlife Service, Albuquerque, New Mexico.

Evans, J. P. & Rutstein, A. N. 2008: Postcopulatory sexual selection favours intrinsically good sperm competitors. Behavioral Ecology and Sociobiology **62**, 1167-1173.

Fedina, T. Y. & Lewis, S. M. 2008: An integrative view of sexual selection in *Tribolium* flour beetles. Biological Review **83**, 151-171.

Fisher, R. A. 1930: The Genetical Theory of Natural Selection. Oxford University Press, Oxford.

Gerhardt, H.C. & Doherty, J. A. 1988: Acoustic communication in the gray treefrog, *Hyla versicolor*: evolutionary and neurobiological implications. Journal of Comparative Physiology A **162**, 261-278.

Geyer, L. & Palumbi, S. R. 2003: Reproductive character displacement and the genetics of gamete recognition in tropical sea urchins. Evolution **57**, 1049-1060.

Gould, J. L., Elliot, S. L., Masters, C. M. & Mukerji, K. J. 1999: Female preferences in a fish genus without male choice. Current Biology **9**, 497-500.

Halliday, T. R. 1990: The evolution of courtship behavior in newts and salamanders. Advances in the Study of Behavior **19**, 137-169.

Hubbs, C. 1959: Population analysis of a hybrid swarm between *Gambusia affinis* and *G. heterochir*. Evolution **13**, 236-246.

Hubbs, C. 2001: Environmental correlates to the abundance of spring-adapted versus stream-adapted fishes. Texas Journal of Science **53**, 299-326.

Hurt, C. R., Farzin, M. & Hedrick, P. W. 2005: Premating, not postmating, barriers drive genetic dynamics in experimental hybrid populations of the endangered sonoran topminnow. Genetics **171**, 55-662.

Irwin, D. E. & Price, T. 1999: Sexual imprinting, learning and speciation. Heredity **82**, 347-354.

Laukaitis, C. M., Critser E. S. & Karn, R. C. 1997: Salivary androgen-binding protein (ABP) mediates sexual isolation in *Mus musculus.* Evolution **51**, 2000-2005.

Mayr, E. 1963: Populations, Species and Evolution. Harvard University Press, Cambridge.

McKinnon, J. S., Mori, S., Blackman, B. K., Kingsley, D. L., Jamieson, L., Chou, J., & Schluter, D. 2004: Evidence for ecology's role in speciation. Nature **429**, 294-298.

McLennan, D. A. & Ryan, M. J. 1997: Responses to conspecific and heterospecific olfactory cues in the swordtail, *Xiphophorus cortezi*. Animal Behaviour **54**, 1077-1088.

McPeek, M. 1992: Mechanisms of sexual selection operating on body size in mosquitofish (*Gambusia holbrooki)*. Behavioral Ecology **3**, 1-12.

Meffe, G. K. & Snelson Jr., F. F. 1989: An ecological overview of poeciliid fishes. In: Ecology and Evolution of Livebearing Fishes (Poeciliidae) (Meffe, G. K. & Snelson Jr., F. F., eds). Prentice Hall, New Jersey, pp. 13-31.

Miyatake, T. & Shimizu, T. 1999: Genetic correlations between life history and behavioral traits can cause reproductive isolation. Evolution **53**, 216-224.

Nosil, P., Crespi, B. J., Gries, R., & Gries, G. 2007: Natural selection and divergence in mate preference during speciation. Genetica **128**, 309-327.

Parker, G. A. 1983: Mate quality and mating decisions. In: Mate choice (Bateson, P. P. G. ed). Cambridge University Press, Cambridge, pp. 141-161.

Pilastro, A., Giacomello, E. & Bisazza, A. 1997: Sexual selection for small size in male mosquitofish (*Gambusia holbrooki)*. Proceedings of the Royal Society of London Series B: Biological Sciences **1385**, 1125-1129.

Pilastro, A. & Bisazza, A. 1999: Insemination efficiency of two alternative male mating tactics in the guppy (*Poecilia reticulata).* Proceedings of the Royal Society of London Series B: Biological Sciences **1431**, 1887-1891.

Plath, M., Makowicz, A. M., Schlupp, I. & Tobler, M. 2007: Sexual harassment in live-bearing fishes (Poeciliidae): comparing courting and noncourting species. Behavioral Ecology **18**, 680-688.

Plath, M., Kromuszczynski, K. & Tiedman, R 2009: Audience effect alters male but not female mating preferences. Behavioral Ecology and Sociobiology **63**, 381-390.

Ptacek, M. 2000: The role of mating preferences in shaping interspecific divergence in mating signals in vertebrates. Behavioral Processes **51**, 111-134.

Rosenfield, J. A., Nolasco, S., Lindauer, S., Sandoval, C. & Kodric-Brown, A. 2004: The role of hybrid vigor in the replacement of Pecos pupfish by its hybrids with sheepshead minnow. Conservation Biology **18**, 1589-1598.

Rundle, H. D. 2002: A test of ecologically dependent postmating isolation between sympatric sticklebacks. Evolution **56**, 322-329.

Rundle, H. D. & Nosil, P. 2005: Ecological speciation. Ecology Letters **8**, 336-352.

Schluter, D. 2003: Frequency dependent natural selection during character displacement in sticklebacks. Evolution **57**, 1142-1150.

Schwartz, J. J. & Henderson, R. W. 1985: A guide to the identification of the amphibians and reptiles of the West Indies exclusive of Hispaniola. Milwaukee Public Museum, Milwaukee

Scribner, K. T. & Avise, J. C. 1994: Population cage experiments with a vertebrate: the temporal demography and cytonuclear genetics of hybridization in *Gambusia* fishes. Evolution **48**, 155-172.

Seehausen, O. & van Alphen, J. J. M. 1998: The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). Behavioral Ecology and Sociobiology **42**, 1-8.

Servedio, M. R. 2001: Beyond reinforcement: the evolution of premating isolation by direct selection on preferences and postmating, prezygotic incompatibilities. Evolution **55**, 1909-1920.

Servedio, M. R. 2007: Male versus female mate choice: sexual selection and the evolution of species recognition via reinforcement. Evolution **61**, 2772-2789.

Shaw, K. A., Scotti, M. L. & Foster, S. A. 2007: Ancestral plasticity and the evolutionary diversification of courtship behavior in threespine sticklebacks. Animal Behaviour **73**, 415-422.

Stamps, J. A. 1977: Social behavior and spacing patterns in lizards. In: Biology of the Reptilia (Gans, C. & Tinkle, D. W., eds). Academic Press, New York, pp 169-204.

Stamps J. A. & Barlow, G. W. 1973: Variation and stereotypy in the displays of *Anolis aeneus* (Saura: Iguanidae). Behaviour **47**, 67-94.

Strecker, U. & Kodric-Brown, A. 1999: Mate recognition systems in a species flock of Mexican pupfish. Journal of Evolutionary Biology **12**, 927-935.

Sublette, J. E. 1990: The Fishes of New Mexico. The University of New Mexico Press, Albuquerque

Tokarz, R. R. 1995: Mate choice in lizards: a review. Herpetological Monographs **9**, 17-40.

Wells, K. D. 1997: Territoriality and male mating success in the green frog (*Rana clamitans*). Ecology **58**, 750-762.

Wilson, R.S., Condon, C. H. L. & Johnston, I. A. 2007: Consequences of thermal acclimation for the mating behaviour and swimming performance of female mosquito fish. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences **1487**, 2131-2139.

Young, J. R., Hupp, J. W., Bradbury, J. W. & Brauns, C. E. 1994: Phenotypic divergence of secondary sexual traits among sage grouse, *Centrocercus urophasianus*, populations. Animal Behaviour **47**, 1353-1362.

Tables and Figures

Table 1. Summary of preference times in seconds and male behavior (Lead chase time (in seconds), copulation attempts and Copulation Success Rate) across all trials by experiment type. Data are presented as Mean + SE (n).

Experiment	Male Species	Female Species *G. affinis*	Female Species *G. nobilis*
Preference times			
Male visual/olfactory preference	*G. affinis*	230.48±26.32 (40)	115.80±16.57 (40)
	G. nobilis	123.10±17.35 (40)	218.30±20.76 (40)
Female visual/olfactory preference	*G. affinis*	188.38±22.68 (35)	122.05±14.71 (35)
	G. nobilis	154.32±23.18 (35)	226.01±23.91 (35)
Lead chase time			
Single Male Open Mating	*G. affinis*	150.05±27.48 (10)	145.95±31.67 (10)
	G. nobilis	163.58±20.15 (10)	169.86±32.26 (10)
Male/Male Competition Open Mating	*G. affinis*	109.92±20.56 (23)	123.62±27.34 (23)
	G. nobilis	98.65±17.84 (23)	92.31±14.98 (23)
Copulation attempts			
Single Male Open Mating	*G. affinis*	4.2±1.02 (10)	3.3±0.97 (10)
	G. nobilis	6.5±2.50 (10)	4.1±0.94 (10)
Male/Male Competition Open Mating	*G. affinis*	7.90±1.75 (23)	6.83±1.79 (23)
	G. nobilis	8.09±2.52 (23)	5.87±1.37 (23)
Copulation Success Rate			
Single Male Open Mating	*G. affinis*	0.54±0.11 (10)	0.25±0.11 (10)
	G. nobilis	0.11±0.10 (10)	0.53±0.11 (10)
Male/Male Competition Open Mating	*G. affinis*	0.44±0.13 (23)	0.19±0.06 (23)
	G. nobilis	0.09±0.03 (23)	0.57±0.10 (23)

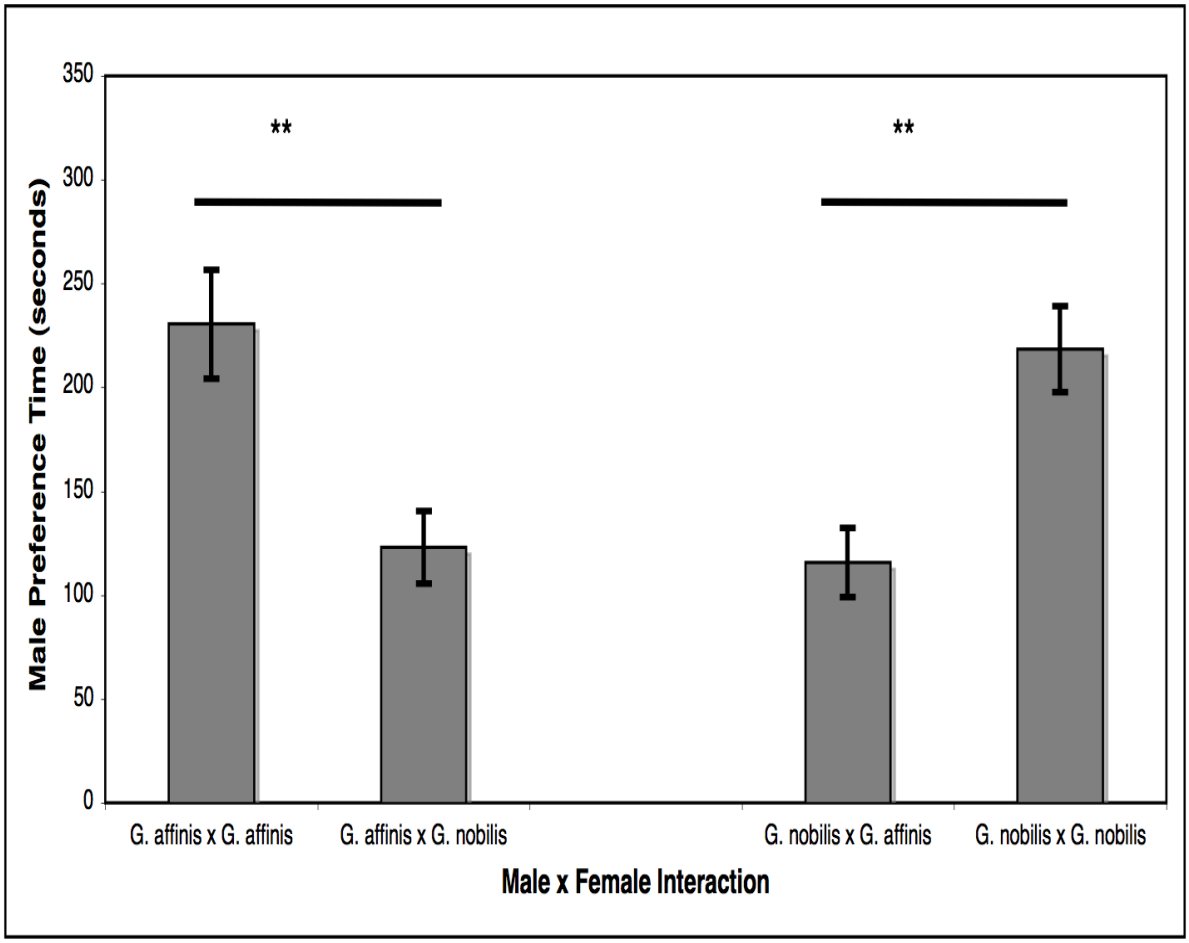


Figure 1. Association time in seconds with standard error bars during visual/olfactory preference tests with females of both species for Gambusia affinis males and G. nobilis males (n = 40 trials, ** = $P < 0.05$).

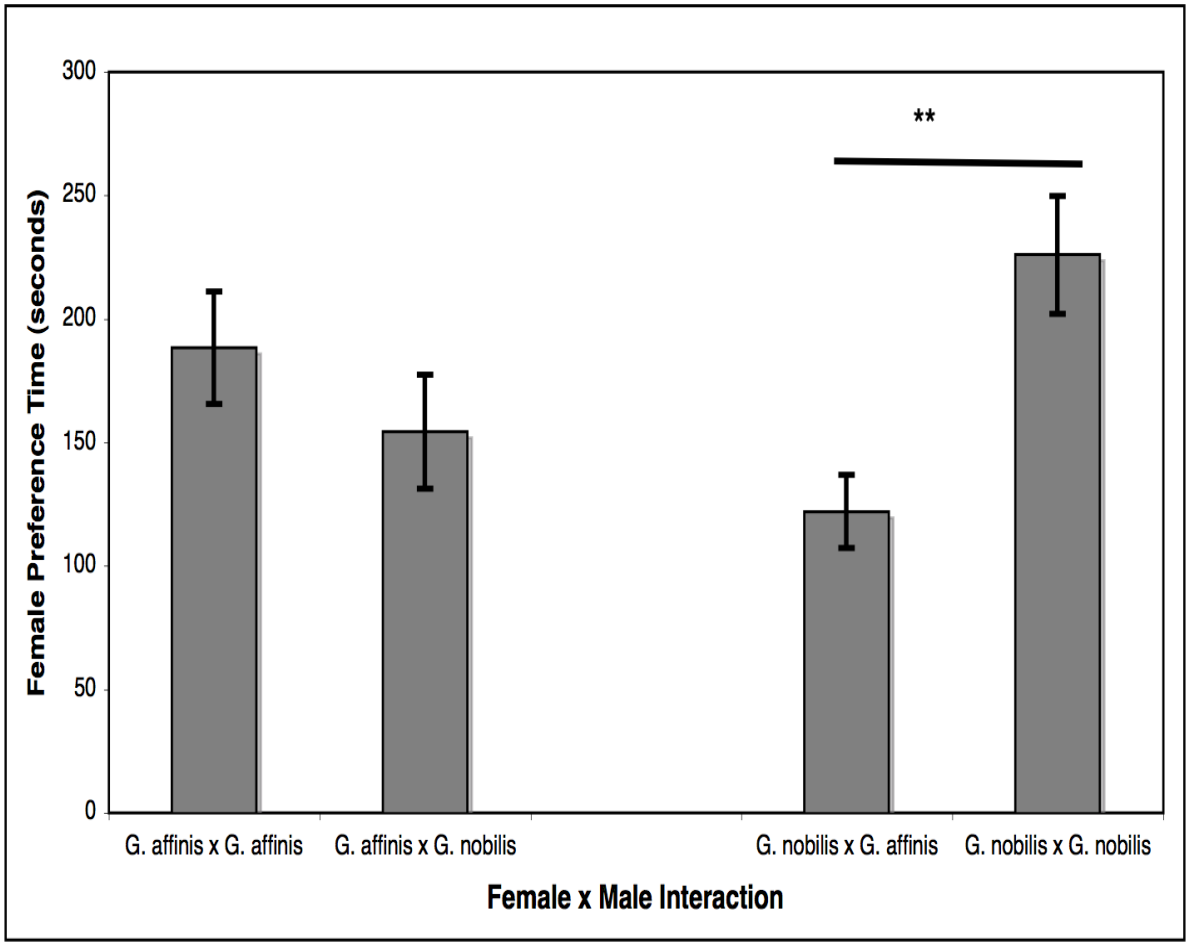


Figure 2. Association time in seconds with standard error bars during visual/olfactory preference tests with males of both species for Gambusia affinis females and G. nobilis females (n = 35 trials; ** = $P < 0.05$).

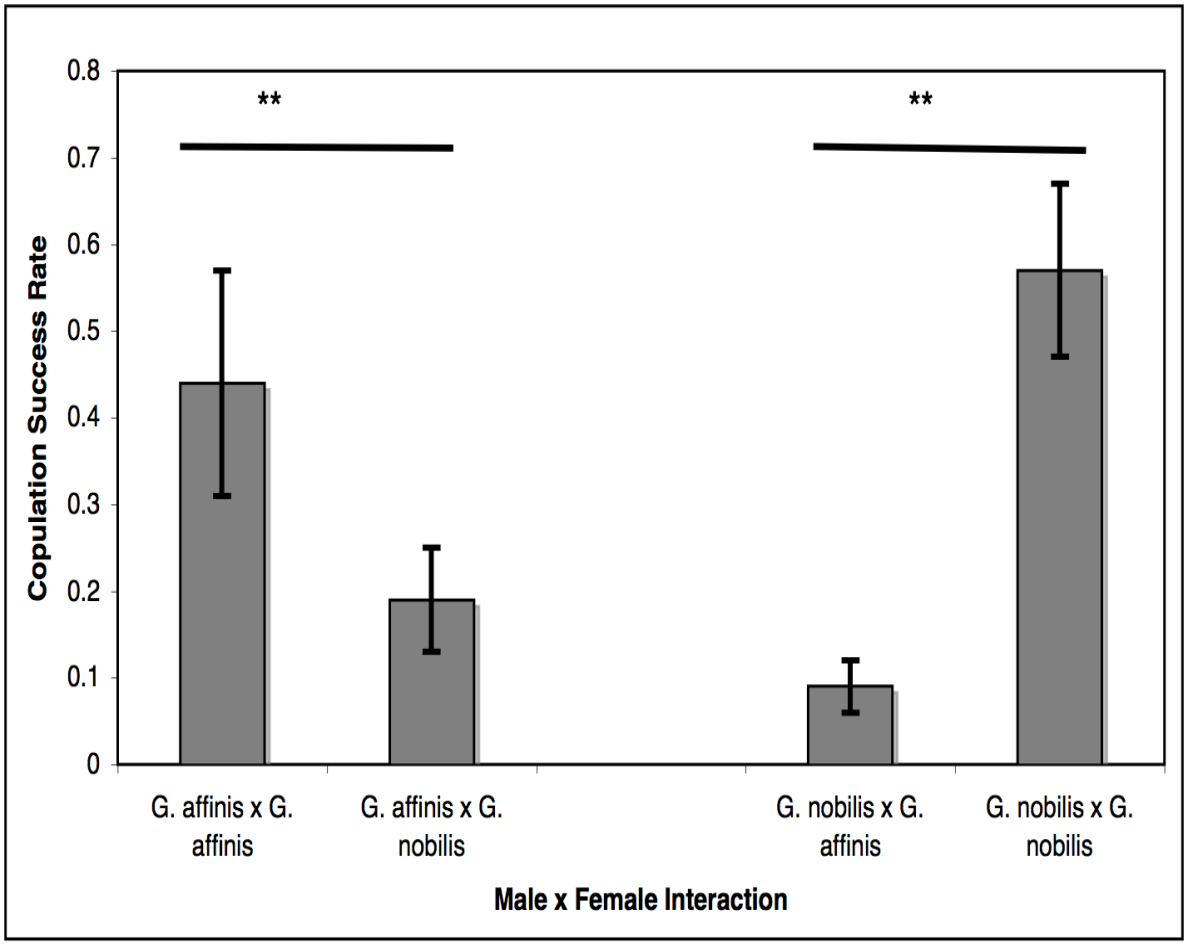


Figure 3. Copulation Success Rate open in mating trials with male-male competition for all female x male interactions (G.a. = G. affinis individual, G.n. = G. nobilis individual; n = 23 trials; ** = $P < 0.05$).

Chapter 2:

Habitat and Life History Differences between Two Species of *Gambusia*

Abstract

Life history strategies reflect maximization of individual fitness in the face of trade-offs such as investment in offspring size or number. Two livebearing fishes of the same genus, *Gambusia affinis* and *G. nobilis* diverged in allopatry and persist in different abiotic and biotic contemporary environments. In our study *G. affinis* were typically found in habitats with high productivity and wide fluctuations in temperature, salinity and dissolved oxygen, whereas *G. nobilis* occurred in spring-fed habitats that were stable in temperature. Heterospecific fish assemblages were also more varied in *G. affinis* habitats than in *G. nobilis* habitats. We collected data on life-history traits: embryo size, brood size, maternal brood reproductive effort, population sex ratios as well as size distributions of adults and juveniles. We found evidence of differences in life history strategies that may reflect a tradeoff between individual productivity and differential mortality rates in different selective environments. There was no difference between species in reproductive effort per brood. They differed, however, in investment strategy. *G. affinis* females produced large broods with small embryos; in contrast, *G. nobilis* females produced larger embryos, but smaller broods. In *G. nobilis,* size of embryos varied, while in *G. affinis* embryo size was constrained. We suggest that at our field site *G. affinis* persists as an annual species with relatively high growth rates and corresponding reproductive patterns. *G. nobilis* females may live multiple years and their reproductive tempo may be slowed as well.

Introduction

An organism's adaptive strategy is expressed in its life history, specifically how it differentially allocates resources to growth, reproduction and survival. Characteristics of individuals that may be strategically adapted to optimize lifetime fitness in a particular environment in the face of trade-offs, which include offspring size, offspring number (per clutch, per year), larval developmental time, adult size at maturation, and lifespan. Predation, competition, resource availability, and the abiotic environment can all influence the life history strategies of organisms (Reznick 1983). Natural selection should favor individuals that maximize fitness in the face of these challenges (Stearns 1992).

Fishes of the family Poeciliidae, which include guppies, mosquitofish and swordtails, present an interesting model system to examine how environmental variables may influence life history strategy. These fishes, found in a wide variety of habitats are small, short-lived, and give birth to live young that feed immediately (Snelson, Jr. 1989). Reproductive investment patterns vary widely across species and genera. Females may invest on a continuum from completely lecithotrophic (providing yolk and incubation only) to matrotrophic (providing nourishment over gestation) (Wourms 1981). There is also evidence for variable and rapid evolutionary changes in embryo and brood sizes between populations within the same species that may be related to stochastic environmental conditions (e.g. Downhower et al. 2000).

Livebearing provides a unique opportunity to accurately assess female reproductive investment across different environmental gradients. Many studies have demonstrated strong effects of environmental variables on life history patterns in poeciliids. In particular, food availability (Reznick 1983; Wurtsbaugh & Cech, Jr. 1983; Smith 1986) salinity (Trexler

1997), predation (Reznick & Endler 1982) and temperature (Wurtsbaugh & Cech, Jr. 1983; Yan 1987) have been examined and a correlation between these factors and life history traits has been documented. Reproductive timing, length of gestation, and offspring size at birth are thought to vary adaptively in response to environmental temperature (Krumholz 1948; Kallman 1975), which is generally recognized as the most important abiotic environmental factor in affecting poeciliid life histories (Snelson, Jr. 1989). Primary productivity in freshwater environments is often dependent on temperature. The influence of other environmental variables, such as predation risk, competition, productivity, seasonality and their interactions must also be considered as the effects of these variables are probably correlated (e.g. Borowsky 1984; Vondracek et al. 1988). Collectively these environmental characteristics shape individual productivity (i.e. defined here as the rate of allocation of energy to offspring and mortality regime). An organism must weigh investment in reproduction relative to the chance of future survival. Natural selection will favor individuals that maximize fitness by acquiring and allocating resources effectively in the face of this tradeoff (Figure 1).

The Pecos gambusia, *Gambusia nobilis* and western mosquitofish, *G. affinis,* in the family Poeciliidae, are fishes found in the arid southwestern U.S. (Hubbs 2001). They are small (<45mm) and may give birth to one to four broods in a breeding season (Rosen & Bailey 1963). The distributional range of *Gambusia nobilis* has been reduced to four remaining areas in the Pecos River Basin (Echelle & Echelle 1980). *Gambusia affinis* is native to warmer regions of the Americas but has been introduced globally to control mosquito populations (Courtenay Jr. & Meffe 1989). It is also a highly effective competitor in most environments and has driven native fish species to endangerment or, in some cases,

to local extinction (e.g. Galat & Robertson 1992; Crivelli 1995). *Gambusia affinis* has invaded three of the four remaining populations of *G. nobilis* and threatens the persistence of this endangered species through competition and hybridization (Echelle & Echelle 1980). The two fishes are closely related and presumably speciated in allopatry as *G. affinis* is not native to *G. nobilis*' recent range (Echelle & Echelle 1980).

At Bitter Lake National Wildlife Refuge (BLNWR, 17 km east of Roswell, NM, N 33.6023141° W 104.4119131° 56.474), the field site for this study, *G. affinis* and *G. nobilis* occur in both allopatry (separate isolated springs, sinkholes and ponds) and sympatry (local coexistence) in some sites. At BLNWR *Gambusia affinis* is found in shallow and disturbed areas that are flooded for waterfowl in the winter and drained for shorebirds in the summer. *Gambusia nobilis* thrives in spring-fed gypsum sinkholes and is thought to be the least saline-tolerant of the *Gambusia* species (Hubbs 2001).

The first documentation of co-occurrence at BLNWR was made in 1938 (Koster personal journals, unpublished). Introduction may have been accidental and has happened repeatedly in the last century (BLNWR survey records, G. Warrick, pers. comm.). Some *Gambusia* species readily hybridize with *G. affinis* (e.g. Meffe & Snelson Jr. 1989; Scribner & Avise 1994) but hybridization between *G. affinis* and *G. nobilis* is infrequent and was previously estimated at 10% in another sympatric location (Blue Spring; Echelle & Echelle 1980). The low occurrence of hybridization in these closely related species suggests a strong degree of reproductive isolation, perhaps facilitated by ecologically driven divergence in life history strategy (Schluter 2000; Coyne & Orr 2004; Rundle & Nosil 2005).

Previous studies have documented morphological divergence in *Gambusia* as a consequence of environmental variables (e.g. Hubbs & Springer 1957; Echelle & Echelle

1986; Langerhans et al. 2004). In the present study, we investigated how the different environments of these two species might be reflected by differences in their life history strategies. We applied several approaches to document the relationship between life history strategies and environmental variables. We collected longitudinal field data as well as life history data from laboratory stocks and museum specimens to quantify life history strategies of these two species and documented how they differ in their contrasting habitats. Stearns (1983) suggested that *G. affinis* have more and smaller offspring, earlier sexual maturity and greater reproductive effort in a fluctuating environment that may include a high rate of predation. *Gambusia* species found in more stable environments with lower primary productivity should have fewer, larger offspring, later sexual maturity and lower reproductive effort. We wished to determine if these patterns are consistent with the populations at BLNWR as such differences in the two species current environments may reflect evolutionary diversification that may contribute to isolation in secondary contact between the endangered *G. nobilis* and its invasive congener.

Methods

Field study. The field portion of this study was conducted during 2008 at BLNWR. Sample sites across the refuge were surveyed every 4 weeks between May and August, spanning the reproductive season of these fishes. Fish were examined from allopatric populations to avoid collection from zones of putative hybridization, where hybrids may be indistinguishable from their pure parental species (Echelle & Echelle 1980). We sampled six *G. nobilis* sites (SH27N, SH27S, SH20, SH37, Lost Spring, SH7) and nine *G. affinis* sites (BC3, U5N, OBBr, OB, Blind, 15MPH, SH3, FB, SC). We sampled a greater number of *G. affinis* sites because they often dry up during the breeding season. These sites were sampled

during each visit if they still contained water and ranged from gypsum sinkhole habitats to drainage ditches. Water quality measurements (temperature, dissolved oxygen and salinity) were measured using an YSI© meter (YSI corporation) during each sampling period between 07:00-09:00 hours. A minnow trap without bait (to avoid complications in a concurrent stable isotope study) was set and retrieved two to three hours later. All fish caught were identified to species and counted. A subset of the *Gambusia* was sexed (male, female, juvenile). Males were identified by the presence of the gonopodium, a modified anal fin that functions as an intromittent organ and is used to inseminate the livebearing females. Females were identified by the presence of the gonopore spot, a black patch marking the site of insemination and an indicator of gravidity (Farr and Travis 1986). Fish that lacked sexual characteristics such as a gonopodium or gonopore spot were categorized as juveniles. Standard length, caudal fin length, depth and gonopodium length (when applicable) were measured on every fifth fish captured in the minnow trap. Fertility of females (degree of abdomen swelling and intensity of gonopore spot) was also noted.

Laboratory breeding. *Gambusia nobilis* and *G. affinis* were collected between 2006-2008 from allopatric populations at BLNWR (*G. nobilis*: SH7, SH37; *G. affinis*: Lower reach of Bitter Creek, Ditches and marshes in southern reach of BLNWR). A subset (n = 50 each per species) of females and males from these populations were transported to the University of New Mexico (UNM) in Breathing Bags™ (Kordon LLC, Hayward, CA) with Bag Buddies™ (Jungle Laboratories, Spectrum Brands, Inc.).

The fish were maintained in 38-l or 76-l aquaria with undergravel-filtration at ambient temperatures between 20 and 26°C and a salinity of 8ppt using CoraLife$_{TM}$ Marine Salt (IL, USA). The fish were kept separate by species in mixed sex groups with

approximately 10 fish in 38-l aquaria and 15-20 fish in 76-l aquaria. The aquaria were either kept in a facility with windows allowing natural light and full-spectrum UV lamps set to the natural light cycle, or in a greenhouse that allowed for full exposure to natural light. Fish were fed daily with a 70:30 mixture of Tetramin flake food (Tetra, Blacksburg, VA, USA) and Freeze-Dried brine shrimp (UT, USA) and were provided with as much food as they would consume in 5 minutes.

During the reproductive season (April-August) females that were gravid were individually placed in livebearing brood chambers floating in a 38 or 76L aquarium at ambient temperatures between 20 and 26°C. All females were kept at similar temperatures to remove any effects from variable temperature environments. These brood chambers are designed to separate neonates from the mother when they are born. *Gambusia* females are known to cannibalize their young after birth if the neonates have no refuge in which to hide (Dionne 1985). When a brood was born, we counted brood size and noted neonate mortality. The mother was weighed using blotted wet weight and measured and placed back in the communal tank to breed again with males from her own species. Brood chambers were examined daily but most neonates were not measured because handling increased the risk of mortality, however, a small subset of broods were sacrificed and weighed using blotted wet weight.

Dissections of fish from museum collections. We performed dissections on females from allopatric populations collected from BLNWR in 1999 and 2000. The fish were collected by Steven P. Platania, American Southwest Ichthyological Researchers LLC, and deposited in the Museum of Southwestern Biology (MSB) at UNM in Albuquerque, NM. They were preserved in 70% EtOH. We sampled a minimum of ten *G. nobilis* females

collected from Sinkhole 7 (SH7) and SH37 in April-July (MSB collection numbers: 43663, 43666, 43640, 43789, 43763, 43637, 43786, and 43760). A minimum of ten *G. affinis* females were sampled from the lower reach of Bitter Creek (BC) from collections in April-August (MSB collection numbers: 46823, 46826, 46848, 46811, 46872, 46791, 46917, and 46896). All females were weighed on an analytical balance, and standard length, caudal fin length and depth were measured in mm to two decimal places. All females were then dissected to obtain information on reproductive effort. Embryos were counted and weighed collectively. We weighed the three largest embryos individually. Maturation state of embryos was also noted. If the embryos had no detectable eyes or other signs of embryonic development they were classified as Stage 1 embryos, or unfertilized eggs (Meffe 1985). If eyes were visible we classified them as Stage 2 embryos.

Data analysis. To characterize the physical characteristics of habitats we computed means and standard errors for all water quality measurements. We pooled the habitat data by species and performed a discriminant function analysis to determine if there were differences in these collective measurements between the habitats of the two species. We computed means and standard errors for all life history characteristics. We calculated the proportion of each size/sex class (male, female and juvenile) from the field demographic data. From the results of the laboratory breeding experiment, we calculated summary statistics for female mass as well as brood size (the number of embryos in a given brood). A subset of recently born broods was sacrificed to obtain measurements on brood mass and neonate mass. From the females in the museum collections we measured female mass, brood mass, brood size and some of the individual embryos for calculating summary statistics. Maternal reproductive effort was calculated as the ratio between brood mass and maternal somatic mass. Average

embryo size was calculated by dividing brood mass by brood size. Only Stage 2 embryos were used for computing average embryo size. Stage 1 embryos were included in calculations for brood size, as we found no evidence of female reabsorption of embryos once incubation begins. For comparison of means in the life history traits between these two species we used a two-tailed t-test. In order to examine trends across months we used a two-way ANOVA. We used an ANCOVA, with female size as a covariate, on log-transformed data to compare the regressions of embryo mass, maternal brood reproductive effort, brood size and brood mass against female somatic mass of the two species. Before performing parametric analyses we examined all data for normality and equality of variances. All data fit normality criteria and when variances were unequal, an alternate t-test, for unequal variances was used.

Results

Habitat characteristics. We found significant differences in the collective water quality measurements between the two types of habitats from where the species were collected ($F_{2,57} = 3.811$, $P = 0.008$). On average, *G. nobilis* habitats had lower means and narrower ranges across habitats and over the breeding season in temperature (°C, $F_{4,57} = 3.763$, $P = 0.029$) and salinity (ppt, $F_{4,57} = 5.013$, $P = 0.01$) than *G. affinis* habitats (Figure 2). *Gambusia affinis* habitats had the highest and lowest recorded temperature, salinity, and dissolved oxygen. Three *G. affinis* habitats dried up completely in late spring and then refilled in July. Fish community assemblages, including potential predators of the two studied species, were generally larger and more varied in *G. affinis* habitats (Table 1).

Demographic patterns during the breeding season. The proportion of *G. affinis* juveniles was highest in June and July and decreased thereafter (Table 2). Museum

dissections revealed that *G. affinis* females reproduced through August. The proportion of juveniles in *G. nobilis* populations was highest in July and decreased thereafter (Table 2). The females were gravid through July but stopped incubating embryos by August. The mean standard length (mm) of *G. affinis* females generally decreased through the season (F_4 = 2.86, n = 57 females, P = 0.024; Figure 3). The mean standard length of *G. nobilis* females, however, did not change significantly throughout the breeding season. (F_3 = 1.09, n = 77 females, P = 0.36; Figure 3).

Maternal investment. *Gambusia affinis* and *G. nobilis* females differed in investment in embryo mass, brood size and brood mass. The two species differed in maternal investment in individual offspring. The results of the ANOVA suggested there is no significant relationship between female somatic mass and Stage 2 embryo mass in *G. affinis* (F_3 = 2.19, n = 16 broods, P = 0.16; Figure 4) but a positive relationship between Stage 2 embryo mass and female mass (F_2 = 3.36, n = 55 broods, P = 0.04; Figure 4). Embryo size remained constant for during the breeding season in *G. affinis* (F_3 = 0.25, P = 0.91) but increased in *G. nobilis* females (F_2 = 0.25, P < 0.0001). Females from the April collections of both species *were* only incubating Stage 1 embryos and Stage 2 embryos were found in the females from the May collection.

The mean mass of Stage 2 embryos from the field collection females differed significantly between the two species (t_{75} = -8.76, P < 0.0001; Table 3). *Gambusia affinis* embryos weighed less than *G. nobilis* embryos. In the laboratory study, embryo mass at birth (neonate size) also differed in the same way between the two species. *G. affinis* neonates (mean = 0.005 $\pm$ 0.0002 g, n = 6 broods) weighed less than *G. nobilis* neonates (mean = 0.01 $\pm$ 0.002 g, n = 5 broods) (t_{11} = -10.37, P < 0.0001).

Brood size and embryo mass appeared to be inversely related for both species (Table 2) and varied across the breeding season. *Gambusia affinis* brood size decreased over the season ($F_4 = 7.22$, $P < 0.0001$; Figure 5a) although embryo mass did not change. In *G. nobilis* mean brood size decreased as embryo mass increased ($F_3 = 6.33$, $P = 0.0007$; Figure 5b). Brood size varied between species with *G. affinis* incubating a significantly larger number of embryos than *G. nobilis* ($t_{72.74} = 8.57$, $P < 0.0001$; Table 3). In both species brood size and female mass were positively correlated, but the regression lines differed in slope ($F_{58} = 5.54$, $P = 0.02$). The species differed in brood size at a standard female mass ($F_{59} = 69.4$, $P < 0.0001$). In the laboratory study brood size at birth also differed between the two species with *G. affinis* females (mean = 18.97 $\pm$ 8.21 embryos) having larger brood sizes than *G. nobilis* females (mean = 6.28 $\pm$ 1.25 embryos; $t_{38.67} = 3.15$, $P = 0.004$).

Gambusia affinis had significantly larger brood mass than *G. nobilis* ($t_{86.05} = 3.27$, $P < 0.002$; Table 3). There was a significant interaction between species and female mass as indicated by the difference in slopes of the regression lines ($F_{58} = 4.86$, $P = 0.03$). Brood mass differed between species (Least square means: *G. affinis* mean = 0.25 g, $n = 16$ broods; *G. nobilis* mean = 0.10 g, $n = 55$ broods; $F_{59} = 12.21$, $P = 0.0009$; Table 3). Maternal reproductive effort of the two species did not differ significantly when female size was controlled ($t_{42.53} = 0.72$, $P = 0.48$; Table 3).

Discussion

The two *Gambusia* species studied here experience different temperature, salinity and community regimes in their respective contemporary habitats at Bitter Lake National Wildlife Refuge. *Gambusia affinis* was found in ephemeral bodies of water with higher mean temperatures and salinity as well as greater fluctuations in these factors over the breeding

season than that of the stenothermal and stenohaline spring-fed habitats of *G. nobilis*. The fish-community in *G. affinis* habitats as well as the predator community assemblage was more varied than that of *G. nobilis***.** Relative maternal reproductive effort for the two species was similar, but they differed in the allocation of resources to the size and number of offspring and number of broods per year (Figure 6). Females of both species invested the same relative amount of energy to reproduction per brood, but the nature of this investment was different. Female *G. nobilis* invested in smaller broods of larger embryos and *G. affinis* invested in larger broods of smaller embryos. *Gambusia nobilis* neonates weighed twice as much as those of *G. affinis*. As brood size in *G. nobilis* decreased over the breeding season, females produced larger embryos. *Gambusia affinis* brood size also decreased over the season, but embryo mass did not change. This is one of the more striking patterns emerging from this study and we suggest this was likely a result of the smaller (i.e. younger) *G. affinis* females reproducing later in the season.

Although these two fishes are found in very different habitats at our study site we cannot be sure these contemporary environments are reflective of the habitats in which they evolved. However, our results seem to fit the expectation of divergence in life history characteristics to optimize survival and reproduction in differing local environments (Schluter 2000). Trade-offs in life history strategies may vary in the face of these different environmental gradients. Differential investments can shape divergence in mortality rates between the two habitats. Temperature can greatly influence life histories of livebearing fishes, particularly with respect to juvenile growth rates. Juvenile poeciliid fishes may grow faster when fed ad libitum in warmer temperatures (i.e. 30°C; Wurtsbaugh and Cech Jr. 1983). Above 30°C, growth may be somewhat inhibited but maturity is achieved faster

(Snelson Jr. 1989). Although temperature can have a profound impact on life histories of aquatic ectotherms (Hochachka & Somero 1984), other environmental characteristics probably act in concert with temperature to shape these patterns. The higher temperatures in these shallow pools may have allowed for high productivity, often leading to high levels of competition and predation. Organisms in such eurythermal environments tend to have fast growth rates and small size at sexual maturity (Stearns 1977). In contrast, stenothermal environments often are less productive but do not experience the fluctuations in water quality associated with eurythermal aquatic habitats. In addition, due to lower productivity there may be fewer predators (Worm et al. 2002). Organisms living in such relatively stable but less productive environments typically have slow growth rates, low mortality, iteroparous reproduction, and long lifespans (Stearns 1977). *Gambusia affinis* and *G. nobilis* life histories in this study were similar to those predicted for these scenarios. The difference in abiotic habitats between *G. affinis* (ephemeral, shallow bodies of water and streams) and *G. nobilis* (unique, spring-fed pools) indicated that temperature may be a strong driver of life history divergence in this system.

Salinity is another factor that may have a significant impact on life history traits of fishes. *Gambusia holbrooki*, the eastern mosquitofish will increase reproductive investment at a cost to somatic condition in habitats with higher salinity (Alcaraz & Garcia-Berthou 2007). Trexler (1997) also demonstrated that sailfin mollies, *Poecilia latipinna*, raised in a low salinity environment with low food provisioning invested in larger embryos. As demonstrated by these studies, food availability has a strong interactive effect with salinity and temperature on life history characteristics of the poeciliid fishes (Reznick & Bryga 1987). Our maternal investment pattern results for both species are consistent with patterns

reported in the literature (Reznick 1983; Meffe 1985; Vondracek et al. 1988; Downhower et al. 2000).

Predation pressure and its influence on differential mortality have been shown to contribute to the evolution of life history characteristics in poeciliids (Reznick 1982; Rodd & Reznick 1997; Walsh & Reznick 2008; Reznick et al. 2008). These predators include a greater number of piscivorous fishes, wading birds and water snakes. Conspecific predation in the form of maternal cannibalism by *G. affinis* females may also have a selective role on offspring. Although we have rarely observed cannibalism of neonates by *G. nobilis* females in laboratory populations, cannibalism by *G. affinis* mothers of young neonates has been extensively reported (Dionne 1985; Hubbs 1991; Benoît et al. 2000). Reznick (1982) demonstrated that guppies, *Poecilia reticulata* found in habitats with greater predation risk have shorter interbrood intervals, early sexual maturity and smaller embryo sizes. Our findings for *G. affinis* populations are consistent with this pattern.

Smaller parental investment per embryo by *G. affinis* may be an adaptive response to locally high temperatures as we expect high growth rates and high juvenile mortality in these environments. By most reports, *G. affinis* individuals live approximately one year (Baird and Girard 1853; Stearns 1983), suggesting an inverse relationship between longevity and growth rate. The mean size of *G. affinis* females was larger earlier in the season and smaller later in the season, suggesting a turnover in the size class (presumably correlated with age) of breeding females from large (2-yr old) to small (1-yr old) females. If size is an accurate indicator of age, then females that bred at the start of the season are most likely 2 yr-old individuals. If temperature and productivity have such a profound effect on growth rates, then *G. nobilis* females may grow more slowly but are more likely long-lived. As survival

rates are probably higher for *G. nobilis* than *G. affinis*, later maturity in the face of slow growth rates may increase female fitness, as the female will be larger and more fecund. We do not have data on female survivorship in the field. However, our size data show that at the start of the season *G. nobilis* females fall into two size classes (small and large; Fig. 3) by the end of the breeding season, all females are in the large size class. We speculate that *G. nobilis* females are living at least three years and reproducing at least two years. Further support for the longevity of *G. nobilis* is demonstrated by the cessation of egg production in females by August, presumably in order to invest in their own somatic tissue presumably to store fat for the winter.

The two species in this study differed in life history traits. Within one breeding season, *G. nobilis* may only manage to give birth to two broods whereas *G. affinis* may give birth to up to five. The high reproductive potential of *G. affinis* allows them to colonize and spread rapidly in new environments (Meffe & Snelson, Jr. 1989). When longevity is factored in, lifetime reproductive investment normalized to adult size may be similar between the two species (Charnov et al. 2007).

If the competitive advantage of *G. affinis* is related to its exploitation of warm, highly productive, ephemeral habitats then it might lose such a competitive edge in the cool, spring-fed habitats of *G. nobilis*. Our data suggest that concern over *G. affinis* invasion into *G. nobilis* habitat may not be threatening to the latter but further study is required. Life history patterns may be plastic and shift in new environments. The *G. affinis* populations we studied here are found on the western most edge of their distribution (Wischnath 1993). Different patterns may be found across the range of this species as their habitat types vary widely (Pyke 2005). Other forces such as genetic drift may be responsible for the current differences

we see in the life history strategies of these two species. Although there are many studies on the evolution poeciliid life histories in response to biotic pressures such as those by Reznick et al. (2008) and others (e.g. Stearns 1983; Meffe 1985; Snelson 1989; Downhower et al. 2000; Langerhans et al. 2004) these studies are mostly concerned with tropical or subtropical fishes. But, there is a dearth of information about the response in life history strategy to environmental changes, particularly in temperate fishes (but see Vondracek et al. 1988; Hubbs et al. 2001). In temperate poeciliid systems such as ours we expect abiotic factors such as temperature to play a large role in life history evolution. Further, comparative and experimental research is needed to elucidate such patterns and determine if, in fact, these life history strategies are adaptive to contemporary environments.

Acknowledgements

Co-author for the *in revision* version of this manuscript is A. Kodric-Brown. We thank J.A, Baker, E.L. Charnov & J.C. Trexler for comments on the conceptual presentation of this work. J.H. Brown, M.J. Ryan, & B.O. Wolf provided editorial comments on the manuscript. L. Snyder, T. Kennedy C.L. Hayes, A. Hung, S. Netz, A. Olson & G. Warrick assisted with data collection. This work was funded by a grant through the New Mexico Department of Game and Fish Share with Wildlife Program to A. Kodric-Brown and D. Swenton, and a National Science Foundation Doctoral Dissertation Improvement Grant, an Alvin and Caroline Groves Research Scholarship (UNM Biology Department), Crawford Scholarship (UNM Biology Department), SRAC grant (UNM Graduate and Professional Student Association, Student Resource Allocations Committee) and GRAC grant (UNM Biology Department, Graduate Resource Allocations Committee) to D. Swenton. Experiments were conducted in accordance with University of New Mexico (UNM)

Institutional Animal Care and Use Protocol number 04MCC006. *Gambusia nobilis*, a federally endangered species, were acquired, maintained and tested in accordance with NM state permit 2968 and federal permit TE676811-0.

References

Alcaraz C, García-Berthou E (2007) Life history variation of an invasive fish (*Gambusia holbrooki*) along a salinity gradient. Biol Cons 139:83–92.

Baird SF, Girard C (1853) Descriptions of new species of fishes collected by Mr. John H. Clark, on the U.S. and Mexican boundary survey, under Lt. Col. Jas. D. Graham. Proc. Acad. Nat. Sci. Phila 6:387–390.

Benoît HP, Post JR, Barbet AD (2000) Recruitment dynamics and size structure in experimental populations of the mosquitofish, *Gambusia affinis*. Copeia 2000:216-221

Borowsky RL (1984) The evolutionary genetics of *Xiphophorus*. In: Turner, BJ (ed) Evolutionary genetics of fishes. Plenum Press, New York, pp. 235-310.

Charnov EL, Warne R & Moses M (2007) Lifetime reproductive effort. Am Nat 170:E129-E142.

Courtenay Jr. WR, Meffe GK (1989) Small fishes in strange places: A review of introduced poeciliids. In: Meffe GK, Snelson Jr. FF (eds) Ecology and evolution of livebearing fishes (Poeciliidae). Prentice Hall, New Jersey, pp 319-331.

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland.

Crivelli AJ (1995) Are fish introductions a threat to endemic fresh-water fishes in the northern Mediterranean region? Biol Cons 72:311-319.

Dionne M (1985) Cannibalism, food availability, and reproduction in the mosquitofish (*Gambusia affinis*): a laboratory experiment. Am Nat 126:16-23.

Downhower, JF, Brown, LP, Matsui ML (2000) Life history variation in female *Gambusia hubbsi*. Environ. Biol. Fishes 58:415–428.

Echelle AA, Echelle AF (1980) Status of the Pecos Gambusia, *G. nobilis*. Endangered Species Report No. 10:1-73, U.S. Fish and Wildlife Service, Albuquerque, New Mexico.

Echelle AF, Echelle, AA (1986) Geographic variation in morphology of a spring-dwelling desert fish, *Gambusia nobilis* (Poeciliidae). Southwest Nat 31:459-468.

Farr JA, Travis J (1986) Fertility advertisement by female sailfin mollies, *Poeciliia latipinna* (Pisces: Poeciliidae). Copeia 1986:467-472.

Galat DL, Robertson B (1992) Response of endangered *Poeciliopsis-occidentalis sonoriensis* in the Rio-Yaqui drainage, Arizona, to introduced *Gambusia affinis*. Environ Biol Fishes 33:249-264.

Hochachka PW, Somero GN (1984) Biochemical adaptation. Princeton University Press, Princeton.

Hubbs C (1991) Intrageneric "cannibalism" in *Gambusia*. Southwest Nat 36:153–157.

Hubbs C (2001) Environmental correlates to the abundance of spring-adapted versus stream-adapted fishes. Tex J Sci 53:299-326.

Hubbs C, Springer VG (1957) A revision of the *Gambusia nobilis* species group, with descriptions of three new species, and notes on their variation, ecology and evolution. Tex J Sci 9:279-327.

Kallman, KD (1975) The platyfish, *Xiphophorus maculatus*. In: King RC (ed) Handbook of genetics, volume 4. Plenum Publishing Corporation, New York, pp. 81-132.

Krumholz LA (1948) Reproduction in the western mosquitofish, *Gambusia affinis affinis* (Baird & Girard), and its use in mosquito control. Ecol Mono 18:1-43.

Langerhans RB, Layman CA, Shokrollahi AM, DeWitt TJ (2004) Predator-driven phenotypic diversification in *Gambusia affinis*. Evolution 58:2305–2318.

Meffe GK (1985) Life history patterns of *Gambusia marshi* (Poeciliidae) from Cuatro Ciénegas, Mexico. Copeia 1985:898-905.

Meffe GK, Snelson Jr. FF (1989) An ecological overview of poeciliid fishes. In: Meffe GK, Snelson Jr. FF (eds) Ecology and evolution of livebearing fishes (Poeciliidae). Prentice Hall, New Jersey, pp 13-31.

Pyke, GH (2005) A review of the biology of *Gambusia affinis* and *G. holbrooki.* Rev Fish Bio Fish 15:339-365.

Reznick DN (1982) The impact of predation on life history evolution in Trinidadian guppies: genetic basis of observed life history patterns. Evolution 36:1236-1250

Reznick DN (1983) The structure of guppy life histories: the tradeoff between growth and reproduction. Ecology 64:862-873.

Reznick DN, Endler JA (1982) The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution 36:160-177.

Reznick DN, Bryga H (1987) Life-history evolution in guppies (*Poecilia reticulata*): 1. Phenotypic and genetic changes in an introduction experiment. Evolution 41:1370-1385.

Reznick DN, Ghalambor CK, Crooks K (2008) Experimental studies of evolution in guppies: a model for understanding the evolutionary consequences of predator removal in natural communities. Mol Ecol 17:97-107.

Rodd FH, Reznick DN (1997) Variation in the demography of guppy populations: the importance of predation and life histories. Ecology 78:405-418.

Rosen DE, Bailey RM (1963) The poeciliid fishes (Cyprinodontiformes), their structure, zoogeography and systematics. Bull Am Mus Nat Hist 126:1-176.

Rundle HD, Nosil P (2005) Ecological speciation. Ecol Lett 8:336–352.

Schluter D (2000) Ecological character displacement in adaptive radiation. Am Nat 156:S4-S16.

Scribner KT, Avise JC (1994) Population cage experiments with a vertebrate: the temporal demography and cytonuclear genetics of hybridization in *Gambusia* fishes. Evolution, 48:155-172.

Smith RB (1986) The effect of food on reproduction in the sailfin molly, *Poecilia latipinna* (Poeciliidae). M. S. Thesis, University of Central Florida.

Snelson Jr. FF (1989) Social and environmental control of life history traits in poeciliid fishes. In: Meffe GK, Snelson Jr. FF (eds) Ecology and evolution of livebearing fishes (Poeciliidae) Prentice Hall, New Jersey, pp. 149-161.

Stearns SC (1977) The evolution of life history traits: a critique of the theory and a review of the data. Annu Rev Ecol Syst 8:145-171.

Stearns SC (1983) A natural experiment in life-history evolution: field data on the introduction of mosquitofish (*Gambusia affinis*) to Hawaii. Evolution 37:601-617.

Stearns SC (1992) The evolution of life histories. Oxford University Press, Oxford.

Trexler J (1997) Resource availability and plasticity in offspring provisioning: embryo nourishment in sailfin mollies. Ecology 78:1370–1381.

Vondracek B, Wurtsbaugh WA, Cech Jr. JJ (1988) Growth and reproduction of the mosquitofish, *Gambusia affinis*, in relation to temperature and ration level: consequences for life history. Environ Biol Fishes 21:45-57.

Walsh MR, Reznick DN (2008) Interactions between the direct and indirect effects of predators determine life history evolution in a killifish. Proc Natl Acad Sci U S A 105:594-599.

Wischnath L (1993) Atlas of Livebearers of the World. Tropical Fish Hobbyist Publications, Inc., Neptune City.

Worm B, Lotze HK, Hillebrand H, Sommer, U (2002) Consumer versus resource control of species diversity and ecosystem functioning. Nature 417:848-851.

Wourms, JP (1981) Viviparity: maternal - fetal relationships in fishes. Amer Zool 21:473-515.

Wurtsbaugh WA, Cech Jr. JJ (1983) Growth and activity of juvenile mosquitofish: temperature and ration effects. Trans Am Fish 112:653-660

Yan HY (1987) Size at maturity in male *Gambusia affinis*. J Fish Biol 30:731-741.

Tables and Figures

Table 1. Fish community assembly data in habitats of Gambusia nobilis and G. affinis during the 2008 breeding season. X marks species presence in habitat. Species abbreviations stand for: Astyanax mexicanus = AM; Cyprinodon carpio carpio = CC; Cyprinella lutrensis = CL; Cyprinodon pecosensis = CP; Dionda episcopa = DE; Gambusia affinis = GA; Gambusia nobilis = GN; Fundulus zebrinus = FZ; Lepomis cyanellus = LC; Lucania parva = LP.

	G. nobilis habitats						*G. affinis* habitats								
Species	SH37	SH7	SH20	SH27N	SH27S	Lost Spring	BC3	U5N	OBB	OB	Blind	15MPH	SH3	FB	SC
AM														X	X
CC								X	X					X	X
CL			X					X						X	X
CP	X	X	X		X	X	X	X					X	X	X
DE						X			X	X					
FZ	X	X													
GA							X	X	X	X	X	X	X	X	X
GN	X	X	X	X	X	X									
LC														X	X
LP									X	X	X	X			

Table 2. Percentage of males, females and juveniles in populations of *Gambusia nobilis* and *G. affinis* in allopatric habitats over the 2008 breeding season at Bitter Lake National Wildlife Refuge.

Species / % Class	May	June	July	August
Gambusia nobilis (N=6 habitats)	(N=284)	(N=83)	(N=633)	(N=226)
% Females	70	82	40	65
% Males	19	10	11	15
% Juveniles	11	8	49	20
Gambusia affinis (N=9 habitats)		(N=291)	(N=71)	(N=100)
% Females	-	13	55	53
% Males	-	49	6	37
% Juveniles	-	38	39	10

Table 3. Brood characteristics for *G. affinis* and *G. nobilis* from dissected museum specimens.

Brood characteristic	*G. nobilis* *n*	Mean (±SE)	*G. affinis* *n*	Mean (±SE)
Brood mass (g)	55	0.10 (0.01)	16	0.25 (0.04)
Brood Size	77	11.17 (0.63)	65	34.48 (2.59)
Stage 2 embryo mass (g)	55	0.01 (0.001)	16	0.006 (0.001)
Maternal reproductive effort	55	0.39 (0.05)	16	0.44 (0.06)

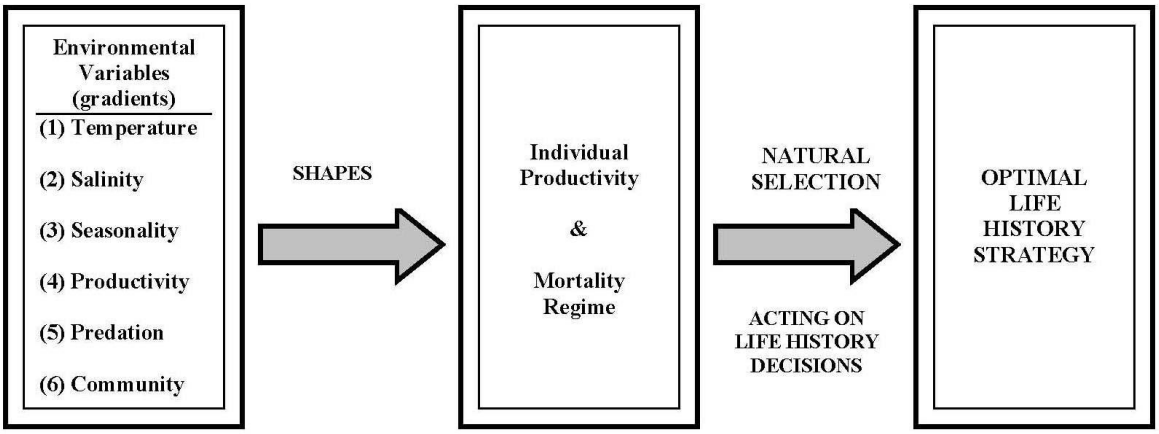


Figure 1. Schematic diagram depicting the evolution of an optimal life history strategy. Environmental characteristics are considered in shaping individual life history phenotypes on which natural selection acts.

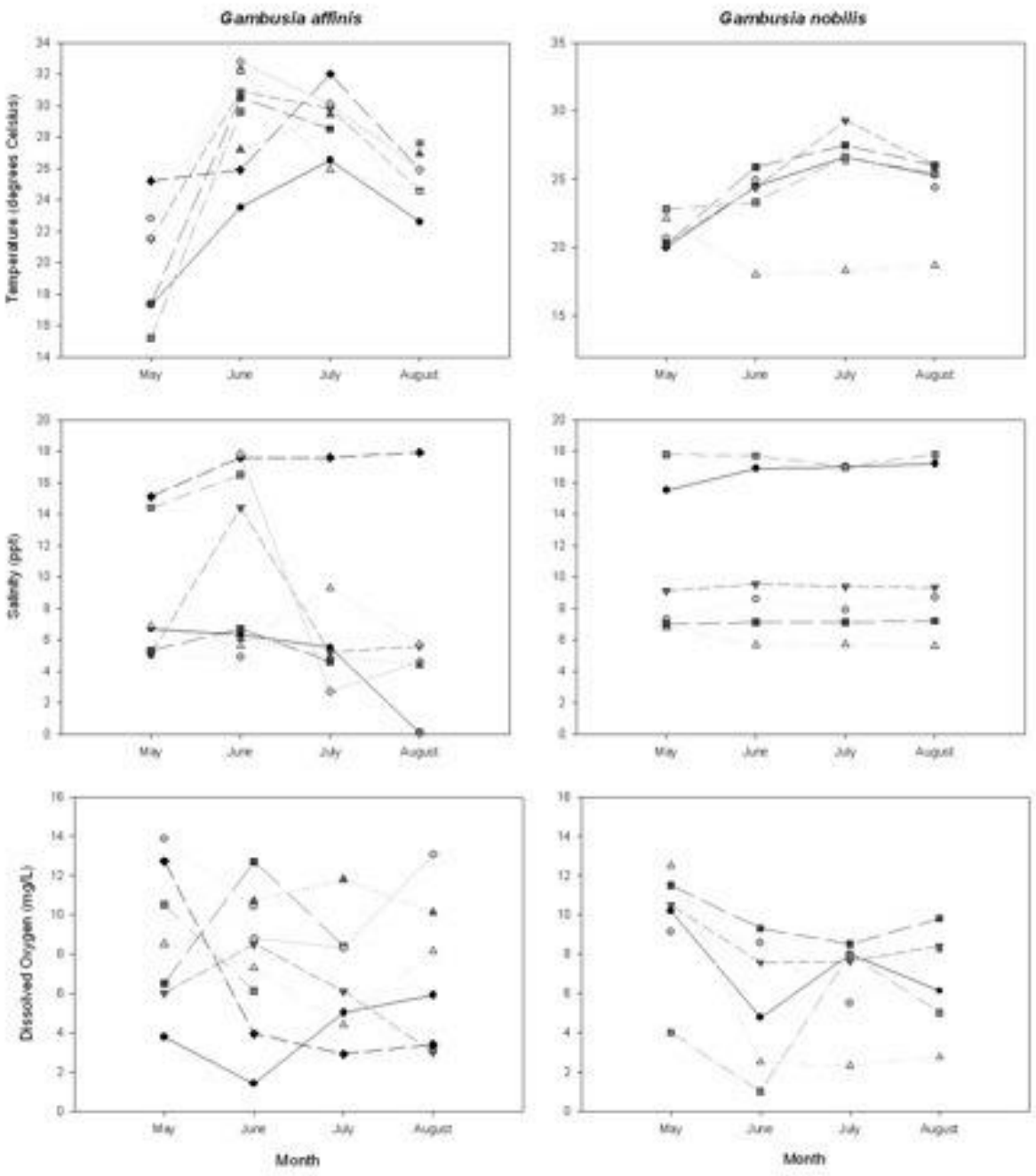


Figure 2. Abiotic habitat characteristics for both species during 2008. Gambusia affinis habitats are in the left column and G. nobilis in the right. Temperature (°C) reading for each sampled habitat (top). Salinity (ppt) reading for each sampled habitat (middle). Dissolved Oxygen (mg/L) reading for each sampled habitat (bottom).

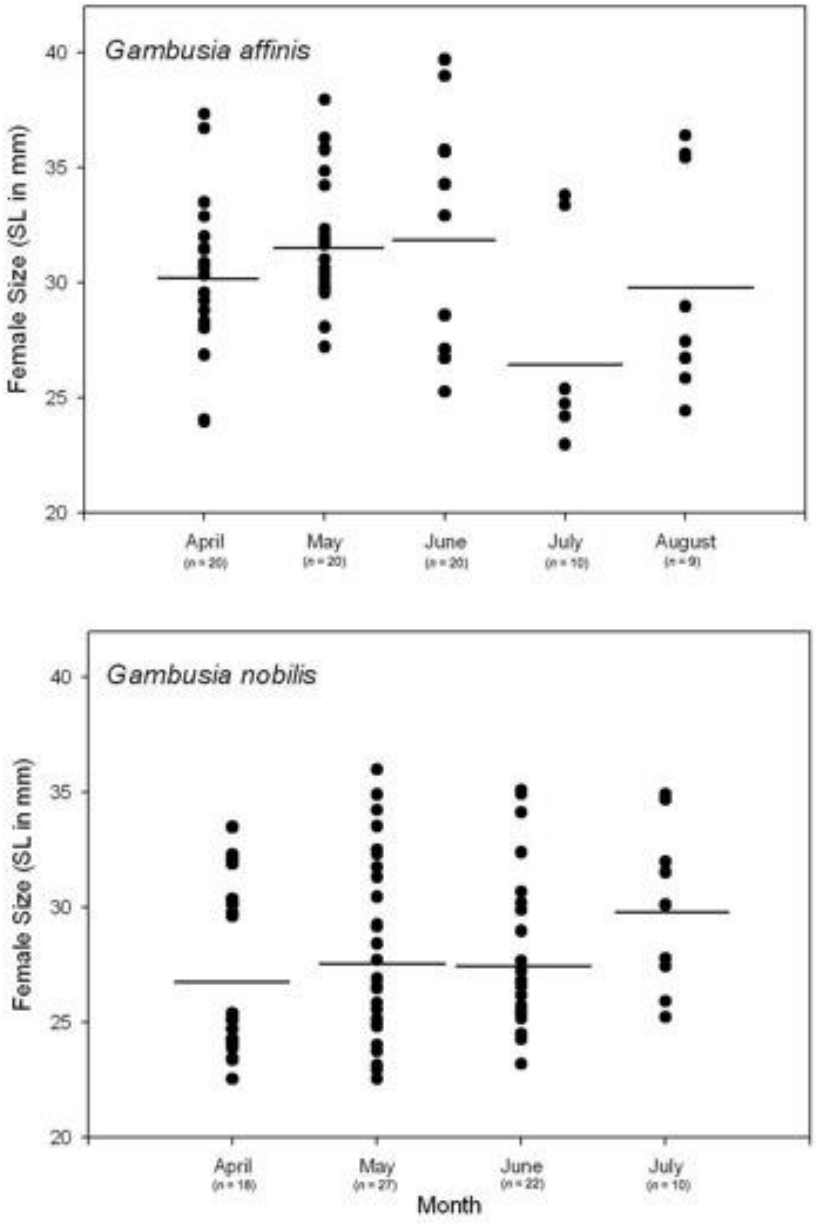


Figure 3. (a) Standard length (mm) distribution of females over the breeding season from museum specimens of Gambusia affinis. (b) Standard length distribution of females over the breeding season from museum specimens of G. nobilis. Cross lines represent the mean female standard length. Sample sizes are in parentheses.

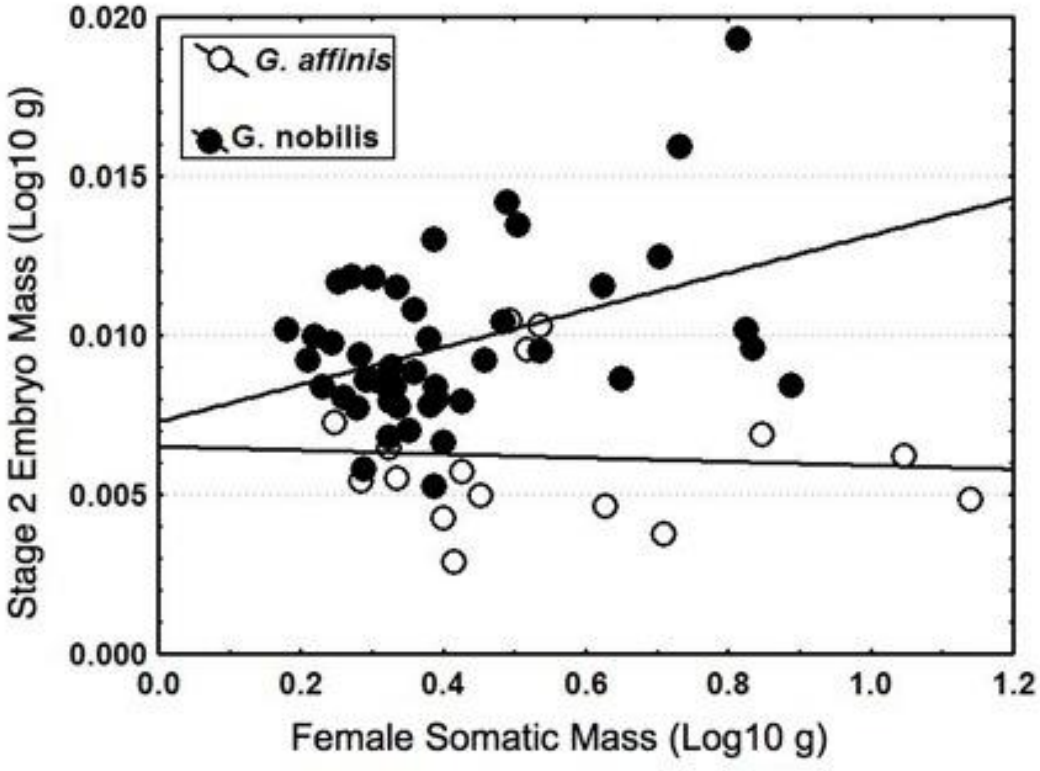


Figure 4. Plot of log female somatic mass (g) by log average embryo mass in her brood (g). Open circles represent Gambusia affinis individuals and filled circles are G. nobilis. All samples are from the museum specimen dissections.

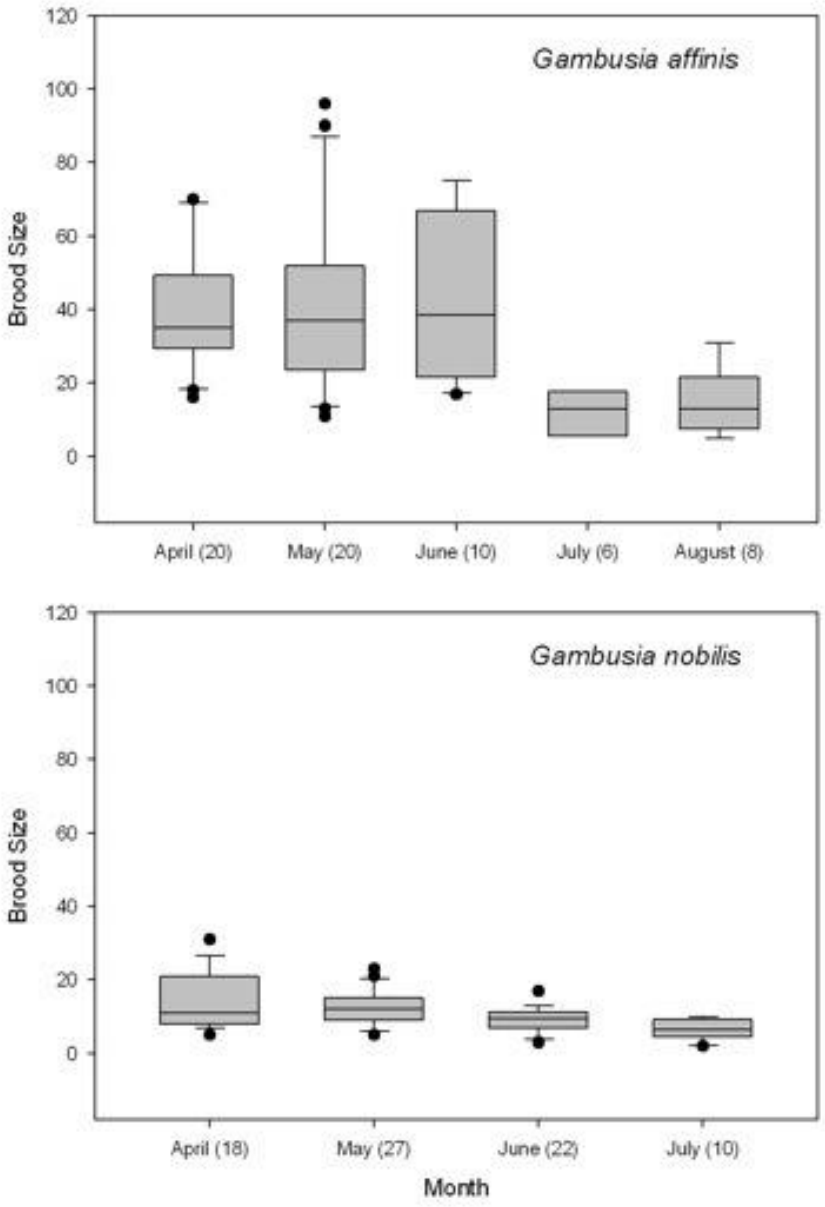


Figure 5. (a) Box plot of brood size from each brood from Gambusia affinis females over the breeding season. (b) Box plot of brood size from each brood from Gambusia nobilis females over the breeding season. All samples are from the museum specimen dissections. Sample sizes are in parentheses.

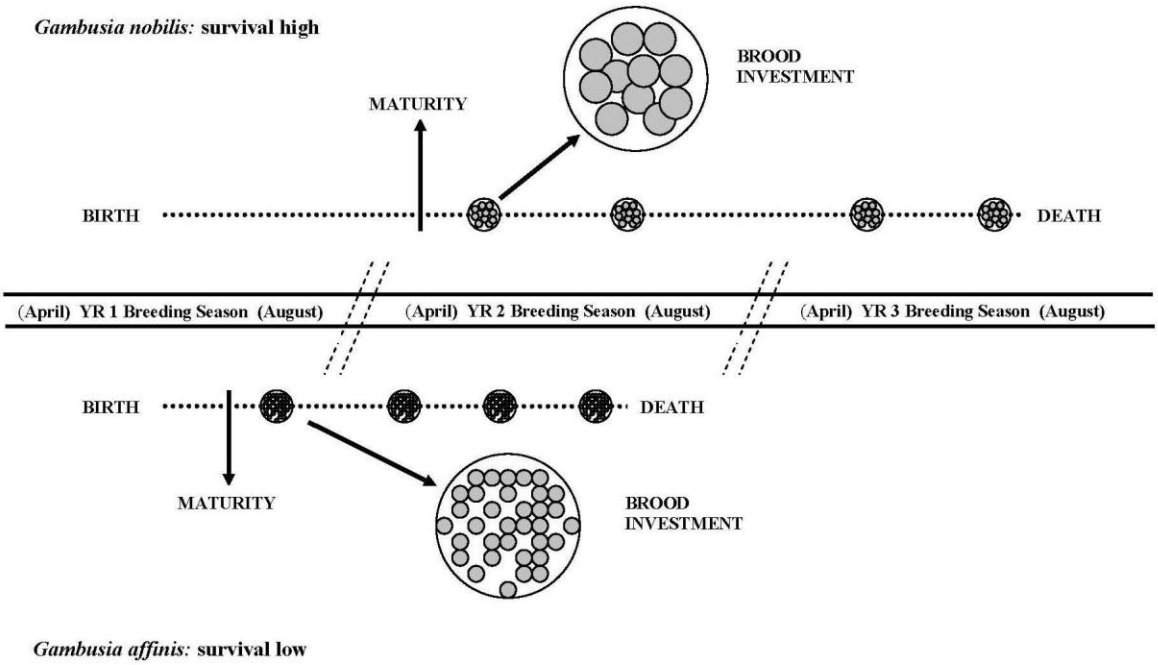


Figure 6. An individual's life history by species. The lifetime reproductive strategy of a Gambusia affinis female (top) and a G. nobilis (bottom) female are depicted over a typical lifetime.

Chapter 3: The Use of $\delta^{15}N$ and $\delta^{13}C$ to Assess Feeding Niche Space of Fishes in a Desert Spring System

Abstract

We investigated the variability in feeding niches of fishes in twelve desert aquatic habitats using stable isotope analysis. We assessed habitats and measured values of $\delta^{13}C$ and $\delta^{15}N$ for plants, macroinvertebrates, and fishes to determine (1) how habitats differ in water quality; (2) if there are differences between fishes in feeding niches across habitats; (3) how trophic niche of the threatened *Cyprinodon pecosensis* varies with biotic and abiotic factors; and (4) if there are dietary differences between *Gambusia nobilis* and *G. affinis* and between the sexes of the sexually dimorphic *Gambusia* species. Habitats varied predictably in water quality and fish assemblages. We found the greatest differences between sinkhole (spring-fed) and stream habitats. Sampling localities with greater biological complexity had lower salinity, temperature and conductivity. Fish values of $\delta^{13}C$ varied between populations as a function of differences in carbon inputs although the relative contribution of aquatic and terrestrial primary producers could not be differentiated. Values of $\delta^{15}N$ varied by community complexity after controlling for $\delta^{15}N_{base}$ differences between sampling localities. $\delta^{13}C$-$\delta^{15}N$ ecological space differed between sinkholes and stream habitats of similar complexity revealing abiotic factors such as differential salinity levels influence food web structure. The isotopic niche of *C. pecosensis* shifted to a higher trophic level and was more depleted in $\delta^{13}C$ but did not expand in simple assemblages. *Gambusia nobilis* fed at a relatively higher trophic level than the invasive *G. affinis* and individuals from their putative

hybrid zone. *Gambusia nobilis* males and females had similar values of $\delta^{15}N$ isotope, although juveniles differed significantly from both, feeding at a lower trophic level.

Introduction

The ecological niche that an organism occupies within a food web is influenced by both biotic factors such as competitive interactions and abiotic factors including temperature. Variation in niche space on spatio-temporal scales may be understood by characterizing these factors (Bearhop et al. 2004). Stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ provide a potentially informative method for assessing trophic interactions and dietary sources. They may be used to elucidate patterns of food web dynamics that are easily overlooked using traditional gut content analyses (Rounick and Winterbourn 1986). $\delta^{13}C$ and $\delta^{15}N$ as well as other isotope values (e.g. H, S, O) may be plotted in Cartesian space to create a δ-space that may provide insight into the ecological niche of an organism (Vander Zanden et al. 2003). $\delta^{13}C$ measurements may allow for the discrimination among energy sources if the sources differ significantly in their values of $\delta^{13}C$ (e.g. terrestrial or aquatic, C3 or C4 plants; DeNiro & Epstein 1981; Minigawa & Wada 1984; Peterson & Fry 1987; Thorp et al. 1998). Measurement of $\delta^{15}N$ in consumer tissues, in contrast, may allow for the inference of food web structure due to its step-wise fractionation at each trophic level (Vander Zanden & Rasmussen 2001). These 'isotope' niche spaces are represented in a bivariate plot with $\delta^{13}C$ for energy source and $\delta^{15}N$ for trophic height. The inter- and intraspecific individual variance in these coordinates may be used to assess niche breadth and trophic position (Bolnick et al. 2002; Bearhop et al. 2004).

$\delta^{13}C$-$\delta^{15}N$ biplots have been increasingly used in studies of fishes as a quantifiable indicator of ecological niche and as a useful tool to document changes in community structure. For example, they have been used to examine competitive interactions (Syväranta & Jones 2008), measure movement patterns (Zeug et al. 2009), and characterize the overall food web structure of aquatic habitats and fish assemblages therein (Vadeboncoeur et al. 2003; Campbell et al. 2005; Schaal et al. 2009; Turner et al. 2010). Many of these stable isotope studies of fish food webs have focused on a few, large bodies of water or on highly connected aquatic habitats such as marine habitats, estuaries, rivers and lakes. Small, natural replicates harboring species of endemics are virtually unstudied. Examination of such systems is particularly important in the face of rapid and increasing anthropogenic alteration of diminishing water resources and the communities therein. Endemics native to small and isolated aquatic habitats are at increased risk of extinction from decreasing water tables, increased eutrophication via human inputs and introduction of non-native species (Williams et al. 1985; Courtenay Jr. & Meffe 1989; Warren & Burr 1994; Kodric-Brown et al. 2007; Gumm et al. 2008). Fishes that occur in desert springs are particularly sensitive to such disturbances (Kodric-Brown & Brown 1993; Kodric-Brown et al. 2007). These spring systems are comprised of a series of small, aquatic habitats with simple communities. These habitats provide natural replicates with which to examine questions regarding variation in fish assemblages without experimental manipulation.

Studies of desert spring fish assemblages such as those by Kodric-Brown et al. have mostly been concerned with extinction risk and persistence of the threatened desert fishes (Kodric-Brown & Brown 1993; Kodric-Brown et al. 2007). Very little is known about these springs and the source of primary productivity that underlies the trophic structure of fish

assemblages therein is of particular interest. The characterization of feeding niches and trophic guilds of threatened desert fishes may contribute to their conservation (Schoenly & Cohen 1991). Sensitive species, such as desert fishes may have highly specific habitat or prey requirements (Hardy et al. 2010). Some researchers have found that indigenous fishes have a strong competition and a high degree of niche overlap whereas some non-native species may exhibit more omnivory, shifts to lower quality diets and broader dietary niches (Echelle et al. 1972; Brun et al. 1990; Declerck et al. 2002). Abiotic factors can also influence the dynamics of a given community. For example, salinity may alter fish assemblages as a function of species-specific tolerances (Echelle et al. 1972; Winemiller & Leslie 1992). In this study we used a desert spring system and examined the variation of feeding niches of several assemblages of fishes using stable isotope analysis.

The field site for this study, Bitter Lake National Wildlife Refuge (BLNWR; Fig. 1) has high habitat diversity at a relatively small spatial scale (9929.37 hectares) providing a tractable system to study the effects of biotic and abiotic factors on feeding niche. There are a number of distinct habitat types with variable water quality including marshes, drainage ditches, creeks, and over 70 spring-fed gypsum sinkholes. What makes this area particularly interesting is the biological diversity across these habitats. Fish assemblages range from one to six species and several localities contain the same fish assemblages. In this investigation we evaluated patterns in $\delta^{13}C$-$\delta^{15}N$ isotope values of fishes as a function of habitat type. We focused on characterizing the feeding niches of two of the most abundant (yet globally endangered) fish species, *C. pecosensis* and *G. nobilis.* We investigated if the trophic level as represented by isotope values of these two species varies between sampling localities as a function of water quality and assemblage complexity. For example, we examined if $\delta^{13}C$-

$\delta^{15}N$ values of fishes vary between sinkhole and stream habitats on BLNWR. We also examined potential niche overlap between the endangered *G. nobilis* and its invasive congener *G. affinis*, western mosquitofish. Additionally, *Gambusia* are sexually size dimorphic, which may result in dietary differentiation between the sexes. Here we ask, specifically: (1) do habitats vary by abiotic factors and primary production and in baseline $\delta^{13}C$-$\delta^{15}N$ levels? (2) Are there differences in $\delta^{13}C$-$\delta^{15}N$ values of common fish species on BLNWR across habitats? (3) Does the trophic niche of *C. pecosensis* expand in the absence of competitors? (4) Are there dietary differences between *Gambusia* congeners and *Gambusia* sexes?

Methods

Study site. The study was conducted at BLNWR**,** 17 km east of Roswell, NM (N 33.6023141° W 104.4119131° 56.474; Fig. 1). We collected tissue from fishes (i.e. caudal fin) to evaluate patterns in $\delta^{13}C$-$\delta^{15}N$ isotope values as a function of habitat type. We also collected samples of macroinvertebrates (whole body), and plants in order to characterize the available ecological niche space in each sampling locality as well as the baseline carbon and nitrogen levels. We collected fish, macroinvertebrate, and plant samples from 12 localities in 2008. Ten sampling localities were gypsum sinkholes (SH 7, 10, 18, 19, 20, 26, 27N, 27S, 37, and Lost Spring;). In addition, we sampled the Oxbow at Hunter's Marsh in the southernmost portion of BLNWR, and the weir at Bitter Creek, a hybrid zone of *Gambusia nobilis* and *G. affinis*. Both of these sampling localities were representative of shallow stream habitats on the refuge. We sampled fishes and primary producers from sampling localities in May, June and July, which span a significant portion of the reproductive season of these fishes (Swenton et al. *accepted*). We measured temperature, dissolved oxygen, conductivity,

and salinity with a YSI© meter (YSI corporation) at each site and during each sampling period between 07:00-09:00 hours.

Sample collection. *Fishes:* At each site one minnow trap was set without bait (to avoid complications in the stable isotope study) within a meter of the shoreline to ensure the highest densities of fishes were collected. Traps were retrieved two to three hours later and all fish were identified to species, measured for standard length, counted and a small (approximately $1cm^2$) caudal fin clip was clipped with dissection scissors from at least five fish from each species present at each site during every sampling period. Fish fin tissue has been shown to have a rapid stable isotope turnover rate and shifts in diet may be detected in a matter of weeks (McIntyre & Flecker 2006). Fish were then released. Fin clips (n = 500) were preserved individually in vials of 95% ethanol. The six most common species sampled were western mosquitofish, *Gambusia affinis*; Pecos Gambusia, *G. nobilis; G. affinis* x *G. nobilis* individuals from the putative hybrid zone; Pecos pupfish, *Cyprinodon pecosensis*; red shiner, *Cyprinella lutrensis*; plains killifish, *Fundulus zebrinus*; and roundnose minnow, *Dionda episcopa*. A subset of each *Gambusia* species and their hybrids was sexed (male, female, juvenile). Males were identified by the presence of the gonopodium, a modified anal fin that functions as an intromittent organ and is used to inseminate the livebearing females. Females were identified by the presence of the gonopore spot, a black patch marking the site of insemination and an indicator of gravidity (Farr and Travis 1986). Fish that lacked sexual characteristics such as a gonopodium or gonopore spot were identified as juveniles.

Macroinvertebrates: Aquatic and habitat associated macroinvertebrates were collected in order to quantify maximum isotopic niche space in a habitat. The macroinvertebrates were caught by dipnet in and around the shoreline of the habitat where

the minnow trap was set. Those found in minnow traps were also used. Macroinvertebrates were taken to the lab, identified to the lowest feasible taxonomic level, typically family, and preserved in 95% ethanol (n = 316). Taxonomic groups identified included: Hydrophilidae, Naucoridae, Corixidae, Odontidae, Anisoptera, Hyallela, Zygopterae, Dytiscidae, and Belostomatidae. In addition we collected mollusks from the family Physidae, and annelids from the family Hirudinidae.

Plants: To obtain food web baseline nitrogen and carbon levels we collected plant material from in and around sampling localities. Fish may feed directly on plants in the water column or else herbivorous invertebrates that fall into the water and the isotopic signatures reflect these carbon inputs. We collected plant samples haphazardly by hand within 2 meters of the shoreline at each site. Terrestrial (e.g. pickleweed, *Tamarisk*, and *Phragmites* spp.), emergent, submerged aquatic plants and algae (e.g. grass and chara, a branched, multicellular algae) were collected. Samples were preserved in 95% ethanol (n = 66).

Stable isotope analysis. All plant and fin clip samples were dried at 40°C for $\geq$ 48 hours. Dried samples were ground into a fine powder, weighed and transferred to tin capsules (Mean mass of measured sample: fishes = 0.57mg; plants = 3.58mg). Carbon isotopic composition was measured using a Finnigan Mat Delta Plus isotope ratio mass spectrometer in the University of New Mexico Earth and Planetary Sciences Mass Spectrometry Laboratory. The precision of these analyses was ± 0.1‰ SD for $\delta^{13}C$. A laboratory standard calibrated against international standards (valine $\delta^{13}C$ -26.3‰ VPDB [Vienna Pee Dee Belemnite Standard] and for $\delta^{15}N$, atmospheric nitrogen) was included on each run in order to make corrections to raw values. Stable isotope ratios are expressed using standard delta

notation (δ) in parts per thousand (‰) as: $\delta X = (R_{sample} / R_{standard} - 1)$ x 1000, where R_{sample} and $R_{standard}$ are the molar ratios of $^{13}C/^{12}C$ of a sample and standard (Sharp 2006).

Data analysis. The average $\delta^{15}N$ and $\delta^{13}C$ of plant samples was calculated for algae, chara, algae combined with chara, and all plant samples for each site by month. To account for differences in baseline nitrogen levels between sampling localities $\delta^{15}N_{base}$ was calculated for each site using the $\delta^{15}N$ from these primary producers (*see* Post 2002a for calculation). All fish $\delta^{15}N$ values used in comparisons between sampling localities were adjusted for $\delta^{15}N_{base}$ values. In addition to $\delta^{15}N$ and $\delta^{13}C$ values for all individuals, we employed three metrics to characterize and examine the isotope $\delta^{13}C$ -$\delta^{15}N$ niche of consumers within these assemblages using Layman et al. (2007): 1) $\delta^{15}N$ range; 2) $\delta^{13}C$ range; 3) mean Euclidian distance to centroid in N-C space.

We conducted statistical analyses using SYSTAT 11 (Cranes Software International Ltd.), VassarStats© (Richard Lowry, Vassar College) and STATISTICA (Statsoft, Inc.). We calculated summary statistics (means, standard errors) of plant and fish samples for $\delta^{15}N$ and $\delta^{13}C$. A two-factor ANOVA (factors of site and time) was used to test for differences between sampling localities in water quality (dissolved oxygen, temperature, salinity and conductivity). Two factor ANOVA analyses were used to test for differences in $\delta^{15}N$ and $\delta^{13}C$ among sampling localities, species across and within sampling localities (factors of site and time). We performed a nested analysis of variance on $\delta^{15}N$ and $\delta^{13}C$ values for fishes from the 9 sampling localities with *C. pecosensis* (adjusted for $\delta^{15}N_{base}$) with site nested in complexity. For differences in centroid location we calculated Euclidian distances and tested if they were significantly different from 0 (after Turner et al. 2010). Before performing parametric analyses we examined all data for normality and equality of variances. All data fit

normality criteria. When variances were unequal or there was a small sample size, non-parametric pairwise tests (Wilcoxon signed rank test) were performed.

Results

Habitats. The 12 sampling localities differed in the composition of fish assemblages (Table 1). Four sampling localities contained only one species (typically *C. pecosensis*) and one, SH27S, contained two species (*C. pecosensis* and *G. nobilis*). Seven of the surveyed habitats contained three or more species. Habitats also varied in abiotic factors (e.g. size, salinity, temperature, dissolved oxygen; Tables 2 & 3). Differences in water quality were apparent across and within sampling localities and time (Fig. 2). The two-factor analysis of variance using the interaction variance as error showed a significant main effect of time and site on dissolved oxygen levels, which decreased over time, and temperature, which increased over time. Salinity differed significantly between sampling localities but not over time. There was no significant effect of time on conductivity; site, however, had a highly significant effect (Table 3). Sampling localities that contained only one or two species had significantly higher average salinity (n = 6 sites, 18.57$\pm$2.42ppt) than sampling localities with three or more species (n = 6, 7.28$\pm$0.50ppt; t = -8.89, $p < 0.001$). The simpler assemblages also had on average higher temperatures (24.85$\pm$0.62 > 22.47$\pm$0.92; t = -2.12, p = 0.0416) and conductivity (28.5$\pm$2.53 > 12.19$\pm$0.57, t = -6.46, $p < 0.001$). There was no difference in dissolved oxygen between Sampling localities with three or more species and those with one or two species (t = -0.35, p = 0.7308).

Primary producers. There was considerable variation in $\delta^{15}N_{base}$ and $\delta^{13}C_{base}$ of primary producers, algae and chara in particular, among sampling localities and habitats (Tables 4 & 5, Appendix 1). Algae and chara had large range of values ranging from almost

no depletion in values of $\delta^{13}C$ to -17‰ VPDB (chara) and -32‰ VPDB (algae; Appendix 1). In general chara was more enriched in the heavy isotope of carbon (x = -9.49‰ VPDB) than the algae (x = -13.36‰ VPDB). We analyzed for differences in $\delta^{13}C$ and $\delta^{15}N_{base}$ values of primary producers, primarily from aquatic sources, over time and across sampling localities using a series of two-factor analyses of variance using the interaction variance as error (Table 5). The two factor-analysis of variance showed a significant effect of time on $\delta^{15}N$ values of algae. Over time the algae tended to become more enriched. There was a highly significant main effect of site on both $\delta^{15}N$ and $\delta^{13}C$ values of chara, of algae, and of algae and chara, collectively. The two factor-analysis of variance showed a significant main effect of time on $\delta^{13}C$ of chara, with increasing enrichment across time. There was a significant interaction between time and site on chara (Table 5). The results of the Wilcoxon Signed Rank tests indicated that $\delta^{13}C$ values from aquatic plant sources differed from the $\delta^{13}C$ value for the most-widespread primary consumers (*C. pecosensis*) at several sampling localities: Bitter Creek Weir ($p = 0.080$), SH 37 ($p = 0.034$), SH 20 ($p = 0.002$) and SH26 ($p = 0.008$; all over sites $p > 0.10$; no data for SH 27N). Aquatic plants were more enriched in carbon and had significantly different $\delta^{13}C$ from terrestrial plant sources ($p = 0.031$).

Fishes. *Gambusia nobilis* and *F. zebrinus* had the highest $\delta^{15}N_{base}$ values and were more enriched by 1-3‰ AIR than other species (*G. affinis, C. pecosensis,* and *C. lutrensis*). The *Gambusia* individuals from the hybrid zones had the lowest $\delta^{15}N_{base}$ values (Fig 4). $\delta^{13}C$ values of fishes did not vary as greatly as $\delta^{15}N_{base}$; however, *Gambusia* individuals from the hybrid zones were the most depleted in values of 13C (Fig 4). We analyzed for differences in $\delta^{13}C$ and $\delta^{15}N_{base}$ values of fishes over time and across sampling localities using a series of two-factor analyses of variance using the interaction variance as error (Table 6). The

ANOVA showed a significant main effect of time, site and the interaction factor of time and site on the $\delta^{15}N_{base}$ values of all fish species. The factors of time and site also had a significant main effect on the $\delta^{13}C$ values of all fishes; however, there was no effect of the time-site interaction factor. The $\delta^{15}N_{base}$ and $\delta^{13}C$ values of *C. pecosensis* differed significantly between sampling localities and over time. The $\delta^{15}N_{base}$ values of *G. nobilis* also differed significantly between sampling localities but not over time (Table 6).

We compared *C. pecosensis* isotope values between sampling localities in complex and simple assemblages. $\delta^{15}N_{base}$ values of *C. pecosensis* differed significantly between simple and complex sampling localities ($t_{142} = -4.48$, $p < 0.0001$; Fig. 3 & Appendix 4). Individuals in simple sites had higher $\delta^{15}N_{base}$ values. $\delta^{13}C$ values of *C. pecosensis* also differed significantly between simple and complex sites ($t_{142} = -3.54$, $p < 0.001$). Individuals in simple sites were more enriched in δ^{13}. The results of the nested ANOVA with site nested in assemblage type examining $\delta^{15}N$ of *C. pecosensis* (adjusted for the $\delta^{15}N_{base}$ of primary producers of each site) showed a significant effect of the factor of site on $\delta^{15}N$ ($F_{7, 135} = 19.011$, $p < 0.001$). A potential effect on $\delta^{15}N$ values of *C. pecosensis* may have been assemblage structure although this result was not statistically significant (simple vs. complex; $F_{1, 7} = 3.626$, $p = 0.098$; Fig. 3). Furthermore, there was a trend for *C. pecosensis* that occur in sampling localities with more than two fish species (SH 20, SH 37 and BCW) to be less enriched in $\delta^{15}N$ (Fig. 3) and shifted to a lower trophic level when competition is present. The $\delta^{15}N$ and $\delta^{13}C$ ranges of *C. pecosensis* within a site were smaller than that of the overall ranges across taxa within a site (Appendix 4). The results of the Euclidian distance analysis also supported the above findings; The mean Euclidean distance of the *C. pecosensis* centroid of any given site to the *C. pecosensis* centroid across all sampling localities was smaller for

simple versus complex assemblages. The mean Euclidean distance of the *C. pecosensis* centroid of any given site to the centroid of all taxa over all sampling localities was also smaller for simple assemblages. This trend was reversed, however, for any given *C. pecosensis* centroid by site when compared to the overall taxa centroid for the same site meaning *C. pecosensis* feeding niche was constrained, presumably by abiotic factors, when competition is absent (Table 7).

To examine whether trophic structure differed between the invasive *Gambusia affinis* and endangered *G. nobilis,* we repeated these models using data from only these two species. $\delta^{13}C$ differed significantly by species ($F_{1, 210} = 11.2$, $p = 0.001$), site ($F_{7, 210}=83.3$, $p < 0.01$) and time ($F_{2, 210}=6.17$, $p = 0.02$). $\delta^{15}N$ also differed significantly between *G. affinis* and *G. nobilis* (Fig. 4), as well as among sites ($F_{7, 211}=80.3$, $p < 0.01$) and time ($F_{2, 211}=22.1$, $p < 0.01$). In both cases, *G. nobilis* was more enriched than *G. affinis* in $\delta^{13}C$. *Gambusia nobilis* was also more enriched than *G. affinis* in $\delta^{15}N$. In *G. nobilis* we found significant differences across sex in $\delta^{15}N$ ($F_{2,124}=4.089$, $p = 0.019$) and $\delta^{13}C$ ($F_{2,124}=5.981$, $p = 0.003$). We performed Tukey's post-hoc tests to determine which groups differed. There was no significant difference between adult males and females in $\delta^{15}N$ (pairwise t-test, $p = 0.861$) but there was a trend towards a difference in $\delta^{13}C$ ($p = 0.063$). Males and juveniles differed in $\delta^{15}N$ ($p = 0.012$) and $\delta^{13}C$ ($p = 0.005$). Females and juveniles differed in $\delta^{15}N$ ($p = 0.020$) but not $\delta^{13}C$ ($p = 0.073$).

Community level patterns. We classified habitats by complexity of fish assemblage. We plotted the $\delta^{13}C$-$\delta^{15}N$ values of the fishes, plants and macroinvertebrates to examine the relative available ecological space available in different types of habitat. Sinkhole habitats that had three or more fish species tended to have greater $\delta^{13}C$-$\delta^{15}N$ ranges

and a greater number of species of plants and macroinvertebrates present than those with only *C. pecosensis* (Fig. 5 & Appendix 4). Those sampling localities with only one fish species, however, also had the highest salinity (≥ 19ppt). In order to examine how the utilized ecological niche space might shift as a function of abiotic rather than biotic factors we further classified complex sites with three or more fish species into sinkhole and stream type habitats. Sinkhole habitats (SH20, SH37, SH7) were spring-fed but had no surface water flow connectivity to other habitats. The salinity was between 5 and 10 ppt at these sampling localities. Stream habitats (BCW, LS, OB) had some degree of flow and were generally interconnected with other habitats. The salinity at these sampling localities was below 5 ppt. We collectively plotted the utilized niche space of plants, macroinvertebrates and fishes in a $\delta^{13}C$-$\delta^{15}N$ space to see if this ecological space may shift as a function of abiotic factors where fish assemblage complexity is held constant (stream vs. sinkhole; Fig. 6). The carbon resource for sinkhole community members is enriched in values of $\delta^{13}C$ relative to stream habitats. Organisms in the stream habitats have higher values of $\delta^{15}N$, including primary producers.

Discussion

In our study we found differences in resource use and trophic structure of fishes between isotopically distinct habitats on BLNWR. Habitats varied somewhat predictably in water quality and fish assemblage. Most notably there were clear differences between stream habitats and sinkholes. Much attention has been paid to examining energy flow in aquatic food webs and fish feeding niches as revealed by stable isotope analyses in riparian, marine and estuary habitats (e.g. Cloern et al. 2002; Syväranta & Jones 2008; Turner et al. 2010). To our knowledge, this is the first study to examine such patterns within and between spring-fed

and stream habitats. As salinity and temperature decreased species diversity increased with the greatest community complexity in stream habitats. Sinkholes with the highest salinity (> 20 ppt) contained only *C. pecosensis* as its fish species, a genus that is generally considered very saline-tolerant. We found limited variation in carbon sourcing across sinkholes suggesting available food sources are very similar in this type of habitat. The lack of complexity at higher salinity may also be attributed to a reduction in number of food types. The greatest differences in carbon sources were observed between sinkholes and stream habitats. The $\delta^{13}C$ of stream habitats was depleted by as much as 10‰ compared to sinkholes presumably from differences in inputs from primary producers. $\delta^{15}N$ values of consumers showed differences in trophic structure between stream and sinkhole habitats, and also among sinkholes. Fishes in sites with two or more fish species had higher $\delta^{15}N$ values relative to sinkholes where *C. pecosensis* occurred singly. Stream habitats showed even greater variation in $\delta^{15}N$ values than sinkholes. The $\delta^{15}N$ values of consumers in sinkholes ranged from 2 to 10‰ AIR and in stream habitats ranged from 4 to 14‰ AIR. This 1-3‰ enrichment in consumer $\delta^{15}N$ regardless of the $\delta^{15}N_{base}$ of the habitat may represent a full trophic level addition. These differences in trophic structure and breadth may be due to the greater biological complexity and lower salinity and temperatures characteristic of stream habitats. Additionally, our results show that the variation in community complexity and abiotic habitat characteristics collectively influence the feeding niches of particularly sensitive fish species on BLNWR. In highly saline sinkholes where *C. pecosensis* occurred singly it generally occupied a higher trophic level. This species was enriched by 1 - 3‰ AIR enrichment in values of $\delta^{15}N$ in these habitats (Fig. 3). When there were three or more fish

species present, *C. pecosensis* fed at a lower trophic level. Endangered *Gambusia*, found in springs, also fed at a higher trophic level than their congener, *G. affinis* and their hybrids.

Habitat variation in water quality and primary producers. Generally carbon sources did not vary considerably over sinkholes, but primary producers in stream habitats exhibited depleted $\delta^{13}C$ values (Fig. 6). There were, however, relatively few species of primary producers in the sinkholes, which generally only contained chara and/or algae for aquatic productivity with occasional pickleweed, sedges or *Tamarisk* growing near the water. Stream habitats contained these species as well as submerged grasses, *Phragmites*, and other vegetation. Although the $\delta^{13}C$ of chara was often more enriched, algae had wider variation in $\delta^{13}C$ with values ranging from 0‰ to 32‰ VPDB across all habitats (Figs. 4 & 5; Appendix 1). This result may be attributed to the low level of water mixing in the sinkholes into the summer. However, patterns in aquatic ecosystems is often complicated by the supplementation of terrestrial carbon inputs (Likens & Bormann 1974; France 1995; Thorp et al. 1998; Ward et al. 2002; Pace et al. 2007). The shift in $\delta^{13}C$ across these habitats may also be a continuum representative of the relative contribution of terrestrial primary production (McCutchan et al. 2003). In a stable isotope study of carbon sources of the fauna of mangrove lagoons in Mexico, Mendoza-Carranza et al. (2010) found high inputs of riparian vegetation such as *Phragmites* in habitats with low connectivity to the larger lagoons with higher inputs of seagrass vegetation (Mendoza-Carranza et al. 2010). . Terrestrial plants on BLNWR use both C3 and C4 photosynthesis and their tissues have $\delta^{13}C$ values that overlap those of algae and chara making it difficult to distinguish between inputs of terrestrial and aquatic production into the food web. Furthermore, organic matter suspended in the water column and potentially taken up by primary producers and consumers may mask the relative

carbon input of aquatic and terrestrial sources (Cloern et al. 2002; Post 2002a). It is probable that carbon in the aquatic system is from algal production, which is very labile in aquatic environments, particularly if the algae take up nitrogen from anthropogenic deposition (Costanzo et al. 2001; Fry 2006). The stream habitats, however, were heavily dominated by semi-submerged *Phragmites*, which were very depleted in values of $\delta^{13}C$ (approximately -24‰ VPDB). Therefore the source of carbon inputs between algae and *Phragmites* is indistinguishable (Fig 4 & Appendix 1).

At BLNWR available ecological niche space increased with community complexity (Fig 6). But our data suggest widely varying $\delta^{13}C$ and $\delta^{15}N$ values across habitat types were also related to abiotic factors affecting primary production. This shift was most marked between stream and sinkhole habitats containing complex fish assemblages. In sampling localities with high salinity, the available ecological niche space decreased. The greatest available niche space was in the low salinity, stream habitats. This finding may also be due to biotic factors. There were fewer species of plants and animals in sinkhole habitats, particularly those of macroinvertebrates and fishes (Appendix 2 & 3). Organisms in the stream habitats had higher values of $\delta^{15}N$. The shift upwards in $\delta^{15}N_{base}$ across all species type (plant and animals) may be reflective of abiotic factors such as differences in the hydrogeology or groundwater inputs of the stream habitats versus the gypsum, spring-fed sinkholes. But stream habitats also had greater food chain length, which is reflected in greater community complexity (Post 2002b). Furthermore, the additional productivity from terrestrial plant inputs may even bolster higher rates of biodiversity via secondary production than might otherwise be expected from an aquatic environment (France 1995; Sabo & Power 2002). We have previously mentioned that stream habitats on BLNWR are often surrounded

by terrestrial plants and *Phragmites*, this additional carbon input may have provided enough productivity to increase these food webs by an additional trophic level as we observed.

Variation in feeding niches of fishes as revealed by stable isotope analyses. Habitats were highly variable in values of $\delta^{13}C$ and $\delta^{15}N$ as were fish feeding niches. Fishes varied greatly in their $\delta^{15}N_{base}$ values on BLNWR ranging from 2 to 16‰ AIR (Fig 4 and Appendix 2). After controlling for differences in $\delta^{15}N_{base}$ between habitats, our results suggest that these differences were driven by interspecific competition and predation. In SH37 and SH7, *Fundulus zebrinus* followed by *G. nobilis* fed at the highest trophic level of the fishes with tissue $\delta^{15}N$ values that were 1 - 4‰ enriched than other species. *Fundulus zebrinus*, the largest of the species sampled (~4-6cm), is piscivorous and was found on the deeper edge of the shallow shelves these fishes occupy. We frequently observed individuals preying on the smaller *C. pecosensis,* which are found next closest to shore but feed in the water column, mostly on algae or suspended organic matter. There was also significant variation in carbon isotope ratios of fish tissues across sites with $\delta^{13}C$ ranging from -27 to -9‰ VPDB across fish species. The shifts in values of $\delta^{13}C$ of fishes varied predictably with base carbon inputs in these habitats. As expected, differences in values of $\delta^{13}C$ are driven by variation in inputs by primary producers. Notably, *Gambusia* from the hybrid zone had $\delta^{13}C$ values ranging from -27 to -17‰ VPDB as they occurred in the *Phragmites*-dominated stream habitats. Those fish species found in the stream habitats, *C. pecosensis* and *D. episcopa*, also had the very depleted values of $\delta^{13}C$.

Studies on large spatial scales such as riparian corridors, large lakes or in marine and estuary systems reveal a strong influence of abiotic factors on energy flow between species (Jackson et al. 2001; Chanton & Lewis 2002). The singular presence of *C. pecosensis* in

many habitats was likely due to abiotic constraints. For example, *C. pecosensis* was the only species found at salinity levels > 20ppt, which is the upper limit of tolerance for *G. nobilis*, its main competitor (Hubbs et al. 2002). The influence of abiotic factors relative on feeding niche can be quite strong. Crow et al. (2010) found that microhabitat use of two sympatric *Galaxias* species was largely determined by abiotic factors in their stream habitat and that interspecific competition did not influence this niche. We found the range of $\delta^{13}C$ values for *C. pecosensis* was narrower in simple fish assemblages than in more complex perhaps due to a decrease in available food resources. *Cyprinodon pecosensis* generally forages on plant material and there was a decrease in diversity of primary producers in sinkholes. The results of the mean Euclidean distance analysis further support the hypothesis that abiotic factors influence the niche space of *C. pecosensis.* In any site where competition was absent the $\delta^{15}N$ and $\delta^{13}C$ centroid of *C. pecosensis* was closer the centroid of all taxa over all sampling localities suggesting *C. pecosensis* had abiotic constraints on its feeding niche (Table 7). Biotic factors, such as competition, however, are also important in shaping food webs and these biotic influences are relatively magnified at smaller spatial scales such as those habitats in our study (Ross 1986; Jackson et al. 2001). In a study of ecological niche of the closely related, *C. rubrofluviatilis*, Echelle et al. (1972) found that the pupfish were niche-displaced in habitats with more competitors. In the presence of competitors at BLNWR, the feeding niche of *C. pecosensis*, too, shifted, suggesting a degree of competitive displacement; when heterospecifics are present they fed at a lower trophic level (Fig. 3). In competition, *C. pecosensis* shifted to feeding a lower trophic level as indicated by a 1 to 4‰ AIR depletion in values of $\delta^{15}N$ in competition.

Invasive species may also alter food web structure and threaten the persistence of native endemics. Vander Zanden et al. (2003) showed shifts in native fish feeding niche from benthic to pelagic in Lake Tahoe with increasing eutrophication and introduction of exotic species. We found significant differences in $\delta^{15}N_{base}$ between the invasive *G. affinis* and the endangered, spring-endemic *G. nobilis*. *Gambusia affinis* inhabited many different habitats of poor water quality but were typically absent from the isolated sinkholes on BLNWR. Rather they were ubiquitous in stream habitats on the refuge, where they have colonized and persisted. The higher trophic level observed in *G. nobilis* compared to *G. affinis* was somewhat surprising. *G. affinis* females are known to cannibalize their live offspring (Hubbs 1991), which may be reflected in a higher trophic level signature; they are also typically larger than *G. nobilis* (Swenton & Kodric-Brown *in review*). We have not observed cannibalism by *G. nobilis* in the field or lab. One possible explanation lies in diet differences. Dragonflies and damselflies are very abundant at BLNWR and in a previous isotope study were found at the highest trophic level in these habitats (*K. Gaines, pers. comm.)*. We observed *G. nobilis* adults feeding on drowned Odonates at the surface of the water column with no competition from other fish species. *G. affinis*, however, must compete with many other fish and other organisms (e.g. turtles, snakes) in their habitats for such high quality food sources. In a series of laboratory experiments by Rehage et al. (2005), the authors determined that invasive *Gambusia holbrooki* fed at higher rates than endangered *Gambusia* spring endemics, but did not differ in diet breadth and prey preference. Their results differed from ours, which are based on wild diets. When given a choice or in direct competition, controlling for habitat variation, there may be differences in competitive ability of these two species.

Both *Gambusia* species are sexually size dimorphic and Taylor et al. (2001) described age and sex structure in *Gambusia* diet, as related to size and gape width. Females may aggressively compete for access to high quality prey items such as insects (D. Swenton. *pers. obs*). Furthermore, males may reduce food consumption upon maturity, and juveniles feed on smaller prey items than adults (Garcia-Berthou 1999; Blanco et al. 2004). Stable isotopes have been used to reveal differences in diet between the sexes in other taxa (Mariano-Jelicich et al. 2008). The results of our study show marked differences between age class, but not sexes in *Gambusia nobilis.* Our data did not support the hypothesis that females feed at a higher trophic level than males, however, both males and females differed significantly in values of $\delta^{15}N$ from those of juveniles. Only males, however, differed from juveniles in values of $\delta^{13}C$. This last finding may be a result of young juveniles still having similar tissue to their mother's in values of $\delta^{13}C$.

In conclusion, our study using stable isotope analysis to characterize trophic dynamics of fish assemblages demonstrated variation in primary productivity across habitats, particularly between sinkholes and streams. This study provides insights as to the trophic structure and dynamics of small, isolated desert spring communities. These are fragile, neglected habitats (Kodric-Brown et al. 2007) and to our knowledge this is the first study to examine the collective influence of water quality and congener assemblage on feeding niche of endemic fishes, particularly in comparison to stream habitats. Studying the dynamics of these communities using stable isotopes provides much needed information regarding the interplay of biotic and abiotic factors on species persistence and food webs in relatively simple assemblages. A detailed understanding of these food webs and the role of abiotic factors in determining the distribution of rare and threatened species are essential in their

conservation (Carreon-Martinez & Heath 2010; Inger et al. 2010). By better understanding factors that affect the distribution of rare organisms such as, competitive interactions as well as energy flow through trophic levels, wildlife management can make better decisions regarding the proper conservation and restoration of native fish species such as those discussed here (Schoenly & Cohen 1991).

Acknowledgements

Co-authors for the *in prep* publication version of this manuscript are T.L. Kennedy & A. Kodric-Brown. We thank J.A. Baker for invaluable statistical and editorial advice for this work. B.O Wolf, J.C. Trexler, & T.F. Turner provided editorial comments on this manuscript. I. Murray, R. Warne & V. Auditori assisted with stable isotope analysis. C.L. Hayes, A. Hung, Z. Taylor & G. Warrick assisted with data collection. A. Mazzarella provided assistance with figures. K.A. Swaim & W.J. Boeing provided data on sinkhole size and depth. L. Schwanz, R. Warne, Y.E. Sawyer, C. Tech, T.B. Helmke, & D.L. Swenton provided editorial comments on the manuscript. A New Mexico Department of Game and Fish Share with Wildlife Program and, a National Science Foundation Doctoral Dissertation Improvement Grant (DEB: 0808508) to A. Kodric-Brown and D. Swenton funded this work. Additional funding was provided to D. Swenton from an Alvin and Caroline Groves Research Scholarship (UNM Biology Department), Crawford Scholarship (UNM Biology Department), SRAC grant (UNM Graduate and Professional Student Association, Student Resource Allocations Committee) and GRAC grant (UNM Biology Department, Graduate Resource Allocations Committee). *Gambusia nobilis* were studied in accordance with NM state permit 2968 and federal permit TE676811-0.

Literature Cited

Bearhop, S., Adams, C. E., Waldron, S., Fuller, R. A. & Macleod, H. 2004. Determining trophic niche width: a novel approach using stable isotope analysis. Journal of Animal Ecology 73:1007-1012.

Blanco, S., Romo, S. & Villena, M-J. 2004. Experimental study on the diet of mosquitofish (*Gambusia holbrooki*) under different ecological conditions in a shallow lake. International Review of Hydrobiology 89:250-262.

Bolnick, D. I., Yang, L. H., Fordyce, J. A., Davis, J. M. & Svanbäck, R. 2002. Measuring individual-level resource specialization. Ecology 83:2936-2941.

Brun, G., Chappaz, R. & Olivari, G. 1990. Modifications in habitat use patterns and trophic interrelationships in the fish fauna of an oligotrophic artificial lake- Sainte-Croix (Provence, France). Hydrobiologia 207:197-207.

Campbell, L. M., Wandera, S. B., Thacker, R. J., Dixon, D. G., & Hecky, R. E. 2005. Trophic niche segregation in the Nilotic ichthyofauna of Lake Albert (Uganda, Africa). Environmental Biology of Fishes 74:247-260.

Carreon-Martinez, L., & Heath, D. D. 2010. Revolution in food web analysis and trophic ecology: diet analysis by DNA and stable isotope analysis. Molecular Ecology 19:25-27.

Chanton, J., & Lewis, F. G. 2002. Examination of coupling between primary and secondary production in a river-dominated estuary: Apalachicola Bay, Florida, USA. Limnology and Oceanography 47:693-697.

Cloern, J. E., Canuel, E. A., and Harris, D. 2002. Stable carbon and nitrogen isotope composition of aquatic and terrestrial plants of the San Francisco Bay estuarine system. Limnology and Oceanography 47:713-729.

Crow, S. K., Closs, G. P., Waters, J. M., Booker, D. J., and Wallis, G. P. 2010. Niche partitioning and the effect of interspecific competition on microhabitat use by two sympatric galaxiid fishes. Freshwater Biology 55:967-982.

Courtenay Jr., W. R. & Meffe, G. K. 1989. Small fishes in strange places: A review of introduced poeciliids. In: Ecology and Evolution of Livebearing Fishes (Poeciliidae) (Meffe, G. K. & Snelson Jr., F. F., eds). Prentice Hall, New Jersey, pp 319-331.

Costanzo, S. D., Udy, J., Longstaff, B., & Jones, A. 2001. Using nitrogen stable isotope ratios ($\delta^{15}N$) of macroalgae to determine the effectiveness of sewage upgrades: Changes in the extent of sewage plumes over four years in Moreton Bay, Australia. Marine Pollution Bulletin 51:212-217.

Declerck, S., Louette, G., De Bie, T. & De Meester, L. 2002. Patterns of diet overlap between populations of non-indigenous and native fishes in shallow ponds. Journal of Fish Biology 61:1182-1197.

DeNiro, M. J. & Epstein, S. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et Cosmochimica Acta 45:495-506.

Echelle, A. A., & Echelle, A. F. 1980. Status of the Pecos Gambusia, *G. nobilis*. Endangered Species Report No. 10:1-73, U.S. Fish and Wildlife Service, Albuquerque, New Mexico.

Echelle, A. A., Echelle, A. F., & Hill, L. G. 1972. Interspecific interactions and limiting factors of abundance and distribution in the Red River pupfish, *Cyprinodon rubrofluviatilis*. American Midland Naturalist 88:109-130.

Farr, J. A, & Travis, J. 1986. Fertility advertisement by female sailfin mollies, *Poeciliia laptinna* (Pisces: Poeciliidae). Coepia, 1986:467-472.

France, R. L. 1995. Differentiation between littoral and pelagic food webs in lakes using carbon isotopes. Limnology and Oceanography. 40:1310-1313.

Fry, B. 2006. Stable Isotope Ecology. Springer Publishers, New York.

Garcia-Berthou, E. 1999. Food of introduced mosquitofish: ontogenetic diet shift and prey selection. Journal of Fish Biology 55:135-147.

Gumm, J. M., Snekser, J. L., & Itzkowitz, M. 2008. Conservation and conflict between endangered desert fishes. Biology Letters 4:655-658.

Hardy, C. M., Krull, E. S., Hartley, D. M., & Oliver, R. L. 2010. Carbon source accounting for fish using combined DNA and stable isotope analyses in a regulated lowland river weir pool. Molecular Ecology 19:197-212.

Hubbs, C. 1991. Intrageneric "cannibalism" in *Gambusia*. The Southwestern Naturalist 36:153-157.

Hubbs, C., Edwards, R. J. & Garrett, G. P. 2002. Threatened fishes of the world: *Gambusia nobilis* Baird & Girard, 1853 (Poeciliidae). Environmental Biology of Fishes 64:428.

Inger, R., McDonald, R. A., Rogowski, D., Jackson, A. L., Parnell, A., Preston, S. J., Harrod, C., Goodwin, C., Griffiths, D., Dick, J. T. A., Elwood, R. W., Newton, J., & Bearhop, S. 2010. Do non-native invasive fish support elevated lamprey populations? Journal of Applied Ecology 47:121-129.

Jackson, D. A., Peres-Neto, P. R., & Olden, J. D. 2001. What controls who is where in freshwater fish communities- the roles of biotic, abiotic and spatial factors. Canadian Journal of Fisheries and Aquatic Sciences 58:157-170.

Kodric-Brown, A. & Brown, J. H. 1993. Highly structured fish communities in Australian desert springs. Ecology 74:1847-1855.

Kodric-Brown, A., Wilcox, C., Bragg, J. G., & Brown, J. H. 2007. Dynamics of fish in Australian desert springs: role of large-mammal disturbance. Diversity and Distributions 13:789-798.

Layman, C. A., Arrington, D. A., Montana, C. G. & Post, D. M. 2007. Can stable isotope ratios provide for community-wide measures of trophic structure? Ecology 88:42-48.

Likens, G. E., & Bormann, F. H. 1974. Linkages between terrestrial and aquatic ecosystems. BioScience 24:447-456.

Mariano-Jelicich, R., Botto, F., Martinetto, P., Iribarne, O., Favero, M. 2008. Trophic segregation between sexes in the Black Skimmer revealed through the analysis of stable isotopes. Marine Biology 155:443-450.

McCutchan Jr., J. H., Lewis Jr., W. M., Kendall, C. K., & McGrath, C. C. 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. Oikos 102:378-390.

McIntyre, P. B. & Flecker, A. S. 2006. Rapid turnover of tissue nitrogen of primary consumers in tropical freshwaters. Oecologia 148:12-21.

Mendoza-Carranza, M., Hoeinguaus, D. J., Garcia, A. M. & Romero-Rodriguez, Á. 2010. Aquatic food webs in mangrove and seagrass habitats of Centla Wetland, a biosphere reserve in southeastern Mexico. Neotropical Ichthyology 8:171.

Minigawa, M., and Wada, E. 1984. Stepwise enrichment of $_{15}$N along food chains: further evidence and the relation between δ^{15}N and animal age. Geochimica et Cosmochimica Acta 48: 1135-1140.

Pace, M. L., Carpenter, S. R., Cole, J. J., Coloso, J. J., Kitchell, J. F., Hodgson, J. R., Middleburg, J. J., Preston, N. D., Solomon, C. T., & Weidel, B. C. 2007. Does terrestrial organic carbon subsidize the planktonic food web in a clear-water lake? Limnological Oceanography 52: 2177-2189.

Peterson, B., & Fry, B. 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18:293-320.

Post, D. M. 2002a. Using stable isotopes to estimate trophic position: models, methods and assumptions. Ecology 83:703-718.

Post, D. M. 2002b. The long and short of food chain length. Trends in Ecology & Evolution 17:269-278.

Rehage, J. S., Barnett, B. K. & Sih, A. 2005. Foraging behaviour and invasiveness: Do invasive *Gambusia* exhibit higher feeding rates and broader diets than their noninvasive relatives? Ecology of Freshwater Fish 14:352-360.

Ross, S. T. 1986. Resource partitioning in fish assemblages: a review of field studies. Copeia 2:352-388.

Rounick, J. S., & Winterbourn, M. J. 1986. Stable carbon isotopes and carbon flow in ecosystems. Bioscience 36:171-177.

Sabo, J. L., & Power, M. E. 2002. River-watershed exchange: Effects of riverine subsidies on riparian lizards and their terrestrial prey. Ecology 83:1860-1869.

Schaal, G., Riera, P., & Leroux, C. 2009. Trophic significance of the kelp, *Laminaria digitata* (Lamour.) for the associated food web: a between-sites comparison. Estuarine, Coastal and Shelf Science 85:565-572.

Schoenly, K. & Cohen, J. E. 1991. Temporal variation in food web structure: 16 empirical cases. Ecological Monographs 61:267-298.

Sharp, Z. 2006. Principles of Stable Isotope Geochemistry. Prentice Hall, New Jersey.

Syväranta, J. & Jones, R. I. 2008. Changes in feeding niche widths of perch and roach following biomanipulation, revealed by stable isotope analysis. Freshwater Biology 53:425-434.

Taylor, R. C., Trexler, J. C. & Loftus, W. F. 2001. Separating the effects of intra- and interspecific age-structured interactions in an experimental fish assemblage. Oecologia 127:143-152.

Thorp, J. H., Delong, M. D., Greenwood, K. S., & Capser, A. F. 1998. Isotopic analysis of three food web theories in constricted and floodplain regions of a large river. Oecologia 117:551-563.

Turner, T. F., Collyer, M. L. & Krabbenhoft, T. J. 2010. A general hypothesis testing framework for stable isotope rations in ecological studies. Ecology 91:2227-2233.

Vadeboncoeur, Y., Jeppesen, E., Vander Zanden, M. J., Schierup, H-H., Christoffersen, K. & Lodge, D. M. 2003. From Greenland to green lakes: cultural eutrophication and the loss of benthic pathways in lakes. Limnology and Oceanography 48:1408-1418.

Vander Zanden, M. J. & Rasmussen. J. B. 2001. Variation in δ15N and δ13C trophic fractionation: Implications for aquatic food web studies. Limnology and Oceanography 46:2061-2066.

Vander Zanden, M. J., Chandra, S., Allen, B. C., Reuter, J. E. & Goldman, C. R. 2003. Historical food web structure and restoration of native aquatic communities in the Lake Tahoe (California-Nevada) Basin. Ecosystems 6:274-288.

Ward, J. V., Tockner, K., Arscott, D. B., & Claret, C. 2002. Riverine landscape diversity. Freshwater Biology 47:517-539.

Warren, M. L. & Burr, B. M. 1994. Status of freshwater fishes of the United States: overview of an imperiled fauna. Fisheries 19:6-18.

Williams, J. E., Bowman, D. B., Brooks, J. E., Echelle, A. A., Edwards, R. J., Hendrickson, D. A. & Landye, J. J. 1985. Endangered aquatic ecosystems in North American deserts with a list of vanishing fishes of the region. Journal of Arizona-Nevada Academy of Science 20:1-62.

Winemiller, K. O. & Leslie, M. A. 1992. Fish assemblages across a complex, tropical fresh-water marine ecotone. Environmental Biology of Fishes 34:29-50.

Zeug, S. C., Peretti, D. & Winemiller, K. O. 2009. Movement into floodplain habitats by gizzard shad (*Dorosoma cepedianum*) revealed by dietary and stable isotope analyses. Environmental Biology of Fishes 84:307-314.

Tables, Appendices, and Figures

Table 1. Fish assemblages of the 12 sampling localities at BLNWR in 2008 with six most abundant species. Complex sites are those comprised of two or more fish species. *Oxbow site is complex but contains other species than those listed here (e.g. *Lucania parva*).

Site	Complex or Simple	*C. pecosensis*	*G. nobilis*	*G. affinis*	*G. nobilis* x *G. affinis*	*F. zebrinus*	*C. lutrensis*	*D. episcopa*
BCWeir	Complex	X	X	X	X			X
SH 37	Complex	X	X			X		
Oxbow*	Complex	X		X				
SH 20	Complex	X	X				X	
SH 27N	Simple		X					
Lost Spring	Complex	X	X					X
SH 27S	Complex	X	X					
SH 7	Complex	X	X			X		
SH 18	Simple	X						
SH 19	Simple	X						
SH 10	Simple	X						
SH 26	Simple	X						

Table 2. Summary of sampled habitat characteristics. Two diameter measurements are provided for non-circular sampling localities. Depth can vary greatly depending on season and time of year, therefore secchi depth measurements are presented as the ranges taken during July & August 2005-2007 (data collected by K.A. Swaim & W.J. Boeing).

Site	Habitat Type	Sinkhole Diameter (m)	Sinkhole Depth (m)
BCWeir	Creek	-	-
SH 37	gypsum/spring-fed sinkhole	59	14.1 - 15
Oxbow	Drainage ditch	-	-
SH 20	gypsum/spring-fed sinkhole	32 x 15	3.2 - 4.15
SH 27N	gypsum/spring-fed sinkhole	14 x 8.5	0.6- 1.15
Lost Spring	gypsum/spring-fed head of creek	-	-
SH 27S	gypsum/spring-fed sinkhole	31 x 21.5	5.5 – 6.5
SH 7	Gypsum/spring-fed sinkhole	41	8.5 – 9.3
SH 18	gypsum/spring-fed sinkhole	9	0.9 -1.8
SH 19	gypsum/spring-fed sinkhole	32.5	2.5 – 3.25
SH 10	gypsum/spring-fed sinkhole	17	2.5 - 3
SH 26	gypsum/spring-fed sinkhole	31	3.5 - 4

Table 3. Results of ANOVA on variation in water quality measurements on Bitter Lake National Wildlife Refuge in 2008. The factors of time (May, June, July) and site were used in the test. Significant results are in bold.

	Water Quality			
	Dissolved oxygen (mg/L)	Temperature (°C)	Salinity (ppt)	Conductivity (S/cm)
Factor				
Time	$F_{2,22} = 16.056$ **$p < 0.001$**	$F_{2,22} = 5.749$ **$p = 0.010$**	$F_{2,22} = 1.502$ $p = 0.245$	$F_{2,22} = 0.156$ $p = 0.857$
Site	$F_{11,22} = 2.444$ **$p = 0.036$**	$F_{11,22} = 2.747$ **$p = 0.021$**	$F_{11,22} = 40.734$ **$p < 0.001$**	$F_{11,22} = 16.297$ **$p < 0.001$**

Table 4. Results of stable isotope analysis on chara and algae in all sampling localities at Bitter Lake National Wildlife Refuge in 2008. Data presented for each month is the mean of chara and algae samples isotope values (pooled sample size). The final mean measurement is the average of the pooled samples taken in May, June and July.

Site	Measurement	May	June	July	Mean
Bitter Creek Weir	$\delta^{13}C$	-24.38 (2)	-26.36 (2)	-23.50 (2)	**-24.75**
	$\delta^{15}N$	9.02 (2)	9.05 (2)	9.81 (2)	**9.29**
Lost Spring	$\delta^{13}C$	-11.82 (2)	-14.30 (2)	-15.54 (2)	**-13.89**
	$\delta^{15}N$	8.14 (2)	10.83 (2)	10.67 (2)	**9.88**
SH 37	$\delta^{13}C$	-12.18 (4)	-5.55 (4)	-5.13 (4)	**-7.62**
	$\delta^{15}N$	7.56 (4)	8.06 (4)	5.09 (4)	**6.90**
Oxbow	$\delta^{13}C$	-17.08 (4)	-16.88 (4)	-17.95 (4)	**-17.30**
	$\delta^{15}N$	5.24 (2)	5.40 (2)	4. 97 (2)	**5.20**
SH 20	$\delta^{13}C$	-8.91 (4)	-7.19 (4)	-9.11 (4)	**-8.40**
	$\delta^{15}N$	4.99 (4)	5.48 (4)	4.17 (4)	**4.88**
SH 27N	$\delta^{13}C$	-10.77 (2)	-9.36 (2)	-10.78 (2)	**-10.30**
	$\delta^{15}N$	2.29 (2)	2.79 (2)	3.29 (2)	**2.79**
SH 27S	$\delta^{13}C$	-15.00 (4)	-11.39 (4)	-13.16 (4)	**-13.18**
	$\delta^{15}N$	5.75 (4)	4.62 (4)	4.33 (4)	**4.90**
SH 7	$\delta^{13}C$	-11.80 (4)	-8.12 (4)	-9.84 (4)	**-9.92**
	$\delta^{15}N$	1.02 (4)	1.25 (4)	1.36 (4)	**1.21**
SH 18	$\delta^{13}C$	-12.41 (2)	-9.92 (2)	-11.00 (2)	**-11.11**
	$\delta^{15}N$	1.34 (2)	1.67 (2)	2.04 (2)	**1.68**
SH 19	$\delta^{13}C$	-11.93 (4)	-11.91 (4)	-11.14 (4)	**-11.66**
	$\delta^{15}N$	4.43 (4)	4.54 (4)	3.99 (4)	**4.32**
SH 10	$\delta^{13}C$	-10.67 (4)	-11.41 (4)	-10.58 (4)	**-10.89**
	$\delta^{15}N$	3.46 (4)	1.52 (4)	2.59 (4)	**2.52**
SH 26	$\delta^{13}C$	-7.20 (4)	-7.95 (4)	-8.01 (4)	**-7.72**
	$\delta^{15}N$	3.64 (4)	3.14 (4)	3.37 (4)	**10.15**

Table 5. Variation in isotope values over time for algae and chara. Site and the interaction between site and date were entered as additional predictors. Significant results are in bold.

	Stable Isotope Measurement					
	$\delta^{15}N$ of chara	$\delta^{15}N$ of algae	$\delta^{15}N$ of algae + chara	$\delta^{13}C$ of chara	$\delta^{13}C$ of algae	$\delta^{13}C$ of algae + chara
Factor						
Time	$F_{2,30} = 0.909$ $p = 0.414$	$F_{2,28} = 5.694$ $\mathbf{p = 0.008}$	$F_{2,79} = 0.456$ $p = 0.636$	$F_{2,30} = 11.281$ $\mathbf{p < 0.001}$	$F_{2,28} = 1.777$ $p = 0.188$	$F_{2,79} = 1.060$ $p = 0.351$
Site	$F_{9,30} = 14.882$ $\mathbf{p < 0.001}$	$F_{8,28} = 34.172$ $\mathbf{p < 0.001}$	$F_{11,79} = 16.086$ $\mathbf{p < 0.001}$	$F_{9,30} = 13.467$ $\mathbf{p < 0.001}$	$F_{8,28} = 63.744$ $\mathbf{p < 0.001}$	$F_{11,79} = 6.731$ $\mathbf{p < 0.001}$
Time*Site	$F_{18,30} = 0.951$ $p = 0.533$	$F_{16,28} = 1.169$ $p = 0.348$	$F_{22,79} = 0.449$ $p = 0.069$	$F_{18,30} = 2.281$ $\mathbf{p < 0.001}$	$F_{16,28} = 1.882$ $p = 0.069$	$F_{22,79} = 0.497$ $p = 0.967$

Table 6. Variation in isotopic values over time for each species collected. For those species collected at multiple sampling localities, site and the interaction between site and date were entered as additional predictors. Significant results are in bold.

	Stable Isotope Measurement					
	$\delta^{15}N$ of all fish spp.	$\delta^{15}N$ of *C. pecosensis*	$\delta^{15}N$ of *G. nobilis*	$\delta^{13}C$ of all fish spp.	$\delta^{13}C$ of *C. pecosensis*	$\delta^{13}C$ of *G. nobilis*
Factor						
Time	$F_{1,460} = 19.8$ $p <$ **0.01**	$F_{1,119} = 5.733$ ***p* = 0.019**	$F_{1,124} = 50.012$ $p = 0.231$	$F_{11,460} = 24.0$ $p <$ **0.001**	$F_{1,119} = 18.12$ $p <$ **0.001**	$F_{1,124} = 8.91$ $p = 0.189$
Site	$F_{11,460} = 101.1$ ***p* < 0.01**	$F_{6,119} = 3.393$ ***p* = 0.003**	$F_{5,124} = 52.292$ ***p* < 0.001**	$F_{11,460} = 102$ $p <$ **0.01**	$F_{6,119} = 16.25$ ***p* < 0.001**	$F_{5,124} = 279.86$ ***p* < 0.001**
Time*Site	$F_{14,460} = 2.64$ ***p* = 0.048**	$F_{14,119} = 3.636$ ***p* < 0.001**	$F_{6,124} = 5.08$ ***p* < 0.001**	$F_{14,460} = 1.552$ $p = 0.067$	$F_{14,119} = 2.09$ ***p* = 0.017**	$F_{6,124} = 3.92$ ***p* < 0.001**

Table 7. Euclidean distances (ED) of *C. pecosensis* centroid of $\delta^{13}C$ x $\delta^{15}N$ by site from the $\delta^{13}C$ x $\delta^{15}N$ centroid of each site for all taxa, the $\delta^{13}C$ x $\delta^{15}N$ centroid of *C. pecosensis* across all sampling localities, and the $\delta^{13}C$ x $\delta^{15}N$ centroid of all taxa across all sampling localities. Sampling localities in bold contain *C. pecosensis* as the single fish species. Sample sizes of all samples over all taxa are in parentheses.

Site (N)	ED of *C. pecosensis* centroid within site to *C. pecosensis* centroid over all sampling localities	ED of *C. pecosensis* centroid to centroid of all taxa centroid within a sampling locality	ED of *C. pecosensis* centroid of given site to centroid of all taxa over all sampling localities
Bitter Creek Weir (82)	6.267	3.369	4.333
SH 37 (108)	3.258	0.615	4.074
SH 20 (85)	1.801	0.256	1.096
SH 27S (91)	3.075	0.604	2.058
SH 7 (127)	2.945	0.851	4.591
Mean of Complex Sampling localities	3.469	1.139	3.230
SH 18 (59)	**1.403**	**1.858**	**2.915**
SH 19 (54)	**0.948**	**2.143**	**2.796**
SH 10 (58)	**1.395**	**1.431**	**2.802**
SH 26 (66)	**1.972**	**3.482**	**2.968**
Mean of Simple Sampling localities	**1.430**	**2.229**	**2.870**

Appendix 1. $\delta^{13}C$ and $\delta^{15}N$ ranges and means for plants with four or more samples across localities at Bitter Lake National Wildlife Refuge in 2008. Data presented are the lowest and highest measurements of $\delta^{13}C$ and $\delta^{15}N$ samples within sites.

Site (N) Species (N)	low $\delta^{13}C$	high $\delta^{13}C$	mean $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$	mean $\delta^{15}N$
Bitter Creek Weir						
algae (4)	-32.53	-15.71	-24.93	7.99	10.05	9.03
Lost Spring						
algae (6)	-16.44	-11.16	-13.88	7.64	11.69	9.88
Oxbow						
chara (6)	-18.55	-16.33	-17.30	4.14	6.65	5.20
SH27N						
chara (6)	-13.64	-6.84	-9.83	1.97	3.88	2.79
SH 37						
algae (6)	-10.35	-9.12	-9.76	3.95	6.92	5.46
chara (6)	-15.33	-0.28	-5.48	0.84	11.03	8.34
SH 20						
algae (6)	-6.61	-2.25	-5.14	4.71	6.24	5.46
chara (6)	-13.72	-10.29	-11.67	3.12	5.08	4.31
SH 27S						
algae (6)	-17.82	-15.83	-16.64	4.45	5.71	5.12
chara (6)	-17.22	-4.80	-9.73	2.14	8.41	4.68
SH 7						
SH 18						
chara (6)	-12.50	-8.77	-11.11	0.54	2.21	1.56
SH 19						
algae (6)	-14.32	-11.60	-12.93	4.07	5.59	4.73
chara (6)	-11.01	-9.94	-10.38	3.56	4.40	3.91
SH 10						
algae (6)	-15.3	-13.59	-14.37	4.35	6.44	5.52
chara (6)	-9.84	-5.53	-7.41	0.66	2.88	0.47
SH 26						
algae (6)	-11.74	-7.70	-9.63	3.09	4.37	3.74
chara (6)	-6.92	-5.04	-5.81	2.57	3.59	3.02

Appendix 2. $\delta^{13}C$ and $\delta^{15}N$ ranges and means for fishes across sampling localities at Bitter Lake National Wildlife Refuge in 2008. Data presented are the lowest and highest measurements of $\delta^{13}C$ and $\delta^{15}N$ samples within sites.

Site (N) Species (N)	low $\delta^{13}C$	high $\delta^{13}C$	mean $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$	mean $\delta^{15}N$
Bitter Creek Weir						
C. pecosensis (5)	-20.88	-17.39	-18.80	8.74	12.05	9.64
Gambusia hybrids (34)	-27.01	-17.72	-21.27	7.32	15.20	11.92
Dionda episcopa (2)	-22.31	-22.23	-22.27	12.73	13.36	13.04
Lost Spring						
G. nobilis (27)	-23.12	-16.26	-20.09	7.30	15.98	13.51
Oxbow						
C. lutrensis (9)	-18.11	-15.84	-16.90	4.14	6.65	5.20
F. zebrinus (2)	-17.14	-15.94	-16.54	7.47	8.04	7.76
G. affinis (18)	-24.57	-14.65	-16.94	5.20	8.92	7.66
SH27N						
G. nobilis (6)	-16.45	-14.43	-15.15	6.32	8.25	7.48
SH 37						
C. pecosensis (15)	-14.90	-9.79	-12.37	8.69	11.45	10.26
G. nobilis (33)	-15.64	-12.74	-14.47	9.95	13.26	11.33
F. zebrinus (24)	-14.56	-10.54	-12.38	6.40	12.34	11.37
SH 20						
C. pecosensis (14)	-19.56	-12.16	-14.70	4.09	7.61	6.29
C. lutrensis (15)	-16.40	-13.21	-14.05	7.53	9.72	8.52
G. nobilis (13)	-15.66	-12.73	-14.23	7.73	9.51	7.51
SH 27S						
C. pecosensis (11)	-15.98	-13.92	-15.16	7.28	10.81	9.35
G. nobilis (36)	-21.77	-14.02	-16.74	8.86	13.05	11.12
SH 7						
C. pecosensis (13)	-20.88	-9.12	-11.49	3.16	10.24	4.60
F. zebrinus (18)	-13.81	-9.69	-11.97	5.26	9.73	7.21
G. nobilis (40)	-16.57	-10.83	-14.18	4.55	9.29	7.66
SH 18						
C. pecosensis (23)	-16.55	-10.96	-16.55	2.68	7.08	5.57
SH 19						
C. pecosensis (17)	-15.77	-10.55	-18.49	6.38	8.95	7.74

Appendix 2. Continued

Site (N) Species (N)	low $\delta^{13}C$	high $\delta^{13}C$	mean $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$	mean $\delta^{15}N$
SH 10						
C. pecosensis (22)	-14.56	-11.26	-12.85	4.09	7.34	5.70
SH 26						
C. pecosensis (16)	-12.02	-9.98	-11.22	7.00	8.46	7.72

Appendix 3. $\delta^{13}C$ and $\delta^{15}N$ ranges and means for any macroinvertebrates with four or more samples across localities at Bitter Lake National Wildlife Refuge in 2008. Data presented are the lowest and highest measurements of $\delta^{13}C$ and $\delta^{15}N$ samples pooled by highest identified taxanomic class within sites.

Site (N) Taxa (N)	low $\delta^{13}C$	high $\delta^{13}C$	mean $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$	mean $\delta^{15}N$
Bitter Creek Weir						
Dytiscidae (5)	-26.27	-20.29	-23.40	6.68	9.73	8.41
Hydrophilidae	-26.46	-15.37	-21.87	3.70	11.47	8.89
Lost Spring						
Hyallela (6)	-17.08	-11.72	-15.42	2.78	10.51	8.50
Hydrophilidae (6)	-22.84	-17.63	-14.92	5.71	11.69	8.06
Oxbow						
-						
SH27N						
Corixidae (11)	-22.47	-11.67	-14.11	2.69	5.56	4.38
Dytiscidae (9)	-18.11	-12.74	-16.01	5.13	6.81	5.58
Hydrophilidae (13)	-24.99	-14.96	-17.72	2.62	11.89	5.69
SH 37						
Anisopterae (4)	-14.32	-11.33	-13.19	6.59	8.56	7.57
Hydrophilidae (5)	-21.59	-11.79	-15.40	4.85	7.48	6.49
Zygopterae (5)	-15.21	-13.2	-14.12	7.73	10.62	9.31
SH 20						
Hydrophilidae (15)	-29.38	-13.12	-19.61	0.44	10.27	3.57
Zygopterae (5)	-17.59	-14.56	-15.56	6.34	6.34	5.96
SH 27S						
Hydrophilidae (6)	-20.69	-15.14	-18.02	4.40	6.89	5.69
SH 7						
Belostomatidae (4)	-22.38	-10.38	-13.84	0.89	13.46	5.56
Hydrophilidae (20)	-28.19	-11.58	-16.09	3.97	8.72	3.51
Zygopterae (9)	-14.33	-12.44	-13.56	3.67	5.84	4.98

Appendix 3. Continued

Site (N) Taxa (N)	low $\delta^{13}C$	high $\delta^{13}C$	mean $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$	mean $\delta^{15}N$
SH 18						
Corixidae (8)	-16.94	-10.60	-12.35	2.59	5.51	3.83
Dytiscidae (5)	-22.13	-14.80	-17.29	1.72	3.67	2.92
Hydrophilidae (10)	-24.02	-13.48	-17.91	3.73	6.14	4.80
SH 19						
Corixidae (8)	-19.43	-9.66	-12.60	4.88	3.19	6.03
Hydrophilidae (11)	-23.22	-14.89	-18.49	2.60	7.56	5.36
SH 10						
Corixidae (5)	-14.00	-11.99	-12.61	0.86	5.19	3.00
Hydrophilidae (7)	-19.29	-14.03	-17.17	3.01	6.4	4.94
Zygopterae (6)	-15.14	-12.9	-14.14	2.69	7.34	5.86
SH 26						
Anisopterae (6)	-18.40	-13.97	-15.68	4.23	6.10	5.21
Corixidae (11)	-20.89	-10.05	-14.06	1.85	6.59	4.48
Dytiscidae (5)	-20.52	-18.28	-19.59	3.72	6.82	5.02
Hydrophilidae (14)	-25.81	-13.13	-19.38	3.32	11.50	6.51

Appendix 4. $\delta^{13}C$ range and $\delta^{15}N$ range for all plants and fish sampled across and within sampling localities containing *C. pecosensis* at Bitter Lake National Wildlife Refuge in 2008. Data presented are the lowest and highest measurements of $\delta^{13}C$ and $\delta^{15}N$ for just *C. pecosensis* individuals within sites and across sites as well as all samples within and across sites. Taxa for overall samples are in parentheses. Sampling localities in italics contain C. pecosensis as the single fish species.

Site (N)	low $\delta^{13}C$	high $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$
Bitter Creek Weir				
C. pecosensis (5)	-20.88	-17.39	8.74	12.05
All samples (82)	-32.53 (algae)	-15.71 (algae)	7.32 (*G. nobilis*)	15.20 (*G. nobilis*)
SH 37				
C. pecosensis (15)	-14.90	-9.79	8.69	11.45
All samples (108)	-15.64 (*G. nobilis*)	-0.28 (chara)	3.95 (chara)	13.26 (*G. nobilis*)
SH 20				
C. pecosensis (14)	-19.56	-12.16	4.09	7.61
All samples (85)	-19.56 (*C. pecosensis*)	-2.25 (algae)	3.12 (chara)	9.72 (*C. lutrensis*)
SH 27S				
C. pecosensis (11)	-15.98	-13.92	7.28	10.81
All samples (91)	-21.77 (*G. nobilis*)	-4.8 (chara)	2.14 (chara)	13.32 (*F. zebrinus*)
SH 7				
C. pecosensis (13)	-20.88	-9.12	3.16	10.24
All samples (127)	-16.57 (*G. nobilis*)	-3.66 (chara)	0.24 (algae)	9.73 (*F. zebrinus*)
SH 18				
C. pecosensis (23)	-16.55	-10.96	2.68	7.08
All samples (59)	-21.57 (*Tamarisk*)	-8.77 (chara)	0.54 (chara)	7.08 (*C. pecosensis*)
SH 19				
C. pecosensis (17)	-15.77	-10.55	6.38	8.95
All samples (54)	-15.77 (*C. pecosensis*)	-9.94 (chara)	3.56 (chara)	8.95 (*C. pecosensis*)
SH 10				
C. pecosensis (22)	-14.56	-11.26	4.09	7.34
All samples (58)	-22.35 (pickleweed)	-5.53 (chara)	1.22 (chara)	7.34 (*C. pecosensis*)
SH 26				
C. pecosensis (16)	-12.02	-9.98	7.00	8.46
All samples (66)	-23.99 (pickleweed)	-5.04 (chara)	2.57 (chara)	8.46 (*C. pecosensis*)

Appendix 4. Continued

Site (N)	low $\delta^{13}C$	high $\delta^{13}C$	low $\delta^{15}N$	high $\delta^{15}N$
Total Range				
C. pecosensis (136)	-20.88	-9.12	2.68	12.05
All samples (730)	-32.53 (algae)	-0.28 (chara)	0.54 (chara)	15.20 (*G. nobilis*)

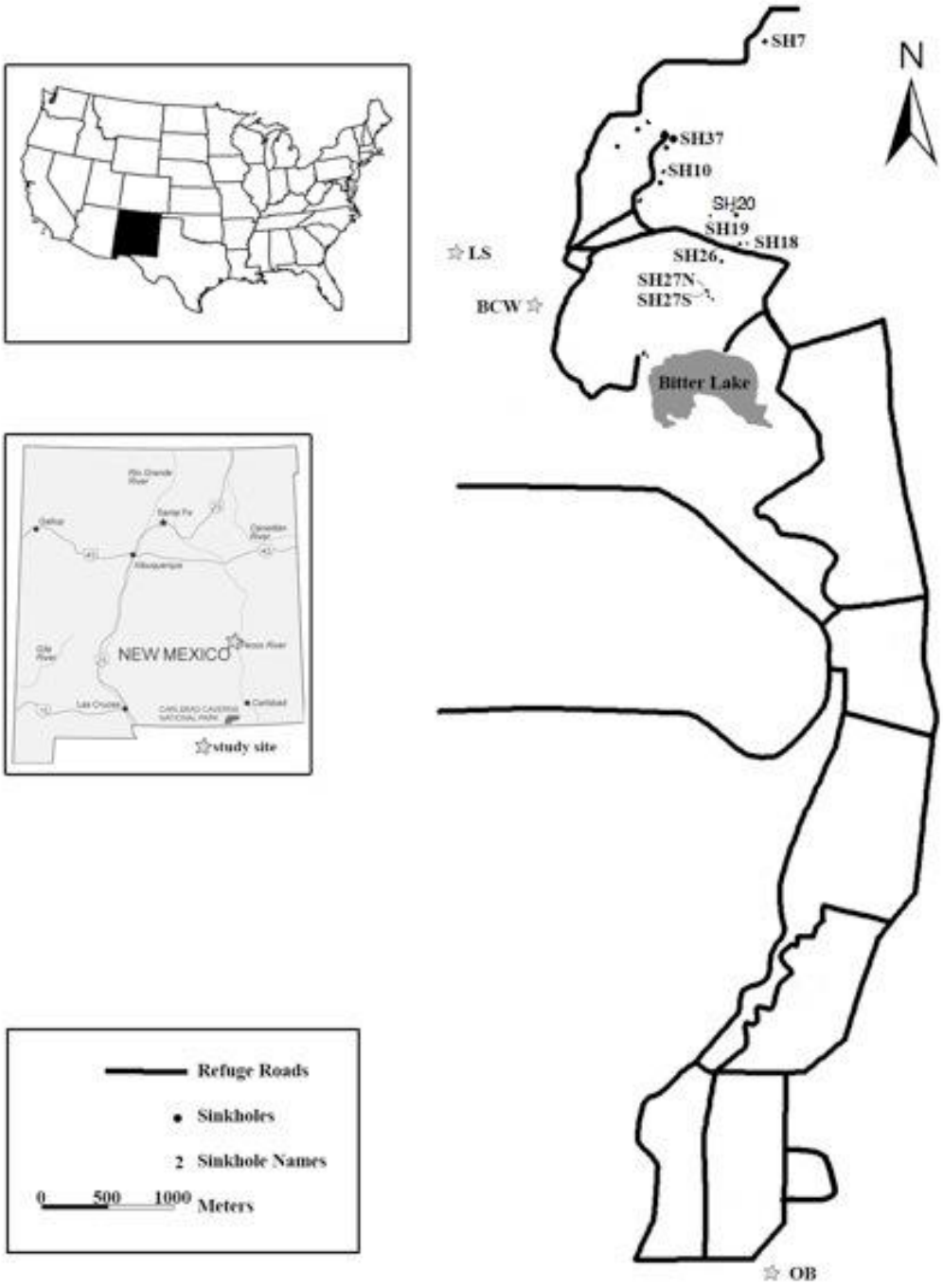


Figure 1. Sampling localities on Bitter Lake National Wildlife Refuge. Starred sampling localities are stream habitats.

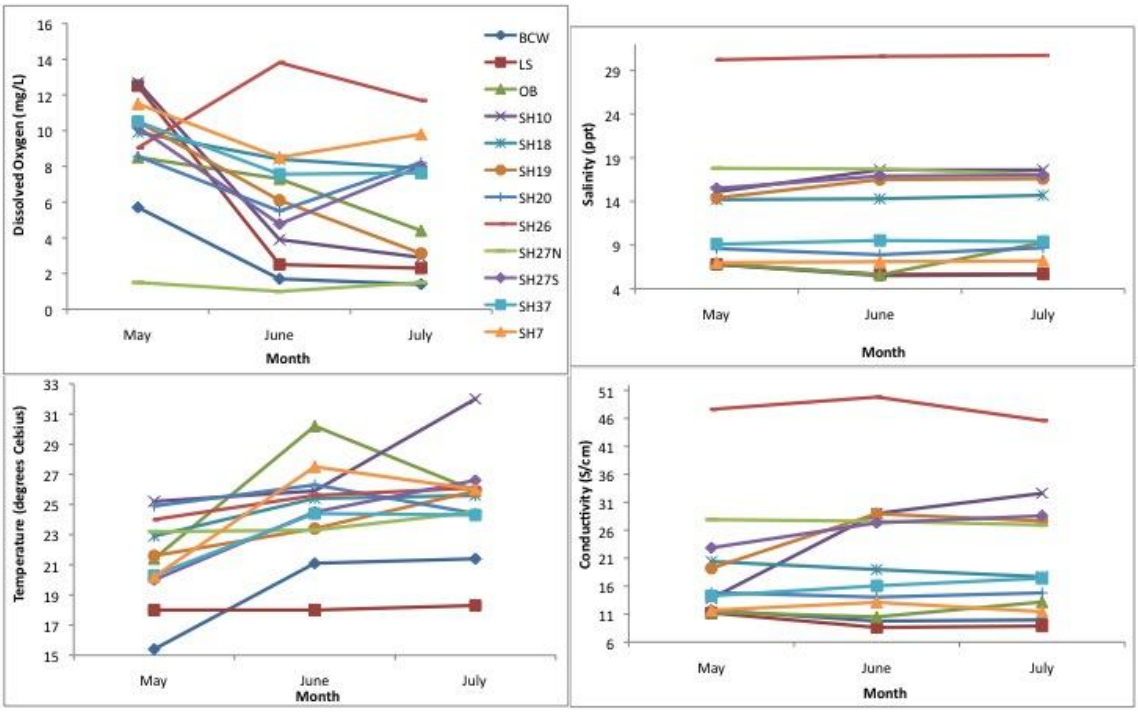


Figure 2. Measurements of habitat variables taken in 2008 across field sampling localities at Bitter Lake NWR in the months of May, June and July: (a) dissolved oxygen (mg/L), (b) salinity (ppt), (c) temperature (°C), and (d) conductivity (S/cm). Line colors correspond to site (see legend).

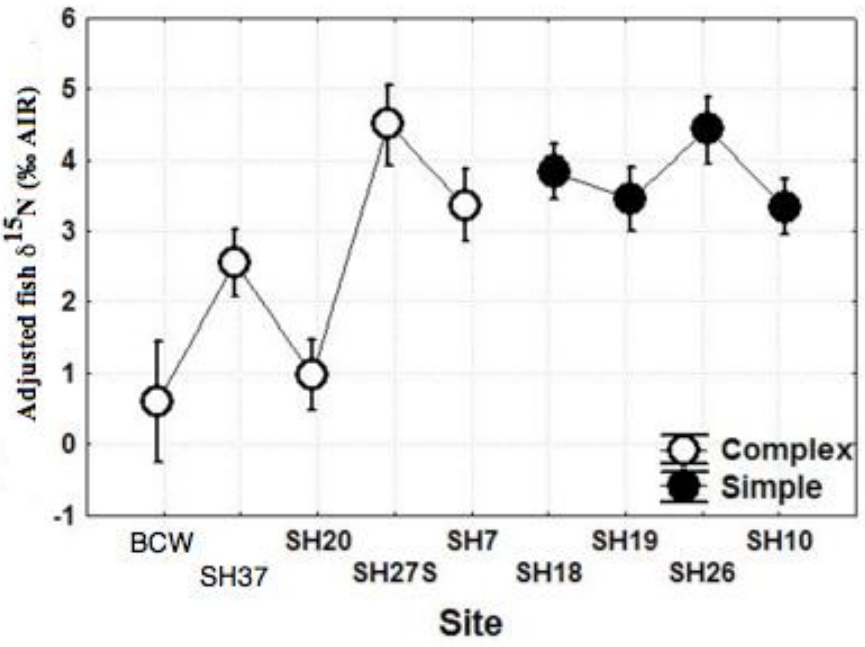
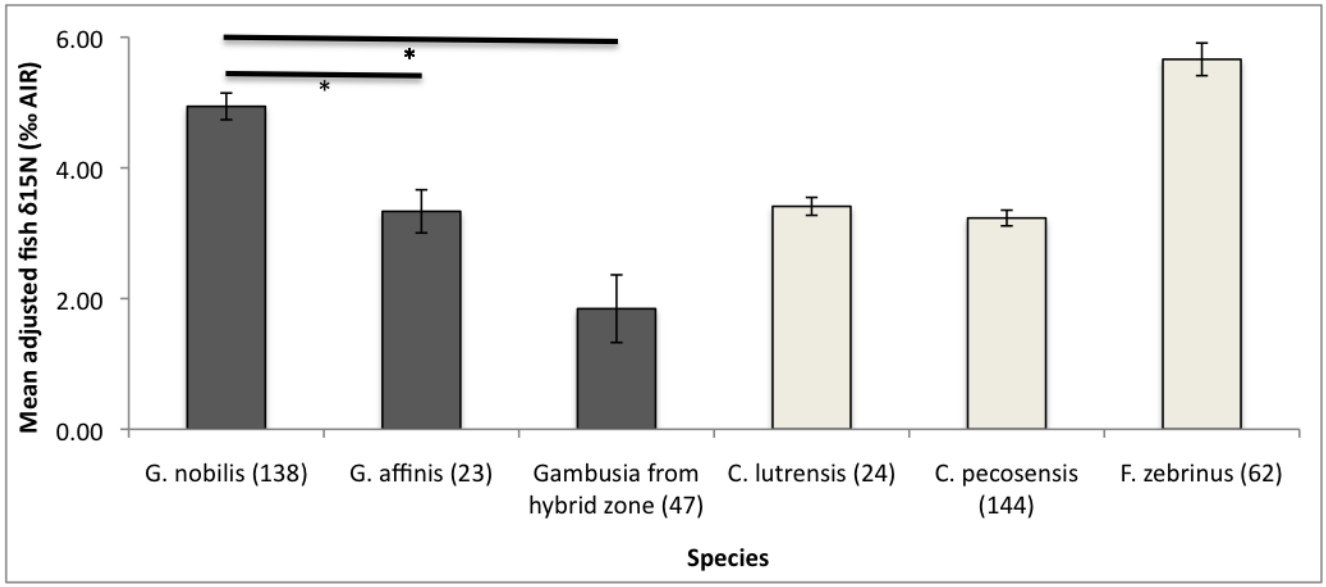


Figure 3. Adjusted $\delta^{15}N$ values for Cyprinodon pecosensis across sampling localities. Means across individuals and months are presented with error bars. Open circles indicate complex sites that have three or more fish species (complex). Closed circles indicate sites that contain only C. pecosensis (simple).



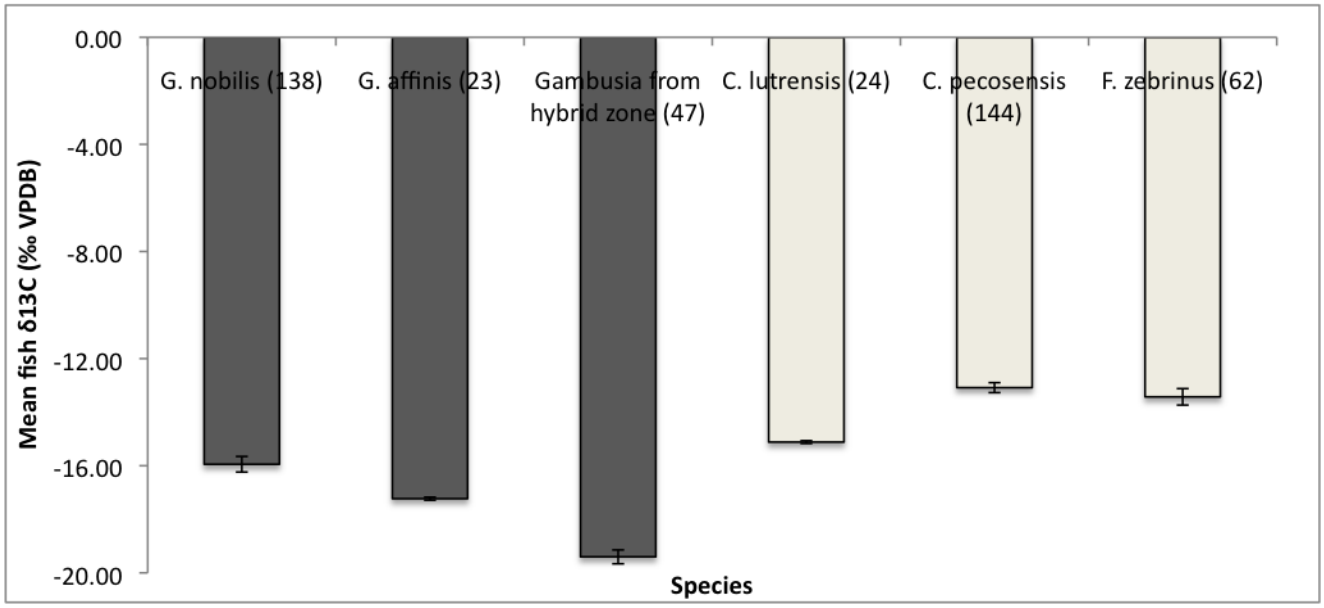


Figure 4. (A) Mean $\delta^{15}N_{base}$ values in ‰ AIR for each fish species surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge with standard error bars. Sample sizes presented. (* = $P < 0.05$). (B) Mean $\delta^{13}C$ values in ‰ VPDB for each fish species surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge with standard error bars. Sample sizes presented.

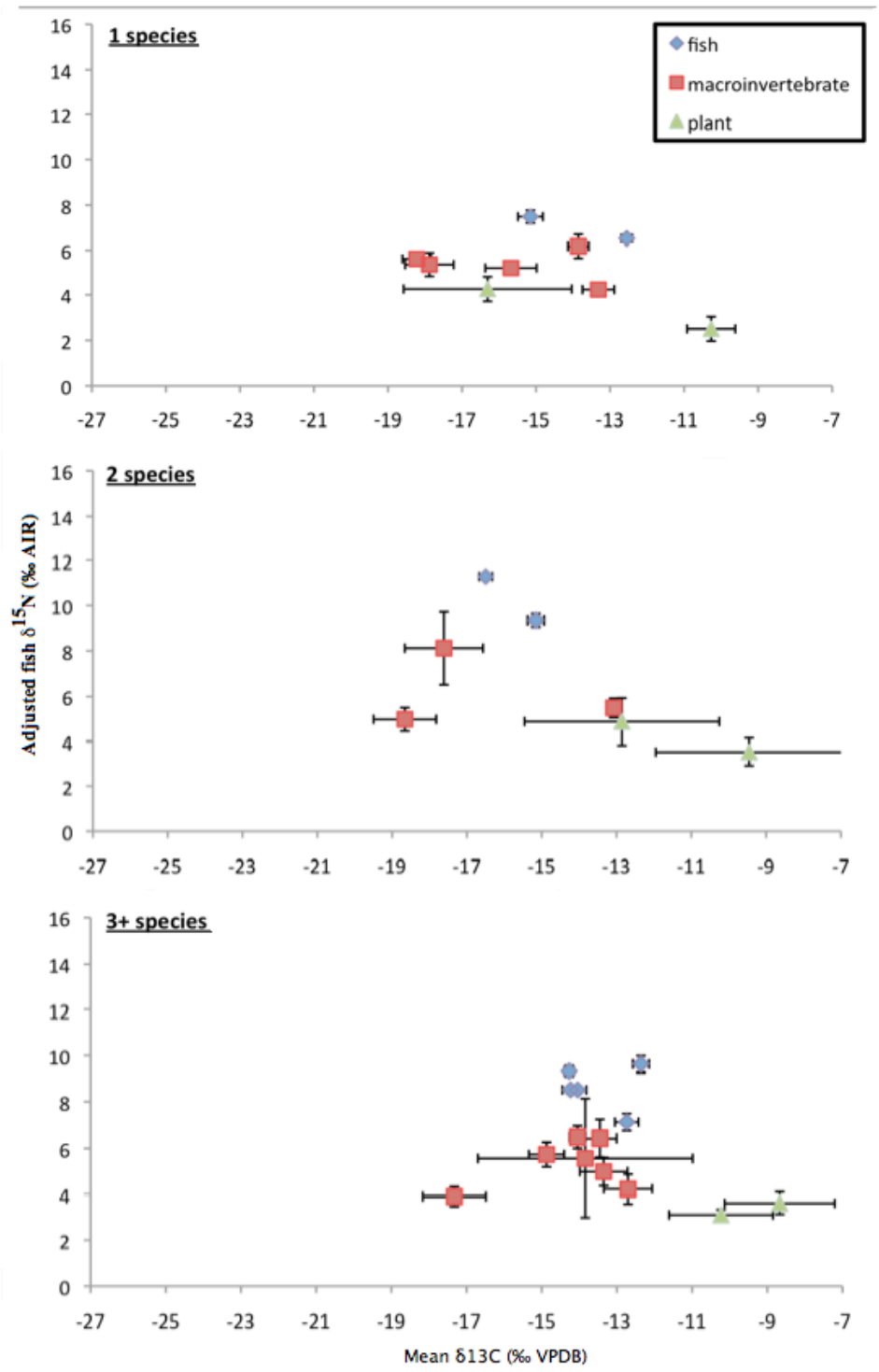


Figure 5. $\delta^{13}C$ and $\delta^{15}N$ biplot showing mean stable isotope values of all fish (diamonds), macroinvertebrate (squares) and plant (triangles) species in all sinkhole sampling localities surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge for (a) sinkholes with

only C. pecosensis, (b) SH27S with only C. pecosensis and G. nobilis, and (c) sinkholes with three or more species. Standard error bars are presented.

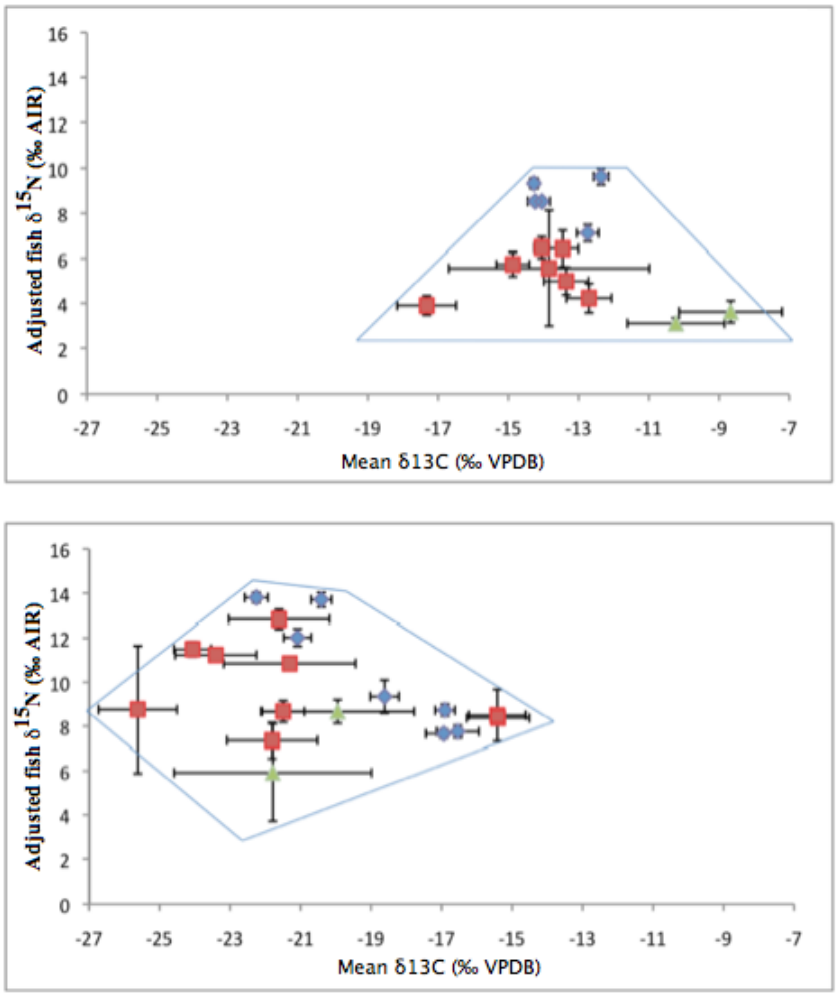


Figure 6. $\delta^{13}C$ and $\delta^{15}N$ biplot showing mean stable isotope values of all fish (diamonds), macroinvertebrate (squares) and plant (triangles) species in all sinkhole sampling localities surveyed in May-July of 2008 at Bitter Lake National Wildlife Refuge for (a) sinkholes with three or more fish species present, (b) stream habitats with three or more species present. Standard error bars are presented.

Chapter 4:

Genetic Characterization of the Endangered *Gambusia nobilis*, the Invasive *G. affinis* and Their Hybrid Zone on Bitter Lake National Wildlife Refuge

Abstract

Endemic desert fishes face a host of threats including hybridization from introduced congeners. *Gambusia nobilis* is restricted to four areas in the southwestern U.S. It is known to hybridize at low levels with the invasive *G. affinis*. We used six microsatellite loci to assess genetic diversity and levels of genetic introgression between these two fishes across a series of geographically clustered allopatric and sympatric populations in New Mexico. There was little genetic diversity and low levels of observed heterozygosity within allopatric sites of *G. nobilis*. A similar pattern was observed for *G. affinis,* however, more allelic richness was revealed. Both species had private alleles, unique to species and some populations. Both species also showed significant genetic structure between populations within and among species. There was only evidence of isolation by distance between *G. affinis* populations. Introgressed individuals comprised less than 30% of populations in putative hybrid zones, which is consistent with previous studies. Individuals in the hybrid zone also showed low levels of observed heterozygosity, which suggested low levels of F1 hybrids. Over 70% of individuals could be assigned with greater than 90% certainty to a parental species' genotype. There was evidence of introgression in putative pure populations of *G. nobilis,* a finding that warrants further investigation. In addition to populations with evidence of introgression from *G. affinis* we also identified populations with rare alleles. These data can be used in the management of *G. nobilis* identification of introgressed populations can be useful in preserving the genome of this fish species by preventing the

spread of individuals from these populations into pure populations. Using individuals from populations with pure and rare species genes in future stocking efforts, however, can increase global genetic diversity of this endangered species.

Introduction

Fishes endemic to the desert southwest account for over half of the federally protected fish species in the United States (Echelle et al. 1989). Across this region, the rapid expansion of metropolitan centers such as Los Angeles, Phoenix and Las Vegas have caused a decline in ground and surface water resources available to natural systems. Desert fishes face a host of other anthropogenic threats to their persistence, including modification of existing habitat (e.g. dams, irrigation channels), eutrophication of water from human inputs (Williams et al. 1985; Warren & Burr 1994), and the introduction of exotic species (Courtenay Jr. & Meffe 1989). Approximately 25-50% of southwestern fishes are nonindigenous (Boydstun et al. 1995; Rahel 2000). The ability of native fishes to persist in the face of this rapidly changing desert ecosystem depends greatly on species and individual variation in traits such as dietary requirements, reproductive investment, and behavior (Meffe 1990; Scribner 1993; Pilger et al. 2010). Genetic variation underlying these characteristics provides the raw material on which selection may act in the face of changing environments.

Characterizing genetic variation within and across populations can enhance the long-term management of threatened desert fishes. Management officials can use genetic information to maintain maximum diversity across current fish populations by preserving those with unique alleles. Meffe (1990) proposed at least six uses of genetic data from fishes in their management and conservation: (1) description of the quantity and distribution of genetic variation in a species; (2) estimation of historical levels of isolation and gene flow;

(3) identification of unique gene pools for special protection; (4) contributions to taxonomic clarifications; (5) information for choosing brood stocks; and (6) monitoring of hatchery populations. In addition, using individuals from populations with high or unique genetic diversity in introduction or restocking efforts can increase species-wide genomic diversity. Genetic characterization can be an invaluable tool in the conservation of fishes of the family Poeciliidae. Fish in this family are small livebearers that are often restricted in their distribution; many are obligate spring endemics and are highly isolated from any source populations. Isolated species often exhibit a low degree of heterozygosity and minimal gene flow between populations, which may lead isolated populations to lose genetic diversity through genetic drift (Echelle et al. 1989).

Species of the genus *Gambusia* (Poeciliidae) have been introduced globally because they are considered useful in mosquito management (Courtenay Jr. & Meffe 1989). The introduced and invasive western mosquitofish, *G. affinis*, and eastern mosquitofish, *G. holbrooki* are highly effective competitors in most environments and have driven native fish species to endangerment or, in some cases, local extirpation (e.g. Courtenay Jr. & Meffe 1989; Galat & Robertson 1992; Crivelli 1995). In addition, hybridization between ecotypically divergent species such as spring- versus stream-adapted forms (Hubbs et al. 2002) of this genus has been observed and documented (e.g. Hubbs 1959; Echelle & Echelle 1980; Scribner & Avise 1993 & 1994; Walters & Freeman 2000). Hybridization between fishes has been well documented across taxa (see Epifiano & Nielsen 2001) and has been suggested as a process that may increase diversity via horizontal transfer of genetic variation (Dowling & Secor 1997). In contrast, however, hybridization may lead to extensive introgression and loss of rare genotypes and species. Avise et al. (1997) demonstrated that

within fifteen years following artificial introduction of spotted bass, *Micropterus punctulatus*, to habitats containing the native small mouth bass, *M. dolomieul*, more than 99% of the sympatric population consisted of the former or individuals resulting from hybridization of the two species. Rapid turnover is also seen in hybridization between *Gambusia* of similar ecotype (e.g. spring-adapted), and genetic introgression may be quick and extensive. Scribner & Avise (1994) documented 80% introgression within one breeding season in experimental ponds between two invasive *Gambusia* species. When an invasive *Gambusia* and a threatened spring obligate *Gambusia* come into contact, however, introgression may be strongly limited by postmating isolating barriers (Davis et al. 2006).

The Pecos gambusia, *Gambusia nobilis*, is a federally endangered poeciliid that is endemic to spring fed-habitats of the Pecos River drainage of southeastern New Mexico and western Texas. They occur in only four areas along the river watershed: (1) Bitter Lake National Wildlife Refuge (BLNWR), New Mexico; (2) Blue Spring near Carlsbad, New Mexico; (3) Diamond-Y Spring, Texas; and (4) springs near Balmorhea, Texas. These four locations are distinct and there is no evidence of gene flow between them as *G. nobilis* has been extirpated from the main stem of the Pecos River for at least 100 years and surface flows have been restricted for at least forty years (Echelle et al. 1989). *Gambusia nobilis* thrive in the stenothermal and stenohaline waters of springs in these areas and have been introduced to different habitats within the four areas where they persist (Bednarz 1979). Established populations of *G. nobilis* in these areas number in the tens to hundreds of thousands (Hubbs et al. 2002), however, it is unclear if restocking and introduction management efforts have affected the genetic diversity within and across populations for this species. Genetic characterization of this endangered species, therefore, is important in

determining the diversity of extant populations. Furthermore, it may elucidate which populations may be utilized as effective management units (MUs) in future restocking efforts as well as conservation of current populations (Hedrick et al. 2006).

Persistence of *G. nobilis* is threatened by a number of factors including lowering of the water table, competition from conspecifics, and hybridization with *G. affinis* (Guillory 1980; Jelks et al. 2008). *Gambusia affinis* and *G. nobilis* are closely related and speciated in allopatry (separate isolated springs, sinkholes and ponds; Hubbs & Springer 1957; Echelle & Echelle 1980). *Gambusia affinis* has invaded three of the four remaining distinct locations in which populations of *G. nobilis* are found (Echelle & Echelle 1980). At Bitter Lake National Wildlife Refuge (BLNWR), the field site for this study, both species occur in both allopatry and in sympatry (local coexistence) in different habitats across the refuge. *Gambusia affinis* is typically found in ephemeral habitats typified by wide daily and seasonal fluctuations of temperature and salinity (Hubbs 2001; Swenton & Kodric-Brown, *in review*). In contrast, *Gambusia nobilis* thrives in spring-fed gypsum sinkholes that are very stable in both temperature and salinity throughout the year (Echelle et al. 1989). This ecological divergence may be important in preventing genetic introgression between these two species and has been suggested as a barrier in other *Gambusia* hybridization systems (Hubbs 1959; Scribner 1993; Scribner & Avise 1993).

The first documentation of sympatry of these two species at BLNWR was in surveys after 1938 (Koster personal journals, unpublished). Introductions may have been accidental and have happened repeatedly and independently in the last century (BLNWR survey records, G. Warrick, pers. comm.). Areas of hybridization are often in creeks where *G. nobilis* is swept from springheads into the more typical habitat of *G. affinis* or in sinkholes

where *G. affinis* has been introduced. Many other *Gambusia* species readily hybridize with *G. affinis* (e.g. Meffe & Snelson Jr. 1989; Scribner & Avise 1994a, b) but hybridization between *G. affinis* and *G. nobilis* is infrequent and was previously estimated at 10% in Blue Spring using allozyme analysis (Echelle & Echelle 1980). Morphological data on individuals in the hybrid zones of BLNWR also suggest a low degree of hybridization but genetic data are lacking (Pers. obs.). Since hybrids can resemble parentals the actual rate of hybridization may be different at BLNWR from that of Blue Spring. The goals of our study were to use microsatellite DNA to quantify (1) the genetic diversity of both the invasive *G. affinis* and the endangered spring-endemic *G. nobilis*, and (2) the degree of genetic introgression between the two species in sympatry at BLNWR.

Methods

Study site and fish collections. This study was conducted at BLNWR, Roswell, NM (N 35° 02.023 W 106° 56.474) in the Pecos river basin (Fig. 1). We collected fin clip samples of both species and their putative hybrids (n=507) for genetic analysis from sixteen sites across their range at BLNWR from May to October in 2007, 2008, and 2009. *Gambusia nobilis* were collected from SH (sinkhole) 37 (n = 64), Lost Spring (LS; n = 34), SH 27S (n = 51) and SH 7 (n = 50). *Gambusia affinis* were collected from Fish Barrier (FB; n = 30), Hunter's Marsh (HM; n = 17), Oxbow Bridge (OBB; n = 34), Oxbow (OB; n = 23), Scout Camp (SC; n = 5), Unit 16 (U16; n = 50) and Unit 5 (n = 5). *Gambusia* individuals were also collected from the potential hybrid zones of Bitter Creek Outflow (BCO; n = 27), Bitter Creek Weir (BCW; n = 70), SH 20 (n = 42), Unit 6 (n = 5) and SH 3 (n = 5). Five sites were gypsum, spring-fed sinkholes in the northern half of BLNWR (SH 3, 7, 20, 27S and 37) ranging between fifteen and fifty-nine meters in diameter. Lost Spring is also spring-fed and

formed from gypsum substrate. Like most spring-fed sinkholes on the refuge it is not enclosed, and flows into Bitter Creek (including the sites BCW and BCO), which is typified by moving water and heavy *Phragmites* vegetation. The creek continues to empty into Bitter Lake, which is named for its highly saline conditions that are inhospitable to the fishes. We also sampled sites in the south of Bitter Lake that were typical of *G. affinis* habitats (shallow with widely fluctuating water conditions; Swenton & Kodric-Brown *in review*; Swenton et al. *in prep*): These sites include OB, OBB, HM, drainage ditches in Units 5 and 16, SC, and FB.

We collected fishes in minnow traps without bait (to avoid complications in a concurrent stable isotope study; Swenton et al. *in prep*) set at each site and retrieved two to three hours later. Each trap was placed within a meter of the shoreline to ensure the highest densities of *Gambusia* fishes were collected, as they typically inhabit shallow, warmer waters. Our trap placement minimizes the capture of heterospecifics, such as *Cyprinodon pecosensis* and *Fundulus zebrinus*, which inhabit the deeper areas of the water column. All fish caught were identified to species by D. Swenton and counted. A subset of the *Gambusia* was sexed (male, female, juvenile) and standard length, body depth, and gonopodium length was measured (e.g. a gonopodium is a modified anal fin that functions as an intromittent organ and is used to inseminate the livebearing females). Females were identified by the presence of the gonopore spot, a black patch marking the site of insemination and an indicator of gravidity (Farr and Travis 1986). Fish that lacked sexual characteristics such as a gonopodium or gonopore spot or visible signs of gravidity were identified as juveniles.

To collect tissue for genetic analysis, we anesthetized or euthanized fish using MS-222, and a small caudal fin clip (approximately $1cm^2$) was removed. These fin clips are non-

destructive and anesthetized fish were given time to recover and released after the tissue was removed. Due to their small size, some juveniles were retained after removal of the entire caudal fin to obtain enough tissue. The fin clip and any whole fish were preserved in 95% ethanol. Because of permit restrictions, we took small collections of the endangered *G. nobilis* relative to numbers of *G. affinis* and *Gambusia* in the hybrid zone.

Molecular methods. Total genomic DNA was isolated by standard phenol-chloroform extraction (Sambrook et al. 1989). Each sample was assayed at seven dinucleotide repeat microsatellite loci developed by Spencer et al. (1999) for use with *G. affinis* and other poeciliid species (*Gafμ1, Gafμ2, Gafμ3, Gafμ4, Gafμ5, Gafμ6,* & *Gafμ7*). Based on initial screening, six loci were consistently scorable and reproducible over multiple assays for both *G. affinis* and *G. nobilis* (*Gafμ6* was removed due to consistent difficulty in reproducing and scoring alleles). Loci were amplified using 10μl polymerase chain reactions (PCR) for *Gafμ4* and *Gafμ5* (1μl DNA, 6.425μl dH_20, 1μl buffer, 0.8μl dNTPs (125μM), 0.35μl each of forward and reverse primers (0.40μM), and 0.075μl *Taq* DNA polymerase). The following microsatellite loci were amplified in 10μl reactions using multiplex PCR: *Gafμ2 + Gafμ7* and *Gafμ1 + Gafμ3* (both using 1μl DNA, 3.425μl dH_20, 2μl buffer, 1μl $MgCl_2$ (2.5mM), 0.8μl dNTPs (125μM), 0.35μl each of forward and reverse primers (0.40μM) for both loci, and 0.075μl *Taq* DNA polymerase). All PCR experiments were run under the following thermal profile: 90 C for 2 min; 30 cycles of 90 C for 30 s, 49-51.9 C (depending on locus) for 30 s, and 72 C for 30 s; 72 C for 10 min. Primers were end-labeled with a florescent dye as follows: Hex (*Gafμ1*, *Gafμ5* & *Gafμ7)* and Fam (*Gafμ2, Gafμ3, Gafμ4* & *Gafμ6*). Nucleotide sequencing and microsatellite screening for this project were

completed on an Applied Biosystems 3100 automated sequencer in the University of New Mexico Molecular Biology Facility and scored using GeneMapper v3.5.

Analysis of genetic variation and structure. We analyzed our microsatellite data for potential genotyping errors such as stuttering, allelic dropouts or null alleles using MICROCHECKER (Van Oosterhout et al. 2004). We used FSTAT v.2.9.3.2 (Goudet 1995) to assay each microsatellite locus, species, and population for number of alleles, private alleles, allelic richness, inbreeding coefficients (F_{IS}) and observed and expected heterozygosity (Goudet 1995). We measured deviations from Hardy-Weinberg equilibrium and tested linkage disequilibrium among loci using GENEPOP 4.0 (Raymond & Rousset 1995).

We characterized hierarchical F_{ST} values and performed pairwise F_{ST} comparisons across localities using FSTAT v.2.9.3.2 (Goudet 1995). We performed analyses of molecular variance (AMOVA) using GenAlEx v.6.1 (Peakall & Smouse 2006). We also determined whether genetic variance could be attributed to differences between populations using pairwise PhiPT values to perform AMOVA analyses. We performed an Isolation by Distance (IBD) analysis to determine if there were any relationships between inferred levels of gene flow and geographic distances between all pairs of sampling localities using a Mantel test with the program Isolation By Distance Web Service version 3.15 using 30,000 randomizations (Jensen et al. 2005). We regressed log-transformed genetic distances (F_{ST} values) against log-transformed geographic distances (estimated as the shortest linear distance between sampling localities; $n = 120$; Slatkin 1993). We also calculated IBD between only pure parental populations of *G. nobilis* and then for only pure parental populations of *G. affinis.* Finally, we conducted a principal component analysis (PCA) with

GenAlEx v.6.1 to visualize the distribution of individual genotypes and populations on principle component axes using the six microsatellite loci. We used this multivariate approach because it to visualize genetic distances among sampling localities.

Population assignments. We used the program STRUCTURE v.2.3.3 (Pritchard et al. 2000) to assign individuals to one of the two species and/or to identify putative hybrids or introgressed individuals. This model-based clustering method may infer population structure and determine an individual's unknown membership to a given population. STRUCTURE provides Bayesian estimates of membership in each of *K* populations. To detect cryptic population structure and detect hybridization in zones of sympatry we used STRUCTURE to estimate *K* for the pooled hybrid zone data. Using the prior assumption that $K = 1$ (as is the case for an unstructured parental, allopatric population), posterior probabilities (Ln likelihood) were assessed for *K* values 1 through 5 in sympatric sites as pure and hybrid population structure is unknown (e.g. it is unclear what proportion of introgressed individuals may be F1 hybrids or backcrosses). This analysis was based on 100,000 Markov chain Monte Carlo iterations after a burn-in period of 50,000 iterations. After determining *K* for hybrid zone populations, we used STRUCTURE to assign individual fish to population (parental species or hybrid/backcross) from genotype data without prior population information. We used *q*-values to estimate cluster (i.e. species) membership (q_i; i = 1 to *K*, where $K = 2$) . In our study q_1 is the estimated proportion of an individual's genome originating from *G. nobilis* and q_2 is the estimated proportion of an individual's genome originating from *G. affinis*. This approach allows for the admixing of genotypes (i.e. hybrids and backcrosses). Any individuals that were not assigned to a pure parental species cluster with 90% confidence ($q \geq 0.90$) were grouped into a hybrid & backcross group for further analysis.

Results

Patterns of genetic diversity & variation. Results from the MICROCHECKER analysis did not provide evidence for null alleles, allelic dropout or stuttering for the six loci used in our study. Observed heterozygosity of parental species and hybrid zone individuals was generally lower than expected heterozygosity (H_E). *Gambusia nobilis*, across populations, had the lowest observed heterozygosity (H_O = 0.1861; *n* = 181), which was lower than expected heterozygosity (H_E = 0.3382). The same pattern was found for populations of *G.* affinis (H_E = 0.6524, H_O = 0.5902, *n* = 148) and for pure parental individuals in the a-priori assigned hybrid zones (H_E = 0.5279, H_O = 0.2853, *n* = 140).

Across all loci, weighted average inbreeding coefficients (F_{IS}) across individuals (*n* = 551) by species had a wide range (Table 1). *Gambusia nobilis* (F_{IS} = 0.451) and pure parental individuals from hybrid zones (F_{IS} = 0.466) were generally deficient in heterozygotes. *Gambusia affinis* individuals had the lowest inbreeding coefficient (F_{IS} = 0.094). Gene diversity was highest for *G. affinis* followed by hybrid zone individuals (0.215-0.853) and lastly by *G. nobilis* (0.037-0.741). Significant departures from H-W expectations from within species comparisons for parentals and hybrid individuals occurred across all loci after Bonferroni correction (Rice 1989; $\alpha < 0.0001$).

Number of alleles and allelic richness across all species and individuals for the six loci used were five (*Gafμ1*; allelic richness = 2.204), eight (*Gafμ2*; allelic richness = 3.473), ten (*Gafμ5*; allelic richness = 5.909), fifteen (*Gafμ3*; allelic richness = 4.736), sixteen (*Gafμ7*; allelic richness = 6.815), and twenty-one (*Gafμ4*; allelic richness = 8.677; Table 2). Number of alleles and allelic richness also varied by species or with individuals of mixed ancestry. *Gambusia nobilis* populations had the lowest number of alleles and the lowest

allelic richness (Table 2). Allelic richness by locus across species was lowest for *Gafμ1* (1.060-2.446) and greatest at *Gafμ4* (4.749-8.348; Table 2). A mean number of 0.0569 private alleles were detected across populations (n = 16 populations, n = 6 loci). Four *Gafμ3* alleles (236, 238, 244 & 254), two *Gafμ2* alleles (106, 158), one *Gafμ*5 allele (250), and three *Gafμ4* alleles (190, 204, 212) were unique to *G. affinis* populations. One *Gafμ2* allele (134), two *Gafμ7* alleles (158, 192), and one *Gafμ*5 allele (240) were unique to *G. nobilis* populations.

Genetic population differentiation. Results of the AMOVA show significant differences in genetic divergence across *G. nobilis* populations are explained significantly by variation among populations (12%) but far more within populations (88%; Table 3). Genetic variation and structure was found between populations of *G. nobilis*. Of 7 possible pairwise comparisons between all pure populations (n = 4), 4 values of F_{ST} were significant after Bonferroni correction at $\alpha = 0.0000417$ level (Table 4). A Mantel test of IBD showed no significant correlation between genetic differentiation and geographic distance across all pairs of *G. nobilis* sampling localities (n = 4; p = 0.834; Table 4).

Results of the AMOVA show significant genetic differences across *G. affinis* populations are explained by variation among populations (10%) but also far more within populations (90%; Table 3). Genetic variation and structure was also found between populations of *G. affinis*. Of 17 possible pairwise comparisons between all pure populations (n = 7), 7 values of F_{ST} were significant after Bonferroni correction at $\alpha = 0.0000417$ level (Table 4). A Mantel test of IBD showed a significant correlation between genetic differentiation and geographic distance across all pairs of *G. affinis* sampling localities (n = 7; p = 0.046; Table 4).

Significant genetic differences across populations of pure parental *Gambusia* individuals in putative hybrid zones are explained by variation among populations (16%) but also far more within populations (84%; Table 3). There was no significant genetic variation and structure found between putative hybrid zone populations. Of 10 possible pairwise comparisons between all putative hybrid populations (n = 5), no values of F_{ST} were significant after Bonferroni correction at $\alpha = 0.0000417$ level (Table 4). A Mantel test of IBD showed no significant correlation between genetic differentiation and geographic distance across all pairs of *G. nobilis* sampling localities (n = 4; p = 0.372; Table 4).

The results of the AMOVA analysis revealed significant genetic variation across individuals from different populations and from different species. Differences among populations explain 24% of the variance (F_{ST}, df = 15, p = 0.010), within populations explain 37% (F_{IS}, df = 491, p = 0.010) and among individuals explain 39% (F_{IT}, df = 507, p = 0.010; Table 5). Results of the AMOVA suggest that there were significant differences in genetic diversity across all *Gambusia* on BLNWR (Table 6). Over all *Gambusia* populations and individuals significant genetic differences are explained by variation among populations (32%) but more by differences within populations (68%; Table 3). Genetic variation and structure was found between species type and between all population types. There were significant genetic differences in values of F_{ST} between parental species in allopatric populations and those in the hybrid zones (Table 6). Of 120 possible pairwise comparisons between all populations, 31 values of F_{ST} were significant after Bonferroni correction at $\alpha = 0.0000417$ level (Table 4). A Mantel test of IBD showed no significant correlation between genetic differentiation and geographic distance across all pairs of sampling localities (n = 120; p = 0.993; Table 4). The first two axes of the PCA analysis (PCI and PCII) of

microsatellite genotypes accounted for 75.42% of total variance, with PCI almost completely separating the two parental species and the hybrid zone populations (Fig. 2).

Species assignment. In the assessment of number of distinct genotypic populations of *Gambusia* (*K*) on BLNWR, the Ln likelihood of the data was -5339.0 (*K* = 2), -5098.3 (*K* = 3), -5033.5 (*K* = 4), and -4917.5 (*K* = 5). Although a slightly smaller likelihood was obtained for *K* = 2 we chose this value following the conservative rationale of the STRUCTURE architects (Pritchard & Wen; STRUCTURE documentation; http://pritch.bsd.uchicago.edu) and Davis et al. (2006). We conducted the simulation on all samples with *K* = 2 (*n* = 551). All but thirty-six fish in parental populations had *q*-values for population assignment >0.90 (q_1 = *G. nobilis* and q_2 = *G. affinis)* and *q* was >0.95. Population assignments were typically far less consistent in putative hybrid zones with many *q*<0.90 (*n* = 50).

Individuals in parental populations of *G. nobilis* (*n* = 204) showed a majority assignment (>88%, *n* = 181) to the *G. nobilis* cluster (Tables 7 & 8). The individuals in parental populations of *G. affinis* (*n* = 154) also showed a majority assignment (>92%; *n* = 142) to the *G. affinis* cluster. The hybrid zone samples generally showed higher rates of admixture and lower assignment consistency than those of the parental populations (Table 8 & Appendix 1). Of the total *Gambusia* individuals collected in the hybrid zone (*n* = 193) 62% were assigned *G. nobilis* identity (*n* = 119), 12% were assigned *G. affinis* (*n* = 24), and 26% were not resolved with $\geq$ 90% probability (*n* = 50; Table 8 & Appendix 1). The unresolved, mixed-ancestry individuals across all populations were identified as hybrids or backcrosses between *G. affinis* and *G. nobilis* and were separated out and pooled together as a hybrid group for genetic variation analyses (*n* = 82; Appendix 1).

For those individuals identified by STRUCTURE as mixed ancestry, the observed heterozygosity was lower than expected, though not as low as those in pure parental individual populations (H_E = 0.6679, H_O = 0.4321, n = 82). Hybrid/backcross individuals (F_{IS} = 0.357) were generally deficient in heterozygotes. Significant genetic differences across populations of hybrid & backcross identified individuals are explained by variation among populations (49%) but equally by differences within populations (51%; Table 3). There were significant genetic differences between pure parental species in allopatric populations, those in the hybrid zones as well as the hybrid & backcross individuals (Table 6).

Discussion

The main objectives of our study were to measure the degree of genetic diversity of *Gambusia nobilis* and *G. affinis* populations on BLNWR and to detect and measure the degree of hybridization in putative sympatric sites. Our results suggest that the allelic diversity and heterozygosity of the endangered *G. nobilis* is relatively lower than that of the invasive *G. affinis* with most genetic variation of the former partitioned among sampling localities. There is evidence for significant population structure between populations within and across species. Only *G. affinis* populations, however, showed any evidence of IBD. Our results provided little evidence for high rates of hybridization, which is congruent to previous studies of *G. nobilis* and *G. affinis.* It may be that most of our introgressed individuals are resultant from maintenance of backcrossed individuals in zones of occasional secondary contact rather than F_1 hybrids

Across all localities examined, we observed a low degree of observed heterozygosity in the endangered *G. nobilis*. This species was previously present in the mainstem Pecos River (*ca* 100ya; Hubbs et al. 2002) but is currently extirpated and is restricted to the

springheads of spring-fed sinkholes and creeks in the watershed. Historically, some sinkholes on BLNWR were connected via canals constructed in the 1930s, but a lowering water table has prevented surface flows between populations for at least forty years (Echelle et al. 1989). Currently, these populations are mostly isolated from surface flow connectivity. Low genetic diversity of *G. nobilis* may be due to range contraction over the last century. Given the degree of geographic isolation between these sites, gene flow is probably highly restricted and may have been subject to drift and fixation over time. This potentially explains the high degree of differentiation among *G. nobilis* populations and the low diversity within populations. Repeated and independent introduction events over the last century on BLNWR have probably facilitated founder effects and genetic bottlenecks in these populations (BLNWR records, G. Warrick, *pers. comm.*). The geographic and temporally haphazard nature of these reintroductions probably also explains the noted lack of IBD between populations of *G. nobilis*. Furthermore, these populations have been mostly isolated in their respective sinkholes with little chance for gene flow exchange since the Pecos River no longer floods and the canals have been filled in.

Extant *G. nobilis* populations can achieve high densities in suitable habitats. An estimated 27,000 individuals inhabit the Bitter Lake National Wildlife Refuge area, and 900,000 inhabit Blue Spring (Bednarz 1975; 1979). The populations at BLNWR, however, are generally diverse across sites but not within sites. Population structure may be due to historic range contractions and probable bottlenecks the species experienced when extirpated from the mainstem of the Pecos River, thereby decreasing gene flow considerably between populations. The isolated spring populations that remain are akin to island populations, which often have lower genetic diversity than mainland source populations (Frankham 1997). We

believe the high degree of differentiation among *G. nobilis* populations represents genetic diversity that should be preserved and increased by introduction of genetically distinct populations to new habitats. We found evidence for private alleles across many loci for both species. These occur, however, at very low frequencies in these populations, often with one or a few individuals with such genotypes in our sampling. However, given the often large population sizes of these fish (thousands and tens of thousands), collection of sufficient individuals with unique genotypes seems feasible. Regardless, given the low allelic diversity of *G. nobilis* populations overall, maintenance of those populations with private or rare alleles should be a priority for management officials (Chase & Knight 2003, Sei et al. 2009). Populations containing such alleles should not only be candidates for conservation, but also used in reintroduction or supplementation efforts to avoid further stocking populations with genetically monomorphic genotypes or those that may be potentially introgressed (e.g. SH27S; Meffe 1990). Currently there is no formal stocking program for *G. nobilis* and the last notable stocking effort on the refuge occurred in the 1980's. The data from this study may be used to identify genetically unique populations, which may be targeted for stocking should more critical habitat be designated for this endangered species.

Gambusia affinis populations are relatively more diverse than its endangered congener; nevertheless, observed heterozygosity is lower than expected heterozygosity. Genetic diversity is probably still low because these fish are also subject to founder effects and genetic bottlenecks on BLNWR as their habitats frequently dry up and flood naturally or for waterfowl management purposes in the southern end of the refuge. Populations may frequently go locally extinct and be re-colonized by even a single pregnant female when habitats become suitable again. In contrast to the range contraction of *G. nobilis*, the genetic

diversity of *G. affinis* may be due to the nature of repeated and independent introduction events but also the movement of these fish in the connected waterways on the southern end of the refuge where *G. affinis* are predominantly found (BLNWR records, G. Warrick, *pers. comm.*). The signature of IBD between *G. affinis* populations is suggestive of movement and gene flow between many of these populations by the fish themselves rather than anthropogenically facilitated transfer as with the spring-endemic, *G. nobilis.*

Previous studies of hybridization between *Gambusia* spp. have demonstrated introgression that is low in some systems and rapid and extensive in others (Echelle & Echelle 1980; Scribner & Avise 1994; Davis et al. 2006). The results of our STRUCTURE analysis suggest that hybridization between the invasive *G. affinis* and the endangered, spring-endemic *G. nobilis* are at low rates on BLNWR. Our findings support an earlier study by Echelle & Echelle (1980) using allozyme data at Blue Spring. They found hybridization rates at approximately 10%. Our data suggest that more than 70% of individuals in hybrid zones on BLNWR can be assigned to one of the parental species with 90% or greater probability. The remaining individuals (<30%) have less clear assignment and may be hybrids or, more likely given the reasonably high assignment values, backcrosses of interbreeding between *G. nobilis* and *G. affinis* (Echelle & Echelle 1980; Davis et al. 2006). This may explain why observed heterozygosity is lower than expected in the putative hybrid zones. Most individuals in this zone are not, in fact, hybrids but can mostly be assigned a *G. nobilis* identity, a species with lowered allelic diversity. Observed heterozygosity was higher when individuals identified as hybrids or backcrosses in the STRUCTURE analysis were considered as a single pooled population. Furthermore, most hybrid individuals appear to be backcrosses rather than F1 hybrids, which could explain lack of heterozygosity in putative

hybrids. We also identified potential hybrid or backcrosses in putative pure *G. nobilis* parental populations, SH27S, in particular. Further examination of these populations, including mtDNA analysis, is warranted to determine if these population genomes are introgressed with the invasive *G. affinis*.

Hybridization between fishes has been extensively documented and subject of increasing study to determine the relative threat it poses to species of concern (Epifiano & Nielsen 2001). Hybridization risk, for example, may affect listing status and recovery plans for endangered species. From the standpoint of protecting the endangered *G. nobilis*, however, there seems to be little immediate threat from hybridization with *G. affinis* under current conditions. These results along with results reported in Echelle & Echelle (1980) are surprising given the long period of contact ($\leq$70 years) between these two short-lived species. Many studies have examined reproductive isolation between divergent stream-adapted and spring-endemic *Gambusia* species, which have provided some evidence for premating reproductive isolation between these two species via ecological speciation (Hubbs 2001; Langerhans et al. 2007; Swenton *in press*; Swenton & Kodric-Brown *in review*). Langerhans et al. (2007) demonstrated divergence in morphology as a product of ecological divergence. In contrast to divergent allopatric habitats of these two species most secondary contact zones resemble habitats more typical of the endangered spring endemic and there is a corresponding skew towards *G. nobilis* genotypes in these sites. As *G. nobilis* has narrow habitat requirements it is unlikely to persist and/or hybridize in those areas typically dominated by the euryhaline and eurythermal tolerant and invasive *G. affinis* (Hubbs et al. 2002; Swenton & Kodric-Brown, *in review*; Swenton et al., *in prep*). For example SH3, which is highly saline ($\geq$ 20ppt) is skewed towards the *G. affinis* genotype, as *G. nobilis*

probably cannot persist for long in this habitat. In addition to ecological barriers there is evidence of premating sexual selection against hybridization mediated by female choice in both species (Swenton, *in press*).

Strong postmating isolating mechanisms may also be at work in this system. There is evidence for divergent selection on life history strategies between these two species that is reflective of the different ecological conditions in which they evolved (Scribner 1993; Rundle & Nosil 2005; Swenton & Kodric-Brown, *in review*). These two fishes also thrive in very different environments, and zones of contact may be suboptimal for the spring endemic *G. nobilis*, or for the stream adapted *G. affinis*. The habitat downstream of springheads, sites typical of hybridization for these fishes, is markedly less stable in temperature and flow (Hubbs 1995). A divergence in life history traits might also lead to divergence in unrelated traits including those related to reproductive compatibility. As in the Langerhans et al. study (2007) the ecologically differentiated *G. nobilis* and *G. affinis* may also be morphologically incompatible in reproduction and/or bear hybrid offspring that are highly unfit relative to their parents.

This study underscores the importance of characterizing the genetic variation of threatened fishes when considering their management, especially in species that have highly restricted populations and specialized habitat requirements (Strayer 2006). Although *G. nobilis* thrives in sinkholes in which it has been introduced, their species-wide persistence may be in question. Despite a large number of individuals, these populations have low allelic diversity consistent with low effective population size. There is little genetic diversity in *G. nobilis* relative to *G. affinis*, the material by which selection can shape these populations in the face of a changing desert environment. Increasing genetic diversity across populations

within a watershed is one means to bolster the persistence of these highly restricted fish species (Calmusso & Rinne 1999). Management officials may offset the current low genetic diversity of *G. nobilis* populations by this approach. The continued identification and preservation of genetically unique populations (e.g. SH7 & SH37) and their supplementation to monomorphic populations or introduction in restocking efforts across the watershed may further the persistence of this endangered desert fish.

Acknowledgements

Co-authors for the *in prep* publication version of this manuscript are T.F. Turner, M.J. Osborne & A. Kodric-Brown. We thank J.C. Trexler, B.O. Wolf, S. Foster, B.B. Barker, Y.E. Sawyer, & T.M. Brennan for helpful editorial comments on this manuscript. T. Diver assisted with genetic analyses. C.L. Hayes, A. Olson, B. Clifford, & G. Warrick assisted with data collection. A. Mazzarella provided assistance with figures. A New Mexico Department of Game and Fish Share with Wildlife Program and, a National Science Foundation Doctoral Dissertation Improvement Grant to A. Kodric-Brown and D. Swenton funded this work. Additional funding was provided to D. Swenton from an Alvin and Caroline Groves Research Scholarship (UNM Biology Department), Crawford Scholarship (UNM Biology Department), SRAC grant (UNM Graduate and Professional Student Association, Student Resource Allocations Committee) and GRAC grant (UNM Biology Department, Graduate Resource Allocations Committee). *Gambusia nobilis* were studied in accordance with NM state permit 2968 and federal permit TE676811-0.

Literature Cited

Avise, J. C., Pierce, P. C., Van Den Avyle, M. J., Smith, M. H., Nelson, W. S., & Asmussen, M. A. 1997. Cytonuclear introgressive swamping and species turnover of bass after an introduction. Journal of Heredity 88:14-20.

Bednarz, J. 1975. A study of the Pecos gambusia. Endangered Species Program, New Mexico Department of Game and Fish, Santa Fe, New Mexico, pp. 1-30.

Bednarz, J. 1979. Ecology and status of the Pecos gambusia, *Gambusia nobilis* (Poeciliidae), in New Mexico. Southwestern Naturalist 24:311-322.

Boydstun, C., Fuller, P., & Williams, J. D. 1995. Nonindigenous fish. In: Our Living Resources: A Report to the Nation on the Distribution, Abundance, and Health of US Plants, Animals, and Ecosystems (LaRoe, E. T., Farris, G. S., Puckett, C. E., Doran, P. D. & Mac, M. J., eds), Department of the Interior, National Biological Service, Washington, D.C., pp. 431-443.

Calmusso, B. & Rinne, J. N. 1999. Native montane fishes of the middle Rio Grande ecosystem: status, threats and conservation. USDA Forest Service Proceedings RMRS-P-&:231-238.

Chase, J. M. & Knight, T. M. 2003. Community genetics: toward a synthesis. Ecology 84:580-582.

Courtenay Jr., W. R. & Meffe, G. K. 1989. Small fishes in strange places: A review of introduced poeciliids. In: Ecology and Evolution of Livebearing Fishes (Poeciliidae) (Meffe, G. K. & Snelson Jr., F. F., eds). Prentice Hall, New Jersey, pp 319-331.

Crivelli, A. J. 1995. Are fish introductions a threat to endemic fresh-water fishes in the northern Mediterranean region? Biological Conservation 72:311-319.

Davis, S. K., Echelle, A. A. & Van Den Bussche, R. A. 2006. Lack of cytonuclear introgression despite long-term hybridization and backcrossing between two poeciliid fishes (*Gambusia heterochir* and *G. affinis*). Coepia 2006:351-359.

Dowling, T. E. & Secor, C. L. 1997. The role of hybridization and introgression in the diversification of animals. Annual Review of Ecology and Systematics 28:593-619.

Echelle, A. A. & Echelle, A. F. 1980. Status of the Pecos Gambusia, *G. nobilis*. Endangered Species Report No. 10:1-73, U.S. Fish and Wildlife Service, Albuquerque, New Mexico.

Echelle, A. F., Echelle, A. A. & Edds, D. R. 1989. Conservation genetics of a spring-dwelling desert fish, the Pecos gambusia (*Gambusia-nobilis*, Poeciliidae). Conservation Biology 3:159-169.

Epifanio, J. & Nielsen, J. 2001. The role of hybridization in the distribution, conservation and management of aquatic species. Reviews in Fish Biology and Fisheries 10:245-251.

Farr, J. A. & Travis, J. 1986. Fertility advertisement by female sailfin mollies, *Poecilia latipinna* (Pisces: Poeciliidae). Coepia 1986:467-472.

Frankham, R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78:311-327.

Galat, D. L. & Robertson, B. 1992. Response of endangered *Poeciliopsis-occidentalis sonoriensis* in the Rio-Yaqui drainage, Arizona, to introduced *Gambusia affinis*. Environmental Biology of Fishes 33:249-264.

Goudet, J. 1995. FSTAT V1.2. A computer program to calculate F-Statistics. Journal of Heredity 86:485-486.

Guillory, V. 1980. *Gambusia nobilis* (Baird and Girard), Pecos gambusia. In: Atlas of North American Freshwater Fishes (Lee, D. S. et al., eds). N.C. State Museum of Natural History, Raleigh, pp 544.

Hedrick, P. W., Lee, R. N. & Hurt, C. R. 2006. The endangered Sonoran topminnow: examination of species and ESUs using three mtDNA genes. Conservation Genetics 7:483-492.

Hubbs, C. 1959: Population analysis of a hybrid swarm between *Gambusia affinis* and *G. heterochir*. Evolution **13**, 236-246.

Hubbs, C. 1995. Springs and spring runs as unique aquatic systems. Copeia 1995:989-991.

Hubbs, C. 2001. Environmental correlates to the abundance of spring-adapted versus stream-adapted fishes. Texas Journal of Science 53:299-326.

Hubbs, C. & Springer, V. G. 1957. A revision of the *Gambusia nobilis* species group, with descriptions of three new species, and notes on their variation, ecology and evolution. Texas Journal of Science 9:279-327.

Hubbs, C., Edwards, R. J. & Garrett, G. P. 2002. Threatened fishes of the world: *Gambusia nobilis* Baird & Girard, 1853 (Poeciliidae). Environmental Biology of Fishes 64:428.

Jelks, H.L., Walsh, S.J., Burkhead, N.M., Contreras-Balderas, S., Diaz-Pardo, E., Hendrickson, D.A., Lyons, J., Mandrak, N.E., McCormick, F., Nelson, J.S., Platania, S.P., Porter, B.A., Renaud, C.B., Schmitter-Soto, J.J., Taylor, E.B. & Warren, M.L. 2008. Conservation status of imperiled North American freshwater and diadromous fishes. Fisheries 33:372-404.

Jensen J. L., Bohonak A. J., Kelley S. T. 2005. Isolation by distance, web service. BMC Genetics 6: 13 v 3.15 http://ibdws.sdsu.edu/

Langerhans, R. B., Gifford, M. E. & Everton, E. O. 2007. Ecological speciation in *Gambusia* fishes. Evolution 61:2056-2074.

Meffe, G. K. 1990. Offspring size variation in eastern mosquitofish (*Gambusia holbrooki*: Poecliidae) from contrasting thermal environments. Coepia 1:10-18.

Peakall, R., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6, 288-295.

Pilger, T.J., Gido, K.B. & Propst, D.L. 2010. Diet and trophic niche overlap of native and nonnative fishes in the Gila River, USA: implications for native fish conservation. Ecology of Freshwater Fish 19:300-321.

Rahel, F. J. 2000. Homogenization of fish faunas across the United States. Science 228:854-856.

Rice, W. R. 1989. Analysis tables of statistical tests. Evolution 43:223-225.

Rundle, H. D. & Nosil, P. 2005. Ecological speciation. Ecology Letters 28:336–352.

Sambrook, F., Fritsch, E. F. & Maniatas, T. 1989. Purification of nucleic acids. In: Molecular Cloning: a Laboratory Manual, 2nd edn (Nolan, C., ed), pp. E.3. Cold Spring Harbor Press, New York.

Scribner, K. T. 1993. Hybrid zone dynamics are influenced by genotype-specific variation in life-history traits: experimental evidence from hybridizing *Gambusia* species. Evolution 47:632-646.

Scriber, K. T. & Avise, J. C. 1993. Cytonuclear genetic architecture in mosquitofish populations and the possible roles of introgressive hybridization. Molecular Ecology 2:139-149.

Scribner, K. T. & Avise, J. C. 1994. Population cage experiments with a vertebrate: the temporal demography and cytonuclear genetics of hybridization in *Gambusia* fishes. Evolution 48:155-171.

Slatkin M. 1993. Isolation by distance in equilibrium and nonequilibrium populations. Evolution 47:264–279.

Spencer, C. C., Chlan, C. A., Neigel, J. E., Scribner, K. T., Wooten, M. C. & Leberg, P. L. 1999. Polymorphic microsatellite markers in the western mosquitofish, *Gambusia affinis*. Molecular Ecology 8:157-168.

Sei, M., Lang, B. K., & Berg, D. J. 2009. Genetic and community similarities are correlated in endemic-rich springs of the northern Chihuahuan Desert. Global Ecology and Biogeography 18:192-201.

Strayer, D. L. 2006. Challenges for freshwater invertebrate conservation. Journal of the North American Benthological Society 25:271-287.

Van Oosterhout, C., Hutchingson, W. F., Wills, D. P. M., & Schipley, P. F. 2004. MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535-538.

Walters, D. M. & Freeman, B. J. 2000. Distribution of *Gambusia* (Poeciliidae) in a southeastern river system and the use of fin ray counts for species determination. Coepia 2:555-559.

Warren, M. L. & Burr, B. M. 1994. Status of freshwater fishes of the United States: overview of an imperiled fauna. Fisheries 19:6-18.

Williams, J. E., Bowman, D. B., Brooks, J. E., Echelle, A. A., Edwards, R. J., Hendrickson, D. A. & Landye, J. J. 1985. Endangered aquatic ecosystems in North American

deserts with a list of vanishing fishes of the region. Journal of Arizona-Nevada Academy of Science 20:1.

Tables, Appendices, and Figures

Table 1. F_{IS} values calculated from six microsatellites of each population on Bitter Lake National Wildlife Refuge.

Species	**Site (N)**	F_{IS}
G. nobilis	SH37 (57)	0.180
	LS (47)	0.393
	SH 27S (50)	0.445
	SH 7 (50)	0.510
G. affinis		
	FB (30)	-0.002
	HM (17)	0.223
	OBB (34)	0.139
	OB (13)	0.235
	SC (5)	0.196
	U16 (50)	0.018
	U5 (5)	-0.144
Putative hybrid sites		
	BCO (27)	0.409
	BCW (114)	0.381
	SH20 (42)	0.226
	U6 (5)	0.087
	SH3 (5)	-0.548

Table 2. Number of alleles and allelic richness of the six microsatellite loci used in this study by pooled groups of individuals. *G.a.* = *G. affinis* individuals from allopatric populations; *G.n.* = *G. nobilis* from allopatric populations; *G.a.* + *G.n.* = *G. affinis* + *G. nobilis* pure parental individuals from putative hybrid zones; hybrids = hybrids & backcrosses identified by STRUCTURE.

	Number of Alleles						**Allelic Richness**					
Species (N)	*Gafu1*	*Gafu3*	*Gafu2*	*Gafu3*	*Gafu5*	*Gafu4*	*Gafu1*	*Gafu3*	*Gafu2*	*Gafu3*	*Gafu5*	*Gafu4*
G.n. (181)	2	4	6	14	7	9	1.060	1.346	2.614	6.269	3.803	4.749
G.a. (148)	4	12	7	11	5	14	2.446	5.661	3.221	6.266	4.080	8.348
G.a. + *G.n* (140)	4	9	4	12	5	14	2.138	3.842	2.419	6.618	5.000	7.589
hybrids (82)	2	7	4	11	7	16	1.999	4.980	3.401	6.369	6.352	8.209

Table 3. Summary of the analysis of molecular variance (AMOVA) within and among populations of *Gambusia* and by species at Bitter Lake National Wildlife Refuge. For the hybrid zone 4 populations were removed because only one hybrid was identified.

Across all *Gambusia* species' populations

Source	df	SS	MS	Est. Var.	%	Stat	Value	*P*-value
Among Pops	15	1187.400	79.160	2.271	32%			
Within Pops	531	2574.840	4.849	4.849	68%	PhiPT	0.319	0.010
***G. nobilis* populations**								
Among Pops	3	87.148	29.049	0.554	12%			
Within Pops	144	741.239	4.188	4.188	88%	PhiPT	0.117	0.010
***G. affinis* populations**								
Among Pops	7	86.423	14.404	0.530	10%			
Within Pops	136	631.633	4.644	4.644	90%	PhiPT	0.102	0.010
Pure parental *G. affinis* and *G. nobilis* individuals in hybrid zones								
Among Pops	4	101.839	25.460	0.924	16%			
Within Pops	140	659.596	4.711	4.711	84%	PhiPT	0.164	0.030
Hybrids and backcrosses								
Among Pops	7	222.401	31.772	3.531	49%			
Within Pops	70	255.650	3.652	3.652	51%	PhiPT	0.492	0.020

Table 4. Pairwise F_{ST} values (below diagonal, calculated from microsatellite data) among populations on Bitter Lake National Wildlife Refuge. Populations are separated by a-priori assigned species type occupying the site. After Bonferroni correction significance value is $\alpha = 0.000417$ level. Significant F_{ST} values are in bold and italics. Pairwise geographic linear distance (m) between sampling localities are above the diagonal.

			POPULATION															
			G. affinis							*G. nobilis*				Hybrid sites				
POPULATION			**FB**	**HM**	**OB**	**OBB**	**SC**	**U5**	**U16**	**SH7**	**SH37**	**LS**	**SH27S**	**SH20**	**U6**	**SH3**	**BCO**	**BCW**
	G. affinis	**FB**	-	5265	5034	4975	106	553	2296	4391	3963	3570	2855	3033	544	4172	2994	3359
		HM	***0.12***	-	865	806	5320	5125	2988	9115	8340	7028	7216	7560	5105	8475	6443	6766
		OB	***0.148***	-	-	95	5102	4983	2747	9116	8408	7221	7258	7578	4962	8563	6609	6954
		OBB	0.015	***0.092***	***0.084***	-	5042	4916	2684	9042	8407	7218	7179	7501	4895	8481	6524	6868
		SC	-	0.12	0.088	-	-	489	2360	4291	3858	3469	2749	2928	484	4067	2909	3258
		U5	0.008	0.1	0.135	0.002	-	-	2247	4223	3686	3112	2537	2770	24	3882	2523	2885
		U16	***0.028***	***0.121***	***0.125***	0.007	0.014	0.029	-	6435	5801	4840	4638	4928	2224	5977	4204	4576
	G. nobilis	**SH7**	***0.506***	0.482	0.43	***0.446***	0.555	0.493	***0.485***	-	1010	2679	1920	1558	4246	1057	2985	2837
		SH37	***0.595***	***0.593***	***0.541***	***0.578***	0.687	0.66	***0.564***	***0.108***	-	1675	1165	930	3708	228	2032	1847
		LS	***0.406***	***0.367***	0.301	0.358	0.4	0.399	***0.408***	0.066	***0.173***	-	1267	1552	3132	1696	635	267
		SH27S	***0.387***	***0.364***	***0.28***	***0.336***	0.394	0.339	***0.387***	0.207	***0.304***	***0.104***	-	388	2561	1349	1226	1261
	Hybrid sites	**SH20**	0.474	0.415	0.334	0.408	0.516	0.485	0.473	0.126	0.177	0.064	0.082	-	2794	1145	1597	1591
		U6	0.151	0.084	0.208	0.14	0.173	0.219	0.21	0.453	0.649	0.348	0.321	0.474	-	3905	2542	2905
		SH3	0.084	0.079	0.007	0.061	0.09	0.007	0.086	0.582	0.644	0.486	0.622	0.509	0.112	-	2113	1892
		BCO	***0.396***	0.372	0.241	0.324	0.383	0.495	***0.386***	0.074	**0.161**	0.055	0.159	0.138	0.462	0.31	-	378
		BCW	0.281	0.238	0.185	0.249	0.274	0.244	0.311	0.126	0.236	0.041	0.06	0.095	0.178	0.295	0.133	-

Table 5. Summary of the analysis of molecular variance (AMOVA) among populations and among and within individuals of *Gambusia* on Bitter Lake National Wildlife Refuge.

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**	**Stat**	**Value**	***P*-value**
Among Pops	15	513.462	34.231	0.516	24%	Fst	0.237	0.010
Among Indiv	491	1212.589	2.470	0.813	37%	Fis	0.491	0.010
Within Indiv	507	428.000	0.844	0.844	39%	Fit	0.611	0.010

Table 6. Pairwise F_{ST} values (below diagonal, calculated from microsatellite data) among pooled populations and type on Bitter Lake National Wildlife Refuge. After Bonferroni correction significance value is $\alpha = 0.00833$ level. Significant F_{ST} values are in bold and italics (*G.a.* = *G. affinis* individuals from allopatric populations; *G.n.* = *G. nobilis* from allopatric populations; *G.a.* + *G.n.* = *G. affinis* + *G. nobilis* pure parental individuals from putative hybrid zones; hybrids = hybrids & backcrosses identified by STRUCTURE).

	G.a.	*G.n.*	*G.a.* + *G.n.*	hybrids
G.a.	-			
G.n.	***0.451***	-		
G.a. + *G.n.*	***0.311***	***0.668***	-	
hybrids	***0.158***	***0.192***	0.068	-

Table 7. Proportion of membership of each population in each of the 2 clusters from STRUCTURE analysis (q_1 = *G. nobilis* parental species assignment and q_2 = *G. affinis* parental species assignment).

Species	**Site**	**N (total)**	q_1	q_2
G. nobilis	SH37	57	0.982	0.018
	LS	47	0.928	0.072
	SH 27S	50	0.900	0.100
	SH 7	50	0.952	0.048
G. affinis	FB	30	0.010	0.990
	HM	17	0.016	0.984
	OBB	34	0.035	0.965
	OB	13	0.125	0.875
	SC	5	0.056	0.944
	U16	50	0.026	0.974
	U5	5	0.015	0.985
Putative hybrids				
	BCO	27	0.811	0.189
	BCW	114	0.754	0.246
	SH20	42	0.970	0.030
	U6	5	0.394	0.606
	SH3	5	0.018	0.982

Table 8. Summary of species assignment of individuals by population using STRUCTURE analysis. q_1 = *G. nobilis* parental species assignment and q_2 = *G. affinis* parental species assignment.

Species	Site	N (total)	q > cutoff	N assigned to parental species	N not assigned to to parental species
G. nobilis	SH37	57	$q_1 > 0.95$	55	2
	LS	47	$q_1 > 0.95$	42	5
	SH 27S	50	$q_1 > 0.94$	34	16
	SH 7	50	$q_1 > 0.94$	49	1
G. affinis	FB	30	$q_2 > 0.90$	30	-
	HM	17	$q_2 > 0.95$	17	-
	OBB	34	$q_2 > 0.97$	31	3
	OB	13	$q_2 > 0.90$	6	7
	SC	5	$q_2 > 0.97$	4	1
	U16	50	$q_2 > 0.95$	49	1
	U5	5	$q_2 > 0.97$	5	-
Hybrid zone	BCO	27	$q_1 > 0.90$	17	8
			$q_2 > 0.90$	2	-
	BCW	114	$q_1 > 0.90$	61	38
			$q_2 > 0.90$	15	-
	SH20	42	$q_1 > 0.90$	40	2
			$q_2 > 0.90$	-	-
	U6	5	$q_1 > 0.90$	1	2
			$q_2 > 0.90$	2	-
	SH3	5	$q_1 > 0.90$	-	-
			$q_2 > 0.90$	5	-

Appendix 1. All individuals without $q \geq 0.90$ inferred assignment to either parental population or a reverse parental assignment from expected population identity. Presented are sample ID, site of origin, q_1 (probability of *G. nobilis* identity) and q_2 (probability of *G. affinis* identity). * =Any assignments with < 0.70 inferred assignment for either species.

***G. nobilis* poulations**			***G. affinis* populations**			**Putative hybrid populations**		
Population_ID	q_1	q_2	**Population_ID**	q_1	q_2	**Population_ID**	q_1	q_2
SH37_1	0.674	0.326*	OBB_2	0.267	0.733	BCO_14	0.588	0.412*
SH37_3	0.818	0.182	OBB_33	0.195	0.805	BCO_15	0.484	0.516*
LS_14	0.269	0.731	OBB_34	0.368	0.632*	BCO_16	0.621	0.379*
LS_26	0.015	0.985	OB_10	0.203	0.797	BCO_17	0.613	0.387*
LS_29	0.284	0.716	OB_12	0.279	0.721	BCO_18	0.537	0.463*
LS_31	0.867	0.133	OB_13	0.217	0.783	BCO_27	0.778	0.222
LS_34	0.594	0.406*	OB_6	0.149	0.851	BCO_5	0.637	0.363*
SH27S_14	0.726	0.274	OB_7	0.111	0.889	BCO_6	0.857	0.143
SH27S_18	0.868	0.132	OB_8	0.200	0.800	BCW_103	0.846	0.154
SH27S_22	0.716	0.284	OB_9	0.349	0.651*	BCW_104	0.839	0.161
SH27S_29	0.723	0.277	SC_4	0.234	0.766	BCW_105	0.836	0.164
SH27S_36	0.475	0.525*	U16_49	0.836	0.164	BCW_106	0.881	0.119
SH27S_37	0.726	0.274				BCW_107	0.829	0.171
SH27S_38	0.705	0.295				BCW_109	0.825	0.175
SH27S_39	0.883	0.117				BCW_111	0.830	0.170
SH27S_40	0.655	0.345				BCW_112	0.841	0.159
SH27S_41	0.895	0.105				BCW_113	0.833	0.167
SH27S_42	0.781	0.219				BCW_114	0.829	0.171
SH27S_43	0.757	0.243				BCW_22	0.336	0.664*
SH27S_44	0.765	0.235				BCW_28	0.768	0.232
SH27S_45	0.501	0.499*				BCW_30	0.310	0.690*
SH27S_46	0.427	0.573*				BCW_45	0.373	0.627*
SH27S_47	0.872	0.128				BCW_49	0.351	0.649*

Appendix 1. Continued

***G. nobilis* poulations**			***G. affinis* populations**			**Putative hybrid populations**		
Population_ID	q_1	q_2	**Population_ID**	q_1	q_2	**Population_ID**	q_1	q_2
SH7_35	0.573	0.427*				BCW_52	0.458	0.542*
						BCW_53	0.809	0.191
						BCW_59	0.878	0.122
						BCW_61	0.422	0.558*
						BCW_62	0.452	0.548*
						BCW_64	0.497	0.503*
						BCW_73	0.497	0.503*
						BCW_74	0.606	0.394*
						BCW_75	0.833	0.167
						BCW_76	0.879	0.121
						BCW_78	0.498	0.502*
						BCW_79	0.832	0.168
						BCW_81	0.842	0.158
						BCW_82	0.831	0.169
						BCW_84	0.433	0.557*
						BCW_87	0.834	0.166
						BCW_91	0.179	0.821
						BCW_92	0.828	0.172
						BCW_94	0.833	0.167
						BCW_95	0.832	0.168
						BCW_97	0.877	0.123
						BCW_98	0.842	0.158
						BCW_99	0.102	0.898
						SH20_34	0.295	0.705
						SH20_8	0.865	0.135
						U6_1	0.148	0.852
						U6_4	0.806	0.194

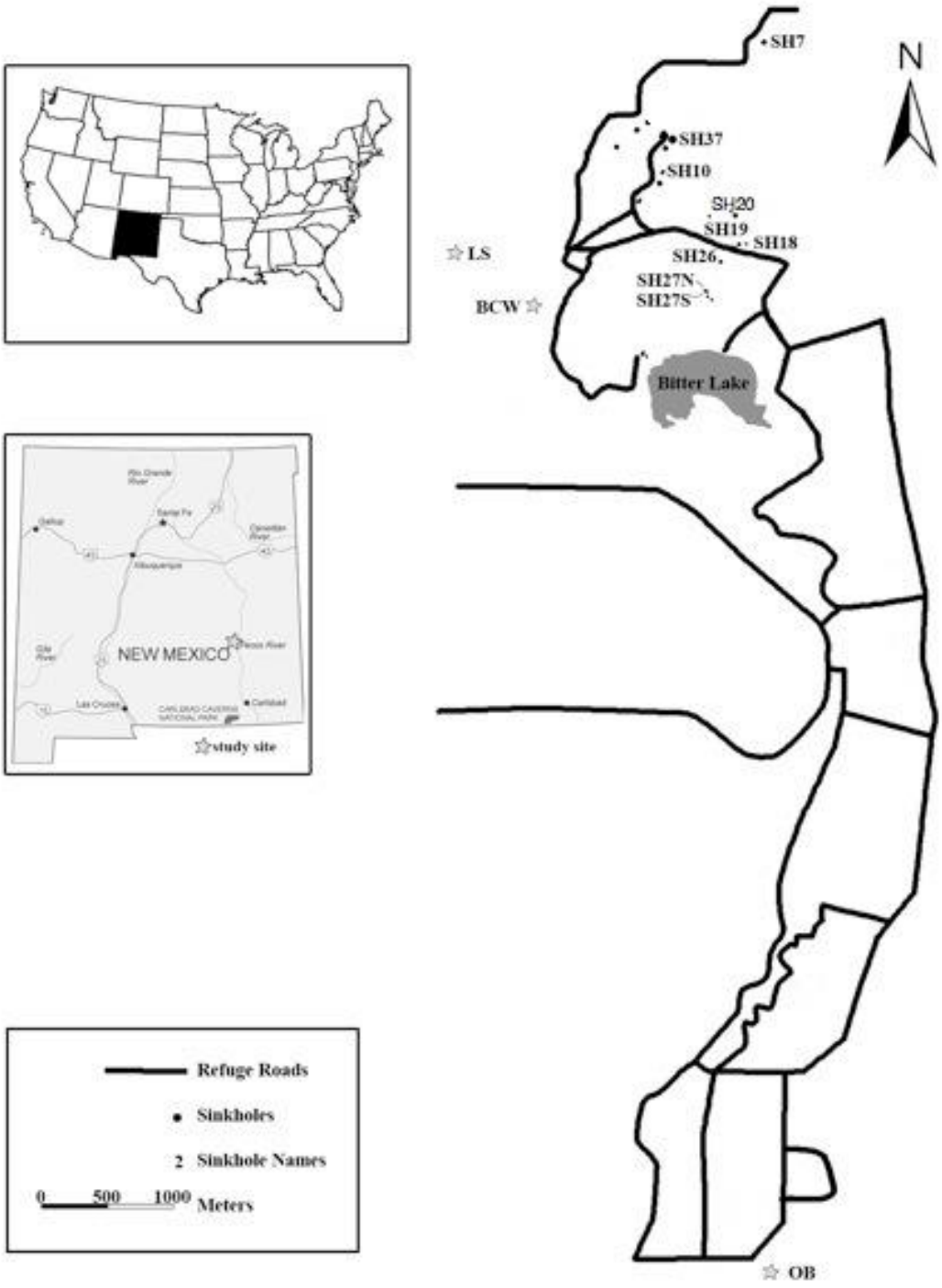


Figure 1. Map of populations sampled on Bitter Lake National Wildlife Refuge. Stars denote non-isolated and/or non-sinkhole populations.

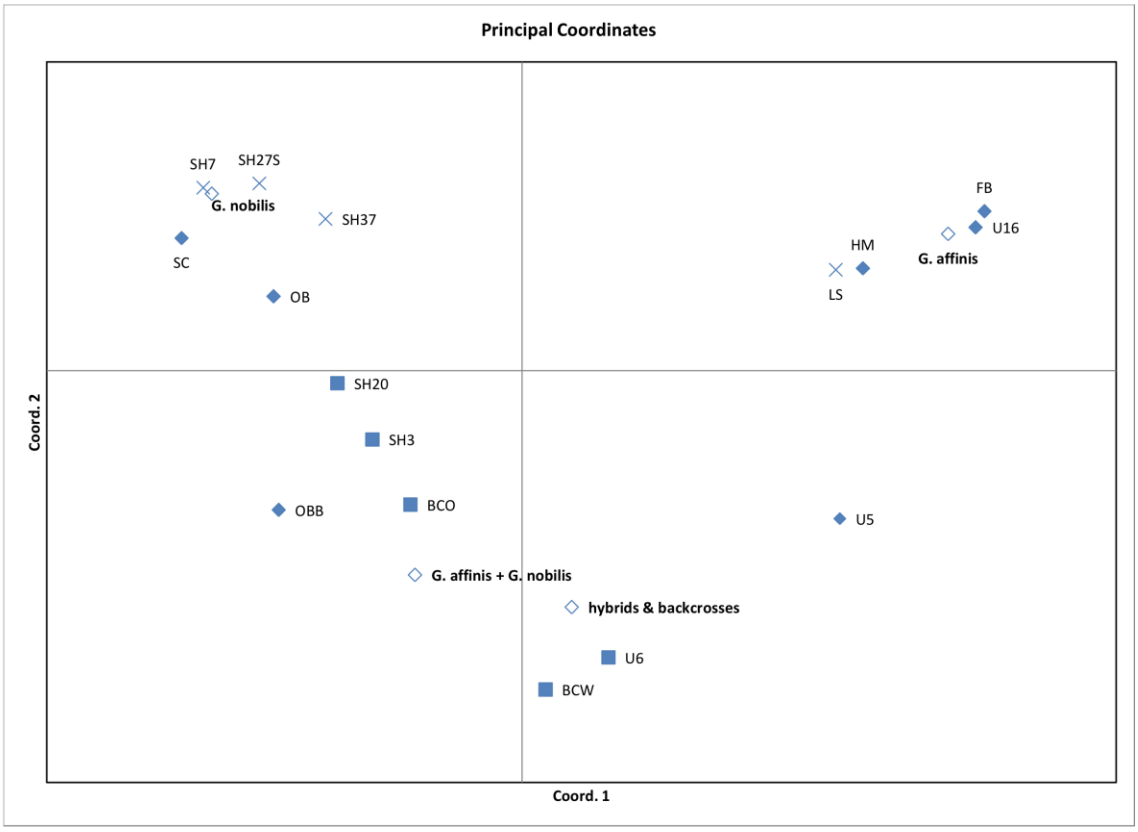


Figure 2. Plot scores of PCI and PCII from the principle components analysis of the multilocus, microsatellite genotype for the 16 populations of G. affinis, G. nobilis and putative hybrid zones on Bitter Lake National Wildlife Refuge. Populations are labeled and marker data points denote species type present (G. affinis = filled diamonds; G. nobilis = cross; hybrid zones = filled squares). Also included are four points of pooled individuals by species/population identity denoted by open diamond markers (pure G. affinis, pure G. nobilis, pure parental individuals in the hybrid zones, and hybrids/backcrosses).