

5-1-2013

LIFE HISTORY AND MORPHOMETRIC VARIATION OF GAMBUSIA NOBILIS AT BITTER LAKE NATIONAL WILDLIFE REFUGE

Alyssa Hopkins

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**LIFE HISTORY AND MORPHOMETRIC VARIATION OF
GAMBUSIA NOBILIS AT BITTER LAKE NATIONAL
WILDLIFE REFUGE**

BY

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THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Master of Science
Biology**

The University of New Mexico
Albuquerque, New Mexico

May, 2013

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ABSTRACT

The Pecos Gambusia, *Gambusia nobilis*, is an endangered, live bearing fish inhabiting sinkholes in a restricted range of the Pecos River Watershed in New Mexico and Texas. The sink holes at Bitter Lake National Wildlife Refuge (BLNWR), Roswell, NM create isolated habitats with varying ecological conditions (habitat size, community composition, dissolved oxygen, salinity and pH). This ecological variation imposes unique selective pressures that may shape differences in life history characteristics and morphology between populations. The goals of this research were to characterize seasonal and population variation in 1) life history characteristics, 2) embryo development patterns, 3) morphology and 4) explore cursory relationships between ecological conditions and *G. nobilis* morphology and life history. Monthly sampling was conducted at BLNWR (May 2011 through April 2012) at sinkholes 7, 27 South, 31, and 37. A trade-off between egg size and brood size was observed from investment in many small embryos to fewer large offspring from April through August. Despite differences in ecological parameters, no significant differences in reproductive traits were observed

between the populations. Interestingly, reproductive effort and average egg size were not correlated with female size. Larger females invested relatively the same amount into reproduction but invested in larger broods rather than increased average egg mass. An analysis of embryo developmental stages identified 1) asynchronous reproduction between females, 2) eggs in multiple stages of development within some gravid females (evidence for superfetation), and 3) increasing average egg mass of later developmental stages suggesting some element of matrotrophy (post-fertilization nutrient transfer). Geometric morphometric techniques were used to evaluate seasonal and population shape differences. During the reproductive season, females tended to be deeper bodied with a posteriorly shifted anal fin. Males and females from sinkhole 31 differed significantly from the other populations; they had deeper body profiles and shorter caudal peduncles than sinkholes 7, 27 South, and 37 which could be related to less predation and higher dissolved oxygen in sinkhole 31. Analyzing life history allocation strategies and identifying seasonal and population differences in morphology and reproduction contributes information that may be important in formulating management strategies and conservation plans for this endangered species.

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CHAPTER 1:

LIFE HISTORY VARIATION OF THE ENDANGERED *GAMBUSIA NOBILIS* (PECOS GAMBUSIA) FROM BITTER LAKE NATIONAL WILDLIFE REFUGE

ABSTRACT

Organisms are limited by resources, that are in turn allocated to aspects of life history, including growth, survival, and reproduction. Although aspects of poeciliid life histories have been studied extensively, variation in allocation to growth and reproduction of endangered and threatened species needs further study. Four ecologically distinct populations of endangered *Gambusia nobilis* from sinkholes at Bitter Lake National Wildlife Refuge in New Mexico were studied. We hypothesized that differences in selective pressures between populations would drive variation in life history and reproductive investment strategies. Female size and condition varied between populations but no significant differences in reproductive traits (brood size and egg mass) were observed. Dissolved oxygen levels had a positive relationship with gravid female size while salinity negatively affected condition. Reproductive strategy shifted over the reproductive season, from investment in many small embryos to fewer large offspring. Reproductive effort did not vary with female size or condition, suggesting that small or poor quality females invested proportionally equally in reproduction as larger or healthier females. Brood size and brood mass increased with female size, but average egg mass remained unchanged. Greater reproductive effort was slightly positively related to the

percent of *Fundulus zebrinus* (a predator) in the sinkhole. Interesting patterns of embryo development were observed which may suggest asynchronous reproduction, some component of superfetation, and post-fertilization nutrient transfer. Females used different allocation strategies to optimize reproductive success while maintaining equal reproductive investment. Understanding ecological effects on life history and potential population variation is important to the conservation efforts of this species.

Keywords:

Gambusia; life history; reproductive investment; embryo

INTRODUCTION

Life history characteristics describe traits related to growth, reproduction, and survival (Roff 1992). These can include growth rate, age and size at sexual maturity, offspring size, number of offspring, inter-brood interval, and lifespan (Roff 1992). Limited resources require trade-offs between investment in one aspect of life history at a cost of investment in another, such as the trade-off between offspring size and number or between growth and size at sexual maturity (Messina and Fox 2001; Roff 1992; Stearns 1989). Selective pressures favor allocation of resources that maximize an organism's lifetime fitness (Roff 1992; Stearns 1989). Evolutionary divergence between populations or species can be driven by selective pressure favoring alternative life history strategies. Some ecological parameters affecting life history traits in fishes include environmental stability (Meffe 1987; Stockwell and Vinyard 2000), predation pressure (Rodd et al. 1997), resource abundance (Reznick et al. 1996), mate availability (Kokita and Nakazono 1998), and abiotic conditions including temperature and salinity (Brown-Peterson and Peterson 1990; Edwards et al. 2006). Ecological variation between localities may result in distinct selective pressures experienced by each population and ultimately lead to divergent evolution or variation in expressed traits by phenotypic plasticity (West-Eberhard, 1989). Natural selection then acts upon the heritable life history traits expressed and drives evolution of populations and species to optimal life history strategies. As one of the major aspects of life history, reproduction requires trade-offs of allocation of resources between investment in current offspring at a cost to female survivorship and investment in future offspring (Stearns 1989). Allocation choices can be based on resource abundance, value of the investment, current and future

environmental conditions, probability of future reproduction, and opportunity costs. For example, foregoing current reproduction in favor of growth reduces fertility at younger ages but increases potential reproductive success in the future. If survivorship is high, or sexual selection for body size is strong, life time fitness would be maximized by delaying reproduction

Poeciliids in general, and *Gambusia* species in particular, have been studied extensively and provide important insights into the types of selective pressures that affect life history traits as well as the tradeoffs in allocation of resources to various aspects of life history. Poeciliids are typically small-bodied and short-lived species, often widely distributed and dominant in habitats with high salinity and temperatures (Rosen and Bailey 1963). They have many unique reproductive adaptations. In males, these include modified anal fin rays and supporting structures forming gonopodia for internal fertilization. Females exhibit a spectrum of reproductive strategies from exclusive lecithotrophy (yolk provides all nutrients for embryos) to complete matrotrophy (females continue to transfer nutrients throughout gestation) (Wourms 1981). Additionally, many poeciliid species exhibit superfetation, gestating multiple broods at different stages of development simultaneously. It is thought that superfetation may allow females to shorten the interbrood interval and temporally disperse resource allocation to offspring, thus overcoming the constraint of body size (Wourms 1981). All but one species are live-bearing, an adaptation that increases offspring survivorship at a cost of reduced fecundity, increased physiological demands, and decreased swimming performance (Goodwin et al. 2002). Live-bearing poeciliids provide a unique opportunity to measure reproductive investment and evaluate ecological effects on resource allocation strategies.

Reproductive investment can vary between closely related species (Swenton and Kodric-Brown 2012) and even between allopatric populations of the same species (Downhower et al. 2000; Langerhans et al. 2010) in response to different environmental conditions.

The effects of ecology on life history have been studied at length in *Gambusia* species. For example, life history traits of *Gambusia* can vary with latitude; *Gambusia holbrooki* from lower latitudes invest more energy in reproduction than did populations from more northern latitudes (Benejam et al. 2009). In environmentally unstable habitats, offspring size is highly variable to maximize the number of surviving offspring (Meffe 1987). *Gambusia affinis* from a thermally unstable environment were in poorer condition, matured at a smaller body size, and invested in smaller embryos than females from more thermally stable environments (Stockwell and Vinyard 2000). Similar results have been obtained from analyses of life history variation along salinity gradients. An analysis of *G. holbrooki* revealed that higher salinity environments were associated with higher reproductive investment, earlier reproduction, larger broods, smaller embryos, and reduced female condition (Alcaraz 2006; Alcaraz and Garcia-Berthou 2007; Brown-Peterson and Peterson 1990). In contrast, some studies suggest that reproductive effort may be inversely related to salinity and that females from high salinity environments may actually produce heavier embryos (Alcaraz 2006; Gomes and Monteiro 2007). Life history strategies also can be shaped by biotic interactions. For example, an increase in population density throughout the reproductive season corresponded to a decrease in fecundity of *G. affinis* (Hughes 1985). Other biotic factors, like the presence of predatory fish in the community, also affect life history strategies. Compared to predator free

populations, greater predation pressure favors investment in many smaller offspring who mature at smaller sizes (Gomes and Monteiro 2007; Reznick et al. 1996).

One species, *Gambusia nobilis* (Baird and Girard 1853), has historically inhabited small springs, sinkholes and seeps throughout the Pecos River watershed but is currently restricted to only four known localities in New Mexico and Texas (Bednarz 1979; Echelle and Echelle 1986; Edwards 2001; Hubbs et al. 1983; Hubbs 2003). *Gambusia nobilis* is sexually dimorphic with males growing little after reaching sexual maturity. Females are larger (18-40mm standard length) with a black gonopore while males are smaller (18-25mm standard length) and have a gonopodium (Bednarz 1979; Edwards 2001; Hubbs et al. 1983; Hubbs et al. 2002; Hubbs 2003; Sublette et al. 1990). There is little courtship behavior observed between males and females; the gonopore is hypothesized to signal fertility to males who actively pursue females or sneak copulation (Leiser et al. 2010). Larger female size allows them to swim faster than males resulting in some female choice and male-male competition (Leiser et al. 2010). Females have a mean brood size of 38 offspring with mean birth weight between 25 and 50 mg and a mean interbrood interval of 52 days (Bednarz 1979; Hubbs 1996; Hubbs 1997, Hubbs 2003). Swenton and Kodric-Brown (2012) observed an inverse relationship between brood size and offspring mass with increasing embryo mass over the reproductive season. The common positive relationship within fishes between female size and brood size has also been noted (Bednarz 1979). The breeding season extends from April through August depending on seasonal conditions and females can produce up to four broods within a season (Rosen and Bailey 1963; Swenton and Kodric-Brown 2012). The

estimated life-span of this species is at least three years (Swenton and Kodric-Brown 2012).

Previous studies of *G. nobilis* have not assessed potential variation in life history between seasons or populations. This is because sampling was concentrated primarily during summer months and at few sites. While *Gambusia* species provide general insight into the maintenance and evolution of life history characters, this research was specifically designed to 1) contribute to the understanding of *G. nobilis* life history, 2) identify potential variation in life history traits between populations and across seasons, 3) document patterns in embryo developmental stages across the reproductive season, and 4) test for relationships between ecological parameters and observed life history strategies. This research meets the goals of the Pecos *Gambusia* Recovery Plan (Hubbs et al. 1983) and may inform conservation efforts targeted at this federally endangered species.

METHODS

Field Collections

This study examined life history traits of four populations of *G. nobilis* at Bitter Lake National Wildlife Refuge (BLNWR) (33°27'32.97" N, 104°24'11.75" W). Three populations (sinkholes 7, 37, and 27 South) were sampled monthly from May 2011 through April 2012. The fourth population (31) was only sampled during June, July, and August with a sample of males obtained in October. Sampling at sinkhole 31 was reduced to minimize impact to other endangered species. Sinkholes 10, 27 North, and 32 were also sampled in the summer months of 2011, but no *G. nobilis* were observed or caught at these sites. Research on endangered species is essential, but can be met with

many challenges including limited sample sizes, limited geographic ranges, and few populations from which to sample. Fish were sampled using mesh minnow traps baited with dry dog food and placed on the shallow underwater ledges of the sinkholes (<1 meters deep during summer months and approximately 5-15 meters deep during winter months (November- March) when the fish populations descend to overwinter). Trapping time varied depending on catch rate in attempt to achieve a sample size of at least ten *G. nobilis* per site per month. During the winter sampling, a rope was attached to both ends of the trap and lowered from opposite banks of the sinkhole (forming a 'V' with the trap at the bottom and rope on each end). To retrieve traps, both lines were pulled simultaneously to raise the trap nearly out of the water and then pulled towards one bank. This technique allowed placement of the trap on the bottom of the sinkhole or on deep underwater ledges and prevented dredging of the sediment and vegetation from the bottom and along the slopes or underwater shelves as the trap was retrieved. For each trapping effort, the number of individuals of each species caught and water quality measurements (YSI hydrolab, model 85) were recorded. Up to ten individuals of *G. nobilis* per site per month (20 from sinkhole 31 in July) were retained and all other fish were released.

Specimen Preparation and Dissections

The *G. nobilis* individuals retained were fin clipped (for preservation of genetic samples), euthanized with MS-222 (Tricaine Methanesulfonate), photographed, placed in 10% formalin, and subsequently dehydrated with an ethanol gradation. The specimens were accessioned at the Museum of Southwestern Biology (MSB) at the University of New Mexico (ACC2011-V:18). A total of 345 specimens (273 females and 72 males)

were collected over the 12 month sampling period. For each individual, sexual maturity was noted, standard length was measured with digital calipers (Mitutoyo Absolute Digimatic), and mass was measured with a digital balance (Sartorius R300S) to the nearest 0.0001 gram. For all individuals, eviscerated mass was measured after removing internal organs and gonads and was used in place of total mass in statistical analyses so comparisons could be made regardless of reproductive status. For females, the brood size was determined by counting all embryos at developmental stage 3 (mature ovum) or greater (Haynes 1995). Brood mass was measured to the nearest 0.0001 grams using the digital balance, and average egg mass was determined by dividing the brood mass by the brood size. A measure of female reproductive investment was estimated by calculating reproductive effort as brood mass \div female eviscerated mass (Swenton and Kodric-Brown 2012). The condition of each individual (sign of relative health) was calculated using Ricker's condition factor, k , [$k = (100 * Weight) \div (length)^b$] (Garcia-Berthou and Moreno-Amich 1993; Samat et al 2008). Condition factor is based on the slope (b) of the regression of the log-log length-weight relationship (generally estimated as $b = 3$ for Fulton's condition factor) which was calculated separately for males and females to account for sexual dimorphism (Garcia-Berthou and Moreno-Amich 1993; Samat et al 2008).

The eggs of all gravid females were staged according to Haynes (1995); Stage 3 and 4 embryos were combined because they could not be reliably distinguished. For each gravid female, the number of embryos at each developmental stage was recorded. To visualize differences and trends in egg development, plots of the number of eggs at each developmental stage per female were made separately for each population which also

indicated the month in which those females were collected. 54.2% of females had broods with at least 2 developmental stages. Of those, 19 females had broods with 3 or more egg developmental stages and were chosen as a subsample to evaluate potential maternal-fetal nutrient transfer. From each of the 19 females, the average egg mass of each developmental egg stage was weighed separately.

Habitat data collected included: air temperature (°C), water temperature (°C), salinity (ppt), dissolved oxygen (mg/l), pH, conductivity (µS:C), sinkhole area (m²), and the day length (hours). The percent of each species comprising each community was determined by dividing the sum of all individuals of each species caught at each site over the twelve month sampling period by the total number of fish caught in that sinkhole. With these data, the relative abundance of each species could be compared regardless of variation in catch rate between seasons and sinkholes.

Statistical Analyses

Each of the aforementioned variables (length, eviscerated mass, condition factor, embryo mass, reproductive effort, brood size, and habitat variables) were plotted against month to ascertain seasonal and among-sinkhole change. Differences between localities were tested via one-way analysis of variance (ANOVA) or a non-parametric analog after data were checked for equality of variance and normality. For these and all subsequent analyses, normality and equal variance assumptions were tested using histograms, Shapiro-Wilkes test, residuals vs expected plots, NQQ plots, and Levene's test. If assumptions were reasonably met a one-way ANOVA with Tukey's multiple comparisons was used, if the equal variance assumption was violated a Welch's one-way ANOVA was performed, and if the normality assumption was violated, a Kruskal-Wallis

with Mann-Whitney multiple comparisons was used with Bonferroni corrections.

Analyses were performed in Paleontological Statistics (PAST version 2.14) statistical software and graphs were generated in Microsoft Excel (Hammer et al. 2001; Microsoft Office Excel 2007).

Life History of G. nobilis

To assess age or size class structure, standard length (mm) and eviscerated mass (g) were plotted against month for each sex with populations denoted. Individual condition (k) was also plotted against month to evaluate potential seasonal fluctuation in relative health. For gravid females, brood size, average egg mass (g), and reproductive effort were plotted against month to determine if potential shifts in reproductive strategy occurred across the reproductive season (April-August determined after dissection by presence of eggs). Linear regression analyses were performed between dependent variables, reproductive traits (brood size, average egg mass, and reproductive effort) and independent variables, both female eviscerated mass and condition, to determine any size-dependent investment, and against each other to evaluate trade-offs between the different aspects of reproduction (trade-off between egg mass and brood size, for example).

Among-Population Variation

Among population and seasonal differences in life history traits were analyzed separately for males and females in consideration of sexual dimorphism. Standard length, mass, and condition factor values for both males and females were analyzed with a Nested ANOVA using the Satterthwaite approximation for unequal sample sizes where months are nested within sites to compare the amount of variation between sinkholes

while accounting for the variation across months (Nested ANOVA's performed in spreadsheet from McDonald 2009). When normality and equal variance assumptions were not met, log transformations were performed prior to Nested ANOVA analyses (assumptions were verified after transformation). If significant differences were identified with the Nested-ANOVA, a one-way ANOVA or non-parametric analog with multiple comparisons was used to determine pair-wise differences between populations. To explore potential differences in reproductive investment, brood size, average egg mass, and reproductive effort were assessed using a Nested ANOVA with the same parameters as above. An analysis of covariance (ANCOVA) was additionally performed on brood size and average egg mass to account for any dependence of reproductive traits on female size which is commonly observed in fishes. For males, the relative length of the gonopodium was calculated by dividing the length of the gonopodium by the total body length. Population differences between relative gonopodium lengths were assessed with an ANOVA and Tukey's multiple comparisons. Additionally, an ANCOVA on gonopodium length adjusted for body length was performed to determine differences between populations while controlling for male body size.

Ecological Effects

Potential relationships between environmental parameters and size, condition, and reproduction were analyzed by generating correlation matrices (separately for males, all females, and gravid females). This exploratory analysis identified some cursory potential relationships between variation in life history traits and the environment.

RESULTS

Environmental variables

Ecological parameters varied seasonally and between sinkholes (Table 1). In all sinkholes, water temperature and day length peaked in the summer months. Water temperature was greatest in August, reaching 30.5°C in sinkhole 7, and least in January, dropping to 8°C in sinkhole 37. The reproductive season occurred during months with at least 13 hours of day light (April thru August). There were no significant differences in pH between sinkholes (ANOVA: $F_{3,36} = 0.5898$, $p = 0.6257$) and, at all sites, pH gradually increased across the sampling period from May 2011 to April 2012. Dissolved oxygen levels (mg/l) were significantly greater in sinkhole 31 compared to the other sinkholes (Kruskal-Wallis: $H_{(3)} = 25.76$, $p < 0.0001$). While dissolved oxygen was relatively stable over time at most sites, values at sinkhole 31 increased drastically from June to August, possibly from increased photosynthesis of the dense aquatic macrophytes. Salinity (ppt), fluctuated very little seasonally, but differed between sinkholes (Welch's ANOVA, $F_{3,11.15} = 595.3$, $p < 0.0001$), with lowest salinity observed in sinkhole 31 and highest salinity in sinkhole 27. Surface areas of sinkholes were estimated from aerial images in Google Earth. Sinkholes varied in size from 407 m² to 2826 m² (Table 1). However, the entire volume of the sinkhole could not be determined without modeling depth. Assemblage composition, calculated as the sum of individuals per species caught over the sampling period divided by the sum of all fish caught, was determined for each sinkhole. Three species (*Gambusia nobilis*, *Cyprinodon pecosensis*, and *Fundulus zebrinus*) were present in sinkholes 7, 27, and 37 and two species were present in sinkhole 31 (*G. nobilis* and *C. pecosensis*). Across all sinkholes, *G. nobilis* was the most abundant species

(comprising 79%- 89%), and each of the other species constituted 7%-14% of assemblages. Where present, *F. zebrinus* was most abundant in sinkhole 37 (14%) and least abundant in sinkhole 27 (8%).

Life History of *Gambusia nobilis*

Life history characteristics documented in this study are summarized in Table 2. Seasonal changes in body size and condition of *G. nobilis* sampled were assessed with scatter plots of eviscerated mass (g), standard length (mm), and condition factor (k) against month (Fig. 1). Size or age class distributions may be incomplete because collection methods were biased toward sexually mature individuals. Across the sampling period, male eviscerated mass and standard length did not appear to vary (Standard Length, Kruskal-Wallis: $H_{(8)}=4.441$, $p=0.7278$; Mass, ANOVA: $F_{7,63}=2.115$, $p=0.0547$), since males grow relatively little after sexual maturity. Male condition was greatest in autumn; a one-way ANOVA between months indicated that condition in September and November were significantly greater than the spring months ($F_{7,63}=14.34$, $p<0.0001$). Among females, eviscerated mass and standard length peaked during summer months; the largest, oldest females were present during the reproductive season but declined from the sample prior to the overwintering period (Fig.1). There was considerable among individual variation in female condition, but no obvious seasonal patterns were evident (Fig. 1). The only significant difference in condition between months were between June and March (Welch's ANOVA, $F_{11,24.46}=4.876$, $p=0.0005$). There was no significant difference in condition between sexually mature and immature females ($T_{272}=0.7708$, $p=0.4418$).

Females were gravid from April through August; although after dissection it was evident that only one female collected in August was gravid. Reproductive investment and strategy changed over the course of the reproductive season (Fig. 2). Brood size peaked in May and declined considerably through July. In contrast, average egg mass increased steadily throughout this time. The relative amount of body mass invested in reproduction (reproductive effort) was lowest in April and increased in May and June. There was considerable variation in all three of these parameters suggesting between-individual variation in reproductive capacity or investment. To examine trade-offs between each of these reproductive traits, linear regressions between each pair of variables were performed (Fig. 2). Positive relationships were observed between reproductive effort and brood size with slope 26.414 ($R^2 = 0.20$, $t_{105}=5.19$, $p<0.0001$) and average egg mass with slope 0.0161 ($R^2 = 0.32$, $t_{105}=7.06$, $p<0.0001$), suggesting that female investment in reproduction consisted of larger eggs and/or larger broods. A slightly negative relationship between brood size and average egg mass with slope $-8.950e^{-5}$ was observed when pooled across the reproductive season ($R^2 = 0.034$, $t_{105}=-1.92$, $p=0.0569$).

One factor that often affects reproductive capacity is female body size. Brood size was positively correlated with female eviscerated mass with slope of 20.858 ($R^2 = 0.198$, $t_{105}=5.10$, $p<0.0001$; Fig. 3A), but neither average egg mass nor reproductive effort were significantly related to eviscerated mass with slopes of 0.0025 and -0.0189 respectively (Avg. Egg Mass: $R^2 = 0.011$, $t_{105}=1.12$, $p=0.2636$, Reproductive Effort: $R^2 = 0.00$, $t_{105}=-0.24$, $p=0.8084$; Fig. 3C-D). Only females from sinkhole 27 showed positive correlation of female eviscerated mass and average egg mass with slope 0.0133

($R^2=0.351$, $t_{32}=4.16$, $p=0.0002$). Brood size was positively correlated with eviscerated body mass suggesting that larger females invested in more offspring, but the relationship with average egg mass indicated that in general female size is not related to egg mass (except in females from sinkhole 27). There does not seem to be any significant relationship between the size of a female and reproductive effort. Interestingly, the condition or relative health of a female was not significantly correlated to the relative amount of body mass invested in reproduction, with average egg mass, or brood size (Reproductive Effort: $R^2= 0.00$, $t_{105}=-0.096$ $p=0.9237$; Avg. Egg Mass: $R^2= 0.008$, $t_{105}=0.926$, $p=0.3566$, Brood Size: $R^2= 0.006$, $t_{105}=0.813$ $p=0.4182$ Fig. 3E,G and H). Females in poor condition invested relatively the same amount in reproduction as did females in better condition.

The developmental stages of all the embryos carried by each gravid female were determined using Haynes (1995) classification of developmental stage (Fig.4). Analysis of developmental stages of embryos revealed that some females had broods that were all at the same developmental stage, some exhibited a gradient of embryos at several consecutive stages, and others had the majority of the brood at late developmental stages but with one or more early stage embryos (especially stage 3 which were mature ova, but may not have been fertilized). Early stage embryos were dominant in April in all populations. Sinkhole 7 had early stage embryos in April and May, while embryos from sinkhole 27 females matured earlier evidenced by late stage embryos occurring as early as May. In June, sinkhole 31 females had many early stage embryos while other populations generally had late stage embryos by this point; sinkhole 31 was the only site in which a gravid female was collected in August. It appeared that females had

asynchronous reproduction because within the same collection (same site and month) there were multiple observations of some females with early stage embryos and some females with late stage. Maternal-fetal nutrient transfer was evaluated by determining increases or decreases in average egg mass throughout gestation. Within each brood the average egg mass increased with developmental stage. In all 19 females showing a gradient of developmental stages, the average mass of eggs in the latest developmental stage was greater than the earliest egg stage, even among the few exceptions where an intermediate stage showed lower average mass (Table 3).

Among-Population Variation

A Nested ANOVA of male log mass, log standard length, and condition revealed no significant differences between populations after accounting for variation between months (Mass: $F_{3,18}=0.80, p=0.8045$, Standard Length: $F_{3,9}=2.36, p=0.1468$, Condition: $F_{3,16}=0.12, p=0.9444$). Males from sinkhole 31 and 27 tended to have the greatest mass and standard length while sinkhole 37 had the least (Table 2). Significant differences between months within populations were identified for male log mass and condition (Mass: $F_{18,49}=21.58, p<0.0001$, Condition: $F_{16,49}=6.12, p<0.0001$) which accounted for 88.53% and 65.76% of the total variance respectively, but not for standard length ($F_{9,49}=1.68, p=0.0732$).

Population differences in female size and condition were also tested. A Nested ANOVA of female eviscerated mass, condition, and standard length suggested that populations differed in both mass and condition (Mass: $F_{3,29}=4.22, p=0.0137$, Condition: $F_{3,28}=4.89, p=0.0076$) which accounted for 19.9% and 18.37% of the variance component respectively, but no differences for standard length were observed ($F_{3,30}=1.64, p=0.2020$).

Among summer females, Eviscerated mass was significantly different among all population pairs except between sinkholes 7 and 37 which had the smallest mass; sinkhole 31 females had the greatest mass (ANOVA, $F_{3,179}=14.76, p<0.0001$; Table 2). Females from sinkhole 31 were in the greatest condition and sinkhole 7 tended to be in the poorest (Welch's ANOVA, $F_{3,97.22}=21.04, p<0.0001$). Among all three variables (mass, condition, and standard length) there were significant differences between months within sites (Mass: $F_{29,237}=6.81, p<0.0001$, Condition: $F_{28,237}=4.20, p<0.0001$, Standard Length: $F_{30,237}=8.98, p<0.0001$).

Variation in reproductive traits between populations was also analyzed. For males, the length of the gonopodium relative to the total body length was calculated and compared across populations. This analysis revealed significant differences in relative gonopodium length only between sinkholes 7 and 31 (sinkhole 31 had significantly shorter gonopodia) (ANOVA, $F_{3,62}=5.79, p=0.0015$) (Table 2). A significant result was also obtained when body size was controlled for using an ANCOVA and adjusted means were compared ($F_{3,61}=5.437, p=0.0022$). Nested ANOVA's were performed on female reproductive data which identified no significant differences between the populations with respect to these variables (Brood Size: $F_{3,10}=0.18, p=0.9076$, Average Egg Mass: $F_{3,11}=0.22, p=0.8788$, Reproductive Effort: $F_{3,10}=0.59, p=0.6326$). There were, however, significant differences between months within sites for brood size, ($F_{10,92}=5.38, p<0.0001$), average egg mass ($F_{11,92}=12.85, p<0.0001$), and reproductive effort ($F_{10,92}=3.48, p=0.0004$), which each accounted for 39.3%, 63.66%, and 26.84% of the variance components.

Ecological Effects

Correlation matrices of ecological and life history measurements were constructed for males, females, and gravid females to determine potential relationships between these variables. Among gravid females, standard length and eviscerated mass were positively related to dissolved oxygen (mg/l) (Standard Length: $R^2=0.13$, $t_{105}=4.0205$, $p=0.0001$; Mass: $R^2=0.17$, $t_{105}=4.6834$, $p<0.0001$), and female condition had a negative relationship with salinity (ppt) ($R^2=0.12$, $t_{105}=-3.7337$, $p=0.0003$). Community composition also seemed to have an effect on mass and condition. Gravid females from populations with relatively greater proportion of *G. nobilis* and *C. pecosensis* tended to have greater mass ($R^2=0.18$, $t_{105}=4.7753$, $p<0.0001$; $R^2=0.17$, $t_{105}=4.5584$, $p<0.0001$) and better condition ($R^2=0.11$, $t_{105}=3.637$, $p=0.0004$; $R^2=0.23$, $t_{105}=5.663$, $p<0.0001$), while these parameters were negatively related to the abundance of *F. zebrinus* (Mass: $R^2=0.19$, $t_{105}=-4.9718$, $p<0.0001$; Condition: $R^2=0.15$, $t_{105}=-4.3789$, $p<0.0001$). Interestingly, females from populations with relatively greater abundance of *G. nobilis* tended to invest slightly less in reproduction ($R^2=0.047$, $t_{105}=-2.279$, $p=0.0247$), as measured by reproductive effort, but there was a slight positive relationship with reproductive effort and the presence of *F. zebrinus* ($R^2=0.042$, $t_{105}=2.1363$, $p=0.0349$) suggesting that females exposed to potential predation invest more in reproduction than do females in a community with fewer predators. Among all females, standard length and mass were positively correlated with water temperatures, which was expected because of shifts in the age structure of the population throughout the year and higher growth rate at higher temperature (Standard Length: $R^2=0.31$, $t_{271}=10.95$, $p<0.0001$; Mass: $R^2=0.24$, $t_{271}=9.37$, $p<0.0001$). Some of these correlations, however, may be confounded by the limited sampled range of each

ecological parameter (only 4 localities), and sinkholes varied in more than one parameter simultaneously with some degree of colinearity observed between variables.

DISCUSSION:

Abiotic and biotic conditions of sinkholes sampled at Bitter Lake National Wildlife Refuge varied in many ways, including dissolved oxygen levels, salinity, habitat size, and community composition. As a result, each isolated population may be exposed to varying selective pressures favoring differential investment of resources allocated to growth and reproduction (Rundle and Nosil 2005; Schluter 2001). We have identified some ways environmental factors may influence and shape life history. Among gravid females, size was positively influenced by dissolved oxygen levels and greater percentage of both *G. nobilis* and *C. pecosensis*. Reduced condition was observed in sinkholes with greater salinity and was negatively related with percent of *F. zebrinus*. This is in agreement with other research; in a recent study at BLNWR, Swaim and Boeing (2008) also found a negative relationship between relative weight and salinity in *G. nobilis* and this trend has been observed in other poeciliids (Alcaraz and Garcia-Berthou 2007; Alcaraz 2006). Interestingly, female reproductive effort was slightly higher in populations with greater percentage of *F. zebrinus*. Many factors not measured in this study could affect growth and reproduction. This includes: parasites, habitat structure, primary productivity, and population density. Common garden experiments are recommended, allowing environmental effects on life history to be more carefully examined and collinear environmental effects to be teased apart.

The age/size structure of the populations cycles throughout the year; large females were present from May throughout the reproductive season but subsequently declined,

presumably from die-offs prior to the overwintering period during which young of year, and likely one year old females, overwintered from November through March. Age structure of males remains difficult to determine because they grow relatively little after reaching sexual maturity. Male and female condition showed among individual variation but no consistent pattern throughout the year. This was surprising as food availability and foraging activity appeared to decline during the overwintering period because they descend toward the bottom of the sinkhole. Even though individuals are relying on fat stores and seemingly very little foraging during the overwintering period, many individuals maintained condition levels observed in summer months. Generally, populations from sinkhole 27 and 31 had greater mass and longer standard length while sinkhole 37 generally had the smallest individuals. Females from sinkhole 31 may have been in better condition in part because they had access to another food resource (aquatic snails) not available in the other three sinkholes (dissection revealed that 26% of individuals from sinkhole 31 contained snails in the intestines).

The objective of this research was to evaluate potential seasonal and population variation in size, condition, and reproductive investment in *G. nobilis* specifically and contribute to the understanding of poeciliid reproductive allocation strategies in general. We predicted significant differences between populations in key life history traits as a result of exposure to different ecological conditions at each site. This was based on the hypothesis that life history traits generally display greater phenotypic plasticity than others (e.g. morphology) because they are more likely to be impacted by local ecological conditions (Moser et al. 2012). Ecological conditions determine, in part, which phenotype is expressed and ultimately which traits are exposed to selective pressures;

in this way, environmentally induced phenotypic plasticity affects the evolution of populations and species (West-Eberhard 1989). Results of this study complement what has already been learned about *G. nobilis* life history and contribute new information about seasonal changes in growth and condition, and variation between populations. One important finding of this study is that despite differences in abiotic factors, reproductive investment of females did not vary significantly among populations.

Contrary to our predictions, no significant differences in reproductive traits (brood size, average egg mass, and reproductive effort) were observed between four populations that vary considerably in biotic and abiotic conditions. This may suggest stabilizing selection in some traits (such as egg mass) or physiological limitations in other traits (brood size) (Ricklefs and Wikelski 2002). An example of stabilizing selection is low variance in egg size of Cuban poeciliids (Ponce de Leon et al. 2011). Mean values for reproductive traits compared between the current study and the results obtained in a recent life history study by Swenton and Kodric-Brown (2012) revealed approximately equal average egg mass, lower reproductive effort, greater brood mass, and larger brood size in the current study. The observation of differences in life history data between the Swenton and Kodric-Brown (2012) and the current study may suggest inter-annual fluctuation of some life history traits.

Trade-offs among reproductive traits were expected, especially between the number and size of offspring. Consistent with Swenton and Kodric-Brown (2012), we observed a shift in the reproductive strategies of females throughout the reproductive season which suggests that the pressures optimizing reproductive success change over time. Females initially invested in larger broods with smaller offspring; while some of

the small offspring may not survive (due to predation, etc.) the females' fitness is maximized by trade-offs with a larger brood. However, by the end of the reproductive season, females shift to smaller broods with larger embryos. Messina and Fox (2001) discuss the trade-off between brood size and offspring number and note that female fitness is maximized at an intermediate offspring size with variation in offspring size expected as a result of differences in local selective pressure. Presumably, the benefits of having larger eggs outweigh the costs of reduced fecundity. This is probably because survivorship of embryos is higher in a site with increased conspecific competition (increasing population density). Also, survival is enhanced for young-of-year fishes that have greater fat stores prior to the overwintering period (Trexler et al. 1992). The common trade-off among fishes between the number of offspring produced and the size of offspring is particularly constrained among live bearing species with limited abdominal space (Goodwin et al. 2002).

An analysis of reproduction by size suggested equal average egg mass, but greater brood sizes for larger females compared to small females. Instead of investing in higher quality offspring (presumably larger eggs), larger females invest in more offspring (larger brood size). Only females from sinkhole 27 showed a positive correlation between eviscerated mass and average egg mass (larger females invested in larger eggs). Interestingly, female reproductive effort was not correlated with condition; females in poor condition invested relatively the same amount into reproduction as did females in better condition. It may be the case that while poor condition females invest the same in reproduction for a single brood, they may have fewer resources to invest in future

reproduction and survival. Further investigation is needed to determine trade-offs between condition and 1) survival and lifespan and 2) female fitness.

The primary male reproductive trait measured was the relative length of the gonopodium; results suggested that males from sinkhole 31 had shorter gonopodia relative to total body length than other populations. The observation that males exposed to greater predation pressure have longer gonopodia is consistent with research of male *Brachyrhaphis episcopa* (Jennions and Kelly 2002). There may be selective pressure for males in environments with increased interspecies competition to have longer gonopodia to increase sneak copulation success rate under competitive conditions (Kelly et al. 2000; Reynolds et al. 1993). However, other researchers have observed shorter gonopodia to increase survival in the presence of predators despite opposing sexual selection for longer gonopodia (Basolo and Wagner 2004; Langerhans et al. 2005; Langerhans 2011).

This study also details embryo developmental stage changes throughout the reproductive season, and identifies some interesting reproductive investment strategies. Wourms (1981) suggests some species within *Gambusia* can occasionally exhibit superfetation- a life history strategy in which females gestate multiple broods simultaneously at different stages of development. Prior to parturition of an older brood, a second brood of eggs mature and are fertilized, thus shortening the interbrood interval (Wourms 1981). Advantages of superfetation include 1) increasing fecundity by distributing embryos temporally to reduce the costs of restricted abdominal space, 2) increasing fitness by reducing the interbrood interval, and 3) increasing reproductive success by investing in some embryos at a future time when resources could be more abundant instead of investing all resources in a single brood (Thibault and Schultz 1978;

Wourms 1981). Analysis of embryo developmental stages revealed differences in the brood composition between females. Some females had broods in which embryos were all at the same developmental stage, others exhibited a gradient of embryos at several consecutive developmental stages, and some females had the majority of the brood at later stages with one or more in early stage. These observations suggest that *G. nobilis* is employing the strategy of superfetation. However, other interpretations of multiple developmental stages include “protracted fertilization” (embryos fertilized over a period of time) and regressing embryos (embryo mortality) (Haynes 1995; Marsh-Matthews et al. 2005). There was no clear progression in developmental stage during the reproductive season; within the same month and sinkhole, some females contained primarily late stage embryos while others had early stage, suggesting asynchronous reproduction between females in this species. Asynchronous reproduction may reduce competition between offspring or may act as a bet-hedging strategy to maximize fitness. Additionally, evidence of average egg mass increasing throughout gestation was observed, suggesting some maternal-fetal nutrient transfer in addition to that provided in the yolk. This cursory analysis of wet weights of developing embryos suggests some component of matrotrophy as part of the life history strategy of *G. nobilis* which supports the conclusions of Marsh-Matthews et al. (2010). Matrotrophy allows females to lengthen the time which they can provide resources to offspring and allows them to adjust resource allocation throughout gestation based on current conditions (Marsh-Matthews et al. 2005). Additionally, it may allow females to increase brood size while not compromising offspring quality by initially having smaller eggs which they provision throughout gestation (Trexler and DeAngelis 2003). Further research is needed to

confirm the degree of superfetation in *G. nobilis* and to further explore maternal-fetal nutrient transfer strategies ie: matrotrophy (nutrient transfer to embryos throughout gestation) or lecithotrophy (all nutrients for embryos supplied in yolk).

Some aspects of this study which may have affected results are limited sample size, limited number of sampled sites, unequal sampling between populations, measurement inaccuracy, and that sampling began in May and concluded the following April. Timing of sampling may have affected results because data are from two distinct reproductive seasons. Additionally there may be some influence of population shared genetic history; in 1980 and 1981 one of the populations (sinkhole 37) was stocked from transplants from two of the other populations (sinkholes 7 and 27) (BLNWR, Personal Communication; Bouma 1984). Despite these challenges, our observations emphasize the importance of tradeoffs and constraints of resource allocation to growth and reproduction and provide important insight into *G. nobilis* life history.

Overall this study suggests that 1) female populations differed in size and condition but not in reproductive traits despite differences in abiotic factors, 2) reproductive strategy shifted over the reproductive season to maximize fitness, 3) average egg size and reproductive effort did not vary with female size or condition; larger females instead invested in larger broods, and 4) there is evidence for asynchronous reproduction among females and some data suggestive of superfetation and matrotrophy.

Understanding effects of environmental change and community structure on reproductive effort is important to the conservation of *G. nobilis*. Stockwell and Henkanaththegedara (2011) provided a list of the greatest threats to poeciliid conservation which included “1) restricted range 2) habitat destruction/degradation 3) overexploitation, and 4) impacts

associated with nonnative species". *Gambusia nobilis* already experiences a restricted range and has been shown to be sensitive to extreme water quality conditions including high temperature and salinity. Most *G. nobilis* habitats are protected, but are still susceptible to changes in abiotic conditions; an understanding of ecological effects on life history is needed to identify changes in habitat that could threaten their survival. Identifying tolerance limits to abiotic conditions and understanding environmental impacts on growth, condition, and reproduction in association with long term habitat monitoring may be critical to predicting and mitigating impacts to the species.

ACKNOWLEDGMENTS:

We would especially like to thank Daniel P. Hopkins, Robert L. Hickey, Kara L. Hickey, Davona J. Hickey, and Kristin Wright who assisted with monthly field work and specimen collection. Thank you also to Kristin Simmons for contributing to the dissection and measurement of specimens. We also appreciate Alexandra M. Snyder and the support of the Museum of Southwestern Biology (MSB) which provided supplies, facilities, and technical support needed to carry out this research. Thank you to Thomas F. Turner and Blair O. Wolf for comments on research design and editing of the manuscript. This project was funded in part by a Graduate Research Project and Travel Grant provided by the Office of Graduate Studies, University of New Mexico.

Specimens were collected under the US Fish and Wildlife Service Native Endangered Sp. Recovery permit #TE28576A-1, US Fish and Wildlife Service Consultation for Section 7 of the Endangered Species Act of 1973 #22420-2011-I-0055, US Fish and Wildlife Service Bitter Lake National Wildlife Refuge Special Use Permit, New Mexico Department of Game and Fish Authorization for Taking Protected Wildlife for Scientific and or Educational Purposes Permit #2968 and #3303 (specimens transferred to researcher), and the University of New Mexico Institutional Animal Care and Use Committee approved protocol # 11-100628-MCC.

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TABLES AND FIGURES:

Table 1 Summary table of ecological characteristics of each habitat. The mean and standard deviation is given for dissolved oxygen (mg/l), salinity (ppt), and pH. The relative abundance of each species per habitat is given as a percentage of the total number of individuals of each species captured compared to the total number of all fish caught.

Variable	Sinkhole 7	Sinkhole 27	Sinkhole 31	Sinkhole 37
Dissolved Oxygen (mg/l)	10.07±0.17	8.10±0.53	15.29±1.91	8.96, ±0.15
Salinity (ppt)	7.22±0.07	17.53±0.29	6.48±0.25	10.30±0.06
pH	6.96±0.23	6.73±0.27	6.38±0.20	6.86±0.20
Surface Area (m ²)	1319	570	407	2826
% <i>G. nobilis</i>	82%	84%	89%	79%
% <i>C. pecosensis</i>	7%	8%	11%	8%
% <i>F. zebrinus</i>	11%	8%	-----	14%

Table 2 Summary table of life history characteristics related to size, condition, and reproduction. For each trait, the mean and standard error is reported for males and females from each population.

Trait	Males				Females			
	Sinkhole 7	Sinkhole 27	Sinkhole 31	Sinkhole 37	Sinkhole 7	Sinkhole 27	Sinkhole 31	Sinkhole 37
Standard Length (mm)	20.22±0.17	21.29±0.37	20.75±0.44	19.71±0.39	26.95±0.59	28.58±0.62	30.99±0.55	26.14±0.48
Eviscerated Mass (g)	0.19±0.01	0.22±0.01	0.23±0.02	0.18±0.02	0.37±0.02	0.46±0.02	0.63±0.03	0.34±0.02
Condition	1.52±0.05	1.52±0.06	1.61±0.07	1.46±0.06	2.25±0.04	2.40±0.04	2.76±0.05	2.40±0.03
Brood Size	-----	-----	-----	-----	16.7±1.9	16.7±1.6	17.7±2.3	14.2±1.2
Average Egg Mass (g)	-----	-----	-----	-----	0.008±0.001	0.009±0.001	0.009±0.001	0.01±0.001
Reproductive Effort	-----	-----	-----	-----	0.26±0.03	0.26±0.02	0.21±0.02	0.31±0.04
Relative Gonopodium Length	0.24±0.003	0.23±0.003	0.22±0.006	0.23±0.004	-----	-----	-----	-----

Table 3 The average egg mass (g) of each developmental stage was measured for 19 females that exhibited more than three stages simultaneously. The average egg mass increased throughout gestation for each brood, except in a few cases where an intermediate stage weighed less than an earlier stage. In all females, the latest developmental stage had a greater average egg mass than the earliest developmental stage.

Individual	Stage 3&4	Stage 5	Stage 6	Stage 7	Stage 8	Stage 9	Stage 10	Stage 11
1	0.0041	0.0046	0.0063					
2	0.0047			0.0062	0.0094			
3	0.0039		0.0040	0.0048				
4	0.0035	0.0023	0.0040	0.0056				
5	0.0022	0.0029	0.0040					
6	0.0053		0.0063	0.0064				
7		0.0040	0.0061	0.0066				
8	0.0052	0.0057	0.0057					
9						0.0053	0.0068	0.0092
10						0.0096	0.0073	0.0112
11	0.0037			0.0038	0.0052	0.0062		
12	0.0036		0.0035	0.0046	0.0065			
13				0.0042		0.0068	0.0090	
14	0.0048					0.0056	0.0086	
15	0.0051					0.0080	0.0086	
16	0.0052	0.0060	0.0070					
17	0.0031			0.0065	0.0061			
18	0.0046	0.0054	0.0057					
19	0.0056	0.0048	0.0064					

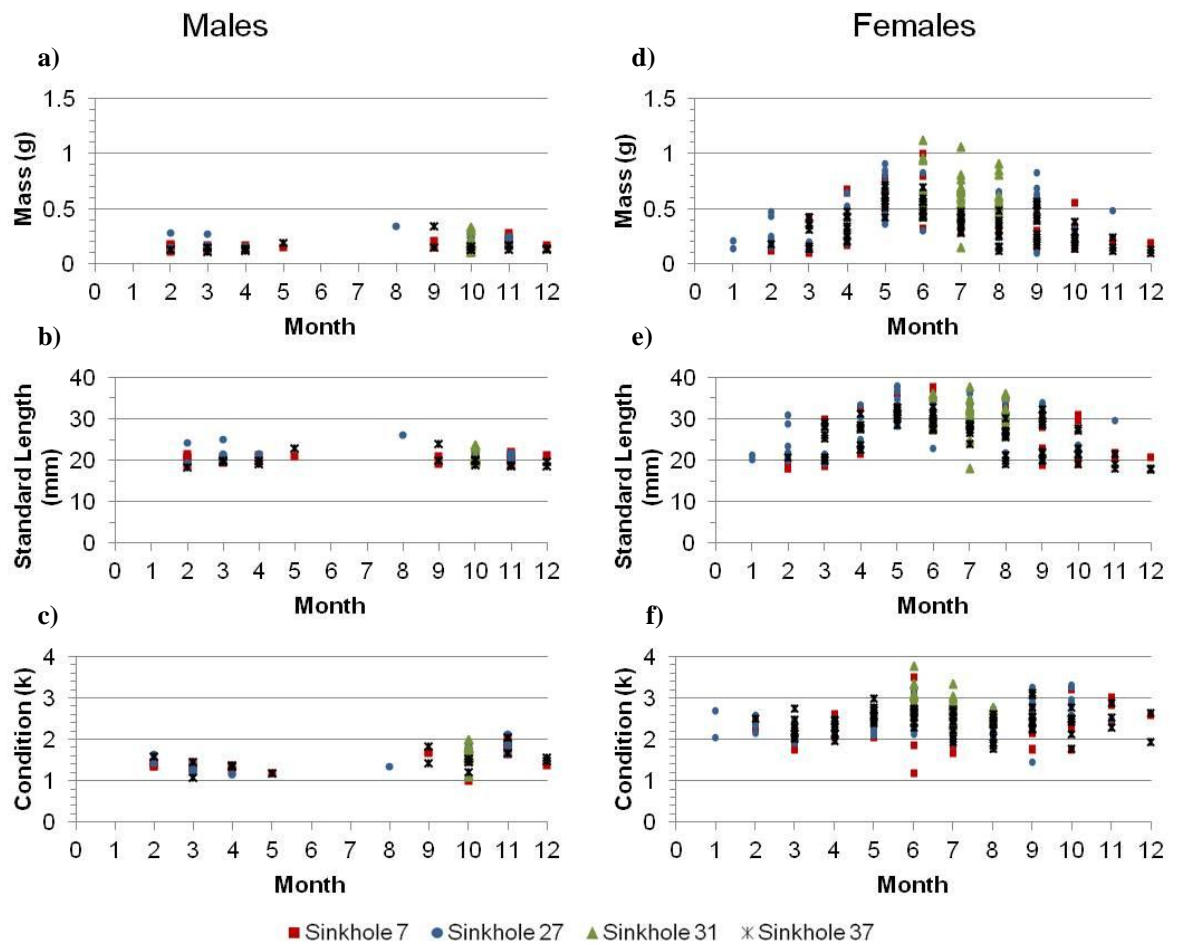


Fig. 1 To visualize changes in size and condition throughout the sampling period, the standard length (mm), eviscerated mass (g), and the condition (k) of individuals for males (a-c) and females (d-f) were plotted against month. The sinkhole from which each individual was sampled is denoted by different symbols. Numbers on X-axis indicate months in calendar year.

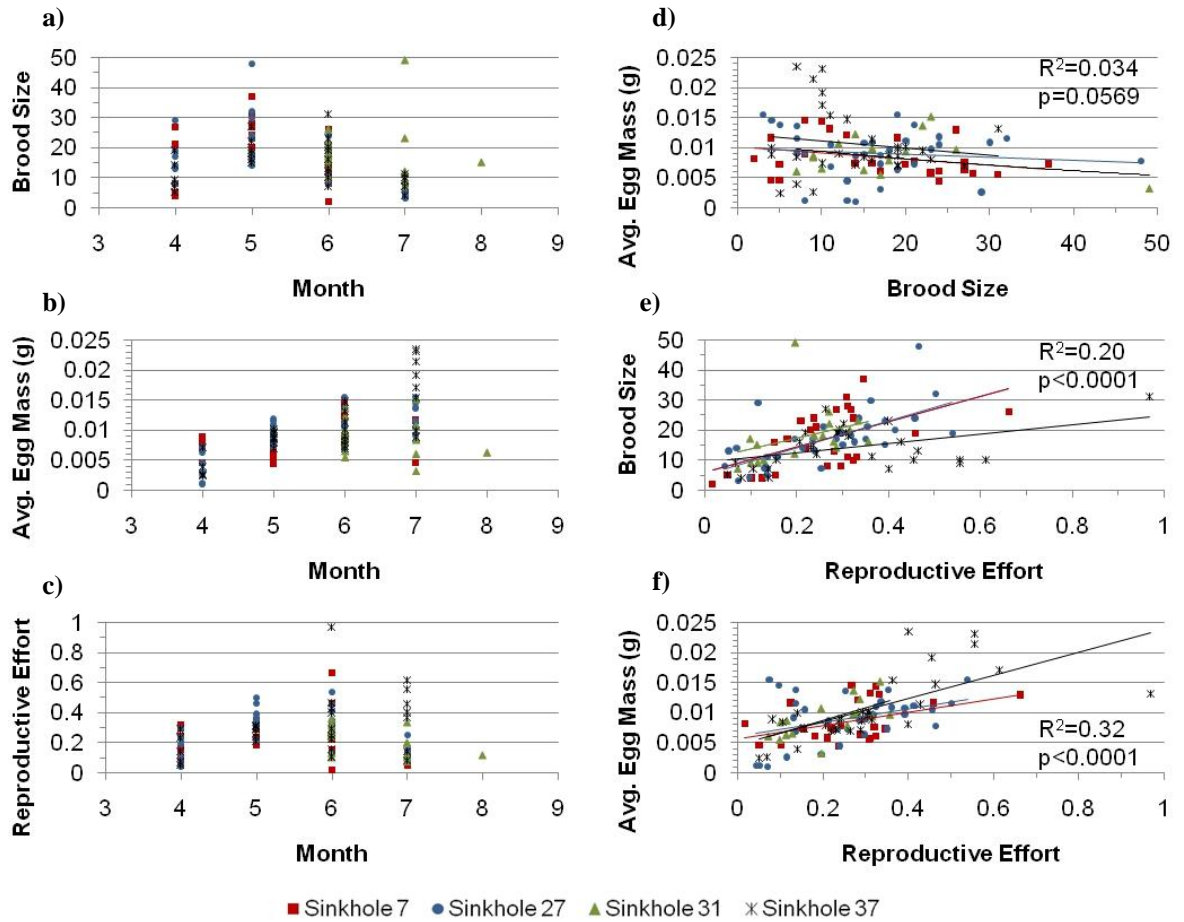


Fig. 2 Reproductive traits a) brood size, b) average egg mass, and c) reproductive effort were plotted against the calendar month in which they were collected. Trade-offs between the reproductive traits were examined with linear regressions of each trait against the others (d-f). A linear trend line is shown for each sinkhole population, but the R^2 and p-value are shown for all populations combined since there was no significant difference between populations for these traits.

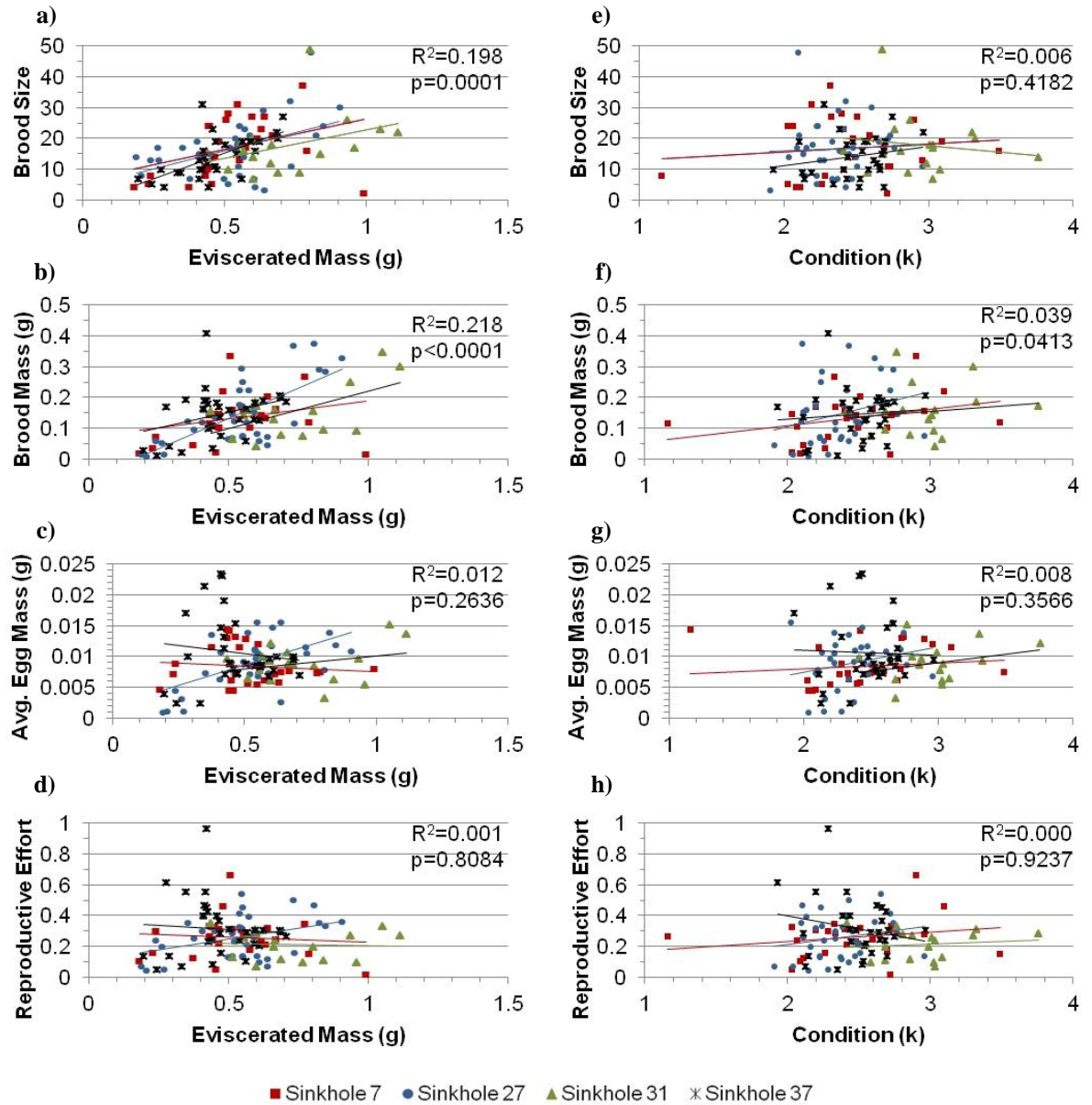


Fig. 3 Reproductive traits were plotted against female eviscerated mass (g) and female condition (k) to determine relationships between female size and condition and reproduction. Larger females tended to have more offspring and an associated greater brood mass (a-b), but average egg mass and reproductive effort were not related to female size or condition (c-d and g-h). Brood size was also uncorrelated with condition, while brood mass may show a slight positive relationship (e-f).

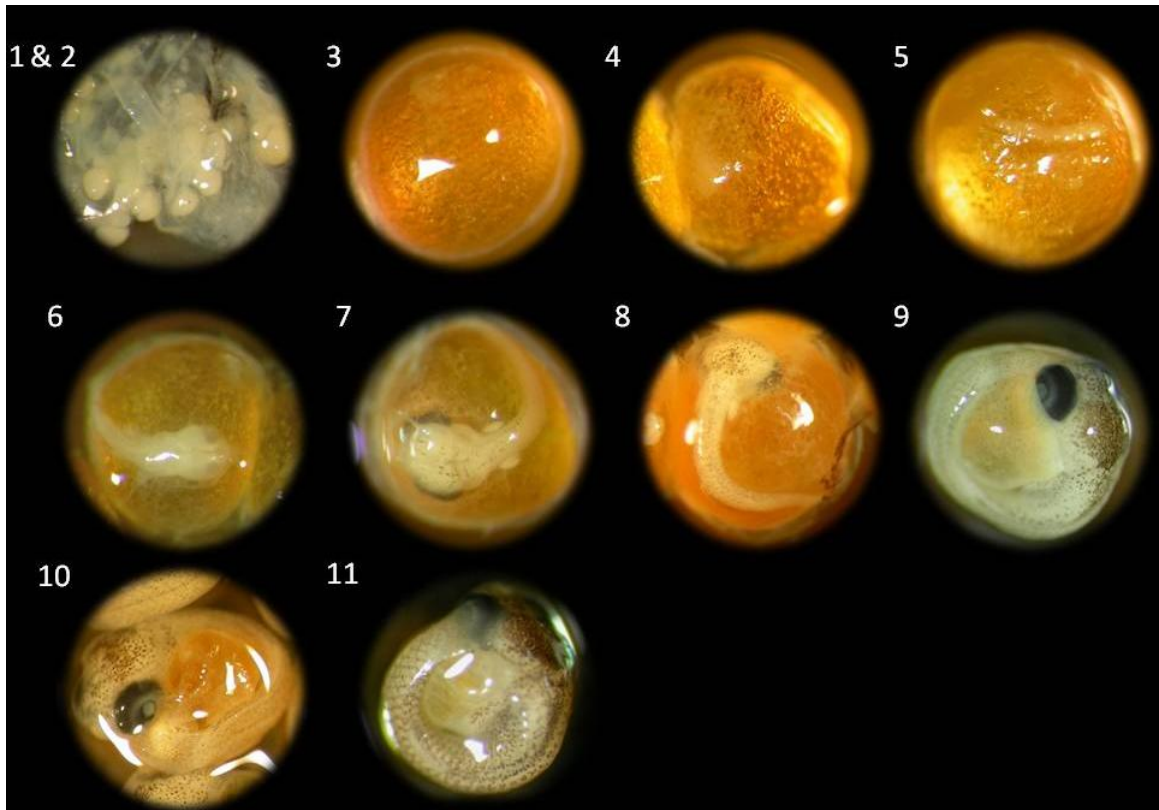


Fig. 4 The embryos of all gravid females were staged according to Haynes 1995, dividing embryos into 11 developmental stages according to the presence of particular features generally related to eye and fin developmental states. In this study, stages 1 and 2 were not counted and stages 3 and 4 were combined because they were not readily distinguished. Images are not to scale.

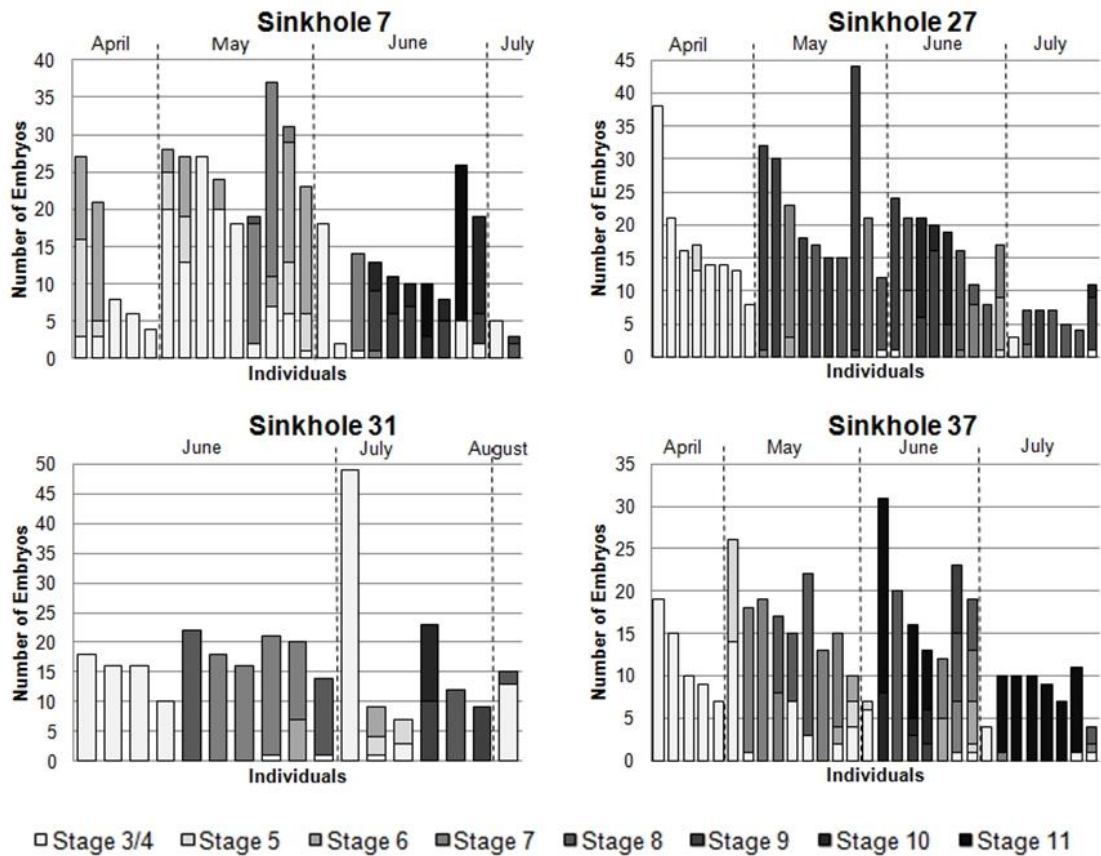


Fig. 5 Embryos of each gravid female were staged and graphs were generated to compare developmental stages within females, within populations, across months, and between sites. The brood composition and size is shown in a single bar per gravid female ordered by the month in which they were collected. The shading of each segment within a bar indicates the developmental stage and the number of embryos within that brood at that stage. Females from each population are plotted separately.

CHAPTER 2:
ECOLOGICAL EFFECTS ON MORPHOLOGICAL VARIATION OF
***GAMBUSIA NOBILIS* (PECOS GAMBUSIA)**

ABSTRACT:

Gambusia nobilis (Pecos Gambusia) are live bearing fishes that inhabit small sinkholes and springs within a restricted range of the Pecos River Watershed in New Mexico and Texas. These habitats are variable in size, salinity, dissolved oxygen and community composition, all of which may expose populations to unique selective pressure that may result in local adaptation. The objective of this study was to test for morphological differences and ecological correlates among four populations of *Gambusia nobilis* at Bitter Lake National Wildlife Refuge near Roswell, NM, USA. Morphological variation was assessed using geometric morphometric techniques based on landmark coordinates digitized from images of individual fish. Shape differed seasonally, likely due to developmental and reproductive changes throughout the year. During the reproductive season, females tended to be deeper bodied with a posteriorly shifted anal fin. One of four populations differed the greatest in morphological traits, particularly body depth, caudal peduncle length, and anal fin placement. Deeper body profiles and shorter caudal peduncles were observed in the population associated with less predation pressure, high

dissolved oxygen, and low salinity. Observed differences in body shape emphasize the importance of managing phenotypic diversity and monitoring habitat quality and community composition associated with conservation efforts for this species.

Keywords:

Gambusia; morphometrics; shape; habitat

INTRODUCTION:

Exposure to varying selective pressures among habitats may lead to behavioral, physiological, or morphological divergence between populations or species (Schluter 2001). These traits may be of genetic nature and are shaped over time by evolution, or populations may react to varying environments through phenotypic plasticity and exhibit flexibility in particular traits that increase fitness under local conditions (Kawecki and Ebert 2004). Environmental conditions that can affect morphology include predation pressure, resource abundance and distribution, and abiotic features like temperature and salinity (Schluter 2001). Langerhans et al. (2003), for example, observed morphological diversification in mouth placement and body depth between conspecific populations of two neotropical fish species in lagoon and river channel habitats which differ in flow and foraging opportunities. Similarly, morphological variation in body depth, caudal peduncle shape, spine lengths, and orbit diameter was observed between populations of three-spine stickleback as a response to varying selective pressures (predation and turbidity) between populations (Webster et al. 2011). *Gambusia affinis* from populations subjected to greater predation had larger caudal peduncle regions and the anal fin position was shifted anteriorly and toward the midline of the body (Langerhans et al. 2004). In isolated populations of pupfish, higher salinity is associated with a more streamlined body (Collyer et al. 2005). Previous research on cichlids has shown that low oxygen environments are associated with more elongated and wider heads that accommodate increased gill tissue needed to maximize oxygen absorption in hypoxic environments (Crispo and Chapman, 2010). It is understood that both genetic divergence and

environmentally induced phenotypic plasticity can contribute to morphological differences between populations and species (Kawecki and Ebert 2004).

Understanding how morphological traits respond to local conditions merits further study, especially among endangered species with limited distributions. *Gambusia nobilis* (Pecos Gambusia) was selected for examining morphological variation because 1) understanding morphological variation is important to the management of this endangered species which is restricted to relatively few localities 2) the populations are demographically isolated, and 3) habitats differ in both abiotic conditions and community structure (i.e. competitors and potential predators). Because habitats vary in ecological parameters and there is little/no gene flow between populations, local adaptation and population divergence in morphological traits are expected.

Pecos Gambusia, *Gambusia nobilis* (Baird and Girard, 1853) are small (25-40 mm standard length), have a deep body and thick caudal peduncle, are relatively short lived, livebearing, and are sexually dimorphic (Bednarz 1979; Edwards 2001; Hubbs et al. 1983; Hubbs et al. 2002; Hubbs 2003; Sublette et al. 1990). Listed as an endangered species in 1970, it is restricted to only four known localities in New Mexico and Texas in the Pecos River watershed (Bednarz 1979; Echelle and Echelle 1986; Hubbs et al. 1983). It inhabits small springs, sinkholes, and seeps at Bitter Lake National Wildlife Refuge (BLNWR) and Blue Spring in New Mexico and Balmorhea springs and Diamond-Y springs in Texas (Bednarz 1979; Edwards 2001; Hubbs 2003).

Across the species' current distribution, populations of *G. nobilis* differ morphologically, but these differences were not correlated with ecological variables (Echelle and Echelle 1986). A principal component analysis of fin ray and scale counts

suggests that there are three morphological subsets of populations within the current distribution: 1) Blue Spring, in NM, 2) Texas populations from Comanche Springs, Phantom Lake, Griffin, and East Sandia near Balmorhea, and 3) populations from Bitter Lake National Wildlife Refuge, NM and Leon Creek, TX (Echelle and Echelle 1986). The morphological variation across the distribution may be due to habitat fragmentation and subsequent divergence; the available habitat of *G. nobilis* has become more restricted over the last century with declining water levels resulting from a drawdown of the water table and diminished flows of the Pecos River (Williams et al. 1985).

Although Echelle and Echelle (1986) demonstrated that there are three primary sets of populations that differ in morphological traits, inter-population variation within these geographic units deserves further research. The focus of this study was to identify morphological variation among populations of *G. nobilis* at Bitter Lake National Wildlife Refuge (BLNWR) and explore how morphological variation is related to ecological conditions. Each of four *G. nobilis* habitats sampled at BLNWR differs in ecological parameters including size, salinity, dissolved oxygen, habitat structure, and community composition (Bednarz 1979; Swaim and Boeing 2008). Selection driven by exposure to varying ecological pressures may have resulted in phenotypic divergence between populations. To assess morphological variation, geometric morphometric methods were utilized. Geometric morphometric methods have been used widely to assess species variation and the effects of ecological factors on morphology in plants and animals including fishes (Clabaut et al. 2007; Hankinson et al. 2006; Love and Chase 2009).

METHODS:

Specimen Preparation

The present study examined geometric morphometric variation among four populations of *G. nobilis* at Bitter Lake National Wildlife Refuge (BLNWR) (33°27'32.97" N, 104°24'11.75" W). Sinkholes 7, 27 South, and 31 (part of the Sago Spring Complex) contain natural populations of *G. nobilis* while Sinkhole 37 was stocked with transplants from sinkholes 27 and 7 (Hubbs et al. 1983; Personal Communication, BLNWR; Bouma 1984).

Monthly samples of the fish community and water quality were collected from May 2011 through April 2012 (Table 1). Fish were sampled using mesh minnow traps baited with dry dog food and placed on the shallow underwater ledges of the sinkholes (0-0.5 meters during summer months and approximately 5-15 meters deep during winter months when the fish populations descend to overwinter). Sinkholes 7, 37, and 27 South were sampled during all 12 months, and from Sinkhole 31, females were only sampled during June, July, and August and a sample of males was obtained in October to minimize impact to other endangered species. The number of captured individuals from each species was recorded to determine the community composition and percent of each species in each sinkhole. Up to ten individuals of *G. nobilis* per site per month (and 20 from sinkhole 31 in July) were retained and all other individuals were released. A total of 345 specimens (273 females and 72 males) were collected over the 12 month sampling period. Specimens were selected randomly, but the sex ratio of collections was biased toward females to increase sample size for a concurrent life history study. *Gambusia nobilis* individuals retained were fin clipped (for preservation of genetic samples),

ethanized with MS-222 (Tricaine Methanesulfonate), photographed, placed in 10% formalin, and subsequently dehydrated with an ethanol gradation for permanent storage. Specimens were accessioned to the Museum of Southwestern Biology (MSB) at the University of New Mexico (ACC2011-V:18).

Morphometrics

Digital images of each specimen were obtained immediately after euthanization to prevent distortion in body morphology resulting from tissue fixation in formalin and dehydration in ethanol. The left lateral side of each individual was photographed using a FUJIFILM FinePix S4000 camera mounted 14 centimeters above the specimen on a portable copy stand (Collyer et al. 2005; Langerhans et al. 2003). Specimens were placed in a small clear plastic dish, with a scale bar and enough water to prevent desiccation while attempting to limit optical distortion.

Morphological variables were obtained by digitizing anatomical landmarks in tps-Dig2 morphometric software (Rohlf 2010). The scale bar was re-measured for each specimen during digitization to adjust the scale associated with each photograph. Landmarks were chosen to reflect easily identifiable and homologous points among all individuals. The landmarks chosen were: 1) most anterior point between maxilla and premaxilla, 2) indentation of the nape, 3) anterior insertion of dorsal fin, 4) posterior insertion of dorsal fin, 5) dorsal insertion of caudal fin, 6) ventral insertion of caudal fin, 7) posterior insertion of anal fin, 8) anterior insertion of anal fin, 9) intersection of operculum and ventrum, and 10) center of the eye (Fig. 1; after Langerhans et al. 2004; Langerhans and Makowicz 2009).

During digitization, 27 of the 345 specimens exhibited spinal deformation, were disfigured during trapping, oriented poorly in the photograph, or were sexually immature (no gonopodium or dark gonopore observable) and were removed from the analysis. Thus sample size consisted of 318 specimens (247 females and 71 males).

Geometric shape variation was analyzed with a Generalized Procrustes Analysis (GPA), (Viscosi and Cardini 2011). GPA separates size and shape variation into two independent components. Size is measured as the centroid size, calculated by summing all of the squared distances between each landmark and the center of the configuration then taking the square root of the sum (Langerhans et al. 2003; Rohlf 1999; Viscosi and Cardini 2011; Zelditch et al. 2004). Raw landmark data is converted into shape configurations that can be analyzed by aligning specimens and removing non-shape variation through a Procrustes least-square superimposition process that scales, rotates, and translates landmark configurations (Adams et al. 2004; Bookstein 1991; Rohlf and Marcus 1993; Viscosi and Cardini 2011; Zelditch et al. 2004). A Procrustes fit of the raw landmark coordinates obtained during digitization of males and females was performed using PAleontological STatistics (PAST version 2.14) morphometric and statistics software (specimens were rotated to the major axis and scaled using the centroid size of each observation with number of constraints adjusted to 4) (Hammer et al. 2001).

Statistical Analyses

A Principal Component Analysis (PCA) and a multivariate analysis of variance (MANOVA) were used as an initial morphometric analysis to determine if the sexes should be analyzed separately. This species displays sexual dimorphism, and as expected

significant morphometric differences between the sexes were obtained. Therefore the sexes were analyzed separately in all subsequent analyses.

To assess population differences in body size, as measured by centroid size, a non-parametric Kruskal-Wallis test with Mann-Whitney pairwise comparisons was performed in PAST on centroid size values (Hammer et al. 2001; Langerhans et al. 2003). Procrustes coordinates representing the shape data were tested for normality using the Shapiro-Wilk normality test in PAST. Ten of 20 shape variables for females and four of 20 shape variables for males were significantly different from a normal distribution, therefore non-parametric MANOVAs were performed to more conservatively assess population differences. Differences in morphology among populations were tested with a non-parametric MANOVA using the Procrustes coordinates of the 10 landmarks for females and males separately, pooling data across months (Michael L. Collyer, Personal Communication; Hammer et al. 2001; Schaefer et al. 2011). The non-parametric MANOVA was calculated with default 9,999 permutations and performed using Euclidean distances. Multiple comparisons were performed with Bonferroni-corrected p-values (Hammer et al. 2001). The mean Procrustes coordinate configuration of each population was determined and plotted against the consensus configuration to illustrate shape difference between each population and the “mean” form of all those sampled. An additional non-parametric MANOVA was performed on females collected only during June, July, and August to assess population differences during the period that collections were made at sinkhole 31. A Non-parametric MANOVA was used to determine if there were significant differences in morphology between fish collected during different seasons pooled across populations

(Feb/ March/ April; May/ June/ July; Aug/ Sept/ Oct; and Nov/ Dec/ Jan). Lastly, shape data was reduced to a single variable (PC1) with Principal Component Analysis and was analyzed with a Nested ANOVA using the Satterthwaite approximation for unequal sample sizes where months are nested within sites to compare the amount of variation between sinkholes while accounting for the variation across months (Nested ANOVA's performed in Excel spreadsheet from McDonald 2009).

The nature of morphological variation was further explored using Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) generated from the covariance matrix of the Procrustes coordinates in PAST (Hammer et al. 2001; Klingenberg 2011). The morphological change associated with a specified principal component value is represented by deformations of the landmarks from the consensus sequence (performed in MorphoJ morphometric software) (Hammer et al. 2001). CVA axes are constructed that best discriminates between pre-assigned groups and addresses the between group variation (distance between groups) relative to the within group variation (size of distribution of each group) (Zelditch et al. 2004).

Regression analyses were performed on primary principal component axis scores of the shape variables against monthly ecological parameters (salinity, dissolved oxygen, pH, sinkhole area, and percent of predators in the community) to determine any potential relationships between habitat and morphology. A principal component analysis was performed on morphological variables (pooled across sites) with season as a classifier to visualize how morphology may change seasonally.

Measurement error

Potential measurement error was estimated as a ratio of the variation among repeated measures on the same specimen to the overall variation among all specimens (Arnvist and Martensson 1998; Yezerinac et al. 1992). The measurement error was estimated following the protocol by Adriaens utilizing tpsUtil, tpsDig, tpsRelw, and tpsSmall software (Accessed 19 October 2012 from: <http://www.fun-morph.ugent.be/Miscel/Methodology/Morphometrics.pdf>; Rohlf 1998; Sadeghi et al. 2009).

A random sub-sample of females and males was chosen (25 females and 10 males); the sub-sample contained specimens from all four sinkholes and from months that spanned the entire year (although not every month was included). After each specimen of the random sample test group was digitized 10 times (10 replicates) the data were examined for digitization error using tps-Small (Rohlf 1998; Sadeghi et al. 2009). The mean Procrustes distance was determined first for the entire random sub-sample data set (separately for each sex; n=250 for females and n=100 for males), then the mean Procrustes distance for the 10 replicates of each individual was calculated and summed across all individuals in the sub-sample.

Variation observed in the entire sub-sample includes both natural and error variation, while variation among the replicates measures variation due to imprecise digitization of landmarks. A ratio of the mean Procrustes distance for replicates to the mean Procrustes distance of the whole sub-sample gives the percentage of variation observed among samples that is due to digitization error (Sadeghi et al. 2009). Among the sub-sample of females, the measurement error rate ranged from 5.31% to 56.66%,

with a mean of 20.03%. The percent measurement error of the male sub-sample ranged from 23.94% to 33.16%, with a mean of 26.91%.

RESULTS:

Principal component analysis showed disparity between male and female shape and MANOVA supported the conclusion that males and females differed significantly in morphology (Wilk's lambda= 0.0520; $F_{16,299}=340.6$, $p =0.0006$). The primary shape differences between male and female mean configurations were in the length of the caudal peduncle and position of the anal fin. Compared to females, males had a longer caudal peduncle and the anal fin was shifted considerably anterior, further increasing the area of the caudal peduncle region.

Centroid size within each population was not normally distributed, likely due to the distinct age-class distributions between summer and winter. Additionally, centroid size for Sinkhole 31 did not have equal variance with the other populations because it was only sampled during June, July, and August when females were the largest, and lacked the variation of small, winter females reflected in the data from the other three sinkholes. Large females were predominant in the summer after overwintering one or more years. Many of the large, older females appeared to decline at the end of the breeding season and the winter population was dominated by young of year and likely one year old females. Kruskal-Wallis tests with Mann-Whitney pairwise comparisons suggested that females from Sinkhole 31 were significantly larger in size (38.88 ± 0.54) than females from the other three sinkholes and that individuals from Sinkholes 7, 27 South, and 37 (31.75 ± 0.77 ; 33.57 ± 0.78 ; and 31.70 ± 0.59 , respectively) were not significantly different from one another with respect to Centroid Size (Kruskal-Wallis:

$H_{(3)}=46.6, p < 0.0001$). When females from June, July, and August (the only months during which sinkhole 31 females were sampled) were analyzed separately, significant differences were observed between all population pairs except between sinkhole 31 and sinkhole 27 (38.88 ± 0.54 ; 38.43 ± 0.65) and between 7 and 37 (35.74 ± 0.61 ; 33.07 ± 0.73) (Kruskal-Wallis: $H_{(3)}=46.73, p < 0.0001$). The analysis of male centroid size suggested that male *G. nobilis* from Sinkholes 7 and 37 (23.74 ± 0.22 ; 23.25 ± 0.44) and from sinkholes 27 and 31 (25.36 ± 0.44 ; 25.11 ± 0.45) also did not differ significantly from one another; sinkholes 7 and 37 and were significantly smaller than those of the other two populations (Kruskal-Wallis, $H_{(3)}=20.80, p=0.0001$).

Non-parametric MANOVA of female shape data indicated some significant differences among populations (NPMANOVA: $F_{76}=10.97, p=0.0001$). Multiple comparisons suggest that each population pair is statistically different except sinkhole 37 compared to sinkholes 7 and 27. Interestingly, the shape of the population from sinkhole 37 was not significantly different from the two source populations (sinkholes 7 and 27) from which sinkhole 37 was stocked in 1980 (BLNWR, Personal Communication; Bouma 1984). An analysis of females collected in only June, July, and August showed that there was a significant difference in shape between each population pair except between sinkholes 7 and 37 (NPMANOVA: $F_{76}=8.49, p=0.0001$). For males, the analysis suggested a significant difference in morphology only between sinkholes 27 South and 37 (NPMANOVA: $F_{76}=2.92, p=0.0006$). Non-parametric MANOVA across seasons suggested significant difference in female morphology between all seasons except Feb/March/April and Nov/Dec/Jan, suggesting seasonal morphological change between winter and summer months (NPMANOVA: $F_{76}=19.27, p=0.0001$). The Nested

ANOVA of female PC1 shape data suggested no significant differences between populations when using the Satterthwaite approximation ($F_{3,29}=2.30, p=0.0985$), but a marginally significant difference without the Satterthwaite approximation ($F_{3,31}=3.09, p=0.0415$). The variance component among groups (sinkholes) was 11.39% while the variance between months within sinkholes accounted for 53.21%; a significant difference among months nested within sinkholes was observed ($F_{29,210}=11.11, p<0.0001$). Among males, the Nested ANOVA of PC1 suggested no significant difference among sinkholes which only accounted for 4.38% of the total variance ($F_{3,10}=1.35, p=0.3175$); there was a significant difference among months nested within sinkholes which accounted for 25.53% of the variance component ($F_{10,49}=2.00, p=0.0280$).

Mean Procrustes coordinates of each population were plotted against consensus configurations to visualize the difference between the “mean” male and female form of all those sampled to the average shape of each population (Fig. 2). These visualizations showed that males and females from sinkhole 31 were deeper bodied and the eyes were shifted more dorsally than other populations. Females from sinkhole 31 had a shortened caudal peduncle and more terminal mouth than other populations. Among females, the mean form of sinkhole 37 most closely resembled the consensus configuration, while females from sinkhole 7 and 27 had a more fusiform profile. Visualization of mean sinkhole 7 females showed an elongated caudal peduncle and an anterior shift in the position of the anal fin, both contributing to a longer caudal peduncle. Similar shape deformations were obtained when females from only June, July, and August were examined as with the complete female dataset. Mean male configuration of sinkholes 27

and 37 indicated a more fusiform body, while mean shape from sinkhole 7 closely resembled the consensus configuration.

The first principal component of female shape data (PC1) explained 39.80% of the variance in shape, PC2 explained an additional 20.90%, and PC3 explained 11.02% of the variation. The first seven principal components explained 89.2% of the total variation in morphology as represented by geometric relationships of landmarks. For males, PC1 accounted for 37.38%, PC2 contributed an additional 23.36%, and PC3 accounted for 11.22% of the total variance. The first seven principal components accounted for a cumulative 89.62% of the total variance.

A bi-plot of PC1 vs PC2, suggested morphological similarity across populations as illustrated by overlap in the convex hulls (Fig. 3). For females, sinkhole 31 had the smallest distribution of points in PC space (i.e., they clustered tightly around negative PC1 values), which possibly reflects lack of seasonal variation from younger females generally collected in winter months. Canonical Variate Analyses showed approximately equal within group variability and sinkhole 31 with the greatest variability between groups (Fig. 3). Wireframe deformations showed how landmark positions shifted between positive and negative values of each principal component (the scale factor for the deformations was +/- 0.08 to increase visibility of the shape changes). Among females, morphological changes associated with PC 1 were primarily shifts in caudal peduncle and anal fin placement. Individuals with negative PC1 scores had shorter caudal peduncles, the anal fin was shifted posteriorly, and the head was shortened. Individuals with increasing values along PC1 had a slightly elongated caudle peduncle, the anal fin was shifted anteriorly, and the body depth was reduced. A principal

component analysis of shape with season as a classifier revealed considerable overlap in the shape distribution of Nov/Dec/Jan and Feb/March/April (Fig. 4). Females from May, June, and July clustered primarily around negative values within the PC1 range and those from Aug, Sept, and Oct showed the greatest variation in extent of PC1 values. Among males, positive PC1 values were associated with a deeper body profile, a superior mouth, and anal fin shifted away from the midline of the body. Individuals in the negative direction of the PC1 axis were more fusiform, had a more terminal mouth, and had the anal fin shifted slightly anterior and toward the midline of the body. It was unclear from a PCA of male shape data if there are any significant seasonal morphological changes.

Principal component values of shape were regressed against ecological parameters (salinity, dissolved oxygen, pH, sinkhole area, and predation pressure) to explore a relationship between shape and habitat attributes (Table 2). The strongest relationship with female shape was with potential predation measured as % *Fundulus zebrinus* in each community ($R^2=0.13$, $t_{243}=6.03$, $p<0.0001$). However, the linear relationship does not seem to fit the distribution well, and the correlation may be driven by the lack of positive PC1 values from sinkhole 31 more common of winter females. When only females from June, July, and August were considered a significant relationship between PC1 and % *Fundulus zebrinus* remained ($R^2=0.087$, $t_{116}=3.32$, $p=0.0012$). Between the populations, *Fundulus zebrinus* had the greatest relative abundance in sinkhole 37 and was absent in sinkhole 31. Body shape was also strongly related to pH that was itself highly correlated with month. Among males, surface area of the sinkhole was most correlated with shape ($R^2=0.12$, $t_{69}=-3.14$, $p=0.0025$) followed by the relative abundance of *F. zebrinus* ($R^2=0.06$, $t_{69}=-2.27$, $p=0.026$).

DISCUSSION:

Geometric morphometric analysis revealed some subtle shape variation among populations of *G. nobilis* at Bitter Lake National Wildlife Refuge, suggesting that exposure to varying abiotic and biotic pressures in isolated sinkholes may have played a role in morphological divergence between populations. Among females, there was significant morphological variation between populations, especially between sinkhole 31 and the others. Females from sinkholes 7, 27 South, and 37 were most similar to the consensus configuration, while the sinkhole 31 population was deeper bodied and had a shorter caudal peduncle. Females from sinkhole 37 did not have significantly different morphology from two source populations from which the sinkhole was stocked (sinkholes 7 and 27). Analysis of male shape indicated that sinkhole 31 males had a larger body size and were deeper bodied than the other populations. Conversely, sinkhole 37 was the most fusiform. Further analyses, however, suggested that after accounting for variation between months, there is no significant difference among populations for the primary principal component (PC1) of shape in either sex. Some seasonal shape variation was observed, and accounted for a large percentage of the total variation in the PC1 of shape in the nested ANOVA analyses.

Among females, significant seasonal changes in shape were observed, suggesting that female *G. nobilis* morphology may change with age, development, size, or reproductive status. PC values of females during reproductive months are associated with deeper body profiles and a posteriorly shifted anal fin which is likely related to increasing abdominal capacity to accommodate gestating broods. This observation raises an important question as to the effect that reproductive morphological change has on

fitness. Gestating females undergo physiological and morphological changes such as increased oxygen consumption, increased energy expenditure, reduced resource acquisition and assimilation, increased size of the abdomen, and shift in position of the anal fin that affect their locomotor performance and swimming ability during pregnancy (Boehlert et al. 1991; Frommen et al. 2009; Weeks 1996). Seasonal changes in morphology may also be tied to development and growth, as size (centroid size) was also very strongly correlated to shape ($R^2=0.65$, $t_{243}=-21.42$, $p<0.0001$). Centroid size was greatest during summer months while smaller and younger females were more dominant in the late fall and winter months. A die-off of older females occurs in the fall with juveniles and younger females overwintering and growing in the spring prior to the next reproductive season.

Ecological pressures are expected to result in changes in morphological traits. Shape variation of *G. nobilis* at BLNWR appears to be related to variation of abiotic factors (i.e., salinity, DO, pH), and biotic factors including potential predation. While determining the ultimate cause of morphological divergence is beyond the scope of this study, I offer several hypotheses for ecological effects on morphology which are based on sinkhole differences in: 1) predation, 2) salinity, and 3) dissolved oxygen. Individuals exposed to greater predation pressure are expected to have larger caudal peduncles which facilitate acceleration and maneuverability, and has been supported in studies of *Gambusia affinis* (Langerhans and Reznick 2010; Langerhans et al. 2004). The fish community of sinkholes 7, 27 South, and 37 consist of *Gambusia nobilis*, *Cyprinodon pecosensis*, and *Fundulus zebrinus*. *Fundulus zebrinus* likely prey on juvenile *G. nobilis* and were observed aggressively chasing and nipping at adult *G. nobilis* during specimen

collection. Female *G. nobilis* from sinkhole 31 are not exposed to *F. zebrinus* and were found to have shorter caudal peduncles, suggesting that in the absence of this predator/they may not experience the same degree of selective pressure for longer caudal peduncles. Analysis of morphometric differences between the sexes revealed a shift in males to a longer and thicker caudal peduncle and an anterior shift of the anal fin. Longer caudal peduncles in males may improve burst speed and increase copulation success, or may increase survival as smaller males may be more susceptible to gape limited predators than females (Hassell et al. 2012; Langerhans and Reznick 2010).

Other ecological parameters may also influence morphology, especially salinity and dissolved oxygen levels which affect physiology and have been identified as limiting factors in the number of habitats suitable for *Gambusia nobilis* and may affect reintroduction efforts (Bednarz 1979). Salinity, for example, has profound effects on growth rates of fish and reproductive investment, influenced by metabolic rate and high costs of osmoregulation (Boeuf and Payan 2001; Trexler and Travis 1990). Similar to results from Collyer et al. (2005), females from the sinkhole with the highest salinity (sinkhole 27), had the most fusiform body profile and those from the sinkhole with lowest salinity (sinkhole 31) were deeper bodied. In environments with low oxygen saturation, some fish may rely on surface breathing and the more oxygenated water at the air/water interface (Lewis, 1970). A smaller angle between the water surface and the fish's position in the water column can be achieved by a more fusiform body and narrower head (Lewis, 1970). The population of *G. nobilis* from sinkhole 31, a habitat with higher dissolved oxygen had a deeper body, while those populations experiencing

lower oxygen saturation levels (sinkholes 27 south and 37) had a more fusiform body profile.

Adaptation in response to unique selective pressures in different environments can lead to phenotypic variation among populations (Langerhans et al. 2003). Ecological effects on morphology have been well documented in teleosts (Collyer et al. 2005; Langerhans et al. 2003; Langerhans et al. 2004; Webster et al. 2011) and can have a significant effect on swimming performance, foraging, reproduction, and predator avoidance in fishes, which ultimately affects the fitness of an individual (Basolo and Wagner 2004; Langerhans and Reznick 2010). While these studies are important, some work may be met with challenges. Research on endangered species can be impacted by limited samples sizes- both of populations and individuals. Circumstances of this study that could potentially confound the results include: limited number of sites sampled, small and unequal sample size of each population, co-variation of multiple ecological parameters, and measurement error of specimen landmarks.

This study identified subtle morphometric variation between populations of *G. nobilis* from four sites at BLNWR. Further areas of research interest include (1) whether the observed morphological differences are due to phenotypic plasticity in response to local environmental conditions, or genetic divergence among isolated populations, (2) how seasonal and population morphological variation affects aspects of fitness in *G. nobilis*, and (3) how tradeoffs among multiple selective pressures, such as reproductive investment, foraging strategies, and swimming performance affect body shape. Local adaptation, driven by selection for traits with greater fitness in local environments, may be constrained by gene flow, genetic drift, and stochastic environments (Kawecki and

Ebert 2004). Thus, not all populations exposed to unique ecological conditions will exhibit differentiation due to local adaptation (Kawecki and Ebert 2004). Common garden experiments may be needed to elucidate genetic and environmental interactions and their roles in local adaptation of *Gambusia nobilis*.

Implications to Conservation

The Pecos *Gambusia* Recovery Plan (1983) calls for maintenance and monitoring of current habitat and populations of *G. nobilis* and reestablishment of populations within the historic range. Not only is suitable habitat needed, but an informed decision about source populations is critical to reintroduction success and maintenance of natural variability. Echelle (1988) suggests that in addition to choosing source populations based on genetic variability, populations exhibiting unique morphologies should also be preserved. Swenton et al. (unpublished) found low heterozygosity and genetic diversity among populations of *G. nobilis* but observed significant genetic divergence across and within populations. Genetic data, in combination with morphometric differences identified in the current study, should be used to maximize and preserve diversity of *G. nobilis* if re-introductions are made to suitable habitat within the historic range. Further research is needed to determine if morphometric variation observed has a genetic basis, or is influenced by ecological conditions.

This morphometric analysis also allows for assessment of past management decisions. For example, in the early 1980's, sinkhole 37 was stocked with individuals from both sinkhole 7 and sinkhole 27. Morphology of the source populations were found to be statistically different from one another, but the sinkhole 37 population was not different from either source population, despite sinkholes 7 and 37 being identified as

genetically unique (Swenton et al. unpublished). At Bitter Lake National Wildlife Refuge, understanding subtle seasonal and population morphological variation may aid management decisions and lead to more successful management and reintroduction initiatives.

ACKNOWLEDGMENTS:

I am very grateful to Daniel P. Hopkins, Robert L. Hickey, Kara L. Hickey, Davona J. Hickey, and Kristin Wright who assisted with monthly field work and specimen photography. I appreciate the important technical and statistical guidance regarding geometric morphometric methods provided by Michael L. Collyer and Rhiannon West. Thank you to Astrid Kodric-Brown for her valuable comments on drafts of this manuscript and mentoring throughout this endeavor. Thank you also to Thomas F. Turner and Blair O. Wolf for comments on research design and editing of the manuscript. I would also like to acknowledge Alexandra M. Snyder and the Museum of Southwestern Biology (MSB) for providing supplies, facilities, and technical support needed to carry out this research. This project was funded in part by a Graduate Research Project and Travel Grant provided by the Office of Graduate Studies, University of New Mexico. Specimens were collected under the US Fish and Wildlife Service Native Endangered Species Recovery permit #TE28576A-1, US Fish and Wildlife Service Consultation for Section 7 of the Endangered Species Act of 1973 #22420-2011-I-0055, US Fish and Wildlife Service Bitter Lake National Wildlife Refuge Special Use Permit, New Mexico Department of Game and Fish Authorization for Taking Protected Wildlife for Scientific and or Educational Purposes Permit #2968 and #3303 (specimens transferred to researcher), and the University of New Mexico Institutional Animal Care and Use Committee approved protocol # 11-100628-MCC.

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TABLES AND FIGURES:

Table 1 Mean ecological parameters of sinkholes at Bitter Lake National Wildlife Refuge. Mean values for each of the ecological parameters were obtained from 12 months of sampling at Sinkholes 7, 27 South, and 37 and from four months at Sinkhole 31. Community composition is reflected by the percentage of each species comprising the total sample from each habitat.

Population	Dissolved Oxygen (mg/l)	Salinity (ppt)	pH	Sinkhole Area (m ²)	% <i>Gambusia nobilis</i>	% <i>Fundulus zebrinus</i>	% <i>Cyprinodon pecosensis</i>
Sinkhole 7	10.074	7.216	6.96	1319	81.72	11.36	6.92
Sinkhole 27	8.103	17.533	6.73	570	84.08	7.88	8.04
Sinkhole 31	15.285	6.475	6.38	407	88.95	0.00	11.05
Sinkhole 37	8.963	10.308	6.86	2826	78.67	13.57	7.75

Table 2 Linear regression analyses of primary principal component scores of shape and ecological parameters. Sample size was 245 females and 71 males.

Ecological Parameter	Sex	r	r ²	t value	P value
Dissolved Oxygen (mg/l)	Female	-0.2273	0.0517	-3.6390	0.0003
	Male	0.0742	0.0055	0.6178	0.5387
Salinity (ppt)	Female	-0.0157	0.0002	-0.2455	0.8063
	Male	0.1268	0.0161	1.0619	0.2919
pH	Female	0.5349	0.2861	9.8690	<0.0001
	Male	-0.3896	0.1518	-3.5135	0.0008
Predation (% <i>Fundulus zebrinus</i>)	Female	0.3610	0.1303	6.0349	<0.0001
	Male	-0.2635	0.0694	-2.2693	0.0264
Sinkhole Surface Area	Female	0.2047	0.0419	3.2598	0.0013
	Male	-0.3533	0.1248	-3.1367	0.0025

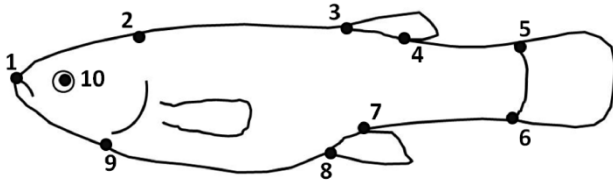


Fig. 1 The location of 10 digitized landmarks employed in this study: 1) most anterior point between maxilla and premaxilla, 2) indentation of the nape, 3) anterior insertion of dorsal fin, 4) posterior insertion of dorsal fin, 5) dorsal insertion of caudal fin, 6) ventral insertion of caudal fin, 7) posterior insertion of anal fin, 8) anterior insertion of anal fin, 9) intersection of operculum and ventrum, and 10) center of the eye.

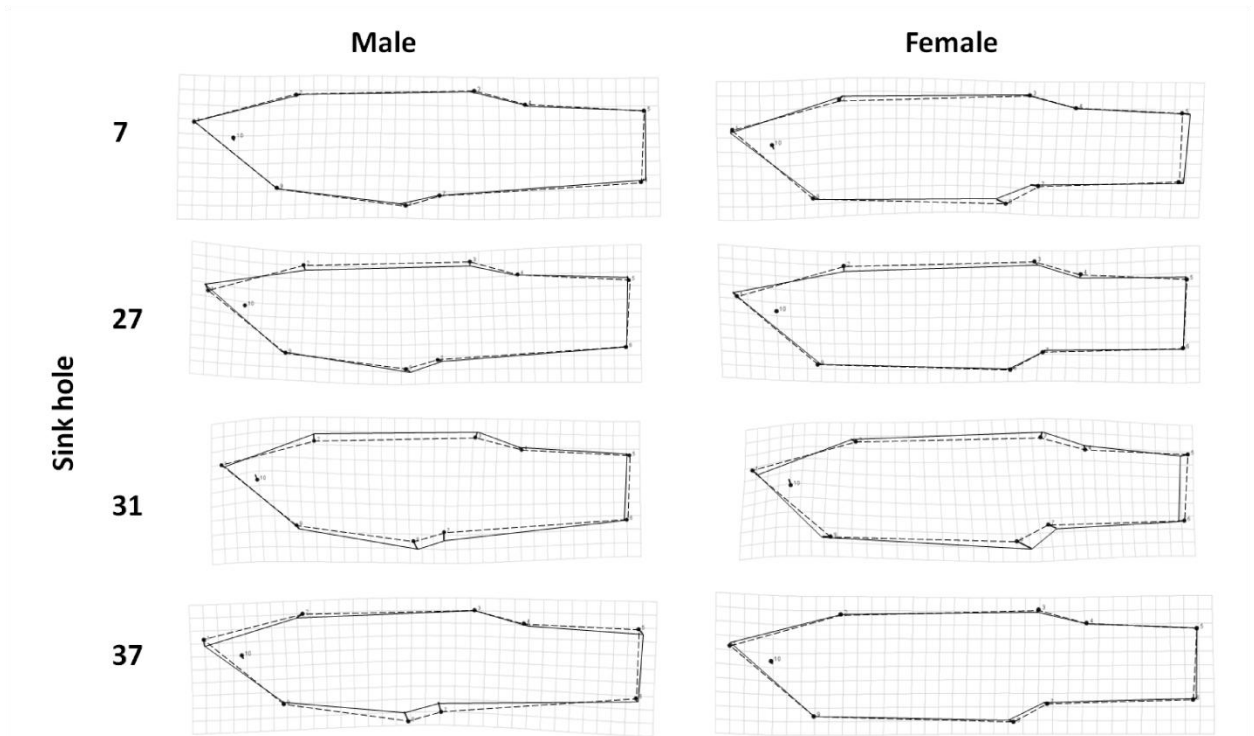


Fig. 2 Grid deformation plots of mean shape difference of each population compared to the consensus configuration for males and females. Dashed lines represent the consensus configuration and solid lines show the shift in shape to the population mean configuration (2x scale for visualization).

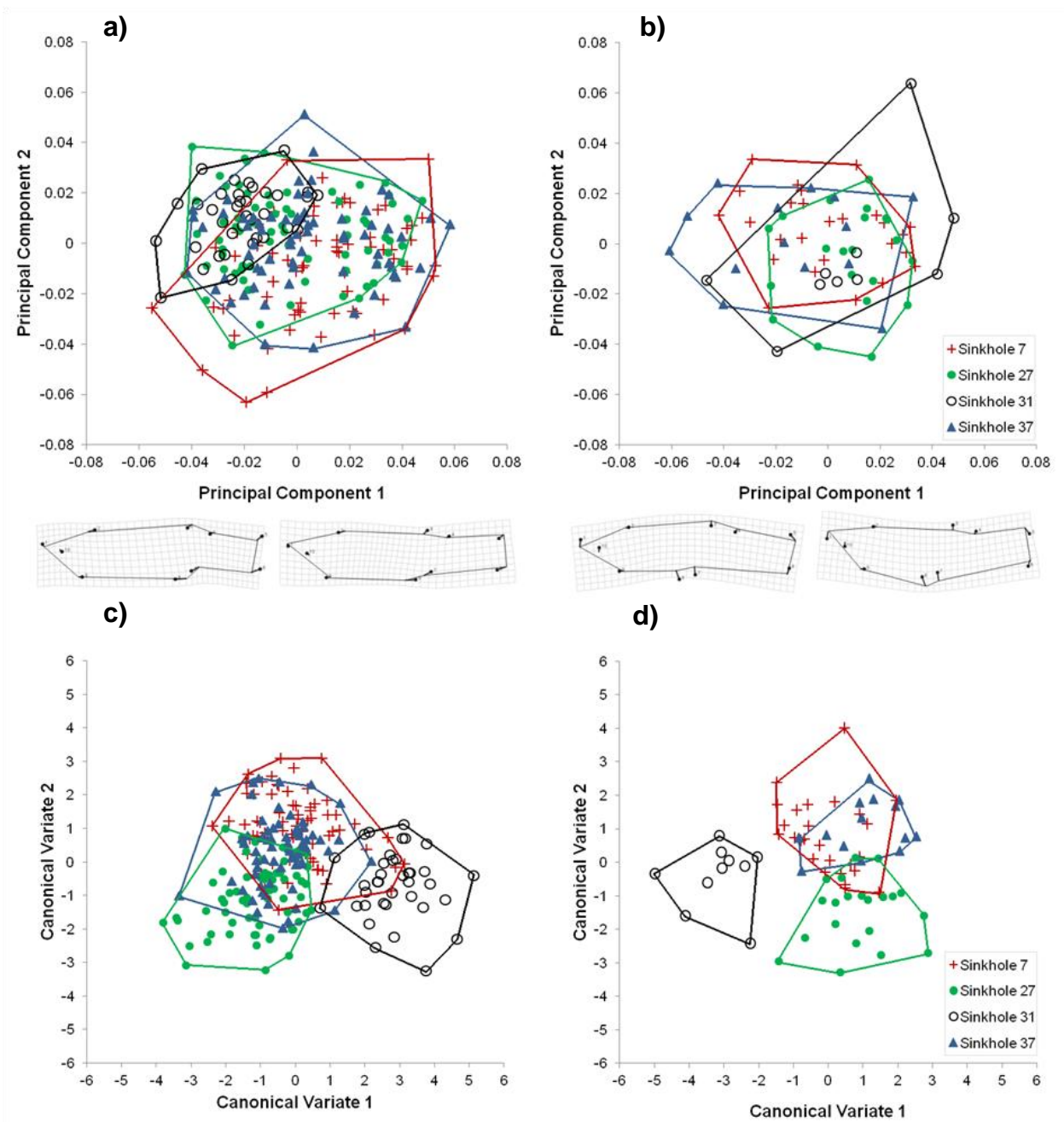


Fig. 3 Principal component analysis of shape variables. Bi-plots of PC1 vs PC2 for (a) females and (b) males shown with convex hulls outlining bivariate distributions. Grid deformations show a shift in shape associated with a + and - 0.08 PC1 value relative to the mean shape at PC=0. Solid lines show the shift in morphology from the consensus configuration represented by solid dots. Bi-plots of canonical variate 1 vs canonical variate 2 for (c) females and (d) males show the axes that explain the greatest and second most amounts of difference between populations given the 20 Procrustes coordinate variables.

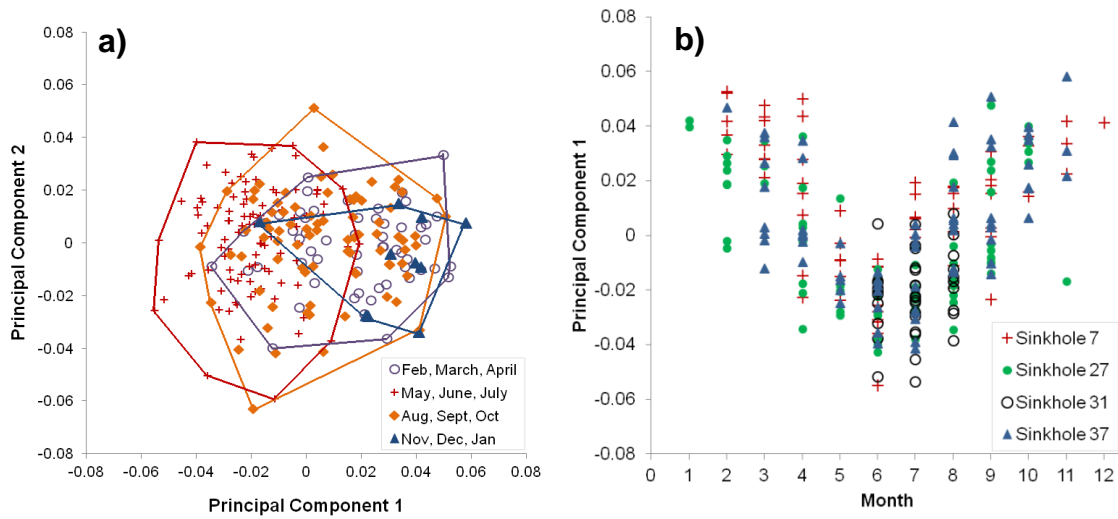


Fig. 4 A principal component analysis of shape variables showing the distribution of variation in shape across seasons. Panel a) Females collected during summer months (May, June, and July) tended to have more negative PC1 values than females collected from November, December, and January. Panel b) shows the distribution of PC1 values over twelve months of the year, illustrating that females (regardless of population) have more negative PC1 values in summertime. Negative PC1 values were associated with a deeper body profile and a shift of the anal fin posteriorly as well as a shortened head and shorter caudal peduncle as seen in Fig. 3.