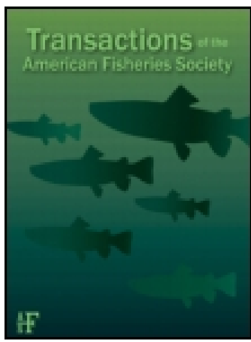

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**ARTICLE**

# Conservation of the Owens Pupfish: Genetic Effects of Multiple Translocations and Extirpations

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***Abstract***
**The Owens Pupfish *Cyprinodon radiosus* represents many of the challenges of managing threatened or endangered species in fragmented refuge populations. All six extant populations of the endangered Owens Pupfish were examined to assess how management practices, including serial translocations and founder events, have influenced the genetic diversity of the species and to make recommendations for future management. Four populations were sampled twice with 3–4 years intervening; two additional populations were sampled once. Populations were genotyped at nine microsatellite loci; estimated effective population sizes ranged from 34.2 to 347.8 individuals based on the linkage disequilibrium method and from 10 to 48 using the sibship assignment method. All of the populations were estimated to have undergone severe bottlenecks, and statistically significant pairwise $F_{ST}$ values increased during the period between sampling. From this data we infer that the individual refuge populations have differentiated and lost genetic diversity and that without intervention they will continue to do so. For the long-term persistence of this species, we recommend founding new populations composed of 30–50 founders from each of the extant populations, regularly translocating up to 10 migrants per generation among stable populations, and maximizing habitat area and quality.**

The habitat for many species is increasingly fragmented and altered, isolating populations, reducing their sizes, and making them more susceptible to stochastic events and extinction (e.g., Gilpin and Soulé 1986). Increasingly, refuge populations are intentionally created to provide insurance against extinction. A sampling of fishes managed this way includes the Mohave Tui Chub *Gila bicolor mohavensis* (Chen et al. 2013), Railroad Valley Springfish *Crenichthys nevadae* (USFWS 2007), Shoshone Pupfish *Cyprinodon nevadensis shoshone* (Castleberry et al. 1990) and Leon Springs Pupfish *Cyprinidon bovinus* and Pecos Gambusia *Gambusia nobilis* (Gumm et al. 2011). However, reliance on refuge populations can create additional management challenges from a genetic perspective. For example, founder events can occur when establishing new populations that reduce genetic diversity, exposing the species to risks such as a reduction in evolutionary potential (Nei et al. 1975; Allendorf 1986) or fitness (Frankham et al. 2002; Reed and Frankham 2003; but see Reed 2010). Further, disjunct refuge populations are often small and may diverge as a result of genetic drift, possibly leading to the fixation of deleterious alleles and general loss of genetic diversity (Frankham et al. 2002). In some cases, refuge populations derive from sequential translocations and founder events, potentially reducing genetic diversity further (Stockwell et al. 1996; Le Corre and Kremer 1998).

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The problems associated with the management of small populations are particularly challenging for aquatic species in deserts; many such populations in western North America are threatened, endangered, or already extinct (Moyle and Williams 1990). The increased risk of extinction is due, in part, to intensifying isolation as climate change desiccated connecting lakes and rivers and habitats shrank during the present interglacial period (approximately the last 12,000–15,000 years; Reheis et al. 2002; Echelle 2008). Today, aquatic species in the desert often inhabit isolated locations characterized by extreme and fluctuating conditions. Such taxa may be made up of only one or a few small populations, making them extinction prone (Meffe and Vrijenhoek 1988). Human activities such as habitat destruction (dewatering, impoundment, or diversion of rivers and springs) and the introduction of exotic species compound this risk of extinction (Meffe 1990).

The history of the Owens Pupfish *Cyprinodon radiosus* demonstrates some of the difficulties of conserving a formerly widespread aquatic species in refuges. The struggle to prevent extinction of the Owens Pupfish has a complicated history, involving rangewide decline, multiple extirpations, founding events, observed demographic bottlenecks, and serial translocations. In this study, nine microsatellite loci were used to assess the genetic variation within and among refuge populations of Owens Pupfish to evaluate how 47 years of interventions and population management have influenced contemporary genetic diversity and make management recommendations for this federally listed species.

Conserving rare aquatic species may include research and management actions such as clarifying taxonomy and defining management and evolutionarily significant units (Moritz 1994; Hurt and Hedrick 2004); restoring habitat, translocating individuals, removing deleterious nonnative species, and performing genetic assessment (e.g., Hurt and Hedrick 2004); monitoring genetic diversity (e.g., Meffe 1990; Minckley 1995; Vrijenhoek 1998), and implementing captive breeding programs (e.g., Vrijenhoek 1998). Stochastic events may have even greater short-term impacts on the survival of species with restricted ranges, so the creation of refuges and habitat protection are critical; however, long-term viability may depend on maintaining genetic diversity, promoting adaptation, and preserving evolutionary processes (Moritz 1999).

## STUDY SPECIES

The Owens Pupfish (Miller 1948) is endemic to the Owens Valley in Inyo County, California. Owens Pupfish are small (<65 mm total length) fish belonging to a group of 16 extant and 3 extinct pupfish species and subspecies found in the American Southwest and northern Mexico (the Desert Pupfish complex; Pister 2001). Once so common they were a food source for Native Americans (Wilke and Lawton 1976), Owens Pupfish were listed as endangered in 1967 (U.S. Office of the Federal Register 1967). Currently, the species persists owing to active management and removal of introduced predators in artificial and seminatural refuges (Table 1; Figure 1).

In habitats with constant temperatures, juvenile Owens Pupfish reach sexual maturity at 3–4 months and can spawn before their first winter, but they rarely live longer than 1 year (Barlow 1961; Soltz and Naiman 1978). In more variable habitats,

TABLE 1. Descriptions of extant Owens Pupfish populations.

| Location | Abbreviation | Habitat description | GPS coordinates | Estimated population size | Date(s) of initial founders | No. of founders | Source(s) of founders |
|---|---|---|---|---|---|---|---|
| Marvin's Marsh | MM | 0.028-ha man-made marsh | 37.504211°N, –118.409978°W | 100–1,000 | 1986 | 1,178 | OVNFS[a] |
| Pond D | PD | 0.004-ha pool in cienega wetland | 37.477180°N, –118.402245°W | 100–1,000 | 1987 | 65 | Old BLM Spring[ab] |
| Pond G–H | PG/H | 0.012-ha pool in cienega wetland | 37.476459°N, –118.401502°W | 100–1000 | | | |
| BLM Spring | BLM | 0.069-ha springbrook | 37.477180°N, –118.402262°W | 1,000–10,000 | 2003 | 2,881 | Mule Spring[a] |
| Well 368 | W368 | 0.020-ha naturalized channel below artesian well | 36.769950°N, –118.125289°W | 100–1,000 | 1986 | 92 | OVNFS[a] |
| Lower Mule Spring Pond | LMS | 0.004-ha man-made pond | 37.106625°N, –118.201794°W | 300–400 | 2007 | 50 | BLM Spring |

[a]Extirpated. OVNFS = Owens Valley Native Fish Sanctuary.
[b]The BLM Spring population used to establish those in Pond D and Pond G–H was extirpated in 1988, and the current BLM Spring population is of a separate lineage.

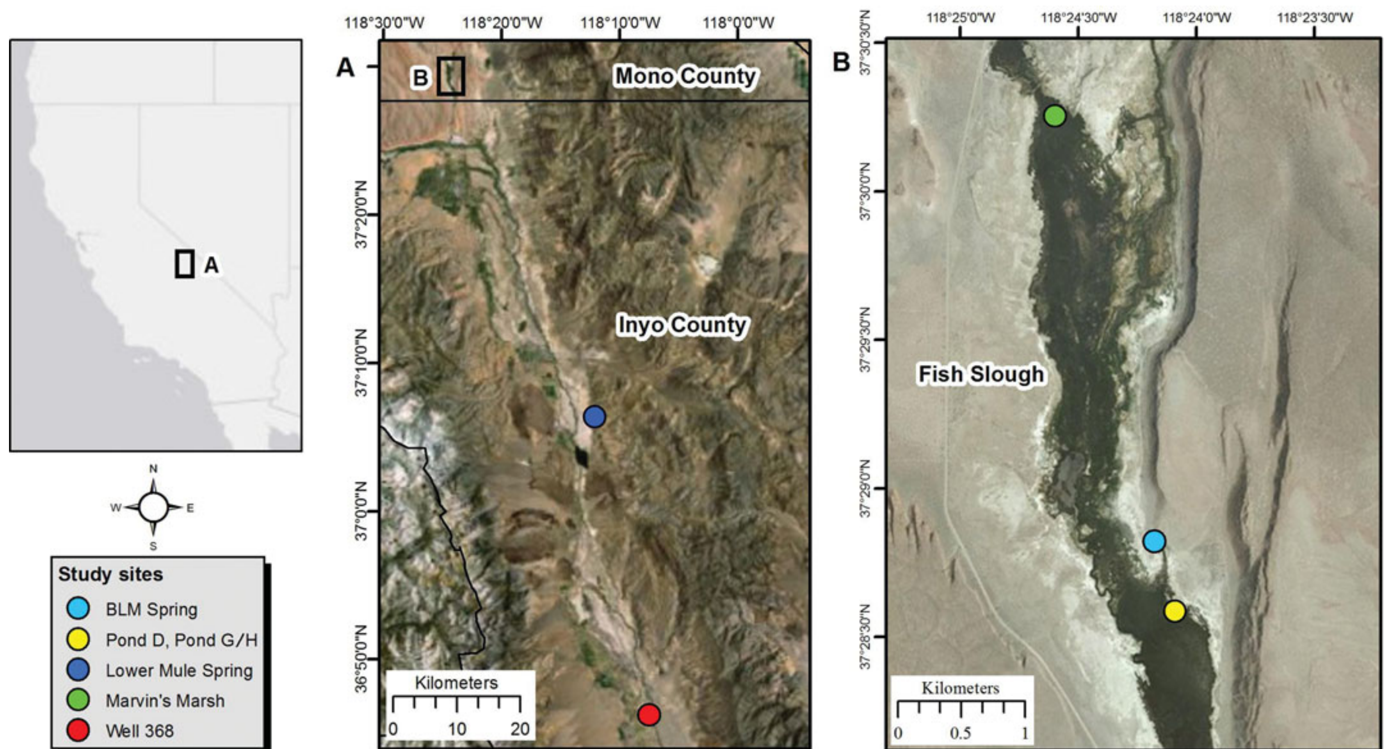


FIGURE 1. Aerial images showing the locations of Owens Pupfish refuge populations in **(A)** the Owens Valley (Inyo and Mono counties) and **(B)** Fish Slough (Mono County). [Figure available online in color.]

fish undergo an annual dormancy period and larvae spawned in autumn will mature the following spring (at 6 months), while those spawned in spring will mature by early summer. The fish in these habitats may live for 2–3 years (Pister 2001). Given the variability of desert aquatic environments, it is probable that Owens Pupfish populations historically underwent large seasonal and interannual variations in size, similar to other Death Valley system pupfishes (Naiman 1976; Sada and Deacon 1994). Under suitable conditions (e.g., no predatory fishes, a permanent water source), Owens Pupfish quickly repopulate a habitat following an observed demographic bottleneck (Young 1976).

Owens Pupfish were thought to be extinct in 1942 (Miller 1969) and did not receive conservation attention until a remnant population of some 200 individuals was documented in 1964 (Miller and Pister 1971). The last approximately 800 descendants of the rediscovered fish were rescued from a drying habitat in 1969 (Pister 1993). Of these, about half were introduced into BLM Spring, from which all extant populations descend. Since then, refuge populations have been repeatedly founded, extirpated, refounded, and at times supplemented with additional fish. California Department of Fish and Wildlife (CDFW) file records show that since 1969, 83 transfers of Owens Pupfish involving 31,995 individuals have occurred among a total of 27 habitats. During this study, five to six Owens Pupfish populations cumulatively occupied 0.125 ha. Each population derived from two to six sequential translocations tracing back to the single population existing in 1964 (Table 1). The refuges included three modified springs (BLM Spring, Lower Mule Spring Pond, and Warm Springs); a flowing artesian well (Well 368); and two cienega—a marshy area fed by springs in the American Southwest—wetlands (Marvin's Marsh and the Letter Ponds). The Letter Ponds cienega is subdivided into seasonally persistent ponds that we referenced by the letters A through H and that share a dynamic hydrologic environment characterized by intermittent interconnections and sometimes unpredictable desiccation. The population at Lower Mule Spring Pond was established during the study period using equal numbers of male and female Owens Pupfish captured for this study from BLM Spring (2007; $N = 50$), augmented by an entire population salvaged and removed from Warm Springs in 2008 ($N = 18$).

## METHODS

Nonlethal caudal fin clips were collected from four disjunct populations of Owens Pupfish (BLM Spring, Marvin's Marsh, the Letter Ponds [represented by Pond D], and Well 368; Table 1; Figure 1) in 2007. A second set of samples was collected during 2010–2011 from the original four locations plus two additional ones: (1) an additional site in the Letter Ponds with individuals from the interconnected Ponds G and H (hereafter, Pond G–H) and (2) Lower Mule Spring Pond.

Unbaited minnow traps were set overnight in July and August among four populations in 2007 (Marvin's Marsh, BLM Spring,

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TABLE 2. Microsatellite loci used in this study. Abbreviations are as follows: $N_A$ = number of alleles, $H_e$ = expected heterozygosity, and $H_o$ = observed heterozygosity. All loci are from Burg et al. (2002).

| Locus | Dye | $N_A$ | $H_e$ | $H_o$ | $F_{IS}$ | Size range (bp) |
|---|---|---|---|---|---|---|
| *AC9* | PET | 3 | 0.299 | 0.273 | 0.087 | 105–115 |
| *AC17* | 6FAM | 6 | 0.643 | 0.631 | 0.019 | 130–154 |
| *AC25* | 6FAM | 4 | 0.465 | 0.447 | 0.038 | 152–270 |
| *AC29* | 6FAM | 3 | 0.609 | 0.589 | 0.033 | 149–159 |
| *AC35* | NED | 4 | 0.461 | 0.466 | −0.011 | 176–184 |
| *GATA2* | VIC | 10 | 0.839 | 0.781 | 0.069 | 167–203 |
| *GATA26* | 6FAM | 11 | 0.361 | 0.354 | 0.018 | 196–312 |
| *GATA39* | NED | 13 | 0.786 | 0.705 | 0.103 | 283–331 |
| *GATA73* | PET | 12 | 0.767 | 0.749 | 0.024 | 264–320 |

Pond D, and Well 368), five populations in 2010 (Marvin's Marsh, BLM Spring, Well 368, Lower Mule Spring Pond, and Pond G–H), and one population in 2011 (Pond D) (Table 1). Fin tissue was obtained from 28 to 57 individuals per site, depending on capture success, and placed in coin envelopes for dry storage.

*Genetic diversity and divergence*.—Whole genomic DNA was extracted from fin clips using the Qiagen DNeasy tissue kit protocol. Nine microsatellite loci developed for Devils Hole Pupfish *Cyprinodon diabolis* (*GATA2*, *GATA26*, *GATA39*, *GATA73*, *AC9*, *AC17*, *AC25*, *AC29*, *AC35*; Burg et al. 2002; Table 2) were used to genotype individual Owens Pupfish. The PCR conditions were identical to those in Burg et al. (2002), with forward primers labeled with VIC, 6FAM, NED or PET fluorophores (Applied Biosystems, Carlsbad, California). One μL of PCR product (diluted with water at a 1:5 ratio) was added to 0.2 μL LIZ600 size standard and 8.8 μL HiDi formamide (ABI) in individual wells on a 96-well reaction plate. The DNA was denatured at 95°C for 3 min before being run on an AB3130XL Genetic Analyzer. The resulting electropherograms were analyzed using GENEMAPPER 4.0 software (Applied Biosystems), with allele sizes being confirmed by visual inspection of the electropherograms.

MICRO-CHECKER (Van Oosterhout et al. 2004) was used to detect the presence of null alleles or genotyping errors. CONVERT software (Glaubitz 2004) was used to create input files for Genetic Data Analysis software (GDA; Lewis and Zaykin 2001), GENEPOP version 4.0 software (updated from Raymond and Rousset 1995; Rousset 2008), and ARLEQUIN (Excoffier et al. 2005). We used GDA to calculate observed and expected heterozygosity ($H_o$ and $H_e$), $F_{IS}$ values, and private alleles. Deviations from Hardy–Weinberg equilibrium were calculated in GENEPOP using the exact test with a Markov chain–Monte Carlo (MCMC) estimator of the probability that the observed sample was taken from a population in Hardy–Weinberg equilibrium (Guo and Thompson 1992). Significant departures from linkage equilibrium were calculated in GENEPOP, followed by a sequential Bonferroni correction to correct for multiple tests (Rice 1989). The default MCMC parameters were used for both linkage disequilibrium (LD) and Hardy–Weinberg tests (dememorization number = 1,000; number of batches = 100; number of iterations per batch = 1,000).

We calculated allelic richness ($R_s$) in HP-Rare (Kalinowski 2005), which uses rarefaction to correct for the increased likelihood of detecting rare alleles with increased sample size (Kalinowski 2004). Pairwise $F_{ST}$ values were calculated using the method of Reynolds et al. (1983) and Slatkin (1995) in ARLEQUIN using 1,000 permutations.

A neighbor joining (N–J) tree was used to visualize genetic distances. Such trees do not necessarily portray evolutionary relationships, just differences in the frequencies of alleles. To construct the N–J tree, the SEQBOOT application in the software package PHYLIP, version 3.69 (Felsenstein 1995) was used to simulate 1,000 data sets before calculating Cavalli-Sforza and Edwards (1967) chord distances (DCEs) for comparison between all pairs of sites in GENDIST (Felsenstein 1995). The main assumption behind DCEs is that differences in allele frequencies are due to genetic drift only. This approach was chosen because it does not assume that population sizes have remained constant or equal over time (Felsenstein 1995) and because Takezaki and Nei (1996) found that DCEs are more likely to recover true tree topology than other genetic distance estimates. Unrooted N–J trees were constructed with the DCE matrices calculated in GENDIST using the NEIGHBOR application in PHYLIP (Felsenstein 1995). We rooted the N–J tree at the midpoint.

*Population bottlenecks and effective population size*.—Effective population size ($N_e$) is a theoretical property of a population that is a function of the rate of genetic drift. Estimating effective population size is of great interest to endangered species managers since $N_e$, rather than census size, describes how a population responds to evolutionary forces. A smaller $N_e$ will result in faster loss of genetic variation due to drift, possibly reducing the adaptive potential of a population. Values of $N_e$ for each population were calculated using two methods: (1) the linkage disequilibrium method ($N_{e(\mathrm{LD})}$; Waples 2006) and (2) the sibship assignment method ($N_{e(\mathrm{SA})}$; Wang 2009).

The $N_{e(\mathrm{LD})}$ method operates on the theory that genetic drift drives the amount of linkage disequilibrium in isolated, randomly mating populations (Hill 1981). Therefore, linkage disequilibrium can be measured and used to retrospectively approximate the $N_e$ of recent generations. The assumptions of this method include random mating, isolation, selective neutrality of the markers used, no genetic structure within the population, and discrete generations. Waples (2006) notes that when the assumption of discrete generations is violated, the estimate reflects the effective number of breeders ($N_B$) that produced the sampled cohort. The program LDNe was used to estimate $N_{e(\mathrm{LD})}$ (Waples and Do 2008). Alleles with frequencies less than 0.02 were excluded from the analysis (as recommended by Waples and Do 2008), and 95% parametric confidence intervals were calculated.

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The $N_{e(\mathrm{SA})}$ method estimates the frequencies of full and half siblings in a cohort, and in turn uses this analysis to derive contemporary $N_e$. The assumptions of the $N_{e(\mathrm{SA})}$ method include a random sample of individuals in the population from the same cohort (rather than parent–offspring relationships), but not random mating. The $N_{e(\mathrm{SA})}$ method can accept samples in which multiple cohorts are included (such as with Owens Pupfish), but power is reduced (Wang 2009). We used the software program COLONY (Jones and Wang 2010) to estimate $N_{e(\mathrm{SA})}$ using the options of female and male polygamy, nonrandom mating, and the option of full-likelihood, medium-length runs.

Directly measuring a population bottleneck is difficult without knowledge of historical population sizes. However, bottlenecks can be inferred using microsatellite data with assumptions regarding microsatellite mutational models (Cornuet and Luikart 1996; Garza and Williamson 2001). We used two different tests to detect population bottlenecks: (1) the Wilcoxon signed-rank test for excess heterozygosity ($H_k$; Cornuet and Luikart 1996) implemented in the software BOTTLENECK (Piry et al. 1999) and (2) the $M$-ratio test (Garza and Williamson 2001) implemented in the software M_P_Val (http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298). Relative to the $M$-ratio test, the $H_k$ test performed in BOTTLENECK detects bottlenecks that are more recent, of lower severity, or for which the prebottleneck value of θ ($\theta = 4N_e\mu$, where μ is the mutation rate) was small. In contrast, the $M$-ratio test is preferred when detecting bottlenecks that are more severe (lasting several generations), the prebottleneck θ is large, or the population has made a demographic recovery (Williamson-Natesan 2005).

The $H_k$ test operates on the theory that during a bottleneck rare alleles are more likely to be lost while common ones are retained and that the latter have proportionately stronger influence on heterozygosity (Cornuet and Luikart 1996). BOTTLENECK creates a null distribution of alleles under mutation drift equilibrium using a chosen mutation model. A Wilcoxon signed-rank test is used to test for significant heterozygosity excess in comparison with the null distribution. We used a two-phased model (TPM) with the parameters recommended in Piry et al. (1999) (variance = 12, proportion of stepwise mutations = 0.95). In addition, we altered the variance (12, 24, or 36) and the proportion of stepwise mutations (0.80, 0.90, or 0.95) to determine the sensitivity of the results to changes in these parameters. Each run consisted of 5,000 iterations.

The $M$-ratio ($M$) is the ratio of the number of alleles at a locus ($k$) over the observed range of allele fragment sizes at that locus ($r$). The value of $M$ will decline after a bottleneck when alleles are randomly lost, opening “gaps” in the expected series of alleles faster than the size range declines. To reduce the likelihood of type I error (a false detection of a bottleneck), we calculated $M$ using conservative values of the proportion of stepwise mutations ($p_s = 0.90$), the average size of non–one-step mutations ($\mathrm{delta}_g = 3.5$), and θ ($\theta = 10$), as recommended by Garza and Williamson (2001). In addition, we varied the values of θ (1, 5, or 10), $\mathrm{delta}_g$ (2.8 or 3.5), and $p_s$ (0.80 or 0.90) to determine the sensitivity of bottleneck detection to changes in these parameters. We inferred a bottleneck when the $M$-ratio of the population was lower than the $M$-ratio expected at equilibrium in 95% of 10,000 simulations ($P < 0.05$). Two loci that did not conform to the mutation models were dropped to calculate the $M$-ratios: *AC35* (which does not have a dinucleotide- or tetranucleotide-repeat motif) and *AC17* (which had a single large allele [270] in one population [Marvin’s Marsh in 2010]).

## RESULTS

### Genetic Diversity and Divergence

Nine microsatellite loci (Table 2) were used to genotype 454 Owens Pupfish individuals. The loci had from three (*AC9* and *AC29*) to 13 (*GATA39*) alleles per locus. Locus *AC25* had the largest size range (166–270 bp), and *AC35* had the smallest size range (176–184 bp) (Table 3). See Table A.1 in the appendix for the allele frequencies and $H_e$ and $H_o$ values for each locus in each sample group of Owens Pupfish.

No significant departures from Hardy–Weinberg equilibrium were detected. In the linkage disequilibrium tests, 25 out of 360 of these tests were significant prior to Bonferroni correction (nominal $P < 0.05$), but none were significant after correction. None of the 25 tests that were significant before the Bonferroni correction involved consistent locus pairs across individual populations or all populations. Marvin’s Marsh (2007) had the fewest alleles ($N_A = 32$) and Pond D (2011) had the most ($N_A = 50$). Expected heterozygosity values ranged from 0.514 in BLM Spring (2007) to 0.597 in Pond G–H (2011; Table 3).

The neighbor-joining tree (Figure 2) had strong bootstrap support for four general groupings representing locations. Additionally, Lower Mule Spring Pond (2010) grouped with BLM Spring (2007 and 2010), with support for a closer relationship with BLM Spring (2007) than BLM Spring (2010).

TABLE 3. Population-genetic parameters for sample groups of Owens Pupfish at nine microsatellite loci; $N$ = sample size and $R_s$ = mean allelic richness.

| Location | Year | $N$ | $H_e$ | $H_o$ | Mean $N_A$ | Private alleles | $R_s$ |
|---|---|---|---|---|---|---|---|
| MM | 2007 | 41 | 0.556 | 0.594 | 3.56 | 2 | 3.41 |
| MM | 2010 | 28 | 0.539 | 0.532 | 3.78 | | 3.75 |
| PD | 2007 | 35 | 0.568 | 0.591 | 4.56 | 2 | 4.44 |
| PD | 2011 | 47 | 0.595 | 0.596 | 5.56 | | 4.91 |
| BLM | 2007 | 49 | 0.514 | 0.531 | 4.78 | 3 | 4.42 |
| BLM | 2010 | 50 | 0.537 | 0.503 | 5.22 | | 4.71 |
| W368 | 2007 | 57 | 0.561 | 0.513 | 5.11 | 0 | 4.54 |
| W368 | 2010 | 48 | 0.541 | 0.565 | 4.78 | | 4.42 |
| PG–H | 2010 | 49 | 0.597 | 0.621 | 4.44 | 1 | 4.24 |
| LMS | 2010 | 50 | 0.524 | 0.524 | 4.78 | 1 | 4.30 |
| Total $N$ | | 454 | | | | | |
| Mean | | | 0.553 | 0.557 | 4.66 | 1.5 | 4.31 |

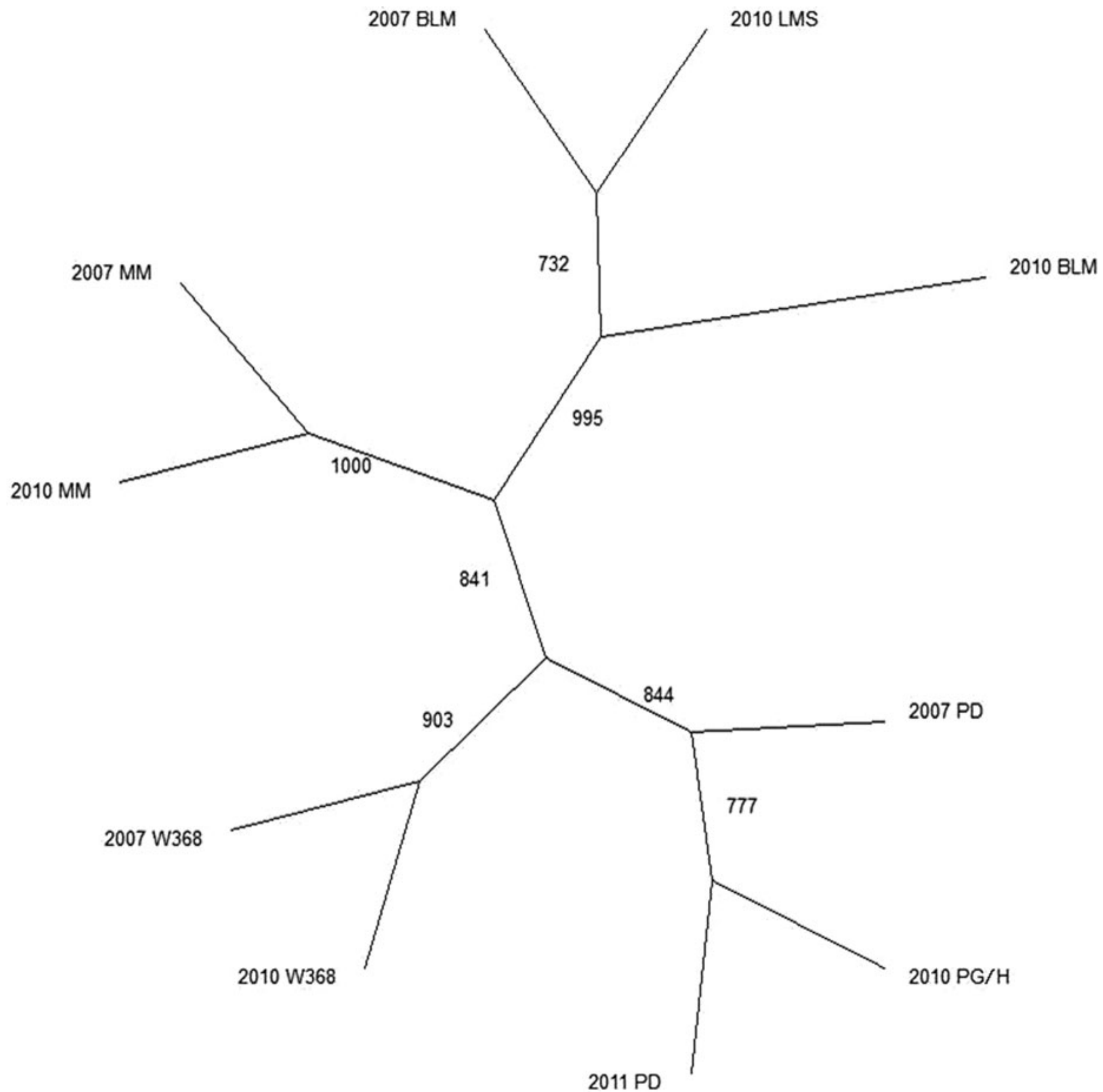


FIGURE 2. Consensus neighbor-joining tree for visualizing the genetic distances between sample years and locations. The tree was created using PHYLIP (Felsenstein 1995); the genetic distances are Cavalli-Sforza and Edwards (1967) chord distances; the tree is unrooted; and boostrap values are based on 1,000 replicates. See Table 1 for the abbreviations of the locations.

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Allelic richness ($R_s$) was calculated with 54 genomic copies, the least number of gene copies found for any locus (both *AC9* and *GATA73*) in any one population (Marvin's Marsh, 2010). The $R_s$ values ranged from 3.41 (Marvin's Marsh, 2007) to 4.91 (Pond D, 2011) (Table 3).

The within-year pairwise $F_{ST}$ values between the locations sampled in 2007 were all significant ($P < 0.05$; Table 4). The within-year pairwise $F_{ST}$ values for 2010–2011 ranged from 0.005 to 0.100, and all but two were significant—that between Pond D (2011) and Pond G–H, and that between BLM Spring (2010) and Lower Mule Spring Pond (2010) (Table 4). The within-year pairwise $F_{ST}$ values between populations increased over time except for that between BLM Spring (2007) and Pond D (2007) ($F_{ST} = 0.095$) and that between BLM Spring (2010) and Pond D (2011) ($F_{ST} = 0.059$).

Except for Pond D ($F_{ST} = 0.034$), no significant pairwise $F_{ST}$ changes were found within populations with repeat sampling (Table 4).

## Population Bottlenecks and Effective Population Size

The $N_{e(\mathrm{LD})}$ estimates ranged from 34.2 (Marvin's Marsh, 2007) to 347.8 (Well 368, 2010), and some of the 95% CIs were

TABLE 4. Pairwise $F_{ST}$ values as calculated in ARLEQUIN (Excoffier et al. 2005). The upper left quadrant shows pairwise $F_{ST}$ values between all locations sampled in 2007. The lower right quadrant shows pairwise $F_{ST}$ values between all locations sampled in 2010–2011. The bottom left quadrant shows pairwise $F_{ST}$ values between locations sampled in 2007 and those locations plus two additional locations sampled in 2010–2011. Values in bold italics are significant ($P < 0.05$) after 1,000 permutations.

| Location and year | MM, 2007 | PD, 2007 | BLM, 2007 | W368, 2007 | MM, 2010 | PD, 2011 | BLM, 2010 | W368, 2010 | PG–H, 2010 | LMS, 2010 |
|---|---|---|---|---|---|---|---|---|---|---|
| MM, 2007 | | | | | | | | | | |
| PD, 2007 | ***0.060*** | | | | | | | | | |
| BLM, 2007 | ***0.059*** | ***0.095*** | | | | | | | | |
| W368, 2007 | ***0.067*** | ***0.051*** | ***0.067*** | | | | | | | |
| MM, 2010 | <0.001 | ***0.065*** | ***0.060*** | ***0.070*** | | | | | | |
| PD, 2011 | **0.043** | ***0.034*** | ***0.074*** | ***0.064*** | ***0.064*** | | | | | |
| BLM, 2010 | ***0.061*** | ***0.080*** | <0.001 | ***0.052*** | ***0.070*** | ***0.059*** | | | | |
| W368, 2010 | ***0.079*** | ***0.067*** | ***0.089*** | 0.001 | ***0.084*** | ***0.077*** | ***0.069*** | | | |
| PG–H, 2010 | ***0.045*** | ***0.031*** | ***0.070*** | ***0.064*** | ***0.061*** | 0.008 | ***0.066*** | ***0.087*** | | |
| LMS, 2010 | ***0.061*** | ***0.094*** | <0.001 | ***0.076*** | ***0.066*** | ***0.067*** | 0.006 | ***0.100*** | ***0.061*** | |

quite wide and included infinity (Table 5). The model returned a negative value for BLM Spring (2010), which we interpreted as infinity (Waples and Do 2008). The $N_{e(SA)}$ estimates ranged from 19 to 29, with 95% CIs much narrower than those for $N_{e(LD)}$ (Table 5).

The TPM model as implemented in the $H_k$ test suggests that only Marvin's Marsh (2007) was declining or had undergone a recent bottleneck (Table 5). This result was not sensitive to changes in variance or the proportion of non-stepwise mutations.

The significance of the comparisons between $M$-values was not sensitive to changes in θ, $p_s$, or delta$_g$. There is generally evidence of a bottleneck if $M < 0.68$ (Garza and Williamson 2001). Marvin's Marsh (2007) was the only population not to show evidence of a bottleneck by this method ($M = 0.688$, $P = 0.076$); the $M$-values in all other populations and years were smaller, showing evidence of bottlenecks.

## DISCUSSION

### Genetic Diversity and Divergence

Each sample of Owens Pupfish is genetically distinct, and we detected private alleles in all populations except Well 368. Owens Pupfish have fewer alleles per locus but higher mean expected heterozygosity (4.66 alleles/locus, mean $H_e = 0.553$; Table 3) than other freshwater fishes that have been studied (7.5 alleles/locus, mean $H_e = 0.46$; DeWoody and Avise 2000). The allelic richness values for Owens Pupfish populations (3.41–4.91) are lower than those for wild populations of Ash Meadows Amargosa Pupfish *C. nevadensis mionectes* (mean = 11.6; Martin and Wilcox 2004) and refuge populations of the Desert Pupfish *C. macularius* (6.8) and Sonoyta Pupfish *C. eremus* (9.1; Koike et al. 2008). Based on these comparisons, Owens Pupfish are less diverse genetically than most other pupfishes.

TABLE 5. Summary of bottleneck and effective population size ($N_e$) tests. For bottlenecks, $M$- and $P$-values are reported for simulations implemented in M_P_Val; significant values are given in bold italics. For the Wilcoxon sign-rank test ($H_k$) of bottlenecks, the table includes $P$-values from BOTTLENECK; the number in bold italics indicates a significant excess of heterozygotes. For effective population size, the $N_{e(LD)}$ values were calculated in LDN$_e$ with 95% parametric confidence intervals and the $N_{e(SA)}$ values were calculated in COLONY with 95% confidence intervals.

| Location and year | $M$ | $P$-value | $H_k$ $P$-value | $N_{e(LD)}$ | 95% Parametric CI | $N_{e(SA)}$ | 95% CI |
|---|---|---|---|---|---|---|---|
| MM, 2007 | 0.688 | 0.076 | ***0.014*** | 34.2 | 16.3–123.0 | 21 | 12–38 |
| MM, 2010 | ***0.572*** | ***0.002*** | 0.213 | 56.1 | 18.0–∞ | 19 | 10–38 |
| PD, 2007 | ***0.571*** | ***0.001*** | 0.248 | 40.1 | 21.2–118.7 | 23 | 13–40 |
| PD, 2011 | ***0.542*** | ***0.002*** | 0.590 | 44.8 | 26.5–95.6 | 28 | 17–48 |
| BLM, 2007 | ***0.509*** | ***<0.001*** | 0.545 | 122.1 | 48.3–∞ | 23 | 14–42 |
| BLM, 2010 | ***0.582*** | ***0.001*** | 0.850 | –221.1 | 315.9–∞ | 29 | 17–48 |
| W368, 2007 | ***0.593*** | ***0.001*** | 0.633 | 60.4 | 34.5–143.9 | 24 | 14–43 |
| W368, 2010 | ***0.567*** | ***0.004*** | 0.590 | 347.8 | 73.1–∞ | 24 | 14–41 |
| PG–H, 2010 | ***0.534*** | ***<0.001*** | 0.180 | 51.8 | 29.2–126.7 | 29 | 18–48 |
| LMS, 2010 | ***0.589*** | ***0.009*** | 0.674 | 42.3 | 25.3–85.9 | 29 | 18–48 |

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Our analyses suggest that Owens Pupfish populations are losing genetic diversity due to population fluctuation, low effective population size, and bottlenecks. However, most populations are currently demographically robust, partly due to intensive habitat management. In 2007, exhaustive trapping found only 38 individuals in Pond D and a bottleneck was detected. However, in 2011 hundreds of fish were unexpectedly captured in Pond D, and the genetic data revealed previously undetected alleles and increases in other measures of genetic diversity. In addition, there was a significant pairwise $F_{ST}$ value between Pond D (2007) and Pond D (2011) and a closer relationship on the N–J tree between Pond G–H (2010) and Pond D (2011) than between Pond D (2007) and Pond D (2011) (Figure 2). We attribute this change to immigration from areas of the Letter Pond cienega during the 4-year interval between samples. Pond D is a small semi-isolated fragment on the fringe of the larger cienega and appears to be dependent on periodic immigration to maintain genetic variation and, perhaps, demographic persistence. Pond G–H apparently hosts more robust pupfish populations. Taken as a whole, the Letter Ponds cienega represents an important genetic and demographic reservoir for Owens Pupfish. The Letter Ponds were established by 65 individuals in 1987 and have received no further population management. Perhaps the pupfish sampled in Pond G–H have undergone fewer bottlenecks and are more representative of past levels of genetic variation in the species. Ironically, BLM Spring (2010) and Pond D (2011) now have the greatest pairwise $F_{ST}$ value despite being the closest geographically.

Further evidence of genetic drift is the observed temporal increase in pairwise $F_{ST}$ value in every case except between BLM Spring and Pond D (which was probably influenced by immigration from Pond G–H). Though only 3–4 years elapsed between repeat sampling and the results could be influenced by the sample effect, our analyses suggest that Owens Pupfish populations are diverging within 3 years (3–9 generations), indicating that genetic drift may have led to rapid divergence of populations within a short period of time.

Though one cannot measure adaptive divergence with these microsatellites, it is possible that selection is contributing to the observed divergence in Owens Pupfish populations, and managers should be aware of the possibility of adaptation to different habitats while managing separate populations. For example, the habitats of the refuge populations are different: Marvin's Marsh is a shallow marshy seep; the Letter Ponds are an interconnected network of ponds and marsh; Well 368 has rapid current, is shallow ($<$0.3 m), and fluctuates seasonally in area; and BLM Spring is a larger, stable, 1-m deep channel. In addition, the refuges variously host nonnative competitors such as the Western mosquitofish *Gambusia affinis* and red swamp crayfish *Procambarus clarki*, which subject some populations to different stressors than others. Though rapid evolution has been shown in some taxa, including Darwin's finches (Grant and Grant 2006), copepods (Hairston and Dillon 1990), and salmonids (Heath et al. 2003), selective forces must be very strong. Collyer et al. (2011) found that the body shape of White Sands Pupfish *C. tularosa* in refuges had diverged from that of the source population over a period of 30 years and that these changes were heritable. However, Martin and Wainwright (2013) show how stabilizing selection for generalist phenotypes could inhibit the evolution of trophic specialization in *Cyprinodon*. Further, pupfish exhibit extreme phenotypic plasticity (Lema 2008), which may itself be under selection. Further research using different markers is needed to determine whether Owens Pupfish are undergoing adaptive divergence due to selection in their respective habitats.

## Population Bottlenecks and Effective Population Size

Population bottlenecks occur when there is a drastic reduction in population size and often result in a loss of genetic variation. Bottlenecks are of conservation concern because they increase genetic drift and the chance of inbreeding, which can reduce diversity, fitness, adaptive potential, population viability, and, by extension, increase the risk of extinction in small populations (e.g., Quattro and Vrijenhoek 1989; Frankham et al. 2002).

Owens Pupfish refuge populations are known to have undergone past demographic bottlenecks (e.g., the observed population low in 1964 of approximately 200 individuals), and genetic effects of these bottlenecks were detected by this study. These results may reflect any number of scenarios, including decline prior to the initial observed bottleneck in 1964, bottlenecks in individual populations subsequent to their founding events, or a combination of the two. Interestingly the $M$-ratio tests detected a historic bottleneck in every population–year combination except for Marvin's Marsh (2007), which was also close to being significant ($P = 0.076$; Table 5). Conversely, the $H_k$ test did not detect recent bottlenecks in any population–year combination except for Marvin's Marsh (2007). Taken together, the $M$-ratio tests provide evidence that most populations underwent severe genetic bottlenecks several generations ago, while the $H_k$ test suggests that these populations are now at mutation-drift equilibrium (or the test may lack power, given our markers).

The linkage disequilibrium estimates have broad confidence intervals and, except for BLM Spring (2010), fall below the thresholds recommended for population maintenance, e.g., 500 (Franklin 1980) to 5,000 (Lande 1995), implying recent and ongoing attrition of genetic variation. The $N_{e(SA)}$ point estimates from the sibship model are consistently smaller than those of $N_{e(LD)}$, falling between 19 and 29 individuals. During the study period, population sizes were estimated for Pond D (2007) by census ($N = 38$), Lower Mule Spring Pond (2010) by mark–recapture (386; 0.95 CI, 297–403), and BLM Spring (2010) by visual estimation ($>$3,000). BLM Spring is also much larger than the other refuge habitats (Table 1), so it is reasonable to expect a larger census size. However, when we collected BLM Spring (2007), we found the population to be critically reduced following intrusion by Largemouth Bass *Micropterus salmoides*. The predatory fish were removed, but we were obliged to wait 4 months for the population to rebound until sufficient numbers of pupfish entered our traps to sample.

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This observed demographic bottleneck may account for the low $N_{e(\mathrm{LD})}$ observed in BLM Spring (2007) compared with BLM Spring (2010). Strangely, the $N_{e(\mathrm{SA})}$ estimates for BLM Spring are similar to those for the other refuges despite large differences in habitat area and census size, and no recent bottleneck was detected with the $H_k$ test despite the observed demographic bottleneck. We suspect that the small number of loci used, low polymorphisms per locus, and both known and unknown violations of the models' assumptions (e.g., discreet generations) reduced the power of the $N_e$ estimation methods.

Given that the methods varied considerably with respect to both the estimates of $N_e$ and the confidence intervals and that some model assumptions may have been violated, we think that managers should use $N_e$ as a guide for Owens Pupfish and similar cases and use caution when interpreting these results. However, by both methods all Owens Pupfish populations have estimated $N_e$ values smaller than those suggested for a healthy population. Maintaining the species as a fragmented set of subpopulations can only continue this phenomenon, despite intensified and improved management of the habitats.

## CONSERVATION IMPLICATIONS

Early actions taken to rescue Owens Pupfish were not guided by genetic considerations, which led to inadvertent losses of genetic variation through founder effects and demographic fluctuation. Genetic considerations are similarly not a major focus of the federal recovery plan (USFWS 1998, 2009), which aims to establish self-sustaining populations that meet certain demographic criteria. When implementing recovery plan objectives, managers should put into practice measures to minimize the loss of genetic diversity specieswide, preserving as much evolutionary potential as possible so that populations can persist and adapt to their environments (Moritz 1999). Two factors complicate this: (1) refuge populations can be unpredictably extirpated or go through major bottlenecks (e.g., BLM Spring [2007]), where no adults were observed at all yet the population recovered in 1 year) and (2) individual refuge populations continue to diverge, most likely through genetic drift, and each (except Well 368) has a portion of the genetic diversity that has been lost by all others (represented by private alleles). Every extant population has been recently and artificially subdivided, and all are subject to potential catastrophic failure, as witnessed at Warm Springs during the period of this investigation. The extensive distribution of private alleles among the existing refuges demonstrates that the system lacks the redundancy necessary to safeguard genetic diversity from predictable but unpreventable stochastic losses. The loss of or a significant bottleneck in any population now would result in the irretrievable loss of a portion of the species' microsatellite variation, taken here as an indicator of potential adaptive variation. The lack of gene flow among populations contributes to low population values for $N_e$ and the accumulation of private alleles. Restoring a level of gene flow among populations would improve the geographic replication of variation (i.e., create "backup" copies of alleles) and reduce its depletion through genetic drift by increasing the global $N_e$ of the species (Rieman and Allendorf 2001), although these would come at the expense of reduced population structure.

To develop effective strategies to preserve genetic diversity in fragmented populations such as the Owens Pupfish, we recommend collaboration between geneticists and managers based on a deep understanding of the natural and population histories of the target species. For Owens Pupfish, for which habitat has been maximized and migration corridors are not viable, we suggest that managers now have three major options: (1) active translocations of individuals, (2) no translocations except in the case of extirpation, and (3) conditional supplementation into declining populations. For all of these options, regular genetic monitoring will ensure that sufficient genetic diversity is maintained or reveal whether continued translocations are necessary. We now outline the pros and cons of each.

### Option 1: Active Maintenance of Gene Flow

Translocation refers to the movement of one or more individuals from one population to another (IUCN 1987). For single-species conservation, the goals for translocation range from maintaining overall population resilience and genetic diversity to genetic rescue (e.g., Hedrick 1995; Westemeier et al. 1998; Tallmon et al. 2004; Weeks et al. 2011). Managers must identify the purpose of translocation, weigh the risks, and determine measures of success (e.g., a target $N_e$ or census size) before acting. Some urge caution before conducting translocations to avoid outbreeding depression (Huff et al. 2011), and in general managers should avoid translocating individuals from dissimilar populations or environments (Edmands 2007). For example, Goldberg et al. (2005) found that outbreeding depression in Largemouth Bass led to increased susceptibility to disease, and Huff et al. (2011) found that outbreeding depression resulted in reduced fitness surrogates, such as the size of young of the year. As discussed, in the case of the Owens Pupfish, population divergence is most likely due to genetic drift. Given that the subpopulations were recently a single global population, outbreeding depression is of less concern and translocations may have the added benefit of increasing the $N_e$ of each population, fostering long-term maintenance of genetic variation. However, large-scale mixing could swamp local adaptation and reduce global $N_e$, and this diversity should be preserved. For Owens Pupfish, we adopt the recommendation of Mills and Allendorf (1996) of 1–10 migrants per generation from each stable refuge population into one or more other refuge population, with the aim of minimizing the loss of polymorphism and heterozygosity while allowing diverged allele frequencies to persist.

### Option 2: No Translocations

The decision not to translocate except in the case of reestablishment has the advantage of preventing potential selective

sweep and/or outbreeding depression caused by mixing populations adapted to different environments. However, small isolated populations may run the risks of inbreeding depression, low $N_e$, reduced genetic variation, and decreased fitness and long-term viability. Evidence for inbreeding depression tends to appear in stressful environments (Armbruster and Reed 2005; but see Marr et al. 2006). Some of the Owens Pupfish refuge populations are in stable springs (e.g., BLM Spring, Lower Mule Spring Pond), so those populations may be less susceptible to inbreeding depression because stable environments are less stressful. In contrast, Well 368, Marvin's Marsh, and the Letter Ponds are spatially and thermally variable (and therefore potentially more stressful), so inbreeding depression may be a greater concern or these populations may be more subject to outbreeding depression if individuals from other populations are added (McClelland and Naish 2007). On the other hand, small Owens Pupfish populations may have gone through purging, a reduction of deleterious alleles resulting in increased fitness and resistance to inbreeding depression. Theory suggests that populations that have gone through multiple generations of inbreeding are more likely to show purging, but experimental evidence provides mixed results (see review by Crnokrak and Barrett 2002). Given the evidence of strong drift in Owens Pupfish populations, their demographic histories, and their reduced genetic diversity, we believe that loss of genetic diversity due to genetic drift and consequent inbreeding depression is a greater risk than outbreeding depression and do not recommend option 2.

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### Option 3: Supplement Declining Populations

If managers deem the risk of outbreeding depression to outweigh the risk of inbreeding depression and choose not to start a full-scale program of regular translocations, we recommend a cautious approach, i.e., if a refuge population is in decline and nongenetic factors such as poor habitat quality have been ruled out, to supplement that population with individuals from other populations. In this case, we again recommend staying within the 1–10 migrants per generation guidelines of Mills and Allendorf (1996); however, sharply declining populations may benefit from more input than stable populations until the recipient population stabilizes.

With all options, catastrophic failure of some Owens Pupfish populations is still likely, if history is any guide. If managers choose to reestablish populations, we recommend using roughly equal proportions of 30–50 individuals from each of the extant populations, limited to no more than 10% of the smallest donor population to minimize genetic or demographic impacts. Thirty or more fish could capture >98% of the global standing genetic variation (Frankel and Soulé 1981) if all founders contribute equally to the next generation (Weeks et al. 2011). After the population is established, continued supplementation of 1–10 individuals per generation from each available source should ensue, along with monitoring.

## CONCLUSION

This study showed that the Owens Pupfish that survived the 1964 bottleneck was fragmented into isolated refuge populations that have lost variation and are diverging through genetic drift. If this trend continues, Owens Pupfish may manifest reductions in individual fitness and evolutionary potential, jeopardizing their continued existence, as has been seen in closely related species. For example, the Devils Hole pupfish is an emblematic western pupfish recognized as being on the brink of extinction, with persuasive evidence that genetic load is playing a decisive role in its fate (Martin et al. 2012). Continentally, seven species and one subspecies of *Cyprinodon* became extinct as early as 1930 and as recently as 1994 (Burkhead 2012). We believe it is important to arrest the erosion of genetic diversity in Owens Pupfish to help prevent this species from joining the list of extinctions. Given the uncertainties and technical difficulties of monitoring fitness and the complicated interactions between selection, drift, inbreeding, and bottlenecks (Bouzat 2010), managers must make decisions to translocate individuals based on logistical constraints, risk assessment, and explicit goals for genetic management. Active management of habitats and gene flow and the establishment of additional refuge populations will be crucial to the long-term maintenance of the species' residual diversity. Genetic data will inform the selection of donor populations and the numbers of individuals for future translocations and provide baseline data for monitoring their effects.

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## Appendix: Detailed Allelic Results

TABLE A1. Allele frequencies and expected heterozygosity ($H_e$) and observed heterozygosity ($H_o$) values for nine microsatellite loci in each sample group of Owens Pupfish. Allele sizes are given in number of base pairs.

| Locus | Allele size | MM, 2007 | MM, 2010 | BLM, 2007 | BLM, 2010 | LMS, 2010 | W368, 2007 | W368, 2010 | PD, 2007 | PD, 2011 | PG–H, 2010 | Overall |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *AC9* | 105 | 0.793 | 0.796 | 0.898 | 0.890 | 0.890 | 0.728 | 0.750 | 0.829 | 0.830 | 0.847 | 0.825 |
| | 109 | | | | 0.010 | | 0.044 | 0.033 | 0.086 | 0.117 | 0.102 | 0.039 |
| | 115 | 0.207 | 0.204 | 0.102 | 0.100 | 0.110 | 0.228 | 0.217 | 0.086 | 0.053 | 0.051 | 0.136 |
| $H_e$ | | 0.333 | 0.331 | 0.185 | 0.200 | 0.200 | 0.420 | 0.393 | 0.303 | 0.298 | 0.272 | 0.293 |
| $H_o$ | | 0.317 | 0.333 | 0.204 | 0.100 | 0.140 | 0.333 | 0.413 | 0.343 | 0.300 | 0.306 | 0.279 |
| *AC17* | 130 | 0.329 | 0.357 | 0.214 | 0.210 | 0.240 | 0.316 | 0.406 | 0.243 | 0.120 | 0.224 | 0.266 |
| | 144 | | | | | | 0.079 | | 0.457 | 0.250 | 0.357 | 0.114 |
| | 146 | | | | 0.020 | 0.020 | | | | | | 0.004 |
| | 148 | 0.415 | 0.429 | 0.673 | 0.660 | 0.690 | 0.500 | 0.500 | 0.257 | 0.543 | 0.347 | 0.501 |
| | 150 | | | | 0.010 | | | | | | | 0.001 |
| | 154 | 0.256 | 0.214 | 0.112 | 0.100 | 0.050 | 0.105 | 0.094 | 0.043 | 0.087 | 0.071 | 0.113 |
| $H_e$ | | 0.662 | 0.655 | 0.493 | 0.515 | 0.468 | 0.639 | 0.582 | 0.674 | 0.627 | 0.704 | 0.602 |
| $H_o$ | | 0.780 | 0.571 | 0.592 | 0.500 | 0.560 | 0.596 | 0.625 | 0.771 | 0.652 | 0.714 | 0.636 |
| *AC25* | 152 | 0.598 | 0.607 | 0.663 | 0.730 | 0.590 | 0.675 | 0.802 | 0.671 | 0.553 | 0.459 | 0.635 |
| | 160 | 0.402 | 0.357 | 0.337 | 0.270 | 0.410 | 0.325 | 0.198 | 0.329 | 0.447 | 0.541 | 0.361 |
| | 166 | | 0.018 | | | | | | | | | 0.002 |
| | 270 | | 0.018 | | | | | | | | | 0.002 |
| $H_e$ | | 0.487 | 0.512 | 0.451 | 0.398 | 0.489 | 0.442 | 0.321 | 0.448 | 0.500 | 0.502 | 0.455 |
| $H_o$ | | 0.415 | 0.536 | 0.510 | 0.380 | 0.460 | 0.404 | 0.354 | 0.486 | 0.511 | 0.469 | 0.452 |
| *AC29* | 149 | 0.329 | 0.482 | 0.429 | 0.290 | 0.440 | 0.188 | 0.167 | 0.271 | 0.128 | 0.235 | 0.296 |
| | 155 | 0.305 | 0.161 | 0.061 | 0.100 | 0.070 | 0.161 | 0.198 | 0.100 | 0.372 | 0.378 | 0.191 |
| | 159 | 0.366 | 0.357 | 0.510 | 0.610 | 0.490 | 0.652 | 0.635 | 0.629 | 0.500 | 0.388 | 0.514 |
| $H_e$ | | 0.673 | 0.625 | 0.558 | 0.539 | 0.567 | 0.519 | 0.535 | 0.529 | 0.601 | 0.659 | 0.581 |
| $H_o$ | | 0.732 | 0.679 | 0.633 | 0.420 | 0.640 | 0.482 | 0.583 | 0.514 | 0.617 | 0.653 | 0.595 |
| *AC35* | 176 | | | | 0.010 | | | | | | | 0.001 |
| | 180 | 0.793 | 0.839 | 0.520 | 0.469 | 0.490 | 0.728 | 0.740 | 0.800 | 0.649 | 0.612 | 0.664 |
| | 182 | | | | | | 0.018 | 0.021 | | 0.021 | | 0.006 |
| | 184 | 0.207 | 0.161 | 0.480 | 0.520 | 0.510 | 0.254 | 0.240 | 0.200 | 0.330 | 0.388 | 0.329 |
| $H_e$ | | 0.333 | 0.275 | 0.504 | 0.514 | 0.505 | 0.408 | 0.399 | 0.325 | 0.475 | 0.480 | 0.422 |
| $H_o$ | | 0.415 | 0.250 | 0.551 | 0.469 | 0.580 | 0.368 | 0.458 | 0.286 | 0.532 | 0.612 | 0.452 |
| *GATA2* | 167 | | | 0.032 | 0.020 | 0.030 | | | 0.029 | 0.106 | 0.041 | 0.026 |
| | 171 | | | 0.053 | 0.040 | 0.040 | 0.009 | | | | | 0.014 |
| | 175 | 0.027 | 0.018 | 0.085 | 0.080 | 0.080 | 0.421 | 0.542 | 0.186 | 0.181 | 0.184 | 0.180 |
| | 179 | 0.054 | | 0.149 | 0.180 | 0.200 | 0.035 | 0.010 | 0.171 | 0.202 | 0.163 | 0.117 |
| | 183 | 0.311 | 0.250 | 0.213 | 0.180 | 0.320 | 0.018 | 0.021 | 0.243 | 0.298 | 0.296 | 0.215 |
| | 187 | | 0.018 | 0.021 | 0.030 | 0.040 | 0.009 | 0.010 | 0.014 | | | 0.014 |
| | 191 | 0.189 | 0.268 | 0.181 | 0.150 | 0.130 | 0.307 | 0.281 | 0.257 | 0.096 | 0.184 | 0.204 |
| | 195 | | | 0.064 | 0.090 | 0.010 | 0.044 | 0.021 | 0.014 | | | 0.024 |
| | 199 | 0.081 | 0.107 | 0.032 | 0.040 | 0.010 | 0.009 | 0.031 | | 0.074 | 0.092 | 0.048 |
| | 203 | 0.338 | 0.339 | 0.170 | 0.190 | 0.140 | 0.149 | 0.083 | 0.086 | 0.043 | 0.041 | 0.158 |
| $H_e$ | | 0.753 | 0.752 | 0.863 | 0.866 | 0.819 | 0.709 | 0.625 | 0.814 | 0.819 | 0.815 | 0.783 |
| $H_o$ | | 0.730 | 0.714 | 0.915 | 0.860 | 0.760 | 0.684 | 0.542 | 0.857 | 0.872 | 0.878 | 0.781 |
| *GATA26* | 196 | | | | | | | | | | 0.010 | 0.001 |
| | 200 | | | 0.031 | 0.040 | 0.050 | 0.026 | 0.031 | | 0.021 | | 0.020 |
| | 204 | 0.792 | 0.839 | 0.898 | 0.820 | 0.890 | 0.789 | 0.729 | 0.686 | 0.670 | 0.765 | 0.788 |
| | 208 | 0.194 | 0.089 | 0.010 | 0.080 | 0.040 | 0.132 | 0.167 | 0.257 | 0.160 | 0.102 | 0.123 |

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TABLE A1. Continued.

| Locus | Allele size | MM, 2007 | MM, 2010 | BLM, 2007 | BLM, 2010 | LMS, 2010 | W368, 2007 | W368, 2010 | PD, 2007 | PD, 2011 | PG–H, 2010 | Overall |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 212 | 0.014 | 0.018 | | | | | | | 0.011 | 0.010 | 0.005 |
| | 224 | | | | | | | | | 0.011 | | 0.001 |
| | 281 | | | | | | 0.009 | 0.031 | | 0.011 | | 0.005 |
| | 285 | | 0.054 | 0.051 | 0.050 | 0.010 | 0.026 | 0.031 | 0.014 | 0.096 | 0.082 | 0.041 |
| | 289 | | | 0.010 | 0.010 | 0.010 | | | | 0.011 | | 0.004 |
| | 297 | | | | | | 0.018 | 0.010 | | | | 0.003 |
| | 312 | | | | | | | | 0.043 | 0.011 | 0.031 | 0.008 |
| $H_e$ | | 0.340 | 0.290 | 0.192 | 0.320 | 0.206 | 0.361 | 0.442 | 0.468 | 0.521 | 0.400 | 0.354 |
| $H_o$ | | 0.417 | 0.321 | 0.204 | 0.360 | 0.220 | 0.316 | 0.458 | 0.457 | 0.404 | 0.429 | 0.359 |
| *GATA39* | 283 | | | | | | | | 0.088 | 0.011 | | 0.010 |
| | 287 | | | | | | | | 0.015 | | | 0.001 |
| | 291 | 0.012 | | 0.0410 | 0.060 | 0.060 | 0.214 | 0.152 | 0.088 | 0.033 | 0.0830 | 0.074 |
| | 295 | 0.317 | 0.375 | 0.020 | 0.050 | 0.040 | 0.018 | 0.098 | 0.265 | 0.402 | 0.250 | 0.184 |
| | 299 | 0.024 | 0.018 | 0.133 | 0.150 | 0.130 | 0.125 | 0.076 | 0.147 | 0.065 | 0.135 | 0.100 |
| | 303 | 0.427 | 0.357 | 0.510 | 0.390 | 0.450 | 0.179 | 0.207 | 0.132 | 0.174 | 0.219 | 0.304 |
| | 307 | 0.207 | 0.250 | 0.214 | 0.270 | 0.220 | 0.402 | 0.391 | 0.191 | 0.261 | 0.281 | 0.269 |
| | 311 | 0.012 | | 0.010 | 0.070 | 0.080 | 0.018 | 0.011 | 0.044 | | | 0.025 |
| | 315 | | | 0.031 | | 0.010 | | | 0.029 | 0.022 | | 0.009 |
| | 319 | | | | 0.010 | | | | | 0.011 | 0.021 | 0.004 |
| | 323 | | | 0.031 | | 0.010 | 0.009 | | | 0.011 | 0.010 | 0.007 |
| | 327 | | | | | | 0.036 | 0.065 | | 0.011 | | 0.011 |
| | 331 | | | 0.010 | | | | | | | | 0.001 |
| $H_e$ | | 0.682 | 0.681 | 0.679 | 0.749 | 0.728 | 0.750 | 0.770 | 0.848 | 0.742 | 0.793 | 0.742 |
| $H_o$ | | 0.683 | 0.536 | 0.612 | 0.760 | 0.620 | 0.732 | 0.783 | 0.824 | 0.696 | 0.771 | 0.702 |
| *GATA73* | 264 | | | 0.023 | 0.010 | 0.010 | 0.028 | 0.044 | 0.063 | 0.067 | 0.031 | 0.028 |
| | 272 | | | | | 0.010 | | | | | | 0.001 |
| | 276 | 0.157 | 0.111 | 0.128 | 0.120 | 0.140 | 0.046 | 0.033 | 0.031 | 0.011 | 0.082 | 0.086 |
| | 280 | 0.300 | 0.389 | 0.465 | 0.410 | 0.360 | 0.157 | 0.244 | 0.359 | 0.211 | 0.204 | 0.310 |
| | 284 | 0.357 | 0.296 | 0.267 | 0.290 | 0.310 | 0.278 | 0.278 | 0.406 | 0.378 | 0.388 | 0.325 |
| | 288 | 0.014 | 0.019 | 0.047 | 0.070 | 0.030 | 0.185 | 0.167 | 0.063 | 0.156 | 0.071 | 0.082 |
| | 292 | 0.171 | 0.167 | 0.058 | 0.060 | 0.140 | 0.250 | 0.189 | 0.078 | 0.133 | 0.224 | 0.147 |
| | 296 | | 0.019 | | | | 0.046 | 0.033 | | 0.011 | | 0.011 |
| | 300 | | | | | | 0.009 | 0.011 | | | | 0.002 |
| | 312 | | | | 0.010 | | | | | 0.022 | | 0.003 |
| | 316 | | | | 0.010 | | | | | 0.011 | | 0.002 |
| | 320 | | | 0.012 | 0.020 | | | | | | | 0.003 |
| $H_e$ | | 0.739 | 0.734 | 0.698 | 0.732 | 0.741 | 0.804 | 0.804 | 0.702 | 0.774 | 0.753 | 0.748 |
| $H_o$ | | 0.857 | 0.852 | 0.558 | 0.680 | 0.740 | 0.704 | 0.867 | 0.781 | 0.778 | 0.755 | 0.757 |

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