# Severe inbreeding, increased mutation load, and gene loss-of-function in the critically endangered Devil's Hole pupfish

David Tian[1], Austin H. Patton[1], Bruce J. Turner[2], and Christopher H. Martin[1]

DT ORCID: 0000-0001-5285-6810
AHP ORCID: 0000-0003-1286-9005
CHM ORCID: 0000-0001-7989-9124

[1]Department of Integrative Biology and Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, CA 94720 USA
[2]Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061 USA

Corresponding Author:
chmartin@berkeley.edu, University of California, Berkeley, Department of Integrative Biology, 3040 Valley Life Sciences Building # 3140, Berkeley, CA 94720-3140

keywords: inbreeding, runs of homozygosity, mutation load, Devils Hole pupfish

**Abstract**

Small populations with limited range are often threatened by inbreeding and reduced genetic diversity, which can reduce fitness and exacerbate population decline. One of the most extreme natural examples is the Devil's Hole pupfish (*Cyprinodon diabolis*), an iconic and critically endangered species with the smallest known range of any vertebrate. This species has experienced severe declines in population size over the last thirty years and suffered major bottlenecks in 2007 and 2013, when the population shrunk to 38 and 35 individuals, respectively. Here we analyzed 30 resequenced genomes of desert pupfishes from Death Valley, Ash Meadows, and surrounding areas to examine the genomic consequences of small population size. We found extremely high levels of inbreeding ($F_{ROH}$=0.34-0.81) and an increased amount of potentially deleterious genetic variation in the Devil's Hole pupfish as compared to other species, including unique, fixed loss-of-function alleles and deletions in genes associated with sperm motility and hypoxia. Additionally, we successfully resequenced a formalin-fixed museum specimen from 1980 and found that the population was already highly inbred prior to recent known bottlenecks. We thus document severe inbreeding and increased mutation load in the Devil's Hole pupfish and identify candidate deleterious variants to inform management of this conservation icon.

## Introduction

Due to declining population sizes and increasing isolation of many species from anthropogenic habitat fragmentation and climate change, understanding the extent and nature of genetic threats in small populations is essential for predicting and increasing population persistence and resiliency [1]. Small and isolated populations often suffer from inbreeding depression, the reduction in fitness caused by increased homozygosity of deleterious recessive alleles or overdominant loci that occurs when closely related individuals breed together [2,3]. Prolonged population decline can result in increased long-term extinction risk due to stochastic demographic events [4], reduced genetic variation for adaptation [5], and decreased efficacy of purifying selection to overpower drift and purge deleterious variants [6]. This reduced efficacy of purifying selection leads deleterious mutations to accumulate more readily in small populations. The burden of accumulated deleterious mutations is known as the mutation load, and can reduce individual fitness [7,8].

Many examples of reduced genetic diversity and inbreeding depression have been documented in the wild [9,10], including Florida panthers (*Puma concolor*) [11], Isle Royale wolves (*Canis lupus*) [12], and mountain gorillas (*Gorilla beringei*) [13]. Severe inbreeding in natural populations is increasingly being documented across a wide range of temporal scales, from ancient bottlenecks [14,15] to recent timescales [12]. Recently, historical museum specimens have been successfully leveraged to investigate temporal changes in inbreeding and genetic diversity, highlighting their utility for investigating the historical dynamics of inbreeding and mutation load in imperiled populations [16,17].

Traditionally, population health and extinction risk was assessed using putatively neutral molecular markers and pedigrees owing to positive correlations between genome-wide

heterozygosity and fitness [18]. Maintaining genome-wide genetic variation is crucial to preserving adaptive potential and preventing inbreeding depression in populations of conservation interest [19] because populations with higher levels of genetic diversity tend to have higher mean fitness and reduced extinction risk [20]. However, several recent studies have suggested that summary statistics of genetic variation do not necessarily accurately reflect population size or extinction risk and that we should also use whole-genome sampling to assess functional genetic diversity and genetic load [17,21]. Genomes enable more detailed measurements of individual inbreeding depression and its genetic basis relative to pedigree-based approaches [22]. Thus, a comprehensive understanding of the evolutionary dynamics of small populations in the wild from a genomic perspective is key to understanding the fate of endangered populations and to inform conservation management.

Here we leverage the unique evolutionary and demographic history of the iconic Devil's Hole pupfish (*C. diabolis*: Fig. 1A) to investigate how isolation and recent population decline have shaped inbreeding and mutation load. Death Valley pupfishes evolved from a common ancestor thousands of years ago when the climate was milder and the region was connected by large inland seas. Populations are now relatively isolated in small desert spring systems and several species are now considered critically endangered [23]. Devils Hole contains the most extreme conditions, including a nearly constant temperature of 34°C [24], absence of direct sunlight during the winter [25] which sharply limits primary production and nutrient availability [26], and dissolved oxygen levels near lethal limits for most fishes (2 – 3 ppm) [25,27]. Surrounding pupfishes in neighboring springs occupy less hypoxic environments, such as *Cyprinodon nevadensis mionectes* in Jackrabbit Spring, due to greater water movement [28].

*C. diabolis* exists at one of the lowest long-term population sizes of any desert pupfish. The population has steadily declined since the late 1990s before reaching lows of 38 and 35 individuals in the spring of 2007 and 2013, respectively (Fig. 1B). *C. diabolis* is restricted to the upper 30 m of Devils Hole, a 3.5 x 22 m water-filled cavern widely believed to be the smallest range of any vertebrate (3.5m x 22m) [29] (Fig. 1C). Population viability analyses in 2014 suggested that the median time to extinction was 26 years [30]. Although this species was previously believed to be isolated in Devils Hole for 10-20 kya (Miller 1981), more recent genome-wide estimates indicate that Devils Hole may have most recently experienced substantial admixture approximately 1-2 kya and that gene flow among these desert oases is surprisingly common [23,32,33].

The continued persistence of *C. diabolis* in the hottest desert on earth in one of the most inhospitable habitats for fishes is extraordinary. Despite its status as one of the world's most endangered species, genetic analyses have so far been limited to delineating phylogenetic relationships, assessing population structure, and measuring genetic diversity with reduced-representation genetic markers [23]. Here we resequenced whole-genomes of *C. diabolis* and several closely related *Cyprinodon* desert pupfishes to investigate how isolation and small population size influence inbreeding and mutation load on a genome-wide scale in this conservation icon.

## Results

### Geography and population structure

We sequenced 30 individuals (8 *C. diabolis*, 13 *C. nevadensis*, 4 *C. salinus*, and one individual each of *C. albivelis, C. eremus, C. fontinalis, C. macularius,* and *C. radiosus*) for our analyses

(Fig. 1D, Table S1, Table S2). After filtering for quality genotypes and exclusion of problematic samples, we retained a total of 6,295,414 SNPs with a mean coverage of 12x. We investigated genome-wide population genetic differentiation among Death Valley desert pupfishes using principal components analyses, corroborating previous results [23] (Fig. 2A). Devils Hole pupfish were substantially divergent from the most closely related neighboring desert pupfish species for genome-wide mean $F_{st}$ estimates (*C. diabolis* vs. *C. nevadensis* = 0.34). Inference of evolutionary relationships among these populations using genome-wide SNP data under the multi-species coalescent using SVDquartets [34,35] and concatenated SNP data across all individuals using IQ-TREE v 1.6.12 [36] confirmed previous findings; *C. diabolis* is sister to *C. nevadensis* and *C. salinus* is sister to both [23]. Our expanded sampling of outgroups also confirmed that the Death Valley clade is most closely related to the geographically proximate Owens pupfish (*C. radiosus*) [37]. ADMIXTURE analyses support *C. diabolis*, *C. nevadensis*, and *C. salinus* as distinct populations. Interestingly, this analysis infers apparent admixture within *C. nevadensis* from neighboring populations as more distantly related desert pupfishes (Fig. 2D); this complex history of admixture may help to explain the lack of phylogenetic resolution within this group.

**Severe inbreeding in Devil's Hole pupfish**

Inbreeding can be identified and quantified through runs-of-homozygosity (ROHs), which are long contiguous tracts of identical haplotypes inherited from a common ancestor [22]. We calculated $F_{ROH}$, an accurate measure of inbreeding, as the summed lengths of ROHs greater than 100 kb divided by the total genome size. We found that *C. diabolis* was highly inbred (mean $F_{ROH}$ = 0.58), significantly exceeding the degree of inbreeding observed in *C. nevadensis* (mean

$F_{ROH}$ = 0.14: Tukey's HSD $P = 8.38 \times 10^{-5}$: Fig. 3). In contrast, ROHs made up less than 10% of the genome in the relatively undisturbed natural spring populations of *C. nevadensis amargosae* and *C. nevadensis nevadensis*, whereas *C. nevadensis shoshone* and *C. nevadensis pectoralis* tended to have a higher $F_{ROH}$ than other *C. nevadensis* species (Fig. 3). These findings are consistent with small census population sizes and intensive management histories as *C. nevadensis shoshone* has experienced extirpation and captive breeding prior to reintroduction in the 1990s and the habitat of *C. nevadensis pectoralis* has undergone extensive habitat modification. [38–40].

We found that the degree of inbreeding in Devils Hole has remained high, from 1980 ($F_{ROH}$ = 0.55; $n$ = 1) to near-present day (2008 – 2012: mean $F_{ROH}$ = 0.58). This suggests that *C. diabolis* was already highly inbred prior to the population decline in the mid-1990s and severe bottlenecks in 2007 and 2013, during which the population size plummeted to 38 and 35 fish, respectively [30] (Fig. 1B).

Shorter ROHs indicate mating between distant relatives or longer-scale historical processes, whereas longer ROHs are indicative of recent inbreeding due to reduced opportunity for recombination [41]. We exploited this signature to estimate the mean number of generations back to the common ancestor of these homologous sequences using the length of ROHs and an assumed recombination rate. Populations with similar high levels of inbreeding can have ROHs that nearly span entire chromosomes [42]. Surprisingly, given the recent severe bottlenecks, we did not find many long ROHs (> 10 Mb) in our *C. diabolis* samples, barring a single exception (Fig. 3D). Instead, much of the cumulative inbreeding is made up of ROHs that are 0.1 – 1 Mb long (Fig. 3B), which corresponds to shared parental ancestry from 11 - 109 generations previous. Our results illustrate that extreme isolation and prolonged small population size have

185 driven *C. diabolis* to become highly inbred and that much of the inbreeding occurred prior to
186 recent bottlenecks over the course of the 20$^{th}$ century.
187
188 **Higher mutation load in Devils Hole pupfish**
189 Small, inbred populations are expected to have higher frequencies and homozygosity of
190 deleterious alleles. However, once deleterious recessive alleles are unmasked, purifying selection
191 can also purge portions of the mutation load [43]. To assess how severe inbreeding in Devil's
192 Hole pupfish has affected mutation load, we calculated the relative proportions of homozygous
193 ancestral, heterozygous, and homozygous derived genotypes across synonymous (SYN), non-
194 synonymous (NSYN), and loss-of-function (LOF) mutations (Fig. 4, Fig. S1). The number of
195 homozygous derived genotypes for LOF variants quantifies load under a recessive model [8,44].
196 Because deleterious alleles tend to be recessive [45,46], LOF homozygous derived alleles are
197 more likely to have a phenotypic effect that leads to a reduction in fitness.
198 *C. diabolis* had significantly lower proportions of heterozygous genotypes than *C.*
199 *nevadensis* across all variant types (SYN: $P = 1.42 \times 10^{-5}$, NSYN: $P = 1 \times 10^{-5}$, LOF: $P = 1.87 \times$
200 $10^{-5}$, Tukey's HSD tests), consistent with our findings of higher inbreeding in this species. *C.*
201 *diabolis* also had significantly higher proportions of homozygous derived LOF genotypes
202 compared to both *C. nevadensis* ($P = 6.61 \times 10^{-4}$) and *C. salinus* ($P = 0.044$). Similarly, *C.*
203 *salinus* also had significantly lower proportions of heterozygous genotypes than pooled *C.*
204 *nevadensis* samples across all variant types (SYN: $P = 2.74 \times 10^{-3}$, NSYN: $P = 3.71 \times 10^{-3}$, LOF:
205 $P = 5.36 \times 10^{-3}$, Tukey's HSD tests), likely reflecting the larger population sizes of *C. nevadensis*
206 populations. There were no significant differences in the proportion of homozygous derived
207 genotypes for SYN and NSYN mutations among the three species.

We also assessed whether there were allele frequency differences among the three species across a comparable set of LOF alleles and found that there were no significant differences among the three species (ANOVA $P = 0.134$), although *C. diabolis* tended to have a higher mean frequency of LOF alleles at 0.63, compared to *C. nevadensis* = 0.54 and *C. salinus* = 0.49. Finally, there was no enrichment of LOF variants in ROHs for most individuals except for three *C. diabolis* individuals that had significantly greater proportions of LOF variants in ROHs than their respective $F_{ROH}$ values (DHP54903: $P = 1.92 \times 10^{-3}$, DHP54913: $P = 4.29 \times 10^{-3}$, DHP54918: $P = 4.71 \times 10^{-2}$). One *C. nevadensis amargosae* individual had a significantly lower proportion of LOF variants in ROHs (CNevAma: $P = 2.01 \times 10^{-2}$).

**Fixed variants unique to Devils Hole**

Devils Hole pupfish clearly harbor a homozygous LOF mutation load greater than neighboring desert pupfish. Thus, we focused on genetic variants most likely to be deleterious to help inform future management of this species; specifically, homozygous derived LOF variants and deletions unique to *C. diabolis*, which are expected to reduce fitness by disrupting gene function.

We identified 11 predicted LOF SNPs in the form of premature stop codons, including several within genes that may affect fecundity or resistance to disease and stress (Table S3). These include *cfap43*, a protein involved in the structure and function of the sperm flagellum axoneme that has been implicated in male infertility [47]. Similarly, reduced sperm motility and abnormal sperm morphology was observed in Florida panthers [11] and lions [48] due to inbreeding.

We also identified 94 deletions unique to *C. diabolis* and focused on the 15 deletions within 2 kb of any annotated genes. Surprisingly, five of the fifteen deletions were involved in

cellular responses to hypoxia (Table S4), including shifts to anaerobic metabolism, erythropoiesis, degradation of misfolded proteins, and regulation of nutrient use and cell fate [49]. These hypoxia-related deletions included an 81 bp deletion in the promoter of *redd1*. During hypoxia, *redd1* inhibits mTORC1 [50,51] through the TSC1/TSC2 tumor suppressor complex to conserve energy and prevent the accumulation of misfolded proteins. Upregulation of a homolog of *redd1* (*ddit4L*/*redd2*) has been implicated in adaptation to hypoxia in shortfin mollies (*Poecilia mexicana*) (Barts 2020) and high-altitude deer mice (*Peromyscus maniculatus*) (Rochette 2021 *pers. comm.*). We also found deletions in *apeh*, an enzyme that destroys oxidatively damaged proteins [53]; t*rim39*, a tripartite motif involved in erythropoiesis [54]; and *slc25a42*, a mitochondrial transporter of coenzyme A [55] associated with mitochondrial myopathy and lactic acidosis in humans [56].

## Discussion

We documented extensive inbreeding, gene loss, and mutation load in the critically endangered Devil's Hole pupfish and identified a set of candidate deleterious genetic variants that can potentially inform future conservation management. We show that *C. diabolis* is significantly more inbred than most neighboring desert pupfish populations. *C. diabolis* was not significantly more inbred than *C. salinus* although our small sample size and high variability make it difficult to infer accurate levels of inbreeding in *C. salinus*. High levels of inbreeding are associated with elevated extinction risk [57,58] and the inbreeding in *C. diabolis* is equal to or more severe than levels reported so far in other isolated natural populations such as Isle Royale wolves [12], mountain gorillas [59], and Indian tigers [60]. Although we were unable to directly measure fitness, the increased inbreeding in *C. diabolis* likely results in a substantial reduction in fitness.

Previous studies have suggested that increases in $F_{ROH}$ have strong negative effects on fitness. For instance, in Soay sheep an increase in $F_{ROH}$ by 10% was correlated with a 60% decline in fitness [61], whereas in helmeted honeyeaters a 9% increase in homozygosity was associated with a reduced lifetime reproductive success of 87-90% [62] . Surprisingly, *C. salinus* also displayed high levels of inbreeding although increased sampling is necessary to obtain reliable estimates of inbreeding.

By successfully sequencing a formalin-fixed historical museum specimen to 10x coverage (out of 8 total attempts) we discovered that inbreeding was already extensive by 1980, suggesting that *C. diabolis* may have an extended history of repeated population bottlenecks. Indeed, the distribution of ROH sizes suggests that a large proportion of homozygous tracts were due to inbreeding that occurred many generations prior to the recent population decline. Furthermore, *C. diabolis* harbors a significantly greater mutation load than either *C. nevadensis* or *C. salinus* (Fig. 4).

Our mutation load results support previous hypotheses that *C. diabolis* harbors a high mutation load due to its relative isolation and small population size [29,63]. Additionally, they are consistent with the observation of rapid allele frequency increases of *C. nevadensis* alleles in a *C. diabolis* refuge population after the accidental introduction of a few individuals [64]. Although recent studies have suggested that small, bottlenecked populations may harbor a lower mutation load due to purging [13,65,66], populations that have experienced recent severe population bottlenecks are likely to maintain a high load, because deleterious variants may reach fixation before purifying selection removes them [67]. Although our measure of mutation load does not directly measure fitness, there is empirical support that LOF mutations are on average deleterious [68].

***Degradation of hypoxia and reproductive pathways in C. diabolis***

We found deleterious variants associated with reproduction and hypoxia genes. For example, we found a fixed LOF variant unique to *C. diabolis* in *cfap43*, a gene associated with sperm morphology and function. Deletions have thus far been rarely studied or quantified in conservation genetics. However, analysis of woolly mammoth genomes from different time points found that a sample dated closer to the time of extinction had accumulated more homozygous deletions than earlier ones [15], suggesting that deletions may be an understudied genetic threat to endangered populations [17].

We found numerous deletions that were unique to the Devil's Hole pupfish. Of these, five were associated with hypoxia, a known environmental stressor in the system, suggesting that *C. diabolis* could be poorly equipped to physiologically deal with the stressful hypoxic environment in Devils Hole. Indeed, previous studies have noted that *C. diabolis* has low fecundity [24,69], low egg viability and juvenile survivorship [29], and lays more eggs at lower temperatures (28 °C) compared to the higher constant temperature of 33 °C in Devils Hole [70]. At present, we cannot rule out the possibility that some of these fixed variants are potentially the result of local adaptation; distinguishing between selection and genetic drift in small populations is extremely difficult because both processes leave similar signatures in allele frequencies [71]. One possibility is that these variants are adaptive in the unique selective environment of Devils Hole and were swept to fixation during initial colonization. Moving forward, functional genetic and eco-physiological tests will be key to understanding the full impacts of these variants. Our results highlight the importance of investigating deletions and structural variants to better understand unique genetic variation in endangered populations.

***Did severe inbreeding cause the recent population decline?***

The Devils Hole pupfish population began to decline in the 1990s from historical population sizes of 200 – 500 to less than 100 individuals for reasons still unknown (although in the most recent census the population has rebounded to 175 fish). Ecological hypotheses include declines in ostracod prey for juvenile pupfish [72,73] and changes in the dominant primary producers from *Spirogyra* algae to diatoms and cyanobacterial mats [74]. Furthermore, climate change may be shortening the seasonal period of optimal hatching conditions on the shallow shelf where *C. diabolis* spawns [75]. The stressful environment of present-day Devils Hole may thus have exacerbated inbreeding depression, which typically increases under environmental stress [76,77].

Alternatively, previous studies speculated that the population has a high genetic load which led to "mutational meltdown" based on the discovery of a dramatic shift towards predominantly *C. nevadensis* ancestry following introduction of a few *C. nevadensis* into a *C. diabolis* refuge population [64]. We estimated effective population size of *C. diabolis* based on the harmonic mean of biannual census data ($N_e^{\text{Spring}} = 122$, $N_e^{\text{Fall}} = 209$) and found that this species has an effective population size far below the suggested minimum for the maintenance of sufficient genetic variation for adaptive capacity [78,79].

*Past and future management actions*

Initial management of the species involved various attempts to create wild refuge populations in the 1950s - 1970s prior to the landmark 1976 U.S. Supreme Court decision mandating a minimum water level, which halted habitat reduction and population decline caused by groundwater pumping[80]. Subsequent attempts to maintain refuge populations of *C. diabolis* in

aquaria and semi-natural outdoor pools largely failed to sustain captive breeding colonies for long periods of time [29,64]. In response to the severe population bottlenecks in the early 2000s, Ash Meadows Fish Conservation Facility was constructed in 2012 to establish a refuge population that more closely mimicked the Devils Hole habitat and the refuge colony now outnumbers the wild population. Although we relied on degraded tissue samples collected from dead pupfish retrieved from Devils Hole for genetic analyses in this study, increasing population numbers may soon allow for tissue-samples or functional genetic approaches in refuge live specimens.

***Conclusion***

Our study adds to a growing number of studies that measure inbreeding and mutation load in wild populations, with a novel focus on gene deletions in a wild highly inbred population [60,66]. The demographic history and genome-wide measures of high inbreeding and mutation load in the Devil's Hole pupfish suggest that the population remains in danger. While successfully sequencing formalin-fixed samples remains difficult, we were able to do so for a single *C. diabolis* specimen from 1980 and found that the population was likely highly inbred prior to the recent bottleneck. Increasing availability of genomic sampling spanning multiple time points for endangered species such as the Devil's Hole pupfish will better inform our understanding of inbreeding, mutation load, and specific putatively deleterious variants in this system, ultimately allowing for conservation management to monitor potentially harmful variation in wild and captive populations over time. Finally, we caution that targeted genetic management of deleterious variants should not be undertaken until candidates have been verified to have fitness consequences.

## Methods

### Samples and sequencing

We sequenced 44 whole genomes including *C. diabolis* (N = 23), *C. nevadensis* (N = 13) and *C. salinus* (N = 4) spanning multiple independent springs, along with closely related desert pupfish species from California, Arizona, and Mexico (Table S1). Of the 23 *C. diabolis* genomes, eight were from historical museum samples, spanning 1937 – 1980, while the rest were non-destructively sampled between 2008 and 2012. Given the critically endangered status of *C. diabolis*, NPS and USFWS staff collected and preserved dead specimens found during routine checks during this period. All other species were collected in the early 1990s [81]. Samples were sequenced on 150 PE runs using an Illumina Novaseq. Historical and several degraded *C. diabolis* samples were prepared using Swift 2S Turbo library kits (Swift Biosciences). All sample metadata are reported in Table S1. Fourteen samples were excluded from downstream analyses due to a low percentage of reads mapping to the reference genome (<70%), improperly paired reads (<70%), or significant amounts of missing data per individual following SNP calling (>80%), presumably due to degradation (Table S2: See supplement for additional details). Following the filtering described above to identify high-quality samples, we retained for all downstream analyses the following 30 samples: 8 *C. diabolis*, 13 *C. nevadensis*, 4 *C. salinus*, and five closely related desert pupfish species (*C. albivelis, C. eremus, C. fontinalis, C. macularius, C. radiosus*) (Fig. 2).

### Alignment and filtering

Raw reads were mapped from all 44 individuals to the *Cyprinodon brontotheroides* reference genome (UCB_Cbro_1.0; total sequence length = 1,162,855,435 bp; scaffold N50 = 32 Mbp) [82] with bwa-mem (v0.7.12; [83]). Duplicate reads were identified using MarkDuplicates and BAM indices were created using BuildBamIndex in the Picard software package (http://picard.sourceforge.net; v2.0.1). We followed the best practices guide recommended in the Genome Analysis Toolkit (v3.5; [84]) to call and refine our single nucleotide polymorphism (SNP) variant dataset using the program HaplotypeCaller. SNPs were filtered based on the recommended hard filter criteria (QD < 2.0; FS > 60; MQ<40; MQRankSum < - 12.5; ReadPosRankSum < −8) because we lacked high-quality known variants. See supplement for additional details. Our final dataset contained 6,295,414 SNPs.

**Population structure**

We pruned SNPs in strong linkage disequilibrium using the LD pruning function (--indep-pairwise 50 5 0.5) in PLINK (v1.9; [85]) leading to the retention of 1,653,597 variants. We then characterized population structure with two approaches. First, we used PLINK to conduct principal component analysis. Second, we used ADMIXTURE (v1.3.0; [86]) to assign individuals to variable numbers of population clusters (K = 1-20). We used the subset parameter in PLINK to randomly select 100,000 SNPs for analysis. We calculated genome-wide $F_{st}$ between *C. diabolis* and *C. nevadensis*, based on the 6.3 million SNP dataset using the weir-fst-pop function in vcftools (v.0.1.15; [87]).

**Phylogenetic inference**

Phylogenies were inferred at the individual and species-level from 1,653,597 LD-pruned SNPs using IQ-Tree (v1.6.12:[36]) and SVDquartets [34,35]. Full details are provided in the supplemental methods.

**Population size**

Census data for 1972 – Spring 2013 was acquired from Beissinger (2014). Data for subsequent counts was based on released NPS news reports. Variance effective population size was calculated as the harmonic mean of spring ($Ne^{Spring}$) and fall ($Ne^{Fall}$) population counts over time.

**Runs of homozygosity**

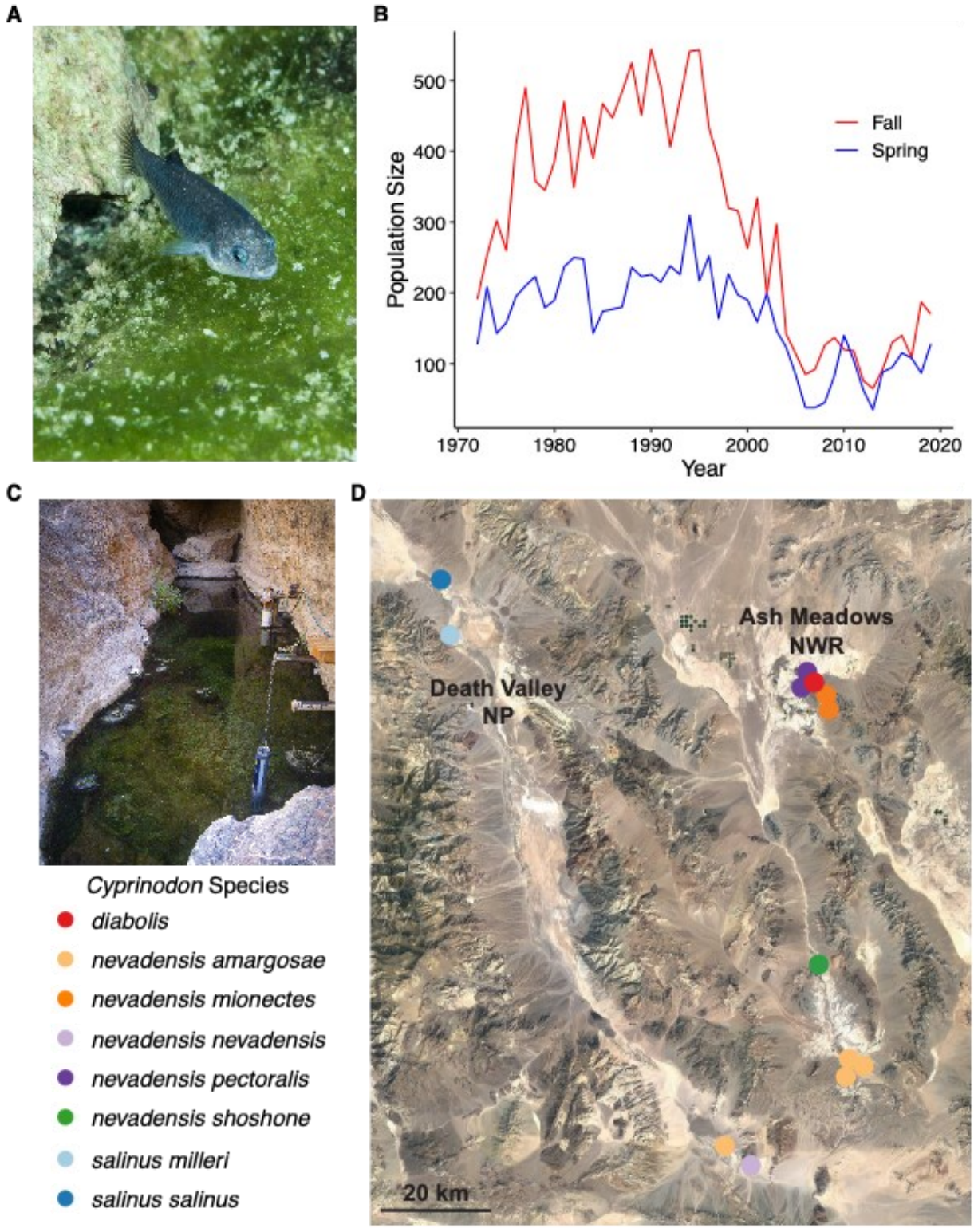
Runs of homozygosity (ROHs) were identified in our Death Valley samples from the full filtered set of 6,295,414 SNPs using the BCFtools/ROH command (v.1.14: [88]), which uses a hidden Markov model to identify homozygous portions of the genome from genetic variation data. To calculate the cumulative fraction of the genome consisting of ROHs ($F_{ROH}$) for each individual, we summed the lengths of identified ROHs with length greater than 100 kb and divided by the total size of the genome (1,162,855,435 bp). We also classified ROHs into various lengths of 0.1 – 1 Mb, 1 – 10 Mb, and greater than 10 Mb and calculated the proportion of the genome that these classes of ROHs comprised in a similar fashion. Although the amount of missing data was significantly associated with our measure of FROH across all samples (Fig. S2), we found no significant association between FROH and depth of coverage or missing data for *C. diabolis* samples alone. Furthermore, we were still able to detect low FROH in two *C. nevadensis* samples with large amounts of missing data. The age of ROHs were estimated as g = 100 / 2*ROH length (cM), where g is the number of generations to the most recent common ancestor

413 [41]. We assumed a generation time of 1 year [29], and an average recombination of ~ 4.6
414 cM/Mb (genetic map length = 5330 cM [89]; *C. brontotheroides* genome size = 1.16 Gb [82]).
415
416 **Measuring mutation load**
417 We categorized variants in coding regions based on their putative effect on the amino acid
418 sequence [i.e. loss-of-function (LOF) or non-synonymous (NSYN)] and whether the alleles were
419 derived with respect to our reference (*C. brontotheroides*) genome using SnpEff [90]. LOF
420 variants were conservatively defined as SNPs that resulted in a premature stop codon [65,91],
421 which are expected to be less prone to misannotation [92]. Unique, putatively deleterious
422 variants were defined as being: (i) homozygous derived, (ii) a gained stop codon, and (iii)
423 present in all of our *C. diabolis* samples for which genotypic information was available and
424 absent in all other non-*C. diabolis* samples in our dataset. See supplement for additional details.
425 We did not rely on direct counts of variants or derived alleles as they are significantly positively
426 correlated with coverage (Fig. S3) and missing data (Fig. S4). Instead, we measured mutation
427 load in terms of the proportions of SYN, NSYN, and LOF genotypes that were homozygous
428 ancestral, heterozygous, or homozygous derived across species (e.g. [91]). This transformation
429 largely corrected for the confounding effect of coverage (Fig. S5) and missing data (Fig. S6) on
430 our estimates of mutation load. We assessed whether mutation loads differed among the three
431 species using an ANOVA in R and used Tukey's HSD tests in R to test for pairwise differences
432 among species [93]. To compare allele frequencies of LOF alleles between our three species, we
433 first identified 62 LOF variants for which there was genotype information for at least four
434 individuals per species and for which the variant was present in at least two of the three species,
435 to control for differences in sampling number among species. We then calculated species-

specific allele frequencies for each of these 62 LOF variants. Differences in average allele frequency of LOF alleles was assessed using an ANOVA in R [93]. We assessed whether LOF variants were enriched in ROHs for each individual by performing binomial tests where the number of successes was defined as the number of LOF variants in ROHs, the number of trials was defined as the total number of LOF variants, and the probability of success was defined as the individual's $F_{ROH}$.

**Unique deletions**

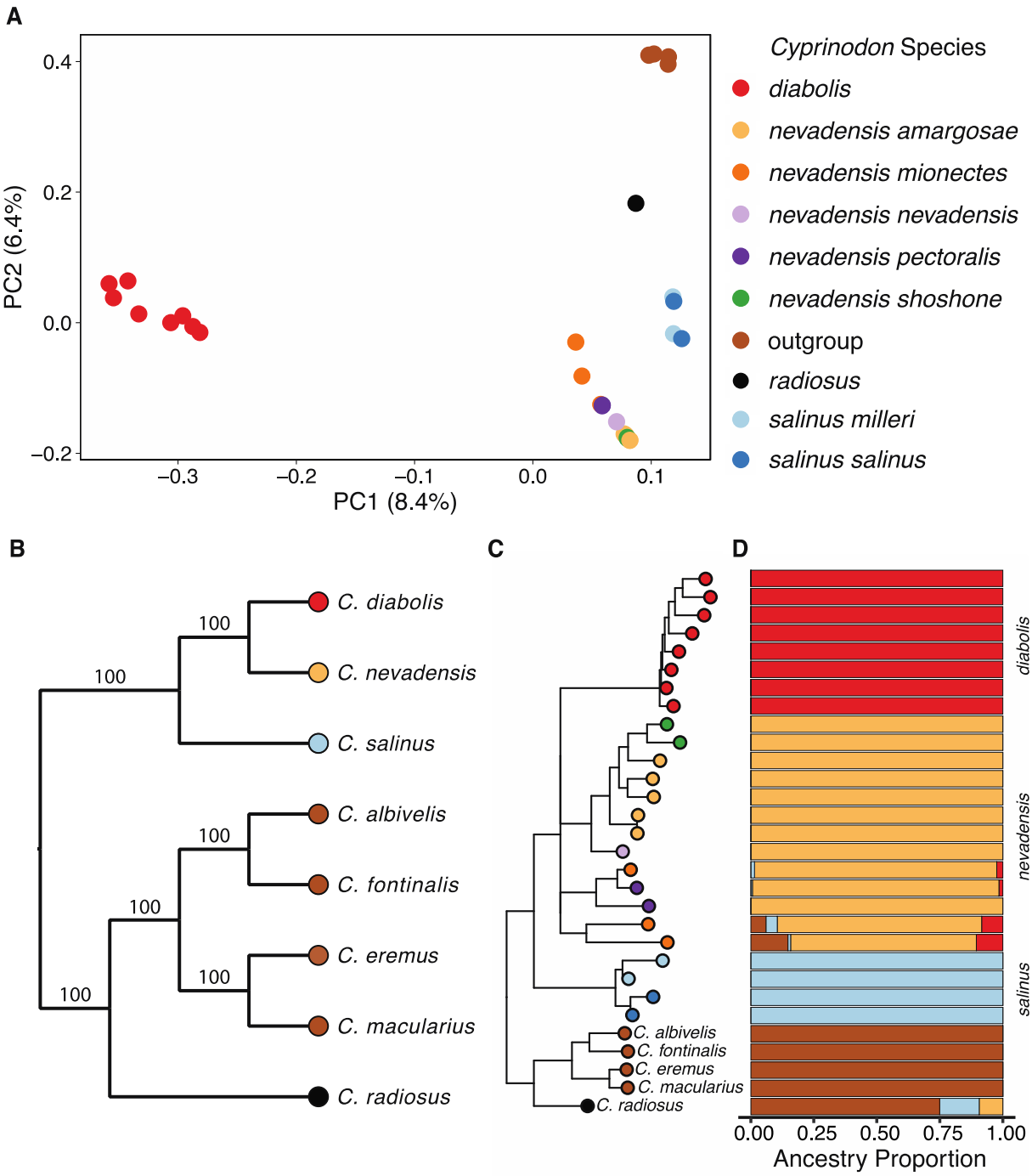
We identified deletions that were unique to *C. diabolis* using DELLY (v0.8.3) [94]. We only characterized deletions that were present in our five highest quality *C. diabolis* samples (DHP1980-5, DHP54903, DHP54913, DHP54917, DIAB54919), but absent in other species. Deletions that were exceptionally large and presumably artifacts were checked for accuracy in IGV and subsequently removed ($n = 4$). We focused primarily on deletions within 2kb of an annotated gene. Deletions were confirmed to be unique to *C. diabolis* by aligning BAM files spanning the deletion and confirming their presence in *C. diabolis* and absence in non-*C. diabolis* samples. We analyzed whether deletions spanned exons, introns, or regulatory regions by BLASTing [95] deleted sequences against the C_variegatus-1.0 (GCF_000732505.1) assembly on Ensembl (release 102; [96]).




454

455 **Figure 1. Sampling locations and recent *C. diabolis* population decline**

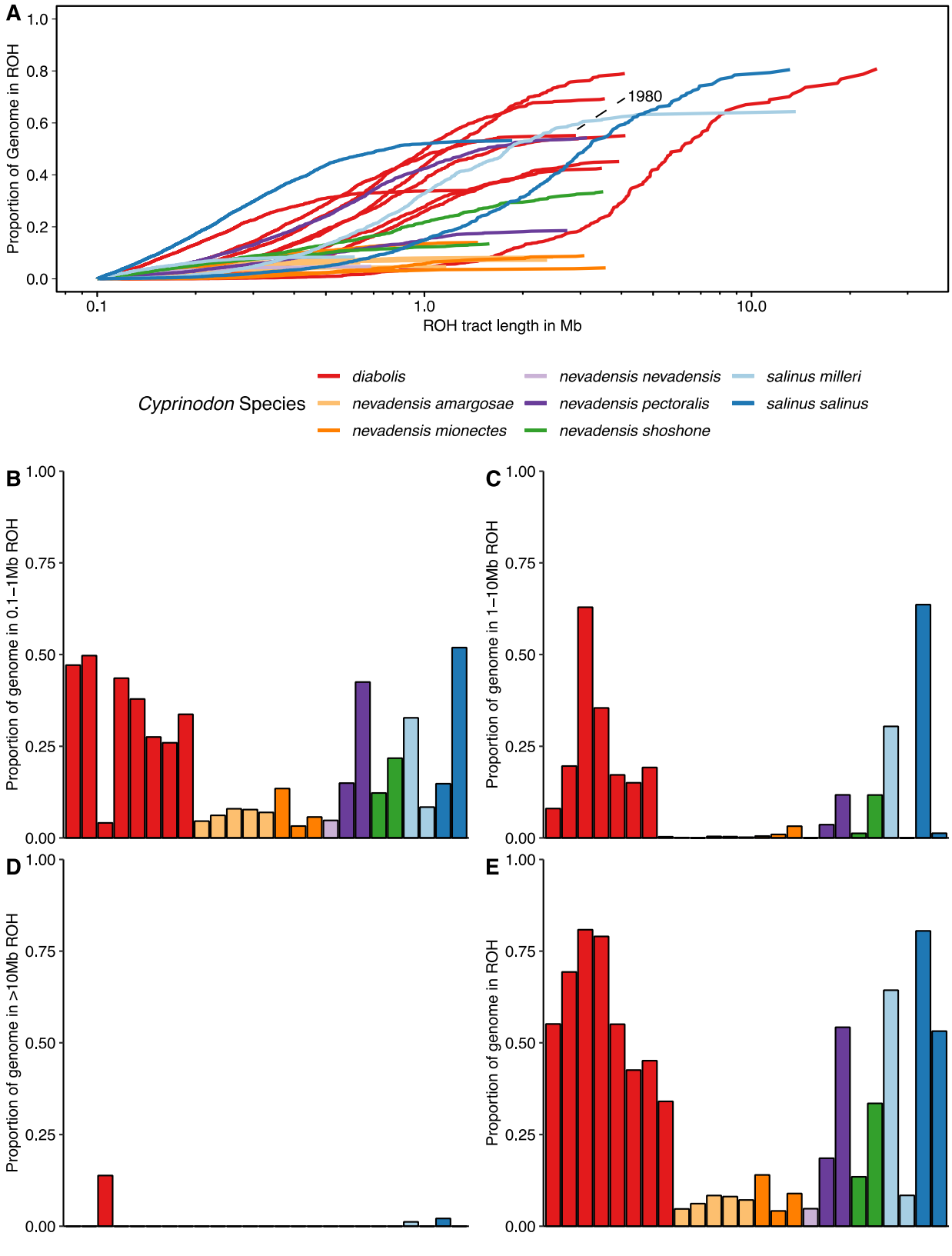
A) Photo of *C. diabolis* by Olin Feuerbacher. B) Biannual population census counts of *C. diabolis* over time. Bottlenecks in 2007 and 2013 reached 38 and 35 individuals, respectively. C) Photo of Devils Hole by USFWS. D) Map of Death Valley NP and Ash Meadows NWR sampling locations.


A
0.4
0.2
0.0
−0.2
PC2 (6.4%)
−0.3
−0.2
−0.1
0.0
0.1
PC1 (8.4%)
Cyprinodon Species
diabolis
nevadensis amargosae
nevadensis mionectes
nevadensis nevadensis
nevadensis pectoralis
nevadensis shoshone
outgroup
radiosus
salinus milleri
salinus salinus
B
C. diabolis
C. nevadensis
C. salinus
C. albivelis
C. fontinalis
C. eremus
C. macularius
C. radiosus
100
100
100
100
100
C
C. albivelis
C. fontinalis
C. eremus
C. macularius
C. radiosus
D
diabolis
nevadensis
salinus
0.00
0.25
0.50
0.75
1.00
Ancestry Proportion

461

462

**Figure 2. Population structure and evolutionary relationships among desert pupfishes** A). Principle component analysis of desert pupfishes revealing substantial population structure among species and populations. B) Species tree estimated by SVDquartets; nonparametric bootstrap support values indicate the percentage of bootstrap replicates supporting monophyly for each clade. C) Maximum-likelihood tree of all 30 samples estimated by IQ-TREE. Two internal branches were collapsed due to low support (ultrafast bootstrap < 95%, SH-aLRT < 80%); all other branch support was unequivocal (UFboot = 100%, SH-aLRT = 100%). D) Ancestry proportions across individuals in Death Valley NP, Ash Meadows NWR, and outgroup desert pupfishes (*C. albivelis, C. eremus, C. fontinalis, C. macularius, C. radiosus*) estimated from a LD-pruned SNP dataset in ADMIXTURE with k = 4. Colors in PCA, trees, and ADMIXTURE designate individuals from different species or populations and correspond to the shared figure legend in A.

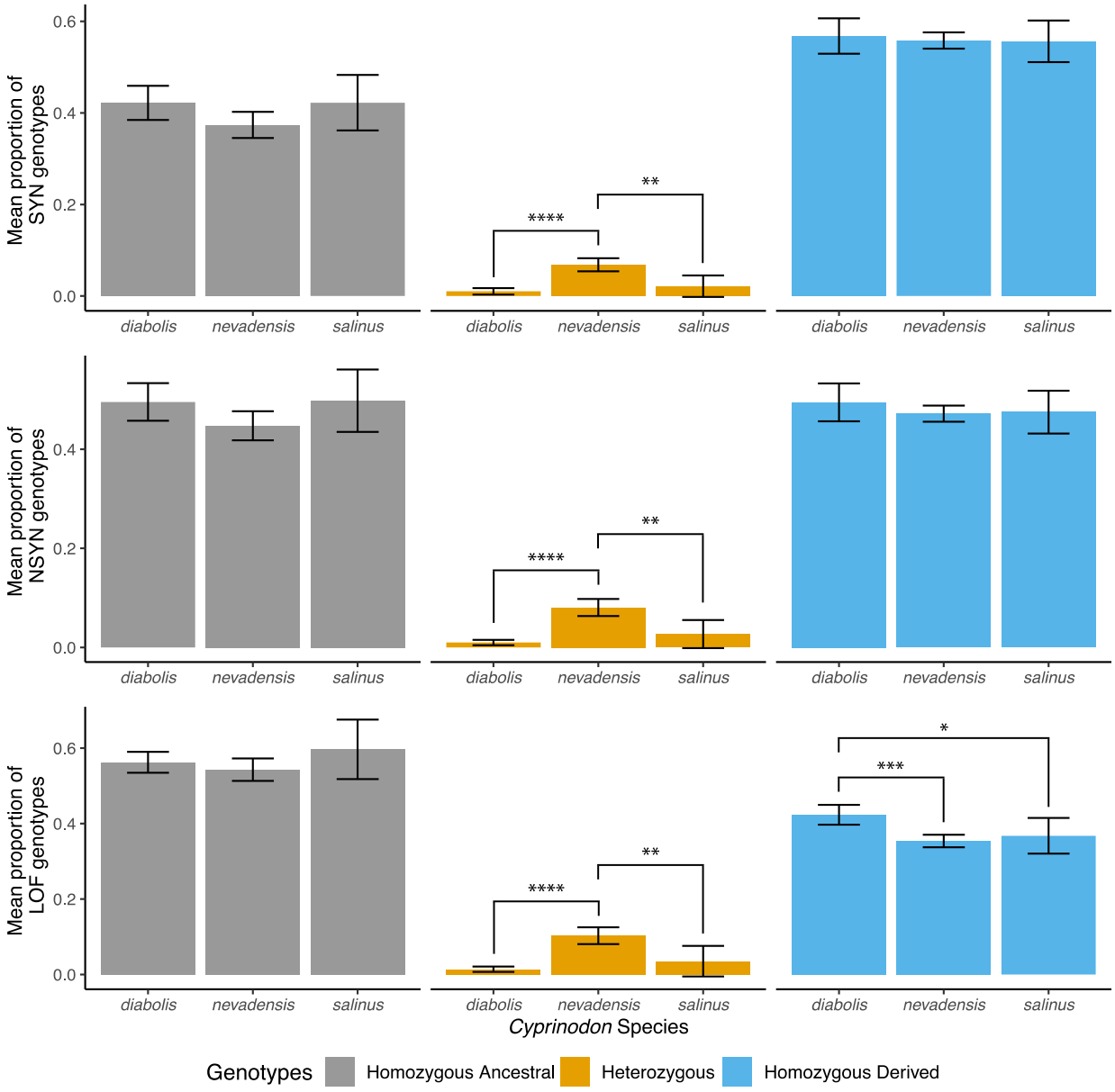

A
Proportion of Genome in ROH
1.0
0.8
0.6
0.4
0.2
0.0
1980
0.1
1.0
10.0
ROH tract length in Mb
Cyprinodon Species
diabolis
nevadensis amargosae
nevadensis mionectes
nevadensis nevadensis
nevadensis pectoralis
nevadensis shoshone
salinus milleri
salinus salinus
B
Proportion of genome in 0.1–1Mb ROH
C
Proportion of genome in 1–10Mb ROH
D
Proportion of genome in >10Mb ROH
E
Proportion of genome in ROH
1.00
0.75
0.50
0.25
0.00

476

477

**Figure 3. Extreme inbreeding in the Devils Hole pupfish**

A) $F_{ROH}$ measured as the cumulative fraction of the genome made up of runs of homozygosity (ROHs) at least 100 kb long. B) Proportion of the genome in ROHs of 0.1 – 1 Mb for each individual. C) Proportion of the genome in ROHs of 1 – 10 Mb for each individual. D) Proportion of the genome in ROHs greater than 10 Mb for each individual. E) Total proportion of the genome within an ROH greater than 100 kb.




485

486 **Figure 4. *Cyprinodon diabolis* suffers from uniquely high mutation load**

487 The mean proportion of homozygous ancestral, heterozygous, and homozygous derived

488 genotypes in *C. diabolis*, *C. nevadensis*, and *C. salinus* with respect to the *C. brontotheroides*

489 genome at segregating sites across all populations in coding regions. Variants were categorized

490 into mutation classes as synonymous (SYN), non-synonymous (NSYN), or loss-of-function

491 (LOF) with Snpeff. Loss-of-function mutations are defined as those that encode a premature stop

codon. Error bars correspond to the 95% confidence interval, spanning the mean ± two standard errors.

**Author Contributions**

BJT and CHM obtained samples. DT wrote the manuscript and performed all genomic analyses. AHP performed phylogenetic analyses. DT, AHP, BJT, and CHM contributed to the development of ideas presented in the study and revised the manuscript. All authors edited and approved the manuscript.

**Competing interests**

The authors declare no competing interests.

**Data and materials availability**

All raw genomic data are archived at the National Center for Biotechnology Information BioProject Database (Accession: PRJNA887195). Genotypic data and population census data are available through the Dryad Digital Repository: https://doi.org/10.6078/D1ND9Q. Scripts are available at https://github.com/tiandavid/Cyprindon_WGS_project/.

**Ethics statement**

Pupfishes were originally collected in the Death Valley region with Endangered Species Permit PRT-769851 and Special Use Permit 56034, U.S. Fish and Wildlife Service; Collecting Permit A9103, Death Valley National Monument, National Park Service; 6840, California, Desert District Office, Bureau of Land Management; S9494, Nevada Department of

Wildlife, and a memorandum of understanding with the California Department of Fish and Game.

**Acknowledgements**

We thank members of the Martin lab for valuable comments and discussions; O. Feuerbacher for providing a photo of *C. diabolis*; T. Echelle for providing specimens of *C. n. pectoralis* from Indian Spring; Lee Simons, the U.S. Fish and Wildlife Service, and the National Park Service for providing *C. diabolis* samples and permits; and the University of California, Berkeley for computational resources. We also gratefully thank Douglas Nelson and the University of Michigan Museum of Zoology for allowing us to sample and sequence specimens from their collection (1980 specimen: UMMZ 213382). This work was funded by the U.S. Fish and Wildlife Service, National Park Service, National Science Foundation DEB CAREER grant #1749764, National Institutes of Health grant 5R01DE027052-02, and the University of California, Berkeley to CHM. DT was supported by a National Science Foundation Graduate Research Fellowship (DGE 1752814).

**References**

1. Frankham R. 1995 Conservation Genetics. *Annual Review of Genetics* **29**, 305–327. (doi:10.1146/annurev.ge.29.120195.001513)

2. Charlesworth B, Charlesworth D. 1999 The genetic basis of inbreeding depression. *Genet Res* **74**, 329–340. (doi:10.1017/s0016672399004152)

3. Charlesworth D, Willis JH. 2009 The genetics of inbreeding depression. *Nat Rev Genet* **10**, 783–796. (doi:10.1038/nrg2664)

4. Melbourne BA, Hastings A. 2008 Extinction risk depends strongly on factors contributing to stochasticity. *Nature* **454**, 100–103. (doi:10.1038/nature06922)

5. Kohn MH, Murphy WJ, Ostrander EA, Wayne RK. 2006 Genomics and conservation genetics. *Trends Ecol Evol* **21**, 629–637. (doi:10.1016/j.tree.2006.08.001)

6. Kimura M. 1957 Some Problems of Stochastic Processes in Genetics. *The Annals of Mathematical Statistics* **28**, 882–901. (doi:10.1214/aoms/1177706791)

7. Agrawal AF, Whitlock MC. 2012 Mutation Load: The Fitness of Individuals in Populations Where Deleterious Alleles Are Abundant. *Annual Review of Ecology, Evolution, and Systematics* **43**, 115–135. (doi:10.1146/annurev-ecolsys-110411-160257)

8. Henn BM, Botigué LR, Bustamante CD, Clark AG, Gravel S. 2015 Estimating the mutation load in human genomes. *Nat Rev Genet* **16**, 333–343. (doi:10.1038/nrg3931)

9. Spielman D, Brook BW, Frankham R. 2004 Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences* **101**, 15261–15264. (doi:10.1073/pnas.0403809101)

10. Crnokrak P, Roff DA. 1999 Inbreeding depression in the wild. *Heredity* **83**, 260–270. (doi:10.1038/sj.hdy.6885530)

11. Johnson WE *et al.* 2010 Genetic Restoration of the Florida Panther. *Science* **329**, 1641–1645. (doi:10.1126/science.1192891)

12. Robinson JA, Räikkönen J, Vucetich LM, Vucetich JA, Peterson RO, Lohmueller KE, Wayne RK. 2019 Genomic signatures of extensive inbreeding in Isle Royale wolves, a population on the threshold of extinction. *Science Advances* **5**, eaau0757. (doi:10.1126/sciadv.aau0757)

13. Xue Y *et al.* 2015 Mountain gorilla genomes reveal the impact of long-term population decline and inbreeding. *Science* **348**, 242–245. (doi:10.1126/science.aaa3952)

14. Palkopoulou E *et al.* 2015 Complete genomes reveal signatures of demographic and genetic declines in the woolly mammoth. *Curr Biol* **25**, 1395–1400. (doi:10.1016/j.cub.2015.04.007)

15. Rogers RL, Slatkin M. 2017 Excess of genomic defects in a woolly mammoth on Wrangel island. *PLOS Genetics* **13**, e1006601. (doi:10.1371/journal.pgen.1006601)

16. Bi K, Linderoth T, Vanderpool D, Good JM, Nielsen R, Moritz C. 2013 Unlocking the vault: next-generation museum population genomics. *Molecular Ecology* **22**, 6018–6032. (doi:10.1111/mec.12516)

17. Díez-Del-Molino D, Sánchez-Barreiro F, Barnes I, Gilbert MTP, Dalén L. 2018 Quantifying Temporal Genomic Erosion in Endangered Species. *Trends Ecol Evol* **33**, 176–185. (doi:10.1016/j.tree.2017.12.002)

18. Hansson B, Westerberg L. 2002 On the correlation between heterozygosity and fitness in natural populations. *Mol Ecol* **11**, 2467–2474. (doi:10.1046/j.1365-294x.2002.01644.x)

19. Kardos M, Armstrong E, Fitzpatrick S, Hauser S, Hedrick P, Miller J, Tallmon DA, Funk WC. 2021 The crucial role of genome-wide genetic variation in conservation. , 2021.07.05.451163. (doi:10.1101/2021.07.05.451163)

20. Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I. 1998 Inbreeding and extinction in a butterfly metapopulation. *Nature* **392**, 491–494. (doi:10.1038/33136)

21. Teixeira JC, Huber CD. 2021 The inflated significance of neutral genetic diversity in conservation genetics. *PNAS* **118**. (doi:10.1073/pnas.2015096118)

22. Kardos M, Taylor HR, Ellegren H, Luikart G, Allendorf FW. 2016 Genomics advances the study of inbreeding depression in the wild. *Evolutionary Applications* **9**, 1205–1218. (doi:10.1111/eva.12414)

23. Martin CH, Crawford Jacob E., Turner Bruce J., Simons Lee H. 2016 Diabolical survival in Death Valley: recent pupfish colonization, gene flow and genetic assimilation in the smallest species range on earth. *Proceedings of the Royal Society B: Biological Sciences* **283**, 20152334. (doi:10.1098/rspb.2015.2334)

24. James CJ. 1969 *Aspects of the Ecology of the Devil's Hole Pupfish (Cyprinodon Diabolis) Wales*. University of Nevada, Las Vegas.

25. Riggs AC, Deacon JE. 2002 Connectivity in Desert Aquatic Ecosystems: The Devils Hole Story. , 38.

26. Wilson, K.P., Blinn, D.W., Herbst, D.B. 2001 The Devils Hole Energetics/Community Relationships: Death Valley National Park, California. Two-Year Progress Report to Death Valley National Park.

27. Gustafson, E.S., Deacon, J.E. 1997 Distribution of larval Devils Hole pupfish, Cyprinodon diabolis Wales, in relation to dissolved oxygen concentration in Devil's Hole. Report to the National Park Service, Death Valley National Park.

28. Madinger HL, Wilson KP, Goldstein JA, Bernot MJ. 2016 Biogeochemistry and Nutrient Limitation of Microbial Biofilms in Devils Hole, Nevada. *wnan* **76**, 53–71. (doi:10.3398/064.076.0107)

29. Deacon JE, Taylor FR, Pedretti JW. 1995 Egg viability and ecology of Devils Hole pupfish: Insights from captive propagation. *The Southwestern Naturalist* **40**, 216–223.

30. Beissinger SR. 2014 Digging the pupfish out of its hole: risk analyses to guide harvest of Devils Hole pupfish for captive breeding. *PeerJ* **2**. (doi:10.7717/peerj.549)

31. Miller, Robert R. 1981 *Coevolution of deserts and pupfishes (genus Cyprinodon) in the American Southwest, in Naiman, R. J., and Soltz, D. L., eds, Fishes in North American Deserts*. See https://azgs.arizona.edu/azgeobib/coevolution-deserts-and-pupfishes-genus-cyprinodon-american-southwest-naiman-r-j-and-soltz.

32. Martin CH, Höhna S, Crawford JE, Turner BJ, Richards EJ, Simons LH. 2017 The complex effects of demographic history on the estimation of substitution rate: concatenated gene analysis results in no more than twofold overestimation. *Proceedings of the Royal Society B: Biological Sciences* **284**, 20170537. (doi:10.1098/rspb.2017.0537)

33. Martin CH, Turner BJ. 2018 Long-distance dispersal over land by fishes: extremely rare ecological events become probable over millennial timescales. *Proc Biol Sci* **285**, 20172436. (doi:10.1098/rspb.2017.2436)

34. Chifman J, Kubatko L. 2014 Quartet Inference from SNP Data Under the Coalescent Model. *Bioinformatics* **30**, 3317–3324. (doi:10.1093/bioinformatics/btu530)

35. Chifman J, Kubatko L. 2015 Identifiability of the unrooted species tree topology under the coalescent model with time-reversible substitution processes, site-specific rate variation, and invariable sites. *Journal of Theoretical Biology* **374**, 35–47. (doi:10.1016/j.jtbi.2015.03.006)

36. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015 IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution* **32**, 268–274. (doi:10.1093/molbev/msu300)

37. Miller RR, Pister EP. 1971 Management of the Owens Pupfish, Cyprinodon radiosus, in Mono County, California. *Transactions of the American Fisheries Society* **100**, 502–509. (doi:10.1577/1548-8659(1971)100<502:MOTOPC>2.0.CO;2)

38. Castleberry D, Williams JE, Sato GM, Hopkins TE, Brasher A, Parker MS. 1990 Status and Management of Shoshone Pupfish, Cyprinodon nevadensis shoshone (Cyprinodontidae), at Shoshone Spring, Inyo County, California. *undefined*

39. Weissenfluh D. 2010 Conservation of Cyprinodon nevadensis pectoralis and three endemic aquatic invertebrates in an artificial desert spring refuge located in Ash Meadows National Wildlife Refuge, Nevada. Thesis. See https://ttu-ir.tdl.org/handle/2346/ETD-TTU-2010-12-1207.

40. Swift CC, Haglund RT, Ruiz M, Fisher RN. 1993 The status and distribution of the freshwater fishes of southern California. *Bulletin of the Southern California Academy of Sciences* **92**, 101–172.

41. Thompson EA. 2013 Identity by Descent: Variation in Meiosis, Across Genomes, and in Populations. *Genetics* **194**, 301–326. (doi:10.1534/genetics.112.148825)

42. Kardos M *et al.* 2018 Genomic consequences of intensive inbreeding in an isolated wolf population. *Nat Ecol Evol* **2**, 124–131. (doi:10.1038/s41559-017-0375-4)

43. Hedrick PW, Garcia-Dorado A. 2016 Understanding Inbreeding Depression, Purging, and Genetic Rescue. *Trends Ecol Evol* **31**, 940–952. (doi:10.1016/j.tree.2016.09.005)

44. Henn BM *et al.* 2016 Distance from sub-Saharan Africa predicts mutational load in diverse human genomes. *Proc Natl Acad Sci U S A* **113**, E440-449. (doi:10.1073/pnas.1510805112)

45. Simmons MJ, Crow JF. 1977 Mutations affecting fitness in drosophila populations. *Annu. Rev. Genet.* **11**, 49–78. (doi:10.1146/annurev.ge.11.120177.000405)

46. Agrawal AF, Whitlock MC. 2011 Inferences About the Distribution of Dominance Drawn From Yeast Gene Knockout Data. *Genetics* **187**, 553–566. (doi:10.1534/genetics.110.124560)

47. Coutton C *et al.* 2018 Mutations in CFAP43 and CFAP44 cause male infertility and flagellum defects in Trypanosoma and human. *Nat Commun* **9**, 686. (doi:10.1038/s41467-017-02792-7)

48. Wildt DE, Bush M, Goodrowe KL, Packer C, Pusey AE, Brown JL, Joslin P, O'Brien SJ. 1987 Reproductive and genetic consequences of founding isolated lion populations. *Nature* **329**, 328–331. (doi:10.1038/329328a0)

49. Lee P, Chandel NS, Simon MC. 2020 Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. *Nat Rev Mol Cell Biol* **21**, 268–283. (doi:10.1038/s41580-020-0227-y)

50. Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, Witters LA, Ellisen LW, Kaelin WG. 2004 Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* **18**, 2893–2904. (doi:10.1101/gad.1256804)

51. DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. 2008 Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev* **22**, 239–251. (doi:10.1101/gad.1617608)

52. Barts, N. 2020 Comparative physiology and the predictability of evolution in extreme environments. Kansas State University.

53. Gogliettino M, Riccio A, Balestrieri M, Cocca E, Facchiano A, D'Arco TM, Tesoro C, Rossi M, Palmieri G. 2014 A novel class of bifunctional acylpeptide hydrolases--potential role in the antioxidant defense systems of the Antarctic fish Trematomus bernacchii. *FEBS J* **281**, 401–415. (doi:10.1111/febs.12610)

54. Yergeau DA, Cornell CN, Parker SK, Zhou Y, Detrich HW. 2005 bloodthirsty, an RBCC/TRIM gene required for erythropoiesis in zebrafish. *Developmental Biology* **283**, 97–112. (doi:10.1016/j.ydbio.2005.04.006)

55. Fiermonte G, Paradies E, Todisco S, Marobbio CMT, Palmieri F. 2009 A novel member of solute carrier family 25 (SLC25A42) is a transporter of coenzyme A and adenosine 3',5'-diphosphate in human mitochondria. *J Biol Chem* **284**, 18152–18159. (doi:10.1074/jbc.M109.014118)

56. Shamseldin HE, Smith LL, Kentab A, Alkhalidi H, Summers B, Alsedairy H, Xiong Y, Gupta VA, Alkuraya FS. 2016 Mutation of the mitochondrial carrier SLC25A42 causes a novel form of mitochondrial myopathy in humans. *Hum Genet* **135**, 21–30. (doi:10.1007/s00439-015-1608-8)

57. Ralls K, Ballou JD, Templeton A. 1988 Estimates of Lethal Equivalents and the Cost of Inbreeding in Mammals. *Conservation Biology* **2**, 185–193.

58. Laikre L, Ryman N. 1991 Inbreeding Depression in a Captive Wolf (Canis lupus) Population. *Conservation Biology* **5**, 33–40.

59. van der Valk T, Díez-Del-Molino D, Marques-Bonet T, Guschanski K, Dalén L. 2019 Historical Genomes Reveal the Genomic Consequences of Recent Population Decline in Eastern Gorillas. *Curr Biol* **29**, 165-170.e6. (doi:10.1016/j.cub.2018.11.055)

60. Khan A *et al.* 2021 Genomic evidence for inbreeding depression and purging of deleterious genetic variation in Indian tigers. *PNAS* **118**. (doi:10.1073/pnas.2023018118)

61. Stoffel MA, Johnston SE, Pilkington JG, Pemberton JM. 2021 Genetic architecture and lifetime dynamics of inbreeding depression in a wild mammal. *Nat Commun* **12**, 2972. (doi:10.1038/s41467-021-23222-9)

62. Harrisson KA *et al.* 2019 Lifetime Fitness Costs of Inbreeding and Being Inbred in a Critically Endangered Bird. *Current Biology* **29**, 2711-2717.e4. (doi:10.1016/j.cub.2019.06.064)

63. Chernoff B. 1985 Population dynamics of the Devils Hole pupfish. *Environ Biol Fish* **13**, 139–147. (doi:10.1007/BF00002582)

64. Martin AP, Echelle AA, Zegers G, Baker S, Keeler-Foster CL. 2012 Dramatic shifts in the gene pool of a managed population of an endangered species may be exacerbated by high genetic load. *Conserv Genet* **13**, 349–358. (doi:10.1007/s10592-011-0289-7)

65. Robinson JA, Brown C, Kim BY, Lohmueller KE, Wayne RK. 2018 Purging of Strongly Deleterious Mutations Explains Long-Term Persistence and Absence of Inbreeding Depression in Island Foxes. *Current Biology* **28**, 3487-3494.e4. (doi:10.1016/j.cub.2018.08.066)

66. Grossen C, Guillaume F, Keller LF, Croll D. 2020 Purging of highly deleterious mutations through severe bottlenecks in Alpine ibex. *Nature Communications* **11**, 1001. (doi:10.1038/s41467-020-14803-1)

67. van der Valk T, Manuel M de, Marques-Bonet T, Guschanski K. 2021 Estimates of genetic load suggest frequent purging of deleterious alleles in small populations. *bioRxiv* , 696831. (doi:10.1101/696831)

68. Eyre-Walker A, Keightley PD. 2007 The distribution of fitness effects of new mutations. *Nat Rev Genet* **8**, 610–618. (doi:10.1038/nrg2146)

69. Liu, R.K. 1969 The Comparative Behavior of Allopatric Species (Teleostei-Cyprinodontidae: Cyprinodon).

70. Burg GC *et al.* 2019 Care and propagation of captive pupfish from the genus Cyprinodon: insight into conservation. *Environ Biol Fish* **102**, 1015–1024. (doi:10.1007/s10641-019-00887-2)

71. Leigh DM, Lischer HEL, Guillaume F, Grossen C, Günther T. 2021 Disentangling adaptation from drift in bottlenecked and reintroduced populations of Alpine ibex. *Molecular Ecology Resources* **n/a**. (doi:https://doi.org/10.1111/1755-0998.13442)

72. Wilson KP, Blinn DW. 2007 FOOD WEB STRUCTURE, ENERGETICS, AND IMPORTANCE OF ALLOCHTHONOUS CARBON IN A DESERT CAVERNOUS LIMNOCRENE: DEVILS HOLE, NEVADA. *Western North American Naturalist* **67**, 185–198. (doi:10.3398/1527-0904(2007)67[185:FWSEAI]2.0.CO;2)

73. Minckley CO, Deacon JE. 1975 Foods of the Devil's Hole Pupfish, Cyprinodon diabolis (Cyprinodontidae). *The Southwestern Naturalist* **20**, 105–111. (doi:10.2307/3670016)

74. Bernot M, Wilson K. 2012 Spatial and temporal variation of dissolved oxygen and ecosystem energetics in Devils Hole, Nevada. *Western North American Naturalist* **72**.

75. Hausner MB, Wilson KP, Gaines DB, Suárez F, Scoppettone GG, Tyler SW. 2014 Life in a fishbowl: Prospects for the endangered Devils Hole pupfish (Cyprinodon diabolis) in a changing climate. *Water Resources Research* **50**, 7020–7034. (doi:10.1002/2014WR015511)

76. Armbruster P, Reed DH. 2005 Inbreeding depression in benign and stressful environments. *Heredity* **95**, 235–242. (doi:10.1038/sj.hdy.6800721)

77. Fox CW, Reed DH. 2011 Inbreeding Depression Increases with Environmental Stress: An Experimental Study and Meta-Analysis. *Evolution* **65**, 246–258. (doi:10.1111/j.1558-5646.2010.01108.x)

78. Franklin IR. 1980 Evolutionary change in small populations. In *Conservation Biology: an Evolutionary–Ecological Perspective*, pp. 135–150. Sunderland, Massachusetts, Sinauer Associates, U.S.A.

79. Traill LW, Bradshaw CJA, Brook BW. 2007 Minimum viable population size: A meta-analysis of 30 years of published estimates. *Biological Conservation* **139**, 159–166. (doi:10.1016/j.biocon.2007.06.011)

80. 1976 Cappaert v. United States. *U.S.* **426**, 128.

81. Duvernell DD, Turner BJ. 1998 Evolutionary genetics of Death Valley pupfish populations: mitochondrial DNA sequence variation and population structure. *Molecular Ecology* **7**, 279–288. (doi:10.1046/j.1365-294X.1998.00347.x)

82. Richards EJ, McGirr JA, Wang JR, John MES, Poelstra JW, Solano MJ, O'Connell DC, Turner BJ, Martin CH. 2021 A vertebrate adaptive radiation is assembled from an ancient and disjunct spatiotemporal landscape. *PNAS* **118**. (doi:10.1073/pnas.2011811118)

83. Li H. 2013 Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv:1303.3997 [q-bio]*

84. DePristo MA *et al.* 2011 A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* **43**, 491–498. (doi:10.1038/ng.806)

85. Purcell S *et al.* 2007 PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *Am J Hum Genet* **81**, 559–575.

86. Alexander DH, Novembre J, Lange K. 2009 Fast model-based estimation of ancestry in unrelated individuals. *Genome Res* **19**, 1655–1664. (doi:10.1101/gr.094052.109)

87. Danecek P *et al.* 2011 The variant call format and VCFtools. *Bioinformatics* **27**, 2156–2158. (doi:10.1093/bioinformatics/btr330)

88. Narasimhan V, Danecek P, Scally A, Xue Y, Tyler-Smith C, Durbin R. 2016 BCFtools/RoH: a hidden Markov model approach for detecting autozygosity from next-generation sequencing data. *Bioinformatics* **32**, 1749–1751. (doi:10.1093/bioinformatics/btw044)

89. St. John ME, Dunker JC, Richards EJ, Romero S, Martin CH. 2021 Parallel genetic changes underlie integrated craniofacial traits in an adaptive radiation of trophic specialist pupfishes. *bioRxiv* , 2021.07.01.450661. (doi:10.1101/2021.07.01.450661)

90. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. 2012 A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly (Austin)* **6**, 80–92. (doi:10.4161/fly.19695)

91. Robinson JA, Ortega-Del Vecchyo D, Fan Z, Kim BY, vonHoldt BM, Marsden CD, Lohmueller KE, Wayne RK. 2016 Genomic Flatlining in the Endangered Island Fox. *Current Biology* **26**, 1183–1189. (doi:10.1016/j.cub.2016.02.062)

92. de Valles-Ibáñez G, Hernandez-Rodriguez J, Prado-Martinez J, Luisi P, Marquès-Bonet T, Casals F. 2016 Genetic Load of Loss-of-Function Polymorphic Variants in Great Apes. *Genome Biol Evol* **8**, 871–877. (doi:10.1093/gbe/evw040)

93. R Core Team. 2021 *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. See https://www.R-project.org/.

94. Rausch T, Zichner T, Schlattl A, Stütz AM, Benes V, Korbel JO. 2012 DELLY: structural variant discovery by integrated paired-end and split-read analysis. *Bioinformatics* **28**, i333–i339. (doi:10.1093/bioinformatics/bts378)

786 95. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 Basic local alignment search
787 tool. *J Mol Biol* **215**, 403–410. (doi:10.1016/S0022-2836(05)80360-2)

788 96. Howe KL *et al.* 2021 Ensembl 2021. *Nucleic Acids Research* **49**, D884–D891.
789 (doi:10.1093/nar/gkaa942)

790