Severe inbreeding and gene loss in the historical and extant population of the critically endangered Devils Hole pupfish

David Tian¹, Bruce J. Turner², and Christopher H. Martin¹

DT ORCID: 0000-0001-5285-6810

CHM ORCID: 0000-0001-7989-9124

¹Department of Integrative Biology and Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, CA 94720 USA

²Department 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, genetic load, Devils Hole pupfish

Abstract

Small populations with limited geographic distributions are predicted to be threatened by inbreeding and lack of genetic diversity, both of which may negatively impact fitness and exacerbate population decline. One of the most extreme natural examples is the Devils Hole pupfish (*Cyprinodon diabolis*), an iconic and critically endangered species with the smallest known habitat range of any vertebrate. This imperiled species has experienced severe declines in population size over the last thirty years and suffered major, repeated bottlenecks in 2007 and 2013, when the population sunk to 38 and 35 individuals, respectively. Here we sequenced contemporary and historical genomes of Devils Hole and neighboring Death Valley and Ash Meadows desert pupfishes to examine the genomic consequences of small population size. We found extreme inbreeding ($F_{ROH} = 0.71 - 0.82$) and increased genetic load in the Devils Hole pupfish. We also document unique fixed loss-of-function (LOF) alleles and deletions in genes associated with sperm motility, stress, and hypoxia within the extant Devils Hole pupfish population that likely reduce fitness. Comparisons between contemporary samples (2008 – 2012) and a genome sequenced from a 1980 formalin-fixed museum specimen suggest that inbreeding has increased 6% as the population has declined, but that many putatively deleterious variants have been segregating in the population since at least 1980. This includes a fixed early stop codon in *cfap43* ($n = 8/8$ samples), which is associated with sperm flagellum defects and causes infertility in humans and mice. Out of ninety-four unique deletions, fifteen were detected within 2 kb of annotated genes. Five have roles in physiological responses to hypoxia and mitochondrial activity, such as *redd1* ($n = 7/7$ samples), suggesting impaired hypoxia tolerance in this species despite the low oxygen concentrations of Devils Hole. We thus document one of the most

extreme inbreeding events in a natural population and a set of candidate deleterious variants to inform management and potential genetic rescue in this conservation icon.

Introduction

Due to the declining population sizes of many species and increasing isolation due to 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 (Frankham 1995). Small and isolated populations are predicted to suffer from inbreeding depression, a reduction in fitness caused by the increased homozygosity of deleterious recessive alleles or overdominant loci (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009). Prolonged population decline can result in increased long-term extinction risk due to stochastic demographic events (Melbourne and Hastings 2008), reduced genetic variation for adaptation (Kohn *et al.* 2006), and decreased efficacy of purifying selection to overpower drift and purge deleterious variants (Kimura 1957) (reviewed by (Frankham 1995)).

Many examples of reduced genetic diversity and inbreeding depression have been documented in the wild such as Florida panthers (*Puma concolor*) (Johnson *et al.* 2010), Isle Royale wolves (*Canis lupus*) (Robinson *et al.* 2019), and mountain gorillas (*Gorilla beringei*) (Xue *et al.* 2015). Inbreeding associated population decline has been identified in the context of ancient bottlenecks (Palkopoulou *et al.* 2015; Rogers and Slatkin 2017) and on recent timescales (Robinson *et al.* 2019, 2021). Nevertheless, identifying inbreeding depression and its genetic basis in wild populations remains challenging due to small sample sizes and a lack of genomic resources. Recent studies of mountain gorillas (van der Valk *et al.* 2019) and Przewalski's horses

(Der Sarkissian *et al.* 2015) have also successfully leveraged museum specimens to measure temporal changes in inbreeding and genomic erosion. This is a powerful emerging approach because genomes from pre-decline museum specimens can serve as a baseline for estimating recent changes in inbreeding and genetic load (Bi *et al.* 2013; Díez-Del-Molino *et al.* 2018) and ultimately assist in restoring genetic diversity to a population (Bell *et al.* 2019).

Traditionally, conservation genetics has relied on neutral molecular markers to measure genetic variation in wild populations as a way of assessing population health and extinction risk, due to positive correlations between heterozygosity and fitness (Hansson and Westerberg 2002). Maintaining genome-wide genetic variation is crucial to preserving adaptive potential and preventing inbreeding depression in populations of conservation interest (Kardos *et al.* 2021). However, several recent studies have suggested that genetic variation summary statistics do not necessarily accurately reflect population size or extinction risk and that we should also leverage genomics to assess functional genetic diversity and genetic load (Díez-Del-Molino *et al.* 2018; Teixeira and Huber 2021). Genomics also allows for more accurate measurements of individual inbreeding relative to pedigree approaches and the opportunity to infer the genetic basis of inbreeding depression (Kardos *et al.* 2016). Thus, a comprehensive understanding of the population and evolutionary dynamics of small population sizes in the wild from a genomic perspective is key to understanding the future of endangered populations and informing conservation management.

Here we leverage the unique evolutionary and demographic history of the Devils Hole pupfish (*C. diabolis*) to investigate how isolation and recent population decline have shaped inbreeding depression and genetic load. While many desert pupfishes are generally isolated with low population sizes, none are as extreme as *C. diabolis*. The habitat of *C. diabolis* is widely

believed to be the smallest in the world for a vertebrate (3.5m x 22m) (Deacon *et al.* 1995) and 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. Population viability analysis models in 2014 suggested that the median time to extinction is 26 years (Beissinger 2014). Although this species was previously believed to be isolated in Devils Hole for 10,000 – 20,000 years (Miller 1981), more recent genome-wide estimates suggest that Devils Hole may have been colonized most recently approximately 1-2 thousand years ago and suggest that gene flow among these desert oases is surprisingly common (Martin *et al.* 2016; Martin *et al.* 2017; Martin and Höhna 2018; Martin and Turner 2018). Furthermore, extremophile conditions include a nearly constant temperature of 34°C (Miller 1948; James 1969), no direct sunlight during the winter (Riggs and Deacon 2002) which limits primary production and nutrient availability (Wilson *et al.* 2001), and low dissolved oxygen levels near lethal limits for most fishes (2 – 3 ppm) (Gustafson, E.S. and Deacon, J.E. 1997; Riggs and Deacon 2002; Sackett *et al.* 2018). The continued persistence of *C. diabolis* in the hottest desert on earth in one of the most inhospitable habitats for fishes is nearly inconceivable.

Despite being one of the world's most vulnerable species, genetic analyses have so far been limited to mitochondrial (Echelle and Dowling 1992), allozyme (Echelle and Echelle 1993), retrotransposon (Duvernell and Turner 1999), and RADseq (Martin *et al.* 2016; Martin and Höhna 2018) approaches. Here we used resequenced whole genomes of *C. diabolis* and several closely related *Cyprinodon* desert pupfishes to investigate how isolation and small population size influence inbreeding and genetic load on a genome-wide scale and across time in this iconic symbol of conservation.

Results

Geography and population structure

Principal components, admixture analyses, and genome-wide mean F_{st} estimates (*C. diabolis*/*C. nevadensis* and *C. salinus* = 0.31) indicate the presence of population structure among Death Valley desert pupfishes, corroborating previous results (Martin *et al.* 2016) (Fig. 1). Differences in individual inbreeding both between and within species also provide evidence for population structure. The *C. diabolis* population is highly inbred while *C. nevadensis* and *C. salinus* populations generally exhibited low to intermediate levels of inbreeding, measured by the cumulative fraction of the genome made up of runs of homozygosity (ROHs) (Fig. 2A). ROHs made up less than 10% of the genome in our *C. nevadensis amargosae* and *C. nevadensis nevadensis* samples, both relatively undisturbed natural spring populations. *C. nevadensis shoshone* and *C. nevadensis pectoralis* tended to have a higher F_{ROH} than other *C. nevadensis* species, consistent with previous surveys that called to attention their small population size and need for protection (Miller 1969) and the intensive management histories of these desert spring populations. Within a species, individuals from different spring populations often revealed contrasting levels of inbreeding, such as between the Indian Spring and School Spring populations of *C. nevadensis pectoralis*, indicating that isolation is strong between different springs with different management histories.

Severe inbreeding in Devils Hole pupfish

Strikingly, more than 70% of the *C. diabolis* genome consists of runs of homozygosity (ROHs), which is indicative of extreme inbreeding. Inbreeding can be identified and quantified through

ROHs, which are long contiguous tracts of identical haplotypes inherited from a common ancestor (Kardos *et al.* 2016). For context, this level of inbreeding is far greater than other documented studies of notable, endangered species such as the Isle Royale wolves (Robinson *et al.* 2019) (< 50%), mountain gorillas (<40%) (van der Valk *et al.* 2019), and extant tigers (<50%) (Armstrong *et al.* 2021), with the exception of one population of lions from the Kathiawar Peninsula of India (90%) that had a population as low as 20 individuals in the early 20th century (de Manuel *et al.* 2020).

The inbreeding observed within our 2008 - 2011 *C. diabolis* samples is ~ 6% more severe than in our 1980 *C. diabolis* sample, which is consistent with the documented population decline that began in the mid 1990s and bottlenecks in 2007 and 2013 when the population size plummeted to 38 and 35, respectively (Beissinger 2014). While shorter ROHs indicate mating between distant relatives or longer-scale historical processes, long ROHs are indicative of recent inbreeding. We can estimate the mean number of generations back to the common ancestor of these homologous sequences based on the expectation that recombination breaks down long ROHs over time (Thompson 2013). We focused our attention on longer ROHs for which we have more confidence in timing and investigated long ROHs that are present in our 2008 – 2011 samples but not our 1980 sample. These long ROHs ranged from 4 – 10 Mb and are estimated to have originated only a few generations prior, suggesting that while the population began to decline a decade earlier, much of the additional inbreeding occurred more recently, due to the 2007 bottleneck. Our results illustrate that extreme isolation and prolonged small population size have driven *C. diabolis* to become one of the most inbred wild populations surviving today. Recent population bottlenecks increased F_{ROH} , but it was already extremely inbred prior to the recent population decline in the 1990s.

Highest genetic load in Devils Hole pupfish

To measure genetic load, we used three summary statistics: the number of variants, derived alleles, and derived homozygous genotypes per individual, per site (Figure 3). We measured these statistics for synonymous, non-synonymous, and loss-of-function (LOF) mutations. *C. diabolis* had significantly more non-synonymous derived alleles ($P = 0.0423$) and derived homozygous genotypes than our *Cyprinodon* desert outgroups ($P = 0.0258$, Tukey's HSD tests). Furthermore, *C. diabolis* had significantly more LOF variants than *Cyprinodon* desert outgroups ($P = 0.0010$, Tukey's HSD test) and *Cyprinodon diabolis*, *nevadensis*, and *salinus* all had more LOF derived alleles than outgroups ($P = 8.36 \times 10^{-5}$; $P = 0.0012$; and $P = 0.0229$; Tukey's HSD test, respectively). We found a similar pattern for LOF homozygous derived genotypes, although in this case *C. diabolis* also had significantly more LOF homozygous derived genotypes than *C. nevadensis* ($P = 0.0036$, Tukey's HSD test).

Fixed loss-of-function variants unique to Devils Hole

We searched for specific genetic variants that are predicted to be deleterious, may explain *C. diabolis* population decline, and can serve as a list of candidate deleterious variants to screen for potential genetic rescue (Table S1). We focused our attention on LOF SNPs and deletions unique to *C. diabolis*, which are expected to be deleterious and likely pose a threat by disrupting gene function.

We found 16 predicted LOF SNPs that were primarily premature stop codons, including several within genes that potentially reduce fecundity and resistance to disease and stress (Table S1). These included *ora1*, a zebrafish olfactory receptor which is predicted to be a reproductive

pheromone because it induces increased oviposition frequency (Behrens *et al.* 2014) and *trim25*, an ubiquitin ligase that promotes innate immune function in zebrafish (Jin *et al.* 2019). We also found a gained premature stop codon in *cfap43*, a protein involved in the conserved structure and function of the sperm flagellum axoneme and implicated in the multiple morphological abnormalities of the flagella (MMAF) infertility phenotype (Tang *et al.* 2017; Coutton *et al.* 2018). In nine men with MMAF that had homozygous mutations in *cfap43*, five had gained a premature stop codon (Coutton *et al.* 2018). Intriguingly, reduced sperm motility and abnormal sperm morphology is also an inbreeding phenotype found in Florida panthers (Johnson *et al.* 2010), East African and Indian lions (Wildt *et al.* 1987b), wild and captive bred cheetahs (Wildt *et al.* 1983, 1987a) and Dryas monkeys (van der Valk *et al.* 2020), which have a homozygous LOF mutation in *sept12* that causes a defective sperm annulus (Kuo *et al.* 2012).

We also searched for variants that may be responsible for recent increased inbreeding depression post 1980 by identifying variants that were heterozygous in our 1980 sample but were homozygous derived in our more recent samples after 2008 (Table S2). We focused our attention on LOF and non-synonymous variants. We found a premature stop codon in *dhx57*, a putative ATP dependent RNA helicase (UniProt 2008), and non-synonymous mutations in *arhgap18*, a GTPase-activating protein that modulates cell signaling (Maeda *et al.* 2011), and *galnt3*, a GalNAc transferase implicated in abnormal calcium phosphate deposits in various tissues (Sun *et al.* 2016). Moreover, we found two non-synonymous mutations that both occurred in *itprid2*, which encodes a protein that serves as a sperm surface antigen involved in cleavage of the fertilized oocyte (Javed and Naz 1992).

Fixed deletions unique to Devils Hole

We searched for unique homozygous deletions in the *C. diabolis* genome that may negatively impact fitness. We identified 94 deletions unique to *C. diabolis* and focused our attention on the 15 deletions within 2 kb of any annotated gene. Surprisingly, 5 of the 15 deletions were involved in responses to hypoxia, mitochondrial activity, and oxidative stress (Fig. 4). Hypoxia leads to a wide suite of adaptive cellular responses that include angiogenesis and erythropoiesis, shifts to anaerobic metabolism, inhibition of translation, degradation of misfolded proteins, and regulation of nutrient use and cell fate (Lee *et al.* 2020).

We found an 81 bp deletion in the promoter of *redd1*, which is activated by HIF1 (DeYoung *et al.* 2008). This deletion knocks out the TATA box and a HRE recognition site where HIF1 is predicted to bind. During hypoxia, *redd1* inhibits mTORC1 (Brugarolas *et al.* 2004; DeYoung *et al.* 2008) through the TSC1/TSC2 tumor suppressor complex to conserve energy and prevent the accumulation of misfolded proteins. Intriguingly, upregulation of a homolog of REDD1 (*ddit4L/redd2*) has been implicated in adaptation to hypoxia in shortfin mollies (*Poecilia mexicana*) from H₂S rich springs (Barts 2020) and high-altitude deer mice (*Peromyscus maniculatus*) (Rochette 2021 pers. comm.). Further evidence that *C. diabolis* is unable to properly inhibit protein translation through mTOR comes in the form of a fixed SNP that leads to an early stop codon in *tbc1d7*, a third core subunit of the TSC1/TSC2 complex whose knockdown leads to decreased association between TSC1 and TSC2 and thus increased mTORC1 signaling (Dibble *et al.* 2012).

Several deletions also likely result in either no protein transcribed or truncated non-functional proteins. *Apeh*, an enzyme acylpeptide hydrolase which destroys oxidatively damaged proteins, contains a fixed 837 bp deletion of exon 13 (Gogliettino *et al.* 2014; Riccio *et al.* 2015). *Trim39*, which in humans has been shown to regulate cell cycle progression and DNA damage

responses, contains a fixed deletion of the last 254 bp of the last exon (Zhang *et al.* 2012b; a). However, *trim39* has undergone multiple rounds of duplications in several teleost lineages; in zebrafish it is known as *bloodthirsty* (*bty*) and is involved in erythropoiesis (Yergeau *et al.* 2005; van der Aa *et al.* 2009). There is also a 283 bp deletion containing the start codon for *slc25a42*, a mitochondrial transporter of coenzyme A (Fiermonte *et al.* 2009; Zallot *et al.* 2013). Mutations in *slc25a42* have been associated with mitochondrial myopathy and lactic acidosis in humans (Shamseldin *et al.* 2016; Iuso *et al.* 2019). *slc25a42* knockdowns in zebrafish cause severe muscle abnormalities that include dorsal curvature, bent tails, and early motor defects (Shamseldin *et al.* 2016).

Discussion

We document extreme inbreeding in the critically endangered Devils Hole pupfish and identify a set of candidate deleterious structural and SNP variants to inform future genetic restoration. We show that *C. diabolis* is highly inbred, more so than all other Death Valley and Ash Meadows desert pupfishes and nearly all other known examples of inbreeding in the wild. By sequencing a historical museum specimen, we found that inbreeding has recently increased 6% from 1980 levels as the population has declined due to the presence of large (4 – 10 Mb) ROHs. Longer ROHs tend to have higher densities of deleterious alleles and larger fitness effects (Stoffel *et al.* 2021a). Although we were unable to directly measure fitness, the increase in inbreeding of 6% from 1980 to 2008 – 2012 likely resulted in a substantial reduction in fitness. Indeed, previous studies have suggested that increases in F_{ROH} have strong negative effects on fitness; in Soay sheep an increase in F_{ROH} by 10% resulted in a 60% decline in fitness (Stoffel *et al.* 2021b) while

in helmeted honeyeaters, a 9% increase in homozygosity reduced lifetime reproductive success by 87-90% (Harrisson *et al.* 2019).

The already substantial amount of inbreeding seen as early as 1980 suggests that *C. diabolis* has likely frequently experienced population bottlenecks in the past prior to their recent population decline and bottlenecks. We know of at least one period of population decline that occurred in the late 1960s to early 1970s when increased groundwater extraction in Ash Meadows reduced water levels and jeopardized a shallow shelf in Devils Hole necessary for feeding and spawning (Deacon *et al.* 1995), leading to a landmark U.S. Supreme Court case over water rights (*Cappaert v. United States* 1976). While additional inbreeding has likely contributed to lower fitness and population decline, it is also important to note that population decline was amplified in 2004 by extreme rain that scoured the shallow spawning shelf in Devils Hole and resulted in the accidental trapping deaths of at least 72 individuals due to a flash flood (Beissinger 2014; Echelle and Echelle 2020). The lek-breeding system of *Cyprinodon* pupfishes also likely produces substantial reproductive skew every generation (Arnold 1972; Kodric-Brown 1977).

C. diabolis harbors higher genetic load than other desert pupfishes for nearly all classes of variation. However, load was generally not significantly different among *Cyprinodon diabolis* / *nevadensis* / *salinus* with the exception of the number of homozygous derived LOF genotypes, for which *C. diabolis* had significantly more than *C. nevadensis*. This implies that *C. diabolis* has a substantial genetic load especially if the LOF variants are recessive and strongly deleterious, and that extinction risk likely remains high (Kyriazis *et al.* 2021). The number of homozygous derived genotypes for LOF variants quantifies load under a recessive model (Henn *et al.* 2015, 2016), which is appropriate given that selection and dominance coefficients are generally

inversely related (Simmons and Crow 1977; Agrawal and Whitlock 2012). Our evidence of higher genetic load in *C. diabolis* supports previous hypotheses of high load in this species due to its isolation and small population size, short generation time (Chernoff 1985; Deacon *et al.* 1995), and the observation that a refuge population of *C. diabolis* that was invaded by a few *C. nevadensis* individuals experienced rapid shifts in the gene pool to *C. nevadensis* alleles, leading to speculations of mutational meltdown (Lynch *et al.* 1995; Martin *et al.* 2012). Although recent studies have suggested that small populations generally have lower load (Xue *et al.* 2015; Robinson *et al.* 2018; Grossen *et al.* 2020), purging is expected to occur over long evolutionary timeframes and thus populations that have experienced recent severe population contraction are likely to have relatively high genetic load as deleterious variants reach fixation before purging removes them, particularly if prior population sizes were much larger (van der Valk *et al.* 2021).

Degradation of hypoxia and reproductive pathways in C. diabolis

We found deleterious variants associated with reproduction and hypoxia and mitochondria stress genes. In particular, we found LOF and non-synonymous SNPs associated with sperm morphology and function (*cfap43*, *itprid2*). 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 close to the time of extinction had accumulated more homozygous deletions (Rogers and Slatkin 2017), suggesting that they may be an understudied genetic threat to endangered populations (Díez-Del-Molino *et al.* 2018). We found that unique, fixed deletions in *C. diabolis* tended to be associated with hypoxia and mitochondrial stress, a known environmental stressor in the system. Our results suggests that *C. diabolis* is poorly equipped to physiologically deal with the stress of low dissolved oxygen in

Devils Hole and that putatively deleterious mutations in key genes likely hamper reproduction and population growth. Inbreeding depression often influences phenotypes such as survival, reproduction, and resistance to disease and environmental stress (Keller and Waller 2002); previous studies noted that *C. diabolis* has low fecundity (James 1969; Liu, R.K. 1969), low egg viability and juvenile survivorship (Deacon *et al.* 1995), and lays more eggs at lower temperatures (28 °C) compared to the higher constant temperature of 33 °C in Devils Hole (Burg *et al.* 2019). These results highlight the importance of investigating deletions and structural variants to more comprehensively understand deleterious variation in endangered populations.

Adaptation to the Devils Hole environment?

However, we cannot rule out that some of these fixed variants are potentially the result of local adaptation, as it is extremely difficult to distinguish between selection and strong genetic drift in small populations because both processes leave similar signatures in allele frequencies (Leigh *et al.* 2021). One possibility is that repeated adaptations have accumulated in hypoxia pathways for the unique selective environment of Devils Hole relative to all other springs. However, we think it more likely that after the first LOF deletion disabled the entire pathway, additional LOF mutations accumulated without functional effects in a neutral manner. Future transcriptomic work will improve our understanding of how functional hypoxia pathways are in *C. diabolis*.

Did mutational meltdown cause the recent population decline?

The DHP population began to decline in the 1990s from historical populations sizes of 200 – 500 individuals to population sizes of less than 100 each year for still unknown reasons. One possible explanation is that changes in environmental conditions, such as reduced nutrient inputs due to

the elimination of wild sheep grazing around Devils Hole after the installation of razor wire fencing around the perimeter. Historical accounts of Devils Hole report thick mats of green macroalgae possibly due to ample manure, rather than the current ecosystem dominated by less-nutritious cyanobacterial mats and possibly decreased dissolved oxygen levels (Madinger *et al.* 2016). Alternatively, previous genetic analyses have speculated that the population has a high genetic load that has led to “mutational meltdown” based on dramatic shifts towards *C. nevadensis* ancestry in a *C. diabolis* x *C. nevadensis* hybrid refuge population (Martin *et al.* 2012). Our historical and modern analyses suggest that severe inbreeding existed before population decline in the 1990s, but that inbreeding and its negative impacts on the population’s fitness has likely been exacerbated due to recent population declines.

Conclusion

Our study adds to a growing number of studies of inbreeding in wild populations in which deleterious mutations have been predicted from sequencing data (Robinson *et al.* 2019; Grossen *et al.* 2020). These studies also indicate that inbreeding depression is stronger in the wild than previously thought. The demographic history and genome-wide measures of genetic load and deleterious variation in the Devils Hole pupfish suggest that the population remains in danger. Our estimates of effective population sizes based on the harmonic mean of biannual census data ($N_e^{\text{Spring}} = 122$, $N_e^{\text{Fall}} = 209$) suggest that *C. diabolis* has an effective size far below suggested minimums for the maintenance of sufficient genetic variation for adaptive capacity (Franklin 1980; Reed *et al.* 2003; Traill *et al.* 2007). Further genomic sampling in the future will better inform our understanding of the mutation load and the genetic basis of inbreeding depression in this iconic conservation symbol.

Methods

Samples and sequencing

We sequenced 44 whole genomes that encompass *C. diabolis*, and many of the *C. nevadensis* and *C. salinus* Death Valley pupfishes spanning multiple independent springs, along with several further removed outgroups from various areas of California, Arizona, and Mexico. Of the 23 *C. diabolis* genomes, 8 were from historical museum samples, spanning 1937 – 1980 while the rest were collected between 2008 - 2012. Given the critically endangered status of *C. diabolis*, NPS and USFWS staff collected dead specimens found during routine checks in this period. *C. nevadensis*, *C. salinus*, and outgroup samples were collected in the early 1990s (Duvernell and Turner 1998). Samples were sequenced to 10x coverage on 150 PE runs using an Illumina Novaseq. Historical and several degraded *C. diabolis* tissues were prepared using Swiftprep library kits. For sampling dates, see extended data.

Alignment and filtering

Raw reads were mapped from 44 individuals to the *Cyprinodon brontotheroides* reference genome (v 1.0; total sequence length = 1,162,855,435 bp; scaffold N50 = 32 Mbp) with bwa-mem (v 0.7.12; (Li 2013)). Duplicate reads were identified using MarkDuplicates and BAM indices were created using BuildBamIndex in the Picard software package (<http://picard.sourceforge.net>; v 2.0.1). We followed the best practices guide recommended in the Genome Analysis Toolkit (v 3.5; (DePristo *et al.* 2011)) 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 (i.e. QD < 2.0; FS > 60; MQ < 40; MQRankSum < - 12.5; ReadPosRankSum < -8 because we lacked high-quality known variants.

390 Variants in poorly mapped regions were removed with a mask file generated from the program
391 SNPable ((Li 2009); k-mer length =50, and ‘stringency’=0.5). Variants were then filtered by
392 minimum (3) and maximum (315) depth based on recommendations (Li 2014). Variants were
393 additionally filtered to remove SNPs with a minor allele frequency below 0.05, genotype quality
394 below 20, or containing more than 50% missing data across all individuals at the site using
395 bcftools (v 1.6; (Li 2011)) and vcftools (v.0.1.15; (Danecek *et al.* 2011)). Our flexible threshold
396 of 50% missing data was necessary given the degraded nature of several samples. Our final
397 dataset contained 6,295,414 SNPs.

399 **Population structure**

400 We pruned SNPs in strong linkage disequilibrium using the LD pruning function (--indep-
401 pairwise 50 5 0.5) in PLINK (v 1.9; (Purcell *et al.* 2007)) leaving 1.6 million variants. We then
402 characterized population structure with two approaches. First, we used PLINK to conduct
403 principal component analysis. Second, we used ADMIXTURE (v.1.3.0; (Alexander *et al.* 2009))
404 to assign individuals to variable numbers of population clusters (K = 1-20). We used the subset
405 parameter in PLINK to randomly select 100,000 SNPs for analysis. F_{st} between *C. diabolis* / *C.*
406 *nevadensis* and *C. salinus* was calculated genome-wide per variant site on the 6.3 million variant
407 dataset using the weir-fst-pop function in vcftools (v.0.1.15; (Danecek *et al.* 2011)).

408 Several samples were excluded from downstream analyses due to a low percentage of
409 reads mapping to the reference genome and properly paired reads, presumably due to
410 degradation. These include 7 out of 8 historical samples sequenced with the exception of one
411 high quality sample (DHP1980-5). Several contemporary samples (DHP54904, DHP54908,
412 DHP54910, DHP54914) were also excluded due to low quality. DHP54909 was excluded

because it did not cluster with the remaining nine *C. diabolis* samples after observing the PCA. DHP54908, DHP54909, and DHP54910 were all collected during the summer of 2009 which may have led to degradation due to the summer heat in Death Valley. The genotypes of unique fixed deleterious variants and deletions were typically invariant or missing in degraded samples. Missing deletions and invariant sites among these samples may be due to degraded samples being more susceptible to contamination by environmental pupfish DNA during the amplification step of sequencing. For DHP54920, a duplicate genome was sequenced, and we only included DHP54920_N2 (higher coverage) in these analyses. We removed DHP54911 due to too much missing data (> 89%).

Out of 8 historical museum samples only one sample from 1980 (DHP1980-5) was successfully sequenced. While reads were obtained from the remaining historical samples, these samples had a low percentage of reads mapping and properly paired reads and tended to be low coverage. This 1980 sample clusters with our high quality 2008 – 2012 samples.

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 (N_e^{Spring}) and fall (N_e^{Fall}) population counts over time.

Runs of homozygosity

Runs of homozygosity (ROHs) were identified using the BCFtools/ROH command, which uses a hidden Markov model to identify autozygous portions of the genome from genetic variation data (Narasimhan *et al.* 2016). For each species within the Death Valley species complex, we

analyzed samples with at least 8X coverage. Samples were run individually and allele frequency was estimated from the AC and AN values within the INFO field of the VCF file. To calculate the cumulative fraction of the genome consisting of ROHs, the identified ROHs were sorted, summed, and divided by the total size of the genome (1,162,855,435 bp). ROHs with a size below 100 kb or a quality score below 50 were filtered out. We calculated the proportion of the genome that was made up of ROHs at least 100 kb long (F_{ROH}) for Death Valley samples with at least 8X coverage.

Timing of inbreeding

We estimated the mean number of generations back to the common ancestor of homologous sequences with the following equation: $g = 100 / 2 * ROH \text{ length (cM)}$, where g is the number of generations (Thompson 2013). Here we assume an average recombination rate across the genome of ~ 4.6 cM/Mb based on an estimated genetic map length of 5330 cM (St. John *et al.* 2021) and genome length of 1.16 Gb in *C. brontotheroides* (Richards *et al.* 2021).

Measuring genetic load

We categorized mutations in coding regions with regards to their effect on the amino acid sequence and whether the alleles were ancestral or derived with respect to the *C. brontotheroides* genome as the reference outgroup. We used Snpeff (Cingolani *et al.* 2012) to identify variants as either loss-of-function (LOF) or non-synonymous (NSYN) to measure genetic load. LOF variants were conservatively defined as SNPs that resulted in a premature stop codon following (Robinson *et al.* 2016, 2018), which are expected to be less prone to misannotation (de Valles-Ibáñez *et al.* 2016). We did not consider splice variants due to possible transcript differences

across *Cyprinodon* species. We also did not include stop loss nor start loss variants in our measurements of load as stop loss variants are expected to have less important phenotypic effects (Richards *et al.* 2015) and start loss variants are difficult to assess due to potential alternative translational initiation at other start sites. Genetic load was measured in terms of three proxies: number of variants, number of derived alleles, and number of derived homozygous genotypes. The number of derived alleles per individual can be used to quantify load under an additive model while the number of derived homozygous genotypes can be used to quantify load under a recessive model. Results were scaled by the number of genotyped sites per individual to account for varying coverage. We assessed whether differences in measures of genetic load among the four species groups were significant using an ANOVA in R and used pairwise Tukey's HSD tests in R to test whether inter-species measurements of load differed.

Unique deleterious variants:

We used the following criteria to identify unique putatively deleterious variants: (i) homozygous, (ii) predicted to be loss-of-function or deleterious based on a Snpeff predicted impact of "HIGH" (Cingolani *et al.* 2012) (start codon loss, stop codon gain, stop codon loss, splice acceptor site variant, splice donor site variant) and (iii) present in at least our five higher quality *C. diabolis* samples (including our 1980 sample) and absent in all other non-*C. diabolis* samples in our dataset. To find unique variants that may be responsible for increased inbreeding depression post 1980, we also identified loss-of-function and non-synonymous variants that were invariant in *C. nevadensis*, *C. salinus*, and outgroups, but heterozygous in DHP1980-5 and homozygous derived in at least the higher quality more recent 2008-2012 *C. diabolis* samples.

Unique deletions

We identified structural variation in the form of deletions that were unique to *C. diabolis* using DELLY (v0.8.3) (Rausch *et al.* 2012) following similar criteria as above (i and iii). We searched for deletions that were present in DHP1980-5, DHP54903, DHP54913, DHP54917, or DIAB54919, and not present in non-*C. diabolis* samples. Deletions that were exceptionally large and presumably artifacts were checked for accuracy in IGV and subsequently removed. We focused our attention on deletions within 2 kb of an annotated gene, based on annotations of the *C. brontotheroides* genome (Richards *et al.* 2021). Deletions were confirmed to be unique to *C. diabolis* by aligning bam files spanning the deletion in both *C. diabolis* and non-*C. diabolis* samples and confirming presence or absence, respectively. We analyzed whether deletions spanned exons, introns, or regulatory regions by blasting deleted sequences against the C_variegatus-1.0 (GCF_000732505.1) assembly on Ensembl (release 102; (Howe *et al.* 2021)).

Figure 1. Geography and genetic structure of desert pupfishes

A) Photo of male *C. diabolis* individual by Olin Feuerbacher. B). Principle component analysis of desert pupfishes revealing genetic population structure. C) Map of Death Valley NP and Ash Meadows NWR sampling locations. Colors in PCA designate individuals from different species. D) Ancestry proportions across individuals in Death Valley NP, Ash Meadows NWR, and outgroup desert pupfishes estimated from a LD-pruned SNP dataset in ADMIXTURE with $k = 4$.

Figure 2. 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. F_{ROH} was calculated for all Death Valley pupfish individuals with at

least 8X coverage. B) Biannual census counts of *C. diabolis* population size over time. Bottlenecks in 2007 and 2013 reduced the population size down to low as 38 and 35 individuals, respectively. C) Distribution of ROH lengths across *C. diabolis* samples from different time points. Greater values of F_{ROH} among recent *C. diabolis* samples relative to 1980 can be primarily attributed to large ROHs (4 – 10 Mb) that are predicted to have arisen recently due to inbreeding within the last 1-3 generations, corresponding with the 2007 bottleneck.

Figure 3. Genetic load across *Cyprinodon* species

The number of synonymous, non-synonymous, and loss-of-function mutations per site, per individual. Genetic load was measured in terms of three proxies: number of variants, number of

derived alleles, and number of derived homozygous genotypes. The number of derived alleles per individual can be used to quantify load under an additive model while the number of derived homozygous genotypes can be used to quantify load under a recessive model. Results were scaled by the number of genotyped sites per individual to account for varying coverage. Loss-of-function mutations are those that encode a premature stop codon. Letters indicate which pairwise comparisons using a Tukey's HSD test were significant.

Gene	Function	Location	Size (bp)	Frequency
<i>adamts15</i>	O-linked glycosylation	deletion in intron 7	55	5/5
<i>apeh</i>	Destroys oxidatively-damaged proteins	exon 13	837	7/7
<i>cnksr2</i>	MAPK/RAS signaling	intron	53	7/7
<i>cyp2a10</i>	Catalyzes the oxygenation of substrates	deletes exon 5/6	980	4/4
<i>fbln7</i>	Angiogenesis in human umbilical vein endothelial cells	7 bp of exon 3, frameshift	51	7/7
<i>fzd1</i>	Regulates cumulus expansion genes, required for normal female fertility in mice	360 bp downstream	66	6/6
<i>nkx2_4</i>	Homeobox protein	deletes 3' UTR	269	6/6
<i>slc25a42</i>	Mitochondrial transporter of coenzyme A	start codon and TATA box in promotor	283	6/6
<i>st6galnac1</i>	Glycosylation of proteins	deletes entire exon 4 and large chunk of intron 3	2747	6/6
<i>tmem206</i>	Acid-chloride channel, under selection in Tibetans for adaptation to high altitude	intron 4	346	7/7
<i>trim39</i>	Erythropoiesis in zebrafish	254 bp of last exon including stop codon, 3'	854	5/5

		UTR		
<i>redd1</i>	Suppresses protein translation to conserve energy	TATA box and hypoxia response element in promotor	81	6/6
<i>rnf220</i>	E3 ubiquitin ligase	1313 bp downstream	139	7/7
<i>skil</i>	Component of SMAD pathway, which regulates cell growth and differentiation.	1830 bp downstream	230	5/5
<i>slc8a1</i>	Required for normal embryonic heart development	1477 bp downstream	61	6/6

Table 1: Unique deletions in *C. diabolis* tend to be involved in hypoxia

Frequencies are based on samples for which there is enough coverage to determine whether the deletion is present or absent. Highlighted genes have functions related to hypoxia and mitochondrial stress. See extended data for details on intermediate scenarios where random spurious reads span the deletion or there is not enough coverage to make a definitive call of deletion presence or absence.

Author Contributions

DT wrote the manuscript and conducted all analyses. BJT and CHM collected the samples. DT 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.

541

542 **Data and materials availability**

543 Genomic sequence data will be archived at NCBI. Additional data will be deposited on the
544 Dryad Digital Repository. All scripts are available on Github (<https://github.com/tiandavid/>).

545

546 **Acknowledgements**

547 We thank members of the Martin lab for valuable comments and discussions; Olin Feuerbacher
548 for providing a photo of *C. diabolis*; T. Echelle for providing specimens of *C. n. pectoralis* from
549 Indian Spring; Lee Simons, U.S. Fish and Wildlife Service, and National Park Service for *C.*
550 *diabolis* samples and permits; and the University of California, Berkeley for computational
551 resources. This work was funded by the U.S. Fish and Wildlife Service, National Park Service,
552 National Science Foundation DEB CAREER grant #1749764, National Institutes of Health grant
553 5R01DE027052-02, and the University of California, Berkeley to CHM. DT was supported by a
554 National Science Foundation Graduate Research Fellowship (DGE 1752814).

555

556 **References**

557 Aa L. M. van der, J.-P. Levraud, M. Yahmi, E. Lauret, V. Briolat, *et al.*, 2009 A large new subset
558 of TRIM genes highly diversified by duplication and positive selection in teleost fish.
559 BMC Biol 7: 7. <https://doi.org/10.1186/1741-7007-7-7>

560 Agrawal A. F., and M. C. Whitlock, 2012 Mutation Load: The Fitness of Individuals in
561 Populations Where Deleterious Alleles Are Abundant. Annual Review of Ecology,
562 Evolution, and Systematics 43: 115–135. [https://doi.org/10.1146/annurev-ecolsys-](https://doi.org/10.1146/annurev-ecolsys-110411-160257)
563 [110411-160257](https://doi.org/10.1146/annurev-ecolsys-110411-160257)

- 564 Alexander D. H., J. Novembre, and K. Lange, 2009 Fast model-based estimation of ancestry in
565 unrelated individuals. *Genome Res* 19: 1655–1664.
566 <https://doi.org/10.1101/gr.094052.109>
- 567 Anthony A. Echelle and Alice F. Echelle, 2020 Cyprinodontidae: Pupfishes, in *Freshwater*
568 *Fishes of North America Characidae to Poeciliidae*, Johns Hopkins University Press.
- 569 Armstrong E. E., A. Khan, R. W. Taylor, A. Gouy, G. Greenbaum, *et al.*, 2021 Recent
570 Evolutionary History of Tigers Highlights Contrasting Roles of Genetic Drift and
571 Selection. *Molecular Biology and Evolution* 38: 2366–2379.
572 <https://doi.org/10.1093/molbev/msab032>
- 573 Barts, N., 2020 Comparative physiology and the predictability of evolution in extreme
574 environments
- 575 Behrens M., O. Frank, H. Rawel, G. Ahuja, C. Potting, *et al.*, 2014 ORA1, a zebrafish olfactory
576 receptor ancestral to all mammalian V1R genes, recognizes 4-hydroxyphenylacetic acid,
577 a putative reproductive pheromone. *J Biol Chem* 289: 19778–19788.
578 <https://doi.org/10.1074/jbc.M114.573162>
- 579 Beissinger S. R., 2014 Digging the pupfish out of its hole: risk analyses to guide harvest of
580 Devils Hole pupfish for captive breeding. *PeerJ* 2. <https://doi.org/10.7717/peerj.549>
- 581 Bell D. A., Z. L. Robinson, W. C. Funk, S. W. Fitzpatrick, F. W. Allendorf, *et al.*, 2019 The
582 Exciting Potential and Remaining Uncertainties of Genetic Rescue. *Trends in Ecology &*
583 *Evolution* 34: 1070–1079. <https://doi.org/10.1016/j.tree.2019.06.006>

584 Bi K., T. Linderroth, D. Vanderpool, J. M. Good, R. Nielsen, *et al.*, 2013 Unlocking the vault:
585 next-generation museum population genomics. *Molecular Ecology* 22: 6018–6032.
586 <https://doi.org/10.1111/mec.12516>

587 Brugarolas J., K. Lei, R. L. Hurley, B. D. Manning, J. H. Reiling, *et al.*, 2004 Regulation of
588 mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor
589 complex. *Genes Dev* 18: 2893–2904. <https://doi.org/10.1101/gad.1256804>

590 Burg G. C., J. Johnson, S. Spataro, A. O’Keefe, N. Urbina, *et al.*, 2019 Care and propagation of
591 captive pupfish from the genus *Cyprinodon*: insight into conservation. *Environ Biol Fish*
592 102: 1015–1024. <https://doi.org/10.1007/s10641-019-00887-2>

593 *Cappaert v. United States*, 1976

594 Charlesworth B., and D. Charlesworth, 1999 The genetic basis of inbreeding depression. *Genet*
595 *Res* 74: 329–340. <https://doi.org/10.1017/s0016672399004152>

596 Charlesworth D., and J. H. Willis, 2009 The genetics of inbreeding depression. *Nat Rev Genet*
597 10: 783–796. <https://doi.org/10.1038/nrg2664>

598 Chernoff B., 1985 Population dynamics of the Devils Hole pupfish. *Environ Biol Fish* 13: 139–
599 147. <https://doi.org/10.1007/BF00002582>

600 Cingolani P., A. Platts, L. L. Wang, M. Coon, T. Nguyen, *et al.*, 2012 A program for annotating
601 and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly (Austin)* 6:
602 80–92. <https://doi.org/10.4161/fly.19695>

603 Coutton C., A. S. Vargas, A. Amiri-Yekta, Z.-E. Kherraf, S. F. Ben Mustapha, *et al.*, 2018
604 Mutations in CFAP43 and CFAP44 cause male infertility and flagellum defects in
605 Trypanosoma and human. Nat Commun 9: 686. [https://doi.org/10.1038/s41467-017-](https://doi.org/10.1038/s41467-017-02792-7)
606 02792-7

607 Danecek P., A. Auton, G. Abecasis, C. A. Albers, E. Banks, *et al.*, 2011 The variant call format
608 and VCFtools. Bioinformatics 27: 2156–2158.
609 <https://doi.org/10.1093/bioinformatics/btr330>

610 Deacon J. E., F. R. Taylor, and J. W. Pedretti, 1995 Egg viability and ecology of Devils Hole
611 pupfish: Insights from captive propagation. The Southwestern Naturalist 40: 216–223.

612 DePristo M. A., E. Banks, R. Poplin, K. V. Garimella, J. R. Maguire, *et al.*, 2011 A framework
613 for variation discovery and genotyping using next-generation DNA sequencing data. Nat
614 Genet 43: 491–498. <https://doi.org/10.1038/ng.806>

615 Der Sarkissian C., L. Ermini, M. Schubert, M. A. Yang, P. Librado, *et al.*, 2015 Evolutionary
616 Genomics and Conservation of the Endangered Przewalski’s Horse. Curr Biol 25: 2577–
617 2583. <https://doi.org/10.1016/j.cub.2015.08.032>

618 DeYoung M. P., P. Horak, A. Sofer, D. Sgroi, and L. W. Ellisen, 2008 Hypoxia regulates
619 TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3
620 shuttling. Genes Dev 22: 239–251. <https://doi.org/10.1101/gad.1617608>

621 Dibble C. C., W. Elis, S. Menon, W. Qin, J. Klekota, *et al.*, 2012 TBC1D7 is a third subunit of
622 the TSC1-TSC2 complex upstream of mTORC1. Mol Cell 47: 535–546.
623 <https://doi.org/10.1016/j.molcel.2012.06.009>

624 Díez-Del-Molino D., F. Sánchez-Barreiro, I. Barnes, M. T. P. Gilbert, and L. Dalén, 2018
625 Quantifying Temporal Genomic Erosion in Endangered Species. *Trends Ecol Evol* 33:
626 176–185. <https://doi.org/10.1016/j.tree.2017.12.002>

627 Duvernell D. D., and B. J. Turner, 1998 Evolutionary genetics of Death Valley pupfish
628 populations: mitochondrial DNA sequence variation and population structure. *Molecular*
629 *Ecology* 7: 279–288. <https://doi.org/10.1046/j.1365-294X.1998.00347.x>

630 Duvernell D. D., and B. J. Turner, 1999 Variation and Divergence of Death Valley Pupfish
631 Populations at Retrotransposon-Defined Loci. *Molecular Biology and Evolution* 16: 363–
632 371. <https://doi.org/10.1093/oxfordjournals.molbev.a026117>

633 Echelle A. A., and T. E. Dowling, 1992 MITOCHONDRIAL DNA VARIATION AND
634 EVOLUTION OF THE DEATH VALLEY PUPFISHES (CYPRINODON,
635 CYPRINODONTIDAE). *Evolution* 46: 193–206. <https://doi.org/10.1111/j.1558-5646.1992.tb01994.x>

637 Echelle A. A., and A. F. Echelle, 1993 Allozyme Perspective on Mitochondrial DNA Variation
638 and Evolution of the Death Valley Pupfishes (Cyprinodontidae: Cyprinodon). *Copeia*
639 1993: 275–287. <https://doi.org/10.2307/1447128>

640 E.T. Arnold, 1972 Behavioral Ecology of two pupfishes (Cyprinodontidae, Genus Cyprinodon)
641 from northern Mexico.

642 Fiermonte G., E. Paradies, S. Todisco, C. M. T. Marobbio, and F. Palmieri, 2009 A novel
643 member of solute carrier family 25 (SLC25A42) is a transporter of coenzyme A and

644 adenosine 3',5'-diphosphate in human mitochondria. J Biol Chem 284: 18152–18159.
645 <https://doi.org/10.1074/jbc.M109.014118>

646 Frankham R., 1995 Conservation Genetics. Annual Review of Genetics 29: 305–327.
647 <https://doi.org/10.1146/annurev.ge.29.120195.001513>

648 Franklin I. R., 1980 Evolutionary change in small populations., pp. 135–150 in *Conservation*
649 *Biology: an Evolutionary–Ecological Perspective*, Sunderland, Massachusetts, Sinauer
650 Associates, U.S.A.

651 Gogliettino M., A. Riccio, M. Balestrieri, E. Cocca, A. Facchiano, *et al.*, 2014 A novel class of
652 bifunctional acylpeptide hydrolases--potential role in the antioxidant defense systems of
653 the Antarctic fish *Trematomus bernacchii*. FEBS J 281: 401–415.
654 <https://doi.org/10.1111/febs.12610>

655 Grossen C., F. Guillaume, L. F. Keller, and D. Croll, 2020 Purging of highly deleterious
656 mutations through severe bottlenecks in Alpine ibex. Nature Communications 11: 1001.
657 <https://doi.org/10.1038/s41467-020-14803-1>

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

661 Hansson B., and L. Westerberg, 2002 On the correlation between heterozygosity and fitness in
662 natural populations. Mol Ecol 11: 2467–2474. [https://doi.org/10.1046/j.1365-](https://doi.org/10.1046/j.1365-294x.2002.01644.x)
663 [294x.2002.01644.x](https://doi.org/10.1046/j.1365-294x.2002.01644.x)

664 Harrisson K. A., M. J. L. Magrath, J. D. L. Yen, A. Pavlova, N. Murray, *et al.*, 2019 Lifetime
665 Fitness Costs of Inbreeding and Being Inbred in a Critically Endangered Bird. *Current*
666 *Biology* 29: 2711-2717.e4. <https://doi.org/10.1016/j.cub.2019.06.064>

667 Henn B. M., L. R. Botigué, C. D. Bustamante, A. G. Clark, and S. Gravel, 2015 Estimating the
668 mutation load in human genomes. *Nat Rev Genet* 16: 333–343.
669 <https://doi.org/10.1038/nrg3931>

670 Henn B. M., L. R. Botigué, S. Peischl, I. Dupanloup, M. Lipatov, *et al.*, 2016 Distance from sub-
671 Saharan Africa predicts mutational load in diverse human genomes. *Proc Natl Acad Sci*
672 U S A 113: E440-449. <https://doi.org/10.1073/pnas.1510805112>

673 Howe K. L., P. Achuthan, J. Allen, J. Allen, J. Alvarez-Jarreta, *et al.*, 2021 Ensembl 2021.
674 *Nucleic Acids Research* 49: D884–D891. <https://doi.org/10.1093/nar/gkaa942>

675 Iuso A., B. Alhaddad, C. Weigel, U. Kotzaeridou, E. Mastantuono, *et al.*, 2019 A Homozygous
676 Splice Site Mutation in SLC25A42, Encoding the Mitochondrial Transporter of
677 Coenzyme A, Causes Metabolic Crises and Epileptic Encephalopathy. *JIMD Rep* 44: 1–
678 7. https://doi.org/10.1007/8904_2018_115

679 James C. J., 1969 *Aspects of the Ecology of the Devil's Hole Pupfish (Cyprinodon Diabolis)*
680 *Wales*. University of Nevada, Las Vegas.

681 Javed A. A., and R. K. Naz, 1992 Human cleavage signal-1 protein; cDNA cloning, transcription
682 and immunological analysis. *Gene* 112: 205–211. <https://doi.org/10.1016/0378->
683 1119(92)90377-2

684 Jin Y., K. Jia, W. Zhang, Y. Xiang, P. Jia, *et al.*, 2019 Zebrafish TRIM25 Promotes Innate
685 Immune Response to RGNNV Infection by Targeting 2CARD and RD Regions of RIG-I
686 for K63-Linked Ubiquitination. *Front Immunol* 10: 2805.
687 <https://doi.org/10.3389/fimmu.2019.02805>

688 Johnson W. E., D. P. Onorato, M. E. Roelke, E. D. Land, M. Cunningham, *et al.*, 2010 Genetic
689 Restoration of the Florida Panther. *Science* 329: 1641–1645.
690 <https://doi.org/10.1126/science.1192891>

691 Kardos M., H. R. Taylor, H. Ellegren, G. Luikart, and F. W. Allendorf, 2016 Genomics advances
692 the study of inbreeding depression in the wild. *Evolutionary Applications* 9: 1205–1218.
693 <https://doi.org/10.1111/eva.12414>

694 Kardos M., E. Armstrong, S. Fitzpatrick, S. Hauser, P. Hedrick, *et al.*, 2021 *The crucial role of*
695 *genome-wide genetic variation in conservation.*

696 Keller L. F., and D. M. Waller, 2002 Inbreeding effects in wild populations. *Trends in Ecology*
697 *& Evolution* 17: 230–241. [https://doi.org/10.1016/S0169-5347\(02\)02489-8](https://doi.org/10.1016/S0169-5347(02)02489-8)

698 Kimura M., 1957 Some Problems of Stochastic Processes in Genetics. *The Annals of*
699 *Mathematical Statistics* 28: 882–901. <https://doi.org/10.1214/aoms/1177706791>

700 Kodric-Brown A., 1977 Reproductive Success and the Evolution of Breeding Territories in
701 Pupfish (*Cyprinodon*). *Evolution* 31: 750–766. <https://doi.org/10.2307/2407437>

702 Kohn M. H., W. J. Murphy, E. A. Ostrander, and R. K. Wayne, 2006 Genomics and conservation
703 genetics. *Trends Ecol Evol* 21: 629–637. <https://doi.org/10.1016/j.tree.2006.08.001>

704 Kuo Y.-C., Y.-H. Lin, H.-I. Chen, Y.-Y. Wang, Y.-W. Chiou, *et al.*, 2012 SEPT12 mutations
705 cause male infertility with defective sperm annulus. *Human Mutation* 33: 710–719.
706 <https://doi.org/10.1002/humu.22028>

707 Kyriazis C. C., R. K. Wayne, and K. E. Lohmueller, 2021 Strongly deleterious mutations are a
708 primary determinant of extinction risk due to inbreeding depression. *Evolution Letters* 5:
709 33–47. <https://doi.org/10.1002/evl3.209>

710 Lee P., N. S. Chandel, and M. C. Simon, 2020 Cellular adaptation to hypoxia through hypoxia
711 inducible factors and beyond. *Nat Rev Mol Cell Biol* 21: 268–283.
712 <https://doi.org/10.1038/s41580-020-0227-y>

713 Leigh D. M., H. E. L. Lischer, F. Guillaume, C. Grossen, and T. Günther, 2021 Disentangling
714 adaptation from drift in bottlenecked and reintroduced populations of Alpine ibex.
715 *Molecular Ecology Resources* n/a. <https://doi.org/10.1111/1755-0998.13442>

716 Li H., 2009 SNPable Regions

717 Li H., 2011 A statistical framework for SNP calling, mutation discovery, association mapping
718 and population genetical parameter estimation from sequencing data. *Bioinformatics* 27:
719 2987–2993. <https://doi.org/10.1093/bioinformatics/btr509>

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

722 Li H., 2014 Toward better understanding of artifacts in variant calling from high-coverage
723 samples. *Bioinformatics* 30: 2843–2851. <https://doi.org/10.1093/bioinformatics/btu356>

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

726 Lynch M., J. Conery, and R. Bürger, 1995 Mutational Meltdowns in Sexual Populations.
727 Evolution 49: 1067–1080. <https://doi.org/10.1111/j.1558-5646.1995.tb04434.x>

728 Madinger H. L., K. P. Wilson, J. A. Goldstein, and M. J. Bernot, 2016 Biogeochemistry and
729 Nutrient Limitation of Microbial Biofilms in Devils Hole, Nevada. wnan 76: 53–71.
730 <https://doi.org/10.3398/064.076.0107>

731 Maeda M., H. Hasegawa, T. Hyodo, S. Ito, E. Asano, *et al.*, 2011 ARHGAP18, a GTPase-
732 activating protein for RhoA, controls cell shape, spreading, and motility. Mol Biol Cell
733 22: 3840–3852. <https://doi.org/10.1091/mbc.E11-04-0364>

734 Manuel M. de, R. Barnett, M. Sandoval-Velasco, N. Yamaguchi, F. Garrett Vieira, *et al.*, 2020
735 The evolutionary history of extinct and living lions. PNAS 117: 10927–10934.

736 Martin A. P., A. A. Echelle, G. Zegers, S. Baker, and C. L. Keeler-Foster, 2012 Dramatic shifts
737 in the gene pool of a managed population of an endangered species may be exacerbated
738 by high genetic load. Conserv Genet 13: 349–358. [https://doi.org/10.1007/s10592-011-](https://doi.org/10.1007/s10592-011-0289-7)
739 0289-7

740 Martin C. H., Crawford Jacob E., Turner Bruce J., and Simons Lee H., 2016 Diabolical survival
741 in Death Valley: recent pupfish colonization, gene flow and genetic assimilation in the
742 smallest species range on earth. Proceedings of the Royal Society B: Biological Sciences
743 283: 20152334. <https://doi.org/10.1098/rspb.2015.2334>

744 Martin C. H., S. Höhna, J. E. Crawford, B. J. Turner, E. J. Richards, *et al.*, 2017 The complex
745 effects of demographic history on the estimation of substitution rate: concatenated gene
746 analysis results in no more than twofold overestimation. *Proceedings of the Royal*
747 *Society B: Biological Sciences* 284: 20170537. <https://doi.org/10.1098/rspb.2017.0537>

748 Martin C. H., and S. Höhna, 2018 New evidence for the recent divergence of Devil's Hole
749 pupfish and the plausibility of elevated mutation rates in endangered taxa. *Molecular*
750 *Ecology* 27: 831–838. <https://doi.org/10.1111/mec.14404>

751 Martin C. H., and B. J. Turner, 2018 Long-distance dispersal over land by fishes: extremely rare
752 ecological events become probable over millennial timescales. *Proc Biol Sci* 285:
753 20172436. <https://doi.org/10.1098/rspb.2017.2436>

754 Melbourne B. A., and A. Hastings, 2008 Extinction risk depends strongly on factors contributing
755 to stochasticity. *Nature* 454: 100–103. <https://doi.org/10.1038/nature06922>

756 Miller R. R., 1948 The Cyprinodont fishes of the Death Valley system of eastern California and
757 southwestern Nevada. undefined.

758 Miller R. R., 1969 CONSERVATION OF FISHES OF THE DEATH VALLEY SYSTEM IN
759 CALIFORNIA AND NEVADA. Conservation of fishes of the Death VallTransactions of
760 the California-Nevada section of the Wildlife Society 107–22.

761 Miller, Robert R., 1981 *Coevolution of deserts and pupfishes (genus Cyprinodon) in the*
762 *American Southwest*, in Naiman, R. J., and Soltz, D. L., eds, *Fishes in North American*
763 *Deserts*.

764 Narasimhan V., P. Danecek, A. Scally, Y. Xue, C. Tyler-Smith, *et al.*, 2016 BCFtools/RoH: a
765 hidden Markov model approach for detecting autozygosity from next-generation
766 sequencing data. *Bioinformatics* 32: 1749–1751.
767 <https://doi.org/10.1093/bioinformatics/btw044>

768 Palkopoulou E., S. Mallick, P. Skoglund, J. Enk, N. Rohland, *et al.*, 2015 Complete genomes
769 reveal signatures of demographic and genetic declines in the woolly mammoth. *Curr Biol*
770 25: 1395–1400. <https://doi.org/10.1016/j.cub.2015.04.007>

771 Purcell S., B. Neale, K. Todd-Brown, L. Thomas, M. A. R. Ferreira, *et al.*, 2007 PLINK: A Tool
772 Set for Whole-Genome Association and Population-Based Linkage Analyses. *Am J Hum*
773 *Genet* 81: 559–575.

774 Rausch T., T. Zichner, A. Schlattl, A. M. Stütz, V. Benes, *et al.*, 2012 DELLY: structural variant
775 discovery by integrated paired-end and split-read analysis. *Bioinformatics* 28: i333–i339.
776 <https://doi.org/10.1093/bioinformatics/bts378>

777 Reed D. H., J. J. O’Grady, B. W. Brook, J. D. Ballou, and R. Frankham, 2003 Estimates of
778 minimum viable population sizes for vertebrates and factors influencing those estimates.
779 *Biological Conservation* 113: 23–34. [https://doi.org/10.1016/S0006-3207\(02\)00346-4](https://doi.org/10.1016/S0006-3207(02)00346-4)

780 Riccio A., M. Gogliettino, G. Palmieri, M. Balestrieri, A. Facchiano, *et al.*, 2015 A New APEH
781 Cluster with Antioxidant Functions in the Antarctic Hemoglobinless Icefish *Chionodraco*
782 *hamatus*. *PLoS One* 10: e0125594. <https://doi.org/10.1371/journal.pone.0125594>

783 Richards S., N. Aziz, S. Bale, D. Bick, S. Das, *et al.*, 2015 Standards and Guidelines for the
784 Interpretation of Sequence Variants: A Joint Consensus Recommendation of the

785 American College of Medical Genetics and Genomics and the Association for Molecular
786 Pathology. *Genet Med* 17: 405–424. <https://doi.org/10.1038/gim.2015.30>

787 Richards E. J., J. A. McGirr, J. R. Wang, M. E. S. John, J. W. Poelstra, *et al.*, 2021 A vertebrate
788 adaptive radiation is assembled from an ancient and disjunct spatiotemporal landscape.
789 *PNAS* 118. <https://doi.org/10.1073/pnas.2011811118>

790 Riggs A. C., and J. E. Deacon, 2002 Connectivity in Desert Aquatic Ecosystems: The Devils
791 Hole Story. 38.

792 Robinson J. A., D. Ortega-Del Vecchyo, Z. Fan, B. Y. Kim, B. M. vonHoldt, *et al.*, 2016
793 Genomic Flatlining in the Endangered Island Fox. *Current Biology* 26: 1183–1189.
794 <https://doi.org/10.1016/j.cub.2016.02.062>

795 Robinson J. A., C. Brown, B. Y. Kim, K. E. Lohmueller, and R. K. Wayne, 2018 Purging of
796 Strongly Deleterious Mutations Explains Long-Term Persistence and Absence of
797 Inbreeding Depression in Island Foxes. *Current Biology* 28: 3487–3494.e4.
798 <https://doi.org/10.1016/j.cub.2018.08.066>

799 Robinson J. A., J. Räikkönen, L. M. Vucetich, J. A. Vucetich, R. O. Peterson, *et al.*, 2019
800 Genomic signatures of extensive inbreeding in Isle Royale wolves, a population on the
801 threshold of extinction. *Science Advances* 5: eaau0757.
802 <https://doi.org/10.1126/sciadv.aau0757>

803 Robinson J. A., R. C. K. Bowie, O. Dudchenko, E. L. Aiden, S. L. Hendrickson, *et al.*, 2021
804 Genome-wide diversity in the California condor tracks its prehistoric abundance and
805 decline. *Current Biology* 0. <https://doi.org/10.1016/j.cub.2021.04.035>

806 Rogers R. L., and M. Slatkin, 2017 Excess of genomic defects in a woolly mammoth on Wrangel
807 island. PLOS Genetics 13: e1006601. <https://doi.org/10.1371/journal.pgen.1006601>

808 Sackett J. D., D. C. Huerta, B. R. Kruger, S. D. Hamilton-Brehm, and D. P. Moser, 2018 A
809 comparative study of prokaryotic diversity and physicochemical characteristics of Devils
810 Hole and the Ash Meadows Fish Conservation Facility, a constructed analog. PLOS ONE
811 13: e0194404. <https://doi.org/10.1371/journal.pone.0194404>

812 Shamseldin H. E., L. L. Smith, A. Kentab, H. Alkhalidi, B. Summers, *et al.*, 2016 Mutation of
813 the mitochondrial carrier SLC25A42 causes a novel form of mitochondrial myopathy in
814 humans. Hum Genet 135: 21–30. <https://doi.org/10.1007/s00439-015-1608-8>

815 Simmons M. J., and J. F. Crow, 1977 Mutations affecting fitness in drosophila populations.
816 Annu. Rev. Genet. 11: 49–78. <https://doi.org/10.1146/annurev.ge.11.120177.000405>

817 St. John M. E., J. C. Dunker, E. J. Richards, S. Romero, and C. H. Martin, 2021 Parallel genetic
818 changes underlie integrated craniofacial traits in an adaptive radiation of trophic
819 specialist pupfishes. bioRxiv 2021.07.01.450661.
820 <https://doi.org/10.1101/2021.07.01.450661>

821 Stoffel M. A., S. E. Johnston, J. G. Pilkington, and J. M. Pemberton, 2021a Mutation load
822 decreases with haplotype age in wild Soay sheep. Evolution Letters 5: 187–195.
823 <https://doi.org/10.1002/evl3.229>

824 Stoffel M. A., S. E. Johnston, J. G. Pilkington, and J. M. Pemberton, 2021b Genetic architecture
825 and lifetime dynamics of inbreeding depression in a wild mammal. Nat Commun 12:
826 2972. <https://doi.org/10.1038/s41467-021-23222-9>

827 Sun L., L. Zhao, L. Du, P. Zhang, M. Zhang, *et al.*, 2016 Identification of two novel mutations in
828 the GALNT3 gene in a Chinese family with hyperphosphatemic familial tumoral
829 calcinosis. *Bone Res* 4: 1–6. <https://doi.org/10.1038/boneres.2016.38>

830 Tang S., X. Wang, W. Li, X. Yang, Z. Li, *et al.*, 2017 Biallelic Mutations in CFAP43 and
831 CFAP44 Cause Male Infertility with Multiple Morphological Abnormalities of the Sperm
832 Flagella. *Am J Hum Genet* 100: 854–864. <https://doi.org/10.1016/j.ajhg.2017.04.012>

833 Teixeira J. C., and C. D. Huber, 2021 The inflated significance of neutral genetic diversity in
834 conservation genetics. *PNAS* 118. <https://doi.org/10.1073/pnas.2015096118>

835 The Universal Protein Resource (UniProt), 2008 *Nucleic Acids Res* 36: D190–D195.
836 <https://doi.org/10.1093/nar/gkm895>

837 Thompson E. A., 2013 Identity by Descent: Variation in Meiosis, Across Genomes, and in
838 Populations. *Genetics* 194: 301–326. <https://doi.org/10.1534/genetics.112.148825>

839 Traill L. W., C. J. A. Bradshaw, and B. W. Brook, 2007 Minimum viable population size: A
840 meta-analysis of 30 years of published estimates. *Biological Conservation* 139: 159–166.
841 <https://doi.org/10.1016/j.biocon.2007.06.011>

842 Valk T. van der, D. Díez-Del-Molino, T. Marques-Bonet, K. Guschanski, and L. Dalén, 2019
843 Historical Genomes Reveal the Genomic Consequences of Recent Population Decline in
844 Eastern Gorillas. *Curr Biol* 29: 165–170.e6. <https://doi.org/10.1016/j.cub.2018.11.055>

845 Valk T. van der, C. M. Gonda, H. Silegowa, S. Almanza, I. Sifuentes-Romero, *et al.*, 2020 The
846 Genome of the Endangered Dryas Monkey Provides New Insights into the Evolutionary

847 History of the Vervets. *Molecular Biology and Evolution* 37: 183–194.
848 <https://doi.org/10.1093/molbev/msz213>

849 Valk T. van der, M. de Manuel, T. Marques-Bonet, and K. Guschanski, 2021 Estimates of
850 genetic load suggest frequent purging of deleterious alleles in small populations. *bioRxiv*
851 696831. <https://doi.org/10.1101/696831>

852 Valles-Ibáñez G. de, J. Hernandez-Rodriguez, J. Prado-Martinez, P. Luisi, T. Marquès-Bonet, *et*
853 *al.*, 2016 Genetic Load of Loss-of-Function Polymorphic Variants in Great Apes.
854 *Genome Biol Evol* 8: 871–877. <https://doi.org/10.1093/gbe/evw040>

855 Wildt D. E., M. Bush, J. G. Howard, S. J. O'Brien, D. Meltzer, *et al.*, 1983 Unique Seminal
856 Quality in the South African Cheetah and a Comparative Evaluation in the Domestic Cat.
857 *Biology of Reproduction* 29: 1019–1025. <https://doi.org/10.1095/biolreprod29.4.1019>

858 Wildt D. E., S. J. O'Brien, J. G. Howard, T. M. Caro, M. E. Roelke, *et al.*, 1987a Similarity in
859 Ejaculate-Endocrine Characteristics in Captive Versus Free-Ranging Cheetahs of Two
860 Subspecies1. *Biology of Reproduction* 36: 351–360.
861 <https://doi.org/10.1095/biolreprod36.2.351>

862 Wildt D. E., M. Bush, K. L. Goodrowe, C. Packer, A. E. Pusey, *et al.*, 1987b Reproductive and
863 genetic consequences of founding isolated lion populations. *Nature* 329: 328–331.
864 <https://doi.org/10.1038/329328a0>

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

Xue Y., J. Prado-Martinez, P. H. Sudmant, V. Narasimhan, Q. Ayub, *et al.*, 2015 Mountain gorilla genomes reveal the impact of long-term population decline and inbreeding. *Science* 348: 242–245. <https://doi.org/10.1126/science.aaa3952>

Yergeau D. A., C. N. Cornell, S. K. Parker, Y. Zhou, and H. W. Detrich, 2005 bloodthirsty, an RBCC/TRIM gene required for erythropoiesis in zebrafish. *Developmental Biology* 283: 97–112. <https://doi.org/10.1016/j.ydbio.2005.04.006>

Zallot R., G. Agrimi, C. Lerma-Ortiz, H. J. Teresinski, O. Frelin, *et al.*, 2013 Identification of mitochondrial coenzyme a transporters from maize and Arabidopsis. *Plant Physiol* 162: 581–588. <https://doi.org/10.1104/pp.113.218081>

Zhang L., N.-J. Huang, C. Chen, W. Tang, and S. Kornbluth, 2012a Ubiquitylation of p53 by the APC/C inhibitor Trim39. *Proc Natl Acad Sci U S A* 109: 20931–20936. <https://doi.org/10.1073/pnas.1212047110>

Zhang L., Y. Mei, N. Fu, L. Guan, W. Xie, *et al.*, 2012b TRIM39 regulates cell cycle progression and DNA damage responses via stabilizing p21. *PNAS* 109: 20937–20942.

Supplemental Figures and Tables

Scaffold	Position	Ref/SNP	Variant Class	Gene	<i>C. diabolis</i> Frequency
1	25026331	G/T	Gained stop codon	<i>cfap43</i>	8/8
3	5977284	G/A	Gained stop codon	<i>lamb1</i>	6/6
4	13853843	C/A	Gained stop codon	<i>herc3</i>	6/6
20	7768	G/A	Splice acceptor site	<i>ankle2</i>	6/6

21	38986750	G/A	Gained stop codon	<i>git1</i>	7/7
26	9468645	G/A	Gained stop codon	<i>dhx57</i>	8/8
27	23697776	T/A	Gained stop codon	<i>acsf2</i>	6/6
34	34643729	G/A	Lost start codon	<i>ora1</i>	6/6
43	10030715	G/A	Gained stop codon	<i>rims1</i>	5/5
43	30333450	G/A	Gained stop codon	<i>protein of unknown function</i>	6/6
47	9410043	G/T	Gained stop codon	<i>neoverrucotoxin subunit beta</i>	6/6
53	23074581	A/C	Lost stop codon	<i>cyth3</i>	7/7
55	6497450	T/A	Lost start codon	<i>acan</i>	5/5
597	2816	A/T	Gained stop codon	<i>tbc1d7</i>	6/6
7768	3929	C/T	Splice donor site	<i>b4galnt1</i>	6/6
10811	499	G/A	Gained stop codon	<i>trim25</i>	5/5

Table S1. Unique, loss-of-function and deleterious variants in *C. diabolis*

Loss-of-function variants that are invariant (0/0 or ./.) in *C. nevadensis*, *C. salinus*, and outgroups, but variant in at least higher quality *C. diabolis* samples. The frequencies of variants among *C. diabolis* individuals with genotype information at the given site are shown. DHX57 is heterozygous in our 1980 sample. See extended data for more information on the specific genotypes of individuals.

Scaffold	Position	Ref/SNP	Variant Class	Gene	Contemporary <i>C. diabolis</i> Frequency
7	20355383	G/C	Non-synonymous	<i>arhgap18</i>	4/4
26	9468645	G/A	Gained stop codon	<i>dhx57</i>	7/7
37	1901551	G/A	Non-synonymous	<i>Protein of unknown function</i>	4/5
52	1780129, 1780297	C/T, G/A	Non-synonymous	<i>itprid2</i>	6/6, 6/6

52	12629951	G/A	Non-synonymous	<i>galnt3</i>	4/4
----	----------	-----	----------------	---------------	-----

Table S2. Unique, putative variants associated with inbreeding depression in *C. diabolis*

Loss-of-function and non-synonymous variants that are invariant (0/0 or ./.) in *C. nevadensis*, *C. salinus*, and outgroups, but heterozygous (0/1) in DHP1980-5 and homozygous derived (1/1) in at least the higher quality more recent 2008-2012 *C. diabolis* samples. The frequencies of variants among *C. diabolis* individuals with genotype information at the given site are shown. Note that there are two separate non-synonymous variants present in *itprid2*. See extended data for more information on the specific genotypes of individuals.