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Genetic and demographic insights into the decline of a captive population of the endangered Hawaiian tree snail *Achatinella fuscobasis* (Achatinellinae)

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Abstract

Hawaiian tree snails in the sub-family Achatinellinae have experienced drastic population declines and range reductions as a result of habitat destruction, over-collection and introduced predators. In 1991, 11 individuals from one of the last remaining populations of *Achatinella fuscobasis*, a federally listed species, were brought to the University of Hawaii's tree-snail captive-rearing facility. After increasing in population size to 441 snails in 2005, the population subsequently declined to only 120 snails by 2014, a 73% reduction. In this study we assessed demographic and genetic changes that occurred in the captive-bred population of *A. fuscobasis*. Demographic data were compared to genetic data collected from 11 microsatellite markers for the F_1 – F_3 generations to investigate the role bottleneck effects such as loss of genetic diversity and inbreeding may have had on the population decline. Demographic measures such as juvenile survival and survival to maturity remained constant for the F_1 and F_2 generations, and significantly decreased in the F_3 generation. There was no difference in genetic diversity measures such as allelic richness, gene diversity, and observed heterozygosity among generations, but all generations were characterized by low genetic diversity and high inbreeding. The drastic population decline appears to have been due to stochastic demographic dynamics characteristic of small populations, with bottleneck effects that occurred before the founding of the *ex situ* population acting in synergy with factors such as disease.

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Introduction

Island endemic species, isolated from the pressures of continental ecosystems, often evolve life history traits, behavior, and immune systems naïve to the presence of mainland predators and diseases (Blackburn et al. 2004, Fordham and Brook 2008, Paulay 1994). Achatinelline tree snails, endemic to the Hawaiian Islands are one such group. Late maturity (5–6 years), low fecundity (1–7 offspring per year) and long lifespans (>10 years) are characteristics of snails within this sub-family (Hadfield 1986, Hadfield et al. 1993, Hadfield and Miller 1989). Unfortunately, achatinelline tree snails have experienced rapid declines and massive range reductions over the last century due to historical habitat loss, predation and over-collection. Currently the more urgent and insidious threat comes from introductions of predators including rats, predatory snails, and chameleons (Hadfield 1986, Solem 1990, Hadfield et al.1993, Hadfield and Saufler 2009, Holland et al. 2010). In light of these declines, in 1981, the entire genus Achatinella, endemic to the island of O`ahu, was listed as Endangered under the US Endangered Species Act (US Fish and Wildlife Service 1993). The formal recognition of these declines elicited emergency conservation efforts that included the establishment of an *ex situ* captive rearing facility at the University of Hawai‘i at Mānoa (UH) (Hadfield et al. 2004). Since 1986 this facility has reared 20 species of Hawaiian tree snails with the ultimate goal of augmenting or reestablishing wild populations. However, heavy predation in remaining wild populations has prevented the release of captive-bred snails, and populations have remained in captivity for multiple generations. Despite initial success and for unknown reasons, most *ex situ* populations at the captive rearing facility have experienced drastic declines in recent years.

In 1991 a population of *Achatinella fuscobasis*, was surveyed in the summit region of Mt. Konahuanui in the southern Ko‘olau Mountains on O‘ahu. This small remnant population was under enormous pressure from introduced predators, so to safeguard it against extirpation a

sample of the population was brought to the lab for captive rearing. The captive population was founded with eleven snails and subsequently increased to 441 snails by 2004. Since 2005 the population has experienced a 73% reduction in size. This decline corresponded with declines in other species reared at the facility (Price and Hadfield 2014).

In this study we investigated potential contributions of demographic and genetic factors to the drastic population decline observed in *A. fuscobasis* between 2005 and 2014. Demographic trends in juvenile survival and survival to maturity were compared among four generations (F_1, F_2, F_3,F_4) for snails born in the captive population. Using 11 microsatellite markers, demographic trends for the first three generations ($F_1 – F_3$) were compared with genetic measures such as heterozygosity, inbreeding coefficient, and genetic diversity.

Materials and methods

Captive propagation methods

Eleven *A. fuscobasis* (10 adults, 1 sub-adult) were brought to the Hawaiian tree snail captive-rearing facility at the University of Hawai`i at Mānoa in 1991 under USFWS permit PRT-826600. In the captive-rearing facility all snails were kept in small plastic cages inside environmental chambers that mimicked environmental conditions experienced by snails in the wild (Hadfield et al. 2004). Cages were cleaned every other week, at which time freshly cut leafy branches from native snail host plants were added to provide grazing opportunities. In addition, snail diets were supplemented with a common native fungus (*Cladosporium* sp.) isolated from the leaves of wild host trees and cultured on calcium-supplemented agar. Through the years, as the abundance of *A. fuscobasis* individuals increased in the laboratory, juvenile snails were separated from adults and moved to separate cages.

During every cage cleaning, demographic data were recorded, including births and deaths, and the total numbers of juveniles (shell length < 9.5 mm), sub-adults (9.5 mm - lipped shell) and adults. Adults stop growing when they form a characteristic thickening around the aperture of their shells, here referred to as the lip. Lip formation is correlated with sexual maturity in achatinelline snails (Pilsbry and Cooke 1912–1914). Every snail that died in the lab was placed in a vial of 95% ETOH for long-term tissue preservation and storage. The species identity, date of death, laboratory population and shell length and width were recorded on each vial. Demographic data obtained from preserved snails were cross-checked with logbook data.

Tissue sample collection, DNA extraction and amplification

A small piece of tissue (10–20 mg) was cut from the foot of preserved, deceased snails with a sterile blade and placed in 100% ETOH in preparation for DNA extraction. Total cell DNA (tDNA) was extracted from all samples using a QIAGEN DNeasy blood and tissue kit, following manufacture's protocols for purification of tDNA from animal tissues (QIAGEN Inc., Valencia CA, USA). TDNA was eluted in 200 µl of QIAGEN's proprietary elution buffer and kept at -20^{o} C until amplification using Polymerase Chain Reaction (PCR).

All samples were genotyped at eleven microsatellite loci (Table 1). For eight previously-published loci (Erickson and Hadfield 2008), we followed the recommended PCR conditions with the addition of 10 µm bovine serum albumin (BSA) to each reaction to bind inhibitor molecules (Kreader 1996, Woide et al. 2010). We additionally amplified three novel primers using the same amplification protocol described in Erickson and Hadfield (2008) with optimized annealing temperatures (Table 2). Genotyping was conducted at the Center for Genomic, Proteomic, and Bioinformatic Research (CGPBR) at the University of Hawai'i at Mānoa. The software Peakscanner version 1.0 (Applied Biosystems 2006) was used to score alleles.

<< Table 1 near here>>

<< Table 2 near here>>

Demographic analysis

Demographic data were collected for 1,417 *A. fuscobasis* that died in the laboratory between 1991 and 2014. In addition, demographic data were collected for 119 live snails at the captive-rearing facility in 2014. Growth curves were used to estimate birth dates for living and deceased snails (Severns 1981, Price and Hadfield 2014). Birth dates were then used to calculate juvenile survival (individuals that lived to at least one year of age), and survival-to-maturity (proportion of individuals that reached sexual maturity). To assess trends in juvenile survival and survival-to-maturity we used the statistical package JMP version 10.0 (2012). Logistic regression analysis was used to test for changes in juvenile and adult survival over time. Chi-square analysis was used to test for changes in survival to maturity among generations.

Genetic analysis

Microsatellite genotypes were collected for 342 snails (27% of the deceased collection) that died in the years 1991 to 2010 ($F_1 – F_3$ generations). The program Microchecker was used to assess scoring problems and to estimate the frequency of null alleles (Van Oosterhout et al. 2004). Because homozygote excess can arise from null alleles (allele scoring, amplification problems, or alleles identical in size but not by decent) and inbreeding (alleles identical by decent), the program INEst was used in addition to Microchecker to independently estimate null allele frequencies. INEst, through an iterative process, simultaneously measures inbreeding and estimates null-allele frequencies providing a more accurate estimate when inbreeding is possible (Chybicki and Burczyk 2009). Using the combined null-allele estimates from both programs, we eliminated four loci from all downstream analyses that had null-allele estimates greater than 25%. The remaining seven alleles had lower estimated null-allele frequencies, were polymorphic, and were therefore used in all further analyses (Table 1).

Microsatellite data were divided into generations spanning 5.59 years using the equation $M - 1 + (1/(1 - v)) = T$ (M = maturation time, v = mean adult survival and T= generation time) (Nunney and Elam 1994). Within each generation the data were further divided into individuals that survived to maturity and those that did not. We were not able to recover DNA from the majority of individuals comprising the founding generation; therefore we did not include founders in this study.

Within each generation, for individuals surviving to maturity and those that did not, we calculated the inbreeding coefficient (F_{is}), observed heterozygosity (H_O), expected heterozygosity (H_E), and genetic diversity using the program Arlequin (Excoffier et al. 2005). Because data divisions had uneven sample sizes, rarified allelic richness (A_R) was calculated using the program HP Rare (Kalinowski 2004, Kalinowski 2005). Individual heterozygosity was calculated for each snail by tabulating the proportion of heterozygous loci per individual. Individual heterozygosity data were then arcsine transformed for analyses. Note the heterozygosity results displayed in table 3 are untransformed values. Allelic richness and individual heterozygosity were compared across generations with an analysis of variance. Within-generation allelic richness and individual heterozygosity of those that survived to maturity was compared to those that did not with t-tests, using the statistical software JMP version 10.0 (2012).

Results

Demographics

In 1991 the captive population of *A. fuscobasis* was founded with 11 individuals and grew at an average rate of 29% per year between 1991 and 2004. The population reached a peak of 441 individuals in 2004 and began a decline in 2005 that continued through the completion of

this study in 2014. The population has declined by 73% from its 2005 peak, resulting in 117 live individuals at the end of 2014 (Figure 1).

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Juvenile survival significantly decreased over time ($r^2 = 0.01$, $P < 0.0001$) and differed among generations (X^2 (3, 1536) = 47.57, P <0.0001). Juvenile survival was comparable in the F1 and F2 generations but dropped in the F3 generation and remained low through the F4 generation (Figure 2A).

Survival to maturity significantly decreased over time ($r^2 = 0.03$ P <.0001) and differed among generations (X^2 (3, 1330) = 57.17, P <0.0001). Survival to maturity was 55% and 53% in the F_1 and F_2 generations, respectively. In the F_3 generation survival to maturity dropped to 32% and finally to 23% in the F_4 generation (Figure 2B).

Genetics

Heterozygosity did not differ among the three generations examined in this study (F = 0.066, df = 2, P = 0.94) (Figure 2C). In the F_2 generation, there was no significant difference in heterozygosity (t = 0.91, P = 0.37) between those that survived to maturity, and those that did not. However, in the F_3 generation those that survived to maturity were less heterozygous than those that did not survive to maturity (t = 2.07, P = 0.041). This comparison was not conducted for the F_1 generation because all genetic samples came from individuals that survived to maturity.

<<Figure 2 near here>>

In the F_2 generation, there was no statistical difference in heterozygosity between juveniles that survived past their first year of life and those that did not (t = 1.72, P = 0.088). Similarly, in the F_3 generation there was no statistical difference between juveniles that survived past their first year of life and those that did not (t = 1.26, P = 0.21). The inbreeding coefficient

(F_{is}) was high in the F_1 generation (> 0.300) and remained high in all three generations, even among snails that survived to maturity (Table 3).

<<Table 3 near here>>

There was no significant difference in allelic richness (A_R) among the three generations ($F = 3.44$, df = 2, $P = 0.956$) (Figure 2D). In addition, there was no statistical difference between A_R of those that survived to maturity compared to those that did not in the F_2 generation (t=1.78, P=0.212) and in the F_3 generation (t=1.81, P=0.355).

Discussion

The recent population declines in the tree-snail captive-rearing facility at the University of Hawaii have raised serious concerns regarding the future of Hawaiian tree snails and our ability to conserve the last remaining species. Declines in *ex situ* populations of any organism may stem from a variety of causes, including but not limited to disease, parasites, sanitation, diet, improper conditions and failure of mechanical life support equipment (Jacobson 1993, Snyder et al. 1996). In addition, erosion of genetic diversity and increased inbreeding are a major concern for *ex situ* populations founded with small numbers of closely related individuals (Freeland 2005). These bottleneck effects in particular, can act synergistically with the above mentioned factors (Frankham 1998).

The demographic measures of population viability assessed, including juvenile survival and survival to maturity, remained relatively stable in the F_1 and F_2 generations, but significantly decreased in the F_3 and F_4 generations. We expected to observe bottleneck effects such as a loss of genetic diversity and increased inbreeding correlating to these declines, but this was not the case (Figure 2). All measures of genetic diversity, including allelic richness (A_R), observed

heterozygosity (H_o), and gene diversity remained relatively stable over 20 years and three generations (Table 3).

This captive population was founded with some of the last known wild individuals of the species. Our data indicate that these founders may have already experienced severe genetic bottleneck effects. In the F_1 generation, we observed low genetic diversity measures, departures from Hardy-Weinberg equilibrium, and high levels of inbreeding. However, despite the indication of a genetic bottleneck, this population grew at a rate of 29% per year from 1991 to 2004 ($F_1 – F_2$ generations) with no changes in genetic trends (Table 3). This rapid rate of increase may have prevented further loss of genetic diversity (Freeland 2005, Kekkonen and Brommer 2015).

There was a significant difference in the observed heterozygosity of the individuals that survived to maturity in the F_3 generation compared to those that did not. Those that survived were significantly less heterozygous than those that died. This is a curious result as individuals with lower heterozygosity tend to be more susceptible to early mortality compared to individuals with higher heterozygosity. For example, Price and Hadfield (2014), in a study of the sister species *Achatinella lila,* determined that individuals with higher heterozygosity were more likely to survive to maturity and produce offspring than individuals with lower heterozygosity. Small populations are susceptible to stochastic demographic change, and it is believed this curious result illustrates the dynamic nature of the interaction between genetic and demographic factors. Small populations experiencing drastic stochastic declines may loose genetic diversity through genetic drift.

It is likely that the *ex situ* decline described here was not a direct result of a further loss of genetic diversity following the severe bottleneck this population experienced. Instead, bottleneck effects that occurred prior to the founding of the laboratory population may have

acted in synergy with other factors, such as disease. Island species often have immune systems naïve to alien diseases and may be highly vulnerable to them (Paulay 1994, Fordham and Brook 2008). In addition, Low genetic diversity and high inbreeding likely increase vulnerability to disease (Khlat and Khoury 1991, Jacobson 1993, Ross-Gillespie et al. 2007). Furthermore, diseases affecting captive populations are often density-dependent; large numbers of individuals kept in close proximity may allow disease to spread quickly among cages or environmental chambers (Anderson and May 1986, Arneberg et al. 1998).

The laboratory population of *Achatinella fuscobasis* grew to 441 individuals. Similarly, Price and Hadfield (2014) report a maximum population size of *A. lila* that was over 600 individuals at the captive-rearing facility. With such high numbers of inbred, immunologically-naïve island taxa, kept in close proximity, the Hawaiian tree-snail captive-rearing facility may be extremely vulnerable to outbreaks of disease and parasites. It is possible that the decline of *A. fuscobasis* was an epidemiological response typical for captive populations. Lafferty and Gerber (2002), in a meta-analysis of parasite and disease effects on populations of rare species, summarized 29 examples of disease-related decline. In all studies a parasite, or a viral, fungal or bacterial pathogen was identified and attributed to population declines of 50 -100%. The only documented extinction of a rare molluscan species in captivity is the complete collapse of a population of *Partula turgida* kept at the London Zoo, which was attributed to a microsporidianparasite (Cunningham and Daszak 1998). Little is known of molluscan diseases and detection is difficult. However, the rapid 73% reduction in population size experienced by *A. fuscobasis* is characteristic of similar disease-induced declines reported by Lafferty and Gerber (2002).

Our demographic and genetic data indicate that despite a genetic bottleneck, captive rearing of *A. fuscobasis* can be successful, as was the case for two generations of laboratory-

reared snails over fifteen years. The captive population of *A. fuscobasis* was initiated with individuals from a genetically bottlenecked population but retained its founding genetic diversity, likely due to rapid growth (Kekkonen and Brommer 2015, Freeland 2005). Our results demonstrate that we need better understanding of the stochastic risks associated with keeping rare, inbred taxa in captive-propagation facilities so we can implement management strategies appropriate to the level of risk. We recommend: (1) that the Hawaiian tree-snail captive-rearing facility undergo a thorough operational review to identify all potential routes of disease transmission, and adopt rigorous hygiene and quarantine protocols. (2) Avoid keeping all individuals in one place by releasing excess individuals back into the wild, or establishing other captive populations at off-site locations. This prevents the proverbial "all eggs in one basket" scenario and will ensure that when a disease or other stochastic perturbation occurs at one locality, the existence of the species is not in jeopardy, as is currently the case with *A. fuscobasis.* 3) Develop a breeding plan by selecting founder snails and making appropriate pairings to maintain maximum genetic diversity.

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Table 1. Locus identification and GeneBank accession number, followed by number of alleles (N_A), size range of alleles, and null allele frequencies estimated by Microchecker and INEST.

Loci Identification, Genbank accession No.)	N_A	Size range (bp)	Microchecker null allele freq.	INEST null allele freq.
AS812 (EU119381)	13	219–337	0.2035	0.003
AS32 (EU119382) *	10	165–210	0.3736	0.416
AS46 (EU119383)	13	219–270	0.286	0.075
AS53 (EU119384)	20	176–296	0.1069	0.054
AS61 (EU119385)	20	157–229	0.1957	0.03
AS62 (EU119386)	8	206–228	0.1794	0.054
AS82 (EU119387) *	31	129–273	0.208	0.284
AS110 (EU119388)*	11	208–283	0.2217	0.353
AS50 (KR872615)	9	315–417	–0.5058	0.057
AS96 (KR872616)	26	208–403	0.1983	0.156
AS100 (KR872617)*	49	217–466	0.2514	0.246

* Indicates loci removed from all further analyses due to high null allele frequencies.

Table 2. Primer information for the three new loci originally identified by Erickson and Hadfield and optimized in this study. Locus name and Genbank accession number are followed by primer sequence, repeat motif, and annealing temperature (T_a).

Loci identification (GenBank Accession no.)	Primer sequence (5' - 3')	Repeat motif	T_a
AS50 (KR872615)	F: CGCGAGCTACGGATATAGGA R: GCGGTTTTGTTGAGATGTCTT	$(AGT)_6$… $(AGT)_3AGA(AGT)_3T$ $GC(AGT)_6ATT(AGT)_6$	54
AS96 (KR872616)	F:CAACAACAATAACAACAACAGCA R: TCCAGGTTGAGACCAACAGA	$(ACT)_3GCTACTACC(ACT)_2CCT(ACT)_{10}$	58
AS100 (KR872617)	F: GAGGTTGTTAGGGTATGGAGATT R: GTTTAGCGCCGGAAAGTTGT	$(ACAT)_{32}AAAT(ACAT)_3AAAT(ACAT)_7ATAT(ACAT)_{10}$	59

Table 3. Genetic measures by generation ($F_1 – F_3$) for captive *A. fuscobasis* including number of individuals (*N*), inbreeding coefficient (F_{is}), observed heterozygosity (H_o), expected heterozygosity (H_E), gene diversity, and allelic richness (A_R). Generations are further subdivided by those that survived to maturity and those that did not. Note, all individuals sampled in the F_1 generation survived to maturity.

Population	N	F_{is}	H_o ± s.d.	H_E ± s.d.	Gene diversity ± s.d.	A_R
F1						
Survived to Maturity	23	0.419*	0.54±0.20	0.74±0.10	0.72±0.43	6.8±2.0
Did not survive to Maturity	--					
F2						
Survived to Maturity	98	0.330*	0.54±0.22	0.69±0.11	0.65±0.37	6.6±2.4
Did not survive to Maturity	84	0.354*	0.52±0.22	0.74±0.11	0.71±0.48	6.9±3.0
F3						
Survived to Maturity	39	0.516*	0.48±0.26	0.67±0.16	0.62±0.37	6.4±3.1
Did not survive to Maturity	74	0.350*	0.54±0.19	0.73±0.10	0.69±0.39	6.9±1.9

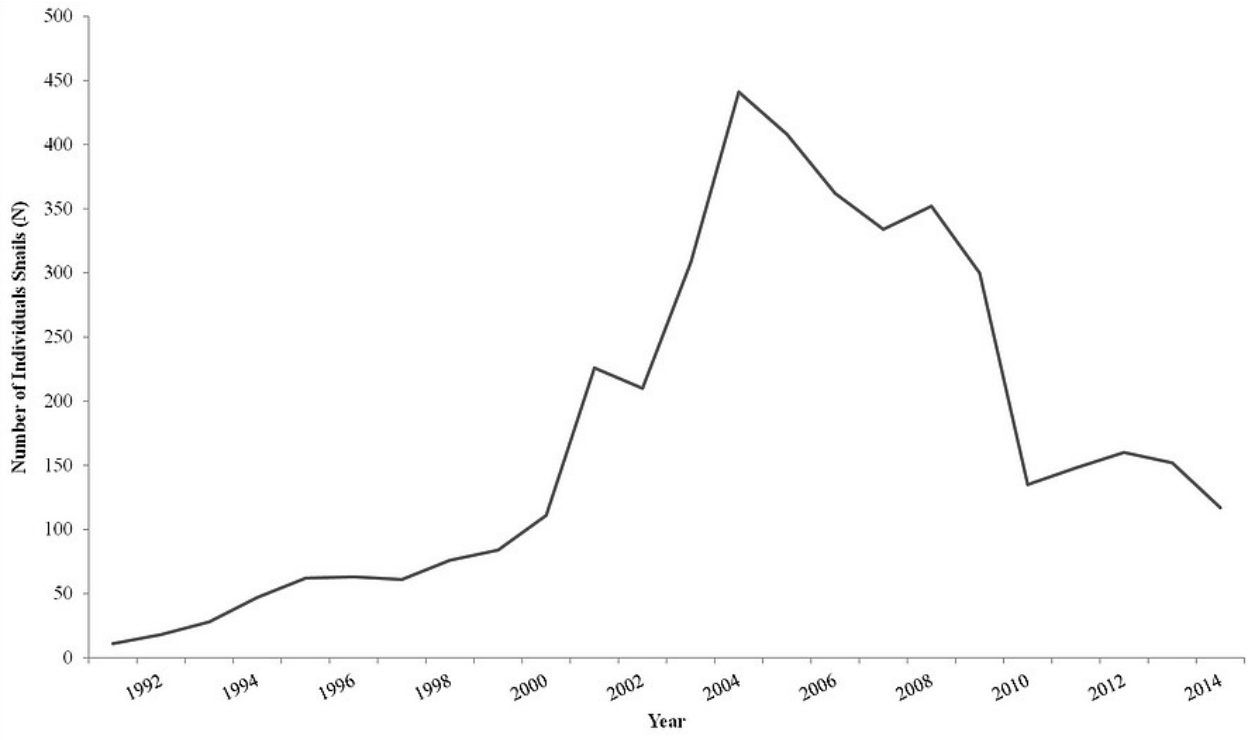

Figure 1. Population trajectory of captive *Achatinella fuscobasis* beginning in 1991 with 11 founding individuals. The population peaked in 2005 with 441 individuals and has since declined to 117 as of December 2014.

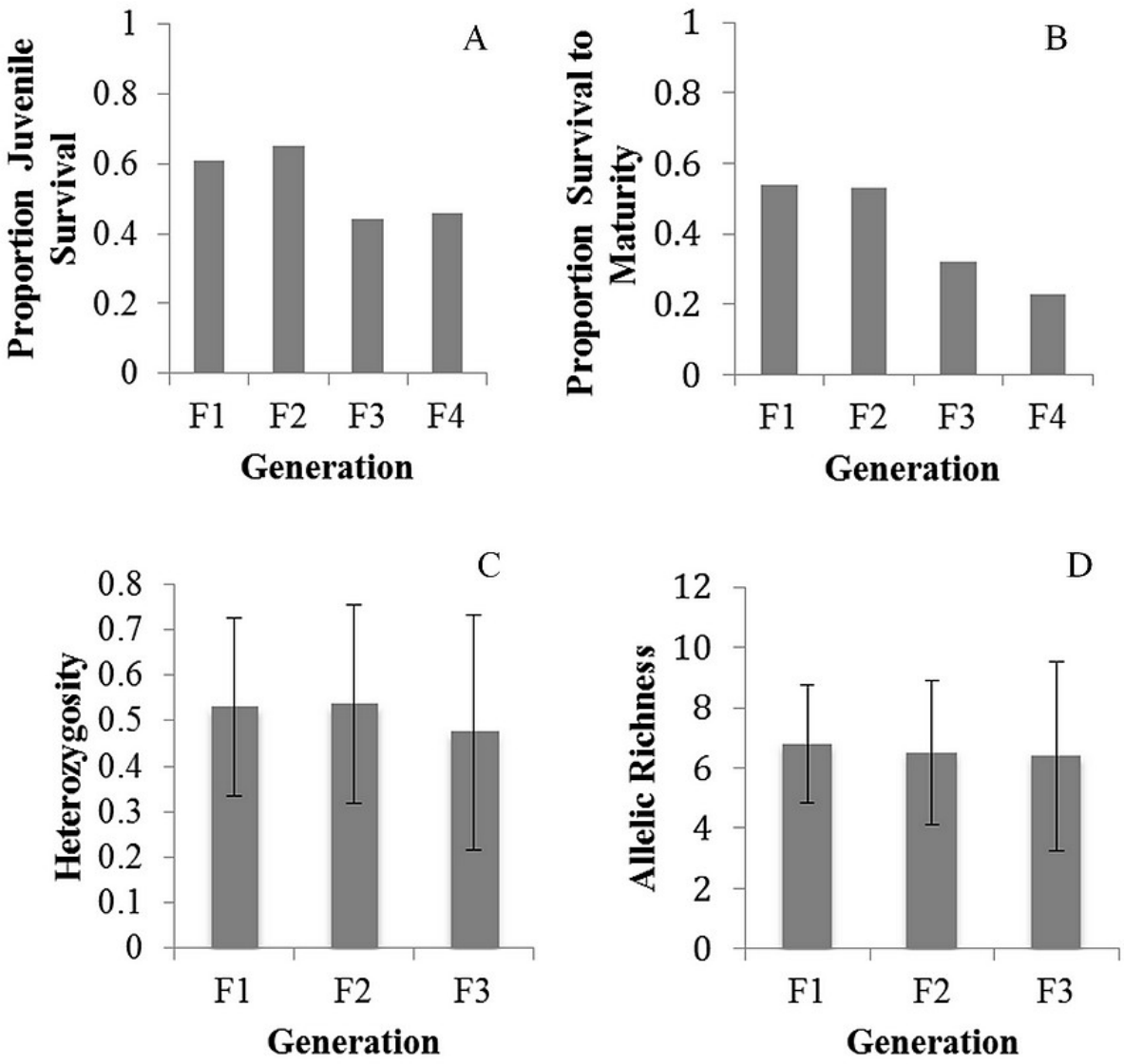

Figure 2. Genetic diversity measures compared to demographic trends. A) Proportion juvenile survival by generation, B) Proportion survival to maturity, C) Heterozygosity by generation, D) Allelic richness by generation. Note, demographic trends continue through the F_4 generation while genetic measures stop at the F_3 generation. Error bars indicate standard deviation.

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