Population genetic diversity and fitness in multiple environments

Jeffrey A. Markert[1,2], Denise M. Champlin[1], Ruth Gutjahr-Gobell[1], Jason S. Grear[1], Anne Kuhn[1], Thomas J. McGreevy Jr[1,3], Annette Roth[2], Mark J. Bagley[2] and Diane E. Nacci[1]

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[1]Population Ecology Branch, Atlantic Ecology Division, US – Environmental Protection Agency, 27 Tarzwell Dr., Narragansett RI USA

[2]Molecular Ecology Research Branch, Ecological Exposure Research Division, US – Environmental Protection Agency, 26 Martin Luther King, Cincinnati OH 45268

[3]Department of Natural Resource Science, University of Rhode Island, Kingston RI 02881

20 Corresponding Author:

Jeffrey Markert – **JeffreyMarkert@gmail.com**
Phone +(401) 782 3153
Fax +(401) 782 9687

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Abstract

Background

When a large number of alleles are lost from a population, increases in homozygosity may reduce individual fitness through inbreeding depression. Modest losses of allelic diversity may also negatively impact long-term population viability by reducing the capacity of populations to adapt to altered environments. However, it is not clear how much genetic diversity within populations may be lost before populations are put at significant risk. Development of tools to evaluate this relationship would be a valuable contribution to conservation biology. We addressed these issues using experimentally manipulated populations of an estuarine crustacean, *Americamysis bahia*. We created replicate cultures with five distinct levels of genetic diversity and monitored them for 16 weeks in both permissive (ambient seawater) and stressful conditions (diluted seawater). The relationship between molecular genetic diversity at presumptive neutral loci and population vulnerability was assessed by AFLP analysis.

Results

Populations with very low genetic diversity demonstrated reduced fitness relative to high diversity populations even under permissive conditions. Population performance decreased in the stressful environment for all levels of genetic diversity relative to performance in the permissive environment. Twenty percent of the lowest diversity populations went extinct before the end of the study in permissive conditions, whereas 73% of the low diversity lines went extinct in the stressful environment. All high genetic diversity populations persisted for the duration of the study, although population sizes and reproduction were reduced under stressful environmental conditions. Levels of fitness varied more among replicate low diversity populations than among replicate populations with high genetic diversity. There was a significant correlation between AFLP diversity and population fitness overall; however, AFLP markers performed poorly at detecting modest but consequential losses of genetic diversity. High diversity lines in the stressful environment showed some evidence of relative improvement as the experiment progressed while the low diversity lines did not.

Conclusions

Taken together, the combination of reduced average fitness and increased variability contributed to realized increases in extinction for very low diversity populations. Modest losses of genetic diversity resulted in measurable decreases in population fitness – these losses were not always detected by AFLP markers.

Background

Decreased population genetic diversity can be associated with declines in population fitness (e.g., [1, 2]). These declines are thought to involve components of the so called genetic 'extinction vortex', which directly ties losses in population genetic diversity to increased extinction risk [3]. In obligately outcrossed species, these losses cause a decrease in individual fitness through the expression of inbreeding depression-like effects, further reducing the effective population size (N_e) and leading to additional increases in autozygosity [4]. The impact of increased autozygosity (or its correlate, the inbreeding coefficient, F_{IS}) on individual fitness has been extensively documented in both laboratory, semi-natural, and natural settings [2, 5-9]. The effects are especially strong in altered or degraded environments [10-12], although the genomic basis of autozygosity-associated fitness differences and heterosis are still debated [13-16]. In addition to increasing individual autozygosity, lost population genetic diversity also reduces the adaptive potential of a population. For populations to persist over longer time-spans, they must have sufficient allelic resources to adjust to novel selective regimes. Forces ranging from invasive parasites and diseases to shifting climatic patterns ensure that environmental conditions will fluctuate temporally and spatially for all populations . Some species have shown a striking capacity to rapidly adapt to novel selective pressures [17, 18] while others have not [19, 20]. Because overall population diversity affects both short-term individual fitness and long-term population adaptive capacity, there is a need to develop an empirical quantitative understanding of the relationship between population genetic diversity and population viability.

Many laboratory models have demonstrated the large role of genetic diversity in increasing population fitness mediated through heterosis, particularly when inbreeding levels are high. In one classic example, Leberg [21] found that populations of mosquito-fish founded with siblings grew more slowly than those founded by unrelated individuals. In a subsequent experiment using non-relatives and experimentally manipulated levels of genetic diversity, Leberg [22] detected no evidence of a relationship between genetic diversity and population fitness. By manipulating N_e while holding the census size constant over three generations in the annual plant *Clarkia pulchella*, Newman and Pilson [23] were able to demonstrate that populations with a small N_e were more than twice as

likely to go extinct as larger populations. Similarly, in a multi-generation experiment using houseflies, Bryant et al. [24] detected clear declines in relative fitness in low founder number populations and in repeatedly bottlenecked populations, even when the bottlenecks were relatively large.

A more direct method for measuring the effect of population genetic diversity on adaptive potential was developed by Frankham et al. [25], who steadily increased the level of an environmental stressor (NaCl) every generation in laboratory *Drosophila* populations. In this study, both mildly bottlenecked and highly inbred populations showed a reduced ability to evolve tolerance to an environmental stressor relative to outbred populations. In order to understand long-term population viability in a changing environment, experimental models that can build upon these results must be developed. Several published studies provide evidence that severely reduced genetic diversity can affect population fitness, but the impacts on population viability of modest (and perhaps more commonly occurring) reductions in genetic diversity are less well characterized. Further, many laboratory studies of evolutionary processes have relied on *Drosophila* or *Tribolium* (e.g. [8, 25, 26]). Both organisms have many experimental advantages but their very high fecundities [27, 28], which can provide for rapid rates of adaptation, make them poor models for vertebrate species with much lower reproductive rates. Laboratory models with lower fecundity may be more directly relevant to vertebrate conservation. Ideally, models of evolutionary genetics should also be able to disentangle the effects of population history and the effects of inbreeding from the adaptive potential represented by genetic diversity *per se*. To do this, they must also allow for fitness to be measured in multiple environments.

Here we present data from laboratory populations of the mysid shrimp (*Americamysis bahia*) a small crustacean native to estuaries along the US East coast [29]. This animal model has several experimental advantages that make it a valuable tool in evolutionary and conservation genetics. Because they are widely used in toxicological studies, optimal culture conditions and demographics are well characterized [30-32]. Time from conception to first mating is approximately three weeks at 25° C and 30 ppt salinity [31, 33]. Mature females can produce a new brood every seven days and provide an unusually high level of brood care for an invertebrate; they incubate a small number of

fertilized eggs in a marsupium for seven days, giving *A. bahia* a reproductive profile more similar to many birds and mammals than to other more fecund invertebrates. Owing to their estuarine habitat, *A. bahia* tolerate a wide range of salinities. In laboratory settings at 25 ° C, *A. bahia* cultures reproduce well in natural seawater with a salinity of 31 parts per thousand (ppt) NaCl, although they are reproductively viable in as little as 10 ppt NaCl [31]. In the wild, *A. bahia* have been collected in waters as low as 3 ppt, although some field surveys suggest they are uncommon below 9 ppt [34].

By simultaneously manipulating the selective environment and genetic diversity under controlled laboratory conditions with replication, we used *A. bahia* cultures to develop a more detailed understanding of the relationship between genetic diversity and population fitness in a changing environment. We also generated AFLP [35] genotypes for many of the populations to determine how well a typical molecular genetic fingerprint analysis predicts meaningful losses of genetic diversity. Our study goal was to determine the general relationship between genetic diversity and fitness in both permissive and stressful environments.

Methods

Collection of stock populations

Americamysis bahia were collected by dragging a fine-mesh net in shallow waters near Biloxi Beach, MS USA (N30.39351, W088.90123) and Navarre Beach, FL USA (N30.38964, W086.83050) during April 2005. Live animals were keyed out under dissecting microscopes at the US-EPA's Gulf Ecology Division in Gulf Breeze, FL USA. Approximately 50 individuals from each collection site were then transported to the US-EPA's Atlantic Ecology Division facilities in Narragansett, RI USA. Populations derived from each of the two collection sites were housed separately in four 80 L tanks with flow-through seawater maintained at 25° C and an ambient salinity of approximately 30 ppt. Animals were fed Selco enriched *Artemia ad lib* [36]. *A. bahia* cultures grew quickly to more than 2000 individuals from each source.

Generation of low diversity lines

Replicate lines with low genetic diversity were generated through a series of population bottlenecks. Individual lines were housed in 9.4 L tanks (environmental conditions as above). In late June 2006, 32 gravid females were selected from each of the two source populations and placed in separate tanks to become founders for 64 low diversity lines (Parental Generation). Following the release of young (F_1 generation), the founding females were removed, and their offspring were allowed to grow to maturity and breed for a period of three weeks. After this time, two gravid F_1 females were selected from each line and remaining individuals were discarded. These F_1 founders were removed after they had released their broods, producing the F_2 generation. After the broods matured and became reproductively active, a single gravid F_2 female was selected to found F_3 and subsequent generations within each line. If the initial founding female was fertilized by a single male (a reasonable assumption given mysid reproductive biology), then pedigree based estimates suggest an average decrease in heterozygosity of 31.25%. Alternatively, the 2-4-2 bottleneck represents a harmonic-mean effective population size of 2.4 individuals and a 50% decrease in heterozygosity relative to the starting populations [37]. Starting with the F_3 generation, random mating was permitted within each line.

Generation of high diversity lines

Dihybrid (2x) Lines - The viable low diversity lines generated through bottlenecks (above) were designated '1x', and represented the lowest level of genetic diversity in our study. Randomly chosen sets of 1x lines were crossed to generate higher diversity levels. Briefly, to generate 2 x lines two gravid females were selected from one of the randomly selected low diversity lines. Their offspring were discarded once they were released, and these mature, now non-gravid females were randomly paired with two males from another low diversity line. Crosses were performed randomly yielding four Navarre x Navarre lines, three Biloxi x Biloxi lines and eight Navarre x Biloxi lines.

6x, 8x and Admixed Lines - Populations containing the genetic equivalent of either six or eight 1x lines were created as the main experiment was established (see below). We created these higher diversity levels by combining individuals from different dihybrid lines. The founding number for each population was 12 individuals, so 6x lines were founded by randomly choosing four individuals from three 2x lines. Similarly, 8x lines were founded by choosing three individuals from each of four unique 2x lines. The founding 2x lines were chosen randomly, with the constraint that any ancestral 1x population could be used only one time within a 6x or 8x population. Lines with the highest level of diversity, "Admixed (AMX)" were obtained by drawing six individuals each from the Biloxi and Navarre stock populations as founders

Salinity and culture

A pilot study demonstrated that reproductive rates for *A. bahia* were similar in ambient seawater and at 10 ppt salinity (data not shown). When the salinity was reduced to 7 ppt, reproduction ceased. Based on this preliminary data, together with published findings [31], and our expectation that low genetic diversity populations would be more sensitive to environmental stress, we chose 9 ppt salinity as the target level of novel environmental stress.

Salinity was maintained by diluting seawater with dechlorinated tap water. Experimental populations were housed in 9.4 L tanks. Both ambient and diluted seawater were available in a flow through system, and we ran water through the tanks for 1 hour in the morning and 1 hour in the evening to ensure precise control of salinities. At the observed flow rate, this was sufficient for more than one complete exchange daily. Tanks were kept in two water tables to ensure uniform temperatures between tables and replicates. Tanks were moved within and between tables weekly to further reduce the potential for position effects. Lights were on a 12:12 light:dark cycle. Light levels were increased and decreased slowly to simulate natural conditions. Salinity was measured using a Hach meter Model 60d. Salinity and temperature were measured daily in a randomly selected 10% of tanks. The mean temperature for all measured tanks was 25.3° C (± 0.027 S.E.). Low salinity tanks were maintained at a mean of 9.4 (± 0.07) ppt.

Normal seawater tanks had an average of 29.4 (± 0.5) ppt. Animals were fed *ad libidum* with Selco-enriched [36] *Artemia* (Aquafauna Biomarine, Hawthorne, CA USA).

Experimental design

Phase 1 - population establishment and expansion

Experimental aquariums were established as matched pairs, one serving as control (permissive environment) and one to be subjected to low salinity (stressful environment). Experimental populations were founded with 12 individuals (see above) and these were allowed to breed and expand for three weeks in a permissive environment (~30 ppt salinity).

Phase 2 – chronic low salinity stress

After this initial census, designated experimental populations were subjected to a stressful environment by gradually reducing the salinity to 9 ppt over the course of four days. Salinity was maintained at this level thereafter. The remaining control tanks were maintained with normal seawater. During the experimental period, a weekly census was conducted in which all individuals were counted and the presence of neonates (animals < 7 days old) was noted.

Fifteen pairs of low diversity (1x) lines were established. We intended to establish these cultures from 15 independently bottlenecked lines, however one of the designated lines went extinct before the start of the experiment, so one of the surviving lines was used twice. Fifteen independent pairs of 2x cultures were also established. Higher diversity levels (6x, 8x and Admixed) were replicated 10 times. The entire experiment contained 120 tanks.

At the end of the 14 week survey period, surviving individuals were preserved in 100% ethanol from each tank for molecular analysis.

Adaptation over time

To estimate the response to selection of each nominal genetic diversity level over the course of the experiment, population sizes in the stressful and permissive environments were compared three weeks (~1 full reproductive cycle) after the

environmental stress was introduced and at the end of the experiment (~3 reproductive cycles later).

Genetic analysis

AFLP genotypes generated from surviving control populations at the end of the experiment were used as a measure of starting genomic diversity for each diversity level. It was not possible to genotype the founding populations at the beginning of the experiment because the low diversity stock lines had only a modest number of individuals, and most of these were required to found the experimental populations. For the lowest diversity lines, the harmonic mean population size was 33.8 individuals, which suggests that the populations would have lost about 2% of their heterozygosity due to genetic drift each generation. In the highest diversity populations, N_e was estimated to be 110.6 individuals, consistent with a decline in neutral locus heterozygosity of less than 1% per mysid generation. Some lines were excluded from the analysis because fewer than ten individuals were available.

Ten individuals were randomly chosen from each line to estimate genetic diversity. DNA was extracted from whole *A. bahia* using DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA). The manufacture's instructions were followed except that we heated the elution Buffer AE to 70° C for ten minutes and incubated the sample with Buffer AE for 5 minutes at room temperature before eluting each DNA sample. Genomic DNA was quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

AFLP analysis followed the procedure of Vos et al. [35], modified to accommodate fluorescent visualization and using the restriction enzyme pair EcoR1/PstI [41]. Total DNA was extracted from *A. bahia* individuals, using the Qiagen DNeasy Tissue kit, and quantitated using PicoGreen (Molecular Probes Inc.). Total genomic DNA (75 – 200ng) was simultaneously digested and ligated in a 15 µl reaction that included 5u each of EcoRI, PstI, and T4 DNA ligase (New England Biolabs), 30 pmoles of each EcoRI and PstI double-stranded DNA adaptor [see 41], 50 ng/ul BSA, and 50 mM NaCl in T4 Ligase buffer (New England Biolabs). Following complete digestion

and ligation at room temperature, products were diluted tenfold into 10 mM Tris pH 7.6, 0.1mM EDTA.

Initial PCR enrichment (pre-amplification) of a subset of fragments (pre-amplification) used 5 µl of the diluted digestion-ligation product as template; 0.5 µM of the EcoR1+A /PstI +C primers (IDT, Coralville, IA) and 0.25 U Taq DNA polymerase (Invitrogen) in 20 µl of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM each dNTP, and 1.5 mM $MgCl_2$. PCR conditions were 2 min at 74° C; 24 cycles of 94°/30 sec, 56°/30 sec; 72°/1 min; followed by 30 min at 72°C. The pre-amplificaation product was then diluted ten-fold with 10 mM Tris pH 7.6, 0.1mM EDTA buffer.

Selective amplification reactions were similar to pre-amplifications, with 3 µl of diluted pre-amplification product used as template and substituting 50 pM of the appropriate FAM–labeled EcoRI +3 / 250pM PstI +2 selective AFLP primers. Three selective primer combinations were used on all samples: EcoRI+ACT–PstI+CT; EcoRI+AGG–PstI+CA; and EcoRI +ATG–PstI+CT. PCR conditions were 2 min at 94°C, 12 cycles of 20 sec at 94°C, 30 sec at 66°C dropping 1°C per cycle, 1 min at 72°C; then 20 cycles of 20 sec at 94°C, 30 sec at 56°C, 1 min at 72°C; followed by 30 min at 72°C. AFLP genotypes were electrophoresed and visualized with an ABI 3730 DNA analyzer.

Bins within the range of 100 to 500 bp [38] were generated for the amplified fragments using GeneMarker® version 1.6 (SoftGenetics LLC®, State College, PA, USA). We manually checked the quality of each AFLP fingerprint and bin using the method described by Whitlock et al. [39] with slight modifications. We removed samples that produced an AFLP fingerprint with less than 20 peaks within the target size range and restricted our analyses to fragments with relative florescence units greater than 100 to reduce background noise. We visually checked the automatically created bins to ensure the bin was centered on the distribution of peaks within the bin and removed bins that had AFLP fragments that differed in size by more than 1 bp. We also deleted bins with fragment-length distributions that overlapped with adjacent bins to reduce the occurrence of homoplasy [38, 40]. The number of initial bins for the three sets of restriction enzymes ranged from 63 to 76 each. We developed an R (http://www.R-project.org/)script to convert the raw peak intensity data output from GeneMarker to a

format compatible for AFLPScore version 1.3 [45]. We scored our raw AFLP data using AFLPScore, normalized our data to the median, filtered our data with a locus selection threshold, and used a relative genotype calling threshold. We tested a range of locus (100 to 1000 bp) and genotype thresholds (1 to 120%) and selected the pair of values that simultaneously minimized the mismatch error rate, minimized the probability of misscoring a presence allele ($\varepsilon_{1.0}$ error rate), and maximized the number of loci retained. We included all pairwise comparisons for the samples that had greater than two replicates in our mismatch analysis. We generated AFLP genotypes for each restriction enzyme pair with the optimized locus selection and genotype thresholds using AFLPScore.

The locus selection threshold was 1000 bp and the genotype threshold was 10% for each restriction enzyme pair. The average mismatch error rate for the three restriction enzyme pairs was 8.3504 ± 1.7367 (SD) and the average $\varepsilon_{1.0}$ error rate was 19.484 ± 2.3992, which retained 59 bins. AFLP based estimates of genetic diversity were calculated using AFLP-Surv v1.0 [38]. AFLP based estimates of genetic diversity were calculated as either the fraction of polymorphic loci within the sample (PLP) or the heterozygosity analogue (H_j).[41]

Statistical analyses

Three different indices of population fitness were evaluated: 1) the number of individuals in the *Last Census* (LC), 2) *Median Population Size* (MPS) using data from all 13 censuses for each experimental tank and 3) the *Reproductive Index* (RI), which was calculated as the number of weeks in which reproduction was observed divided by the total number of weeks that the population survived for each population.

Statistical relationships among fitness, genetic diversity (treating levels 1x, 2x, 6x, and 8x, and Admixed as ordinal categorical data), and environmental stress were evaluated using general linear models. All calculations were performed using either JMP 7.0 or SAS 8.0 (SAS institute, Cary NC).

Results

Fraction of bottlenecked lines surviving

A substantial proportion of the bottlenecked lines did not survive long enough to be used in the main experiment. Of the 64 lines initially started, only 14 achieved a population size sufficient to provide the 2 founders required to generate dihybrid lines and to supply 24 founders for the main experiment.

Molecular estimates of genomic diversity

The 1X lines had an average PLP of 35.6 and an average H_j of 0.14. The Admixed lines had an average PLP of 52.1 and an average H_j of 0.19. Nominal genetic diversity explained a moderate amount of variation in AFLP diversity estimates (PLP Spearman's $\rho = 0.67$, $p < 0.0001$; H_j Spearman's $\rho = 0.44$, $p = 0.0043$). In post-hoc tests, neither estimator was effective at differentiating among the three highest genetic diversity treatments; however the 1X , 2X and higher diversity lines were distinguishable from each other when PLP was used to estimate genetic diversity (Table 1).

Population growth in permissive conditions

Abundance after three weeks of culture under permissive conditions (Phase 1) was significantly affected by nominal genetic diversity level (Spearman's $\rho = 0.68$, $p < 0.0001$, Table 2). Population sizes increased from 12 individuals to an average of 18.6 individuals in the low diversity lines (1X) and to 79.3 individuals in the highest diversity populations (AMX). All treatments differed from each other, except 6X and 8X. Variance was unequal among treatments ($p = 0.0244$) with the coefficient of variation inversely related to genetic diversity (Table 2, Figure 1).

AFLP diversity estimated as PLP explained a modest percentage of the variation in abundance after three weeks in permissive conditions (adjusted $R^2 = 0.24$, $p < 0.0001$). AFLP diversity estimated as H_j explained less but still significant abundance variation (adjusted $R^2 = 0.16$, $p < 0.0001$).

Population fitness, environmental stress and genetic diversity

A model including genetic diversity and environmental stress explained the majority of the variation in MPS during the chronic low salinity experiment (Phase 2) (adjusted $R^2 = 0.74$, $p < 0.0001$). Both factors contributed strongly to the relationship (environment $F = 127.8$, $p < 0.0001$; diversity $F = 53.3$, $p < 0.0001$). Treatment means ranged from 9.7 individuals (low salinity, 1X) to 123.2 individuals (normal salinity, AMX). There was no significant interaction between salinity stress and genetic diversity level ($F = 0.59$, $p = 0.66$). Therefore, the relative relationship of population sizes among genetic diversity levels was maintained, even though values within a diversity level decreased in the stressful environment (Figure 2). The last census size ranged from a mean of 2.50 individuals (low salinity, 1X) to 84.4 individuals (normal salinity, Admixed). An additional model for MPS that included the results of the first census (Phase 1, pre-stress) as a covariate also explained much of the variation in MPS (Adj $R^2 = 0.78$, $p <0.0001$). Abundance at initiation of experimental treatments was a significant covariate ($F = 16.5$, $p < 0.0001$). In this more complex model, there was a significant interaction between this initial abundance and genetic diversity level ($F = 4.0$, $p = 0.0043$), but no interaction between environment and genetic diversity class ($F = 0.78$, $p = 0.536$). In this model both nominal diversity level ($F = 13.1$, $p< 0.0001$) and environment ($F = 22.3$, $p < 0.0001$) were significant individually.

A model including nominal genetic diversity and environmental stress explained 53% of the observed variation in LC ($p < 0.0001$). Genetic diversity ($F = 50.8$, $p < 0.001$) and environmental stress ($F = 21.4$, $p < 0.001$) were both statistically significant, and there was no significant interaction between these variables ($F = 0.54$, $p = 0.71$).

Stress and genetic diversity explained much of the variation in RI (adjusted $R^2 = 0.58$, $p < 0.0001$). Both factors were statistically significant (stress $F = 95.4$, $p < 0.0001$; genetic diversity $F = 14.3$, $p < 0.0001$) with a marginally insignificant interaction between these two factors ($F = 2.18$, $p = 0.075$). The fraction of weeks during Phase 2 when young were observed ranged from 0.27 to 0.97 across treatments. Variance was unequal among treatments ($p < 0.0001$), and higher at lowest diversity and under stressed conditions (Figure 2). Variance remained unequal despite attempted transformations. Variation (expressed as the coefficient of variation) in all three fitness proxies is

summarized in Table 2, and the distribution of individual replicate values is shown in Figure 1.

Population fitness, environmental stress and molecular diversity

The effects of AFLP diversity (estimated for each individual replicate using either PLP or H_j) and environmental stress were evaluated for three different fitness proxies: MPS, LC, and RI.

A significant portion of the variation in MPS is explained by a model incorporating AFLP diversity measured as PLP and environmental stress (adjusted R^2 of 0.53, $p < 0.0001$). Both variables were statistically significant (stress $F = 59.1$, $p < 0.0001$; PLP $F = 32.9$, $p < 0.0001$), and there was no significant interaction between the two terms ($F = 0.04$, $p = 0.83$). Similar results were obtained when H_j was substituted for PLP (adjusted $R^2 = 0.51$, $p < 0.001$; stress $F = 56.1$, $p < 0.0001$; H_j $F = 27.2$, $p < 0.0001$; stress*H_j $F = 0.26$, $p = 0.61$).

Models evaluating the effect of AFLP diversity and environmental stress on the LC fitness proxy were also significant overall (adjusted $R^2 = 0.37$, $p < 0.0001$ using PLP, adjusted $R^2 = 0.38$, $p < 0.0001$ using H_j). There was no interaction between genetic diversity and stress in either model using PLP (PLP $F = 11.8$, $p < 0.0001$; stress $F = 36.8$, $p = 0.001$; stress*PLP $F = 0.58$, $p = 0.45$) or H_j (H_j $F = 13.5$, $p < 0.0004$; stress $F = 37.2$, $p < 0.0001$; stress*H_j $F = 0.0009$, $p = 0.97$).

Similarly, both PLP and H_j explained a significant fraction of the variation in RI (PLP Adj $R^2 = 0.53$, environment $F = 75.6$, $p < 0.0001$, PLP 15.6, $p = 0.0002$), (H_j Adj $R^2 = 0.52$, environment $F = 73.9$ $p < 0.0001$, H_j $F = 14.0$, $p = 0.0002$). Neither genetic diversity estimator had a significant interaction with environmental stress.

Observed population extinctions

Population extinctions were rare during the course of the study, and were confined to the low diversity populations (Table 2). Three out of 15 1X populations went extinct even under permissive conditions. Median time to extinction for these populations was seven weeks. By contrast, 11 of 15 1X populations went extinct under stressful conditions, with a median extinction time of nine weeks (three of these 11

extinct lines also went extinct under permissive conditions). Only a single 2X population went extinct in the low salinity treatment at 11 weeks.

AFLP data were available for nine of the 15 pairs in the lowest diversity 1X treatment. The remaining six pairs could not be surveyed due to extinction or low survivor numbers in the control line. The lines that went extinct had a mean PLP of 32.5 vs. 39.4 for the surviving lines, although this difference was not significant ($p = 0.17$). H_j in extinct lines was 0.11 and 0.18 in surviving lines, and the difference was statistically significant ($p = 0.014$).

Adaptation over time

After three weeks exposure to low salinity (Week 6 of the experiment), the average 1X population in this stressful environment had a census size 57% smaller than those in the high salinity control environment, while the high diversity AMX lines were 24% smaller in the stressful environment. At the end of the experiment (Week 16), the average 1X stressed population was 94% smaller than its control, whereas the average AMX population reared in low salinity was only 7% smaller than the average control population. The relative decline in performance of the salinity stressed 1X populations was partly driven by the extinct lines; however, when these were excluded the net decline relative to the control population was still 83% (Table 2).

Discussion

In conservation biology, there is a critical need to develop a quantitative understanding of the relationship between measurable genetic diversity and population viability. It is well accepted that when enough genetic diversity is lost from a population, the negative effects include inbreeding depression (a result of reduced average individual heterozygosity) and reduced adaptive capacity (a result of lost allelic variation). This inverse relationship between genetic diversity and population viability is a central pillar of conservation genetics. However the question, "How much genetic diversity is enough?" is unresolved. This question also pervades the broader field of population viability analysis where definitions of quasi-extinction thresholds are operational, at best. Massive losses of genetic diversity are clearly harmful, but are smaller losses dangerous?

If so, are molecular tools able to detect these losses before the population spirals inescapably down the extinction vortex? Neutral molecular markers would be useful tools to rapidly survey genetic diversity in wild populations of concern. Since AFLP markers can survey genome scale diversity in a wide variety of organisms without the extensive development time and expense required for single nucleotide polymorphisms, microsatellites, or other molecular markers, they have the potential to be an important tool in measuring critical losses of genetic diversity. However in this mysid experimental system, the specific set of AFLP markers surveyed displayed only moderate statistical power to detect ecologically important reductions in genetic diversity.

Reduced diversity and population fitness

It has been repeatedly demonstrated that the impact of inbreeding depression varies with environment [42, 43], and the negative effects of high levels of inbreeding may be masked by permissive environments or when a direct comparison with outbred individuals is not possible [6, 12]. However, the population level consequences of increased individual inbreeding and reduced population allelic diversity have not been as well characterized. In our simplified laboratory system, reduced population genetic diversity tended to decrease mean population fitness, although this decrease was not always statistically significant in all post-hoc tests. As expected, average population fitness in the stressful environment was always lower than fitness in the permissive environment for a given level of genetic diversity. Interestingly, there was no statistical interaction between nominal genetic diversity and any of our fitness indices, indicating that *relative* performance among genetic treatments is similar in both environments (but always lower in the stressful environment). Genetic diversity and environmental quality may, of course, interact in a more complex way in natural systems.

A modest amount of neutral locus genetic diversity (as estimated with AFLP genotypes) was explained by nominal diversity level. The overall relationship is in the expected direction; however, post hoc tests (Table 1) reveal that estimates of both PLP and H_j based on our final set of 59 screened AFLP markers do not reliably detect differences between the three highest nominal diversity levels. Similarly, both estimators explain only a modest amount of the variation in the three fitness indexes. Despite the

lack of a detectable molecular genetic difference, the observed mean fitness was always lower in 8X populations than in AMX populations in the stressful environment and for two of the three fitness proxies in the permissive environment. Post-hoc tests showed these differences were statistically significant for two of the three proxies in the stressful environment. We could detect no statistically significant difference in the mean AFLP diversity estimates between 6X, 8X and AMX lines (Table 1). In our study system, AFLP markers detected large decreases in genetic diversity but missed more modest losses. This may have important implications for the application of AFLP genotypes to problems in conservation genetics: the set of AFLP markers we analyzed did not reliably detect reductions in genetic diversity that clearly impacted population fitness, especially under environmental stress.

Inbreeding and genetic rescue

A striking aspect of our study was the strong manifestation of inbreeding depression and the power of hybrid rescue. The stock populations were very susceptible to inbreeding depression, and the modest level of inbreeding employed in this study had a dramatic effect on population health. The clearest evidence for the effects of inbreeding on *A. bahia* populations was obtained before the formal experiment started. In order to generate the 1X lines used in this study, we started with 64 founding lines. Fully three quarters of these lines failed to generate the 26 individuals that were required to found the experimental lines after several months in culture. Some early losses may also be due to bad demography – initial brood sizes are small in young mysid females, so demographic stochasticity could have caused lineage extinction. However many lines that survived failed to thrive during more than four months under permissive conditions. Thus, inbreeding effects were a major determinant of the number and types of lines available for our main experiment making it necessary to construct experimental populations using only the modest number of lines that were *most resistant* to inbreeding depression. This result is typical of animals with large, panmictic populations [24, 44].

Because we constructed our higher diversity populations by combining different numbers of low diversity lines, our study may be viewed as a series of replicated ‘genetic rescue’[45] experiments (albeit with very high immigration rates, comparable to [44]).

Population fitness was substantially improved when two or more 1X lines were combined, and in almost all cases, the 'rescue' was successful. Only a single 2X population went extinct in the stressful environment. Within our system, nominal genetic diversity was an important predictor of population fitness for most levels of genetic diversity. In both environments and for all three of the fitness proxies, the 2X lines performed better on average than the 1X lines, and the 6X lines performed better than the 2X lines. The difference was not always statistically significant in post-hoc comparisons for each proxy at each level (Figure 1), but the relative performance was as expected. Further, the high diversity AMX populations were generally more fit than any of the lower diversity populations.

Surprisingly, we did not detect a statistically significant difference between the 6X and 8X populations in any of the fitness assays or by using molecular markers. We note that the best performing 8X were superior to the best performing 6X populations, however the worst performing 8X populations were inferior to the worst performing 6X populations. Because the 8X lines were founded with only three individuals from each of four founding 2X lines, it is possible that some of the founding lines did not establish themselves in some 8X populations. In any case, genetic differences between these two genetic classes are expected to be quite small. Even for a locus that is fixed for alternate alleles in the 1X populations, expected heterozygosity of 6X and 8X populations would only differ by 4% on average (H = 0.833 and 0.875, respectively [46]). The actual heterozygosity difference is likely under 2% since 1X lines would have experienced only a 30% to 50% reduction in heterozygosity relative to the founding stock populations.

Diversity, selection and adaptation.

Many studies have focused on the individual fitness consequences of inbreeding in benign and stressful environments due to inbreeding depression effects [42] but this is only one way that genetic diversity affects extinction risk. It also is important to determine the consequences of reduced genetic diversity for the capacity of the population to adapt to a novel environment. Even modest losses of genetic diversity may result in a reduced ability to adapt to environmental change, yet the short-term impact of such losses may be minimal if populations are maintained in stable environments or if the

loss does not cause detectable inbreeding depression-like effects. The long-term impact of moderate losses on population persistence can best be measured by estimating generational changes in population fitness in multiple environments. The mysid experimental system demonstrates that both population fitness and inter-population variability are influenced by genetic diversity, and that both fitness and variability are influenced by environmental stress.

To assess the strength of selection in the stressful environment, we calculated the ratio of populations in the stressful environment to those in the permissive environment three weeks (~1 mysid generation) after the stressful environment was introduced. We hypothesized that the relative proportions should be similar at both time points if inbreeding and heterosis are influencing the relationship, but that when adaptation has occurred, population sizes in the stressful and permissive environments will grow more similar over time. We found that after three weeks of selection the 1X population sizes in the stressful environment were 57% smaller than those in the permissive environment, while the AMX population sizes were only 24% smaller in the stressful environment. These declines represent the selection pressure imposed by the stressful environment. After ten more weeks of selection, the AMX population sizes in the stressful environment were only 7% lower than those in the permissive environment while the 1X population sizes were 94% lower (Figure 2). Therefore, the low diversity populations did poorly in the stressful environment early in the experiment and grew progressively worse as the experiment proceeded. By contrast, the high diversity populations were relatively less disadvantaged early on and even showed some improvement by the end of the experiment. In the AMX lines the level of improvement did not rise to statistical significance; however, the trend was consistent with the one predicted by evolutionary adaptation (and some stressed populations even outperformed their matched controls), suggesting that simple heterosis may not be the only force operating in populations with high genetic diversity. However these results should be interpreted with some caution as the high diversity populations may have been close to the carrying capacity of the habitat in both normal and low salinity environments.

In our mysid data set, nominal genetic diversity was an important predictor of variability between populations within an environmental treatment, with lower diversity

populations having more inter-population variability than higher diversity populations. Population size (either median or final) was also notably lower in low genetic diversity populations, so a much higher fraction of low diversity lines are likely to fall below the minimum number of individuals required to maintain population viability [47]. In general, temporal variation in abundance within a single population is expected to increase the chances of population loss [47, 48], so these results indicate that genetic diversity is an important component of extinction risk.

Conclusions

Using the mysid experimental system, we found that: 1) reduced population genetic diversity reduces population fitness in both permissive and stressful environments, 2) even some modest reductions in genetic diversity can reduce the value of some fitness proxies, especially in stressful environments, 3) environmental stress and genetic diversity appear to independently influence population fitness, 4) AFLP genotypes detected large reductions in population genetic diversity, but did not reliably detect modest reductions in genetic diversity that may influence population fitness and many more AFLP loci than are commonly used would be necessary to detect these losses, 5) low diversity populations show more inter-population variability than high diversity populations for most estimates of population fitness, and 6) high diversity populations may show some capacity to adapt to the stressful environment, but low diversity populations may not.

In natural populations, the nature of the relationship between population fitness and genetic diversity will obviously depend on the specifics of the environment and the organism. Genetic diversity may not always enable populations to persist, but a lack of diversity essentially guarantees that adaptation to altered environments will not occur. Despite the importance of diversity for population survival, our understanding of the relationship between diversity and long-term population viability is limited. Studies in simplified laboratory environments, such as the one described here, can be used to determine a baseline for the relationship between diversity and population risk under the best possible conditions (i.e., with the least environmental variation) and provide an

important way to assess molecular tools that are potentially useful in conservation biology.

List of Abbreviations

AMX = Admixed lines

H_j = Heterozygosity estimate derived from dominant molecular markers.

LC = The Last Census, the number of individuals in an aquarium at the end of the experiment.

MPS = Median Population Size for a single line over the course of the experiment.

NI = Net Increase in population size after three weeks in permissive conditions.

PLP = Proportion of Loci Polymorphic, the fraction of AFLP bands that vary within an experimental populations.

RI = Reproductive Index, the fraction of census weeks in which neonates were observed

TTE = Time To Extinction.

Author Contributions

JAM, DEN and MJB took the lead in designing overall experiment, while AK-H, JSG and DMC provided critical insights into key sections of the design. DMC A K-H and JM also conducted an extensive series of pilot studies that made this project possible. RG-G designed methods that allowed us to precisely maintain the experimental environment. TJM- optimized objective AFLP scoring parameters, produced AFLP genetic diversity estimates, wrote the AFLP scoring section. AR managed the collection of molecular data and wrote the molecular section of the AFLP methods. All Narragansett based authors participated in weekly censuses and daily culture activities. All authors contributed to the writing of the paper.

Figure 1 - Population fitness, estimated with Median Population Size (A), Last Census size (B), and Reproductive Index (C). Paired box plots define the median and middle two quantiles in stressful (left) and permissive environments (right). Lower case letters unite groups that are not statistically distinguishable using post-hoc tests (Tukey's LSD) at $\alpha = 0.05$.

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Figure 2 - Ratios of census sizes in the stressful environment to those in the permissive environments for each diversity class after three weeks in the selective environment (left box plot) and at the end of the experiment (right box plot). The box plots enclose the central two quantiles and show the group medians. The inset shows the average percent decline in census size in the stressed populations relative to the control populations. Asterisks indicate when the ratios are significantly distinguishable using the Wilcoxon signed rank test.

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Nominal Diversity	Avg PLP	Sig?	Avg H_j	Sig?
1x	35.6 ± 7.3	A	0.14 ± 0.05	A
2x	43.1 ± 9.2	B	0.16 ± 0.03	B
6x	49.2 ± 7.1	C	0.19 ± 0.02	C
8x	47.8 ± 5.1	C	0.18 ± 0.03	B,C
Admixed	52.1 ± 4.6	C	0.19 ± 0.02	C

Table 1 – Estimates of average neutral locus genetic diversity – Values are shown ± 1 S.D. using AFLP genotypes calculated as the percentage of polymorphic loci (PLP) or the heterozygosity analogue (H_j). Letters in the "Sig?" column unite groups that are not statistically distinguishable using Tukey's LSD.

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Diversity Level		*NI*	*NI* C.V.	Average *MPS*	*MPS* C.V.	Average *RI*	*RI* C.V.	Average *LC*	*LC* C.V.	% Extinct
1X	**Permissive**	6.6	1.59	42.4	0.67	0.69	0.49	34.29	0.91	20
	Stressful			9.50	1.07	0.27	0.77	2.50	2.44	73
2X	**Permissive**	19.7	0.77	60.2	0.31	0.90	0.09	44.53	0.42	-
	Stressful			19	0.64	0.38	0.44	6.20	1.27	7
6X	**Permissive**	33.7	0.49	95.30	0.19	0.93	0.09	66.30	0.37	-
	Stressful			49.70	0.5	0.68	0.34	33.60	0.58	-
8X	**Permissive**	33.3	0.43	94.90	0.22	0.94	0.06	63.60	0.49	-
	Stressful			47.40	0.38	0.57	0.41	28.44	0.63	-
AMX	**Permissive**	67.3	0.28	123.20	0.2	0.97	0.04	84.40	0.39	-
	Stressful			77.40	0.21	0.73	0.22	65.60	0.47	-

Table2 – Averages and coefficients of variation for each treatment – The net increase (NI) was calculated after three weeks in permissive conditions. MPS is the median population size calculated using weekly census data from 13 post stress weekly censuses. RI is the reproductive index – the fraction of census weeks in which reproduction was observed. LC is the population size at the last census.

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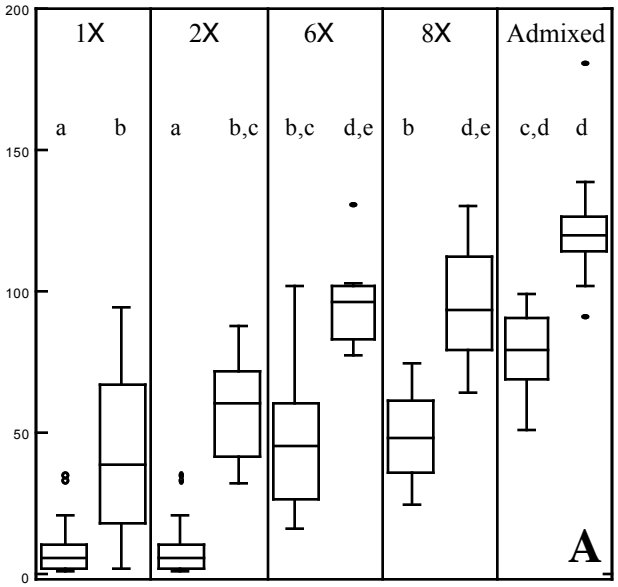
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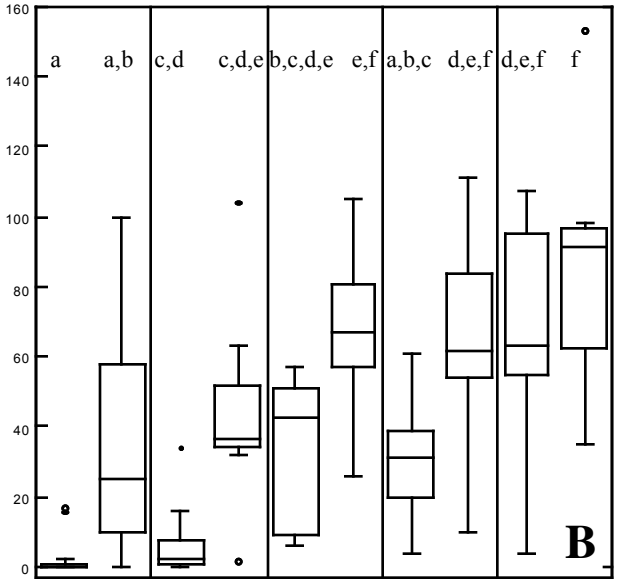
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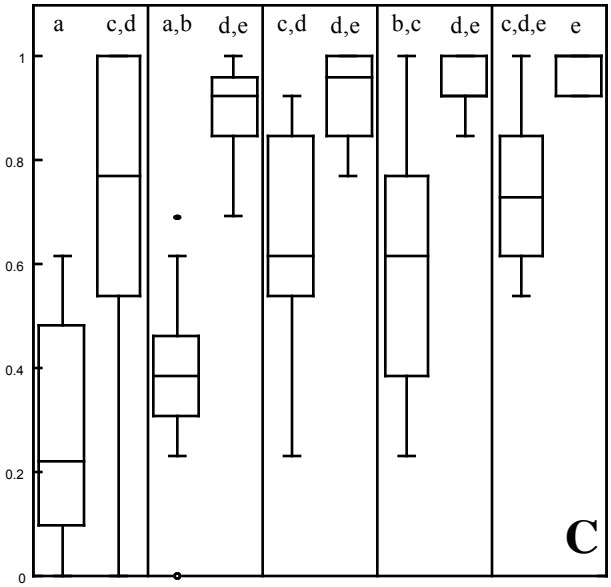
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Figure 1

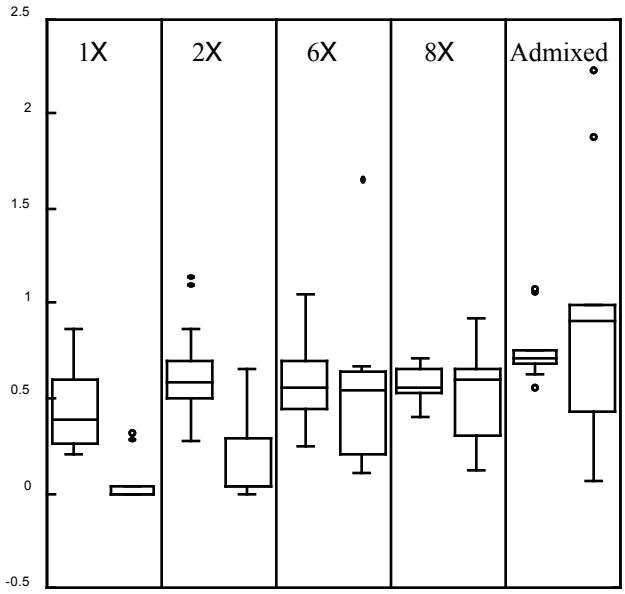

Figure 2