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Levels of inbreeding depression over seven generations of selfing in the androdioecious clam shrimp, *Eulimnadia texana*

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Abstract

Androdioecy (mixtures of males and hermaphrodites) is a rare mating system in both plants and animals. Theory suggests that high levels of inbreeding depression can maintain males in androdioecious populations if hermaphrodites commonly self-fertilize. However, if inbreeding depression (δ) can be 'purged' from selfing populations, maintaining males is more difficult. In the androdioecious clam shrimp, *Eulimnadia texana*, δ is estimated to be as high as 0.7. Previous work suggests that this high level is maintained in the face of high levels of inbreeding due to an associative overdominance of fitness-related loci with the sex-determining locus. Such associative overdominance would make purging of inbreeding depression difficult to impossible. The current experiment was designed to determine if δ can be purged in these shrimp by tracking fitness across seven generations in selfing and outcrossing treatments. Evidence of purging was found in one of four populations, but the remaining populations demonstrated a consistent pattern of δ across generations. Although the experimental design allowed ample opportunity for purging, the majority of populations were unable to purge their genetic load. Therefore, δ in this species is likely due to associative overdominance caused by deleterious recessive alleles linked to the sex determining locus.

Introduction

Androdioecy is a rare reproductive form in which males and hermaphrodites coexist (Pannell, 2002). The relative rarity of androdioecy is consistent with models that predict it to be an unstable form of reproduction (Lloyd, 1975; Charlesworth, 1984; Charlesworth & Charlesworth, 1987; Pannell, 1997). Yet, androdioecious systems do persist in nature (reviewed in Pannell, 2002). Because androdioecy is predicted to be unstable, examinations of the described examples of androdioecy might prove to be most informative to our understanding of biparental and uniparental reproductive tactics.

One well-studied androdioecious animal species is the clam shrimp *Eulimnadia texana* Packard. A genetic analysis of *E. texana* (Sassaman & Weeks, 1993) revealed that populations are comprised of self-compatible hermaphrodites and males. Hermaphrodites are either of two reproductive types: one that produces only hermaphroditic offspring when selfed or crossed with a male (termed monogenic hermaphrodites), and one that produces hermaphrodites and males in a 3 : 1 ratio when selfed and a 1 : 1 ratio when crossed with a male (termed amphigenic hermaphrodites). Hermaphrodites cannot cross with other hermaphrodites (Sassaman & Weeks, 1993). Thus, *E. texana* has a unique, genetic sex-determining mechanism in which males successfully coexist with two forms of hermaphrodites (i.e. a modified form of androdioecy).

This mating system is intriguing because, although self-fertilization is common (inbreeding coefficients ranging from 0.20 to 0.97; Sassaman, 1989; Weeks & Zucker, 1999), androdioecy is still maintained.

Several previous studies have shown cumulative inbreeding depression (δ) to be substantial in *E. texana*, ranging from 0.5 to 0.7 (Weeks *et al.*, 1999, 2000, 2001b). These levels of δ should be great enough to maintain

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androdioecy in this species (Otto *et al.*, 1993), even with low outcrossing rates and reduced male longevity (Weeks *et al.*, 2001a,b), as long as inbreeding depression is not substantially 'purged' from populations after continued inbreeding, as could occur if inbreeding depression were caused by many recessive deleterious alleles scattered throughout the genome (Charlesworth & Charlesworth, 1987; Lande *et al.*, 1994).

However, we have good reason to believe that δ in *E. texana* is maintained by a type of associative overdominance of deleterious recessive alleles with the sex determining locus (or loci). If sex is determined not by one, but rather by many linked loci in *E. texana*, there should be selection for reduced crossing over between these loci (Bull, 1983), which could in turn generate a large linkage group (Weeks *et al.*, 1999, 2000, 2001a). If this hypothesized linkage group contained a number of fitness-related loci, then these deleterious alleles would be inextricably linked to sex determination, making δ very difficult to purge. Such a scenario would facilitate the maintenance of males and outcrossing in these shrimp (Weeks *et al.*, 2001a).

If associative overdominance causes inbreeding depression in *E. texana*, then δ should not decline or be 'purged' over time (Charlesworth & Charlesworth, 1987). On the other hand, if the circumstantial evidence for associative overdominance is incorrect, and in fact δ is the result of a number of unlinked, deleterious recessive alleles, then continued inbreeding should largely purge δ, which would warrant a reevaluation of the importance of inbreeding depression in the maintenance of androdioecy in this system. The current project was therefore undertaken to assess the likelihood of purging in *E. texana* by comparing outcrossed to selfed replicate populations of clam shrimp reared for seven generations. Because the ability to purge may be limited by the extent of previous inbreeding (Husband & Schemske, 1996), we compared levels of inbreeding depression in two sets of populations that were expected to have experienced high and low levels of inbreeding in the wild to note whether δ can be purged in these shrimp over seven generations of self-fertilization, and whether a previous history of inbreeding mitigated this purging.

Materials and methods

Rearing protocol and data collection

Four populations of clam shrimp were used in this study (see also Weeks *et al.*, 1999): three sites in New Mexico, (JD1, JT4 and SWP5), all within Doña Ana Co. (south-central New Mexico), and one site in Arizona (WAL) in Cochise Co., near the south-east base of the Chiricahua mountains. These four populations were specifically chosen to attempt to capture the extremes of levels of outcrossing, using two metrics thought to be correlated with outcrossing rate in these shrimp: male frequency (Sassaman, 1989; Knoll, 1995; Weeks *et al.*, 1999; Weeks & Zucker, 1999) and level of expected heterozygosity (H_e; Weeks *et al.*, 1999; Weeks & Zucker, 1999). JD1 and JT4 have a combination of low male frequency (17 and 22%, respectively) and low H_e (0.19 and 0.15, respectively; Weeks *et al.*, 1999; Weeks & Zucker, 1999). SWP5 and WAL have the highest male frequencies reported (31 and 25%, respectively; Sassaman, 1989; Knoll, 1995; Weeks *et al.*, 1999; Weeks & Zucker, 1999) and the highest reported H_e (0.23 and 0.37, respectively; Weeks *et al.*, 1999; Weeks & Zucker, 1999).

Twenty amphigenic egg banks from each population ('lineages'; determined by being heterozygous at one or more 'marker' loci closely linked to the sex-determining locus) generated in a previous study (Weeks *et al.*, 1999) were hydrated using filtered tap water and transferred to 37-L aquaria (Fig. 1). Hydrations occurred in ten time blocks of two lineages per population per time block (eight hydrations per time block for 80 total hydrations). Shrimp in these aquaria were raised under 'standard' conditions (aquaria were under continuous light using Durotest sunlight-simulating fluorescent bulbs, kept at 25–27 °C water temperature, and had continuous aeration; see Weeks *et al.*, 1999). Each aquarium was fed 40 mL of baker's yeast solution (1 g dried yeast per 100 mL water) per day. Just prior to sexual maturity, up to 50 offspring from each lineage were isolated in 500-mL cups (Fig. 1) with approximately 12 g of finely sifted soil (<125 μm diameter). The soil was collected from a site near the New Mexico sites listed above, but in an area known to be free of branchiopod cysts (>1500 L of soil hydrated over 6 years has produced no clam shrimp). This 'shrimp-free' soil was used for all isolations. Offspring in all cups were fed 1 mL of baker's yeast solution per day. When the offspring matured, males were removed and discarded (Fig. 1), whereas the hermaphroditic offspring were allowed to produce selfed eggs for up to 1 week after isolation. A total of 1721 hermaphroditic offspring were isolated (JD1 = 381, JT4 = 433, SWP5 = 460, WAL = 447) from a total of 67 amphigenic lineages (JD1 = 15, JT4 = 16, SWP5 = 18, WAL = 18). After the eggs were collected, hermaphroditic offspring were then frozen for enzyme electrophoresis (Fig. 1).

Hermaphroditic offspring were assayed for the marker locus of their original hermaphroditic parent (i.e. lineage) using cellulose acetate electrophoresis [heterozygous marker locus was either *Fum* (fumarate hydratase, EC 4.2.1.2), *Idh*-1 or *Idh* -2 (isocitrate dehydrogenase, EC 1.1.1.42); for methods see Richardson *et al.*, 1986]. Such assays allow separation of monogenic (homozygous for the marker locus) from amphigenic hermaphrodites (heterozygous for the marker locus; Weeks *et al.*, 1999). All gels were run using 'Buffer C' from Richardson *et al.* (1986). Out of a total of 1649 offspring that were successfully scored, 1161 were scored as amphigenics (70%; A in Fig. 1) and 488 were scored as

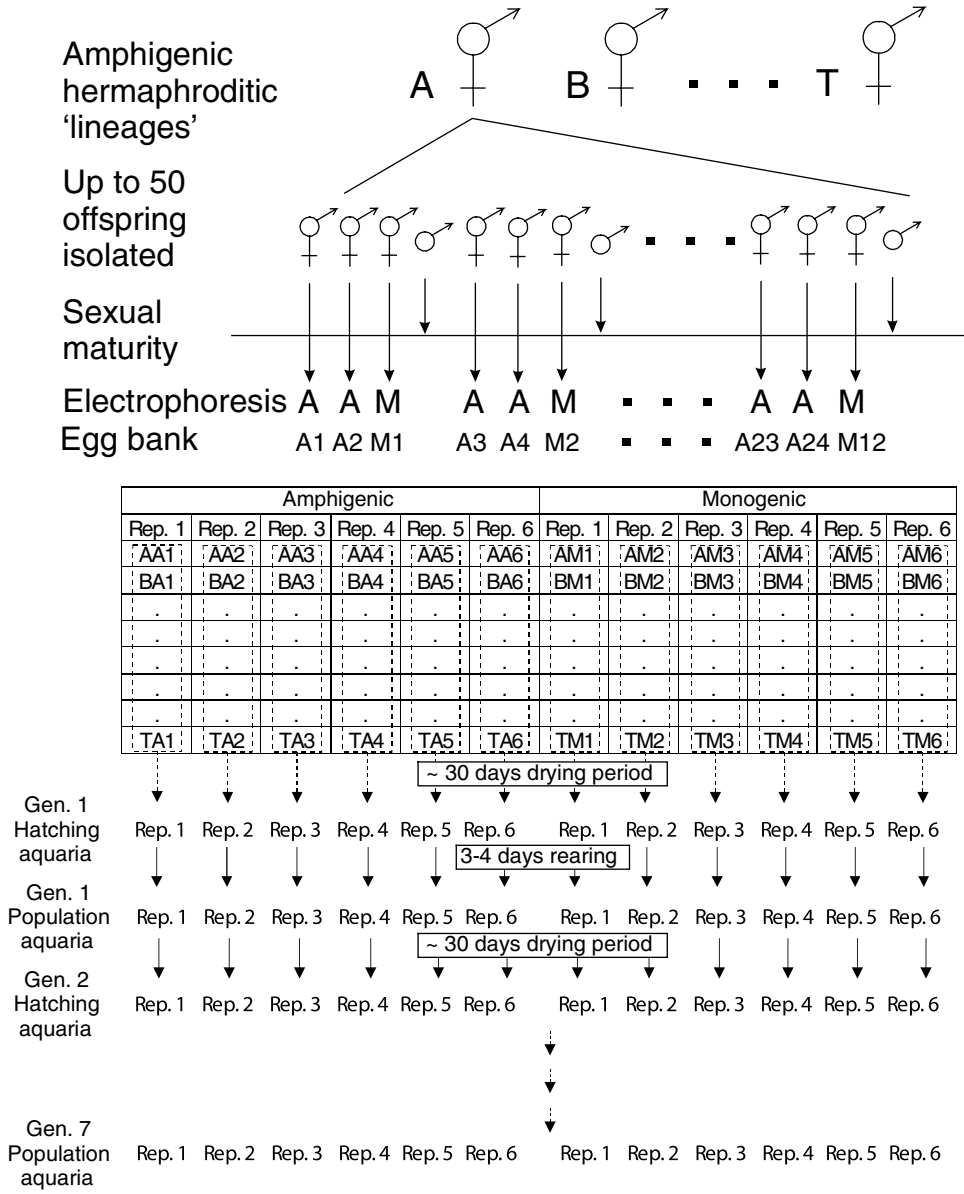

Fig. 1 Experimental design. 'Amphigenic lineages' refers to the 20 original amphigenic egg banks hydrated per population from which up to 50 offspring were isolated. Males were discarded at sexual maturity, and egg banks were collected from the remaining hermaphrodites. Hermaphrodites were electrophoretically scored, and then assigned a hermaphrodite type (A, amphigenic; M, monogenic) based on their allozyme patterns at three loci: *Fum*, *Idh*-1, *Idh*-2. Egg banks were then assigned a number, and stored for later hydration. Six replicates of each hermaphrodite treatment (monogenic and amphigenic) were started with the stored egg banks from each lineage as outlined in the table.

monogenics (30%; M in Fig. 1), which was significantly different ($\chi^2_{(1)} = 10.38$; $P < 0.01$) from the 2 : 1 ratio expected among hermaphroditic offspring from selfing amphigenics (Sassaman & Weeks, 1993). However, this 3% deviation from expectation is reasonable when considering the lower survival of monogenics relative to amphigenics (Weeks *et al.*, 1999, 2001a), and that electrophoretic typing was done on fully grown adult shrimp.

Once electrophoretically typed, egg banks were numbered according to amphigenic lineage (A–T) and hermaphroditic type (A or M, Fig. 1), and stored until egg banks were collected from all 20 lineages per population. To maximize the opportunity for purging (Wang, 2000), the goal was to attain six egg banks of each hermaphroditic type from each of 20 separate lineages to allow genetic diversity within replicate aquaria even when maintaining some consistency among aquaria (Fig. 1).

After all 20 lineages per population were hydrated, the resulting egg banks were dried for at least 30 days. Only 11–15 of the 20 lineages per population produced the requisite six monogenic and six amphigenic egg banks (monogenic egg banks were the limiting factor) to fill the 12 replicates (Fig. 1). Therefore, after the 30-day drying period, egg banks from these 11–15 lineages were combined into each replicate, 37-L aquarium (Fig. 1).

The 11–15 egg banks were combined by hermaphroditic type (monogenics vs. amphigenics), as outlined in Fig. 1, for a total of 48 replicate aquaria: six replicates of two breeding treatments for each of the four populations. The monogenic treatments were started with 100% monogenic offspring produced by selfed monogenic hermaphrodites that 'breed true' (i.e. they produce only monogenic hermaphrodites and no males, thus disallowing cross-fertilization; Sassaman and Weeks, 1993). Therefore, these treatments were consistently inbred throughout the seven generations of the experiment, and are hereafter referred to as the 'selfed' treatments. The amphigenic treatments began with 25% monogenic, 50% amphigenic and 25% male offspring, being produced by selfing amphigenic hermaphrodites (Sassaman & Weeks, 1993). Therefore, the second and subsequent generations were produced via natural levels of outcrossing, and are hereafter referred to as the 'outcrossed' treatments.

The hatching nauplii were reared under 'standard' conditions, with 40 mL of baker's yeast solution added daily. At days 3–4, up to 200 juveniles were randomly chosen to be transferred to a second 37-L ('population') aquarium with 500 mL of shrimp-free soil (Fig. 1). This began experimental 'Day 1' for each population aquarium. The shrimp were sexed and checked for eggs. These densities (5–6 individuals per litre) are within the natural range reported for these shrimp (0.25–7 individuals per litre; Medland, 1989; Mackay *et al.*, 1990). The water from the 'hatching' aquarium was also transferred with the juveniles.

In the population aquaria, the shrimp were again raised under standard conditions, and also were fed 40 mL of baker's yeast solution daily. At days 4, 8 and 12, two measures were recorded: (a) population estimates using fish-net sweeps of each aquarium and (b) egg production in hermaphrodites. For the population estimates, three sweeps of the aquarium were taken, each sweep being for a fixed length of time (30 s). The total number of shrimp caught was used as the population size estimate. Egg production was measured by taking images (using NIH IMAGE software) of up to ten shrimp per aquarium. Each gravid hermaphrodite had two images taken, one on each side of the shrimp, and then the shrimp were returned to the aquaria. From these images (because the carapace is clear), eggs could be counted directly through the carapace (Weeks *et al.*, 1997). At the end of the experiment (day 12), all remaining shrimp were captured and counted. The soil and associated eggs were again dried for 30 days, and then rehydrated for the following generation (Fig. 1). The above procedures were then again followed for a total of seven generations.

The design of this experiment relies on the observation that three electrophoretically scored loci are tightly linked to the sex-determining locus (Weeks *et al.*, 1999). Homozygous offspring resulting from the selfing of an amphigenic heterozygous for *Fum, Idh*-1 or *Idh*-2 should be either male or monogenic (Sassaman & Weeks, 1993; Weeks *et al.*, 1999). However, some crossing-over between these loci and the sex determining locus can occur (Weeks *et al.*, 1999), thus allowing mistakes when using this method for scoring monogenics. In fact, six of the 24 'monogenic' aquaria (JD1, 1; JT4, 0; SWP5, 2; WAL, 3) had one or more amphigenic egg banks mistakenly added to the aquarium, resulting in male 'contamination' in these replicates. We therefore removed these replicates from all analyses because the presence of males made them neither monogenic-only nor amphigenic-only treatments (i.e. leaving a total of 42 replicates: 24 outcrossed and 18 selfed). In the 24 amphigenic (outcrossed) replicates, sex ratio in generation one was found to be 21.7 ± 2.6% (1 SE) males, which is close to the expected 25% males produced during the selfing of an amphigenic (a slightly lower proportion of males is expected due to higher male mortality; see Sassaman & Weeks, 1993).

Statistical procedures

It is important to note that the outcrossed aquaria were mixtures of males, monogenics and amphigenics (the natural outcome of this mixed mating system; Weeks & Zucker, 1999). Therefore, among the outcrossed offspring, measures of population size included males, monogenics and amphigenics, whereas egg production measures included monogenic and amphigenic hermaphrodites. Among the monogenic offspring, all measures only represented monogenic hermaphrodites. Additionally, because the first generation of both selfed and outcrossed treatments was produced by selfing of monogenic or amphigenic hermaphrodites, respectively (see above), comparisons for the effects of continued selfing vs. natural outcrossing were made for generations two to seven only. The results of generation one are reported elsewhere (Weeks *et al.*, 2001a).

All data were analysed using the statistical program JMP (SAS Institute, 2003). The models included three fixed effects: generation (2–7), population (JD1, JT4, SWP5 and WAL) and breeding treatment (outcrossed and selfed). Hereafter, these three fixed effects are abbreviated as Gen, Pop and Trt, respectively. Because populations were not chosen at random, but rather were grouped into low (JD1 and JT4) vs. high (SWP5 and WAL) genetic diversity sets, Pop was considered a fixed effect. Additionally, although generations were linked via parent to offspring inheritance, all other aspects of

replicates (e.g. soil, water, aquaria) were unrelated across generations. Therefore, Gen was considered a fixed rather than a repeated measures effect.

'Survival' of replicate aquaria was analysed using a logistic regression analysis (SAS Institute, 2003), with those replicates extant at the end of the seven generations considered as 'surviving' and all others as 'extinct,' compared between outcrossed and selfed treatments.

Net reproductive rates (R) were calculated per aquarium by constructing life tables from population size estimates and average egg production at days 1, 4, 8 and 12. These two metrics were multiplied and then summed across days to calculate R. These data were analysed with a three-way ANOVA, and needed square-root transformations to normalize residuals. The three-way interaction (Gen*Pop*Trt) was not significant, and thus was not included in the final analysis.

Proportion male within the outcrossed treatments was assessed per population across the seven generations of the experiment using a two-way ANOVA with population and generation as the two main effects. Multiple comparisons were made using a Tukey's HSD comparison (SAS Institute, 2003). Sex ratios were measured at time of sexual maturity (day 4 of experiment), and thus a single measure was generated per replicate aquarium. Analyses were performed both on arcsine-square-root transformed and non-transformed data, weighting proportions by the numbers of shrimp per replicate aquarium. Because both analyses gave virtually identical results, the results for the nontransformed analysis are reported herein.

Results

Out of the initial 42 replicate aquaria, 13 went extinct in the seven generations of the experiment. The number of replicate aquaria that went extinct differed between breeding treatments ($\chi^2_{(1)} = 4.96$; $P = 0.0259$), with more of the selfed replicates going extinct than the outcrossed replicates (Fig. 2). Interestingly, there was a consistent pattern among populations in that the two more genetically diverse populations (high diversity – SWP5 and WAL) had a greater proportion of selfed treatments go extinct ($\chi^2_{(1)} = 4.91$; $P < 0.05$) than in the two more genetically homogeneous populations (low diversity – JT4 and JD1; $\chi^2_{(1)} = 1.11$; n.s.; Fig. 2).

Overall performance in these treatments was compared using net reproductive rates, calculated using numbers of surviving shrimp and average egg production estimates per replicate aquarium. Average net reproductive rates did not include those replicates that went extinct (i.e. once a replicate aquarium went extinct, its zero net reproductive rate was not included in the average for that treatment combination), and therefore this measure should detect a purging in fitness accompanying extinction of lower-fit shrimp in extinct replicate aquaria. Net reproductive rates (R) for surviving replicates did not differ across generations or among populations, nor did these two independent variables interact (Table 1). There was no evidence that the two high diversity populations (SWP5 and WAL) had greater R than the two low diversity populations [JD1 and JT4; $F_{(1,165)} = 0.004$; n.s.]. Outcrossed treatments had significantly greater R than selfed treatments (Table 1, Fig. 3), but there was no significant breeding treatment by generation interaction (Table 1), and thus no suggestion of an overall reduction in the fitness differences between selfed and outcrossed treatments over time (Fig. 3).

Tests of differences between breeding treatments within populations showed outcrossed treatments had either similar or greater fitness than the selfed treatments in JD1, JT4 and SWP5, but fitness differences were minimal in WAL (Fig. 4). The within population difference between breeding treatments was only significant in SWP5 [SWP5: $F_{(1,45)} = 4.86$, $P < 0.05$; JD1: $F_{(1,53)} = 2.42$, n.s.; JT4: $F_{(1,51)} = 2.32$, n.s.; WAL: $F_{(1,41)} = 0.3133$, n.s.].

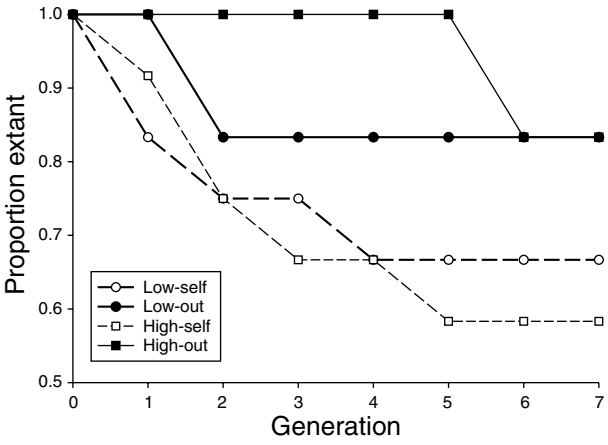

Fig. 2 Proportion of original replicate aquaria surviving over the seven generations of the experiment, grouped by level of genetic diversity (JD1 and JT4 = low diversity = circles; SWP5 and WAL = high diversity = squares), and portrayed for selfed (Self = open symbols and dashed lines) and outcrossed (Out = solid symbols and solid lines) treatments.

Table 1 ANOVA results for net reproductive rates (square-root transformed) per replicate aquarium. Significant P values are in bold.

Source	d.f.	Sum of squares	F ratio	Prob > F
Generation (Gen)	5	7901	1.04	0.3989
Population (Pop)	3	9390	2.05	0.1089
Gen*Pop	15	14 116	0.61	0.8588
Breeding treatment (Trt)	1	8178	5.36	**0.0219**
Gen*Trt	5	1171	0.15	0.9788
Pop*Trt	3	5991	1.31	0.2736
Error	165	251 938		

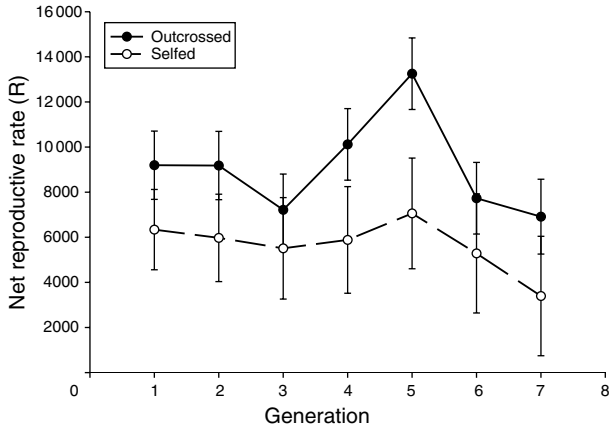

Fig. 3 Net reproductive rates for the outcrossed and inbred treatments averaged across populations. Error bars portray one standard error of the mean.

Table 2 ANOVA results for sex ratios in the outcrossed treatments. Significant *P* values are in bold.

Source	d.f.	Sum of squares	*F* ratio	Prob > *F*
Generation (Gen)	5	10.49	2.93	**0.0162**
Population (Pop)	3	16.74	7.78	**<0.0001**
Gen*Pop	15	11.80	1.10	0.3677
Error	108	77.43		

Table 3 Percent male for each population averaged across generations. Percentages with same letters are not significantly different at the $P = 0.05$ level.

Population	Average percent male	Standard error
JD1	22%[a]	2%
WAL	17[ab]	2
SWP5	14[bc]	2
JT4	10[c]	2

Sex ratios in the outcrossed treatments differed among populations and across generations (Table 2). Starting sex ratios in the outcrossed treatments were ~25% (see above), as expected among offspring from selfing amphigenics (Sassaman & Weeks, 1993). However, in all four populations, percentage male dropped in the first two generations of outcrossing, and then generally stabilized at anywhere from 10 to 22% (Table 3, Fig. 5). These differences among populations were significant (Table 3) and did not significantly change over generations (Table 2). The percentage male in these laboratory populations was generally lower than found in natural populations for all but the JT4 replicate aquaria (Fig. 5), suggesting that these laboratory treatments likely caused greater selfing than in natural populations.

Discussion

The conventionally accepted cause of inbreeding depression (δ) is that the expression of deleterious recessive or partially recessive alleles in homozygous form causes reduced fitness among the offspring of inbred parents (partial dominance model; Charlesworth & Charlesworth, 1987). If this is true, then the model predicts that many of these alleles (at least those of large effect; Lande *et al.*, 1994) should be 'purged' by natural selection in the first several generations of continued inbreeding. If such

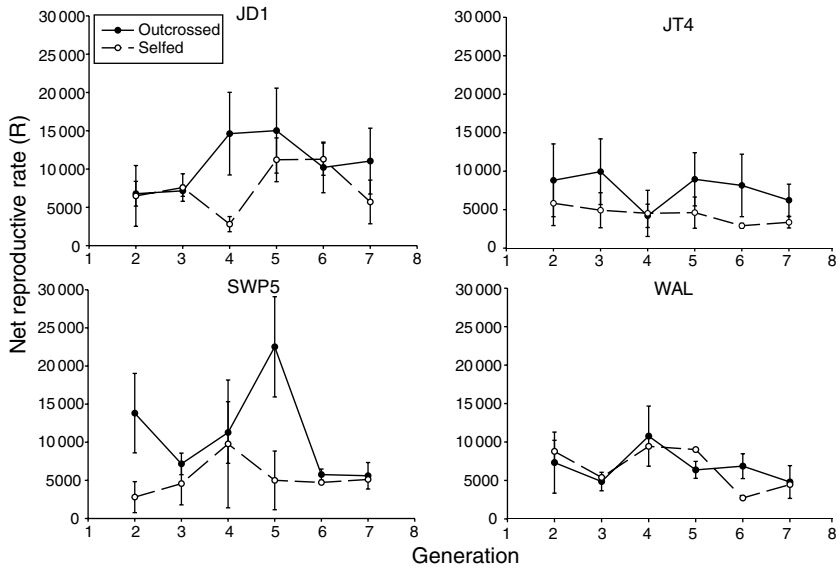

Fig. 4 Net reproductive rates of the four populations across the seven generations of the experiment. Error bars portray one standard error of the mean.

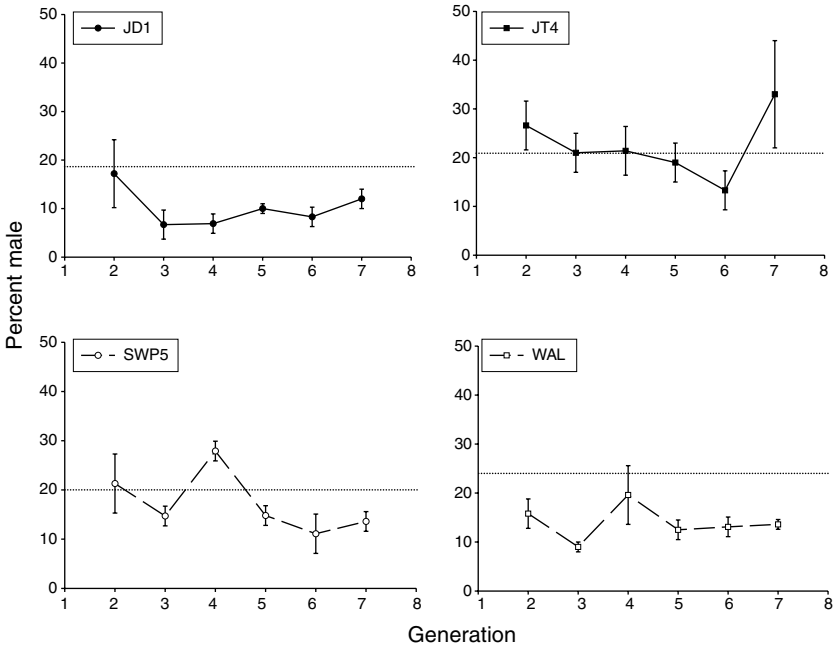

Fig. 5 Percentage males in outcrossing treatments for all four populations across the seven generations of the experiment. Dotted lines indicate natural percentage males for each population (from Weeks *et al.*, 1999).

purging is common, then estimates of the importance of δ in shaping the evolution of life history characters, such as mating systems, should be made only after such purging has had the opportunity to occur. On the other hand, if inbreeding depression is caused by deleterious recessive alleles clustered into a large linkage group (associative overdominance; Ohta, 1971), as has been suggested in previous experiments on *E. texana* (Weeks *et al.*, 1999, 2000, 2001a), then purging of δ would be unlikely (Ohta, 1971) and the previous estimates of δ would be considered robust (Weeks *et al.*, 1999, 2000, 2001b). The current experiment was therefore undertaken to measure inbreeding depression over multiple generations to note either a decline of inbreeding depression over time (consistent with the partial dominance model) or a persistently high level of inbreeding depression (consistent with associative overdominance) in *E. texana*.

Results indicative of purging of inbreeding depression were limited. Clearly, when averaging across all four populations, inbreeding depression was not purged, and in fact remained relatively consistent across the seven generations of the experiment (Fig. 3). However, δ was lower in these population comparisons than previously estimated: δ was estimated to be 0.34 when averaging across all four populations, and 0.41 if the potentially 'purged' WAL population (see below) was not included in the calculation of δ. These values are much lower than the 0.5–0.7 previously reported for these same populations (Weeks *et al.*, 1999, 2000, 2001b). Such a difference is reasonable as these previous estimates strictly compared known selfed to known outcrossed shrimp, where herein the 'outcrossed' treatment included some level of selfing (see discussion below). Thus, the absolute value of δ found in the current experiment is not important, but rather the relative affects of continued inbreeding on the magnitude of δ over time. This project does illustrate that inbreeding depression continued to be an important parameter that was not purged over time, when averaging across populations.

However if we look within populations, we do find some evidence for purging via extinctions of replicate aquaria, specifically within the WAL population. Such extinctions could indicate a type of 'among lineage' purging that could leave the remaining lineages at a higher average fitness due to the loss of the poor performers (Wang, 2000). This type of among-lineage purging was suggested in comparable studies of flour beetles (Pray & Goodnight, 1995) and crickets (Roff, 2002). In the current experiment, the two most genetically diverse populations, SWP5 and WAL (Weeks *et al.*, 1999), had significantly more selfing replicates go extinct relative to outcrossing replicates (75 vs. 17% and 67 vs. 17%, respectively). In the two more genetically homogeneous populations (JD1 and JT4), there was no significant difference in extinctions between breeding treatments (20 vs. 17% and 50 vs. 17%, respectively; Fig. 2). In WAL, the selfing replicates remaining in the experiment did have a similar fitness to their outcrossing counterparts after these replicate aquaria went extinct (Fig. 3), suggesting that among-lineage purging of δ may be possible in this more diverse population. The WAL population has the highest genetic diversity and the second highest proportion of males of the populations so far surveyed (Sassaman, 1989; Weeks *et al.*, 1999; Weeks

& Zucker, 1999), and thus may be more likely to harbour deleterious recessive alleles of large effect that can be effectively purged by continued selfing. Ironically, WAL is the population in which inbreeding depression might feasibly be eliminated with a few generations of inbreeding, but is also a population with a high proportion of males, suggesting that such purging has not affected male abundance in the wild. This may be due to differences in relative levels of inbreeding depression: lifetime inbreeding depression has been estimated to be the highest in this population (0.66–0.69) relative to all others examined (Weeks *et al.*, 2001b). Thus, although a few generations of intense inbreeding may eliminate inbreeding depression in WAL, avoidance of such inbreeding may be strongly selected, and could explain the maintenance of a higher level of males relative to other populations of *E. texana*.

Purging of inbreeding depression was not evident, though, in the other three populations. In SWP5, even with the high replicate extinction rate (Fig. 2), the remaining selfing aquaria continued to have lower fitness than their outcrossed counterparts, possibly due to the random fixation of deleterious alleles in the selfing replicates (termed 'local drift load', Whitlock *et al.*, 2000; or 'among-population inbreeding depression', Keller & Waller, 2002). In JD1 and JT4, there was no significant difference in selfing relative to outcrossing extinctions, and the outcrossing replicates had either similar or greater fitness across generations than the selfing replicates (Fig. 4). Thus, although there is some evidence of purging via replicate extinctions in WAL, there was no other corroborating evidence of purging in any of the remaining three populations.

One caveat that needs to be mentioned regarding the current estimates of purging is that the 'outcrossing' treatments were not as strictly outcrossed as in other, similar multi-generational tests of inbreeding depression (Barrett & Charlesworth, 1991; Pray & Goodnight, 1995; Carr & Dudash, 1997; Dudash *et al.*, 1997; Willis, 1999; Roff, 2002). Because the current experiment estimated inbreeding depression under the more realistic situation of shrimp in small populations (relative to shrimp isolated in cups), natural levels of outcrossing accompanied the comparisons of selfed to 'outcrossed' treatments. Such an experimental design, however, raises two important questions for a test of purging of inbreeding depression: (a) Was the level of outcrossing consistent across generations? (b) Was the level of outcrossing in the laboratory populations similar to those from the field? To address these questions, male frequency (which is known to be related to outcrossing rates, Sassaman, 1989; Weeks & Zucker, 1999) in the outcrossing treatments was compared across generations. It was clear from Fig. 5 that male proportion initially declined (from the ~25% in generation one), but then basically stabilized (with a couple of temporary spikes) for the remainder of the experiment in all four populations. If male proportion were to have shown a consistent reduction over time, then the differences between 'outcrossed' and selfed treatments would have diminished, and such a scenario could have led us to overestimate the importance of potential purging. This was clearly not the case in these populations. For the second question, three of the four populations (JD1, SWP5 and WAL) had consistently lower male proportions than have been reported for wild populations (Weeks *et al.*, 1999; Weeks & Zucker, 1999; Fig. 5). Therefore, for these three populations, the differences between outcrossed and selfed treatments were likely lower than one would find in the field, potentially biasing our results towards finding purging by decreasing the fitness differences between outcrossed and selfed breeding treatments in the laboratory. Thus, our finding of an inbreeding depression 'purge' in WAL should be considered a liberal estimate, with the caveat that the lack of a fitness difference between breeding treatments may under-represent true fitness differences in the field.

The lack of appreciable purging is not unique to this experiment. Byers & Waller (1999) surveyed 52 studies for evidence of purging of inbreeding depression. They included studies that compared levels of inbreeding depression among taxa, among populations and among lineages within populations. Byers and Waller grouped studies on the basis of 'study type', to note whether the method of estimation (inbreeding depression estimated from single-generation laboratory studies, multiple-generation laboratory studies or estimated from natural differences in level of inbreeding among field populations) affected their conclusions. Overall, only 38% of these experiments found any evidence for purging of the genetic load with continued inbreeding. The majority of the studies surveyed (36) were single-generation comparisons and only 14 of these (39%) noted purging. Fourteen studies followed populations for multiple generations (arguably the best method for detecting purging), and only five of these (36%) had any evidence consistent with purging. Additionally, the level of comparison (taxa, population or lineage) did not affect these conclusions (43, 38 and 33% supported purging, respectively). Byers and Waller conclude that 'purging is an inconsistent force within populations', which might be an understatement given the lower likelihood of publication for negative results.

Is seven generations enough time to detect a purge? In inbreeding experiments where populations are selfed, heterozygosity should be reduced by 50% each generation of selfing. Thus, the selfed treatments should have had only <1% of their original heterozygosity at the completion of the experiment, which should be sufficiently low to expose most recessive alleles to homozygosity. Additionally, several other studies have detected purging in less than seven generations. Barrett & Charlesworth (1991) noted significant purging in the hyacinth *Eichhornia paniculata* in only five generations of selfing. Similarly, Dudash *et al.* (1997) found purging in

some lineages of *Mimulus guttatus* in five generations of selfing. Pray & Goodnight (1995) also found some lineages of the flour beetle, *Tribolium castaneum*, purged inbreeding depression across a number of traits in only four generations of sib-mating. In one of the most dramatic examples, Saccheri *et al*. (1996) detected purging in only two generations of inbreeding in the butterfly *Bicyclus anynana*. Thus, seven generations of selfing should be sufficient to detect purging in most species, and our findings of limited purging of inbreeding depression in the current study should be considered robust.

A persistent level of δ across multiple generations of inbreeding is consistent with the notion that associative overdominance causes inbreeding depression in *E. texana*. Previous research provides circumstantial evidence for associative overdominance due to selection for limited crossing over among the sex determining loci, which in turn causes a large linkage group (Weeks *et al*., 1999, 2000, 2001a). Indirect evidence for such a linkage group has been suggested in two separate observations. First, amphigenic offspring have significantly greater survival and reproductive output than monogenic offspring within single clutches produced by selfing amphigenic hermaphrodites (Weeks *et al*., 2000, 2001a). Such a repeatable fitness difference among offspring from the same selfing event can be best explained if either the sex-determining locus affects hermaphroditic fitness directly, or more likely if one or more linked loci are affecting fitness. Second, three of seven variable electrophoretic loci are all linked to one another and to the sex-determining 'locus' (Weeks *et al*., 1999). The odds of encountering such a high proportion of closely linked loci at random are extraordinarily low, and so it is more likely that these loci are a part of a larger linkage group. These results, combined with the general lack of purging in the current experiment, all suggest a large linkage group containing several fitness-related loci is a major contributor to δ in this species.

Deleterious fitness-related loci mutating within this linkage group would be difficult to purge, and thus may tend to accumulate. Deleterious alleles outside this linkage group should be capable of being purged, and any detectable inbreeding depression in a regularly selfing species would likely be due to those deleterious loci contained within the linkage group. Thus, continued inbreeding depression in the majority of populations of *E. texana* is likely caused by a form of associative overdominance of a linkage group that contains the sex-determining region of these shrimp.

The upshot of this for the *E. texana* system is that inbreeding depression estimates found in previous studies (Weeks *et al*., 1999, 2000, 2001a,b) should be considered robust, and the maintenance of androdioecy in *E. texana* should therefore continue to be considered largely shaped by the detriments of producing offspring via self-fertilization.

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