



# Are all-hermaphroditic populations of *Eulimnadia texana* Packard, 1871 (Branchiopoda: Spinicaudata) resistant to invasion? Implications for the maintenance of androdioecy

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## ABSTRACT

Androdioecy (males and hermaphrodites) is a rare breeding system in multicellular organisms, found mostly in barnacles and branchiopod crustaceans. The most speciose and longest-lived androdioecious clade is the genus *Eulimnadia* Packard, 1874 (Branchiopoda, Spinicaudata), the clam shrimps, consisting of over 50 species that have maintained androdioecy for an estimated 24–180 million years. Many populations of *Eulimnadia* nevertheless comprise entirely “monogenic” hermaphrodites. Hypotheses proposed to explain the relative stability of androdioecy (sexual conflict, overdominance, and metapopulation model) differ in their predictions of the resistance of existing all-hermaphrodite populations to invasion of males and hermaphrodites. We tested whether all-hermaphroditic populations of *Eulimnadia texana* Packard, 1871 may be resistant to male invasion by adding males and “amphigenic” hermaphrodites to all-hermaphrodite, monogenic populations that have been inbred for eight generations. All-hermaphrodite populations of *E. texana* that have been selfing for multiple generations are easily invaded by males, both directly and indirectly. The addition of males also increased the productivity of these experimental treatments, suggesting a selective benefit to outcrossing and thus to males. These results do not align with the sexual conflict nor the overdominance models, but are consistent with the metapopulation model of the maintenance of androdioecy.

**Key Words:** breeding systems, metapopulation, reproductive assurance, sexual conflict

## INTRODUCTION

Androdioecy (populations of males and hermaphrodites but lacking females) is a rare breeding system in multicellular organisms (Charlesworth, 1984; Pannell, 2002; Weeks *et al.*, 2006a; Weeks, 2012). Such rarity fits models that predict it to be a short-lived “transitional” breeding system (Lloyd, 1975; Charlesworth & Charlesworth, 1978; Charlesworth, 1984). Branchiopod crustaceans in the genus *Eulimnadia* Packard, 1874, however, do not fit this pattern of rare, short-lived androdioecy (Weeks *et al.*, 2006b; 2008). These clam shrimps belong to over 50 species (Brtek, 1997; Reed *et al.*, 2015) and the clade has maintained androdioecy for 24–180 million years (Weeks *et al.*, 2006b).

Even though androdioecy is the dominant breeding system in *Eulimnadia* (Weeks *et al.*, 2009), several *Eulimnadia* populations (Sassaman, 1995; Weeks *et al.*, 2008), and even one species (*E. agassizii* Packard, 1874; Zinn & Dexter, 1962; Smith, 1992; Weeks *et al.*, 2005), are entirely hermaphroditic. It is unclear

whether these all-hermaphroditic populations have lost males through selection or if random processes such as genetic drift or limited colonization have led to their loss.

To understand the latter option, one needs to understand the unique genetic sex determination of these clam shrimps and their ecology. Sex in *Eulimnadia texana* Packard, 1871 is genetically determined via a Z/W sex chromosome system: ZZ individuals are males, ZW “amphigenic” hermaphrodites and WW “monogenic” hermaphrodites (Sassaman & Weeks, 1993; Weeks *et al.*, 2010). Both types of hermaphrodites can either outcross with males or can self-fertilize but cannot fertilize one another because of the absence of claspers in hermaphrodites that are needed for pairing during mating. Clam shrimps live in small, temporary freshwater pools that commonly form after rains, and are found on all continents except Antarctica (Dumont & Negrea, 2002). Such pools are commonly short-lived, with new pools arising regularly and then infilling with sediments over time. Colonization of new pools and local population extinctions are therefore likely common for clam

shrimps (Vanschoenwinkel *et al.*, 2010; Stoch *et al.*, 2016). When colonization of new habitats occurs by only a few clam shrimp (or possibly a single one), all individuals of a population can be hermaphroditic because they were started with only WW monogenic hermaphrodites (Pannell, 1997). If no further colonization occurs, these populations will maintain all-hermaphroditic individuals only due the vagaries of their initial colonization (Pannell, 1997, 2002). Because of this unique sex-determining mechanism, Pannell (1997) predicted that *Eulimnadia* populations should be a mixture of androdioecious (i.e., males + hermaphrodites) and hermaphrodite-only populations in what he termed the metapopulation model for the maintenance of androdioecy.

Hermaphrodite-only populations could have purged males through selection (Otto *et al.*, 1993; Pannell, 2008; Chasnov, 2010). Chasnov (2010) posits the sexual conflict model in which many generations of self-fertilization are assumed to purge genetic load and thus inbreeding depression (Barrett & Charlesworth, 1991; Byers & Waller, 1999), and with it any selective benefits for hermaphrodites to outcross with males. An alternate argument is that males could be lost if their ability to successfully mate falls below two times the inbreeding depression that selfing hermaphrodites may experience (Otto *et al.*, 1993; Pannell, 2008). In both of these arguments, male-less populations would reflect selection against males, and such populations would be resistant to male colonization.

We tested whether male-less populations of *E. texana* are indeed resistant to male colonization. Because male colonization in this species can either be direct (i.e., by colonizing ZZ individuals) or indirect (i.e., by colonizing ZW individuals that then produce males via selfing), we subjected established populations of all-monogenic *E. texana* to invasions by either males or amphigenic hermaphrodites. Experimental populations over the course of three generations were monitored to determine if males would successfully invade existing monogenic pools. Population sizes over the three generations were also monitored to determine if these colonization treatments conferred increased growth rates over time. The results were also compared to models of the maintenance of androdioecy to note which model best explained the observed invasion patterns.

## MATERIALS AND METHODS

Sediment containing resting eggs of *E. texana* from a previous experiment (Weeks, 2004) was used for the initial populations. The previous experiment yielded four different all-monogenic hermaphroditic populations (started with 11–15 monogenic lines per population) from three localities in New Mexico and Arizona, southwestern USA that were established by repeated inbreeding over the course of seven generations (Weeks, 2004). Resting eggs from this experiment were hydrated and allowed to hatch (beginning the eighth generation of selfed offspring, which is recorded herein as Generation 1). Four additional “transfer” tanks with sediment (known to be free of resting eggs) were simultaneously hydrated, as was sediment from wild populations of the same localities as the inbred populations, to supply either males or amphigenic hermaphrodites for the experimental manipulations.

Prior to maturity (~4 d post-hatching), all individuals were removed from their hatching tank with nets. Up to 200 of these juveniles from each hatching tank were moved to the egg-free transfer tanks noted above. The male or amphigenic shrimp from the wild tanks were added to the experimental tanks when both types of individuals and the experimental shrimp had reached sexual maturity (~5 d). Four males or five hermaphrodites were added. In these populations, ~20% of the hermaphrodites are monogenics, and thus when randomly selecting five hermaphrodites, on average four of them are expected to be amphigenic (Weeks *et al.*, 1999; 2014). This number reflects the low migration rate (0.6 individuals per generation) observed in natural populations (Weeks &

Duff, 2002). The day the shrimp were counted and transferred to the experimental tank is referred to as Day 1 of the experiment.

Population size assessment occurred on days 4, 8, and 12. Three, 30-second sweeps through each tank with a dip net provided estimates of population size. The tanks were completely sampled and all shrimp removed on day 12.

Sediment from the tanks containing resting eggs of the previous generation was allowed to dry for a minimum of 30 days. The sediment was mixed well and divided into three equal portions. One third was mixed with egg-free sediment to 500 ml of total volume, hydrated, and monitored for hatched nauplii. The remaining two thirds of the sediment were retained for future use.

Prior to maturity, up to 200 individuals were transferred with their water to tanks containing egg-free sediment and allowed to mature (Generation 2 treatment). Individuals were netted, counted, and sexed every four days. This was continued for one additional generation, or Generation 3. The entire design of four populations bred through three generations for each type of introduction (male or amphigenic) was replicated three times, and males and hermaphrodites in each tank were recorded for all three generations.

### Statistical analyses

We assessed the numbers of males at Day 4 between addition treatments (male *versus* amphigenic) and over generations using a mixed-model, two-way ANOVA. The number of males was square-root transformed, which normalized residuals. Populations were considered blocks, and the three replicate hydrations for each population were nested within populations and considered random effects.

We used a repeated-measures MANOVA on total population counts at the three repeated population samples (days 4, 8, and 12) to assess within-generation population size. The overall model was the same as noted above for males: Addition and Generation were the main effects, Population was the blocked effect, and Replicate hydrations were nested within Population. The repeated measure was termed Time.

The effects of amphigenic- and male-addition treatments on population growth were assessed by regressing percent population change in size from one generation to the next (1 → 2 or 2 → 3) on the proportion of added males or amphigenic hermaphrodites at the start of the treatment.

## RESULTS

Out of 72 total tank hatches (four populations × two introduction types (male/amphigenic) × three generations × three replicate hatches), 52 hatches yielded adult clam shrimp. Population sizes in the various treatment combinations ranged from 4 to 200 (our pre-determined cap) with an average population size of 114 individuals.

Males were present in several populations within two generations of male and amphigenic introductions. In Generation 3, males were found in 6 out of 8 male-introduced populations (75%) and in 4 out of the 9 amphigenic-introduced populations (44%). Males significantly increased in number over the course of two generations in both additions (Table 1). Although the number of males present in Generation 3 male-introduction populations appeared to be greater, on average, than those found in Generation 3 amphigenic-introduction populations (Fig. 1), this difference was not significant (i.e., no Addition by Generation interaction; Table 1).

The results of a repeated measures MANOVA with Population as a block effect and hatching-replicate nested within Population confirmed that population sizes declined steadily over time since hatching (Table 2; Fig. 2). There was nevertheless no difference in population size over time between male- or amphigenic-addition

treatments or generations (Time × Addition and Time × Generation; Table 2).

Population sizes were boosted by the addition of amphigenic hermaphrodites. The percent of hermaphrodites seeded into the Generation 1 was associated with a significant increase ( $P = 0.022$ ) in population size (% change) from the Generation 1 to Generation 2 in the amphigenic-addition treatments (Fig. 3A).

Among male-addition lineages, the proportion of males seeded into the population in Generation 1 significantly ( $P = 0.014$ ) impacted the change in the size of the overall population from that generation to the next. When males were initially in a higher proportion (Generation 1), those populations had a greater percent increase in total population size from Generation 1 to Generation 2 (Fig. 3B).

Because the self-fertilizing hermaphrodites that were seeded into Generation 1 were capable of producing male offspring, we also examined how the percent of males in Generation 2 of the amphigenic-addition treatments influenced the change in population size in the following generation. Although male proportions were low in Generation 2, the higher the male proportion

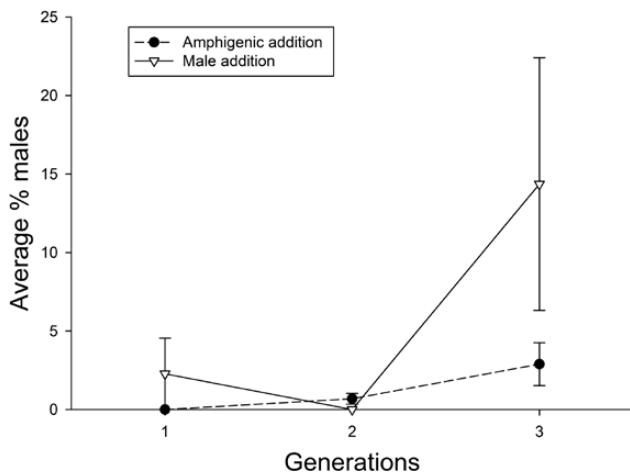
in Generation 2, the larger the population size increase from Generation 2 to Generation 3 ( $P = 0.011$ ; Fig. 3C).

DISCUSSION

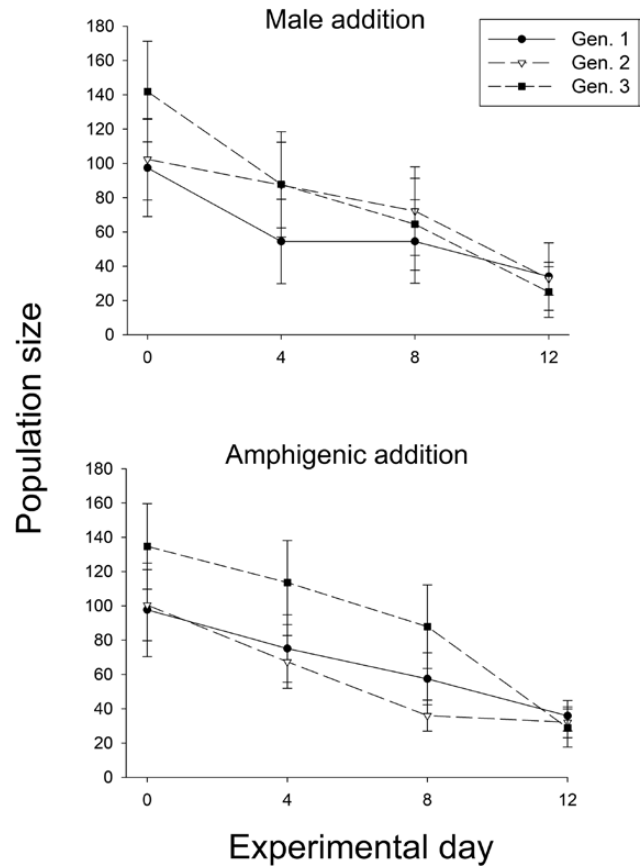
The common observation of male-less populations (and even species) in *Eulimnadia* could be due to selection against males in these populations (Otto *et al.*, 1993; Pannell, 2008; Chasnov, 2010) or to the colonization of these populations by a few monogenic hermaphrodites (Pannell, 1997). Another possibility is that small populations of clam shrimps may lose the Z chromosome via genetic drift. The latter two hypotheses suggest a “random” cause of a lack of males whereas the former postulates direct negative selection against males. These various alternatives can be easily assessed by introducing males to all-monogenic populations of *Eulimnadia*.

**Table 1.** ANOVA results comparing number of males present in each treatment over generations. Replicate hydrations were nested within Populations, which had a variance component (using restricted maximum likelihood estimates) of 0.177 and accounted for 17.6% of the total variation. The Addition treatment was males or amphigenic hermaphrodites.

Source	df	df Denominator	F-ratio	P
Addition	1	41.35	0.1492	0.7013
Generation	2	33.41	12.3769	0.0001
Addition × Generation	2	33.41	0.7688	0.4716
Population	3	5.124	1.6859	0.2819



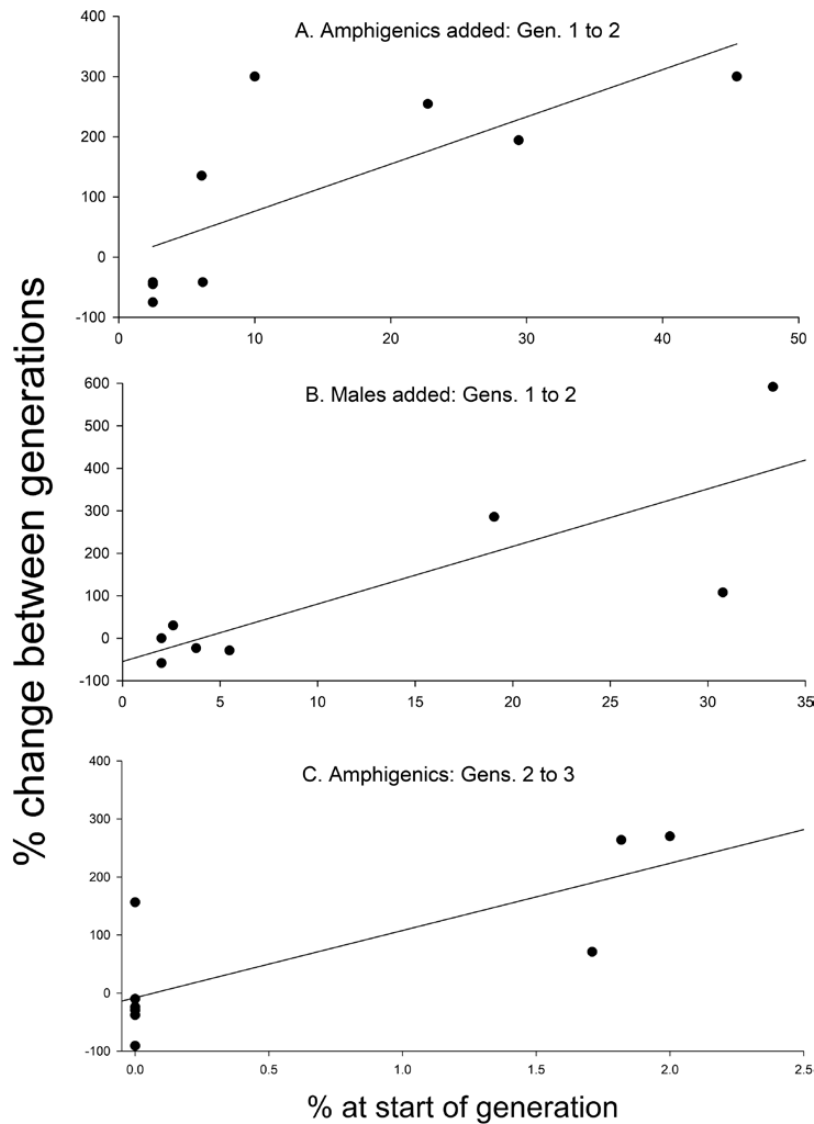
**Figure 1.** Average male proportions of male- and amphigenic-addition treatments over three generations. Error bars represent one standard error.



**Figure 2.** Average population sizes of male- and amphigenic-addition treatments across three generations and over experimental days. Error bars represent one standard error.

**Table 2.** MANOVA table examining the effect of Time (4, 8, or 12 days) by generation (1, 2 or 3) on population sizes among different addition treatments (males or amphigenic). There were four starting “Populations” of monogenics, and each Population was hydrated three separate times (Replicates).

Source	df	df Denominator	F-ratio	P
Time	3	33	23.5287	0.0001
Time × Addition	3	33	1.5353	0.2237
Time × Generation	6	66	1.5875	0.1647
Time × Addition × Generation	6	66	1.2732	0.2818
Time × Population	9	80.464	1.5385	0.1487
Time × Replicate (Population)	21	95.308	1.6290	0.0583



**Figure 3.** The effect of amphigenic addition into Generation 1 on the percent change in total population size from Generation 1 to Generation 2. R-squared, 0.487, slope = 0.07033,  $df = 7$ ,  $P = 0.02197$  (A). The effect of male addition into Generation 1 on the percent change in total population from Generation 1 to Generation 2. R-squared, 0.6086, slope = 0.04903,  $df = 6$ ,  $P = 0.01368$  (B). The effect of males in Generation 2 of the amphigenic-addition treatments on the percent change in total population size from Generation 1 to Generation 2. R-squared, 0.5686, slope = 0.005375,  $df = 7$ ,  $P = 0.01148$  (C).

All-hermaphroditic populations of *E. texana* were clearly invisable by males both through direct male introductions and by indirect introductions of amphigenic hermaphrodites (which produce males via selfing). Male-addition treatments may have been somewhat more effective in establishing males (Fig. 1), but males persisted in the otherwise monogenic-only experimental populations in both treatments. Indirect colonization via the introduction of amphigenic hermaphrodites into all-monogenic populations was also found to successfully establish males in a previous study of *E. texana* (Weeks, 2009), which underscores the robustness of the current findings. Not only did males successfully establish via direct or indirect colonization, but their presence increased the success of the populations in direct proportion to male abundance (Fig. 3). Both results suggest male-less populations of species of *Eulimnadia* are not the result of selection against males. Instead, these populations have either likely been colonized by one or a few monogenic hermaphrodites (and never subsequently been colonized by males or amphigenic hermaphrodites) or have lost the Z chromosome due to severe genetic drift in very small populations. We believe that the egg banks typical of branchiopod crustaceans

(Brendonck & De Meester, 2003) and the unique ZW genetic sex determination of these clam shrimps make the latter possibility less likely, and thus favor the former colonization argument.

The various models of the maintenance of androdioecy (Otto *et al.*, 1993; Pannell, 1997; 2008; Chasnov, 2010) can also partially be assessed using these data. The sexual conflict model (Chasnov, 2010) predicts that established monogenic populations would be resistant to amphigenic hermaphrodite invasion because monogenics have higher fitness due to purging of their genetic load. Males are predicted to invade because of “forced copulations” with monogenic hermaphrodites. We observed that males can invade and mate, although we have not measured specific behavioral resistance to outcrossing on the part of monogenics. Furthermore, amphigenic hermaphrodites successfully established in the amphigenic addition treatments, both herein and in a previous study (Weeks, 2009). The successful invasion of amphigenic hermaphrodites contradicts the predictions of the sexual conflict model in clam shrimps.

An assumption of the sexual conflict model is that an established monogenic population will have purged any potential

inbreeding depression. This would create highly fit animals resistant to outcrossing with males and able to outcompete amphigenic hermaphroditic invaders. Because the monogenic populations in our experiment had been inbreeding exclusively for eight generations prior to the experimental migration treatments, hermaphrodites had ample opportunity to purge inbreeding depression if such purging is possible (Barrett & Charlesworth, 1991; Byers & Waller, 1999; Miller & Hedrick, 2001). Weeks (2004), however, showed a lack of purging in these populations, and the positive correlation of % male with % increase in population size after male addition in our experiments (Fig. 3) indicate that monogenic populations do not easily purge genetic load. The observation that inbreeding depression has not been purged is likely related to viability genes in the linkage group associated with sex determination: alleles located near the sex-determining linkage group are subjected to reduced recombination rates (Weeks *et al.*, 2010) and as a result are hypothesized to not be easily purged, even if they confer a notable fitness detriment (Weeks *et al.*, 1999; 2010).

The clear success of amphigenic invasion in our study, combined with previous data suggesting a restricted capacity to purge inbreeding depression in the sex-determining linkage group, suggests that the sexual conflict model does not well explain the maintenance of androdioecy in *E. texana*. The model provides a better explanation for androdioecy in other species, particularly nematodes (Chasnov, 2013).

Otto *et al.* (1993) and Pannell (2008) developed comprehensive models to explain the maintenance of androdioecy in *Eulimnadia*. Both models (that of Pannell (2008) is patterned from the earlier model of Otto *et al.* (1993)) provide a broad spectrum of options dependent on the range of values of inbreeding depression, relative male mating success, differences between males and hermaphrodites in survivorship, whether there may be sperm limitation in hermaphrodites and the relative fitness of ZW and WW hermaphrodites. These broad options cannot be fully assessed using our data. A variant of Pannell's model (articulated in Chasnov, 2010) nevertheless suggests that males are maintained merely by a substantial viability difference between ZW (amphigenic) and WW (monogenic) hermaphrodites. In this variation, males are ineffective at outcrossing but are still maintained in populations at ~25% abundance due to a type of "overdominance" of the ZW relative to both the ZZ and WW individuals (Pannell, 2008; Chasnov, 2010). We refer to this variant as the Overdominance Model. The clear ability of males to outcross successfully in the male-addition treatments combined with the observation that such outcrossing clearly increased fitness in these experimental populations suggests that this Overdominance Model is not realistic.

The model that best explains our findings is the Metapopulation Model of Pannell (1997), which specifically posits that androdioecious species will be a mix of androdioecious and all-hermaphroditic populations in a larger metapopulation. The all-hermaphrodite populations should be the result of colonization by WW hermaphrodites and should be prone to "conversion" to androdioecious populations by immigration of either ZZ or ZW individuals. These predictions are clearly borne out in *Eulimnadia*, which comprise androdioecious and all-hermaphrodite pools (Sassaman, 1995; Weeks *et al.*, 2008) and are invulnerable by males (Fig. 1) as well as amphigenic hermaphrodites (Fig. 1 and Weeks, 2009). The Metapopulation Model is thus the most realistic model so far explaining the long-term persistence of androdioecy in *Eulimnadia*. Further experiments, in which males are added to all-hermaphroditic populations of other *Eulimnadia* species, would provide further tests of these ideas.

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