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Implications for the Maintenance of Androdioecy in the Freshwater Shrimp, *Eulimnadia texana* Packard: Encounters between Males and Hermaphrodites Are Not Random

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Abstract

Androdioecy is a mixed-mating system in which there are males and hermaphrodites but no pure females. Few species exhibit such a mating system. *Eulimnadia texana* is a branchiopod crustacean that has recently been identified as an androdioecious species. This system is ideal for testing questions related to the evolution of sexual reproduction. We are testing a model that predicts androdioecy to be a stable mixed-mating system under certain conditions. Specifically, we investigated whether encounters between males and hermaphrodites are random or if either sex seeks out the other for mating. Focal male or hermaphrodite clam shrimp were presented with stimulus shrimp of the other sex or kept alone. Swimming speed and time spent within different areas of a test chamber were recorded. Males did not alter mean swimming speed or spend more time than expected by chance near partitioned hermaphrodites. Hermaphrodites, however, decreased mean swimming speed in the presence of males and also spent more time than expected by chance near partitioned males, suggesting that hermaphrodites respond to male chemical and/or visual stimuli. Modified swimming behaviour probably facilitates inter-sexual contact, thereby increasing opportunities for outcrossing above that expected by random encounters.

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Introduction

Sexual reproduction for dioecious animals requires that the two sexes are able to locate and recognize potential mates. Differences in the relative costs of parental

care or differences in the sex ratio and reproductive rates are expected to result in intra-sexual competition for access to mates. The sex that is more common, has the lower parental investment, or has the higher potential reproductive rate, often males, is expected to evolve traits that would increase its ability to compete with others of the same sex for the opportunity to mate with members of the other sex (Trivers 1976; Gwynne 1991; Clutton-Brock & Parker 1992). Since a major component of sexual reproduction is the successful location of a mate, searching behaviour should be under strong selection pressure (Parker 1978). We would expect this to be especially true in 'mixed-mating' systems (mixtures of self-fertilization and out-crossing). Androdioecy is one such rare mixed-mating system where males and hermaphrodites, but no true females, coexist (Charlesworth 1984). Furthermore, in some androdioecious systems, hermaphrodites cannot out-cross with other hermaphrodites, but can self-fertilize or mate with males. In these systems, males are not only competing with each other for potential mates, but are also faced with the additional task of pursuing hermaphrodites which do not require male sperm to fertilize their eggs and may, therefore, be less willing to mate. There may thus be particularly strong selective pressure on males to locate and identify receptive mates (Jarne and Charlesworth 1993).

The branchiopod crustacean *Eulimnadia texana* has such a specialized androdioecious system of reproduction. This species is a small conchostracan shrimp which inhabits ephemeral freshwater ponds throughout the south-western United States (Sassaman 1989). Hermaphrodites can self-fertilize or out-cross with males, but they are unable to out-cross with other hermaphrodites because they lack the male's specialized mating appendages (Sassaman and Weeks 1993). Males are often rare relative to hermaphrodites, with populations containing approximately only 25% males on average (Sassaman 1989; Weeks and Zucker 1999).

Otto et al. (1993) developed an analytical population genetics model to predict the equilibrium frequencies of mating types that are stable in the *Eulimnadia texana* mixed-mating system. In this model, hermaphrodites can choose either to self-fertilize or to out-cross with a male. Out-crossing is dependent on the likelihood of the two sexes encountering each other. The model assumes that encounters between males and hermaphrodites are directly proportional to the frequency of males in the population. Once an encounter is made, a successful mating will occur a certain proportion of the time. If no male encounters a hermaphrodite then she will self-fertilize a proportion of her own eggs.

Encounters may simply be random, analogous to movement described by the molecular gas laws where the shrimp are particles that randomly bump into each other in a homogenous 3-dimensional environment (Brewer 1998), or may be directed, with one or both sexes modifying their behaviour to increase mating opportunities. Here, we examine encounter behaviour between males and hermaphrodites in *Eulimnadia texana* by comparing their swimming speeds and locations in the presence or absence of the other sex.

Methods

Two populations of *Eulimnadia texana* were used in this study. The first population (JT4) was collected from a natural depression used by cattle, located on the USDA-ARS Jornada Experimental Range, near Las Cruces, Doña Ana County, New Mexico, USA. The second population (WAL) was collected from a human-made cattle tank located near Portal, Cochise County, Arizona, USA. At each site several soil samples were collected from the dry surface (top 2 cm), returned to the laboratory and stored for future use. Study populations were reared by hydrating 30 ml of WAL or 100 ml of JT4, using charcoal-filtered tap water. The soil was first sieved through 270 μm mesh to remove the eggs of *Triops* sp., which feed on clam shrimp larvae. On the second day, larvae were separated from the settled soil. Filtered water from the other site (i.e. water incubated for 24 h on soil and then filtered through a 63-μm sieve to remove any branchiopod larvae) was added to each population to ensure that both populations were reared under common conditions. For the first 3 days, populations were fed bakers yeast (Fleischmann's) and were maintained at 28–30°C using 100-W light bulbs. On the fourth day, just before they reached sexual maturity, shrimp were identified to sex using a dissecting microscope. Males and hermaphrodites were moved to separate tanks, maintained at summertime light/dark cycles under fluorescent light, and were fed ground TetraMin fish-flakes daily until behavioural observations were conducted 48–72 h later.

We quantified searching behaviour in three different ways: swimming speed; relative distances travelled during an 8-min trial; and relative time spent near and away from conspecifics of the opposite sex.

Swimming behaviour in the presence and absence of conspecific stimulus shrimp of the other sex was characterized by videotaping focal animals. The experimental set-up consisted of a Plexiglas container placed on a light-table with a video camera recording from above. The container (30 cm long by 15 cm wide by 2 cm deep, holding 1 cm of filtered tap water) had a transparent bottom and opaque sides to improve image capture quality. The container was divided into two sub-chambers by a transparent barrier perforated by 10 1-mm holes located 2 mm from the bottom, which allowed both visual and chemical communication between shrimps on each side. The observation chamber, into which the focal animal was released, measured 25 cm by 15 cm by 2 cm, and the stimulus chamber, which confined either five stimulus shrimps of the other sex or was empty, measured 5 cm by 15 cm by 2 cm.

Sexually mature 6–7-day-old focal animals were individually introduced into the far end of the observation chamber with or without stimulus shrimps of the other sex present in the stimulus chamber and were video-taped for 8 min. To ensure that focal hermaphrodites would be receptive to males, they were encouraged to drop their clutch of eggs about 30 min before the trials by isolating them in a small amount of water. Hermaphrodites that are nearly ready to release their clutch do so within a few minutes under these conditions, and are then again recep-

tive for mating. After a trial was run, the focal animal was moved to a 30-ml cup, provided with yeast solution for food, and left undisturbed for 2–3 h. After each trial the container was emptied and rinsed with 70% ethanol. After the rest period the focal animal was again videotaped as previously, but with the alternate treatment type. About half the animals were tested with no stimulus shrimp (treatment = 'absence') first and half with five stimulus shrimps of the other sex (treatment = 'presence') first. A total of 34 males each from JT4 and WAL, and 38 hermaphrodites each from JT4 and WAL, were tested. Videotapes of focal animals were analyzed using Dynamic Animal Movement Analyzer (DAMA) software developed by Hoy et al. (1993). This program tracks a moving object by comparing the previous reference image with each newly captured video image. Images were captured every 0.1 s. Distance was calculated using the DAMA program as the square root of $((x_2–x_1)^2 + (y_2–y_1)^2)$. Mean speed was calculated as the sum of distances divided by the sum of time intervals.

From the 8-min trials, mean swimming speed of focal animals in the presence of stimulus shrimps and when no animals were present, was determined. Data required square-root transformation to normalize distributions and equalize variances among populations. A 2-way multivariate ANOVA, with population (WAL vs. JT4) and sex (male vs. hermaphrodite) as the two main effects and treatment (presence vs. absence) as the repeated-measure variable, was used to test for differences between treatments (overall $\alpha = 0.05$).

The relative time spent swimming was determined by tallying the total number of position changes each focal animal made during the 8-min trial. Differences between the sexes and difference within a sex between treatments were compared using the Mann–Whitney U-test ($\alpha = 0.05$) because the data were not normally distributed. Means were similar to medians for all data, and so means and standard errors are reported for all non-parametric tests.

To determine whether focal animals spent more time swimming in the third of the observation chamber nearest the stimulus animals, relative to the third furthest away, we analyzed DAMA-generated images of tracks created by the shrimp using the 'thick' option of the 'line profile' analysis of ImagePro Plus Software (Media Cybernetics, version 1.1 1991). This technique, often used to measure DNA gel density, compares the intensity of pixels in a 'line' of a defined thickness. Here, a 'thick' line was defined as one third the length of the observation chamber (i.e. 8.33 cm). Because the tracks are black against a white background, we could use the 'thick line' density as a relative comparison of how much area the animal traversed in each third of the chamber. The mean density (i.e. the amount of black) of the 'thick line' region adjacent to the stimulus chamber was compared with that of the 'thick line' region furthest from the stimulus chamber. Data could not be normalized with transformations and were, thus, analysed using the non-parametric Wilcoxon signed-rank test for paired samples and comparing mean track coverage between the two distant thirds of the observation chamber.

Results

Although hermaphrodites ($x \pm SE = 5.04 \pm 0.05$ mm, $n = 76$) tended to be slightly longer than males (4.9 ± 0.07 mm, $n = 68$), there was no significant correlation between body length and swimming speed for either sex or population (all Spearman rank correlation coefficients $r_s < 0.12$ and all $p > 0.50$). Additionally, because there was no difference in swimming speed between populations within a sex (Table 1), the data for the two populations were pooled for further tests.

Mean ($\pm$SE) swimming speed of males (7.56 ± 0.60 mm/s) was significantly greater than that of hermaphrodites (4.24 ± 0.36 mm/s; Table 1). Hermaphrodites markedly decreased their mean swimming speed in the presence of males (Table 1, Wilks' $\lambda = 0.975$, $p > 0.06$, Fig. 1).

Based on numbers of position changes, males were far more active than hermaphrodites (Table 2). Hermaphrodites spent less time swimming and more time resting on the bottom of the observation chamber than did males in both the pre-

Table 1: MANOVA calculations for swimming speed. Treatment refers to either the presence or absence of stimulus shrimp separated from a focal shrimp by a perforated Plexiglas partition (see Fig. 2). Population is JT4 or WAL. Sex is male or hermaphrodite. Data were square-root transformed to equalize variances

Parameter	Value*	df	F	Prob > F
population	0.999	1	0.001	0.9702
sex	0.719	1	55.084	< 0.0001
population * sex	0.996	1	0.564	0.4540
treatment	0.905	1	14.801	0.0002
treatment * population	0.995	1	0.682	0.4104
treatment * sex	0.975	1	3.571	0.0608
treatment * population * sex	0.959	1	6.091	0.0148

Table 2: Mean $\pm$ (SE, n) relative position changes as a measure of swimming activity. Treatment refers to either the presence or absence of stimulus shrimps separated from a focal shrimp by a perforated Plexiglas partition (see Fig. 2). Population is JT4 or WAL. Sex is male or hermaphrodite. Total refers to sex pooled across population and treatment

	Male	Hermaphrodite
JT4 absence	2207.8 (114.7, 25)	1488.8 (109.9, 27)
JT4 presence	2074.4 (90.9, 23)	1326.1 (130.0, 21)
WAL absence	2060.8 (134.7, 16)	1376.4 (169.6, 17)
WAL presence	2148.8 (143.2, 12)	1375.1 (183.4, 10)
Total	2127.2 (58.4, 76)	1402.6 (69.5, 75)

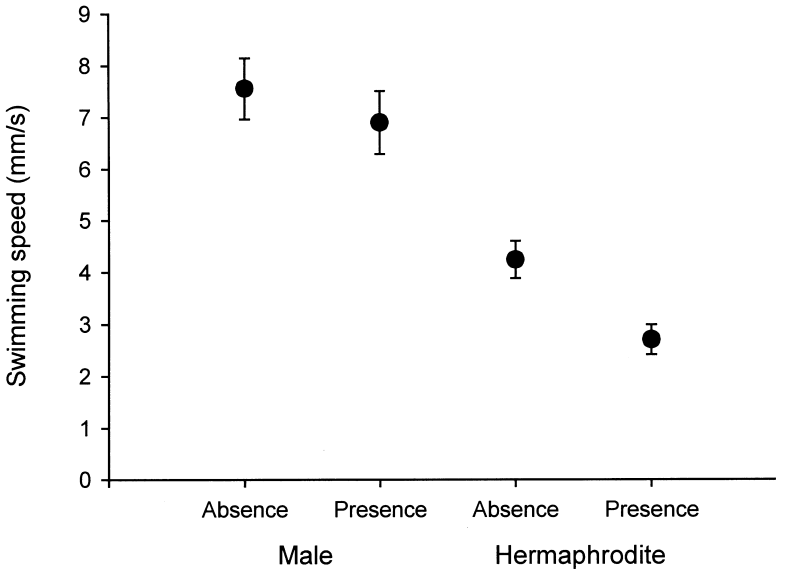

Fig. 1: Mean (± SE) swimming speed of focal male (n = 68) and hermaphrodite (n = 76) shrimps in presence and absence treatments. In the absence treatment, no stimulus shrimps were present. In the presence treatment, five shrimps of the other sex were separated from the focal animal in the stimulus chamber by a perforated Plexiglas partition (see Fig. 2)

sence (Mann–Whitney U-test, $U = 666.5$, $df = 31$, $p < 0.0001$) and absence treatments (Mann–Whitney U-test, $U = 2306.5$, $df = 44$, $p < 0.0001$). Mann–Whitney U-tests for the other factors of population and presence/absence of the alternate were not significant (lowest p-value > 0.4).

The 'thick' line (density) analysis, comparing estimates of amount of time spent in the thirds of the observation chamber closest to and furthest from the stimulus chamber, revealed that males spent equal time in each third whether stimulus hermaphrodites were present or absent (Wilcoxon paired signed-rank test, $Z = -18.5$, $df = 60$, $p = 0.316$ and $Z = 79$, $df = 70$, $p = 0.684$, respectively). Hermaphrodites in absence treatments also did not swim significantly more on one side of the chamber or the other (Wilcoxon paired signed-rank test; $Z = 61.5$, $df = 64$, $p = 0.071$). However, in the presence of males, hermaphrodites swam significantly more in the third of the chamber nearest the stimulus males compared to the third furthest from the stimulus males (Wilcoxon paired signed-rank test; $Z = 516.5$, $df = 58$, $p = 0.0001$) (see Fig. 2b vs. d).

Discussion

Male *Eulimnadia texana* swam approximately twice as fast as hermaphrodites in both treatments. Gerritsen's (1980) model of random encounters between pre-

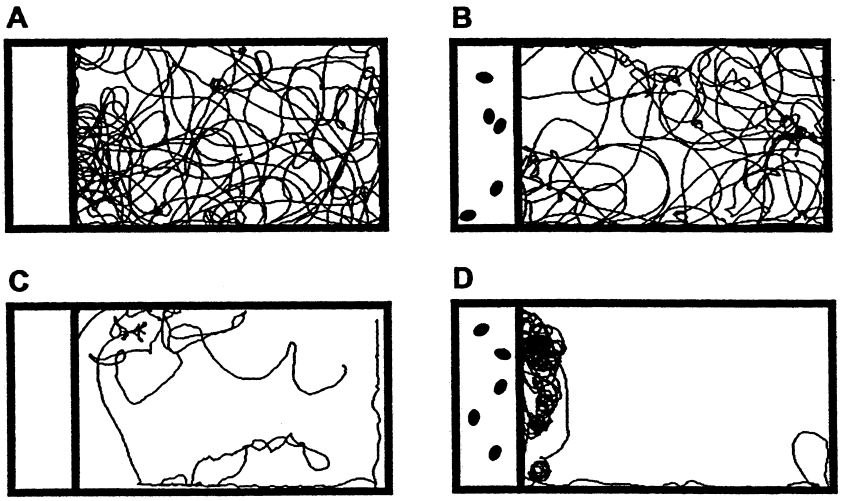

Fig. 2: Typical swimming tracks shown by (a) male *Eulimnadia texana* in absence treatment, (b) males in presence treatment, (c) hermaphrodites in absence treatment, and (d) hermaphrodites in presence treatment. All tracks were from 8-min observation periods. Drawings of test chamber are to scale (outer dimension = 30 × 15 cm); stimulus shrimps in (b) and (d) are larger than in life

dators and prey in three-dimensional space predicts that increasing predator swimming speed greatly increases encounters with slower moving prey. By analogy, if we think of males as predators seeking hermaphroditic prey, increasing swimming speed would increase encounter rates by males and, thus, opportunities for mating. Increasing encounter rates in this way has been empirically supported for other aquatic crustaceans, including copepods (Watras 1983; Yen 1988) and cladocerans: *Bosmina* (Kerfoot and Peterson 1980) and *Daphnia* (Brewer 1998). Our results suggest that *Eulimnadia texana* males also use this tactic to increase their potential encounter rate with slower moving hermaphrodites. Our observation that males did not alter their swimming speed or spend more time near stimulus hermaphrodites suggests that males are not able to discriminate between the presence or absence of hermaphrodites, and only haphazardly encounter hermaphrodites.

Observations by Knoll (1995) and Hollenbeck (1998) indicate that males often clasp onto other males upon encounter, but usually quickly release them, suggesting that they do not discriminate between males or hermaphrodites until post-encounter. This is also true of male *Daphnia pulicaria*, another branchiopod crustacean (Brewer 1998). Similar to *Eulimnadia texana*, *Daphnia* males are in low proportion in the population. Male *Daphnia pulicaria* were unable to identify females at long distances and only recognized females after initial contact with them. In natural populations of *Eulimnadia texana,* the proportion of hermaphrodites usually ranges between 60 and 90% of the individuals (Sassaman 1995; Weeks and Zucker 1999), and total population density is often high, frequently

greater than 500 individuals per square metre (MacKay et al. 1990). Therefore, it is likely that males encounter sufficient hermaphrodites solely by random encounter and increased swimming speed. This is true in some other arthropod mating systems where breeding densities are high (Telford and Dangerfield 1993; Brewer 1998). Many male crustaceans, including amphipods, decapods and copepods, are capable of locating mates via chemosensory cues (Dunham 1978). However, this is not the case for male *Daphnia pulicaria* (Brewer 1998), nor does it seem to be the case for male *Eulimnadia texana*. We suggest that males do not require any special sensory mechanism to locate hermaphrodites, because hermaphrodites usually occur in high frequencies.

Hermaphrodites spent less time swimming than males in the current study. They also spend a greater proportion of time gathering food than males (Knoll 1995; Zucker et al., unpubl. data). Because energy costs increase with the square of swimming speed (Gerritsen 1980), the differences in swimming speeds between the two sexes may simply reflect a sex difference in the trade-off between fecundity and mating activity, in that hermaphrodites may be conserving energy for egg production that males are able to expend in seeking mates.

We found that the behaviour of hermaphrodites differs significantly from that of males. Contrary to expectations, it appears that it is hermaphrodites, rather than males, which are altering their behaviour in the presence of the other sex. The fact that when stimulus males are present hermaphrodites swim to the area of the chamber near the males and tend to remain there, and that many hermaphrodites did not explore the observation chamber in the absence of stimulus shrimps as much as males did (Fig. 2), suggests to us that hermaphrodites are capable of locating males. Pheromones may be involved as they are for other small crustaceans (Hoffman 1983; Bell 1991), but this experiment was not designed to discern whether hermaphrodites are using pheromones or some combination of mechanical, visual and chemical cues to locate males. In addition, our experiment does not allow us to eliminate the possibility that hermaphrodites are attracted to both sexes rather than specifically to males. Studies currently underway to ascertain the stimulus channel used by hermaphrodites to locate males and to determine whether the stimulus is uniquely produced by males or by clam shrimp in general, found no attraction of either sex to members of their own sex (Medland et al., unpubl. data).

If inbreeding depression is high, as was found in *Eulimnadia texana* in a previous study (Weeks et al. 1999), and if males are rare, then factors that allow hermaphrodites to increase encounter rates (and thus their likelihood of out-crossing) should be favoured. It is possible that hermaphrodites perceived conditions in the stimulus chamber where there were no males as 'rare male' conditions and their greater swimming speed represents a tactic to increase encounter rates, much as the males are doing under all conditions. Since no pheromone or visual cues were present in the absence treatment, hermaphrodites would have no conspecific cues to locate potential mates. Under these conditions, the hermaphrodites may be switching their encounter tactics from one of keying in on males to that of more rapid random movements, as we observed.

According to the model of Otto et al. (1993), out-crossing is dependent on the likelihood of the two sexes encountering each other. We specifically tested whether encounters were random or directed. We have demonstrated that males and hermaphrodites are apparently using different tactics to locate potential mates – males moving rapidly but at random and hermaphrodites moving slowly but in a directed manner. These tactics suggest that *Eulimnadia texana* has evolved encounter behaviours that increase the likelihood of out-crossing.

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