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Barriers to outcrossing success in the primarily self-fertilizing clam shrimp, *Eulimnadia texana* (Crustacea, Branchiopoda)

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Abstract. The expected proportion of males in androdioecious populations (those comprised of males and hermaphrodites) largely depends on the fertilization opportunities of males. If male mating opportunities are low due to restricted access to hermaphroditic eggs, then populations will be hermaphrodite-biased. Hermaphrodites have two mechanisms available to limit male mating success: (1) pre-mating barriers to outcrossing, in which hermaphrodites choose not to pair with males and (2) post-mating barriers to outcrossing, in which hermaphrodite sperm has greater access to eggs than male sperm. In this study, we measured male mating success in the androdioecious clam shrimp *Eulimnadia texana* when pre-mating barriers to outcrossing were removed. These branchiopod crustaceans are small (5–8 mm), filter feeders that live in ephemeral pools in the deserts of the southwestern United States. Using genetic markers, we measured male mating success in laboratory experiments in two populations of these shrimp. We correlated mating success with clasping time, clasping during egg transfer, and male thrusting during egg transfer. Males fertilized an average of 24–40% of the hermaphrodites' eggs. Outcrossing success was positively correlated with clasping duration, and was nearly an order of magnitude higher for males thrusting during egg transfer relative to thrusting at other times during pairing. Because these estimates of mating success were similar to previously reported estimates (in which both pre- and post-mating barriers to outcrossing were potentially important), we deduced that pre-mating barriers to outcrossing do not greatly decrease male outcrossing success in *E. texana*; the low fertilization (25–50% of available eggs) by males is thus due to post-mating barrier(s) to outcrossing.

Additional key words: Mating behavior, androdioecy, mate guarding, branchiopods

Mating systems that combine some proportion of self-fertilization with some proportion of outcrossing (termed "mixed mating systems") have long intrigued biologists (Lloyd 1975; Charlesworth 1984; Jarne & Charlesworth 1993; Pannell 1997). One type of mixed mating system is found in some androdioecious species, in which populations consist of self-compatible hermaphrodites and males but lack true females. Androdioecious mating systems are rare, with only a few plant species and fewer animal species exhibiting this reproductive mode (Charlesworth 1984; Jarne & Charlesworth 1993; Pannell 2000, 2002). If a hermaphrodite can efficiently pass on its genes through self-fertilization (100% of its genes via selfing relative to only 50% via mating with males), then why are males maintained in these systems?

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The advantage of increased gene transmission in species with self-fertilization is thought to be countered in many species with mixed mating by inbreeding depression (Lande & Schemske 1985), with high levels of inbreeding depression selecting for higher male proportions in androdioecious populations (Lloyd 1975; Charlesworth 1984). Avoidance of inbreeding depression must be coupled with high male mating success for males to be maintained in androdioecious species (Lloyd 1975; Charlesworth 1984; Otto et al. 1993). If the operational sex ratio (the ratio of receptive hermaphrodites to sexually active males) is low because the number of receptive hermaphrodites allows only limited mating opportunities for sexually active males, then males are at a disadvantage (Emlen & Oring 1977). The reduced male fertilization opportunities in this case would reduce male relative to hermaphrodite fitness, and thus should bias the population toward greater numbers of hermaphrodites (Lloyd

1975; Charlesworth 1984). Hermaphrodites may keep receptivity low by self-fertilizing eggs, either by choosing not to pair with males or by using self sperm preferentially to male sperm. The former would be a "pre-mating barrier" to outcrossing, whereas the latter would be a "post-mating barrier," similar to cryptic female choice (Eberhard & Cordero 1995), except with the hermaphrodite choosing between self versus male sperm rather than a female choosing among sperm from several males.

One androdioecious system that has been well described is found in the clam shrimp *Eulimnadia texana* PACKARD 1871. In this species, males coexist with simultaneous hermaphrodites of two phenotypically similar but genetically different types: amphigenic and monogenic hermaphrodites. Sex appears to be controlled by a single genetic locus (Sassaman & Weeks 1993), with a recessive allele coding for males and a dominant allele coding for hermaphrodites. The homozygous dominants are monogenic hermaphrodites, the heterozygotes are amphigenic hermaphrodites, and homozygous recessives are males (Sassaman & Weeks 1993). Males comprise ~20% of the population, while amphigenics comprise ~70% and monogenics make up the remaining ~10% (Weeks et al. 1999). This mating system is intriguing because, although inbreeding depression ranges from 50–70% (Weeks et al. 1999; Weeks et al. 2000a), self-fertilization is common with inbreeding coefficients ranging from 20–97% (Sassaman & Weeks 1993; Weeks & Zucker 1999). Thus, although mating with males should produce more viable offspring, male outcrossing success appears to be limited by one or more factors.

Hermaphrodites can either outcross with males or fertilize their own eggs, but are not cross-compatible with other hermaphrodites. The exact mechanism of sperm transfer has not been determined, nor is it known how much control the hermaphrodites have over with whom they mate. Numerous clutches of eggs can be produced during a hermaphrodite's reproductive lifetime (Weeks et al. 1997). Hatching is usually within a day of hydration, and sexual maturity occurs within 4–8 days in the laboratory and 5–6 days in the field (Vidrine et al. 1987). At sexual maturity, the first two pairs of phyllopod appendages in males are modified into clasper-like appendages which are used to grasp onto the hermaphrodites during mating. The lack of claspers in hermaphrodites disallows pairing, which explains the inability of hermaphrodites to cross with one another.

A previous study (Weeks et al. 2000b) revealed that *E. texana* is not capable of sperm storage, and estimated the percentage of eggs sired by males at ~40%. Although lower rates of fertilization due to sperm limitation (caused by either small male size or depleted sperm reserves due to previous matings) have been reported in other crustaceans (MacDiarmid & Butler 1999; Sparkes et al. 2002), such limitation reduces fertilization by 18–40%, which cannot explain the 50–75% reduction previously reported in *E. texana*. Additionally, the previous study paired clam shrimp of similar ages and sizes, and had only one hermaphrodite per male, all of which should have reduced the importance of sperm limitation in males (Weeks et al. 2000b). These factors suggested that causes other than sperm limitation were responsible for the very low male success, and suggested that control of fertilization success by the hermaphrodite was the most likely candidate for the low outcrossing rates.

In the previous experiment on male fertilization success (Weeks et al. 2000b), males were paired with hermaphrodites for three days, the resulting eggs were hatched, and the proportion of outcrossed offspring was estimated using genetic analyses. Because shrimp were not continuously observed for the three days of the pairings, we could not distinguish whether the resulting 40% outcrossing rate was due to 40% of the clutches being fertilized by a male (which fertilized 100% of the eggs) or to 100% of the clutches being fertilized, each at a siring rate of 40%, or some combination of these two extremes. The former suggests that hermaphrodites were behaviorally limiting outcrossing (a pre-mating barrier to outcrossing), allowing male access to less than half of the clutches. The latter suggests that hermaphrodites either preferentially used self sperm to fertilize most of their eggs or that high sperm competition limited fertilization with sperm from males (a post-mating barrier to outcrossing).

Herein we tested whether the previously reported ~40% male outcrossing success was due to post-mating barriers to outcrossing by calculating male outcrossing success in single clutches when successful mating behavior was observed. We confined our observations to cases in which matings occurred (i.e., no pre-mating barrier to outcrossing) to determine if the proportion outcrossed increased when pre-mating barriers were removed. Three mating behaviors were also recorded: (1) duration the male was clasped to the hermaphrodite's carapace during coupling; (2) whether the male was attached to the hermaphrodite during egg transfer (eggs moved from the ovotestis to the brood chamber); and (3) whether the male was thrusting his telson between the valves of the hermaphrodite's carapace during egg transfer. To evaluate the possibility that male behavior modified outcrossing proportion when males successfully mated, we compared outcrossing success to these three mating behaviors.

Methods

Rearing protocol

Soil containing clam shrimp eggs was collected near Portal in Cochise Co., Arizona (previously reported as the "WAL" population) and from Doña Ana Co., New Mexico (previously reported as the "JT4" population). Samples were transported to the University of Akron in Akron, Ohio. Sub-samples (250 ml) of soil were hydrated using "standard conditions": soil was placed in filtered tap water in aquaria under continuous light (Durotest sunlight-simulating fluorescent bulbs) and aeration at 25–27°C, (see Sassaman & Weeks 1993; Weeks et al. 1997). These conditions provide growth and survival rates that best mimic natural rates (Weeks et al. 1997).

Before they reached sexual maturity (at ~3–4 days), hatched shrimp were randomly chosen for individual isolation in 500 ml plastic cups filled with filtered tap water and with ~12 g of finely sifted soil (<125 μm diameter; Marcus & Weeks 1997; Weeks & Zucker 1999). This soil was collected from a locale nearby the JT4 site, but in an area known to be free of branchiopod cysts. Shrimp in all cups were fed 1 ml of Baker's yeast solution (1 g dried yeast per 100 ml water) per day. Before sexual maturation (4–6 days of age), the shrimp were sexed and the males were discarded. The hermaphrodites were allowed to produce self-fertilized clutches for 7–14 d, after which the adults were frozen for gel electrophoresis. The clutches were allowed to slowly air-dry and stored for future use (see below).

Hermaphrodites were assayed using cellulose acetate (CA) electrophoresis (Richardson et al. 1986). Shrimp were scored for 5 polymorphic loci: *Fum* (fumarate hydratase, EC 4.2.1.2), *Idh*-1 and *Idh*-2 (isocitrate dehydrogenase, EC 1.1.1.42), *Mpi* (mannose-phosphate isomerase, EC 5.3.1.8), and *Pgm*-1 (phosphoglucomutase, EC 5.4.2.2). Gel electrophoresis was done using Buffer C according to protocols reported by Richardson et al. (1986). Because *Fum*, *Idh*-1, and *Idh*-2 are known to be linked to the sex-determining locus, we can use heterozygosity for any of these three loci to screen for amphigenic hermaphrodites (Weeks et al. 1999). Monogenic hermaphrodites are always produced via a self-fertilization event that renders the sex-determining locus homozygous for the dominant, hermaphrodite-determining allele (Sassaman & Weeks 1993). Because crossing over between the sex determining locus and the linked enzyme loci occurs only ~1% of the time (Weeks et al. 1999), monogenic individuals should only rarely be heterozygous for any of these three electrophoretic loci (homozygotes for all three can be either monogenic or amphigenic). Thus, we can screen for amphigenic individuals using these three electrophoretic loci with an accuracy rate >95%. Amphigenic and monogenic individuals can be heterozygous or homozygous for the loci that are unlinked to the sex determining locus (*Mpi* and *Pgm*-1; Weeks et al. 1999).

From these electrophoretically-scored hermaphrodites, we chose pairs that were both heterozygous for one or more of the sex-linked loci (*Fum*, *Idh*-1, and *Idh*-2) but that were also alternate homozygotes for at least one of the 5 assayed loci. This pairing assured a choice of two amphigenic hermaphrodites (and thus the production of males among the selfed offspring of both egg clutches), and that outcrossing between offspring from the two hermaphrodites would be genetically marked, and thus distinguishable from a selfing event using CA electrophoresis (Sassaman & Weeks 1993; Weeks et al. 2000a). Pairings were only done within populations (i.e., no crosses were made between JT4 and WAL shrimp).

After air drying for at least 30 d, egg banks generated from each hermaphrodite in the above pairs were hydrated. The resulting nauplii (50–100 per egg bank) were transferred into 14 l plastic tubs containing water from aquaria with 500 ml of WAL soil. The water was filtered using a 63 μm mesh to remove all branchiopod shrimp. When clam shrimp in each tank grew to near sexual maturity (determined on the basis of clasper formation in males), males from one family group (n = 10–20) were placed in 10 l plastic tubs with hermaphrodites (n = 20–50) from an alternate family group (29 family groups were measured; see Table 1). Once shrimp were transferred, the tubs were visually scanned for matings, searching for males clasped onto hermaphrodites without eggs in their brood chamber. Once a pairing was noted, the couple were removed from the tub and placed into a small dish for more detailed observation. [Note: usually this movement of shrimp did not affect mating events. In those few cases where the movement did affect the coupling, the male would release the hermaphrodite and the observation was terminated.] For all studies, the only pairings used were when those where the male was observed actually initiating the mating.

Behavioral data collected were: (1) total time clasped during the entire mating event, (2) presence or absence of the male during egg transfer, and (3) presence or absence of thrusting of the male's telson between the "valves" of the hermaphrodite's carapace during egg transfer. Similar male behaviors have been found to be useful for comparing mating strategies in isopod and amphipod crustaceans (Jormalainen 1998).

When the male detached from the hermaphrodite and egg transfer to the brood chamber had occurred, mating was considered complete. The male was dis-

carded, the hermaphrodite was isolated in a 500 ml cup with no soil (to facilitate egg removal), and checked twice daily for production of a clutch of eggs. Once the clutch was laid, the hermaphrodite was removed from the cup and discarded. The eggs were dried over a 30 day period.

After drying, egg banks generated from each mating were hydrated as described above. When egg banks hatched, the resulting nauplii were transferred into 10 l plastic tubs containing filtered WAL water, and again reared in conditions described above. When shrimp in each tank grew to the minimum size required for CA electrophoresis (i.e., near sexual maturity) they were frozen for later genetic analyses. Homozygotes for the marker locus were categorized as "selfed" while heterozygotes were categorized as "outcrossed" (Weeks et al. 2000a).

Statistical analysis

All data were analyzed using the statistical program JMP (SAS Institute 2003). Weighted averages for percentage outcrossed were calculated per population (observed % outcrossing per clutch weighted by the number of offspring scored in each clutch). To test for the effects of population (WAL and JT4) and thrusting behavior during egg transfer on outcrossing success, we used analysis of covariance, with number of offspring outcrossed as the dependent variable, total number of offspring scored as the covariate, and population or "thrusting" as the independent variables. The relationship of number outcrossed to total number was uniform among the independent variables (i.e., the assumption of "homogeneity of slopes" in the ANCOVA was met). Such an ANCOVA approach is more statistically robust than using the proportion outcrossed as the dependent variable in an ANOVA because the former allows specific tests of the assumption of homogeneity of slopes, which is merely assumed in the latter test. Both number outcrossed and total number of offspring were square-root transformed to normalize residuals.

Similarly, to test for a correlation between outcrossing and total time clasped, residual values from a regression of the number of outcrossed shrimp on total offspring number (both square-root transformed) were regressed against total cumulative time clasped.

Results

Outcrossing

A total of 88 egg banks from matings were generated from observed matings and hydrated to assess outcrossing rates. Only 29 of these 88 egg banks (~33%) resulted in hatched nauplii. Such a low hatching rate is characteristic of desert-dwelling conchostracans (Brendonck 1996). From these 29 matings, more eggs were found to be fertilized by the hermaphrodite selfing than by outcrossing with a male, with outcrossing rates varying from 0–100% (Table 1). For JT4, 39.6% of the eggs were estimated as outcrossed while in WAL, only 24.1% were outcrossed (Table 1). The difference in outcrossing between populations was not statistically significant (Table 2). Because self-fertilization reduces egg viability (Weeks et al. 1999; Weeks et al. 2001), the outcrossing estimates in this and previous studies may be biased upwards due to greater hatching and survival of outcrossed relative to selfed eggs.

Behavioral observations

Of the 29 above matings, only 19 provided data on clasping during egg transfer. Because only two of 19 males were not clasped during egg transfer, the sample size to detect the effect on success of outcrossing while not clasped was too low for a meaningful comparison. Of the two that were not clasped during egg transfer, one had 0% outcrossing and the other had 57% outcrossing, so it appears that males do not need to be clasped during egg transfer to successfully outcross (but see below).

In 15 of the 29 matings, the presence or absence of thrusting by the male while the hermaphrodite extruded eggs and moved them to the brood chamber was determined. During pairing, males grasped onto the hermaphrodite's carapace such that the two ventral portions of the body were aligned (Fig. 1). Two types of thrusting behaviors were noted. The more common thrusting behavior was a very brief bending of the body wherein the telson was bent towards the hermaphrodite's ventral surface followed by a very rapid extension of the body (Fig. 1A). We termed this thrusting "Type 1" and observed many such thrusts (5–10) in rapid succession followed by a lack of activity by both individuals. Type 2 thrusting was only observed once per mating, and consisted of the male bending his body toward the hermaphrodite and successfully opening the valves of the carapace with the telson (Fig. 1B). When the male was in this position, the telson was kept between the valves of the carapace and the body was flexed several times (3–5). Usually, shortly thereafter the male would release the hermaphrodite, but some males would continue to clasp the hermaphrodites for several minutes. Type 2 thrusting is assumed to be the only point at which sperm transfer occurs, primarily because the male does not successfully insert his telson between the valves of the cara-

Table 1. Distribution of outcrossing rates in 29 clutches measured. Outcrossing (out) was determined by noting the presence (= outcrossing) or absence of male alleles in the offspring of each of the 29 hermaphrodites (using cellulose acetate electrophoresis to score alleles).

Pop	Rep	# self	# out	% out
WAL	1	25	0	0
WAL	2	14	0	0
WAL	3	2	0	0
WAL	4	1	5	83
WAL	5	3	0	0
WAL	6	2	6	60
WAL	7	15	6	29
WAL	8	15	12	44
WAL	9	3	0	0
WAL	10	39	0	0
WAL	11	7	0	0
WAL	12	1	0	0
WAL	13	3	0	0
WAL	14	4	3	33
WAL	15	0	1	100
WAL	16	0	6	100
WAL	17	4	4	40
WAL	18	8	2	20
WAL	19	3	0	0
WAL	20	2	0	0
WAL	21	1	0	0
JT4	1	14	8	35
JT4	2	10	4	28
JT4	3	3	1	20
JT4	4	11	7	39
JT4	5	0	14	100
JT4	6	13	44	77
JT4	7	66	0	0
JT4	8	7	4	33

pace during Type 1 thrusting. In our observations, the hermaphrodites were essentially passive throughout the thrusting episodes.

Type 2 thrusting was often, but not always, associated with the hermaphrodite extruding eggs into the brood chamber (Fig. 1B). Nine of the 15 males exhibited Type 2 thrusting during egg transfer, and these males fertilized a greater number of offspring than those that did not exhibit Type 2 thrusting during egg transfer (Table 2; Fig. 2). All males performed Type 2 thrusting, and thus this comparison quantifies those males specifically using Type 2 thrusting during egg extrusion relative to those that used Type 2 thrusting at other periods during pairings.

The cumulative time the male was clasped with a hermaphrodite was found to be positively correlated with outcrossing success ($F_{1,24} = 8.99$; $p = 0.0062$), although most of this correlation was due to two pairings in which males were clasped for over an hour and a half (Fig. 3).

Table 2. ANCOVA results for number of outcrossed offspring sired per male in the two populations (WAL and JT4), and comparing males that were thrusting during egg transfer to those that were not (Thrust). The covariate was total offspring measured. Significant p-values are in bold. The number of outcrossed offspring sired per male did not significantly differ between populations, but did depend on whether or not the male was thrusting during egg transfer.

		Sum of		
Source	df	Squares	F-ratio	p-value
Population				
Total #	1	4.69	2.42	0.1320
Population	1	6.40	3.30	0.0808
Error	26	50.43		
Thrust				
Total #	1	0.99	0.88	0.3674
Thrust	1	6.87	6.06	**0.0299**
Error	12	13.60		

Discussion

The proportion of males that can coexist with hermaphrodites in an androdioecious population is directly related to the availability of outcrossing opportunities for males (Lloyd 1975; Charlesworth 1984). If the operational sex ratio is such that very few eggs are available for outcrossing, then male proportions should be low in most populations. In crossing experiments with *E. texana,* outcrossing rates have been measured at approximately 40% of the total fertilized offspring (Weeks et al. 2000b). This level of outcrossing could either be due to the low propensity of hermaphrodites to mate with males (pre-mating barrier to outcrossing) or to increased self fertilization by hermaphrodites (post-mating barrier to outcrossing), or some combination of these two factors.

In the current experiment, we eliminated the possibility of male rejection by the hermaphrodite by only examining outcrossing proportions from pairings in which the male was clearly seen thrusting the telson between the valves of the hermaphrodite's carapace (previously defined as a "mating"; Knoll 1995). With the pre-mating barrier to outcrossing eliminated, we once again evaluated outcrossing rates, and by comparing observations from previous studies (Crosser 1999; Weeks et al. 2000b) that included both pre- and post-mating barriers with observations including only post-mating barriers, we could deduce the relative contribution of both barriers to total outcrossing rates in these shrimp (Table 3).

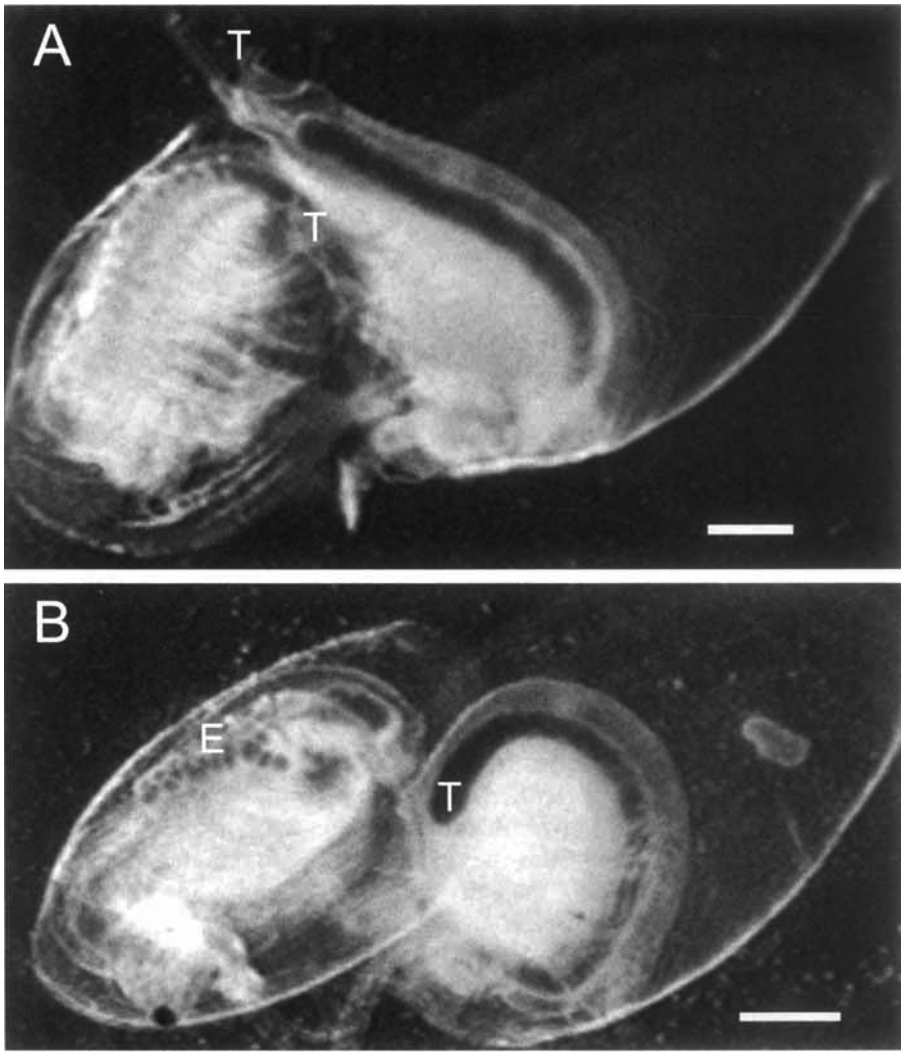

Fig. 1. Photographs of male and hermaphrodite of *E. texana* in copula illustrating Type 1 (A) and 2 (B) thrusting behavior. The male is on the right and the hermaphrodite is on the left. Both are head-down. In (A) the male has unsuccessfully attempted to thrust his telson (T) between the valves of the hermaphrodite's carapace. In (B) The male has successfully thrust his telson (T) between the valves of the hermaphrodite's carapace. The male moves his body in short pulses while the hermaphrodite is extruding eggs (E) into the brood chamber (on the dorsal surface of the body directly underneath the carapace). Scale bar = 1 mm.

With the pre-mating barrier to outcrossing removed, we again measured low outcrossing rates for clasped males. If pre-mating barriers to mating were substantial in *E. texana,* we expected the outcrossing rates in the current project to be substantially greater than those measured in the previous experiments. Thus, because our current estimates of outcrossing from shrimp that were observed to be paired and in which the males were actively thrusting between the hermaphrodite's carapace (Type 2 thrusting) were actually lower than the previously reported outcrossing rates (Table 3), all of our laboratory estimates of low outcrossing (47% and 32% in JT4 and WAL, respectively) are most likely due to some form of post-mating barriers to outcrossing. Such barriers may include sperm competition or preferential use of self over male sperm. We cannot completely deduce the importance of pre-mating barriers in natural populations, but in our laboratory pairings, it appears that hermaphrodites likely have little chance to avoid males.

This result is in agreement with anecdotal observations of sexual encounters in *E. texana.* Although some hermaphroditic resistance to male clasping has been noted in *E. texana,* this resistance rarely results in the dislodging of the male by the hermaphrodite. A comparable lack of female control over mate pairing has been reported in isopod crustaceans with a similar mating system (Manning 1975; Jormalainen & Merilaita 1995).

Regardless of the overall importance of potential pre-mating barriers to outcrossing in natural systems, the very low outcrossing success observed in actively mating pairs requires explanation. These low outcrossing proportions are likely explained by one of two factors: (a) self-sperm is preferentially used over male sperm, or (b) male sperm, although more abundant

Fig. 2. Comparison of mean outcrossing success of males found thrusting while hermaphrodites transferred eggs to brood chamber (Y) and those thrusting at other times during the reproductive cycle (N). Error bars portray one standard error of the mean. *Numbers of outcrossed offspring adjusted for total offspring sampled (see text for further explanation).

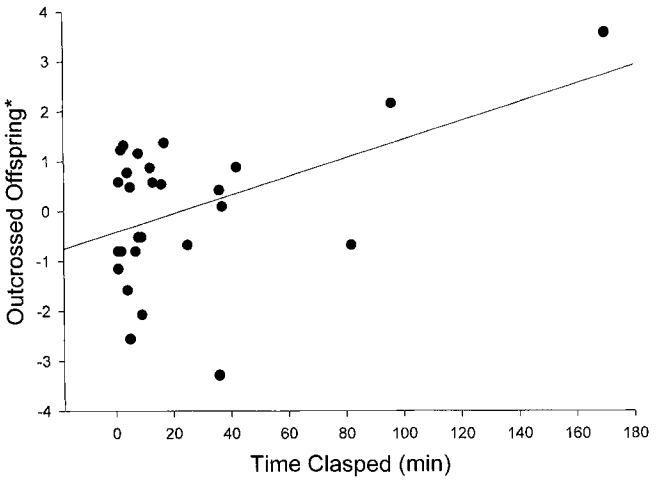

Fig. 3. Outcrossing success as a function of the total time the male was clasped with the hermaphrodite. *Numbers of outcrossed offspring adjusted for total offspring sampled (see text for further explanation).

(Zucker et al. 1997), is competitively inferior to hermaphroditic sperm in egg fertilization. At this point, the exact mechanism of egg fertilization is unknown. The low outcrossing proportions may be due to a type of "priority effect," wherein the self-sperm have first access to the eggs, and thus are more effective than the male sperm. In some self-compatible hermaphroditic plants, self pollen is deposited on stigmas before flowers open for outcross pollinations (termed "prior autonomous self-pollination"; Lloyd & Schoen 1992). Such self-pollination mechanisms are thought to be selected to assure offspring production when males are rare, but can have the negative side effect of reducing outcrossing rates when males are present even if outcrossing is beneficial (i.e., when inbreeding depression levels are high). For example, the self-compatible plant *Aquilegia canadensis* has outcrossing rates of only 25% even though inbreeding depression ranges from 86–100% (Herlihy & Eckert 2002). Therefore, in organisms that have undergone selection for mechanisms promoting reproductive assurance, the resulting reproductive mechanisms (e.g., prior autonomous self-pollination) may limit outcrossing rates even if outcrossing provides the highest offspring fitness.

The observation herein that males using Type 2 thrusting during egg transfer had an almost ten-fold greater outcrossing success than males using Type 2 thrusting at other periods of the reproductive cycle (Fig. 2) may shed light on the mechanism of post-mating barriers to outcrossing in this species. If sperm transfer is most important during the actual extrusion of eggs, we conclude that male fertilization must occur externally because sperm cannot move into the hermaphroditic gonopore while eggs are actively being extruded. If males were fertilizing eggs internally, then males thrusting before egg extrusion should have had higher outcrossing success relative to those thrusting while eggs were being extruded. Because this was not the case, we presume that males must be fertilizing eggs externally. External fertilization has been reported in several brooding crustaceans by the males pumping sperm into the marsupium directly after egg extrusion (Jormalainen 1998). In a related hermaphroditic clam shrimp, *Limnadia lenticularis,* meiosis was found to be completed while the eggs were in the brood chamber (Zaffagnini 1969), suggesting that fertilization is also external in this species. If the same is true for *E. texana,* then fertilization must take place externally.

If eggs are externally fertilized, how does a hermaphrodite self-fertilize? Scanabissi and Mondini (2002) have suggested that in *Limnadia lenticularis,* self-sperm actually invade the egg shell in the ovotestis by dissolving the outer layers of the egg shell directly after the "egg shell substance" coats the egg. By being able to invade the egg shell within the ovotestis, self-sperm would have the aforementioned competitive superiority by being able to begin the fertilization process before male sperm was available.

Scanabissi and Mondini (2002) additionally suggest that for *L. lenticularis,* a delayed meiosis keeps the eggs from being fertilized until they are in the brood chamber. If this is also true for *E. texana,* then there may be a narrow "window of opportunity" directly

after the eggs are extruded that male sperm can invade the egg shell. Such a window would explain why, in the current study, males clasping for a longer period of time had higher outcrossing success: greater time clasped would be more likely to include the "window of opportunity" of being clasped when eggs are transferred to the brood chamber. However, a greater amount of sperm may also be transferred when clasping continues for a longer period of time. Therefore, a fertilization mechanism akin to that found in *L. lenticularis* could explain two important observations of the current study: both the low outcrossing success of males and the higher outcrossing success of males thrusting while eggs were being transferred to the brood chamber.

The proposed fertilization mechanism poses two major obstacles for males in this system. First, if outcrossing success is maximal only for a short window during the reproductive cycle (as is the case in several isopods; Jormalainen 1998), then the mating opportunities in *E. texana* may be a mere fraction of what one might expect on the basis of the high hermaphroditic frequency. A male would need to clasp a hermaphrodite during that short time window to be successful. This may explain the "mate guarding" behavior (i.e., clasping onto a hermaphrodite for an extended period) described in this species (Knoll & Zucker 1995): the optimal male strategy may be to mate guard because successful fertilization is restricted to a short interval during the critical moment of egg extrusion (Parker 1974; Ridley 1983; Jormalainen 1998). In such a situation, a conditional male mating strategy may be optimal: when males are rare, males sample multiple hermaphrodites to find individuals at the correct time in their reproductive cycle, but when the probability of finding an unpaired, receptive hermaphrodite (e.g., extruding eggs) is low, males "mate guard" to maximize their fertilization success (Yamamura & Jormalainen 1996). Such a dependence of male strategies on previous experience has been found in both vertebrate and invertebrate systems (Dunham & Hurshman 1990; Hasselquist & Bensch 1991; Jormalainen & Shuster 1999). For example, male isopods in the genus *Thermosphaeroma* mate-guarded females longer when competing with other males relative to being with females alone (Jormalainen & Shuster 1999).

The second barrier to high male frequencies in androdioecious populations, given the proposed fertilization mechanism, is due to the above inferred self relative to male sperm preference. Even if a male can find a receptive hermaphrodite at the appropriate time of egg extrusion, it appears that maximal outcrossing success will be constrained to <50% (Table 3) due to presumptive reduced competitiveness for male relative to self sperm.

The low male fertilization success measured in this and previous experiments in *E. texana* has been interpreted as indicative of some form of hermaphroditic control over male access to eggs. However, it is possible that hermaphrodites allow free access, but that males either do not or cannot supply sufficient sperm to fertilize all or most of the available eggs per clutch. Such sperm limitation has been documented in dioecious species, in which males fertilize less than the full complement of available eggs (MacDiarmid & Butler 1999; Sparkes et al. 2002). For example, female isopods in the genus *Lirceus* had an average of 18% reduction in fertilized eggs when paired with males that had mated with another female immediately before (Sparkes et al. 2002). Also, male size can limit egg fertilization in female spiny lobsters (*Panulirus argus*), with large male lobsters fertilizing up to 40% more eggs than smaller males (MacDiarmid & Butler 1999). Although the possibility does exist for male sperm limitation in *E. texana,* we do not believe that these previously reported causes of sperm limitation are important in our current comparison for the following reasons. First, males and hermaphrodites were of identical sizes in this and the two previous experiments (Crosser 1999; Weeks et al. 2000b), and were chosen during their peak reproductive period, which is directly after sexual maturity (Weeks et al. 1997). Second, although the current experiment did not control for previous mating history in the males (since they were a random draw from a larger group, and thus could have mated before entering the experiment), the two previous experiments paired single males with single hermaphrodites for multiple days (Crosser 1999; Weeks et al. 2000b), which eliminates the possibility of sperm limitation due to previous mating(s). Because male sperm limitation in *E. texana* would need to range from 50–75% (Table 3), which is much greater than that reported in other crustaceans (MacDiarmid & Butler 1999; Sparkes et al. 2002), and because the experimental design limited any potential sources of male sperm limitation, we inferred that the low fertilization rates were primarily due to hermaphroditic control over fertilization rates, which appears to be via some form of sperm competition.

The current project suggests that post-mating barriers to male outcrossing are high in *E. texana.* The picture emerging from the combined studies of *E. texana* is that pre-mating barriers, in the form of hermaphroditic exclusion of males, do not significantly reduce male fertilization success. It is unclear why such post-mating barriers exist in *E. texana,* and it is possible that these barriers are side-effects of selection

Table 3. Percent outcrossing and standard errors (se) for three separate projects measuring male fertilization success. N = number of clutches measured. Offspring scored (OS) is the total offspring reared from each population. The percent outcrossing (% out) in the current project (where only post-mating barriers to outcrossing were allowed) is similar or lower than outcrossing in two previous experiments (where both pre- and post-mating barriers were allowed), which suggests pre-mating barriers are insignificant in reducing male fertilizations success in *E. texana*.

Source	% out	se	N	OS
Current Project				
JT4	39.6	12.8	8	206
WAL	24.1	6.5	21	197
Weeks et al., 2000b				
JT4	51.5	14.3	10	196
WAL	33.6	7.1	20	560
Crosser, 1999				
JT4	53.0	14.7	8	117
WAL	26.9	12.5	12	119
Total				
JT4	47.0	7.8	26	519
WAL	31.9	4.4	53	876

for reproductive assurance via self-fertilization in these temporary-pool specialists. Nevertheless, the low mating success of males may help explain the highly skewed sex ratios observed in this species (ranging from 0–30% males; (Weeks & Zucker 1999), and may play a major role in the maintenance of mixed selfing and outcrossing in a species with such high levels of inbreeding depression (Weeks et al. 1999; Weeks et al. 2000a; Weeks et al. 2001).

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