Invertebrate Biology 121(1): 66–72.

Impact of males on variation in the reproductive cycle in an androdioecious desert shrimp

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Abstract. In the clam shrimp *Eulimnadia texana* and some other species of desert ephemeral pool-dwelling branchiopod crustaceans, males coexist with hermaphrodites. The hermaphrodites can mate with males or can fertilize their own eggs but cannot mate with other hermaphrodites. Understanding the evolutionary dynamics of this mixed mating system, known as androdioecy, requires a basic knowledge of the reproductive behavior of this species. Here we describe the reproductive cycle of hermaphrodites when isolated and when in the presence of a male. Videos were analyzed to provide a description of egg movement from the ovotestes to the brood chamber. Through time-lapse photography, we determined that paired hermaphrodites carried their brood longer and swam fast for a greater duration than did isolated hermaphrodites. Isolated hermaphrodites dug more preliminary burrows before burying their clutch and had longer inter-clutch intervals than did paired hermaphrodites. These observations suggest that hermaphrodites may behave in ways that maximize the likelihood of mating, and that males may interfere with hermaphrodites during egg laying.

Additional key words: androdioecy, mating behavior, clam shrimp, *Eulimnadia texana*, Branchiopoda, Conchostraca, Crustacea

We have little knowledge of the behavior of some animal species which exhibit unusual mating systems, yet these groups may hold a key to our understanding of the evolution of the more common mating systems. Androdioecy, in which males and hermaphrodites coexist, is one such mixed mating system that is rare in both the plant and animal kingdoms (Jarne & Charlesworth 1993), but was described in the clam shrimp *Eulimnadia texana* (PACKARD 1871) by Sassaman & Weeks (1993). In *E. texana,* hermaphrodites may fertilize their own eggs or mate with males but cannot outcross with other hermaphrodites. We are currently exploring the evolutionary maintenance of males in this system (Weeks & Zucker 1999; Medland et al. 2000; Weeks et al. 1999, 2000a,b). Studies on classification dominate our knowledge of this group, but even that aspect of their biology is controversial (Sassaman 1995; Spears & Abele 2000). Recent studies have explored life history and related phenomena (Marcus & Weeks 1997; Weeks et al. 1997). As little is known about the reproductive biology of *E. texana,* we describe here the first detailed behavioral observations of its reproductive cycle.

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E. texana is a small branchiopod crustacean encased in a translucent bivalve-shaped carapace (carapace length to 8 mm). The shrimps are found in temporary ponds and depressions throughout the southwestern U.S. (Sassaman 1989). Resting eggs, which contain embryos, hatch within a day after summer rains flood the depression (MacKay et al. 1990). The shrimps reach sexual maturity in 4 to 7 days depending on water temperatures (Strenth 1977; Weeks et al. 1997) and live from 1 to 3 additional weeks if the pond does not dry up first (Weeks et al. 1997; Zucker et al. 2001). Hermaphrodites carry a clutch of eggs in the fold of their carapace (Fig. 1A) before depositing the eggs in the soil. Males have two pairs of claspers (Fig. 1B) used to hold onto the carapace of hermaphrodites during mate guarding and mating (Fig. 1C). Eggs typically go through a drying period before they hatch and may remain dormant but viable for a decade or more. Thus, only a single generation occurs per wetting event even when the pool is flooded long enough for several generations to mature.

Methods

Description of mating

Dry soil containing eggs of the clam shrimp *Eulimnadia texana* was collected from a cattle tank located

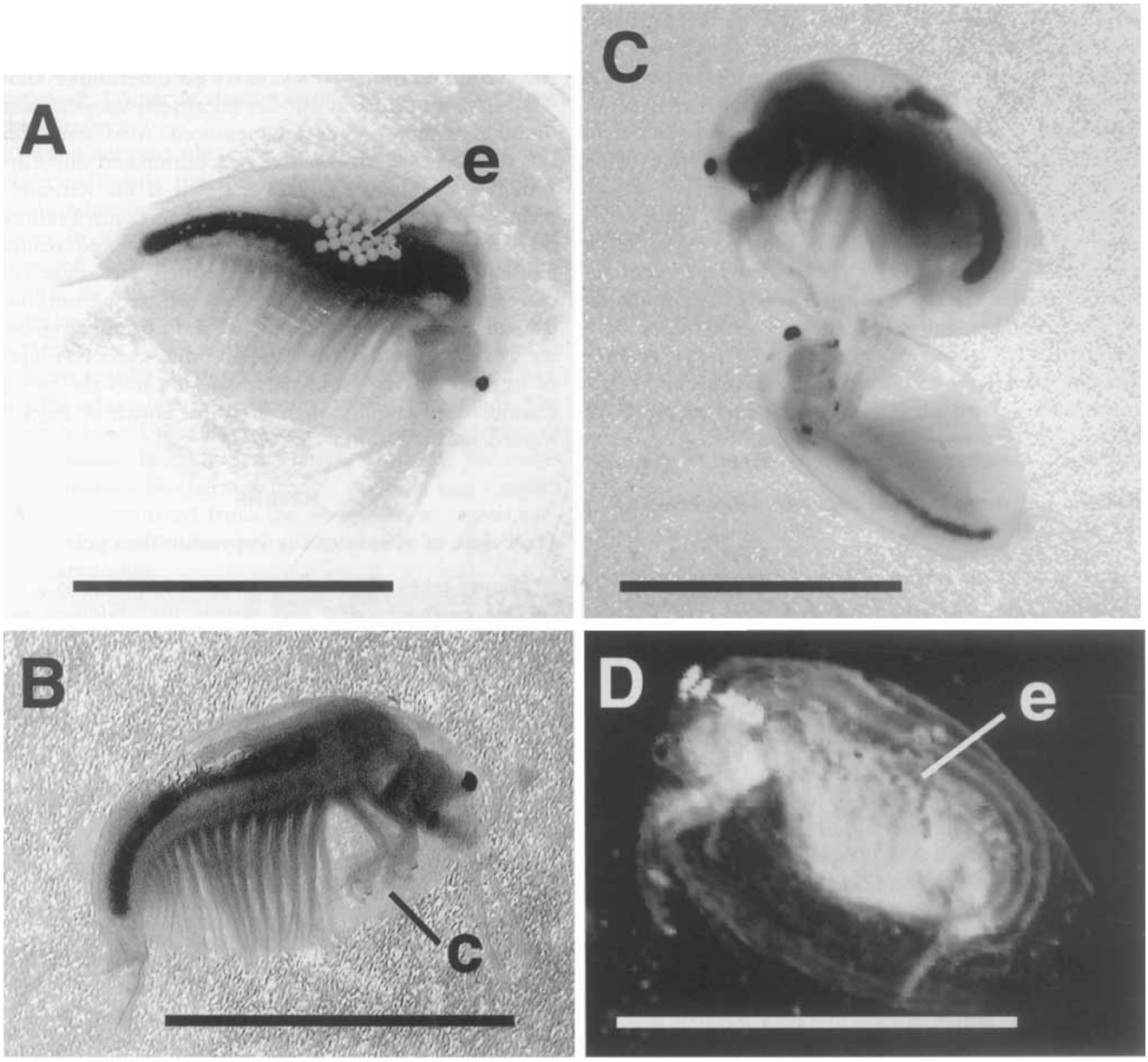

Fig. 1. The clam shrimp *Eulimnadia texana*. **A.** Hermaphrodite brooding a clutch of eggs (e). **B.** Male showing his claspers (c). **C.** A male (below) clasped to the carapace of a hermaphrodite (above) before mating. **D.** Hermaphrodite showing eggs (e) in transit, moving out of the left ovotestis (along side the digestive tract) and into the brood chamber. Photo in D taken from video tape. Scale bars, 5 mm. Photos A, B, and C taken by NZ; photo D taken by LGM.

4 km north of Portal Road (Road 533) near Portal, Arizona, USA. The soil was stored in double freezer-strength locking plastic bags in the dark, under ambient room temperature and humidity in the laboratory of SCW at The University of Akron for several months before use. Approximately 500 ml of soil was hydrated in each of several 38-liter rearing aquaria in a temperature-controlled room (27°C), with constant aeration and 24-h lighting (Duratest Full Spectrum Lighting). Each tank was supplied with 20 ml of a yeast suspension made by mixing 1 g of dried baker's yeast (Fleischmann's™) with 100 ml of filtered tap water (Weeks et al. 1997, 1999). Under these conditions, nauplii hatched within 24 h and reached sexual maturity within 5 days.

Just before producing their first clutch, mating pairs (5–8 days old) were removed from the rearing aquaria and placed in glass dishes 55 mm in diameter. A small amount of tank water (~3 ml) was also transferred with the mating pair. The small amount of water lim-

ited the movement of the shrimps, yet allowed for swimming in a plane suitable for the computer video imaging system (a COHU high performance CCD camera model 4915 attached to a Navitar lens system). This setup was held in place by an Olympus dark-field base (model SZH-D). The video feed was split (using a Video Accessory Corp. splitter) between an image-grabber card (Scion LG-3) attached to a computer running NIH Image software (adapted for the PC by Scion Corporation, 1997) and a VHS video tape recorder. Mating pairs were observed under 10× magnification on the computer and recorded at 33.3 frames/s on a standard VHS video tape recorder. Mating behavior and egg transfer were observed successfully for 3 hermaphrodites, with observation periods ranging from 30 to 120 minutes.

Events and timing of the reproductive cycle

Dry soil containing resting eggs of the clam shrimp *Eulimnadia texana* 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 and stored in the dark in a plastic garbage can at temperatures of 27 ± 2°C and ambient humidity in the laboratory of NZ at New Mexico State University for several months before use. Approximately 200 ml of soil was hydrated in 8 liters of aged tap water in each of several plastic rearing tanks and placed under heat lamps to maintain a water temperature of 28 ± 2°C. Shrimp hatched in about 24 h and were fed initially on dried baker's yeast (Fleischmann's™) dissolved in water and later on ground fish flake food (TetraMin™).

At about 5 days old, just before producing their first clutch (as determined by the presence of large eggs in the ovotestes, which are readily seen through the carapace with a hand lens, Zucker et al. 1997), isolated hermaphrodites (n = 13) or hermaphrodite/male pairs (n = 10) were placed individually in a 250 ml translucent cup with 75 ml of soil covered by 100 ml of aged tap water. Each hermaphrodite or hermaphrodite/male pair was video-taped from above in its cup continuously for 72 h, using time-lapse photography (a 120-min VHS tape set to last 72 h on a Panasonic Time Lapse Video Recorder model AG-6040 attached to a Panasonic B/W camera model WV-BP110). This allowed us to videotape the complete first, second, and (in most cases) third reproductive cycles of each hermaphrodite. A time generator mark embedded on the tape allowed us to determine the duration of each activity when the tapes were viewed on a frame-by-frame, slow-motion video player (Panasonic model AG-1970).

To avoid pseudoreplication in our statistical analyses, we are reporting only the events associated with the second reproductive cycle for each hermaphrodite. The second cycle was chosen because it was the least variable of the three cycles measured. Also, removing the first cycle from the analyses eliminated any confounding problems that might occur if the hermaphrodites were mated before we isolated them. Preliminary analysis using all three cycles showed results similar to those from the second clutch only; thus, our conclusions were not affected by considering only the second clutch. We define the second reproductive cycle as starting when the hermaphrodite's second clutch of eggs is moved from the ovotestes into the brood chamber and ending when the third clutch of eggs is moved into the brood chamber.

Results

Overview of events of the reproductive cycle

Mating takes place when a male clasps on to a receptive hermaphrodite and thrusts his abdomen between the hermaphrodite's folded carapace releasing sperm within; or a hermaphrodite may self-fertilize its own eggs. At the same time, the hermaphrodite moves the clutch of eggs from the paired ovotestes (which parallel the digestive tract on each side with the bulk of the organ producing eggs and only a small posterior region producing sperm, Zucker et al. 1997) to the brood chamber in the fold of the carapace. The eggs are brooded for less than a day, during which early development takes place. During this time, the hermaphrodite swims about at its normally slow pace (relative to males, Medland et al. 2000). Concurrently, a new clutch of eggs is maturing in the ovotestes. A few hours before the brooded clutch is ready to be to be laid, the hermaphrodite increases its swimming speed and then digs one or several burrows prior to depositing the eggs within the last to be dug. Within a few minutes to hours, the hermaphrodite re-mates or self-fertilizes its next clutch of eggs. The hermaphrodite molts ~11 min before fertilizing its next clutch (Knoll 1995).

Pairing and egg release into the brood chamber

Hermaphrodites had eggs present and visible in the ovotestes at the beginning of each observed mating, but had no eggs in the brood chamber. During pairing, the male grasped the edge of one fold of the hermaphrodite's carapace, most often ~⅔ down the length of the hermaphrodite's body. While clasping, but before the hermaphrodite transferred eggs into the brood chamber, the male thrust at irregular times, forcing his

telson between the sides of the hermaphrodite's folded carapace. During this time, males were often observed moving up and down the edge of the hermaphrodite's carapace, primarily staying within the posterior half of the carapace.

We witnessed one case in which egg transfer was observed while the male was still clasping the hermaphrodite (which we interpret as outcrossing) and 2 cases in which eggs were transferred after the male released its hold on the hermaphrodite (which we interpret as selfing). In the former case, when the hermaphrodite began transferring eggs during pairing, the male at first thrust at irregular intervals, then thrust in a more regular pattern, finally ending by briefly holding his telson inside the hermaphrodite's carapace. The male released the hermaphrodite upon completion of egg transfer. In each of the latter two cases, the males had released the hermaphrodite prior to egg transfer and were removed from the glass dish to prevent interference with video-taping the transfer of eggs in the hermaphrodite.

During egg transfer, as the eggs were released from the ovotestes to the brood chamber, they were gathered into a mass in a space enclosed by several of the phyllopods. The eggs were held here for a short time (1–2 s) before being guided into the brood chamber, one row of eggs moving up along each side of the body (Fig. 1D). As the eggs entered the brood chamber, they were adjusted by the hermaphrodite with continual shifting of the eggs from side to side, and forwards and backwards, apparently allowing the eggs to contact the hair-like projections of posterior phyllopods that were directed into the chamber. This adjustment continued until all the eggs were tightly packed into the chamber about 5 to 10 s after the last egg was released from the ovotestes.

Egg transfer was considered to begin with the release of the first egg from the gonopore and to end when the final egg entered the brood chamber (Fig. 1D), including a 1–2 s transit time between gonopore and brood chamber, but not including the 5–10 s of packing time. Transfer of the eggs from the ovotestes into the brood chamber (Fig. 1D) took 24 s for an 80-egg clutch in the outcrossed hermaphrodite, compared to 23 s for a 30-egg clutch and 71 s for a >150-egg clutch in the 2 hermaphrodites that selfed. Thus, the fastest transfer rate of 3.3 eggs/s occurred in the outcrossed hermaphrodite while the selfed hermaphrodites transferred eggs at rates of 1.3 and about 2.1/s.

Brooding of eggs and swimming

Isolated hermaphrodites (n = 13) carried each clutch (i.e., were gravid) for an average of 15.0 h (range 10.0–20.4 h), whereas paired hermaphrodites (n = 10) were gravid significantly longer, averaging 20.1 h (range 13.9–30.0 h) (Fig. 2A; Wilcoxon Rank Sums, df = 1; Z = 3.00784, p = .003). Hermaphrodites often increased their rate of swimming before depositing their clutch (12 of the 13 isolated and 9 of the 10 paired hermaphrodites). From the tapes of paired individuals, the "fast" swimming of hermaphrodites appeared to be about as rapid as the normal male swimming speed but we did not quantify either rate. Paired hermaphrodites swam fast for significantly longer periods (mean 2.5 h; range 0.0–5.5 h) than did isolated hermaphrodites (mean 0.73 h; range 0.0–4.1 h) (Fig. 2B; Wilcoxon Rank Sums, df = 1, Z = 2.63118, p = .009).

Digging of burrows and egg deposition

Gravid hermaphrodites, but neither males nor non-gravid hermaphrodites, were observed to dig in the soil in the observation cups. Isolated hermaphrodites dug significantly more burrows (n = 13; mean 4.4; range 1–11) than did paired hermaphrodites (n = 10; mean 1.6; range 1–2) (Fig. 2C; Wilcoxon Rank Sums, df = 1, Z = −2.90075, p = .004), but there was no difference in the duration of the final digging bout during which the eggs were deposited (Fig. 2D; Wilcoxon Rank Sums; df = 1, Z = −0.26691, p = .790).

Inter-clutch interval and cycle duration

The inter-clutch interval (as defined here, time from emerging from burying a clutch to moving the next clutch into the brood chamber) was significantly longer for isolated hermaphrodites (n = 13; mean 5.4 h; range 1.7–9.2 h) than for paired ones (n = 10; mean 2.4 h; range 0.8–5 h) (Fig. 2E; Wilcoxon Rank Sums, df = 1, Z = −2.48915, p = .013). Nonetheless, the total reproductive cycle for isolated vs. paired hermaphrodites showed no significant difference (Fig. 2F; Wilcoxon Rank Sums, df = 1, Z = 0.22225, p = .824).

Discussion

By clasping the posterior half of the carapace of the hermaphrodites, males appeared to be positioning themselves such that thrusting would occur near the opening of the gonopore between the 10th and 11th pair of phyllopod legs where eggs are released from the ovotestis (Zucker et al. 1997). This would place the sperm near the eggs as soon as they are released. Knoll (1995) also observed male thrusting during egg transfer and assumed that sperm release and fertilization were taking place during that time. Neither we, nor Knoll (1995), observed any structure that could be

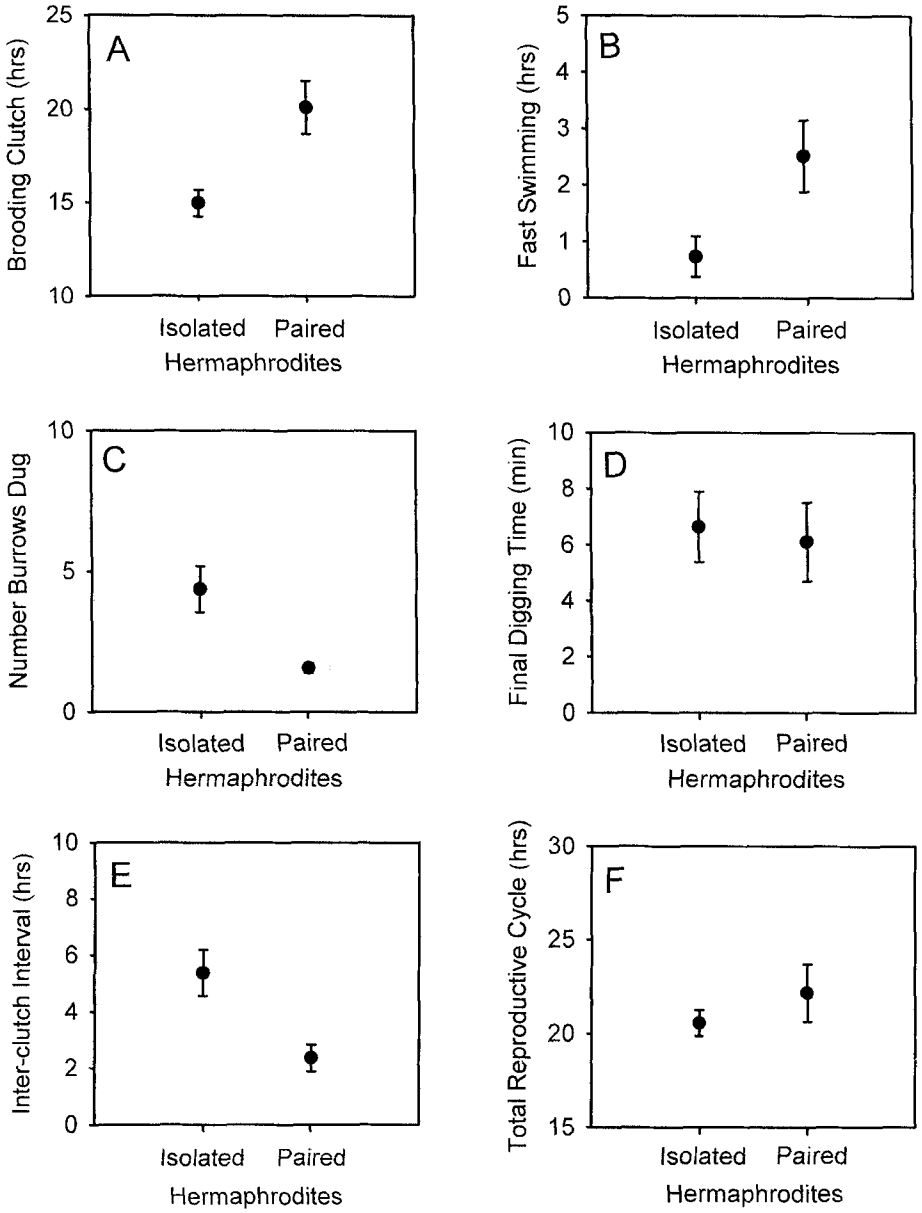

Fig. 2. Comparison between isolated (n = 13) and paired (n = 10) hermaphrodites of *E. texana*. **A.** Duration of brooding. **B.** Time spent swimming fast before egg laying. **C.** Number of burrows dug before egg laying. **D.** Duration of the final digging bout before egg laying. **E.** Interval between laying one clutch of eggs and brooding the next. **F.** Total duration of the reproductive cycle. Error bars, s.e.

interpreted as a spermatophore delivered to the phyllopods of the hermaphrodite as described by Strenth (1977). However, it is conceivable that the mass of eggs the hermaphrodite was seen to ball up with its phyllopods in this study is what Strenth reported as a spermatophore.

The switch from intermittent male thrusting to a rhythmic pattern that occurred during egg transfer is interpreted by us to be the period when outcrossing typically occurs. While we have evidence that some outcrossing can take place even when a male releases a hermaphrodite before egg transfer, we have observed an 8-fold higher outcrossing success rate if the male remained clasped and thrusted during the time of egg transfer (S.C. Weeks, C.L. Marquette, & E. Latsch, unpubl. data). If we are correct in assuming that fertilization is external, and that the eggs have a narrow window for successful fertilization (after release from the ovotestes but before the egg shell hardens), then a male that transfers sperm too early may not be very successful at outcrossing. Further studies are needed to pinpoint the time of sperm transfer by males and its relationship to successful fertilization.

We speculate that the longer time that hermaphrodites carried their clutches when a male was present is an artifact of our experimental setup. In our setup, there was one male to one hermaphrodite in a small container. In nature, however, males are rare, usually

representing <30% of the population (Strenth 1977; Weeks & Zucker 1999). In the wild, hermaphrodites about to dig a burrow and deposit their clutch would probably not be harassed by males, as the males would likely be seeking out receptive hermaphrodites. Knoll (1995) found that an unreceptive hermaphrodite would struggle with a clasping male, resulting in the male releasing it. In our cups, males attempted to clasp the lone hermaphrodites repeatedly throughout the reproductive cycle. Therefore, the longer time these hermaphrodites spent in "fast" swimming before egg deposition might represent the hermaphrodite's attempt to evade the male. This could increase the time a hermaphrodite carries its clutch before successfully digging a burrow and depositing the eggs.

We do not know the function of the fast swimming we observed by hermaphrodites near the end of brooding. Typically, while carrying a clutch, hermaphrodites spend most of their time swimming slowly or resting at the bottom of the tank (Zucker et al. 2001). Males, on the other hand, swim significantly faster than do hermaphrodites (Medland et al. 2000) and for significantly longer periods of time (Zucker et al. 2001). Further observations of hermaphrodite swimming behavior in larger tanks with more individuals would shed light upon whether this behavior and its duration is simply an artifact of the current experimental setup or an attempt to evade males around the time of egg deposition.

Knoll (1995) observed a few occasions in the laboratory in which gravid hermaphrodites dug burrows and one case in which the hermaphrodite emerged from the burrow without its clutch. Neither we nor she observed males digging. The present study confirms that gravid hermaphrodites do, indeed, dig burrows for depositing eggs. In fact, all hermaphrodites dug at least one burrow before depositing their eggs and all eggs were deposited in a burrow. Most hermaphrodites dug in several different areas in the hour or so before depositing their eggs, but paired hermaphrodites dug in only 1 or (at most) 2 areas. These paired hermaphrodites, which brooded for longer periods, might have been forced to postpone egg deposition until the last minute because of constant harassment by the male, resulting in fewer burrows being dug. Unpaired hermaphrodites often returned to the same 2 or 3 burrow sites to continue some digging before settling on one site for depositing all their eggs. At this point we do not know whether the hermaphrodites were exploring for some specific condition(s) and, if so, what those might be.

From our study it appears that depositing a clutch of eggs in a burrow is an obligate behavior for these shrimps. Two, non-mutually exclusive functions for burying eggs are (a) a predator-defense tactic and (b) a bet-hedging mechanism. The tadpole shrimp *Triops*, a larger branchiopod crustacean, is often found in the same pools as *Eulimnadia* and probably would prey upon the eggs if they were simply left on the substrate surface, for *Triops* forages by rummaging on the surface for organic matter. Also, if all eggs were left at the substrate surface, then most, if not all, might hatch during the next wetting event. Many such events produce enough water to stimulate hatching but insufficient water for the shrimp to reach sexual maturity (Zucker, unpubl. obs.). Branchiopod eggs do not hatch under very low oxygen and/or light levels (Brendonck 1996), so eggs buried in burrows might not hatch even if wetted. Desert sand storms and the activities of birds and mammals on the surface would likely churn up buried eggs at different times exposing only a fraction at a time. Thus, by burying eggs, the hermaphrodites might be hedging their bets since not all eggs would hatch during any given wetting event, leaving some for future, perhaps more productive rains.

Mating with a partner resulted in a significantly shortened inter-clutch interval, a potential benefit of outcrossing over selfing in this system. For a species that is sexually mature for only 1–2 weeks and produces a new clutch about once a day (Weeks et al. 1997; and herein), a 3-h reduction in each inter-clutch interval could result in additional clutches over the lifetime of a hermaphrodite. Even one additional clutch would represent a significant increase in net reproductive rate, especially since clutch size increases significantly with hermaphrodite age/size (Knoll & Zucker 1995; Weeks et al. 1997). Further studies should examine whether a clasping male stimulates a hermaphrodite to move its developing eggs into the brood chamber earlier, or whether the lack of a male results in the hermaphrodite postponing egg release from the ovotestes as long as possible to maximize the chances of encountering a male and achieving outcrossing.

While the inter-clutch interval for hermaphrodites was shorter in the presence of males, the total duration of the reproductive cycle was the same for isolated and paired hermaphrodites. This is due to the longer brooding period of paired hermaphrodites. If we are correct in our speculation that the extended brooding time was an artifact of our experimental setup (as males were constantly harassing the hermaphrodites while they were trying to dig and deposit their clutches), then total cycle length would normally be significantly shorter for paired hermaphrodites. More research is needed to determine why paired hermaphrodites hold on to their clutches for longer periods before we can say with confidence that outcrossing results in shorter

reproductive cycles allowing for more clutches in the lifetime of a hermaphrodite.

The above observations have revealed several interesting differences between selfing and outcrossing hermaphrodites that invite further exploration. For example, do mated hermaphrodites transfer eggs from the ovotestes to the brood chamber at a faster rate than for selfed hermaphrodites? Also, do mated hermaphrodites have, under more natural conditions, a brooding period similar to that of selfed hermaphrodites, contrary to our results when the shrimps were placed in a very small container with no escape from the male? If so, then because mated hermaphrodites appear to have a shorter inter-clutch interval, their total reproductive cycle would also be shorter. In a time-compressed environment such as a temporary desert pool, any behavioral variants that would shorten the breeding cycle (thereby allowing more bouts of egg production per lifetime) should be strongly selected for. Therefore, should these results and speculations be supported with additional evidence, they would point to advantages in outcrossing.

Acknowledgments. We thank two anonymous reviewers for suggestions that improved the manuscript. Funding was provided by NSF grants IBN-9631042 to NZ and IBN-9614226 to SCW.

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