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Intersexual conflict in androdioecious clam shrimp: Do androdioecious hermaphrodites evolve to avoid mating with males?

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Abstract

A recent sexual conflict model posits that a form of intersexual conflict may explain the persistence of males in androdioecious (males + hermaphrodites) populations of animals that are being selected to transition from dioecious (gonochoristic) mating to self-compatible hermaphroditism. During the evolutionary spread of a self-compatible hermaphrodite to replace females, the selective pressures on males to outcross are in conflict with the selective pressures on hermaphrodites to self. According to this model, the unresolved conflict interferes with the evolutionary trajectory from dioecy to hermaphroditism, slowing or halting that transition and strengthening the otherwise "transitory" breeding system of androdioecy into a potentially stable breeding strategy. Herein, we assess this model using two dioecious and two androdioecious clam shrimp (freshwater crustaceans) to ask two questions: (1) Have hermaphrodites evolved so that males cannot effectively recognize them?; and (2) Do androdioecious hermaphrodites avoid males? Androdioecious males made more mistakes than dioecious males when guarding potential mates suggesting that androdioecious males were less effective at finding hermaphrodites than dioecious males were at finding females. Similarly, in a three-chambered experiment, focal hermaphrodites chose to aggregate with their same sex, whereas focal dioecious males chose to aggregate with the alternate sex. Together, these two experiments support the sexual conflict model of the maintenance of androdioecy and suggest that hermaphrodites are indeed evolving to avoid and evade males.

KEYWORDS
Eulimnadia texana Packard, mate guarding, mating success, reproductive evolution, sexual aggregation

1 | INTRODUCTION

Sexual selection was initially developed to address the evolution of extreme male secondary sexual traits and extravagant displays (Darwin, 1859, 1871). These are often costly to the male but important for successful mating. The general idea is that females who are attracted to these males will gain a benefit (directly or indirectly), and thus a positive feedback (that enhances male and female fitness) can be maintained (Andersson, 1994). An alternative view, termed antagonistic selection (Partridge & Hurst, 1998), considers a different scenario: the increase in fitness of one sex does not necessarily imply an increase in fitness in the opposite sex (Daly, 1978; Parker, 1979).

Recently, increased attention has been devoted to sexually antagonistic selection, precipitated by the recognition of widespread "intersexual conflicts" and their effects on evolution. The concept is not new (Bateman, 1948; Parker, 1979; Trivers, 1972; Williams, 1966), but recent theoretical models, and the use of empirically innovative tools, have renewed interest in the topic (e.g., Arnqvist, 2006; Arnqvist & Rowe, 2005; Chapman, 2006; Friberg, 2005; Hosken & Snook, 2005; Rowe & Day, 2006).

Traditionally, two outcomes for intersexual conflicts have been considered. (1) Resolution of the conflict or (2) Escalation (runaway process). The runaway process (outcome 2) should, by definition, produce an accelerated rate of evolution. This acceleration has been observed, for example, in the evolution of male genitalia in species with internal fertilization (Eberhard, 1985, 1996). In this study, we intend to examine a recent theory (Chasnov, 2010) that postulates the opposite: intersexual conflict can retard (or even halt) evolutionary development, in this case, of mating systems. Specifically, Chasnov proposes that antagonism between the sexes slows or stops the full evolutionary transition from dioecy (males and females) to hermaphroditism and instead maintains the mating system of androdioecy (males and hermaphrodites).

Specific environmental conditions, such as “reproductive assurance” (Baker, 1955), might favor a shift from dioecy to hermaphroditism (Pannell, 2002). According to theory, mutant hermaphrodites capable of facultative self-fertilization should spread within a dioecious population with low outcrossing possibilities (Pannell, 1997; Wolf & Takebayashi, 2004). Such incipient hermaphrodites are likely to quickly outcompete one or the other sex to form gynodioecy (hermaphrodites outcompete males and coexist with females) or androdioecy (hermaphrodites outcompete females and coexist with males). Thus, initially a coexistence of the original single-sex individuals with hermaphrodites is likely. However, models predict this coexistence to be short lived as the system fully transitions to hermaphroditism (Charlesworth, 1984). Nevertheless, androdioecious systems persist in nature (see reviews for plants and animals; Pannell, 2002; Weeks, Benvenuto, & Reed, 2006; Weeks, 2012), with the longest-lived androdioecious clade (25–100+ million years) being freshwater crustaceans in the genus *Eulimnadia* (Weeks, Sanderson et al., 2006; Weeks, Chapman et al., 2009).

Chasnov’s (2010) model assumes that androdioecy evolves because of reproductive assurance and assumes that inbreeding depression is not as important as assumed in other models of androdioecy (Otto, Sassaman, & Feldman, 1993; Pannell, 2002; Wolf & Takebayashi, 2004). Hermaphrodites can invade and replace females when the benefits of reproductive assurance outweigh the extra costs of producing sperm and when self-fertilized offspring “are of reasonable fitness” (i.e., have low inbreeding depression; Chasnov, 2010; pg. 541). Reproductive assurance can be seen as directly proportional to the amount of times that females/hermaphrodites cannot find a mate, commonly due to low population size. Chasnov further predicts that, although the initial mutant hermaphrodite is likely to be receptive to fertilization by males, such a “receptive” hermaphroditic type (i.e., receptive to mating with males) should be replaced by an “unreceptive” type if inbreeding depression is <0.5. This may occur quickly, if inbreeding depression is generally low, or may occur over a longer period if inbreeding depression is initially high but then is reduced below 0.5 via purging of genetic load (Barrett & Charlesworth, 1991; Lande, Schemske, & Schultz, 1994). It is under this second scenario (i.e., after the spread of unreceptive hermaphrodites) that Chasnov (2010) envisions the potential for sexual conflict. Males would have a strong impetus to mate with unreceptive hermaphrodites if these were the only mates available, while unreceptive hermaphrodites would have an asymmetrically lower pressure to avoid outcrossing with males (i.e., the cost of outcrossing for a hermaphrodite would not be as high as the cost of a male being unable to mate at all).

Two animal systems have been thoroughly investigated for the evolution of androdioecy (Chasnov, 2010): clam shrimp in the genus *Eulimnadia* (esp. *Eulimnadia texana* Packard) and nematodes in the family Rhabditidae [esp. *Caenorhabditis elegans* (Maupas)]. In both clades, the ancestor has been determined to be dioecious (Kiontke et al., 2004; Sassaman, 1995; Weeks, Sanderson et al., 2006), suggesting an evolutionary trajectory from dioecy to hermaphroditism. Chasnov (2010) suggests that in both *E. texana* and *C. elegans* this evolutionary trajectory has been “frozen” in the normally transitory androdioecious breeding system due to intersexual conflict between males and hermaphrodites.

In the nematodes, several studies of Chasnov’s (2010) model have been done on *Caenorhabditis elegans* and *C. briggsae* Dougherty & Nigon, both of which have been compared to the closely related dioecious species, *C. remanei* (Sudhaus) and *C. brenneri* Sudhaus & Kiontke. In these androdioecious nematodes, hermaphrodites lose many “female traits” when compared with dioecious females, making the hermaphrodites hard to recognize by males (Chasnov, 2010; Chasnov, So, Chan, & Chow, 2007; Chaudhuri et al., 2015). In addition, males of *C. elegans* and *C. briggsae* seem to have trouble copulating with hermaphrodites, whereas males of the dioecious species easily mate with their respective females (Garcia, LeBoeuf, & Koo, 2007; Kleemann & Basolo, 2007), and nematode hermaphrodites no longer respond to a factor males produce to nullify female resistance (Chaudhuri et al., 2015; Garcia et al., 2007). Some hermaphrodites also appear to expel some or all of the semen after mating occurs (Barker, 1994; Kleemann & Basolo, 2007). In addition, males in androdioecious populations cannot recognize hermaphrodites from a distance (Chasnov et al., 2007). It would seem that in *Caenorhabditis*, androdioecious hermaphrodites are “reluctant” mates compared to dioecious females, and hermaphrodites seem to have evolved adaptations to help them avoid males (Chasnov, 2010).

Similar studies have been conducted in the branchiopod crustaceans, but none directly assessing Chasnov’s (2010) predictions in these shrimp. Like the Rhabititdae nematodes, these crustaceans are differentiated based on their mating systems with both dioecy and androdioecy found throughout the family Limnadiidae (Weeks, 2012). In particular, *Eulimnadia* species have an androdioecious mating system where hermaphrodites can either outcross with males or self-fertilize (Sassaman & Weeks, 1993). Intersexual conflicts during mate guarding are common in clam shrimp (Benvenuto & Weeks, 2011, 2012). Chasnov (2010) suggests that similar sexual conflicts occur even before mate guarding: hermaphrodites might not be selected to outcross with males at all. Thus, males might be maintained in the population through sexual conflict and their ability to ensure copulation, even though hermaphrodites would be best suited by self-fertilizing.

The following study focused on two questions to address Chasnov's (2010) model in these clam shrimp: (1) Have hermaphrodites evolved so that males do not effectively recognize them? and (2) Do androdioecious hermaphrodites avoid males? To assess these questions, mating behaviors in androdioecious clam shrimp were compared with the same behaviors in dioecious clam shrimp. If (1) is true, then dioecious males should more effectively recognize females for mate guarding while androdioecious males should be more prone to making mistakes. If so, dioecious males should be more frequently found guarding their opposite sex compared to androdioecious males, and the latter should be more likely to mistakenly "guard" other things (e.g., other males or debris). If (2) is true, then androdioecious hermaphrodites should avoid males at a higher rate than dioecious females. These two questions were addressed in two separate experiments described below.

2 | METHODS AND MATERIALS

2.1 | General methods

A drawback of studies of rare breeding systems, such as androdioecy, is the inability to do a phylogenetically controlled comparison. In clam shrimp, there is only a single genus that is androdioecious (*Eulimnadia*) but many that are dioecious (Sassaman, 1995; Weeks, Sanderson, Zofkova, & Knott, 2008). Given this constraint, we nonetheless replicated our comparison of reproductive types (dioecious vs. androdioecious) to disassociate reproductive mode comparisons from species differences. We compared two dioecious species [*Eocyzicus argillaquus* Timms & Richter and *Paralimnadia stanleyana* (King)] to two androdioecious species (*Eulimnadia texana* and *Eulimnadia dahli* Sars) in the studies outlined below.

Encysted eggs of all four species were hatched from previously collected field soil samples (Table 1). Soil was put into the bottom of a 5-L tank until approximately 5 mm depth of the bottom was covered. Each individual soil sample containing the species' eggs was put in separate tanks and then hydrated with deionized water. Each tank was kept under 24-hr Durotest sunlight-simulating fluorescent lights and had an air-stone to provide aeration (Weeks, Marcus, & Alvarez, 1997). Once per day the tanks were fed a portion of food mixture made with a 0.5 g brewer's yeast and 0.5 g of ground algae fish food flakes dissolved in 100 ml of water. Any hatched tadpole shrimp (Notostraca) were removed from the tanks, as these shrimp are predators on the clam shrimp. The clam shrimp were then allowed to sexually mature before being used in the experiments. Shrimp are considered mature once the presence of claspers on males and eggs on hermaphrodites/females were seen (Weeks et al., 1997).

Once maturity was reached, males and females were marked so they could be easily visually differentiated. For marking, the clam shrimp were placed under a dissecting microscope and the water was removed from around the carapace using a pipette; the carapace was further dried off using kimwipes. A thin paintbrush was then dipped into nail polish and a series of small marks were painted on the carapace to differentiate individuals within males and females. After the marking was done, a small amount of water was reintroduced around the shrimp until the mark completely dried. Once the mark dried, the shrimp was returned to a temporary holding container momentarily until it was used for the experiment. Such carapace marking does not alter clam shrimp behaviors (Benvenuto, Knott, & Weeks, 2009).

2.2 | Experiment 1: mate guarding

To observe the mate guarding behavior of clam shrimp, a 1-L beaker was filled with 900 ml of water from the species' rearing tank. To this environment, 10 previously molted carapaces and 10 pieces of debris (leaf litter and/or small sticks that were approximately the size of a carapace) were added. Five males and five female/hermaphrodites of the same species were then haphazardly collected from the temporary holding tank of marked individuals (mentioned above) using a large-bore pipette and placed into the observation environment. Each of the males was marked with a different pattern of paint dots on their carapace using nail polish (as noted above) so the focal and other males could be differentiated from each other during the observation. Lastly, the focal male was added to the environment and allowed to acclimate for approximately 2 min. Afterwards, the focal male was observed for 20 min and scored (using the JWatcher computer application; Blumstein & Daniel, 2007) based on the following behaviors: guard same sex, guard opposite sex, guard debris, and guard molted carapace. "Guarding" was defined as a clasping of one of the above objects during the observational period. JWatcher recorded the number of times a behavior occurred as well as the duration of guarding. Once a trial was completed, the focal male was

TABLE 1 Location of clam shrimp soil samples

Species	Reproductive mode	Location name	Coordinates
Eocyzicus argillaquus	Dioecious	Cullimbin pools 1 & 5, Australia	S30°50.972 E117°14.751′
Paralimnadia stanleyana	Dioecious	Kanangra Walls Pool 1, Australia	S33°59.933′ E150° 5.133′
Eulimnadia texana	Androdioecious	Wallace (previously WAL, Sassaman & Weeks, 1993), Arizona, USA	N31°57.387′; W109°08.998′
Eulimnadia dahli	Androdioecious	The humps pool 3 & 6, Australia	S32°19.034 E118°57.525′
		Elachbutting pool 5 & 6, Australia	S30°35.984; E118°36.447′

removed and returned to a separate tank so as to not be used again and a new focal male of that same species was used in the set up for the next trial. This same method was used until several males of the each species were measured. The set up would then be reset for the next species, with new water, debris, carapaces, and individuals and the above procedure would be repeated again.

2.3 | Experiment 2: sexual aggregation behavior

The following methods were adapted from Medland, Zucker, and Weeks (2000) for quantifying clam shrimp swimming behavior in the laboratory. To test whether hermaphrodites/females and/or males aggregate with the same or opposite sex, shrimp were placed in a three-chambered (larger center chamber + two smaller peripheral chambers) Plexiglas container with the following dimensions: Overall—35 cm long × 15 cm wide; center chamber—25 × 15 cm; and each peripheral chamber—5 × 15 cm. All chambers were 3 cm deep. The perimeter of the whole container was opaque and the dividers between sub-chambers were clear with small holes (1 mm diameter) to allow for both visual and chemical cues between the shrimp in the different chambers. The center chamber was marked with two lines drawn on the bottom of the tank allowing the identification of three equal portions of the central chamber.

The Plexiglas container was then filled with water from the species' rearing tank. Five haphazardly selected hermaphrodites or females (based on species) were then added to one of the peripheral chambers and five randomly selected males were added to the opposite peripheral chamber. A focal hermaphrodite/female or male was then added to the center chamber and allowed to acclimate for 2 min. After the acclimation period, JWatcher (Blumstein & Daniel, 2007) was used to score which third of the center chamber the focal clam shrimp was in for a total of 10 min. JWatcher totaled the time a focal shrimp entered each third of the central chamber. This design allowed the focal individual to make a choice to aggregate near the same sex, the opposite sex, or to have no preference. After being used as a focal individual, the clam shrimp was returned to a separate tank, to eliminate the possibility of being used again. After each behavioral observation, the chamber was physically reoriented to remove any preference for a certain side of the experimental setup.

2.4 | Statistical analyses

For Experiment 1, behavioral observations were conducted for the two dioecious species (*Eocyzicus argillaquus*, $n = 55$; *Paralimnadia stanleyana*, $n = 35$) and the two androdioecious species (*Eulimnadia texana*, $n = 50$; *Eulimnadia dahli*, $n = 46$). Counts of the number of times and what (same sex, opposite sex, debris, or empty carapace) an individual guarded were used to perform the analyses. First, a chi square was run to determine whether there was a difference between the two species in guarding behavior within each reproductive type. There were no significant differences in guarding behavior between either of the two androdioecious species ($\chi^2_{(3)} = 1.76$; $p = .62$) or the dioecious species ($\chi^2_{(3)} = 6.97$; $p = .07$). Thus, the results were combined for the two species within each reproductive type (androdioecious and dioecious) and a second a chi square was run to determine whether there was an overall difference in guarding behavior between reproductive types. All pairwise comparisons of reproductive types (i.e., *E. argillaquus* vs. *E. texana*, *E. argillaquus* vs. *E. dahli*, *P. stanleyana* vs. *E. texana*, and *P. stanleyana* vs. *E. dahli*) showed the same significant (all $p < .0001$) pattern as the combined results.

For Experiment 2, observations were collected for two dioecious species (*Eocyzicus argillaquus*, $n = 55$; *Paralimnadia stanleyana*, $n = 56$) and two androdioecious species (*Eulimnadia texana*, $n = 63$; *Eulimnadia dahli*, $n = 53$). To analyze these data, the time a focal individual spent in the third of the chamber closest to the same sex was subtracted from the amount of time an individual spent in the third of the chamber nearest the opposite sex. Hence, if the value is positive, the focal individual aggregated near the opposite sex and if the value is negative, the focal individual aggregated near the same sex. A value near zero suggests no preference of either sex. ANOVAs were then computed to assess the difference in male and females/hermaphrodites in time spent near either sex. As in Experiment 1, the two species within reproductive type were compared with each other to note any species-specific behavioral differences. There was no significant difference between species either for the androdioecious ($F_{1,112} = 0.715$; $p = .3996$) or the dioecious species ($F_{1,107} = 1.640$; $p = .2030$). Thus, the results were combined for each species within reproductive type. Next, a two-way ANOVA was run to assess behavioral differences in where the shrimp aggregated in the focal chambers between reproductive types (androdioecious compared with dioecious) and sexes (male compared with females/hermaphrodites). These data met the assumptions of ANOVA: the residuals were normally distributed and the variances were homogeneous among treatments.

3 | RESULTS

3.1 | Experiment 1: mate guarding

The reproductive types significantly differed in how they guarded ($\chi^2_{(3)} = 92.38$; $p < .0001$), with the androdioecious shrimp making many more guarding mistakes than the dioecious clam shrimp (Figure 1). The androdioecious males mistakenly guarded debris, empty carapaces, and the same sex in about two-thirds of the guarding attempts, and only correctly guarded hermaphrodites about one-third (32%) of the time (Figure 1). The opposite was true for the dioecious males: they guarded females almost two-thirds of the time (64%) while making guarding mistakes slightly over a third of the time (Figure 1). In fact, the androdioecious species guarded empty carapaces, the opposite sex, and the same sex almost equally, whereas the dioecious males clearly were the most effective at finding their opposite sex (Figure 1). In none of the mistaken guarding attempts were there signs that the males were in any way consuming the debris or molted carapaces. The total

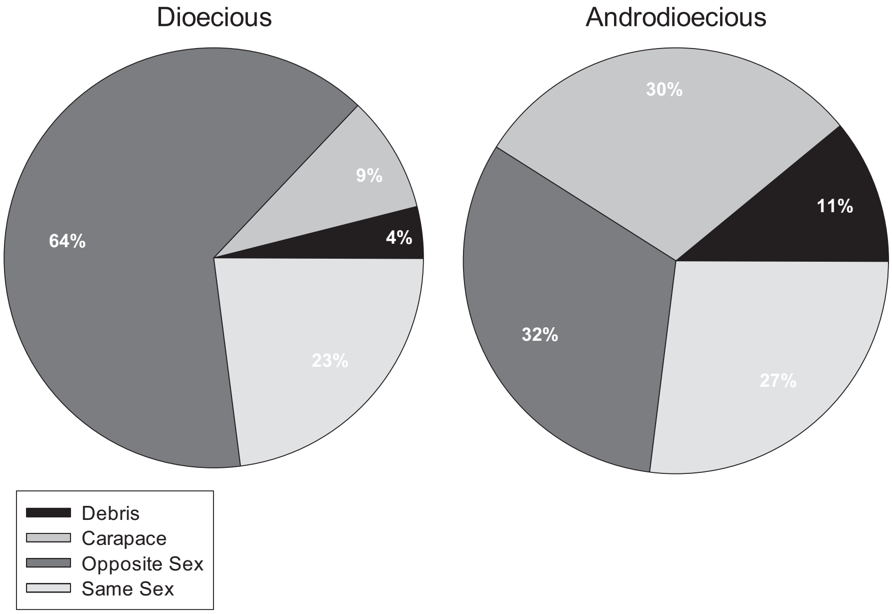

FIGURE 1 Comparison of 96 androdioecious (*Eulimnadia texana*, *n* = 50; *Eulimnadia dahli*, *n* = 46) and 90 dioecious (*Eocyzicus argillaquus*, *n* = 55; *Paralimnadia stanleyana*, *n* = 35) males guarding hermaphrodites and females, respectively. Guarding behavior is reported as the proportion of total guard attempts in each of four categories. Total guarding attempts: 247 in dioecious species and 633 in androdioecious species

TABLE 2 Two-way ANOVA of time spent in the chamber for each reproductive type and focal sex

Main effect	*df*	SS	*F*-ratio	Prob > *F*
Reproductive type	1	97.45	3.67	.0567
Focal sex	1	827.38	31.16	<.0001
Reproductive type * Focal sex	1	0.09	0.003	.9549

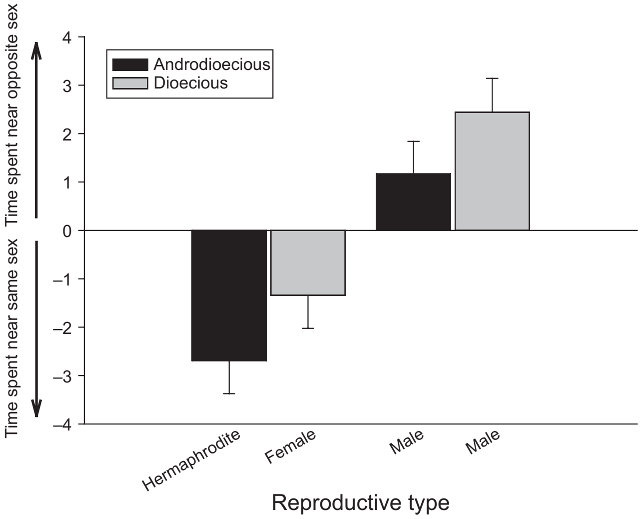

FIGURE 2 Relative time (time spent near the opposite sex minus time near the same sex, in minutes) for each sex in the two reproductive types. Relative time of zero suggests no preference of opposite or same sex. Error bars depict 1 standard error of the mean

guarding attempts were also noticeably different from dioecious species guarding 247 times and androdioecious species guarding 633 times.

3.2 | Experiment 2: sexual aggregation behavior

The time spent aggregating near the opposite sex differed in the two reproductive types (Table 2; Figure 2). In both reproductive types, the males tended aggregate near the opposite sex, whereas the females/hermaphrodites tended to aggregate near their own sex ("Focal Sex" effect, Table 2; Figure 2). There was a marginally significant reproductive type effect (Table 2) with androdioecious individuals tending to aggregate nearer the same sex, whereas dioecious individuals tending to aggregate nearer the opposite sex (Figure 2). However, this result was mainly driven by androdioecious hermaphrodites having a greater affinity for other androdioecious hermaphrodites than females being attracted to other females while dioecious males were more strongly attracted to females than androdioecious males were attracted to hermaphrodites (Figure 2). In fact, the only sex/reproductive type combinations that showed a significant tendency toward one sex or the other (i.e., that the Opposite–Same significantly different from zero) were androdioecious hermaphrodites being more attracted to other hermaphrodites and dioecious males being more attracted to dioecious females (Figure 2).

4 | DISCUSSION

Herein, we found evidence consistent with the notion that clam shrimp hermaphrodites are evolving to evade males. To address the first of our two questions, we observed mate guarding behavior and found androdioecious clam shrimp guarded significantly differently than dioecious clam shrimp (Figure 1). The androdioecious males guarded empty carapaces, other males, and hermaphrodites at almost the same rates, suggesting that androdioecious males cannot easily recognize the opposite sex. On the other hand, dioecious males guarded females much more often than anything else

(Figure 1), showing that dioecious males can effectively find their opposite sex, although occasional mistakes are still made. In addition, the total amount of guarding attempts was noticeably higher in androdioecious clam shrimp (633 times) compared to dioecious clam shrimp (247 times). If androdioecious hermaphrodites are indeed losing their female cues, it is possible that androdioecious males are confused in what they are clasping and therefore must make more attempts at guarding until they find their intended target.

These findings mirror results comparing dioecious and androdioecious nematodes: dioecious *Caenorhabditis* females secrete pheromones that can attract males from a distance, whereas androdioecious *Caenorhabditis* hermaphrodites have lost that chemical attraction (Chasnov et al., 2007). Other evidence of a loss of female mating behaviors in *Caenorhabditis* hermaphrodites include rapid hermaphroditic movement when in the presence of males and expulsion of semen after mating with males (Chasnov, 2010; Chaudhuri et al., 2015). *Eulimnadia texana* hermaphrodites have been documented to attempt to "kick off" attached males (Weeks, Marquette, & Latsch, 2004), but no corresponding assessments have been made in dioecious clam shrimp to note whether this is a general clam shrimp behavior or is something specific to androdioecious hermaphrodites. Overall, the differences in male behaviors when encountering hermaphrodites relative to females are consistent with Chasnov's (2010) predictions that androdioecious hermaphrodites should evolve to evade males, perhaps by losing the characteristics that make hermaphrodites easily identifiable to the opposite sex.

To address the second question, we observed clam shrimp in a divided chamber to detect any evidence for sexual aggregation behavior. Androdioecious hermaphrodites were strongly attracted to other hermaphrodites, whereas androdioecious males were weakly (but not significantly) attracted to the hermaphrodites (Figure 2). The dioecious females were weakly (marginally significantly) attracted to other females and the dioecious males were strongly attracted to females. This evidence shows that dioecious males are able to find the opposite sex more readily than androdioecious males, possibly because hermaphrodites are no longer producing chemical attractants that androdioecious males can respond to. Similar evidence suggesting hermaphrodites are evolving to avoid males was noted by the tendency of hermaphrodites to stay closer to other hermaphrodites and away from males. This male avoidance behavior is mirrored in *C. elegans* hermaphrodites, which move so quickly away from males that their behavior is described as "sprinting" away from males (Kleemann & Basolo, 2007). Female clam shrimp also stayed closer to their own sex but not nearly to the same extent as seen in the hermaphrodites (Figure 2). The lack of female attraction to males differs from other studies of nematodes wherein females are attracted to males whereas hermaphrodites are not (Chaudhuri et al., 2015). Overall observations from this second experiment are most easily explained by hermaphrodites actively avoiding males (question 2 above), supporting the prediction that androdioecious hermaphrodites should evolve to avoid males to increase their rate of self-fertilization (Chasnov, 2010).

Other research has touched on the question as to whether androdioecious clam shrimp are evolving to avoid males. Contrary to Chasnov's (2010) theory (and our current research), there seems to be some evidence that androdioecious *Eulimnadia* hermaphrodites do prefer to outcross. Females in dioecious clam shrimp populations will not move their eggs into their brood chamber until a male is present (Weeks et al., 2008), and because egg fertilization only occurs in the brood chamber (Weeks et al., 2004), such female behavior is consistent with females maximizing outcrossing opportunities. *Eulimnadia* hermaphrodites that are isolated do move eggs into their brood chamber in the absence of males, but do so more slowly than hermaphrodites in the presence of a male, which has been interpreted as a "desire" to outcross with males (Zucker, Aguilar, Weeks, & McCandless, 2002). *Eulimnadia* males swim faster while hermaphrodites swim slower in the presence of males, which has been proposed as a hermaphroditic strategy to increase the likelihood of encountering a male (Medland et al., 2000). In this same study, Medland et al. (2000) found that hermaphrodites were attracted to being near males in an observation chamber. Hermaphrodites of *Eulimnadia texana* also kick and resist mate guarding less when they are closer to being receptive (Benvenuto & Weeks, 2011). The above examples point toward hermaphrodites "preferring" to outcross, which is inconsistent with our current findings.

However, the above studies do not necessarily imply that hermaphrodites are attracted to males. Although isolated hermaphrodites do wait longer before they move their eggs into their brood chamber than paired hermaphrodites (Zucker et al., 2002), they nonetheless will move the eggs in the absence of males; dioecious females will never move their eggs without a male present (Weeks et al., 2008). This difference suggests that androdioecious hermaphrodites are evolving away from the behavior of "waiting" for males. The fact that hermaphrodites still "wait" for some time may be a legacy of the previous behavior of "waiting" indefinitely. In terms of hermaphrodites swimming slower than males (Medland et al., 2000), it remains unclear whether androdioecious males have increased their swimming speed to increase the probability of contacting a hermaphrodite (Medland et al., 2000) or if hermaphrodites swim more slowly to be easily found by males. A simple comparison of swimming rates in dioecious vs. androdioecious males could easily address these two alternatives. In addition, the Medland et al. (2000) finding that hermaphrodites were "attracted" to males could be due to a flaw in their experimental design. In their experiment, hermaphrodites were not given a choice between the same or opposite sex; it is possible that their finding of an attraction of the hermaphrodite to the opposite sex may in fact have been an attraction to movement or other clam shrimp. In the current experiment, when both opposite and same sex options were presented simultaneously, the androdioecious hermaphrodites clearly preferred their same sex (Figure 2), suggesting they are not attracted to males when given a distinct choice. Overall, even though previous studies have reported evidence that hermaphrodites "want" to outcross, these previous studies, combined with the current evidence, are not definitively counter to Chasnov's (2010) prediction that hermaphrodites should evolve to avoid mating with males.

Additional evidence also points toward androdioecious clam shrimp evolving to primarily self-fertilize. Weeks, Crosser, Gray, Matweyou, and Zucker (2000) showed that even when mating occurs, less than half of the eggs are actually outcrossed by the male. However, it is not known if this is from inviable male sperm (Weeks, Reed, Ott, & Scanabissi, 2009) or hermaphrodite choice. There are also some clam shrimp populations which are purely hermaphroditic (Weeks, Posgai, Cesari, & Scanabissi, 2005; Weeks et al., 2008), which could be because these populations have evolved from an androdioecious ancestor by successfully eliminating outcrossing and thus losing males. *Eulimnadia agassizii* Packard is one such species in which no males have been found (Weeks et al., 2005). In addition, there are several populations within various *Eulimnadia* species (Weeks, Sanderson et al., 2006) that have no males. In all these cases, it is possible that hermaphrodites have been selected to avoid males, although it is also possible that males may have been lost due to their poorer dispersal abilities (Pannell, 1997, 2002). Future studies of hermaphroditic mating behavior that compares hermaphrodites from androdioecious populations to those from hermaphroditic populations would shed light on these two options.

Chasnov (2010) theorized that in androdioecious systems, if the benefits of selfing are greater than the benefits of outcrossing, then androdioecious hermaphrodites should evolve away from outcrossing. One such way a hermaphrodite could evolve away from outcrossing is by becoming unrecognizable to males by losing female characteristics to evade males. Another way to evolve away from outcrossing would be to avoid males all together. Together, the two experiments in this study show androdioecious hermaphrodites evading and avoiding males at a higher rate than their dioecious close relatives. In addition, previous clam shrimp research is also consistent with androdioecious hermaphrodites evolving away from characteristics that promote outcrossing (Benvenuto & Weeks, 2011; Weeks et al., 2000; Zucker et al., 2002). This combined evidence supports Chasnov's (2010) theory.

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