Sex bias in interspecific copulation between the hermaphroditic freshwater snails, *Physa acuta* and *P. pomilia* (Physidae)

Amy R. Wethington[1], Deborah L. Kirkland[2], and Robert T. Dillon, Jr.[3]

[1]Department of Biological and Physical Sciences, Chowan University, Murfreesboro, North Carolina 27855, U.S.A.

[2]Department of Biological Sciences, University of Alabama, Box 870345, Tuscaloosa, Alabama, 35487, U.S.A.

[3]Department of Biology, College of Charleston, Charleston, South Carolina 29424, U.S.A.

Correspondence, Amy R. Wethington: wethia@chowan.edu

Abstract. Previously-published mate-choice experiments have uncovered significant prezygotic reproductive isolation between South Carolina populations of *Physa acuta* (Draparnaud, 1805) and *P. pomilia* (Conrad, 1834). Here we report the results of similar tests returning no evidence of such barriers between an Alabama population of *P. pomilia* and three separate populations of *P. acuta*, two from Alabama and one from South Carolina. However, heterogametic copulations did demonstrate both a significant sex bias and a significant size bias. The smaller *P. acuta* was more likely to copulate in the male role and hence less exposed to any potential fitness decrements due to the reduced viability and sterility of the hybrids produced. Our observations on defeated mating attempts suggested that *P. acuta* may be more aggressive than Alabama *P. pomilia* in mating contests (its smaller size perhaps contributing to greater agility) and hence more likely to prevail in the male role. The absence of a sex bias in our previously-published mate-choice observations involving South Carolina *P. pomilia* and *P. acuta* may be attributable to our use of size-matched snails, which equalized the agility of the prospective copulants, thus perhaps prolonging the mating contests, and ultimately lessening the likelihood that any mating took place at all.

Key words: Sexual isolation, reinforcement, mate-choice tests, gender conflict, pulmonate gastropods.

Speciation in animal populations is typically thought to begin with the evolution of reproductive incompatibility in allopatry, after which prezygotic reproductive isolating mechanisms may evolve, possibly reinforced by natural selection (Coyne and Orr 2004). Thus in well-studied systems it is not unusual to discover populations of biological species demonstrating incomplete prezygotic reproductive barriers, some pairs having evolved sensory or behavioral cues to reduce the frequency of interspecific copulation, and others copulating indiscriminately (e.g., *Drosophila* Gleason and Ritchie 1998, finches Grant and Grant 2002).

Physa acuta (Draparnaud, 1805) and *P. pomilia* (Conrad, 1834) are closely-related freshwater pulmonate gastropod species that separate into two monophyletic groups based on sequence data with ranges broadly overlapping throughout the southern United States (Wethington and Lydeard 2007). They differ in minor details of shell morphology, penial anatomy, and life history (Wethington *et al.* 2009). *Physa acuta* demonstrates a "weedy" life history strategy, populations reaching maximum density in more unpredictable or disturbed habitats, such as farm ponds or impoundments. *Physa pomilia* populations are more typical of stable habitats, such as the quiet backwaters of rivers or permanent streams. Hybrids between *P. acuta* and *P. pomilia* are sub-vital and sterile (Dillon *et al.* 2007). But although the mate-choice experiments of Dillon and colleagues (2007) returned evidence of

significant prezygotic reproductive barriers between South Carolina populations of *P. acuta* and *P. pomilia*, no such evidence was returned by the experiments of Dillon *et al.* (2011) matching South Carolina *P. pomilia* with a population of *P. acuta* from Pennsylvania. This suggested to Dillon and colleagues that the behavioral differences leading to assortative mating among the South Carolina populations might represent adaptations, being reinforced by natural selection.

Here we extend this line of research to Alabama, performing mate-choice tests to include an Alabama population of *Physa pomilia* and two Alabama populations of *P. acuta*, as well as our South Carolina *P. acuta*. We also expand our observations to gather data on the polarity of interspecific copulation, the relative sizes of the copulants involved, and the frequency at which copulations may be entirely defeated by rejective behaviors.

Physid snails are simultaneously hermaphroditic at adulthood, female function being added to male function around week 7–8 in laboratory culture (Wethington and Dillon 1993). They are preferential outcrossers, suffering a significant reduction in fitness if compelled to self-fertilize in isolation (Wethington and Dillon 1997, Jarne *et al.* 2000). Copulation is not reciprocal. Pairs of snails typically engage in contests to determine which individual will initially assume the male role, after which swapping may occur (Facon *et al.* 2008). By comparing snails of differing reproductive histories, Wethington

and Dillon (1996) adduced evidence that such contests are attributable both to (conventional) sexual conflict and to a phenomenon they termed "gender conflict" between simultaneous hermaphrodites vying to assume a male role.

In addition to prior reproductive history, the relative size of prospective copulants can influence the gender initially assumed by copulating freshwater pulmonate snails, the smaller partner more often mating in the male role (Ohbayashi-Hodoki *et al.* 2004, Norton *et al.* 2008). DeWitt (1991, 1996) correlated such observations with the size advantage model for simultaneous hermaphrodites (Charnov 1982, Angeloni *et al.* 2002). He documented four behaviors that *Physa* may display in their attempts to discourage copulation as a female: shell shaking, increasing the distance from the gonopore, covering the gonopore with the shell, and biting the partner's preputium.

All of our mate-choice experiments published to date have been specifically designed to test for reproductive isolation among populations of *Physa*, and hence have involved size-matched snails in an explicit effort to factor out effects such as those described by DeWitt. Few of the previous reports have included data on the directionality of any heterogametic copulations that may have been observed. Here we add these layers of observation in an effort to cast additional light on the nature of prezygotic reproductive barriers between *P. acuta* and *P. pomilia*.

METHODS AND METHODS

We collected approximately 50 adult snails from each of four natural populations, the identities of all of which were confirmed by dissection. *Physa pomilia* was sampled from the type locality of the species: Randons Creek, 12 km S of Claiborne, Monroe County, Alabama (31.4387°N, 87.5445°W). This population was previously designated "P" by Dillon *et al.* (2007), and "alpom" in the mtDNA sequence analysis of Wethington and Lydeard (2007). Our population "*acuta*-A" was sampled from Lake Palmer on the University of Alabama campus (33.2151°N, 87.5467°W), and our population "*acuta*-T" was sampled from Cypress Pond, southeast of Tuscaloosa, Alabama (33.0659°N, 87.6373°W). Our control population "*acuta*-C" was sampled from the main pond at Charles Towne Landing State Park, west of the Ashley River in Charleston, South Carolina (32.8062°N, 79.9862°W). This population has previously been designated "Ctl" by Dillon and Wethington (1995), "C" by Dillon *et al.* (2002, 2005), "A" by Dillon *et al.* (2004, 2007) and "*acuta*-C" by Dillon *et al.* (2011). Twenty 16S and CO1 mtDNA sequences from this population were published by Wethington *et al.* (2009). The habitat has been described by Dillon and Dutra-Clark (1992).

Wild-collected snails were returned to the laboratory, isolated for a minimum of seven days in 10 oz. (210 ml) clear polyethylene drinking cups of aerated, filtered pond water with Petri dish covers, and fed a commercial *Spirulina*-based flake fish food, finely ground. All cultures took place at approximately 23° C in a 12:12 light cycle. Snails demonstrating the ability to lay viable eggs served as experimental animals for our mate-choice tests.

We performed three separate mate-choice experiments to assess prezygotic reproductive isolation: *pomilia* x *acuta*-C, *pomilia* x *acuta*-T, and *pomilia* x *acuta*-A. Each experiment comprised two trials involving five adult snails per population. Since statistics to measure sexual isolation in simultaneous hermaphrodites have conventionally been calculated on the basis of the first successful copulation initiated by each snail in the male role (to avoid double-counting), the maximum number of mate-choice observations in each experiment was 20. Note that our experimental design yields a slightly unbalanced expectation, each snail being presented with five heterogametic partners but only four homogametic.

For each trial, the two groups of five snails were measured with calipers, painted with a dot of fingernail polish (either pink or silver) and numbered with an ultra-fine Sharpie pen. They were then placed together in a glass container 19 cm in diameter and 6.5 cm high, elevated on a glass plate platform. Approximately 750 ml of filtered pond water was added to the container, bringing the depth to approximately 2.7 cm. We then monitored mating activity over a period of two hours, recording every copulation attempt. When a successful copulation was observed, defined as the complete insertion of the penis of one partner into the mantle cavity of a second, both snails were identified and their gender roles recorded. Snails were not removed or disturbed during the two-hour observation period, however, and were often observed to engage in multiple mating interactions. Thus we were also able to record the total number of defeated mating attempts in each experiment--those mating interactions that did not ultimately culminate in copulation by either partner.

We tested the hypothesis of prezygotic reproductive barriers between *Physa pomilia* and each of the three *P. acuta* populations using the Index of Pair Sexual Isolation (IPSI), a statistic designed to remain unbiased should two populations demonstrate differing mating propensities (Perez-Figueroa *et al.* 2005, Carvajal-Rodríguez and Rolán-Alvarez 2006). We then tested the (one-tailed) hypothesis that smaller snails might be more likely to copulate in the male role with a chi-square goodness-of-fit test on the entire data set, combined over all three experiments. Focusing finally on the subset of copulations between heterogametic partners, we used a goodness-of-fit chi square statistic to test for evidence of bias in the gender assumed by the two species and a matched-pair t statistic to test the (one-tailed) hypothesis that the snails copulating in the male role tended to be smaller.

RESULTS

Table 1 shows our primary data sorted by experiment and the relative size of the copulants. The average size of the 30 *P. pomilia* ultimately involved in our experiment was 8.26 mm (range 6.15–11.15), as compared to 7.99 mm (range 6.00–9.50) for the 10 *acuta*-C, 7.83 mm (range 6.00–10.10) for the 10 *acuta*-A, and 5.47 mm (range 4.40–6.25) for the 10 *acuta*-T. Only 7 of the 20 snails tested in the *pomilia* x *acuta*-C experiment had copulated in the male role when the two hour time limit expired, 12 (of 20) in the *pomilia* x *acuta*-T experiment, and 15 (of 20) in the *pomilia* x *acuta*-A experiment. The 14 copulations totaled for *pomilia* x *acuta*-A in Table 1 exclude one homogametic pairing identically matched in size.

Table 1 reflects no evidence of assortative mating by population or by the relative size of the copulants. The IPSI index in the *pomilia* x *acuta*-T experiment was 0.26 ($p = 0.184$) and in the *pomilia* x *acuta*-A experiment IPSI = 0.20 ($p = 0.221$). The *pomilia* x *acuta*-C experiment returned more heterogametic copulations observed than homogametic copulations. Combining the three independent tests of the same hypothesis using the Fisher method (Sokal and Rohlf 1995: 794) yielded a nonsignificant result ($\chi^2 = 6.70$, 6 *df*). Summed over all three experiments, a test of the hypothesis that males tended to be the smaller of the two partners yielded a goodness-of-fit $\chi^2 = 2.46$, also not significant.

Focusing on the subset of 16 heterogametic copulations, however, Table 2 does demonstrate a significant bias toward the male role in *Physa acuta* ($\chi^2 = 4.00$, $p < 0.05$). And the average size of the male copulants in this subset (mean = 7.11 mm, range 4.90–9.50) was significantly smaller than the snails ultimately copulating as female (mean = 7.80 mm, range 5.65–9.65), our paired t-test returning t = 1.89 (one-tailed,

Table 2. The subset of copulations shown in Table 1 involving heterogametic partners.

	Male smaller	Male larger	Total
Male *acuta*	9	3	12
Male *pomilia*	2	2	4
Totals	11	5	16

$p = 0.039$). It would appear that smaller *acuta* tend to prevail over larger *pomilia* in interspecific gender conflicts.

We observed all of the rejective behaviors described by DeWitt (1991) during the course of these experiments, as well a variety of others. Often snails mounted as females were observed to crawl out of the water in an apparent attempt to dislodge an eager partner. Biting (or perhaps head-butting) was applied to all the exposed surfaces of prospective suitors, including the foot as well as the preputium. "Dancing," a term coined by us, was applied to situations where a pair of prospective copulants pressed their feet together and twisted about in a vigorous fashion. Such "dancing" typically seemed to result when a pair of snails nearly matched in their size encountered each other head-on, and vied to assume the upper (male) position.

The number of entirely defeated mating attempts, culminating in no copulation by either partner, exceeded the tally of successfully copulating partners in all three experiments. Table 3 shows that individual *Physa acuta* sampled from all three populations were significantly more rejective than *P. pomilia*. While only three *pomilia* x *pomilia* homogametic pairings ultimately failed to yield a successful copulation over all 12 hours of observation combined, 24 pairs of *acuta* ultimately defeated each other (Fisher's $p = 0.0012$) . This figure is comparable to the 23 *pomilia* x *acuta* heterogametic pairings that did not ultimately culminate in copulation by either partner (Fisher's $p = 0.074$).

DISCUSSION

Our previously-published observations on hybrid fitness led us to expect that some prezygotic reproductive isolation

Table 1. The distribution of initial copulations observed in mate-choice tests involving Alabama *Physa pomilia* and three populations of *P. acuta*.

	Male smaller	Male larger	Total
pomilia x *acuta*-C			
Homogametic	1	1	2
Heterogametic	4	1	5
pomilia x *acuta*-T			
Homogametic	4	3	7
Heterogametic	4	1	5
pomilia x *acuta*-A			
Homogametic	4	4	8
Heterogametic	4	2	6
Totals	21	12	33

Table 3. Defeated mating attempts (not culminating in copulation by either partner) observed in mate-choice tests involving *Physa pomilia* and three populations of *P. acuta*.

Experiment	*pomilia* x *pomilia*	*pomilia* x *acuta*	*acuta* x *acuta*
pomilia x *acuta*-C	1	4	3
pomilia x *acuta*-T	2	14	10
pomilia x *acuta*-A	0	5	11
Totals	3	23	24

might be evident between Alabama populations of *Physa pomilia* and *P. acuta*, not perhaps apparent in interactions between Alabama *pomilia* and our standard population of *P. acuta* from South Carolina (Dillon *et al.* 2007, 2011). The mate-choice experiments we report here do not, however, reflect any evidence of assortative mating between any pair of populations tested. The behavioral mechanisms that seem to have evolved to lessen the frequency of copulation between South Carolina populations of *P. acuta* and *P. pomilia* did not appear effective in our samples from Alabama.

Although our experiments are patterned closely after the "mate-choice tests" that have seen broad application to measure sexual isolation between populations of a great variety of dioecious animals, each of our observations is not a "choice" in any sense, but rather the outcome of a contest (Wethington and Dillon 1996). The victor obtains the male role. The data in Table 3 demonstrate that Alabama *pomilia* mate more often with *acuta* than with other *pomilia*. But the data in Table 2 suggest that ultimately, it is *Physa acuta* (smaller, and perhaps more agile) that tends to prevail as male more often.

The observation that males tend to be eager and indiscriminant in their mating, while females tend to be passive and choosy, has been a familiar one since the days of Darwin. Bateman (1948) suggested that the asymmetry in the energetic investments required in the production of egg and sperm should lead to greater sexual selection among males than females, a theory that was extended to hermaphrodites by Charnov (1979). The phenomena of egg trading (Crowley *et al.* 2007) and sperm trading (Michiels *et al.* 2003, Anthes *et al.* 2006) have been viewed as adaptations to resolve the (male-female) sexual conflicts in simultaneous hermaphrodites, and direct contests such as those observable in freshwater pulmonates typically ascribed to (male-male) gender conflict (Facon *et al.* 2007, 2008).

In dioecious animals, one well-documented basis for female choosiness is the suite of mating cues offered by courting males (e.g., appearances, behaviors, chemicals), with selection acting to refine the female's ability to recognize mates yielding maximum fitness for her offspring (Milam 2010). This is the theoretical basis of mate choice tests (Bateson 1983). Although such selective forces also certainly apply to simultaneous hermaphrodites (Pelissie *et al.* 2012), it seems possible that in the 16 cases documented in Table 2, eagerness for victory in a (male-male) gender conflict has led to defeat in a (male-female) sexual conflict.

In direct contrast to the results reported here, the mate-choice experiments involving South Carolina populations we reported in 2007 did return evidence of reproductive isolation between *pomilia* and *acuta* while reflecting no sex bias in heterogametic pairings. Only 11 of the 41 copulations logged by Dillon and colleagues (2007) were heterogametic, five with

pomilia as male and six with *acuta* as male, not statistically different from 1:1. But the snails we selected for our earlier experiments were size-matched as closely as possible, in an explicit effort to factor out size-related differences in aggression or agility. In equalizing the body sizes of all prospective copulants, we almost certainly extended the contests among heterogametic partners, ultimately lessening the likelihood that copulation took place in either direction at all.

LITERATURE CITED

Angeloni, L., J. W. Bradbury, and E. L. Charnov. 2002. Body size and sex allocation in simultaneously hermaphroditic animals. *Behavioral Ecology* **13**: 419–426.

Anthes, N., A. Putz, and N. K. Michiels. 2006. Sex role preferences, gender conflict and sperm trading in simultaneous hermaphrodites: A new framework. *Animal Behaviour* **72**: 1–12.

Bateman, A. 1948. Intra-sexual selection in *Drosophila. Heredity* **2**: 349–368.

Bateson, P. 1983. *Mate Choice*. Cambridge University Press, Cambridge United Kindgdom.

Carvajal-Rodríguez, A. and E. Rolán-Alvarez. 2006. JMATING: A software for the analysis of sexual selection and sexual isolation effects from mating frequency data. *BMC Evolutionary Biology* **6**: 40.

Charnov, E. L. 1982. *The Theory of Sex Allocation*. Princeton University Press, Princeton, New Jersey.

Coyne, J. and H. Orr. 2004. *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Crowley, P. H. and M. K. Hart. 2007. Evolutionary stability of egg trading and parceling in simultaneous hermaphrodites: The chalk bass revisited. *Journal of Theoretical Biology* **246**: 420–429.

DeWitt, T. J. 1991. Mating behavior of the freshwater pulmonate snail, *Physa gyrina. American Malacological Bulletin* **9**: 81–84.

DeWitt, T. J. 1996. Gender contests in a simultaneous hermaphrodite snail: A size-advantage model for behavior. *Animal Behaviour* **51**: 345–351.

Dillon, R. T. and A. V. C. Dutra-Clark. 1992. *Biomphalaria* in South Carolina. *Malacological Review* **25**: 129–130.

Dillon, R. T., Jr., C. Earnhardt, and T. Smith. 2004. Reproductive isolation between *Physa acuta* and *Physa gyrina* in joint culture. *American Malacological Bulletin* **19**: 63–68.

Dillon, R. T., Jr., J. D. Robinson, T. P. Smith, and A. R. Wethington. 2005. No reproductive isolation between freshwater pulmonate snails *Physa virgata* and *P. acuta*. *Southwestern Naturalist* **50**: 415–422.

Dillon, R. T., Jr., J. D. Robinson, and A. R. Wethington. 2007. Empirical estimates of reproductive isolation among the freshwater pulmonate snails *Physa acuta, P. pomilia*, and *P. hendersoni*. *Malacologia* **49**: 283–292.

Dillon, R. T., and A. R. Wethington. 1995. The biogeography of sea islands: clues from the population genetics of the freshwater snail, *Physa heterostropha*. *Systematic Biology* **44**: 401–409.

Dillon, R. T., Jr., A. R. Wethington, and C. Lydeard. 2011. The evolution of reproductive isolation in a simultaneous hermaphrodite, the freshwater snail *Physa*. *BMC Evolutionary Biology* **11**: 144.

Dillon, R. T., Jr., A. R. Wethington, J. M. Rhett, and T. P. Smith. 2002. Populations of the European freshwater pulmonate *Physa acuta* are not reproductively isolated from American *Physa heterostropha* or *Physa integra*. *Invertebrate Biology* **121**: 226–234.

Facon, B., V. Ravigne, and J. Goudet. 2008. Gender-role alternation in the simultaneously hermaphroditic freshwater snail *Physa acuta*: Not with the same partner. *Behavioral Ecology and Sociobiology* **62**: 713–720.

Facon, B., V. Ravigne, L. Sauteur, and J. Goudet. 2007. Effect of mating history on gender preference in the hermaphroditic snail *Physa acuta*. *Animal Behaviour* **74**: 1455–1461.

Gleason, J. and M. Ritchie. 1998. Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex: Do sexual signals diverge the most quickly? *Evolution* **52**: 1493–1500.

Grant, B. R. and P. R. Grant. 2002. Lack of premating isolation at the base of a phylogenetic tree. *The American Naturalist* **160**: 1–19.

Jarne P., M.-A. Perdieu, A.-F. Pernot, B. Delay, and P. David. 2000. The influence of self-fertilization and grouping on fitness attributes in the freshwater snail *Physa acuta*: population and individual inbreeding depression. *Journal of Evolutionary Biology* **13**: 645–655.

Michiels, N. K, A. Raven-Yoo-Heufes, and K. K. Brockmann. 2003. Sperm trading and sex roles in the hermaphroditic opisthobranch sea slug *Navanax inermis*: Eager females or opportunistic males? *Biological Journal of the Linnean Society* **78**: 105–116.

Milam, E. L. 2010. *Looking for a Few Good Males: Female Choice in Evolutionary Biology*. Johns Hopkins University Press, Baltimore, Maryland.

Norton, C. G., A. F. Johnson, and R. L. Mueller. 2008. Relative size influences gender role in the freshwater hermaphroditic snail, *Helisoma trivolvis*. *Behavioral Ecology* **19**: 1122–1127.

Ohbayashi-Hodoki, K., F. Ishihama, and M. Shimada. 2004. Body-size dependent gender role in a simultaneous hermaphrodite freshwater snail, *Physa acuta*. *Behavioral Ecology* **15**: 976–981.

Pelissie, B., P. Jarne, and P. David. 2012. Sexual selection without sexual dimorphism: Bateman gradients in a simultaneous hermaphrodite. *Evolution* **66**: 66–81.

Perez-Figueroa, A., A. Caballero, and E. Rolán-Alvarez. 2005. Comparing the estimation properties of different statistics for measuring sexual isolation from mating frequencies. *Biological Journal of the Linnean Society* **85**: 307–318.

Sokal, R. R. and F. J. Rohlf. 1995. *Biometry, Third Edition*. W. H. Freeman, New York.

Wethington, A. R., and R. T. Dillon, Jr. 1993. Reproductive development in the hermaphroditic freshwater snail, *Physa*, monitored with complementing albino lines. *Proceedings of the Royal Society (London) B* **252**: 109–114.

Wethington, A. R. and R. T. Dillon, Jr. 1996. Gender choice and gender conflict in a non-reciprocally mating simultaneous hermaphrodite, the freshwater snail, *Physa*. *Animal Behaviour* **51**: 1107–1118.

Wethington, A. R., and R. T. Dillon, Jr. 1997. Selfing, outcrossing, and mixed mating in the freshwater snail *Physa heterostropha*: Lifetime fitness and inbreeding depression. *Invertebrate Biology* **116**: 192–199.

Wethington, A. R. and C. Lydeard. 2007. A molecular phylogeny of Physidae (Gastropoda: Basommatophora) based on mitochondrial DNA sequences. *Journal of Molluscan Studies* **73**: 241–257.

Wethington, A. R., J. Wise, and R. T. Dillon, Jr. 2009. Genetic and morphological characterization of the Physidae of South Carolina (Gastropoda: Pulmonata: Basommatophora), with description of a new species. *The Nautilus* **124**: 282–292.