Reproductive isolation between *Physa acuta* and *Physa gyrina* in joint culture*

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Abstract: Recent laboratory tests of postzygotic reproductive isolation in physid snails, although providing fresh insight into the evolution of an important model organism, have focused on reproductively compatible populations of *Physa acuta*. Here we extend such studies to include a population of *Physa gyrina* known to be incompatible with *P. acuta*. Reared in pairs, the median age of first reproduction in a laboratory population of *P. acuta* originating from Charleston, South Carolina, USA was nine weeks. Over the next ten weeks of reproduction, the laboratory population of *P. acuta* posted a mean fecundity of 61.9 embryos per pair per week, with a mean F_1 viability of 63% and 100% F_1 fertility. Individual *P. acuta* reproduced by self-fertilization when reared with *P. gyrina* in no-choice mating experiments. Their median age at first reproduction was delayed to 10.5 weeks, their fecundity was 36.4 embryos per parent per week, and F_1 viability reduced to 26%. These figures were not significantly different from the reproductive success of individual *P. acuta* self-fertilizing in isolation (median 11 weeks at first reproduction, 37.4 embryos per parent per week, 37% hatchling viability, 88% F_1 fertility). Laboratory populations of *P. gyrina* originating from Hot Springs, Virginia, USA, were not as well adapted to our culture conditions as *P. acuta*. Pairs did not initiate egg laying until a median age of 11.5 weeks, after which their mean fecundity was only 21.2 per pair per week over ten weeks, with an F_1 viability of 33.5% and 100% F_1 fertility. When reared with *P. acuta* in joint culture, individual *P. gyrina* did not reproduce successfully. Thus the effects of joint culture with *P. gyrina* were negligible for *P. acuta* but ruinous for *P. gyrina* reared with *P. acuta*. These results have important implications for the interpretation of experiments involving postmating reproductive isolation with no-choice design.

Key words: *Physa*, Basommatophora, Pulmonata, mating, speciation

Freshwater pulmonates of the family Physidae may simultaneously be counted among America's best-known and least-known gastropods. Their adaptability to laboratory culture has led to great strides in our understanding of genetics (Dillon and Wethington 1992, 1994, Monsutti and Perrin 1999), morphology (DeWitt *et al.* 1999), life history (Rollo and Hawryluk 1988, Crowl and Covich 1990, McCollum *et al.* 1998), ecology (Brown *et al.* 1994, Turner *et al.* 2000, Bernot and Turner 2001), reproductive biology (Jarne *et al.* 2000, Wethington and Dillon 1993, 1997), and behavior (Covich *et al.* 1994, Wethington and Dillon 1996, DeWitt 1996, Turner *et al.* 1999, McCarthy and Fisher 2000). For a review see Dillon (2000). Yet their genetic diversity and phenotypic plasticity has resulted in confusion regarding the specific identity of even the most widespread American physid taxa.

Recently we have initiated a program of laboratory breeding experimentation designed to assess reproductive isolation among a variety of physid populations worldwide. We have established that two of the nominal species most common in North America, *Physa heterostropha* (Say, 1817) and *Physa integra* (Haldeman, 1841), are conspecific with European populations of *Physa acuta* (Draparnaud, 1805) (Dillon *et al.* 2002). Our no-choice mating experiments yielded no evidence of delay in maturity or reduction in fecundity, F_1 viability, or F_1 fertility among hybrids of six populations (two of each species) below incross controls. Experimental crosses such as these will, however, be more reliably interpreted given the benefit of a "negative control." Thus the purpose of the present experiment was to document the reproductive activity of pairs of physids known to be reproductively isolated when cultured in a no-choice design.

Physa acuta is a member of the subgenus *Costatella* Dall, 1870 (Burch and Tottenham 1980), characterized by a two-part penial sheath. *Physa gyrina* (Say, 1821), a member of the subgenus *Physa* (*s.s.*), has a three-part penial sheath. The animals are similar in their overall morphology; *P. gyrina* maturing at a slightly larger size and bearing a more rounded shell with more convex apical whorls. Individuals of the two species will copulate, although our preliminary observations have suggested that the only progeny born are the products of self-fertilization, rather than hybridization (Wethington *et al.* 2000).

The most likely outcome of jointly culturing a pair of non-hybridizing physids in a no-choice design would seem

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to be reproduction below that posted by control pairs of either species. Self-fertilization, which would be the only reproductive option expected in this experimental situation, is known to engender delayed age at first reproduction, reduced fecundity, and reduced hatchling viability in physids generally (Jarne *et al.* 1993, 2000, Wethington and Dillon 1997). Moreover, a pair of mismatched *Physa* Draparnaud, 1801 might be expected to compete with each other for food and other resources, and perhaps interfere with the self-fertilization process through false copulation, yielding a reduction in fecundity below even control individuals self-fertilizing in isolation.

It is also possible, however, that self-fertilization in one or both individuals might be "socially facilitated" by a second snail present in joint culture, even if not conspecific. Vernon (1995) observed that, although self-fertilization reduces reproductive success in the planorbid *Biomphalaria glabrata* (Say, 1818), the reproductive output of snails reared in pairs but prevented from cross fertilizing by a nylon mesh barrier may approach that of outcrossing pairs. The ordinarily self-fertilizing terrestrial pulmonate *Balea perversa* (Linné, 1758) enjoys increased longevity and reproductive success when cultured with a partner, even though paired snails do not copulate (Baur and Baur 2000). Such social facilitation might also occur between snails as similar as *Physa acuta* and *Physa gyrina*.

Our investigation thus included four treatments: an experiment and three controls. The reproductive success of the *acuta* × *gyrina* experiment was compared to *acuta* × *acuta* controls, *gyrina* × *gyrina* controls, and a self-fertilizing control of *Physa acuta* reared in isolation. This design allowed us to identify social facilitation even in the reduced reproductive output expected from a pair of non-hybridizing species.

METHODS

Our population of *Physa acuta* was collected at Charles Towne Landing State Park (32°49′N, 79°59′W), west of the Ashley River within the city limits of Charleston, South Carolina. Animals from this population (previously identified as *Physa heterostropha* [Say, 1817]) have been the subject of many of our past studies on the genetics (Dillon and Wethington 1994, 1995) and reproductive biology (Wethington and Dillon 1991, 1993, 1997) of the genus *Physa*. Our population of *Physa gyrina* was collected in the town of Hot Springs, Virginia, approximately 100 meters downstream from the origin of naturally-heated waters inside "The Homestead" resort (38°36′N, 79°30′W). This is the type locality of *Physa aurea* (Lea, 1838), now recognized as a subspecies of *P. gyrina* (Burch and Tottenham 1980).

Our standard culture vessel was a transparent polyethylene 295.73 ml (10 US oz.) drinking cup, which we filled with approximately 210 ml of aerated, filtered pond water and covered with a 95 × 15 mm polystyrene Petri dish lid. The food was O. S. I. *Spirulina* Aquarium Flake Food, sold in pet stores primarily as a diet for herbivorous aquarium fishes. All experiments took place at room temperature, approximately 23°C.

We isolated ten wild-collected snails from each of the two study populations in separate cups, collected egg masses, and reared the offspring to 3 mm shell length, approximately three weeks post-hatching, with weekly water change. These two sets of ten wild-conceived but laboratory-born sibships (A1-A10 and G1-G10) constituted the P generation for the four treatments (one experiment and three controls) we report here.

Each treatment was composed of ten replicates. Control A was a set of ten pairs of unrelated *Physa acuta* (A1 × A2, A2 × A3, . . . , A10 × A1). Control G was similarly constituted for *Physa gyrina* (G1 × G2, G2 × G3, . . .). The AG experiment was a set of ten cups of *P. acuta* paired with *P. gyrina* (AG1, AG2, . . . , AG10). The As control was a set of ten cups with isolated *P. acuta* snails (A1, A2, . . . , A10).

Each replicate received a water change and fresh food every seven days, at which time the sides of the cup were inspected for egg masses. If egg masses were present, we counted all embryos and transferred the adults to a fresh cup. Eggs were monitored until hatching, generally about two weeks, and all viable, crawling F_1 juveniles were counted. Replicates were terminated upon the mortality of either adult individual. For statistical analysis of fecundity (egg production) and F_1 viability (hatching success), week 1 was set separately for each treatment as the first week in which eggs were laid in three or more replicates. Fecundity and F_1 viability were subsequently recorded for ten weeks.

The central tendency of age at first reproduction was compared among the A, AG, and G treatments by dividing at the pooled median and testing the resulting 3 × 2 contingency table using chi-square. The AG experiment was compared to the As control in age at first reproduction using a similar median-based approach, although a Fisher's exact test was employed rather than chi-square, because of the former test's improved power. We compared the fecundity of the A, AG, and G treatments using two-way analysis of variance, with week and treatment the independent variables and embryos as the dependent variable (Statistica release 5.5, StatSoft 1994). Overall (ten-week) F_1 viability was compared among these treatments using analysis of covariance, with treatment the independent variable, viable hatchlings the dependent variable, and embryos the covariate. *Post hoc* tests were performed using Tukey's "highly significant difference" (HSD) tests for unequal sample sizes (Spjotvoll and Stoline 1973). A second ANOVA and a second ANCOVA were used

to compare the fecundity and F_1 viability of treatment A to treatment As. Leading (pre-maturity) zeros were not included in any ANOVA or ANCOVA, nor were post-mortem zeros included, although internal zeros (i.e., reproductive failure by mature, apparently healthy snails) were analyzed.

To assess F_1 fertility in the AG, A , and G treatments, F_1 hatchlings were reared from each of three separate unrelated replicates to size 3 mm (AG1, AG2, AG3, A12, A34, A56, G12, G34, G56). These were paired across replicates within treatment in time series—one early pair from eggs laid around week 1, one middle pair produced around week 5, and one late pair produced around week 10. Each treatment thus yielded nine F_1 pairs. For example, treatment A yielded: A12 × A34 early, A12 × A34 middle, A12 × A34 late, A12 × A56 early, . . . , A34 × A56 late. Nine pairs were likewise constituted for treatments G and AG, and the total of 27 pairs of F_1 snails were reared to adulthood with weekly feeding and water change. We recorded the date at which embryos and viable F_2 hatchlings were produced by each pair.

A larger sample of 56 F_1 progeny from the AG1, AG2, and AG3 treatments was reared to 4-5 mm shell length, at which time they were frozen in 100/µl of tissue buffer for analysis by protein electrophoresis. Populations of *Physa acuta* and *Physa gyrina* share no alleles at six of the seven polymorphic allozyme loci routinely surveyed in our laboratory, including 6-phosphogluconic acid dehydrogenase (6Pgd) and isocitrate dehydrogenase (Isdh). We used horizontal starch gel electrophoresis in an aminopropylmorpholine pH 6 buffer system (Clayton and Tretiak 1972) to assess the allozyme phenotype of all putative F_1 hybrids at these two loci. Details regarding our electrophoretic methods, including a description of our equipment and recipes for stains and buffers, have been previously published (Dillon 1992, Dillon and Wethington 1995).

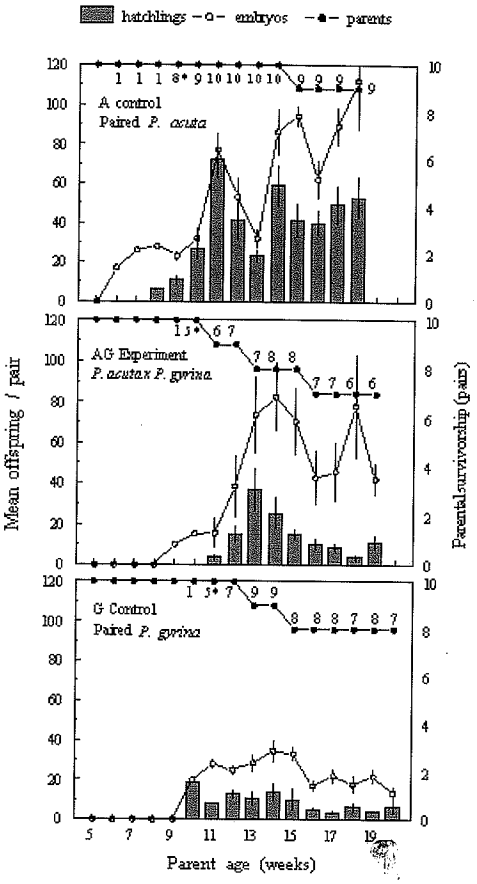

Figure 1. Production of embryos and viable hatchlings as a function of parental age (weeks post hatching) for ten pairs of *Physa acuta* (A control), ten pairs of *Physa gyrina* (G control) and ten pairs of *P. acuta* × *P. gyrina* (AG Experiment). The bars are standard errors of the mean. The number of reproducing pairs is given with parental survivorship (right axis). Asterisks* denote week 1 for analysis of variance.

RESULTS

Data on the production of F_1 progeny by the 10 pairs of parents in the A control, the G control, and the AG experiment are compared in Figure 1. The first pair of A parents laid eggs in week 6, although the median age at first reproduction was 9 weeks. Setting the ninth week of the treatment as week 1 for the purpose of analysis, over 10 weeks the mean weekly production of embryos was 61.9 per pair, and for hatchlings 39.1 per pair (63.0% viability). All 9 pairs of F_1 progeny successfully reproduced, laying eggs at a median age of 8 weeks that hatched to viable F_2 progeny a median of 2 weeks later.

The reproductive success of our G control of *Physa gyrina* was not as great as typically posted by *Physa acuta* under our culture conditions. Egg laying commenced at week 10 and reached a median between weeks 11 and 12 of the treatment. Setting week 11 to start, the mean fecundity over 10 weeks was only 21.2 embryos per pair per week, and the mean weekly F_1 hatchling yield was only 7.2 (33.5% viability). One F_1 pair was terminated by mortality, but the remaining 8 pairs laid viable eggs at a median age of 8 weeks that hatched to viable F_2 progeny at week 10.

Reproductive success in the AG experiment was generally intermediate between the A control and the G control. Egg laying began at week 9 and reached a median between weeks 10 and 11, yielding a mean fecundity of 36.4 per pair per week over 10 weeks. Hatchling production averaged only 9.3 per pair per week, for a 25.6% F_1 survival rate over that period. One pair of F_1 snails retained for testing ultimately proved sterile, but the remaining 8 pairs reproduced on the

same schedule as the A and G controls: egg laying at a median of 8 weeks and viable F_2 hatchlings at week 10.

Our comparison of age at first reproduction in treatments A, AG, and G (Fig. 2) revealed a significant difference in central tendency ($\chi^2 = 9.02$, $p = 0.011$). Seven of the pairs of *Physa acuta* in control A reproduced before any of the pairs of *P. gyrina* in control G, with the AG experiment intermediate. Analysis of variance also uncovered a significant difference in fecundity (Table 1), HSD *post hoc* tests confirming that the A control produced significantly more embryos than the AG experiment ($p = 0.0149$), which yielded more embryos than the G control ($p = 0.0001$). Analysis of covariance (Fig. 3) returned a significant difference between treatments ($F = 16.8$, $p = 0.000$). HSD *post hoc* tests showed that the 63% F_1 viability posted by the A control was significantly greater ($p = 0.0001$) than either the 33% of the G control or the 26% of the AG experiment, which did not differ ($p = 0.8196$).

Protein electrophoretic analysis of F_1 progeny from the AG experiment revealed all viable offspring to be the products of self-fertilization by the *Physa acuta* parent. The 56 offspring we examined were distributed evenly across the ten weeks of reproduction in three replicates and were entirely homozygous for the *P. acuta* markers 6pgd100 and Isdh100 (Dillon and Wethington 1995). No hybrid progeny were recovered, nor did the *Physa gyrina* parent apparently reproduce successfully by self-fertilization.

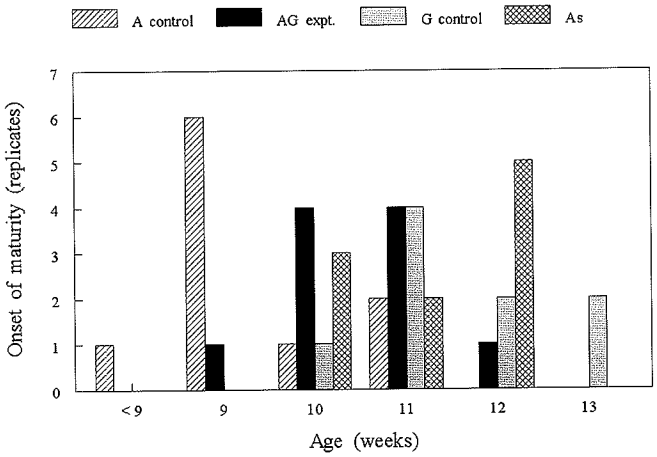

Figure 2. The number (of 10) replicates laying their first eggs as a function of parental age (weeks post-hatching) for pairs of *Physa acuta* (A control), pairs of *Physa gyrina* (G control), isolated individual *P. acuta* (As) and the *P. acuta* × *P. gyrina* experiment (AG).

Table 1. Results of analysis of variance comparing the fecundities measured over 10 weeks in the A control, the G control, and the AG experiment.

Effect	df effect	MS effect	df error	MS error	F	p-level
treatment	2	36,399	211	1,301	27.97	0.000
week	9	3,791	211	1,301	2.91	0.003
t × w	18	4,266	211	1,301	3.28	0.000

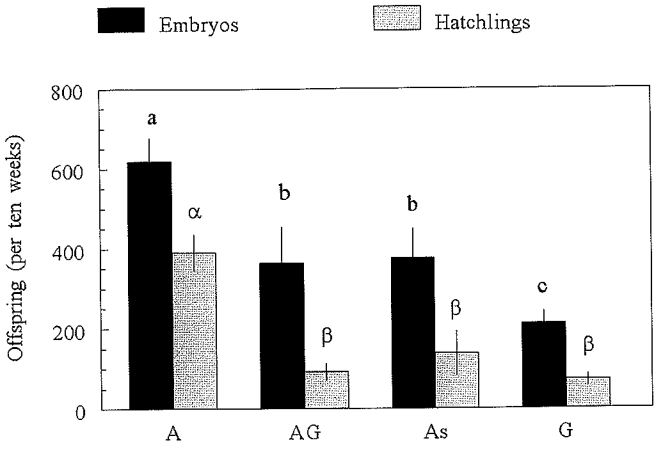

Figure 3. Total ten-week fecundity and yield of viable hatchlings (mean ± sem) for pairs of *Physa acuta* (A), pairs of *Physa gyrina* (G), the *P. acuta* × *P. gyrina* experiment (AG), and isolated individual *P. acuta* (As). Values significantly different by post hoc tests are designated with different lower case letters in Arabic for embryos or Greek for hatchlings.

Reproduction in the ten individual *Physa acuta* isolated for the As control is shown in Figure 4. Egg laying commenced at week 10 and reached a median at week 11. Taking week 10 as a start, the mean fecundity over ten weeks was 37.4 embryos per parent per week, yielding a weekly average of 13.9 hatchlings per parent for a 36.9% F_1 viability. Comparison of the AG experiment to the As control showed no difference in age at first reproduction (Fisher's exact probability = 0.65), fecundity ($p = 0.6795$, Table 2), or hatchling viability ($F = 0.82$, $p = 0.38$, Fig. 3).

DISCUSSION

The reproductive success we recorded for pairs of *Physa acuta* in these experiments (a median age of 9 weeks at first reproduction and 61.9 embryos per pair per week, 63% viability) was similar to that recorded for the Charleston population by Dillon *et al.* (2002). The present figures are lower than those reported for Charleston *P. acuta* by Wethington and Dillon (1997), but the 1997 experiments involved mated singletons (rather than pairs) and took place over the lifetime of the animals, rather than ten weeks.

Apparently our Virginia population of *Physa gyrina* is not as well-adapted to standard culture conditions as is

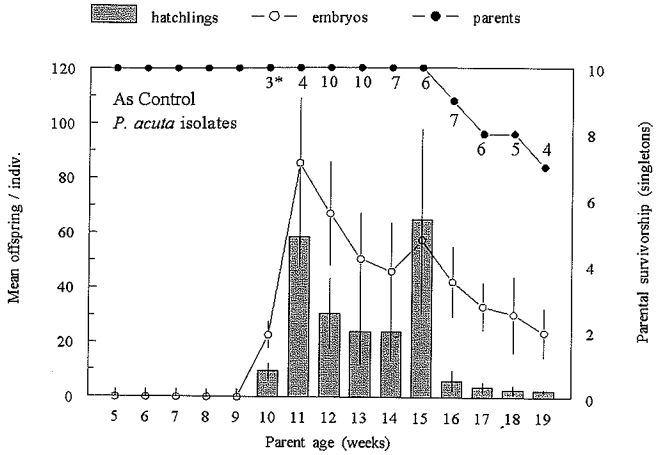

Figure 4. Production of embryos and viable hatchlings as a function of parental age (weeks post hatching) for ten individual *Physa acuta* reared in isolation (As control). The bars are standard errors of the mean. The number of reproducing individuals is given with parental survivorship (right axis). Asterisk* denotes week 1 for analysis of variance.

Table 2. Results of analysis of variance comparing the fecundities measured over 10 weeks in the As control and the AG experiment.

Effect	df effect	MS effect	df error	MS error	F	p-level
treatment	1	475	132	2,771	0.17	0.68
week	9	2,922	132	2,771	1.05	0.40
t × w	9	3,942	132	2,771	1.42	0.18

Physa acuta. Its reproductive record, which began at a median age of 11 weeks and featured only 21.2 embryos per pair per week with a 33.5% viability, was significantly below A control *P. acuta*. Indeed, the fecundity and F_1 viability posted by outcrossing pairs of *P. gyrina* was even below figures posted by our As self-fertilizing *P. acuta*.

Although copulation has been observed between *Physa acuta* and *Physa gyrina*, our results suggest that postmating reproductive isolation between the two species is complete. We cannot rule out the possibility that subviable F_1 hybrids (and pure *P. gyrina* as well) may have been born but were unable to compete with contemporaneous cohorts of pure *P. acuta*. In any case, all the progeny we recovered from ten weeks of joint culture were the products of self-fertilization by the *P. acuta* parent.

As has been previously reported (Wethington and Dillon 1997), individual *Physa acuta* isolated in our As control and forced to self-fertilize displayed delayed age at first reproduction (median age 11 weeks) and much-reduced F_1 viability (36.9%). There was no significant difference in the reproduction of As isolates and the self-fertilizing *P. acuta* cultured jointly with *Physa gyrina* (10.5 weeks, 36.4 embryos/week, 25.6% viability). Apparently, joint culture has neither a positive nor a negative effect on *P. acuta*. Culture with an individual *P. acuta* seems to be quite deleterious for *P. gyrina*, however, effectively shutting down whatever (relatively low) reproduction it would have otherwise achieved.

The results of this investigation offer no evidence of social facilitation between the two species of *Physa*. They do, however, contain an important cautionary message for future studies of postmating reproductive isolation in laboratory cultures of pulmonates. By none of the measures of fitness we employed here—age at first reproduction, fecundity, F_1 viability, and F_1 fertility—were the results of the AG experiment significantly depressed below either of the two controls. Our finding that the survivorship of the outcrossed F_1 progeny of *Physa gyrina* from the G control was not significantly greater than that of the selfed progeny of *Physa acuta* from the AG experiment was especially surprising. Had we not examined the allozyme phenotypes of the F_1 progeny via protein electrophoresis, and discovered that no hybrids were being produced, the reproductive isolation displayed between species as different as *P. acuta* and *P. gyrina* might have been missed entirely.

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