

NO REPRODUCTIVE ISOLATION BETWEEN FRESHWATER PULMONATE
SNAILS *PHYSA VIRGATA* AND *P. ACUTA*ROBERT T. DILLON, JR.,* JOHN D. ROBINSON, THOMAS P. SMITH, AND
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ABSTRACT—Mate choice tests provided no evidence of prezygotic reproductive isolation between a population of *Physa virgata* (Gould, 1855) collected from its type locality in the Gila River of Arizona and *Physa acuta* (Draparnaud, 1805) from a control site in Charleston, South Carolina. Reared in a no-choice experimental design, 10 outcross Arizona \times South Carolina pairs initiated reproduction at approximately the same age as Arizona \times Arizona controls, and earlier than South Carolina \times South Carolina controls. Parents in the outcross experiment did not differ significantly from either control in their median weekly fecundity across 10 weeks of observation, yielding an F₁ generation with significantly improved viability. We detected no evidence of reduction in F₁ fertility. Thus, *P. virgata*, the most widespread freshwater gastropod of the American Southwest, should be considered a junior synonym of the cosmopolitan *P. acuta*.

RESUMEN—Pruebas de la elección de pareja no proveyeron ninguna evidencia de aislamiento reproductivo precigótico entre una población de *Physa virgata* (Gould, 1855) colectada en su tierra típica del Río Gila de Arizona y *Physa acuta* (Draparnaud, 1805) de un lugar control en Charleston, South Carolina. Cultivados en un diseño experimental de “no-elección,” 10 parejas exogámicas de Arizona \times South Carolina iniciaron reproducción a aproximadamente la misma edad que los controles de Arizona \times Arizona y antes que los controles de South Carolina \times South Carolina. A través de 10 semanas de observaciones, los padres del experimento exogámico no se diferenciaron significativamente de cualquier control en la mediana de la fecundidad semanal, produciendo una generación F₁ con viabilidad significativamente mejorada. No detectamos ninguna evidencia de la reducción en la fertilidad F₁. Como resultado *P. virgata*, el gastrópodo de agua dulce más extendido en el suroeste de América del Norte, se debe considerar como el sinónimo menor de la cosmopolita *P. acuta*.

Pulmonate gastropods of the family Physidae are the most abundant and widespread snails of American freshwaters (Burch, 1982). Modern reviews generally recognize approximately 40 species in United States, with another 40 subspecies and forms, organized into genera and subgenera by penial morphology (Te, 1978; Burch and Tottenham, 1980; Turgeon et al., 1998). Recently, however, it has become apparent that the number of biological species in the Physidae has been overestimated. Dillon et al. (2002) could detect no reproductive isolation among the 3 most common members of the subgenus *Costatella* worldwide: *Physa acuta* (described from France), *P. heterostropha* (from the American South and East) and *P. integra* (from the American Midwest), suggesting that

the latter 2 nomina should be considered junior synonyms of the former. Dillon and Wethington (2004) obtained similar results for 5 species of the subgenus *Physella*, including the widespread American *Physa gyrina* and *P. ancillaria*. Reproductive isolation seems, however, to be complete between physids of different subgenera (Dillon et al., 2004). Based on these results, as well as on an extensive survey of mitochondrial DNA sequence divergence, Wethington (2003) reduced the number of North American physid species to around 10, and the number of higher taxa to 2 (Wethington and Guralnick, 2004; Wethington and Lydeard, in press).

Among the nominal species proposed for synonymy under *P. acuta* by Wethington (2003)

was the most widespread physid of the American Southwest, *Physa (Costatella) virgata* (Gould, 1855). There have been several thorough studies of the population biology of *P. virgata* (Crowl and Schnell, 1990; Brown et al., 1994), including investigations regarding its resistance to heat and drought (Crowl, 1990; Stanley et al., 1994) and population grazing effects (Carman and Guckert, 1994; King-Lotufu et al., 2002). Substantial literature has focused on interactions between *P. virgata* and its many predators (Crowl and Alexander, 1989; Alexander and Covich, 1991; Covich et al., 1994; Moser and Willis, 1994; Langerhans and DeWitt, 2002). Laboratory populations are used as models in parasitological experiments (Weir and Sullivan, 1989; Sullivan et al., 1995; Orta and Sullivan, 2000). *Physa virgata* also has served as a model organism for the study of thermal pollution (McMahon, 1985). A general review of the biology of the Physidae can be found in Dillon (2000:66–70).

Physa virgata was originally described by A. Gould (1855) from the Gila River. It bears a penial complex indistinguishable from the cosmopolitan *P. acuta* and is not distinct from *P. acuta* in DNA sequence for the 2 mitochondrial genes examined by Wethington (2003). The only unique characteristic of *P. virgata*, the presence of “variegated coloration” on its shell, has not been considered diagnostic by subsequent authors (e.g., Bequaert and Miller, 1973; Wu and Beetle, 1995).

The purpose of this paper is to report a series of laboratory experiments assessing whether a *P. virgata* population, collected from its type locality, is specifically distinct from a control population of *P. acuta* from South Carolina. We used mate choice tests to prospect for evidence of prezygotic reproductive isolation and tested for postzygotic isolation by comparing the reproductive output of incross controls to outcross experiments paired in a no-choice design.

Mate choice tests have been used to evaluate prezygotic reproductive isolation in *Drosophila* for many years (Coyne and Orr, 1989, 1997), and a large literature has accumulated (Bateson, 1983). Such tests have previously been used to examine specific relationships in the freshwater pulmonate snail *Biomphalaria* (Parasense, 1956), land snails and slugs (Murray and Clarke, 1968; Baur and Baur, 1992), and

the intertidal prosobranch snail *Littorina* (Pickles and Grahame, 1999; Rolan-Alvarez et al., 1999). The use of no-choice tests to evaluate postzygotic reproductive isolation also was pioneered with fruit flies (Wu and Palopoli, 1994) and has been widely applied to other taxa (e.g., Sasa et al., 1998; Price and Bouvier, 2002). Such tests have previously been used to explore specific relationships in the freshwater pulmonate snail *Bulinus* (Wu, 1973) and in the marine prosobranch *Lacuna* (Langan-Cranford and Pearse, 1995). Coyne and Orr (2004) offered a general review of experimental approaches to species determination.

METHODS—In May 2002, we collected a sample of *P. acuta* from the pond at Charles Towne Landing State Park, within the city limits of Charleston, South Carolina (32.8062°N, 79.9862°W). This is our standard population C, from which many of the laboratory lines previously involved in our studies of the reproductive biology of *Physa* have originated, including our mating experiments with near-topotypic *P. acuta* from France (Wethington and Dillon, 1991, 1993, 1996, 1997; Dillon et al., 2002; in press).

Gould (1855) provided no more specific type locality data for *P. virgata* beyond “in the River Gila.” The Gila River is today intermittent through most of southern Arizona, and we sampled several localities south of Phoenix without success in May 2002. We ultimately discovered a large and apparently healthy population of *Physa* in San Carlos Reservoir at Coolidge Dam, Gila County, Arizona (33.1840°N, 110.5233°W).

Our standard culture vessel was a transparent polyethylene 10-ounce drinking cup, filled with approximately 210 mL of aerated, filtered pond water, and covered with a 95-mm × 15-mm polystyrene Petri-dish lid. The food was O.S.I. *Spirulina* Aquarium Flake Food (Ocean Star International, Hayward, California), sold in pet stores primarily as a diet for herbivorous aquarium fishes. All experiments took place at approximately 23°C. We initially isolated 10 wild-collected snails from each study population in separate cups, collected egg masses with weekly water change, and reared the offspring to 2-mm shell length, approximately 3 weeks post-hatching (well in advance of maturity). These unrelated sets of wild-conceived but laboratory-born sibships were designated A1 through A10 and C1 through C10. From them we took isolates for the mate choice tests and pairs of parents for the study of postzygotic reproductive isolation.

For mate choice tests, large samples of juvenile snails from both populations were reared to maturity over the course of 8 to 10 weeks isolated in individual cups, with weekly feeding and water change. We

performed 3 trials, each involving 10 adult-A snails and 10 adult-C snails of approximately the same shell sizes. Snails were blotted dry and marked with a small dab of fingernail polish according to the population of origin. Then all 20 individuals were simultaneously introduced into a 2-L glass beaker (filled with 1,400 mL of filtered, aerated pond water) and placed on a glass table to facilitate observation.

We monitored mating activity for 6 hours. When a snail first successfully copulated as male (defined as the complete insertion of its penis into the gonopore of a partner), it was removed from the beaker, its shell marked with a dot of white correction fluid, and returned. Each individual was often involved in many matings over the 6 hours of observation, both in the male and in the female role, but only its first successful copulation in the male role was recorded. Note that this design yields a slight bias toward heterogametic pairings, not 1:1 but rather 9:10.

Each of the 3 trials involved 20 fresh, unmated snails. We pooled the results across trials to yield mate choice observations on 30 population-A snails and 30 population-C snails, presented with approximately equal numbers of A and C partners. A chi-square statistic was calculated from the resulting 2×2 contingency table, normalized by $4/N$, as a measure of sexual isolation (Gilbert and Starmer, 1985).

For no-choice tests of postzygotic reproductive isolation, control cups were set up using 10 parental pairs of unrelated lines within each population, $A1 \times A2$, $A2 \times A3$, . . . , $A10 \times A1$ and $C1 \times C2$, $C2 \times C3$, . . . , $C10 \times C1$. An experimental cross was set up with 10 cups paired across populations, $A1 \times C1$, $A2 \times C2$, . . . , $A10 \times C10$. Each pair of parents received a water change and fresh food every 7 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 2 weeks) and all viable, crawling F_1 juveniles were counted. Observation was terminated upon the death of either parent in a pair.

Any difference in the central tendency of age at first reproduction between the 10 outcross pairs and either set of 10 control pairs was tested by calculating a combined (20-pair) median and comparing counts above and below that median using Fisher's exact test.

For statistical analysis of fecundity and F_1 viability, week 1 was established separately for each set of 10 pairs as the first week in which eggs were laid by 3 or more pairs of parents. Embryos and viable hatchlings were subsequently counted for 10 weeks. We then averaged the embryo production of each pair of parents across its lifetime, ignoring any leading (pre-maturity) zeros and any postmortem zeros, while including as "0" any failure to reproduce by

viable, mature pairs. We used a Kruskal-Wallis nonparametric ANOVA to test whether any significant difference existed in the median weekly mean fecundity of the 10 outcross pairs and the 2 sets of 10 control pairs. Similarly, we averaged the counts of F_1 hatchlings within pairs across weeks, ignoring zeros not corresponding to embryo production, and divided each pair mean by its mean embryo production to obtain pair mean F_1 viability. We used a second Kruskal-Wallis nonparametric ANOVA to test whether any significant difference existed in the median weekly mean F_1 viability posted by the 10 outcross pairs and the 2 sets of 10 control pairs.

To assess the fertility of putative hybrid offspring, F_1 hatchlings (both control and experimental) were reared from each of 3 separate unrelated pairs to a size of 2 mm. These were crossed in time series: 1 early pair from eggs laid around week 1, 1 middle pair produced around week 5, and 1 late pair produced around week 10, to yield 9 F_1 pairs. So if the putative hybrid progeny were reared from pairs $A1 \times C1$, $A2 \times C2$, and $A3 \times C3$, they were crossed as $AC1 \times AC2$ early, $AC2 \times AC3$ early, $AC3 \times AC1$ early, $AC1 \times AC2$ middle, $AC2 \times AC3$ middle, . . . , $AC3 \times AC1$ late. Nine crosses were likewise constituted for controls A and C, and the total of $3 \times 9 = 27$ crosses of F_1 snails reared to adulthood for each experiment, 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 F_1 progeny from 3 outcross pairs ($A1 \times C1$, $A2 \times C2$, and $A3 \times C3$) were reared to 4-mm to 5-mm shell length, at which time they were frozen in 100 μ L of tissue buffer for analysis by allozyme electrophoresis. We have identified 12 enzyme-encoding loci at which allozyme variation is interpretable as the product of codominant alleles segregating in Mendelian fashion (Dillon and Wethington, 1994). These are aconitase (*Acon*), esterases (3 loci: *Est1*, *Est3*, *Est6*), glucose phosphate isomerase (*Gpi*), isocitrate dehydrogenase (2 loci: *Isdh1* and *Isdh2*), leucine aminopeptidase (*Lap*), mannose phosphate isomerase (*Mpi*), phosphoglucomutase (2 loci: *Pgm1* and *Pgm2*), and 6-phosphogluconate dehydrogenase (*6pgd*). We used horizontal starch gel electrophoresis in an aminopropylmorpholine pH 6 buffer system to resolve allozyme variation at the *Gpi*, *Isdh*, and *6pgd* loci; a Tris-Citrate pH 6 buffer system for *Acon*, *Mpi*, and *Pgm*; and a TEB8 system for *6pgd*, *Lap*, and *Est*. 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).

RESULTS—The design of our mate choice tests yielded a great deal of copulatory activity in the initial minutes of each trial, individuals

TABLE 1—Results of the mate choice tests between populations of *Physa virgata* collected from its type locality in the Gila River, Arizona (A), and *Physa acuta* from a control site in Charleston, South Carolina (C).

| | C males | A males | Totals |
|-----------|---------|---------|--------|
| C females | 13 | 6 | 19 |
| A females | 16 | 8 | 24 |
| Totals | 29 | 14 | 43 |

typically becoming involved in multiple pairings. Most snails seemed eager to mate in the male role, while displaying a variety of rejective behaviors (e.g., evasive maneuvers, shell shaking) in the female role, as has been described by DeWitt (1991, 1996). The levels of copulatory activity slowed as an increasing fraction of the population mated successfully in the male role. Ultimately only 1 of 30 South Carolina snails failed to mate as a male, but 16 of 30 Arizona snails failed to do so.

The first choice of the 43 snails successfully mating as males was entirely without regard to the population from which the female originated (Table 1). Both South Carolina and Arizona snails copulated as males, with slightly more Arizona mates than South Carolina mates, but the contingency chi-square testing for sexual isolation was not significant ($\chi^2 = 0.015, 1 df$).

Parental survivorship was good through the duration of the no-choice tests for postzygotic reproductive isolation (Fig. 1). Both the A × C outcross experiment and the Arizona control first reproduced at a median age of 5 weeks after the initiations of their respective trials at 2 mm (Table 2). The median age of first reproduction in the South Carolina control was 8 weeks, a significant delay (Fishers Exact $P = 0.0198$). The Kruskal-Wallis statistic testing for a difference in fecundity among the outcross and the 2 controls was 3.72, not significant by chi-square approximation ($P = 0.155$). The 3 treatments were significantly different, however, in F_1 viability ($KW = 6.47, P = 0.039$), with the 76% hatchling survivorship posted by the A × C outcross better than either control.

Electrophoretic analysis of allozyme variation revealed relatively little genetic divergence between control samples from the Arizona and

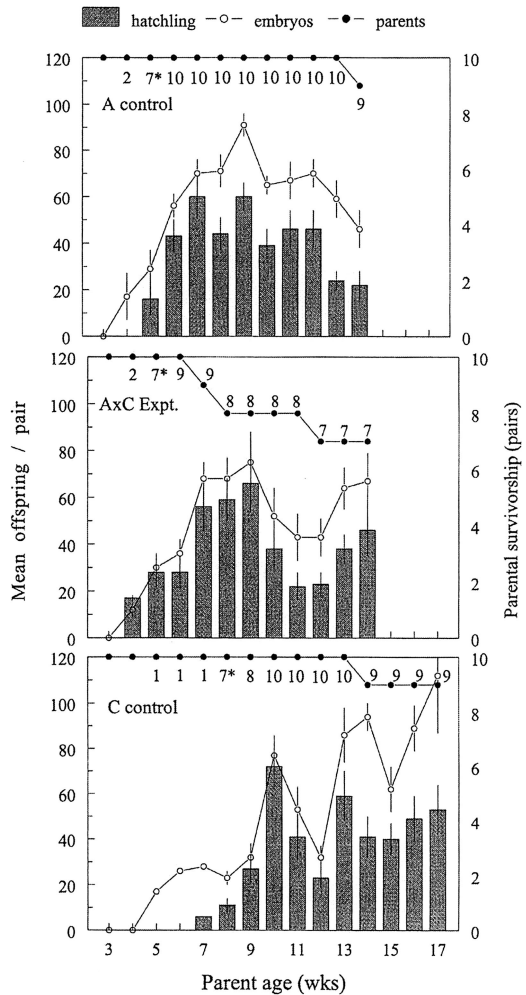


FIG. 1—Production of embryos and viable hatchlings as a function of parental age (weeks post hatching) for 10 pairs of Arizona *Physa* (A control), 10 pairs of South Carolina *Physa* (C control) and 10 Arizona × South Carolina pairs (A × C Expt). The bars are standard errors of the mean. The number of reproducing pairs is given with parental survivorship (right axis). Asterisks (*) denote week 1 for analysis of variance.

South Carolina populations. The Arizona population seemed fixed for allele 103 at the *Lap* locus, where the South Carolina population showed both alleles 100 and 103 in roughly equal frequency. The Arizona population showed a strong polymorphism for alleles 94 and 100 at the *Isdh* locus, where the South Carolina population was nearly fixed for *Isdh*100. Markers at these 2 loci were sufficient to con-

TABLE 2.—Summary statistics (medians, ranges) on reproduction in 3 laboratory populations of *Physa*.

| | Arizona control | | South Carolina control | | A × C outcross | |
|--|-----------------|--------------|------------------------|---------------|----------------|--------------|
| | Median | Range | Median | Range | Median | Range |
| Week at first reproduction | 5 | 4 to 6 | 8 | 5 to 10 | 5 | 4 to 7 |
| Weekly mean fecundity (embryos) | 64.6 | 43.7 to 73.8 | 66.9 | 19.4 to 104.4 | 50.2 | 15.5 to 77.5 |
| Weekly mean F ₁ viability (%) | 65 | 40 to 83 | 58 | 45 to 86 | 76 | 59 to 94 |
| Week of F ₂ hatch | 10 | 7 to 14 | 10 | 10 to 14 | 10 | 7 to 17 |

firm that 100% of the F₁ progeny reared from the 3 putative outcrosses tested were indeed hybrids.

We could detect no reduction in F₁ fertility. One pair of F₁ individuals in the Arizona control died early, but the remaining 8 pairs survived to reproduce successfully, as did all 9 of the A × C hybrid pairs and South Carolina control pairs. Eggs hatched to viable F₂ progeny at a median age of 10 weeks in all 3 treatments (Table 2).

DISCUSSION—The interactions we observed in our mate choice experiments were typically characterized by a great deal of shell-shaking and evasive maneuvering among prospective copulants. Wethington and Dillon (1996) have attributed such behavior to 2 sources—sexual conflict and gender conflict. Sexual conflict results from the widely observed tendency for males to be eager to mate and females to be choosy, as described by Bateman (1948). Gender conflict, which is peculiar to non-reciprocally mating simultaneous hermaphrodites such as *Physa*, results from the tendency for both copulants to elect the male role. Successful copulation among pulmonate snails requires that the snail acting as male overcome resistance of both origins.

Gender conflict disappears when at least one of the prospective copulants has recently mated as a male (Dillon and Wethington, 1996). Thus, aggressive interactions declined over the 6-hour duration of our mate choice tests, to the point that all snails with any tendency to mate had encountered multiple receptive partners, and no further attempts were observed. But although 29 of the 30 snails originating in South Carolina population C were ultimately observed mating as males, only 14 the 30 snails from Arizona population A did so. The origin of this differential tendency to mate as male (termed “vigor” by Gilbert and Starmer, 1985) is obscure. As a consequence, however, Arizona snails demonstrated less male-male gender conflict, making them easier to mount as females. Both South Carolina and Arizona snails demonstrated a slight bias to mate as male with Arizona partners, and no prezygotic reproductive isolation resulted.

Our no-choice test for postzygotic reproductive isolation between the South Carolina and Arizona populations yielded results similar to

those obtained by Dillon et al. (2002) and Dillon and Wethington (2004). The South Carolina control lagged significantly behind the Arizona control in age at first reproduction, suggesting a difference in the adaptation of the 2 populations to our standard culture conditions. The A \times C outcross experiment was not significantly different from at least one control by 3 of the 4 fitness criteria measured (age of first parental reproduction, parental fecundity, and F₁ fertility) and significantly greater than either control in F₁ viability. Thus, our experiments returned no evidence of postzygotic reproductive isolation between population C and population A.

The population of *Physa* inhabiting the type locality of *P. virgata* seems to be conspecific with a population of known *P. acuta* from Charleston, South Carolina. This result, taken together with the absence of any consistent morphological, allozyme, or DNA differentiation between the 2 nominal species (Wethington, 2003; Wethington and Lydeard, in press), suggests strongly that *P. virgata* be considered a junior synonym of *P. acuta*.

Dillon et al. (2002) synonymized the most common physid nomen of the American East and South (*P. heterostropha*), as well as the most common physid of the North and Midwest (*P. integra*), under the European *P. acuta*. Paraense and Pointier (2003) have synonymized the common Caribbean *Physa cubensis* under *P. acuta* as well. Now it would seem that the most common physid of the American West is not different from *P. acuta*. It seems possible that most populations of physids in the subgenus *Costatella* might ultimately be shown to be conspecific under the nomen of Draparnaud (1805).

Rapid growth, self fertility, and high reproductive rate are but 3 from a long list of adaptations especially suiting *P. acuta* for dispersal and colonization. Successful invasions of Africa, Asia, and Australia in the last 40 years have earned *P. acuta* a nomination as "the world's most cosmopolitan freshwater gastropod" (Dillon et al., 2002). Given the levels of interpopulation gene flow implied by this large and expanding range, the absence of a reproductive barrier between a pair of *Physa* populations sampled from regions merely as distant as Arizona and South Carolina might, in retrospect, be unsurprising.

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