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# Populations of the European freshwater pulmonate *Physa acuta* are not reproductively isolated from American *Physa heterostropha* or *Physa integra*

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**Abstract.** It has long been speculated that *Physa acuta,* a pulmonate snail widespread and invasive in fresh waters of the old world, may have originated in North America. But the identification of a new-world cognate has been complicated by the confused systematics and taxonomy of the Physidae in America. More than 40 species of physids are currently recognized in the United States, many with variable and overlapping morphology. We have previously established that premating reproductive isolation is negligible among physid snails. Here we report the results from no-choice crosses each involving 2 populations of the widespread American species *Physa heterostropha* and *Physa integra,* both with each other and with *P. acuta,* designed to compare measures of reproductive success between species and between populations within species. Samples of *P. acuta* were collected from France and Ireland, *P. heterostropha* from eastern Pennsylvania and South Carolina, and *P. integra* from southern Indiana and northern Michigan. The 6 intrapopulation controls varied quite significantly in their survival, age at first reproduction, parental fecundity, $F_1$ viability, and $F_1$ fertility under our culture conditions. Measures of survival and reproduction in the 6 interpopulation crosses were generally intermediate, but in no case significantly worse than the more poorly performing control. Thus we were unable to detect evidence of reproductive isolation among our 6 populations of snails from 2 continents. All should be referred to the oldest available nomen, *P. acuta.*

*Additional key words:* Gastropoda, *Physella,* snails, invasion, hybridization

The most abundant and widespread freshwater gastropods of North America belong to the pulmonate family Physidae (Burch 1982). In certain lakes and quiet rivers, grazing by physid snails may demonstrably affect the biomass and composition of epibenthic algae and macrophytes, exerting indirect effects throughout the ecosystem (Kehde & Wilhm 1972; Sheldon 1987; Lowe & Hunter 1988; Swamikannu & Hoagland 1989; Brown et al. 1994). Physid snails themselves may comprise a substantial portion of the diet of a variety of fishes (Martin et al. 1992; Turner 1996; McCollum et al. 1998), crayfishes (Chambers et al. 1990; DeWitt et al. 1999; Turner et al. 1999; McCarthy & Fisher 2000), and other predators (Kofron & Schreiber 1985; Brown & Strouse 1988). North American physid populations have served as model organisms for influential studies of interspecific competition (Brown 1982), life history evolution (Rollo & Hawryluk 1988; Crowl & Covich 1990), and population genetics (Dillon & Wethington 1995). The ecology of physid snails has been reviewed (Dillon 2000).

*Physa (Physella) heterostropha* (Say 1817) has been called "the most misunderstood mollusk in America" (Baker 1928). It is certainly among the most common and widely distributed, its 3 subspecies together ranging from the Atlantic provinces of Canada south to the Bahamas and west through the Mississippi drainages to Kansas and Texas (Burch & Tottenham 1980; Clarke 1981). Described from Philadelphia by Thomas Say (1817), *P. heterostropha* was the first North American physid to reach formal description. Almost equally common and widely distributed is *Physa (Physella) integra* (Haldeman 1841), whose 3 subspecies generally range across more northern and western regions of North America: Quebec and Manitoba south to Kentucky and Colorado (Burch & Tottenham 1980; Wu 1989). Haldeman first described *P. integra,* postdated in an 1842 publication, with "Sent to me from Indiana by Mrs. Say" as its geographical distribution. The type locality must almost certainly be

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New Harmony, Indiana, where Thomas Say's wife resided from 1833 to 1841.

Haldeman did not distinguish *P. integra* from *P. heterostropha* in his original description. Subsequent authors (Clarke 1981; Jokinen 1992) have generally noted that *P. integra* bears a heavier shell than *P. heterostropha,* marked with faint white shell bands or varices, which are peculiar to the species. Baker (1900) also noted a difference in radular cusp number between the 2 species, as well as the presence of "spiral lines" on the shell of *P. integra,* but not *P. heterostropha.*

In addition to *P. heterostropha* and *P. integra,* ~40 other species of physid snails are currently recognized from North America (Turgeon et al. 1998). Many of these are extremely difficult to distinguish from one another, identifications resting on (at best) minor aspects of shell morphology. As a result, the attribution of a specific identity to most populations of the most common freshwater snails on the American continent is currently problematic.

Although the center of physid diversity is American, several well-characterized members of the family inhabit Europe. The most widespread of these is *Physa (Physella) acuta* DRAPARNAUD 1805, originally described from the Garonne River of France. The distinction between *P. acuta* and *P. heterostropha* is unclear. The shells of the latter have been reported to bear more rounded whorls (Macan 1977), although a second American species, *P. gyrina,* may have been confused with *P. heterostropha* in some cases (Anderson 1996). Anderson suggested that *P. acuta* and *P. heterostropha* might be distinguishable by certain details of penial morphology, although his more recent observations have led him to reconsider (pers. comm.).

These morphological characters are variable, however, and in some cases insufficient to distinguish *P. acuta* from *P. heterostropha* (Te 1978; Gloer & Meier-Brook 1994). So given the invasive habit of *P. acuta,* and its subsequent spread around the world, it has commonly been speculated that *P. acuta* might not be native to Europe, but rather may have originated in North America (Brackenbury & Appleton 1991; Hofkin et al. 1992; Brown 1994).

If *P. acuta* is in fact an American native, it is not clear which of the many American species might be its cognate. So the purpose of the present investigation is to estimate levels of reproductive isolation among European populations of *P. acuta,* American populations of *P. heterostropha,* and American populations of *P. integra,* and to compare any reproductive isolation observed within species to that displayed between the 3 nominal species. Broadly, our approach is to compare intrapopulation control crosses to intercrosses between pairs of populations in age at first reproduction, fecundity, and fertility under constant conditions. The details of our experimental design have been guided by two peculiarities in the reproductive biology of the study organism: the absence of sexual isolation and the likelihood of self-fertilization.

As is generally true of pulmonate gastropods, snails of the genus *Physa* are preferentially outcrossing simultaneous hermaphrodites, male function developing slightly before female function (Wethington & Dillon 1993, 1996). Snails reared in isolation are capable of self-fertilization, but demonstrate delayed onset of egg-laying, reduced fecundity, an increased proportion of inviable eggs, and reduced $F_1$ survival (Wethington & Dillon 1997). Self-fertilization also may occur at low frequencies in naturally outcrossing populations with no apparent effect on fitness, however.

As might be predicted from the above, allozyme data suggest that wild populations of *Physa* in South Carolina reproduce primarily by outcrossing, with occasional evidence of self-fertilization at low frequencies (Dillon & Wethington 1995). Some European populations of *P. acuta* may demonstrate higher selfing rates in the wild (Monsutti & Perrin 1999). But the laboratory experiments of Jarne et al. (2000) with *P. acuta* from France, broadly similar to those of Wethington & Dillon (1997) with *P. heterostropha* from South Carolina, confirmed that populations of *P. acuta* reproduce primarily by outcrossing.

Wethington et al. (2000) have evaluated premating reproductive isolation among conspecific populations of *Physa* from South Carolina, Virginia, and Michigan, and between *P. heterostropha* and the anatomically distinctive *P. gyrina.* In each trial, 3 sexually mature snails (a homogamic pair and a heterogamic individual) were observed until first mate choice. With 6–18 trials per experiment, none of 6 experiments yielded any evidence of assortative mating among individuals from these diverse populations. In the cases where *P. heterostropha* were observed to receive sperm from *P. gyrina,* allozyme electrophoresis demonstrated that all putative $F_1$ offspring were the product of self-fertilization rather than outcross, confirming that postmating reproductive isolation between these two species is complete.

The absence of any evidence for premating reproductive isolation between strikingly divergent species of *Physa* prompted us to select what might conventionally be called a "no choice" design for the breeding experiments we describe here. But strictly speaking, each member of a pair of snails does have a choice: to outcross, self-fertilize, or forego reproduction. Thus we have focused our analysis on those elements of fitness that might distinguish between self-

ing and outcrossing, e.g., a delay in parental reproduction or a reduction in parental fecundity. In addition, we use allozyme markers to confirm that a small sample of $F_1$ from each putative outcross is not the result of self-fertilization.

## Methods

### Culture

Samples of *Physa acuta* were collected from Saint-Martin-de-Londres, southern France, in a small wadi called Rieutort, and at the Glastry Clay Pits in County Down, Northern Ireland. Snails from cultures originating in these 2 populations will be referred to by single-letter abbreviations "F" and "I," respectively. Our cultures of *Physa heterostropha* originated from Philadelphia, Pennsylvania, in the Schuylkill River at Fairmount Park (hereafter "P") and Charleston, South Carolina, in the largest pond at Charles Towne Landing State Park ("C"). Our lines of *Physa integra* originated from a pond near the "Roofless Church" in New Harmony, Posey County, southern Indiana ("N") and from Douglas Lake, Cheboygan County, northern Michigan, at the University of Michigan Biological Station ("D").

Our culture methods were generally those of Wethington & Dillon (1991, 1993, 1997). Wild-collected adults from the 6 populations were isolated in transparent plastic cups of aerated, filtered pond water (210 ml), with disposable Petri-dish lids, and fed a commercially available brand of green flake fish food, with a spirulina base. All animals were cultured at room temperature (~23°C) in a 12/12 daylight cycle. Animals were shifted to new cups every 7–10 days as egg masses were laid, and the water changed. The progeny of 10 adults from each population, wild-conceived but laboratory-born, were designated the "parental generation" for the experiments we report here.

We initiated our 6 intrapopulation controls by pairing parental snails (as 2-mm juveniles, ~3 weeks posthatch) from the 10 different (wild-collected) mothers in round-robin fashion. For example, the Charleston control was comprised of 10 pairs of snails: C12, C23, C34, and so forth up to C01. Our 6 interpopulation crosses (termed CP, DN, FI, NP, FP, and FN) were also initiated with 10 pairs of parents (as 2-mm juveniles) from the 10 different (wild collected) mothers, and labeled (for example) CP1, CP2, CP3, and so forth up to CP0. The 60 pairs of control snails and 60 intercross pairs were cultured in 10 cups as described above.

Each cup was monitored with food and water change every 7 days initially, and at 10–12-day intervals as the animals aged. If either member of the pair died during the first 2 weeks, both were replaced from backup cultures. But the death of either snail during later weeks terminated the pair. If embryos were produced, the parents were transferred to a fresh cup and the date and number of embryos recorded. This "parental phase" of the investigation was continued until mortality reduced the number of pairs to <5 in the cross with the poorest survival. The embryos were held to hatch and the number of viable, crawling $F_1$ juveniles counted for all 10 lines within all 12 crosses.

The $F_1$ progeny were retained from the first 3 lines of each intercross (e.g., CP1, CP2, CP3) and from the first 3 lines of each control cross not sharing a parent (e.g., C12, C34, C56). These were paired (as 2-mm juveniles) between parents within lines to verify $F_1$ fertility. For example, in the CP intercross, $F_1$ snails were paired as CP1 × CP2, CP2 × CP3, and CP1 × CP3. For the C controls, $F_1$ snails were paired as C12 × C34, C34 × C56, and C12 × C56. Each of these 36 (12 × 3) crosses to verify $F_1$ fertility was performed in an early-middle-late time series. That is, we combined a single pair of $F_1$ individuals conceived early in the investigation, a second pair conceived about midway through the parental phase of the investigation, and a third pair conceived in the final weeks of the parental phase. Then a total of 108 (36 × 3) pairs of snails were reared in the "$F_1$ phase" of the investigation, cultured as above, and the date of initial egg laying and hatch of viable $F_2$ progeny recorded.

### Allozyme analysis

Because of the potential for self-fertilization, additional samples of $F_1$ progeny were reared to 4–5-mm shell length and outcrossing was verified by allozyme electrophoresis. We have documented polymorphism at 7 enzyme-encoding loci in our 6 populations: aconitase, esterase-3, esterase-6, isocitrate dehydrogenase, leucine aminopeptidase, 6-phosphogluconate dehydrogenase, and phosphoglucomutase-2 (Dillon & Wethington 1995). Codominant alleles segregating in Mendelian fashion appear to control allozyme phenotype in each of these cases (Dillon & Wethington 1994). So upon reaching sufficient body size for analysis, at least 12 putative $F_1$ animals were sampled from each intercross (4 early, 4 middle, 4 late) and allozyme phenotype determined by horizontal starch gel electrophoresis as detailed in Dillon (1992). We used the AP6 buffer to resolve *Pgm2* and *Isdh,* and the TEB8 buffer for *Est3, Est6,* and *Acon.* Both buffer systems were used for *6pgd* and *LAP.* Note that the (necessarily outcrossing) ratio of 1 heterozygote: 1 homozygote can be distinguished from a (potentially selfing) 1:2:1 phenotypic ratio at better than 95% confidence with 11 progeny ($0.75^{11} = 0.042$). The (necessarily outcrossing) observation of all-heterozygous progeny can be

distinguished from 1:2:1 with a sample of only 5 individuals ($0.50^5 = 0.031$).

### Statistical analysis

For clarity, we refer to a set of 10 intercross pairs, together with its 2 corresponding sets of 10 intrapopulation controls, as a single "experiment." For example, the N × P experiment involved 10 pairs of N parents, 10 pairs of P parents, and 10 NP intercross pairs.

Reproduction was not in any experiment initiated in the same week for the interpopulation cross and its 2 corresponding controls. Unadjusted analysis of variance in fecundity was thus precluded by the existence of entirely empty early cells in every experiment. We therefore set "age level 1" independently for all 6 controls and all 6 intercrosses as the first week in which embryo production was recorded for 3 or more pairs of snails, and we analyzed age at first reproduction separately from fecundity.

Age at first reproduction was recorded (in weeks) for each pair of parents, and the median and range calculated across each of the 6 controls and 6 intercrosses. Note that this age does not include the (~3) weeks required for hatchlings to grow to 2-mm shell length in our culture conditions. Then a grand median age at first reproduction was calculated over each experiment, the 30 pairs cast into a table with 6 cells (cross by early/late), and tested using chi-square statistics with 2 degrees of freedom. For example, over all 30 observations in the N × P experiment, the grand median age at first reproduction was 3.5 weeks. Then the 30 observations were cast into a table with 3 rows (N, P, NP) and 2 columns (greater than 3.5 and less than 3.5) and tested for a difference in central tendency with a contingency chi-square.

For each of the 6 experiments, the mean fecundity (from first reproduction to death or termination) displayed by the 10 pairs of intercross parents was compared to control fecundity using two-way ANOVA, with age level and population the independent variables and embryos the dependent (Statistica release 5.5, StatSoft 1994). Given a significant population effect, *post hoc* comparisons were made using Tukey's "highly significant difference" (HSD) test for unequal sample sizes (Spjotvoll & Stoline 1973). To avoid confounding fecundity with survival or age at first reproduction in this analysis, parental fecundity was calculated only from viable, demonstrably mature pairs. That is, no zeros were entered into the fecundity analysis for either early (immature) or late (postmortem) weeks, although internal zeros (i.e., pairs of apparently healthy, mature snails not laying embryos) were included.

For each of the 6 controls and 6 intercrosses separately, we summed both the embryos and the viable hatchlings counted across all dates for each of the 10 pairs of parents. We then tested for a significant difference in $F_1$ viability for each experiment using one-way ANCOVA, with total viable hatchlings as the dependent variable and embryos as the fixed covariate. If a significant difference among the 3 crosses was uncovered, we again performed *post hoc* comparisons using Tukey's HSD test for unequal sample sizes.

## Results

The 6 control crosses posted very significant differences in reproductive output under our culture conditions. Median age at first reproduction ranged from 3 weeks in populations N and P to 14 weeks in population F (Fig. 1). Control fecundities ranged from over 50 embryos/pair/period as posted by populations P, N, and I to only 17.2 embryos/pair/period as recorded by population D (Figs. 2, 3). Embryo viability ranged from 69% in population D to 93% in population F (Fig. 4).

Parental survival also varied strikingly among the control crosses. The sixth and seventh pairs of parental snails for the I controls died between day 144 and day 156, bringing to a close the parental phase of this investigation at day 150. Six pairs of D parents had also died by this juncture, 4 without issue. But only a single pair of C parents, 2 pairs of F parents, and 3 pairs of N and P parents had died by day 150, all after reproduction. Control snails from the D line continued to display poor survival in our culture conditions through the second generation. Of the 9 pairs of D controls tested for $F_1$ fertility, only a single pair survived to produce $F_2$ progeny.

For 5 of the 6 interpopulation crosses, age at first reproduction was intermediate between the 2 controls (Fig. 1). The only exception was in the cross of populations N and P, where the median for both controls was 3 weeks and that of the NP intercross 4 weeks, an insignificant difference.

Parental fecundity from the 6 interpopulation crosses was compared to control fecundity (Fig. 3). In 2 of the 6 experiments (D × N and F × I) intercross fecundity was intermediate between the controls, and significantly different from both. In the remaining 4 experiments, intercross fecundity was below control fecundity, but not significantly worse than the more poorly performing control cross.

The mean total embryos laid by the parental generation for each cross, and their corresponding yields of viable $F_1$ hatchlings, are given in Fig. 4. ANCOVA returned no significant difference in $F_1$ survival in 4 experiments (C × P, N × P, F × P, and F × I). In the

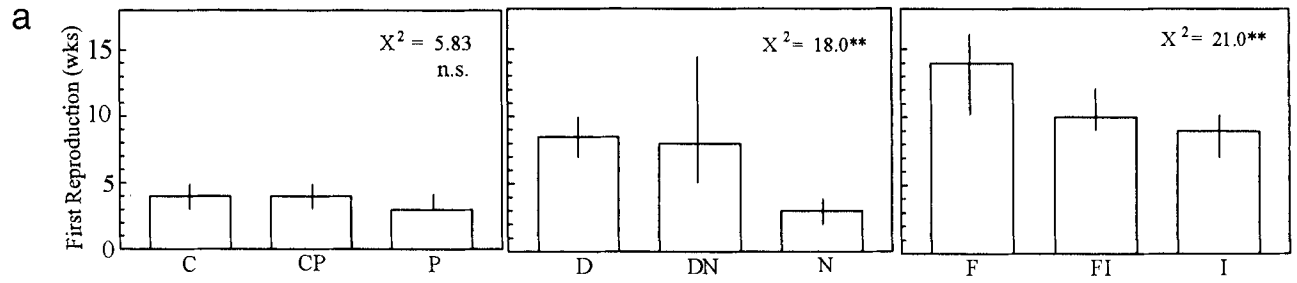



**Fig. 1.** Median and range in age at first reproduction for the 6 experiments. The chi-square testing for heterogeneity in central tendency has 2 degrees of freedom (**p < .01).

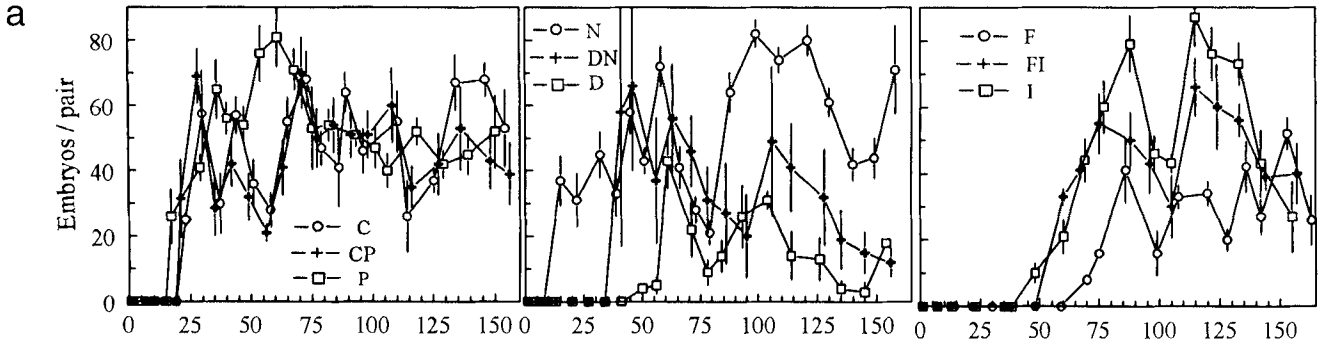



**Fig. 2.** Mean parental fecundity (and SE) as a function of time (days) for the 6 experiments.

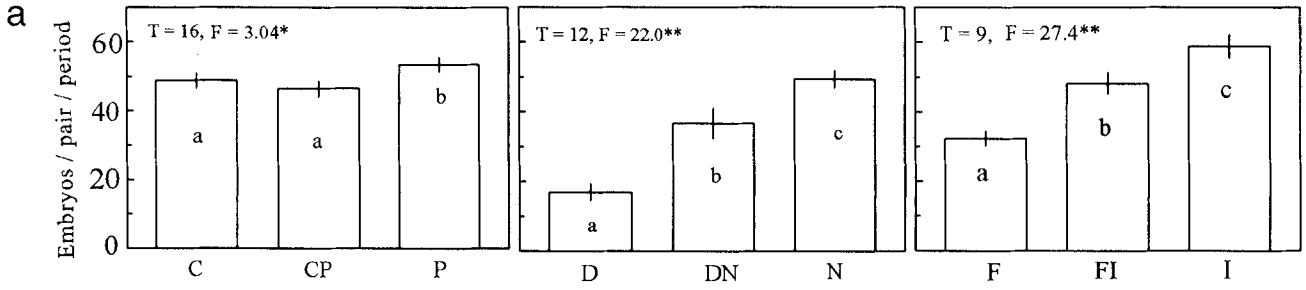



**Fig. 3.** Grand mean parental fecundity (and SE) over the subset (T) of age levels analyzed by two-way ANOVA, together with F for the population effect (T-1 and 383 df). Since F was significant in all 6 experiments (*p < 0.5, **p < .01), results of *post hoc* tests are shown, with significantly different means denoted by different lower-case letters.

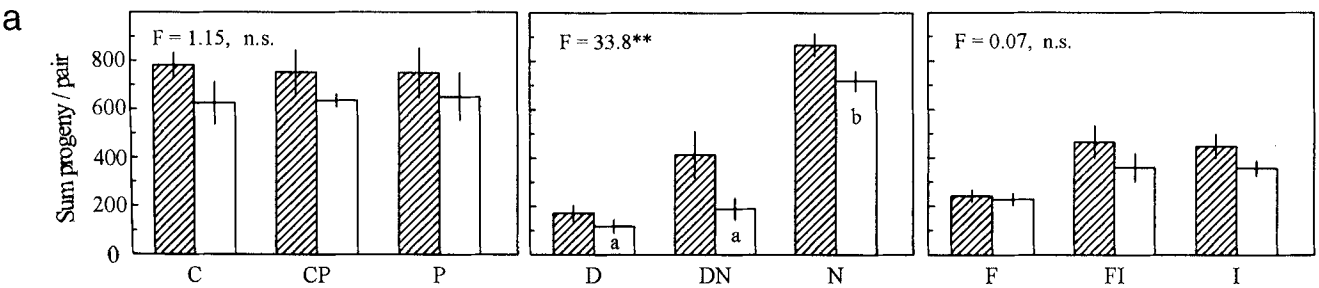



**Fig. 4.** Comparisons of $F_1$ viability for the 6 experiments, with mean (and SE) embryos/pair shaded and mean viable hatchlings unshaded. The value of F is from ANCOVA (2, 26 df). Means significantly different by *post hoc* tests (**p < .01) are denoted by different lower-case letters.

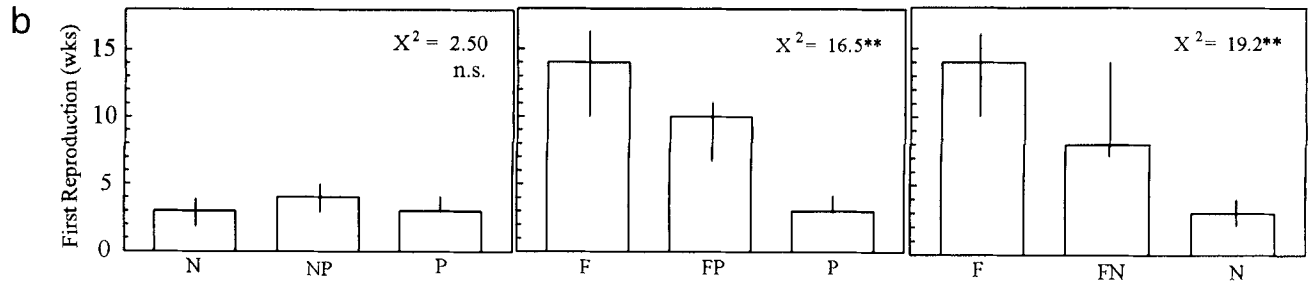
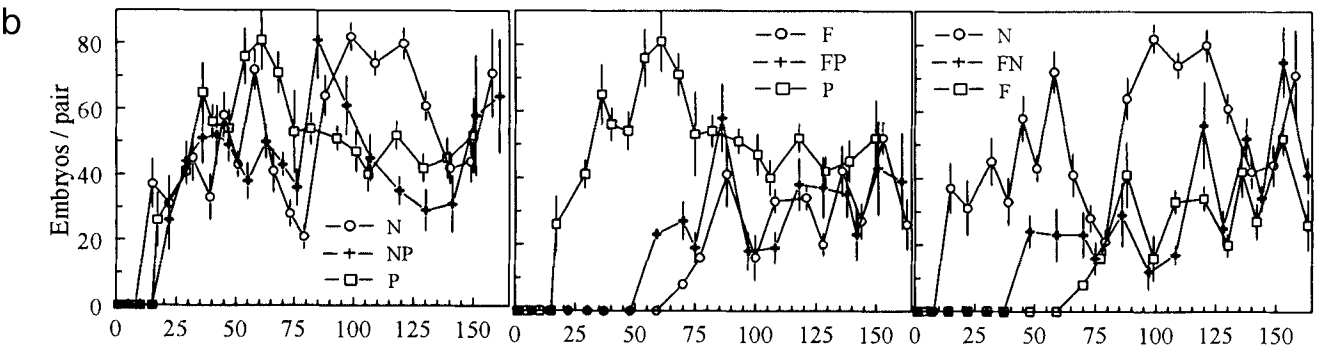



**Fig. 1.** Continued.

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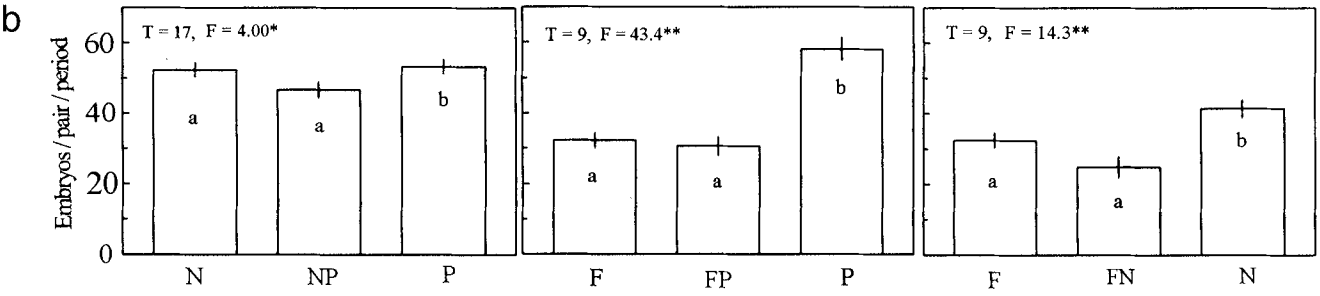


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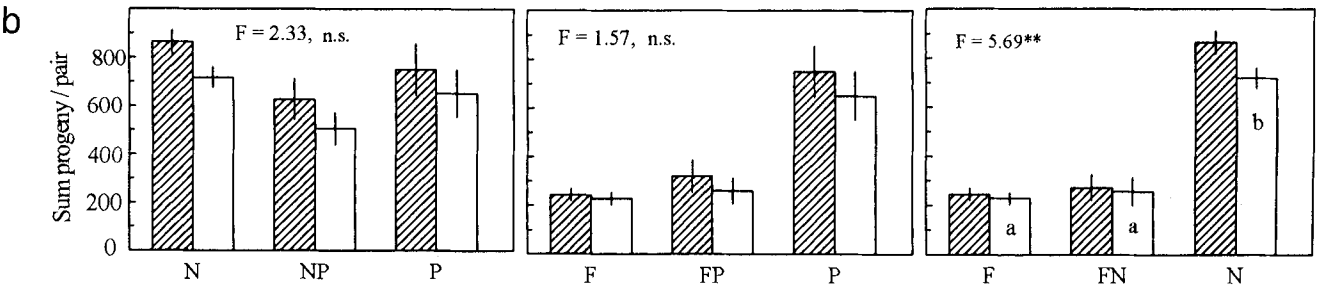


**Fig. 4.** Continued.

D × N experiment, *post hoc* tests showed $F_1$ survival to be indistinguishable in D controls and DN intercrosses (69% and 46%) but significantly worse than the 83% posted by the N controls ($p < .0001$). In the F × N experiment, the FN intercross and F control were not significantly different (93–94%), but both were significantly better than the N controls ($p < .0001$). In no case did $F_1$ survival reflect any evidence of reproductive isolation.

For all 6 interpopulation crosses, the possibility of parental reproduction by self-fertilization was rejected at the 95% confidence level by electrophoretic analysis of allozyme phenotype in more than 11 of the $F_1$ progeny. At a minimum of one locus, all 6 × 3 samples of $F_1$ offspring retained for testing were missing one or both of the homozygous classes expected to segregate from self-fertilization.

All 9 pairs of $F_1$ snails ultimately laid eggs hatching to viable $F_2$ progeny in 5 intercrosses and 5 controls. The median times to reproduction in the $F_1$ phase of this investigation were similar in all cases to those posted during their parental phases. The only exceptions were noted in the DN intercross, where one pair of $F_1$ snails died without issue at week 6, and in the D control, where only a single pair of $F_1$ snails survived to produce viable $F_2$ progeny at week 15.

## Discussion

Although reared in a constant environment, our 6 control populations displayed strikingly different life history responses. The most productive population was *Physa integra* from its type locality, N, with a modal age of 3 weeks at first reproduction and an average 52.3 embryos/pair/period over 17 age levels. The other population of *P. integra,* population D, performed the most poorly overall, beginning reproduction at a mode of 8.5 weeks and averaging only 16.9 embryos/pair/period over 12 age levels. The European populations of *P. acuta* both initiated reproduction at later dates, (the I mode was week 9, the F mode week 14) although both populations were quite fecund once mature. We think these differences reflect genuine genetic diversity among our populations. It would be surprising if 6 populations adapted to such a diversity of environments, ranging across 20 degrees of latitude and 80 degrees of longitude, had not undergone substantial genetic divergence.

But none of this genetic diversity seems correlated to reproductive isolation. Reading Figs. 1–4 vertically, note that the experiments reported in the left 3 columns are nominally intraspecific (*P. heterostropha, P. integra, P. acuta*), and those in the right 3 columns are nominally interspecific. No significant fitness decrement is displayed by the intercrosses shown in the right 3 columns in any case.

Our experimental design allowed us to measure reproductive isolation by any or all of four separate criteria. First, reproductive isolation might be prezygotic, such that outcross fertilization is reduced or precluded. A pair of snails isolated in this fashion might nevertheless reproduce by self-fertilization (Wethington et al. 2000), but in such cases, we would expect reproduction to be delayed (Wethington & Dillon 1993, 1997). However, we found no evidence that age at first reproduction in the 6 interpopulation crosses was significantly delayed behind the slower of the 2 corresponding controls (Fig. 1). The intermediate values for median age at first reproduction observed in most experiments are a likely consequence of the ability of smaller snails to copulate as males before they have attained a size sufficient to lay eggs (Wethington & Dillon 1993).

Second, it seems possible that reproductive isolation might manifest itself as a reduction in the fecundity of intercrossed snails below controls. This could result from prezygotic factors; a reduction in the fecundity of self-fertilizing individuals of *Physa* is well documented (Wethington & Dillon 1997; Jarne et al. 2000). Or postzygotic factors, such as reduced or incomplete embryonic development, might also be involved. But again, we found that, after the onset of egg laying, the fecundity of the DN and FI intercrosses was intermediate between their corresponding controls (Fig. 3). Fecundity was somewhat reduced in the CP, NP, FP, and FN intercrosses, although not significantly below the more poorly performing control.

Third, postzygotic reproductive isolation might manifest itself in reduced hybrid viability. But no significant difference was noted in $F_1$ survival in 4 of the 6 experiments (Fig. 4). DN hybrid survival was reduced, although not significantly worse than the more poorly performing control, and FN hybrid survival was improved, although not significantly better than the better performing control.

Finally, postzygotic reproductive isolation might be apparent in a fertility reduction among $F_1$ hybrids. But all the $F_1$ progeny we tested proved fertile, showing no obvious delay in reproduction or reduction in fertility to the termination of this investigation.

We therefore conclude that these 6 populations, sampled from diverse environments across the breadth of North America and Europe, are conspecific. The nomina *P. heterostropha* and *P. integra* are junior synonyms to *P. acuta* DRAPARNAUD 1805.

Although the experiments we report here do not bear directly on the origin of *P. acuta,* several lines of evidence support the hypothesis that the species is native to North America and introduced elsewhere. North American populations seem relatively stable and morphologically diverse; their range is largely prehistoric

and taxonomists have recognized numerous subspecies and forms. Elsewhere the species seems more morphologically uniform and biologically invasive. Its date of first record in the Garonne River corresponds to an era of great trade between France and the young United States, when Bordeaux (at the mouth of the Garonne) was a primary port-of-entry. If the species was in fact transported from North America, it is ironic that its description in France preceded its description in Philadelphia by 12 years.

Most authors attribute the modern spread of *P. acuta* to aquarium hobbyists, and to trade in aquatic plants for water gardens. The species seems to have first spread through Mediterranean regions (e.g., Eleutheriadis et al. 1993; Yousif et al. 1993; Montanini et al. 1998) and then more slowly into northern Europe, appearing in Belgium in 1869 (Adam 1960) and in Poland around 1909 (Serafinski et al. 1989). Today it is found widely in Africa (Appleton & Branch 1989; Madsen & Frandsen 1989; Kristensen & Ogunowo 1992; Brown 1994), southern Asia (e.g., Brandt 1980; Tanveer & Kahn 1991; Ali 1995; Raut et al. 1995), Australia (Endersby 1990), and Japan (Gotoh & Kawata 2000). When to this range is added most of the continent of North America, *P. acuta* might reasonably be nominated as the world's most cosmopolitan freshwater gastropod.

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