Genetics, Shell Morphology, and Life History of the Freshwater Pulmonate Limpets *Ferrissia rivularis* and *Ferrissia fragilis*

Robert T. Dillon, Jr. and Jacob J. Herman
Department of Biology, College of Charleston
Charleston, South Carolina 29424 USA
E-mail: dillonr@cofc.edu

ABSTRACT

The weak and variable nature of the shell morphometric criteria by which the North American ancylid limpets *Ferrissia rivularis* and *Ferrissia fragilis* are distinguished has led to speculation that they may be ecophenotypic variants. The shells of *F. rivularis* that we collected from a rocky river in the upper piedmont of South Carolina were significantly higher and wider per length than those of an *F. fragilis* population collected from a weedy ditch in the coastal plain. Both populations laid singleton eggs approximately 0.6 mm diameter in culture, hatching in one week and maturing in four-five weeks at 2.0 - 3.0 mm shell length. Adult *F. rivularis* reared in our laboratory environment were morphologically indistinguishable from *F. fragilis*, suggesting that the differences in shell shape displayed by their wild-collected parents may have little additive genetic component. Both populations were fixed for identical allozyme alleles at nine loci, with two alleles segregating at a tenth, *IsdhF*. The absence of *IsdhF* heterozygotes in wild-collected samples of 104 *F. rivularis* individuals ($p = 0.52$, $q = 0.48$) and 102 *F. fragilis* individuals ($p = 0.14$, $q = 0.86$), together with no evidence of outcrossing in laboratory breeding experiments, suggests obligate self-fertilization. Thus in the absence of any reliable morphological distinction, we propose that *F. fragilis* be considered a junior synonym of *F. rivularis*.

INTRODUCTION

Pulmonate limpets of the family Ancylidae are inconspicuous but common inhabitants of freshwater benthic environments worldwide (Hubendick 1964, 1967, and 1970). The well-studied *Ferrissia rivularis* of North America reaches maximum abundances on stones in rivers, streams, and the littoral zone of lakes (Burky 1971, Keating and Prezant 1998) where it grazes on periphytic algae, especially diatoms. *Ferrissia fragilis* (Tryon 1863) more commonly inhabits macrophytes and organic debris in lentic environments (McMahon 1976, Jokinen 1978), although its apparent preference for diatoms is similar (Blinn et al. 1989).

Like all pulmonate gastropods, ancylids are simultaneously hermaphroditic, although male and female functions may develop according to slightly differing schedules (Russell-Hunter and McMahon 1976). Copulation has been described, and growth, reproduction, and life history patterns have been documented for *F. rivularis* (Burky 1971). Both a simple annual life cycle and two non-overlapping generations per year have been reported.

The most recent systematic review of the North American Ancylidae was that of Basch (1963). He recognized 11 species in four genera: *Rhodacmaea* (3), *Laevapex* (2), *Hebetancylus* (1), and *Ferrissia* (5), distinguishing species primarily by slight variation in shell morphology and habitat. But Basch expressed considerable uncertainty regarding the specific taxonomy, noting that "ecological phenotypes are numerous, and the plasticity of the shell has been remarked upon many times." More modern studies of DNA sequence divergence have indeed suggested that the family may have been overly split (Walther et al. 2006a and 2006b). The North American *F. fragilis* has apparently invaded Europe and East Asia, where it has been re-identified under other specific

nomena and even re-assigned to different genera (Walther et al. 2006b and 2006c).

In South Carolina, rapidly flowing rivers and streams of the upper piedmont are inhabited by *F. rivularis*, while *F. fragilis* is common in the swamps and ditches of the coastal plain. The former species was characterized by Basch (1963) as bearing a robust shell with an elevated apex, while the latter was identified by a shell that is not robust, with an apex depressed or moderately elevated. But through the middle of the state run many rivers where current is low or variable, and in those waters are found populations of *Ferrissia* bearing shells morphologically intermediate between *F. rivularis* and *F. fragilis*. This suggested to us that the distinction between the two nominal species might be ecophenotypic in origin.

The purposes of this study were twofold. First, taking as our inspiration previous experiments on other freshwater pulmonate gastropods (Arthur 1982, Lam and Calow 1988), we reared populations of the two nominal species from egg to adulthood under controlled conditions in the laboratory, qualitatively assessing the genetic components of shell morphology, growth, and reproduction by holding the environment constant. The second aim of the present study underwent modification as our investigation proceeded. We initially planned to test for evidence of reproductive isolation between our populations of *F. rivularis* and *F. fragilis* using methods similar to those developed for use with the freshwater pulmonate snail *Physa* (Dillon et al. 2002, 2004, 2005 and 2007). Our preliminary surveys to identify genetic markers at allozyme-encoding loci, however, returned unexpected evidence of complete self-fertilization in our wild populations. This prompted us to recast our experiments as an investigation of whether the levels of self-fertilization we observed in the wild might be obligate or facultative (Städler et al. 1993 and 1995).

METHODS AND MATERIALS

Sampling sites

F. fragilis was collected from Potato Creek in Clarendon County, South Carolina (33.5735°N, 80.2926°W). The water body at the point of our sampling was about 3-4 m wide with a sand/mud bottom and negligible current. Limpets were collected primarily on the loose, floating stems of alligator weed (*Alternanthera sp.*), which were carried whole to the laboratory and trimmed with scissors. *F. rivularis* was collected from the North Saluda River in Greenville County (35.1271°N, 82.4264°W). The river was about 8-10 m wide at the point of our sample with rapid current over a bottom of sand and cobble. Limpets were lifted by scalpel blade from small rocks.

Culturing

Culture techniques were adapted from those of Wethington and Dillon (1993 and 1996) for *Physa*. For our initial comparisons of growth and reproduction in *F. rivularis* and *F. fragilis*, sets of five wild-collected limpets were placed into low, 260 mL polyethylene drinking cups containing 150 mL of filtered, aerated pond water. Petri dishes were used as covers. Seven such cups were initially established for *F. rivularis*, and 18 for *F. fragilis*. Culture was at room temperature (approximately 20°C) with a 12:12 light cycle. The food provided was a commercially-available green flake fish food with a *Spirulina spp.* base, very finely ground.

Eggs were laid in most cups within the first seven days of culture. Cups were drained, and adults were removed by trimming with scissors the small sections of cup wall on which they were adhering. Shell measurements were taken as described below, and adults were frozen for genetic analysis. The remaining portions of each cup wall (bearing eggs) were placed in new polyethylene cups with 150 mL of pond water and cultured as above, with water change and feeding every three-four days. Shell lengths

were measured for ten largest individuals in randomly chosen cups every week (Durrant 1976). After six weeks, adults of the laboratory-reared generation were trimmed from their cups and measured as below.

Culture techniques were similar for our hybridization experiments. We initially established 21 cups, each containing two wild-collected *F. rivularis* and two wild-collected *F. fragilis*. Cups were trimmed and adults removed at day 13, by which time egg laying had occurred. Then the first generation (wild-conceived but laboratory-born) was cultured for four-five weeks as above until the eggs of a second (laboratory-conceived) generation were observed in the cups. Adults of the first generation were removed by trimming, the portions of the cup wall bearing their laboratory-conceived eggs were transferred to fresh cups, and this second generation was reared to adulthood as before. Second generation progeny were tested for hybridity using allozyme methods as described below. A second hybridization experiment was conducted with 19 cups each containing one *F. rivularis* and one *F. fragilis*. Culture techniques through first and second generations were identical to those described above in all respects.

Shell morphometrics

Three shell measurements were taken on samples of both nominal species collected from the wild and reared in culture. The standard length was taken as the longest shell dimension, width was the maximum dimension perpendicular to length in the plane of the substrate, and height was the maximum dimension perpendicular to length and perpendicular to the plane of the substrate (McMahon 2004). Measurements were made using an ocular micrometer at 12x magnification on living animals manipulated on the cut fragment of the cup wall to which they were adhering. Sample sizes were N=26 for both wild-collected populations, N=15 for cultured *F. rivularis*, and N = 30 for cultured *F. fragilis*. The cultured individuals were measured six weeks post-hatch.

Differences in shell shape between our *F. fragilis* and *F. rivularis* populations were tested with analysis of covariance using the separate slopes model (JMP version 7). Four analyses were run, testing for differences in shell width and testing for differences in shell height (holding length as covariate) both on wild-collected populations and on populations reared in the laboratory.

Allozyme variation

Individual limpets were ground in 10 µL of 0.05 M tris tissue buffer 7.4 using a heavy gauge dissecting needle, the point of which had been trimmed blunt. The samples were thrn centrifuged briefly, and the supernatant was pipetted into the sample wells of 12% starch gels (Sigma Starch S5651) prepared using three different buffers -- the AP6 buffer of Clayton and Tretiak (1972), the TC6.8 buffer of Mulvey and Vrijenhoek (1981), and the TEB8 buffer of Shaw and Prasad (1970, "number III"). Horizontal electrophoresis was performed using equipment and techniques as described by Dillon (1992).

Gels were stained to reveal the activity of seven different enzymes. The AP6 gel was stained for glucose phosphate isomerase (*Gpi*), leucine aminopeptidase (*Lap*), isocitrate dehydrogenase (*Isdh*), and 6-phosphogluconate dehydrogenase (*6pgd*). The TC6.8 buffer was stained for *Gpi*, *Isdh*, phosphoglucomutase (*Pgm*), and mannose-phosphate isomerase (*Mpi*). The TEB8 gel was stained for *Gpi*, *Lap*, *6pgd*, and esterases (*Est*). Recipes for all enzyme stains are available in Dillon (1992).

Städler et al. (1993, 1995) have confirmed that inheritance of allozyme phenotype at the *Gpi* and *Lap* loci conforms to expectation for codominant Mendelian alleles. Breeding experiments with other freshwater pulmonates have also confirmed Mendelian

inheritance at *Pgm*, *6Pgd*, and the *Est* loci (Mulvey and Vrijenhoek 1984, Mulvey et al. 1988, Dillon and Wethington 1994). No experimental confirmation of Mendelian inheritance has been published for *Isdh* or *Mpi*, but our unpublished observations using variation at these loci to mark breeding experiments in *Physa* spp. have not suggested any unusual inheritance patterns. Wild populations of *Physa* typically show all expected genotypes for all these loci (Dillon and Wethington 1995 and 2006).

We initially screened for genetic variation in samples of N = 15 limpets wild-collected from each population, *F. rivularis* and *F. fragilis*. Subsequent analyses of larger samples focused on *IsdhF*, the only locus at which our initial survey uncovered evidence of genetic variation. Ultimately our sample sizes at the *IsdhF* locus were 104 wild-collected *F. rivularis* and 102 wild-collected *F. fragilis*. We used identical techniques to assess the hybridity of 61 second-generation limpets reared in culture.

RESULTS

The shell dimensions of *F. rivularis* and *F. fragilis* collected from the wild are compared in Figure 1. Limpets inhabiting rock substrates in the rapidly-flowing North Saluda River had shells more acutely conic than those of the populations inhabiting macrophytes and debris in the static waters of Potato Creek. Analysis of covariance returned significant values of t -- testing for an effect due to species in both shell height and shell width, holding length constant (Table 1). Qualitatively the shells borne by our Potato Creek population of *F. fragilis* were thin and translucent, while those of the *F. rivularis* population in the North Saluda River appeared thicker, stronger, and opaque.

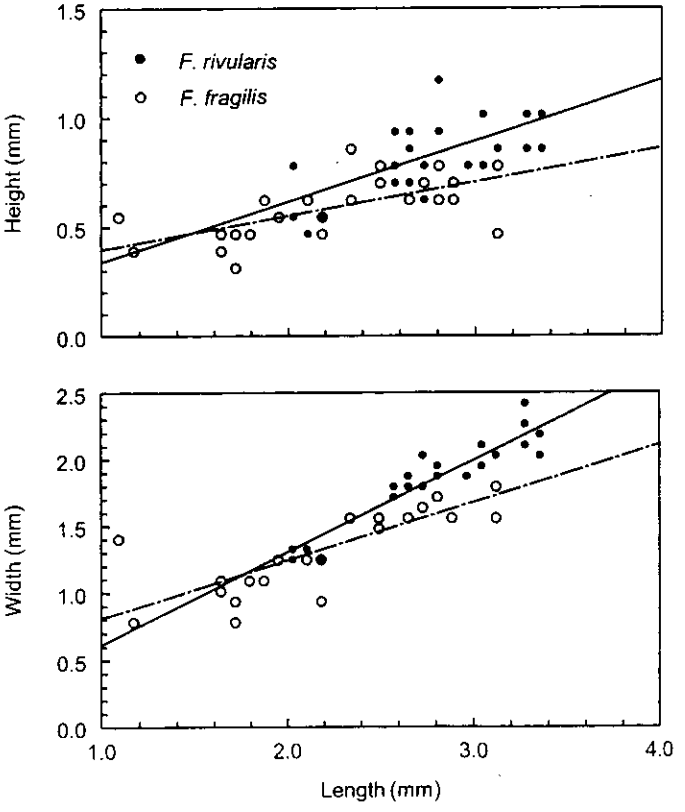

Figure 1. Shell height and width as functions of shell length in wild-collected populations of *F. rivularis* and *F. fragilis*.

Adult limpets began depositing eggs on the walls of their culture vessels within the first three days of their removal from the wild. Eggs were approximately 0.6 mm in diameter and laid singly, with negligible capsule. They hatched in approximately seven days. Both *F. rivularis* and *F. fragilis* reached adulthood between weeks four and five post-hatch (Fig. 2). The first eggs appeared on day 29 for *F. fragilis* and day 34 for *F. rivularis*, when maximum shell lengths ranged from 2.0 to 3.0 mm.

The shell width and height of cultured *F. fragilis* and *F. rivularis* are compared as a function of shell length in Figure 3. Analysis of covariance uncovered no significant effect attributable to species in either shell height or width in our laboratory-reared samples (Table 1). Qualitatively, all limpets hatched and reared in our artificial environment, regardless of parentage, bore extremely thin, nearly transparent shells.

Our initial screening of 15 wild-collected limpets from both populations uncovered no evidence of genetic variation at six of the seven enzyme systems we examined, representing eight putative loci -- *Gpi, Lap, Mpi, 6pgd, Pgm,* and *Est* (3 loci). Gels stained to disclose *Isdh* activity revealed two zones of activity, a (apparently monomorphic) zone migrating toward the anode (*IsdhS*) and a dimorphic zone (*IsdhF*) migrating cathodally. Both our *F. rivularis* and our *F. fragilis* populations contained mixtures of animals bearing bands migrating approximately 37 mm cathodally and 40 mm cathodally. Focusing on *IsdhF* and enlarging our sample size, we ultimately counted 54 individuals with the 37 mm band and 50 with the 40 mm band in the *F. rivularis* population, ($p_{37} = 0.519$ and $q_{40} = 0.481$). In our *F. fragilis* population we counted just 14 individuals with the 37 mm band and 88 with the 40 mm band, ($p_{37} = 0.137$ and $q_{40} = 0.863$). These frequencies were different significantly at the $P<0.001$ level (Yates corrected $\chi^2 = 32.3$, 1 df).

We observed reduced second generation survivorship in both trials of our hybridization experiment, ultimately recovering progeny from only 24 of the 40 cups initiated. The 16 successful cups yielded a total of 61 progeny in the second generation among them, with sibships ranging from one to nine. Analysis of allozyme phenotype at the *IsdhF* locus revealed that 60 of these 61 progeny bore single bands migrating at 40 mm. One sibship of four offspring contained three individuals with the 40 mm band and a single individual with the 37 mm band. No putative heterozygotes were observed.

Table 1. Statistics from four analyses of covariance (separate slopes), testing for differences in shell height and shell width between *F. rivularis* and *F. fragilis*, with shell length as a covariate. Degrees of freedom were 3,48 for the comparisons of wild-collected animals, and 3,41 for comparisons of animals from culture. Asterisks designate significance at the 0.05, 0.001, and 0.0001 levels, respectively.

	Height, wild	Width, wild	Height, culture	Width, culture
R^2	0.66	0.86	0.27	0.74
F	31.2***	100.6***	5.00*	38.2***
Intercept (t)	1.62	1.27	2.47*	3.86**
Species (t)	-3.41*	-4.06**	-0.42	-1.98
Length (t)	6.10***	12.53***	3.87**	10.4***
S x L (t)	-1.73	2.84*	0.29	-0.77

DISCUSSION

The growth rates demonstrated by both our populations in culture were similar to those of Burky (1971), who reported no definitive adult size in his New York populations of *F. rivularis* but suggested a minimum size at maturity of 2.5 mm. They are also quite similar to those of Basch (1959), who reported a 2.0 mm size at maturity and a generation

time of one month in his cultured population of *Ferrissia "shimekii"* (a synonym of *fragilis*) originating from a roadside pond in southern Michigan.

Our observation that eggs were laid as singletons by both our cultured lines is not unexpected for *Ferrissia*. Burky (1971) reported that one of his New York populations of *F. rivularis* averaged 3.1 embryos/capsule, while the other averaged only 1.2 embryos/capsule. Basch (1959) stated that eggs were always deposited singly in his cultured population of *F. fragilis*.

Our experiments returned no evidence that the morphological differences observable in wild populations of *F. rivularis* and *F. fragilis* have an additive genetic component. The acutely conic apex that distinguished the shells of the limpets from the North Saluda River disappeared in their laboratory-reared progeny. This suggests that variance in shell height and width in our populations may be plastic responses to current, substrate, or some other aspect of the riverine environment.

Ecophenotypic plasticity in shell shape is a well documented phenomenon in freshwater pulmonates (Arthur 1982, Lam and Calow 1988, Langerhans and DeWitt 2002). English populations of *Ancylus fluviatilis* sampled from riverine habitats tend to bear higher shells per standard width than populations sampled from lake shores (Durrant 1975, Sutcliffe and Durrant 1977), and Irish populations of *A. fluviatilis* sampled upstream bear higher shells than those sampled downstream (McMahon and Whitehead 1987). McMahon (2004) documented striking interannual variation in shell morphometric ratios calculated from a single population of *Laevapex fuscus* monitored over 15 years, strongly suggesting that such variance is not additively genetic.

Interpopulation variation in calcium metabolism has also been well documented in ancylid limpets (Russell-Hunter et al. 1967). Much of the variance in the $CaCO_3$ fraction of total shell weight in the 25 Irish populations of *A. fluviatilis* studied by McMahon and Whitehead (1987) was attributable to ecophenotypic plasticity. Such observations would account for the qualitative differences between fragile and robust shells often noticed in American *Ferrissia* populations sampled from the wild.

Our initial survey for allozyme variation returned surprisingly little evidence of genetic divergence between our *F. rivularis* and *F. fragilis* populations. The two populations appeared to be fixed for identical alleles at nine enzyme-encoding loci, including three *Est* loci and one locus each at *Gpi*, *Lap*, *6pgd*, *Pgm*, *Mpi*, and *IsdhS*. German populations of *A. fluviatilis*, by contrast, vary at five loci (Städler et al. 1995).

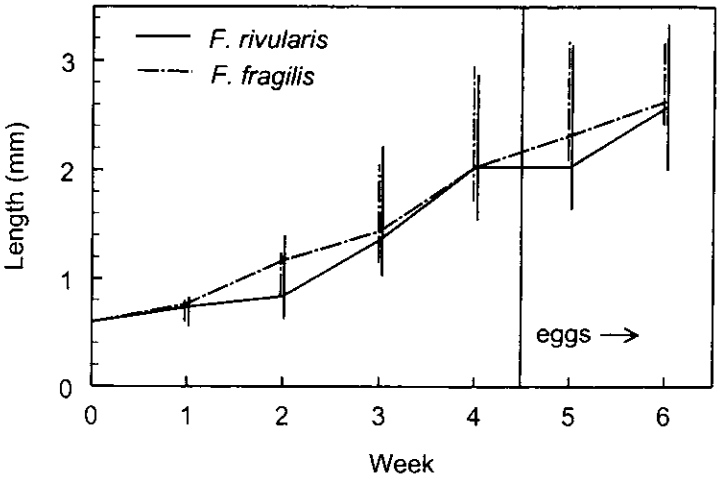

Figure 2. Median and range in shell length for the ten largest limpets in randomly chosen sibships over six weeks of culture.

Natural populations of *A. fluviatilis* do include rare heterozygotes. The breeding experiments of Städler et al. (1993 and 1995) estimated outcrossing rates of 2.3% and 10.3%. The complete absence of heterozygotes at the *IsdhF* locus in both of the limpet populations we sampled in South Carolina, however, constitutes strong evidence of self-fertilization in American *Ferrissia*. Our populations appear to contain mixtures of two non-recombining genomes, one marked by *IsdhF-37* and the other marked by *IsdhF-40*. The latter genome appears to be significantly more common in the population we sampled from Potato Creek.

We observed no copulation during the course of any of our breeding or rearing studies, nor did we recover any heterozygotes in the second generation of our hybridization experiments. Any conclusion that self fertilization is obligate in *Ferrissia* populations must be qualified, however, by evidence that the two genomes were not equally adapted to our culture conditions. Although all our crosses were initiated with equal numbers of wild-collected *F. rivularis* and *F. fragilis* parents, 60 of the 61 second-generation offspring we recovered were homozygous for the *IsdhF-40* allele. The single sibship of four that included one *IsdhF-37* homozygote is consistent with expectation if self-fertilization were obligate, however, rather than facultative.

The distribution of self-fertilization and outcrossing in freshwater gastropods have been the objects of a great deal of research (Jarne 1995, Jarne and Auld 2006, Jordaens et al. 2007). Most pulmonate snails seem to be preferential outcrossers, reproducing by self-fertilization only if no mate is available with a concomitant reduction in fitness

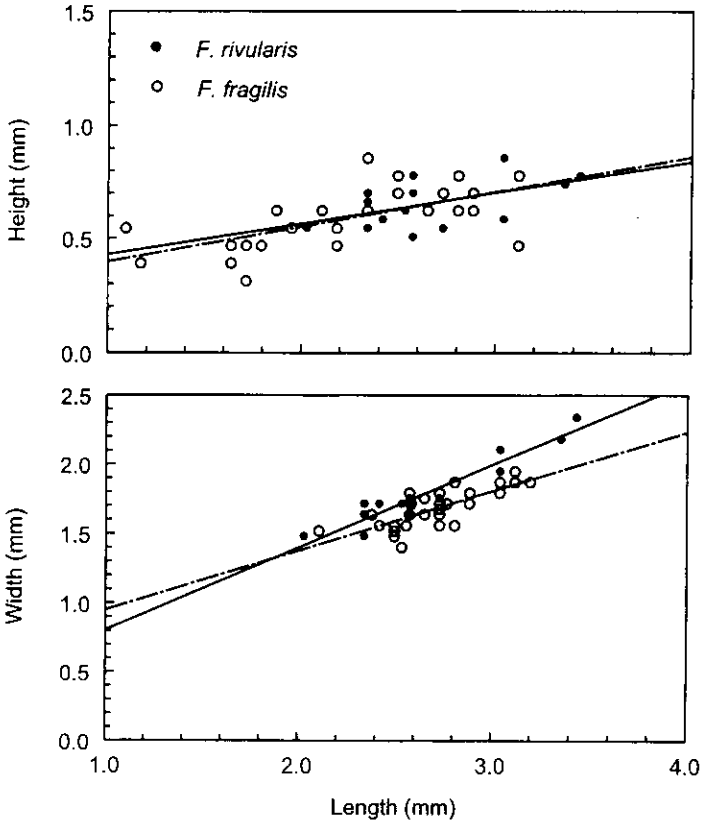

Figure 3. Shell height and width as functions of shell length in samples of *F. rivularis* and *F. fragilis* raised in culture.

(Jarne et al. 1993, Wethington and Dillon 1997, Coutellec-Vreto et al. 1998). Among the basommatophoran pulmonates, preferential selfing has been demonstrated only in certain populations of planorbids (Doums et al. 1996, Charbonnel et al. 2005) and lymnaeids (Trouvé et al. 2003), as well as in the *A. fluviatilis* populations previously noted.

Walther (2007) reported DNA sequence results suggesting that North American *Ferrissia* populations can be divided into two widely-distributed clades, one including nominal *F. rivularis* and the other *F. fragilis*. Samples of two individuals from each of our two populations shared identical sequences at the nuclear 28S locus and varied minimally at ITS-2, suggesting that all four individuals were referable to the *F. fragilis* clade (pers. comm., A Walther[a]). It is possible, however, that Walther's *F. fragilis* and *F. rivularis* clades correspond to the genomes marked by our *IsdhF-37* and *IsdhF-40* alleles. The chance of matching four *IsdhF-37* individuals or four *IsdhF-40* individuals in two limpets drawn from each of our two populations would be approximately 18%.

The situation in American *Ferrissia* thus seems quite similar to that of the better-studied *A. fluviatilis* in Europe, where phenotypic plasticity produces populations with acutely conic shells in riverine environments and lower shell heights in lentic environments and where variable $CaCO_3$ metabolism yields shells of differing thickness. All such populations have nevertheless always been referred to the single specific nomen, "*fluviatilis*." The recent discovery that *A. fluviatilis* populations are largely self-fertilizing has led to the description of numbered "clades," not new species (Pfenninger et al. 2003).

In summary, the evidence we have obtained strongly pointing to self-fertilization in American populations of *Ferrissia* voids the biological species concept, and necessitates a retreat to the morphological. And since our experiments have returned no evidence that previously described morphological differences have a heritable component, we suggest that *F. fragilis* (Tryon 1863) be considered a junior synonym of *F. rivularis* (Say 1817).

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[a] Andrea Walther, Department of Biological and Allied Health Sciences, Ohio Northern University, Ada, OH. 45810

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