Research Note

Sperm storage and evidence for multiple insemination in a natural population of the freshwater snail, *Physa*

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Abstract. Although a number of estimates are available regarding the capacity of freshwater pulmonates to store sperm after laboratory mating, few such data are available for wild-collected snails, where the recency of mating is necessarily unknown. We collected 35 *Physa heterostropha pomilia* (Conrad) from a local population and held each in isolation for 60 days, rearing all egg masses. We then used protein electrophoresis to determine the LAP genotype of each parent and a sample of its offspring. Five of the parental snails (total 60 day fecundities from 300 - 600 progeny) were found to be homozygous at the LAP locus yet producing approximately 50% heterozygous offspring. In 4 of these 5 cases, no significant difference was detected in offspring genotype frequencies over 60 days, suggesting both that mating has generally been recent and that reservoirs of stored sperm are, as a rule, large. In the fifth case, the frequency of heterozygous offspring increased significantly, suggesting multiple insemination. Multiple insemination and sperm storage have obvious adaptive significance to colonizing species such as freshwater pulmonates.

The capability to store sperm, especially sperm contributed by multiple partners, has the potential to lessen the severity of genetic drift by increasing the effective population size represented by a small number of survivors or colonizers. And the capacity of an individual pulmonate snail to store sperm can be prodigious. In *Bulinus*, laboratory mating has been reported to provide enough sperm to fertilize from 1000 - 2000 eggs up to 4400 eggs, depending particularly upon the reproductive condition of the snail acting as female (Rudolph, 1983; Rudolph and Bailey, 1985). In *Biomphalaria*, longevity of stored sperm has been variously estimated as 25 - 68 days (Paraense, 1955), 42 days (Richards, 1973), and more than 100 days (Monteiro *et al.*, 1984). Vianey-Liaud *et al.* (1987) reported a mean of about 50 days with a range from 3 days to 127. Cain (1956) reported some exogenously fertilized egg production by *Lymnaea stagnalis* Say up to 116 days after isolation. All these studies have involved lab crosses with pigment variants (usually albinism) as a genetic marker. Rollinson and Wright (1984) used isozyme markers to demonstrate sperm storage up to 70 days after laboratory mating of Mauritian *Bulinus*.

The effect of a delay in oviposition by the mother (as by a severe winter or desiccation on a birds foot, for example) has been investigated by Rudolph and Bailey (1985). Apparently *Bulinus* can store viable exogenous sperm through a minimum of seven weeks of starvation, eight weeks of low temperature (10 - 15°C) or four weeks of desiccation.

Rudolph and Bailey (1985) also reported some fairly strong evidence that *Bulinus* can store sperm from more than a single male simultaneously. Although short-lived copulatory plugs have been described in several pulmonate species, behavioral observations nevertheless suggest that multiple insemination could be common in laboratory situations (Rudolph, 1979a, b; van Duivenboden and ter Maat, 1988). Duncan (1959) reported that he had not observed reciprocal copulation in either *Physa fontinalis* (L.) or *P. acuta* (Drap.). But we have observed both reciprocal copulation and multiple mating behavior in our laboratory populations of *Physa*, without genetic markers for confirmation.

Using isozyme markers, Mulvey and Vrijenhoek (1981) found strong evidence of multiple paternity in clutches of eggs laid by isolated wild-caught *Biomphalaria*. It could not be determined, however, if the sperm used to fertilize these eggs came from two different exogenous sources, or from a combination of one outcross and selfing. It does in fact seem that fertilization can proceed with endogenous and exogenous sperm simultaneously, at least in some situations (Paraense, 1955; Monteiro *et al.*, 1984; Rollinson, 1986). But multiple insemination has been conclusively documented by Rollinson *et al.* (1989), using stocks of *Bulinus cernicus* (Morelet) homozygous for three different alleles at the Gpi locus.

As important as they are, data from lab crosses such as these do not directly address the likelihood of genetic drift after a population crash or founder event. A second set of

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variables is involved: how recently will an arbitrarily chosen pulmonate snail have mated? On the average, how healthy and fecund will it be? Rollinson (1986) has performed experiments bearing on this question using several species of African *Bulinus*. He isolated individual wild-caught snails and collected and reared their eggs up to 60 days. He then examined isozyme phenotype of both parent and offspring at four polymorphic loci. The large majority of these parents did lay demonstrably outcrossed eggs, apparently using stored sperm. Stored sperm was used for at least 41-44 days. Intriguingly, Rollinson found one *Bulinus scalaris* (Dunker) clearly producing offspring from two different fathers.

The great majority of all observations to date have involved lab matings of the tropical planorbids *Bulinus* and *Biomphalaria*. So to test the generality of the sperm storage phenomenon in natural populations of temperate pulmonates, we performed an analysis similar to Rollinson's on a local population of *Physa*.

METHODS

Physa heterostropha pomilia (Conrad) is a widespread and variable species, found throughout eastern North America (Wurtz, 1949; Burch and Tottenham, 1980). Recently Te (1978, 1980) has suggested that this taxon, along with the majority of North American species, be separated into the resurrected genus *Physella*. But we agree with Taylor (1988) that the distinction between *Physella* and *Physa* (s.s.) is more properly at the rank of subgenus.

We collected *Physa heterostropha pomilia* from a pond at Charles Towne Landing, a state park within the city limits of Charleston, South Carolina. The snails did not appear to be common on this nor on any subsequent trip, and we felt it possible that individual encounters and matings could be infrequent in this population. Voucher specimens have been deposited in the Academy of Natural Sciences of Philadelphia.

For an initial survey of polymorphism, we homogenized 62 snails in a 7% sucrose solution, buffered at pH 7.4 with 0.05 M tris (hydroxymethyl) aminomethane and H_3PO_4, to which xylene cyanole had been added as marker. Samples were centrifuged and horizontal starch gel electrophoreses was performed on the supernatant using standard techniques (Dillon and Davis, 1980; Dillon, 1982, 1985). The 14% starch gels were made of 3 parts Sigma starch: 1 part Electrostarch and AP6 buffer, diluted 19:1. AP6 buffer is 0.04 M citric acid (monohydrate) adjusted to pH 6 with N−(-3-aminopropyl) morpholine (Clayton and Tretiak, 1972). Gels were run for approximately 4.5 hr at 40 volts under refrigeration. They were then sliced and stained for leucine aminopeptidase (LAP) using the recipe of Shaw and Prasad (1969).

Two isozymes were found to be segregating in a fashion consistent with Hardy Weinberg expectation in the Charles Towne Landing population, at gene frequencies of 0.58 and 0.42. We have designated the more common isozyme "LAP 100", and the isozyme migrating 3 mm faster in our gel conditions "LAP 103".

We returned to Charles Towne Landing in May, 1989, and collected 35 adult *Physa*, placing each in a separate 10 oz plastic cup of pond water with a plastic petri dish cover. We fed them commercial Tetra-Min "Conditioning" food for plant-eating fish (Jennings *et al.*, 1970), and changed the water with fresh, aerated pond water periodically. Each parent was checked daily and transferred to a new cup when an egg mass was produced, in a fashion similar to that of Rollinson (1986). Water was changed once a week for the adults (along with newly laid egg masses) and once every other week for the juvenile snails when they had grown to a size at which this could be done safely.

The experiment was terminated in July (after 60 days), by which time 29 of the parents had reproduced, some prolifically and others much less so. We then determined the genotypes of each parent and a sample of early laid offspring at the LAP locus. Of the 15 LAP homozygotes identified, four were producing all homozygous progeny and the remainder were producing high frequencies of heterozygotes, obviously using exogenous sperm. We did not find any case where a homozygous parent was producing entirely heterozygous offspring, as could be expected from a single outcross to the opposite homozygote. But we selected for further study the five largest sibships from the 11 including heterozygotes. We then compared the frequency of heterozygotes in 20 to 30 offspring from the earliest egg masses in these sibships to a similar sample from the last laid sibships.

RESULTS

Data on the fecundity of these five snails (A through E) during the 60 day study period are presented in figure 1. The figure shows the cumulative number of juveniles surviving to countable size. It can be seen that all parents continued to lay eggs throughout the entire period, although the rate slowed, especially after day 45. Total viable egg masses ranged from 15 to 19, and the total countable offspring ranged from about 300 to 600.

Table 1 shows the frequency of heterozygotes in 20 to 30 offspring of the first laid sibships and the frequency of heterozygotes in similar sized samples from the last laid sibships. Genotype frequencies were not significantly different from 1:1 (chi square, Yates corrected) in any of the five groups of first laid offspring. This is consistent with Mendelian expectation if each mother had mated with a single heterozygous father.

Table 1 also shows that in four of the five cases, there was no significant difference between the genotype frequencies in the first laid sibships and the last laid sibships.

Table 1. LAP genotypes among the offspring of five parent *Physa*, first laid sibships compared to last laid sibships.

Parent	LAP Genotype	Day Number of Oviposition	Number of Homozygotes	Number of 100/103 Heterozygotes	chi-square
A	103/103	4,9	20	15	1.44
		58,60	16	5	
B	100/100	7,11	16	17	6.63**
		51,52,53,56	2	18	
C	100/100	2,8	17	13	0.36
		56,57,58	11	7	
D	103/103	2	19	15	1.04
		44,51,56	9	14	
E	103/103	7	13	17	0.13
		51,52,56	12	11	

**P < 0.01

The one significant value of chi square (contingency test, Yates corrected) is shown in the offspring of parent B, where there was an unexpected excess of heterozygotes among the offspring laid in the final days of the experiment.

DISCUSSION

We do not know whether the total fecundities reflected in figure 1 overestimate or underestimate production by a single *Physa* founding a new population in the wild. On the one hand, we attempted to provide food in excess and protected the juveniles from predation. But competition among juveniles could have been an important factor in our plastic cups, especially on occasions when as many as three egg masses were laid overnight.

Although it can be seen that all parents continued to lay eggs for the entire 60 day period, there was a reduction in the number of viable embryos per egg mass through time. For example, the last three egg masses laid by snail E, on days 57, 58, and 60, contained no viable embryos at all. Qualitatively these results are similar to those obtained by Duncan (1959) with English *Physa fontinalis*, although *P. heterostropha pomilia* fecundities seem to be higher. As previous work suggests a gradual shift from exogenous to endogenous sperm (Paraense, 1955; Cain, 1956), we initially interpreted this observation as evidence of depleted sperm stores. But Table 1 shows that after 60 days and as many as 600 offspring, none of these individuals seems to have exhuasted its reservoir of stored sperm. In retrospect, our decision to terminate the experiment was premature.

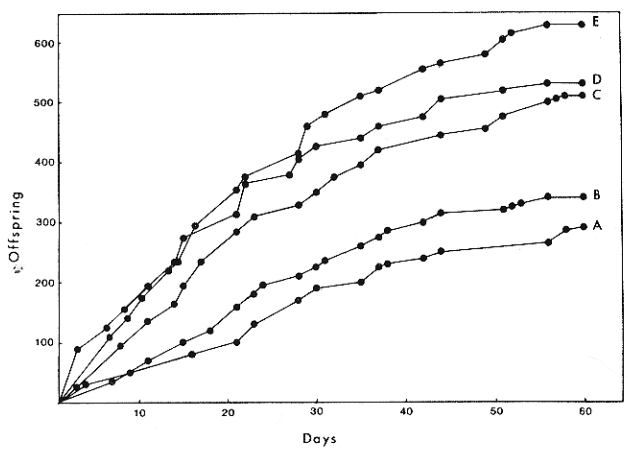

Fig. 1. Cumulative 60-day fecundity (offspring surviving to countable size) of the five *Physa* examined for sperm storage.

The excess of heterozygotes observed among the progeny of parent B is not easily explained as the result of a single pair mating. If the offspring were fathered by a single heterozygous individual, one would expect a 1:1 ratio of homozygous to heterozygous progeny. This is indeed the ratio observed in all early sibships, laid on days 2 through 11 (Table 1). But the sample of snail B progeny from days 51, 52, 53, and 56 contained 18 heterozygotes and only 2 homozygotes, very significantly different from 1:1. Even combining all 53 progeny examined from parent B, one still obtains a significant excess of heterozygotes (goodness-of-fit chi square = 4.79). Nor can these results be explained by a single cross to a homozygous father, as no homozygous progeny would have been expected at all. To argue that self fertilization played any role, one would need to postulate that parent B was at least partly self fertilizing to start but increasingly shifted to exogenous sperm as the days in isolation passed, quite counter to all previous observations on other pulmonates.

By far the most likely explanation for the results from parent B is multiple insemination by both a heterozygote and a homozygote for the opposite allele. The first eggs seem to have been fertilized by sperm from the former, and the last eggs by the latter, in a fashion similar to that inferred for *Biomphalaria* (Mulvey and Vrigjenhoek, 1981) and well documented for *Bulinus* (Rudolph and Bailey, 1985; Rollinson, 1986; Rollinson *et al.*, 1989). Clearly more work is called for, possibly using multiple loci as markers for different parents. But if multiple insemination is in fact

widespread, individual pulmonate snails surviving colonization events, hard winters or severe storms could potentially represent a great deal of genetic variation indeed.

Given the apparently large capacity *Physa heterostropha pomilia* displays for sperm storage, it would be interesting to see how frequently an average snail mates, and in what capacity. The great majority of freshwater pulmonate species show either simultaneous development of both reproductive tracts, or are slightly protandric (Russell-Hunter and McMahon, 1976; Rudolph, 1983). The male organs develop before the female organs in *P. fontinalis*, although environmental conditions in the wild do not in general favor mating until both organ systems are mature (Duncan, 1959). It is not clear whether fully mature pulmonates of any sort prefer to mate as a certain sex, whether a snail's sexual role can change with size or sperm stores, or how often snails switch roles in single encounters. Multiple insemination introduces questions of sperm competition and sperm "sharing" (Monteiro *et al.*, 1984; Vianey-Liaud *et al.*, 1987).

ACKNOWLEDGMENTS

We thank Charles Towne Landing State Park and Mike Dorn for access to the *Physa* population and Dr. Margaret Mulvey for her advice on pulmonate culture.

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Date of manuscript acceptance: 10 September 1990