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Reproductive development in the hermaphroditic freshwater snail *Physa* monitored with complementing albino lines

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SUMMARY

We estimated age and size at onset of functional outcross- and self-fertility in three isofemale lines of *Physa heterostropha* carrying the recessive, non-allelic genes, *alb1* and *alb2*. 'Experimental' snails of these lines were reared from 2 weeks of age to adulthood and periodically introduced to mature, complementing 'challenge' snails. The three lines differed significantly in the size at which they matured, but not the age, under our culture conditions. Male reproductive maturity was reached at a mean of 5.7 weeks and female reproductive maturity at 7.3 weeks ($n = 50$). These ages correspond to mean shell lengths of 5.3–6.2 mm as male and 6.9–7.6 mm as female. Over all three lines, first production of viable offspring by self-fertilization occurred at a mean age of 22 weeks ($n = 113$). Again a significant line effect on size at reproduction was detected, with mean size at onset of self-fertilization ranging from 8.1 mm to 8.7 mm. Autosterility in the three lines ranged from negligible to 44%. Among autofertile snails, we identified cases of outcross male-sterility, outcross female-sterility, and outcross double-sterility. Of 46 individuals demonstrating male function before female, two passed through brief periods of self-fertilization before outcrossing as females. We also identified three individuals maturing simultaneously in both capacities and one individual that matured as female before male. So, although the situation is complex, we suggest that 'simultaneous hermaphroditism' provides the best description of the reproductive biology of *Physa*. The great diversity of reproductive allocation described here implies considerable potential for life-history evolution in these snails.

1. INTRODUCTION

Basommatophoran pulmonate snails are among the more common and conspicuous inhabitants of the world's fresh waters. Because of their ubiquity and relative ease of culture, pulmonates have been the subject of a wide variety of inquiry at the behavioural, ecological, physiological, and cellular levels (Fretter & Peake 1975, 1978). Particularly well studied have been the planorbids and lymnaeids serving as intermediate host for parasitic disease (Malek & Cheng 1975). General reviews of reproduction in freshwater pulmonates are provided by Duncan (1975) and Geraerts & Joosse (1984).

Thorough anatomical descriptions of pulmonates include those of Crabb (1927), Duncan (1958, 1960), Bayne (1973) and Paraense (1976). The gonad is called an 'ovotestis', and both egg and sperm are carried through an hermaphroditic duct into a region termed the vesicular seminales, where endogenous sperm (autosperm) is believed to be stored. From here the female and male systems branch, the male system being joined by a prostate gland and leading into the penial complex. The female system consists of an albumen gland and oothecal gland (or oviduct) which leads to the vagina. Along the vagina is a bursa copulatrix for storage of foreign sperm (allosperm).

Before a completely hermaphroditic adulthood, maturing pulmonates may pass through a developmental stage during which they are functionally male. Duncan's (1959) histological studies of *Physa fontinalis* suggested that male maturity was attained in the winter and female maturity in the spring, but he noted that environmental conditions in the wild would not in general favour mating until both organ systems mature. Spermatogonia develop about two weeks before oogonia in *Lymnaea stagnalis* (Duncan 1975), and male maturity also precedes female in the freshwater limpet, *Laevapex fuscus* (Russell-Hunter & McMahon 1976). Rudolph (1983) observed that *Bulinus globosus* are able to copulate successfully as males before their female systems mature. In contrast to these studies, however, Richards (1962) found that the female organs of the planorbid *Gyraulus* developed after 17 d but that 60 d were required for male maturity.

The ability of freshwater pulmonates to reproduce by self-fertilization was first noted by Colton (1918), and with occasional exceptions (DeLarambergue 1939) seems universal (Vianey-Liaud 1976; Paraense & Correa 1988; Jarne & Delay 1990). Selfing individuals

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typically begin to lay eggs much later than paired snails and produce fewer viable young (DeWitt 1954; DeWitt & Sloan 1959; van Duivenboden 1983; Jarne & Delay 1990; Jarne *et al.* 1991). Some authors have noted improved survivorship in selfing pulmonates, however. Van Duivenboden *et al.* (1985) considered the time *Lymnaea stagnalis* spent in copulation detrimental to egg laying, noted that outcrossing snails died earlier, and concluded that self-fertilization was beneficial. And Noland & Carriker (1946) found that *Lymnaea stagnalis* laid more eggs when selfing than when allowed to outcross. Thus selfing seems to have a negligible or even positive effect on fitness, at least under laboratory conditions (Smith 1981).

An isolated pulmonate snail that has begun to self-fertilize generally switches to fertilization by allosperm immediately after copulation with a partner. Egg masses laid by albino *Biomphalaria glabrata* after copulation with a pigmented partner contain either 100% pigmented offspring or an increasing percentage of pigmented offspring in successive egg masses (Paraense 1955; Richards 1973). Rollinson *et al.* (1989) noted that 70% of outcrossed *Bulinus cernicus* switched to allosperm immediately.

In 1989 we began a research program to study the reproductive biology of *Physa heterostropha pomilia* (Conrad). Rearing experiments suggested that *Physa* collected from the wild may carry allosperm for over 60 days (in quantities sufficient to fertilize a minimum of 300–600 eggs) and that multiple insemination may occur (Wethington & Dillon 1991). Albinistic individuals were discovered at low frequencies among the offspring of four adults kept for these experiments, perhaps the result of low, background levels of selfing by heterozygotes. Albinism was shown to be inherited as a recessive trait at two complementing loci (Dillon & Wethington 1992).

All studies on the onset of reproductive maturity in pulmonates to date have been histological or behavioural. Here we use albino lines as tools to describe the onset of functional reproductive maturity and self-fertilization, and to assess rates of sterility.

2. MATERIALS AND METHODS

Experimental animals for these studies were taken from three of the isofemale lines characterized by Dillon & Wethington. These lines were founded from small numbers of albino offspring (perhaps only single individuals) produced by three different wild-collected females in the summer of 1989. Lines 27 and 29 are fixed for a recessive albino allele at the *alb1* locus, and line 7 is homozygous recessive for albinism at the complementing locus *alb2*. These lines have been mass-cultured in large containers since their initial bottlenecks, so that further inbreeding before the inception of these experiments in 1990 should have been minimal.

The experimental design involved 73 pairs of snails consisting of an adult 'challenge' snail from one albino line and a maturing 'experimental' individual from a second, complementing line. 'Challenge' snails were reared to adulthood in isolation, and were producing viable albino offspring at time of pairing. Experimental snails were isolated at about age 2 weeks and introduced for a period of 24 h weekly into a vessel containing the challenge individual. When the challenge snail first began to lay eggs that developed into pigmented offspring, the experimental snail was judged mature in male function, and when the experimental snail itself began producing pigmented offspring, it was considered mature as an outcrossing female.

Initially we had some concern regarding possible delays between successful outcrossing and the production of pigmented embryos. But five pairs of complementing challenge snails, placed together for 24 h, all produced 100% pigmented offspring in their next egg mass. We were also concerned that the disparity in the sizes of the experimental and challenge snails might influence onset of maturity. But 45 complementing pairs of 2-week-old juveniles reared together produced pigmented young at ages and sizes consistent with those observed in the main experiment.

For use as challenge snails, we isolated 107 two-week-old juveniles from isofemale line '7', 53 juveniles from isofemale line '27', and 54 individuals from isofemale line '29'. The snails were reared in 10 oz. (approximately 220 ml) clear plastic cups of pond water with Petri dish covers, at room temperature and ambient light. They were fed commercial Tetra-Min 'Conditioning' food for plant-eating fish once a week (Jennings *et al.* 1970), with fresh pond water (aerated, filtered through an 80 μm mesh) in alternate weeks. Culture vessels were checked weekly for the presence of egg masses and all snails laying egg masses were measured with an ocular micrometer or calipers. Snails were considered available for use as 'challenge' individuals when the viability of their self-fertilized egg masses was verified, as judged by the presence of actively crawling young.

The first 37 challenge snails, including an approximately equal number of *alb1* (line 7) and *alb2* (line 27 or 29) individuals, became available at about age 20 weeks. A similar number of two-week-old virgin snails were isolated from complementing cultures to become experimental snails. Once a week, each experimental snail was measured with an ocular micrometer and paired with its challenge snail partner for 24 h. Egg masses laid by both the challenge and experimental snails over the following 6 d were saved and scored as to pigmentation. This was accomplished on embryos as early as 4 d after laying by noting eye coloration through a dissecting microscope. A second set of 36 pairs of snails was started 2.5 weeks after the first, for a total of 73 trials.

Each experiment was terminated when pigmented embryos were discovered among the offspring of both experimental and challenge snail, or when either member of the pair died. If both snails had not produced pigmented embryos after 15 weeks, outcross sterility on the part of at least one of the partners was considered probable. All data from such pairs were excluded from analysis of maturation time. Most of the putatively outcross-sterile individuals were paired with new individuals of proven reproductive ability to verify the nature of the sterility.

The three lines were compared in the age and size at which they matured in a variety of capacities by using one-way analysis of variance (SYSTAT; Wilkinson 1988).

3. RESULTS

(a) *Self-fertilization*

Table 1 shows statistics on age and size at production of first offspring by self-fertilization in a total of 113 snails from three lines. An analysis of variance testing for a significant line difference in age at earliest successful self-fertilization gave an F-ratio of 0.67, not significant (with 2 d.f., $p = 0.51$). Over all three lines, first onset of selfing occurred at age 14 weeks and

Table 1. *Statistics on age and size at first production of viable eggs by self-fertilization*

	line 7	line 27	line 29	total
n	59	16	38	113
mean age/weeks	22.0	22.9	21.6	22.0
(s.d.)	(3.1)	(6.54)	(3.39)	(3.83)
range	14–29	16–42	16–32	14–42
mean size/mm	8.7	8.7	8.1	8.5
(s.d.)	(0.78)	(1.21)	(0.85)	(0.91)
range	7.3–10.5	7.1–11.2	6.8–11.2	6.8–11.2

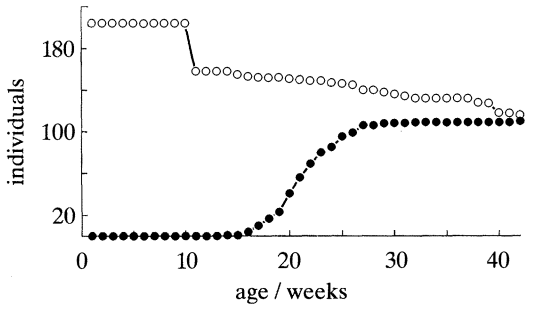

Figure 1. Cumulative onset of successful self-fertilization (filled circles) in the 214 snails reared in isolation. Survivorship curve (open circles) does not reflect survivorship of snails removed to serve as challenge individuals in the outcross study.

extended to 42 weeks (figure 1), with 21.9 weeks the mean age at which the snails began to self-fertilize successfully. There were, however, very significant line differences in shell size at onset of selfing ($F = 5.55$, $p = 0.005$). Table 1 shows that mean size at onset of self-fertilization in line 29 was 8.1 mm, a value much smaller than observed for lines 7 and 27.

Figure 1 also shows the combined survivorship of all challenge snails over the 42 week course of the experiment. (High apparent mortality at week 10 is an artefact of data collection.) Mortality complicates the estimation of autosterility rates. A glance at figure 1 shows that percentage autosterility ranges from 100% if estimated at week 1 to a very low figure if estimated at week 42. We selected week 30 as a fair time at which almost all individuals had an opportunity to self-fertilize but before the base population against which a frequency might be calculated had begun to shrink noticeably. There were 135 snails which survived to age 30 weeks, by which time 110 had successfully reproduced by self-fertilization. (One individual from each of the three lines first selfed at a later date.) A total of 12 out of 70 (17%) of line 7 individuals and 12 out of 27 (44%) of line 27 individuals had not reproduced by that time and may be judged autosterile, but autosterility in line 29 was negligible. Of 38 line 29 individuals surviving to week 30, the single putatively autosterile individual subsequently reproduced successfully.

(*b*) *Outcrossing*

Table 2 shows statistics on age and size at outcrossing maturity in 50 experimental snails from sets 1 and 2, categorized by line. An analysis of variance comparing the three lines in age at attainment of male function did not suggest significant differences ($F = 2.04$, $p = 0.14$), nor did a test comparing lines in age at onset of female function ($F = 1.11$, $p = 0.34$). Summed over all three lines and both data sets, mean age at attainment of male function was 5.7 weeks, and age at attainment of female function 7.4 weeks.

Line differences in shell size at onset of outcrossing maturity were more striking than line differences in age, however. Analysis of variance testing for a difference among lines in size at onset of male maturity gave a highly significant result ($F = 9.43$, $p < 0.001$). In this case line 7 attained male function at a substantially larger mean size than lines 27 or 29. The difference in size at attainment of female function was also large, although not quite significant ($F = 2.97$, $p = 0.06$).

Results of the two sets of outcrossing experiments are shown separately in figure 2, combining experimental snails of lines 7, 27, and 29. (Combining lines would be legitimate when age is plotted on the abscissa, although not when size is plotted.) It is clear that the onset of outcrossing male function generally preceded female

Table 2. *Statistics on age and size at onset of outcrossing maturity*

(Included are experimental snails ultimately maturing as both male and female in data sets 1 and 2 combined.)

	as male			as female			total	
	line 7	line 27	line 29	line 7	line 27	line 29	male	female
n	21	15	14	21	15	14	50	50
mean age/weeks	6.0	5.7	5.8	7.7	7.1	7.3	5.7	7.4
(s.d.)	(1.08)	(0.90)	(1.64)	(1.64)	(0.97)	(1.00)	(1.24)	(1.31)
range	4.5–9.0	4.0–8.5	4.0–10.5	6.0–12.0	5.5–9.0	6.0–9.5	4.0–10.5	5.5–12.0
mean size/mm	6.2	5.3	5.5	7.6	7.4	6.9	5.7	7.3
(s.d.)	(0.58)	(0.45)	(0.88)	(0.94)	(0.88)	(0.48)	(0.75)	(0.85)
range	5.2–7.6	4.6–8.2	4.4–7.8	5.9–9.9	6.3–9.7	5.9–7.5	4.4–7.8	5.9–9.9

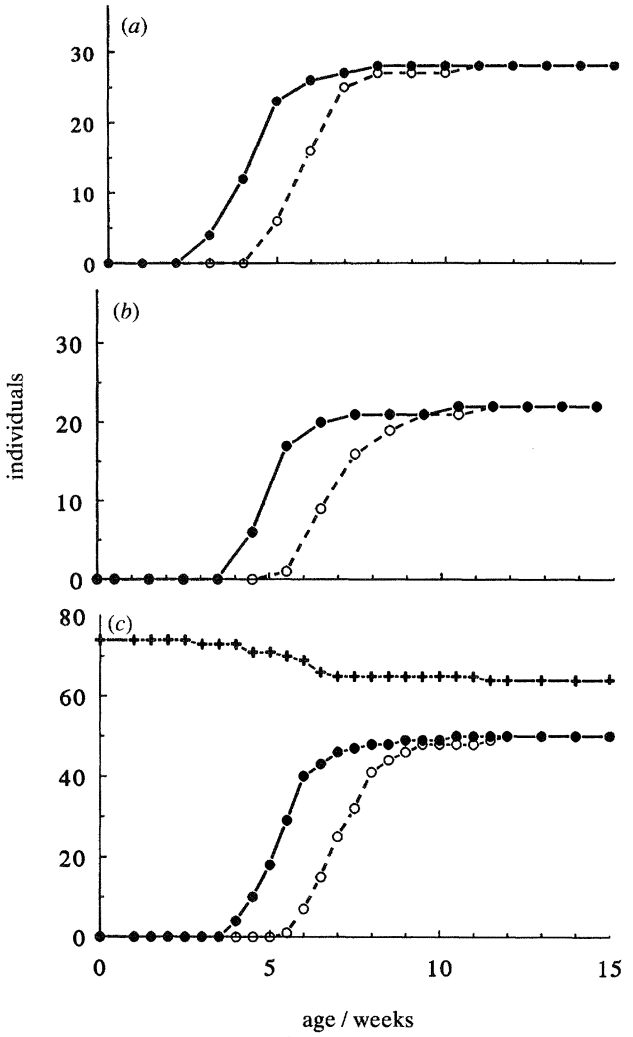

Figure 2. Cumulative onset of male (filled circles) and female (open circles) function in experimental snails of data sets (*a*) 1, (*b*) 2, and (*c*) both combined. The composite graph also shows the combined survivorship of either member of a pair over the 15 weeks of the outcross study (crosses).

function by about two weeks under our culture conditions. However, data set 2 snails matured substantially later than set 1 snails in both capacities.

Table 2 shows that no additional snails matured in either capacity after age 12 weeks. A total of ten deaths occurred among either challenge or experimental snails over the first 12 weeks of these experiments. Then setting aside the 50 fertile pairs ultimately involved in the maturation study, the remaining 13 pairs manifested outcross sterility by at least one partner. Crosses of individuals from most of these pairs to complementing snails of proven reproductive ability allowed us to infer four cases of male-sterility and one case of double-sterility among the experimental snails. Among challenge snails (all demonstrably autofertile) we inferred three cases of male outcross-sterility, two cases of female outcross-sterility, and one double outcross-sterility.

It is interesting to note that juvenile growth averaged almost exactly 1 mm per week in our culture conditions, so that mean shell size in millimetres at reproductive maturity almost exactly equaled mean age at maturity in weeks. In figure 3 all 50 experimental

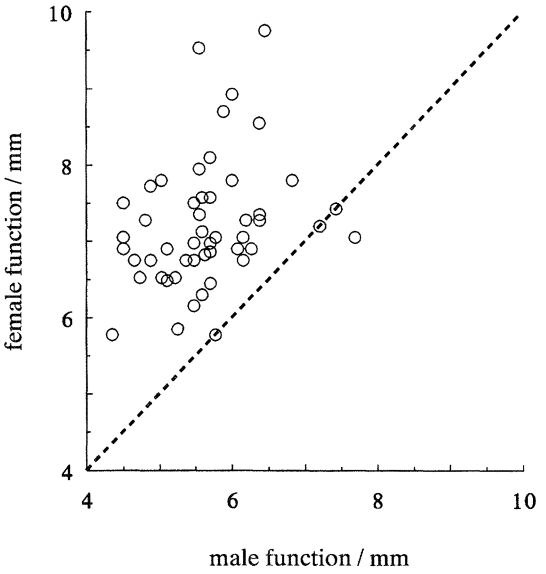

Figure 3. Shell size at which each of 50 snails from data sets 1 and 2 attained male and female function. Data on the diagonal mark individuals maturing in both capacities simultaneously.

snails are plotted by the size at which they matured in both capacities. The data averages presented in table 2 and figure 2 obscure our observation that, although 46 experimental snails did achieve male function before female, three individuals matured simultaneously in both capacities and one matured as a female first. The 46 individuals achieving male function first included two that passed through a period of self-fertilization before outcrossing as females.

4. DISCUSSION

The maturation process in *Physa* is complex. Figures 1 and 2 show no clear threshold size or age by which male function, female function, or self-fertility can be predicted, even under laboratory conditions. Both genetic and environmental components of the variance may be identified. Comparing data sets 1 and 2, a two-week delay in age at onset of both male and female function was evident in all three lines. This is almost certainly due to temperature or some other aspect of our culture technique.

Small but significant differences in the size at maturation among the three lines almost certainly have a genetic basis. Our observations suggest that growth in line 7 snails was more rapid, such that they matured at the same age but at a slightly larger size than lines 27 or 29. Growth seemed to slow earlier in line 29 snails than in lines 7 or 27, such that line 29 snails tended to reproduce by self-fertilization at a smaller size. This is consistent with our observation of higher autofertility in line 29, as these snails would be expected, on average, to allocate more energetic resource to reproduction than to growth in later weeks.

Individual *Physa* do, in general, pass through a purely male stage of about 2 weeks duration before onset of hermaphroditism. This phenomenon, often referred to as 'slight protandry' (Russell-Hunter & McMahon 1976), has been described in *Lymnaea*,

Bulinus, and English *Physa fontinalis*. But Hoagland (1984) argues that the term 'protandry' is correctly applied only to organisms that function first as male and then subsequently as female, and should not be used to describe animals simultaneously making eggs and sperm. She points out that if we apply the label 'simultaneous hermaphrodite' only to those animals that produce eggs and sperm precisely at the same time, 'we will have defined the term almost out of existence'.

Physa would seem to provide an excellent illustration of the difficulties inherent in labelling reproductive function for, among 50 snails, we did identify three that matured in both male and female function within the same week and one individual that matured first as a female. We suggest that 'simultaneous hermaphroditism', broadly defined, describes this situation best.

We also observed that two line 7 individuals passed through periods of self-fertilization after attaining outcross-male function but before outcrossing as females. These individuals both matured as males at 5.5 weeks, as expected. One individual produced viable self-fertilized eggs at 6.5 weeks (7.1 mm) and first outcrossed as female shortly thereafter (8.5 weeks, 8.9 mm). The other individual produced self-fertilized eggs at age 7.5 weeks (8.0 mm) and showed much-delayed outcross female maturity (11.5 weeks, 9.1 mm). Although both these snails successfully reproduced by self-fertilization at much younger ages than any observed in the main selfing experiment (table 1), their sizes were within expected ranges.

Shell growth rates, initially about 1.0 mm per week in our culture conditions, slowed markedly when the snails achieved the capability of outcrossing as females at a mean age of 7.4 weeks. If no partners were made available, reproduction was delayed another 14.5 weeks, or 1.2 mm of growth, on average. We did not collect fecundity data and thus have no direct evidence regarding the relative fitnesses of selfing against outcrossing snails. But in the wild, a 14 week delay would greatly increase the risk of death before onset of reproduction.

Our estimates of autosterility are probably below that prevailing in natural populations, because we used snails from isofemale lines with a previously demonstrated ability to self-fertilize. The genetic component of autosterility is clearly demonstrated by the range of the values we obtained among lines, from negligible to 44%, under relatively constant environmental conditions.

Outcross male sterility seems to be moderately common in *Physa*, even among individuals with proven ability to self-fertilize. The phenomenon of aphally, complete absence of male reproductive structures, has been well documented in the African planorbid pulmonate *Bulinus* (Jarne *et al.* 1992; Schrag & Reed 1992). Aphallic individuals have generally proven to be both self-fertile and outcross-female fertile, as we have observed in *Physa*. We do not yet have anatomical data on our experimental animals, however.

We discovered one self-fertile 'double outcross-sterile' individual among our challenge snails, unable to copulate as a male with a partner subsequently shown to be outcross-female fertile and unable to serve as female to a partner of demonstrated male-fertility. This has interesting population genetic implications. Although our data are from laboratory lines, apparently natural *Physa* populations may include both obligate self-fertilizers and individuals completely incapable of self-fertilization. Estimates of inbreeding are generally calculated over entire populations; rarely are detailed data available to show heterogeneity in the likelihood of inbreeding among individuals. To the extent of its heritability, the intrapopulation variation in mating system discovered here implies considerable potential for life-history evolution.

Although fully mature *Physa* are hermaphroditic, pairs of snails do not mate reciprocally. One snail mounts the shell of the other and unilaterally serves as male, after which sex roles may be reversed, but often are not (Rudolph 1979*a*, *b*; van Duivenboden & ter Maat 1988; DeWitt 1991). Mating behaviour thus adds a layer of complexity to the question of reproductive function in *Physa* not addressed by the present study. We have recently completed a series of gender-choice experiments involving individual snails of varying maturity and reproductive history (Wethington & Dillon 1993). Ultimately we feel that, by virtue of its physiological and behavioural plasticity in reproduction, as well as its ease of culture and genetic variability, *Physa* may become a model organism for the study of sex allocation generally.

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