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Sperm Competition in a Plethodontid Salamander: Preliminary Results

LYNNE D. HOUCK, *Department of Biology, University of Chicago, 940 East 57th Street, Chicago, Illinois 60637, USA.*

STEPHEN G. TILLEY, *Department of Biological Sciences, Smith College, Northampton, Massachusetts 01060, USA.*

STEVAN J. ARNOLD, *Department of Biology, University of Chicago, 940 East 57th Street, Chicago, Illinois 60637, USA.*

Female plethodontid salamanders are capable of storing viable sperm throughout the many-month courtship season that precedes annual (or biennial) oviposition. Sperm are kept in a specialized female organ, the spermatheca, and eggs are not fertilized until just prior to oviposition. Sperm storage permits the decoupling of insemination and oviposition in many amphibians, sometimes for periods of weeks, months or even years (Boisseau and Joly, 1975). This situation enables a female to oviposit in a secure site without the physical presence of a male.

The decoupling of insemination and fertilization has a different significance for the male: insemination does not assure paternity. Even during the relatively short reproductive period of

aquatic-breeding salamanders (e.g., many species of ambystomatids and salamandrids), each female can pick up sperm masses from multiple spermatophores deposited by different males (Arnold, 1976; Rafinsky, 1981). The prolonged courtship season (10 mo or more) for many plethodontids provides a more extended period during which a female could be inseminated by multiple males. Few data for plethodontids are available concerning the number of multiple inseminations in the field, however, although Stebbins (1954:89) recorded multiple inseminations for *Ensatina eschscholtzii*. Our laboratory observations of many other plethodontid species (including *Plethodon jordani, Desmognathus ochrophaeus, D. wrighti, D. fuscus, Dendrotriton bromeliacia, Bolitoglossa franklini, B. occidentalis, B. resplendens, B. rostrata, Pseudoeurycea brunnata,* and *P. rex*) indicate that a female usually can be inseminated more than once, and perhaps more than 10 or 15 times during one season. We assume, then, that it is not unusual for a female to be inseminated by at least two different males in the field. Indirect evidence to support this assumption of multiple inseminations is provided by Tilley and Hausman (1976). Their electrophoretic analysis of field-collected *Desmognathus ochrophaeus* females and their broods showed that, as a minimum estimate, at least 7% of all individual clutches examined were sired by more than one male. Labanick (1983), using color pattern markers, also found that two of eight *D. ochrophaeus* clutches examined had multiple sires.

The combination of sperm storage and multiple inseminations indicates the potential for sperm competition in plethodontid salamanders. Sperm competition is defined as the competition within a single female between the sperm from two or more males for the fertilization of the ova (Parker, 1970:527). Sperm competition has been well documented for a variety of animals, especially for many insect species (Parker, 1970; Walker, 1980; Gwynne, 1984). The particular outcome of sperm competition (first male, last male, or mixed male paternity) might influence the evolution of male courtship behavior. If first male paternity is the case, for example, one might expect intense competition for uninseminated females.

Until recently it has been impossible to analyze sperm precedence in salamanders. One needs (1) to know the number and timing of inseminations, as well as the identity of male mates, for each female; (2) to be able to retrieve a naturally-laid clutch for each female, since hormonally induced ovulation frequently results in infertile ova; (3) to raise each clutch to hatching, and (4) to identify the male parent for each hatchling. We have been able to complete all these steps with one plethodontid species, *Desmognathus ochrophaeus*. Large numbers of individuals can be maintained in the laboratory, and courtship and insemination can be scheduled and recorded for known male-female pairs. Under certain conditions, mated females will oviposit spontaneously in the laboratory. Eggs easily are raised to hatching, and crosses can be designed that reveal paternities using electrophoresis of allozymic markers.

We report our preliminary results here to eliminate two possible outcomes of sperm competition (first male and last male paternity), and to describe techniques potentially applicable to other salamander species.

The *D. ochrophaeus* were collected from two different populations in the Appalachian Mountains: Population A = Unaka Mountain, Unicoi Co., Tennessee (collected August 1980); Population B = Mt. Rogers, Grayson Co., Virginia (collected April 1981). Tilley et al. (1978) found that these two populations are nearly fixed for alternative electromorphs at three enzymatic loci: *Idh-1, Ldh-2,* and *Ldh-2,* and *Pgi.* The relative mobilities of the two electromorphs at *Idh-1* are too similar to permit reliable discrimination between homozygotes and heterozygotes but, during the present study, we discovered that the populations also are nearly fixed for alternative variants at *Ldh-1.* By simultaneously scoring the *Ldh-1, Ldh-2,* and *Pgi* zymograms for all specimens, we were able to determine unambiguously whether a hatchling had been sired by a male from Population A or Population B.

Within one week of collection, salamanders were brought to the laboratory where they were housed individually at 15–16°C on a natural (Chicago) photoperiod and fed *Drosophila* spp. *ad libitum.* Courtships were staged by placing a known female and a known male together overnight in the same cage. Insemination easily is scored the following morning since the sperm mass remains visible in the female's cloaca for approximately 24 hr following sperm transfer (pers. obs.). The last insemination for each female was by a male not from her own population (i.e., a female from Population A was last inseminated by a male from Population B). The number of preceding inseminations in the field is unknown for six of the seven females for which we have data. The seventh female (A-1) became sexually mature (developed large, yolky ova which were visible through the body wall) in the laboratory. Some salamanders were used for courtship experiments unrelated to this study and so the total number of laboratory inseminations for the seven females varies. Although we cannot separately identify each sire using electrophoresis, we can distinguish individuals from each of the two populations. Offspring which have alleles at all three loci that are rare in their mother's population (but common in the last male's population) are sired by the last male.

Oviposition was promoted by simulating a natural situation where a female is in an undisturbed, protected place. Each female was placed in a plastic container (31 × 17 × 9 cm) which was lined with coarse gravel and contained one or two small flat rocks, a clump of moist sphagnum moss, and two crumpled moist paper towels. Containers

housing females were stacked and covered with a blanket to reduce daytime light and visual disturbances. Boxes were not disturbed for about six weeks, although they were checked occasionally to make sure that they were sufficiently moist.

On 27 July 1981, boxes were examined to see whether females had oviposited. Females guarding eggs were removed and frozen for subsequent electrophoresis. Tilley (1972) found low laboratory mortality of *D. ochrophaeus* eggs when each clutch was attended by a female. Brooding females sometimes eat eggs, however, so we used the following procedure. Egg clutches were removed from the oviposition boxes and transferred to small perforated plastic containers without tops (Tissue Tek capsules, TM-Miles Laboratories, Inc.) which were then placed in individually labelled Petri dishes (with lids) for care and observation. Petri dishes were either glass or plastic (60 mm or 100 mm dia) and were lined with filter paper or paper towels (cut to fit) moistened with aged tap water. Several Petri dishes with eggs were set inside a covered plastic box (31 × 17 × 9 cm) which had been lined with moist paper towels to insure a humid environment. Eggs were kept at 15–16°C in the same room in which adults were kept. To prevent fungal growth on the eggs, each clutch was washed daily for at least the first month of development and then every 2–3 days until hatching. To wash eggs, we used forceps to lift the Tissue Tek capsule containing eggs out of the Petri dish. The entire capsule was then submerged for about 5 sec in a small glass of either aged tap water or 0.1% hydrogen peroxide. (It was thought that the hydrogen peroxide would inhibit fungal growth, but washing with aged water alone seemed equally successful.) If hydrogen peroxide was used, eggs were then immediately rinsed in aged tap water. During washing, each egg was deliberately moved, either by the motion of submerging the capsule or by gently prodding the egg with closed, blunt forceps. Excess water was drained from the capsules by blotting them briefly on a paper towel before each capsule was returned to its Petri dish. The filter paper or paper towel in each Petri dish was replaced at each washing. Petri dishes were replaced with new or clean dishes if eggs in the old dish were spoiling or if fungal growth was a problem.

Hatchlings and adults were frozen until needed for electrophoresis. Each individual was scored at three loci (*Ldh-1, Ldh-2,* and *Pgi*) using techniques described by Tilley et al. (1978).

The history of inseminations and paternity of offspring are given in Table 1 for each of seven females. In the single case (female A-1) where the female was inseminated only once, all 13 surviving offspring were sired by a male from Population B. In all other cases, the last male to inseminate a female sired some but not all offspring. Paternity for "last males" ranged from 5% (female A-5) to 69% (female B-1).

TABLE 1. History of laboratory inseminations and paternity of resulting offspring for 7 female *Desmognathus ochrophaeus*. All females except A-1 almost certainly were inseminated in the field an unknown number of times before being collected.

Females:	No. of inseminations in laboratory by: Pop. A male	Pop. B male	Initial clutch size	Total no. surviving offspring	No. offspring sired by: Pop. A male	Pop. B male
Population A						
A-1	0	1*	14	13	0	13
A-2	4	1*	7	7	6	1
A-3	11	1*	12	11	9	2
A-4	6	1*	17	17	14	3
A-5	1	1*	15	14	10	4
Population B						
B-1	1*	0	27	26	18	8
B-2	1*	0	24	22	1	21

* Last male to inseminate this female.

Our results show that, for females having more than one mate, neither the first nor the last male to inseminate the female sires the entire clutch. Female A-3, for example, was inseminated eleven times in the laboratory by males from Population A before she was inseminated for the last time by one male from Population B (Table 1). Despite this number of inseminations by males from her own population, two of her 11 offspring were sired by the Population B male. This shows that the first male—or even the first 11 males—to inseminate a female may sire offspring, but their inseminations do not prevent sperm from subsequent mates from fertilizing at least some ova. Conversely, last male paternity is excluded since in none of the six females with multiple inseminations did the last inseminating male sire the entire clutch.

We conclude then that mixed male paternity is the outcome of sperm competition in *D. ochrophaeus*. Our data do not address the precise nature of mixed male paternity, however, so we do not know whether sperm from the first insemination is disproportionately represented, or whether sperm from all males is used randomly during fertilization. The number of offspring sired by the last male, for example, may be inversely related to the female's total number of prior inseminations. If this were so, we would expect that female B-1 had relatively fewer inseminations in the field than female B-2 prior to the final insemination by a Population A male. Ideally, one should be able to assign paternity of each offspring to an individual male, rather than identify a sire only according to his population. This would be possible if potential sires were screened before mating, and

if there were sufficient electrophoretic differences to identify individuals.

In this preliminary study we used sires from two different populations because of their electrophoretic differences. Zygotes from between-population matings could be less viable than those produced by parents from the same population, and the surviving offspring might therefore represent a biased sample of fertilized ova. All of the eggs laid by the *D. ochrophaeus* in our study showed embryonic development, and zygote mortality was low (Table 1). Possible differences in viability between inter- and intra-populational zygotes could be evaluated in future studies, however, by comparing initial clutch sizes and final number of hatchlings of females from Population A and from Population B that each received the same sequence of sires (e.g., four sires (= inseminations): A, A, A, B).

It is not known whether sperm competition, and thus paternity, also might be affected by the relative amount and age of a sire's sperm, or whether a male's sperm supply is depleted by the recent deposition of other spermatophores. Larger males may produce more sperm per spermatophore than smaller males, or sperm from recent inseminations may be more likely to fertilize ova than sperm from a female's first insemination; the latter could be tested by experimentally varying the time between inseminations. Also, viable sperm from the past season may be retained even after oviposition (Houck and Schwenk, 1984).

Our preliminary results document the potential for sperm competition in *D. ochrophaeus* but do not resolve the nature of mixed male paternity. Further laboratory experiments are necessary, but we also require basic information on the number and rate of inseminations for females in natural populations. Such data are as yet unavailable for any terrestrial plethodontid, and the interpretation of laboratory results ultimately depends on understanding male-female interactions in the field.

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