

Topping off: A mechanism of first-male sperm precedence in a vertebrate

Adam G. Jones*, Erika M. Adams, and Stevan J. Arnold

Department of Zoology, 3029 Cordley Hall, Oregon State University, Corvallis, OR 97331

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Competition among the sperm of rival males is an important evolutionary phenomenon in many organisms. Yet, despite extensive research on sperm competition in some vertebrate taxa, very little progress has been made on this topic in amphibians. Urodele amphibians (newts and salamanders) are of particular interest to theories of sperm competition because most urodele females—in contrast to other vertebrate females—control the transfer of sperm from the male. Here we present a molecular study of sperm precedence and storage patterns in the rough-skinned newt (*Taricha granulosa*). First, we used microsatellite markers to show that female newts typically use sperm from 1–3 males under natural and seminatural conditions. Second, we mated experimental females sequentially to two males and collected fertilized eggs in a temporal series. Patterns of paternity were consistent with first-male sperm precedence and complete mixing of sperm within the female. This simple pattern of sperm usage, best described as “topping off,” is consistent with the expectation from sexual conflict theory that free female choice before insemination eliminates selective pressures for the evolution of complex patterns of paternity manipulation involving cryptic female choice.

Sexual selection is an important facet of the evolutionary process. Although many of the key aspects of sexual selection occur before mating, research over the last three decades (1–3) has led to the understanding that competition among sperm within a female’s reproductive tract can be vital to the ultimate outcome of mating competition. A central goal of sperm competition research in recent years has been to quantify the proportion of offspring sired by the second of two males mated sequentially to a female, a value known as P_2 (4). Unfortunately, such an approach to the problem often says very little about the actual mechanism of sperm competition, because multiple distinct mechanisms can lead to the same value of P_2 (5). Here we describe a scheme of sampling and analysis, involving the temporal sampling of eggs as they are laid, by which a molecular study of P_2 can lead to additional insights regarding the mechanisms of sperm dynamics within the female.

The focal organism for this study was the rough-skinned newt, *Taricha granulosa*. This species provides a useful model for studies of sperm competition for several reasons. First, females receive sperm during a short receptive period at the beginning of the reproductive season. Females then lay eggs singly over the course of several weeks to months, fertilizing them with stored sperm. Second, females lay large numbers of eggs, a characteristic that permits a description of the change in P_2 over time. Such data are relevant to the pattern of sperm stratification within a female’s spermathecae (6). Third, the transfer of sperm in newts is indirect. Before insemination, the male must unclasp the female to deposit a spermatophore on the substrate in front of her (7). The unrestrained female then has the option of either picking up the spermatophore (using her cloaca) or moving away and ending courtship (7, 8). This indirect transfer of sperm, which is typical of many urodeles, gives female newts greater control over sperm acquisition than females of other vertebrate taxa with internal fertilization, and may be a major factor in the evolution of sperm usage patterns.

Finally, *T. granulosa* is of interest because there have been numerous papers written about patterns of sperm precedence in newts and salamanders (9), yet no definitive molecular studies of precedence patterns have been performed. Previous molecular studies in these taxa have suffered from small sample sizes and other limitations that led to ambiguous results (10, 11). Thus, urodele amphibians represent a major vertebrate lineage, with a system of sperm transfer that is unique among vertebrates, for which almost no hard data are available regarding sperm precedence.

Nevertheless, predictions have been made with respect to the nature of sperm competition in newts (9). For newts of the genus *Taricha* in particular, a laboratory-based study of courtship behavior (8) has led to the belief that females do not typically receive sperm from multiple males in nature. Hence, in the words of Halliday (9), “sperm competition appears to be rare or nonexistent in *Taricha*.” However, this extrapolation from laboratory observations may be premature, because there are no data regarding rates of multiple mating in natural populations of *Taricha*. If *Taricha* females were to mate with multiple males, we might expect last-male precedence in this species on the basis of two arguments. First, mating involves a period of preinsemination clasp, followed by insemination and a lengthy period of postinsemination clasp (8). If this postinsemination period of amplexus represents mate guarding, then we might expect such a behavior to evolve in a species with last-male sperm precedence (5). Second, some aspects of the mating behavior of related newts of the genus *Triturus* imply that these species experience last-male precedence (9, 12, 13).

To address the aforementioned predictions, we conducted three distinct studies. In the first two studies, microsatellite markers were used to document patterns of multiple mating in female newts that had mated under either natural or seminatural conditions to verify that sperm competition actually does occur in this species in nature. In the third study, experimental crosses were conducted in which a focal female was mated sequentially to two males. Fertilized eggs were collected as they were laid, resulting in a temporal series of samples from each female, and the parentage of offspring was determined by using microsatellite markers. By collecting eggs in a temporal series, we were able to address the hypothesis that sperm mix randomly within the female’s spermathecae, because if sperm are stratified we expect a change in the proportion of offspring sired by each male as successive groups of eggs are laid. The high fecundity of newts facilitates investigation of this hypothesis with greater statistical power than is possible for most other vertebrates.

Materials and Methods

Paternity in Seminatural Breeding Assemblages. For this experiment, we collected unmated female newts on rainy nights in early February 1999 (during their migration to breeding ponds) by

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*To whom reprint requests should be addressed. E-mail: jonesa@bcc.orst.edu.

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walking the Midge Cramer Path near the Benton County Fairgrounds (44° 33' 56" N, 123° 19' 17" W) in Corvallis, OR, and picking up female newts crossing the path. We collected males by submerging plastic minnow traps in flooded areas flanking the path. To each of six water-filled cattle tanks (approximately 1,500 liters in volume) we added eight males and eight females, and to each of six other tanks we added eight males and three females. Newts were allowed to mate freely within each tank from 22 February to 6 May, when the males were removed from the tanks. On 19 May, each female ($n = 66$) was isolated in a plastic shoebox filled with water and given an injection into the body cavity of 10 μ l of a 0.5 mg/ml solution of a luteinizing hormone-releasing hormone (LH-RH) analog, des-Gly¹⁰-[D-His(Bzl)⁶]LH-RH ethylamide (referred to hereafter as LH-RH) to induce egg laying. Fifty-seven of these females laid large numbers of fertilized eggs. We monitored the eggs for several weeks, and collected either hatchlings or eggs containing well-developed embryos for microsatellite analysis.

Mating Patterns in a Natural Population. Field-mated female newts were collected from a natural population during the egg-laying phase of their reproductive cycle by submerging minnow traps in a pond (44° 41' 18" N, 123° 12' 29" W) on the E. E. Wilson Wildlife Area near Corvallis, OR (Benton County), from 4 April 2000 to 9 June 2000. Captured females ($n = 43$) were placed in water-filled plastic shoeboxes and injected with 10 μ l of LH-RH. Eggs from 30 of these females were raised to hatching, and the hatchlings were frozen for microsatellite assay.

Sperm Precedence Experiment. To assess patterns of sperm precedence in newts, we collected males and females (as described above) from the Midge Cramer Path on 5 February 2000. Each experimental female was placed with a male in a water-filled 38-liter aquarium. Females were scored every 12 h for insemination. We removed the males from the tanks of inseminated females and waited until the sperm cap was no longer visible in the female's cloaca, at which time a second male was placed in the tank with the female. Seven females (of the 10 that mated twice and laid eggs during our experiment) received sperm from the first two males placed in her tank. In three cases (F07, F16, F26), however, the female appeared uninterested in the second male, so we rotated additional males through each female's tank until a male inseminated her. The results of these three trials did not seem to differ in any way from the other seven. To simulate the natural interval between mating and egg laying (14), we held those females successfully inseminated twice in isolation for at least 20 days and then injected them with 10 μ l of LH-RH (0.5 mg/ml). Rough-skinned newts lay eggs singly in vegetation over a period of several weeks, so we were able to collect eggs in temporal groups by checking a female's tank daily for the presence of eggs. For ease of presentation, we partitioned our results into two temporal groups, but we found identical results when we analyzed the data with greater subdivision. Newly laid eggs were removed to water-filled shoeboxes and raised to hatching. Hatchlings were preserved at -80°C for the molecular analyses.

Microsatellite Analyses. Microsatellite markers were used to assess parentage and to document patterns of multiple mating. Up to six highly polymorphic microsatellite loci were used to genotype parents and progeny. For adult newts, we extracted DNA from excised tail tips (2–5 mm in length) by using a standard proteinase K, phenol/chloroform procedure. For newt hatchlings and eggs, we used the fish embryo adaptation (15) of the single-tube DNA extraction procedure developed for *Drosophila melanogaster* (16). Details of primer sequences, PCR conditions, and PCR fragment analysis have been described (17).

To document patterns of multiple mating in our first two

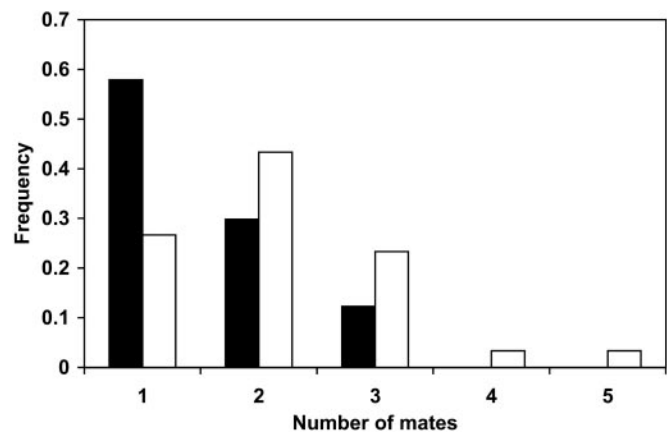


Fig. 1. Frequency histogram of female newt mating behavior. Solid bars show results from females ($n = 57$) that mated and produced offspring as part of laboratory breeding aggregations consisting of eight males with either eight or three females. Open bars show results from naturally inseminated females ($n = 30$) that were collected from the field.

experiments, we assayed a random sample of an average of 24 offspring from each female. In the seminatural breeding assemblages, paternity was assigned by complete exclusion for 99.1% of the 1,357 offspring genotyped, resulting in a very precise estimate of multiple mating by females. In the wild-caught females, three loci (*Tgr14*, *Tgr10*, and *Tgr06*) were used to determine the minimum number of males contributing sperm to each family. Simulations were run to investigate our probability of detecting multiple mating when females mate with from one to four males by using the computer program GERUDSIM1.0 (18), and in all cases these probabilities were above 0.99, assuming equally shared paternity.

For the sperm precedence experiment, we first assayed all males and females by using six microsatellite loci. For each clutch, we chose the loci that would differentiate the two experimental males and assayed all hatchlings produced by the focal female (a total of 1,724 hatchlings for the entire experiment) to yield the exact number of offspring fathered by each male. Ninety-two percent of eggs laid survived to hatching, so differential hatching success was not a meaningful source of error in this experiment. One of the females (F06) produced some eggs ($n = 29$) that had not been fertilized by either of the experimental males, suggesting that she had stored sperm from inseminations that had occurred before her collection. Even in this case, however, we were certain of the final male with which the female was paired, so the results are still relevant to the analysis. We also detected some embryos that had apparently received mutant alleles ($n = 8$), so parentage was assigned for these individuals on the basis of additional microsatellite loci.

Results

Our results show clearly that sperm competition is a common occurrence in rough-skinned newts. In both natural and seminatural settings, female newts frequently used sperm from multiple males (Figure 1). This result flies in the face of conventional wisdom, which held that *Taricha* females do not mate with multiple males during the course of the breeding season (8, 9). Furthermore, the majority of multiply inseminated females appeared to have mated with just two males, suggesting that our experimental investigation of sperm precedence patterns, which used two males per female, reflects a biologically relevant pattern of mating in this species.

Two main results are apparent from our controlled laboratory crosses. First, *T. granulosa* exhibits first-male sperm precedence.

Table 1. Patterns of sperm precedence in the rough-skinned newt

Female	Days between inseminations	First set laid		Second set laid		Totals		χ^2 test <i>P</i> value
		<i>n</i>	<i>P</i> ₂	<i>n</i>	<i>P</i> ₂	<i>n</i>	<i>P</i> ₂	
F02	14.5	76	0.41	84	0.30	160	0.35	0.14
F05	4.9	132	0.20	81	0.19	213	0.20	0.73
F06	5.2	48	0.02	64	0.05	112	0.04	0.46
F07	17.6	66	0.35	78	0.27	144	0.31	0.30
F08	4.0	77	0.38	81	0.37	158	0.37	0.94
F15	5.0	107	0.27	95	0.28	202	0.28	0.83
F16	34.8	104	0.23	120	0.14	224	0.18	0.09
F19	4.5	127	0.30	99	0.29	226	0.30	0.92
F26	29.5	85	0.27	61	0.15	146	0.22	0.08
F27	16.6	77	0.21	62	0.26	139	0.23	0.48
Mean	13.7	89.9	0.26	82.5	0.23	172.4	0.25	

Fertilized eggs were collected in a temporal series and are presented here in two sets defined by the order in which they were laid. Shown are the total numbers of hatchlings obtained from each sample (*n*) and the proportion of hatchlings fertilized by each female's final mate (*P*₂). The final column shows the *P* value for a χ^2 test of the hypothesis that *P*₂ and laying order are independent.

In all cases, the second male fertilized less than half of a female's eggs (i.e., $0.04 \leq P_2 \leq 0.37$; Table 1). Our overall estimate of *P*₂ is 0.25 ± 0.03 (mean \pm SE). Second, we found no strong evidence for changes in *P*₂ as the eggs were laid (Table 1). Despite very large samples of offspring from each female and hence high statistical power, we were unable to reject the hypothesis that laying order and *P*₂ were statistically independent by using a χ^2 test for each female's offspring (Table 1). We also tested for a trend in *P*₂ over time, considering our entire data set simultaneously, by using a linear model for categorical data (procedure CATMOD in SAS), and failed to reject the null hypothesis that *P*₂ remained constant (*P* = 0.09). These analyses provide strong evidence that the sperm from rival males are not stratified within the female's spermathecae.

In addition, we found no significant relationships between *P*₂ and other measured variables, including insemination interval (*n* = 10, *r* = 0.14, *P* = 0.70), first male size (*n* = 10, *r* = 0.45, *P* = 0.19), second male size (*n* = 10, *r* = 0.47, *P* = 0.17), and male size difference (*n* = 10, *r* = 0.04, *P* = 0.92). These observations are consistent with random usage of sperm with respect to the phenotype of the sires.

Discussion

The results of this study show clearly that the potential for sperm competition is very high in natural populations of *T. granulosa*. A large percentage of females used sperm from more than one male to fertilize eggs. The idea that each female typically receives sperm from a single male was based on an extrapolation from laboratory observations of newt mating behavior (8, 9). Such extrapolations plainly should be treated with caution, a sentiment that underscores the need for additional studies of newt and salamander mating systems in nature. The bulk of knowledge regarding mating behavior in urodele amphibians comes from studies of animals in the laboratory (19), and molecular markers should play a central role in future efforts to connect these observations to the events that actually occur in natural populations.

This study also demonstrates that the assay of offspring in temporal groups based on fertilization order can lend additional insights into the mechanisms of sperm competition beyond merely characterizing the value *P*₂ for each female. In the case of the rough-skinned newt, our results clearly demonstrate, given the mating conditions used in this experiment, a first-male advantage with an absence of sperm stratification.

Taken together, our observations suggest an uncomplicated mechanism of sperm usage in *T. granulosa*. The patterns of sperm precedence and sperm stratification are consistent with a

model in which a female accepts a large amount of sperm from her first mate, and, if additional space remains in her spermathecae, she then seeks additional mates until she has no need for further sperm. Once this "topping off" of the female's storage organ is complete, sperm from the males mix freely within the spermathecae until they are used to fertilize the eggs as they are laid. This interpretation is also consistent with anatomical observations of sperm storage in *Notophthalmus* and *Triturus*, close relatives of *Taricha*. The spermathecae consist of a series of simple glandular tubules, throughout which sperm are visible as scattered, tangled clusters (20, 21), which present no apparent barriers to sperm mixing.

These data are perhaps best interpreted in light of sexual conflict theory and "sexual dialectics" (22). A female rough-skinned newt can control both her choice of mates and the conduct of insemination. Males congregate in ponds in large numbers at the onset of the breeding season, whereas females trickle into the pond in small numbers as they become receptive (23). Each female finishes her mating activity within a short period, after which she is no longer sexually receptive (8). The resulting male-biased operational sex ratio presumably facilitates the acquisition of preferred males by females (24). Most sexual selection is probably by means of male–male competition (9), but females in amplexus appear able to affect the outcome of competition by assuming a rejection posture that signals disinterest to the male (8) or by moving to attract the attention of unpaired males that then try to physically dislodge the paired male (A.G.J., unpublished observation). Furthermore, because sperm transfer is indirect, females always have the option of ending courtship before insemination.

In a system with so much female control over insemination, we might expect patterns of sperm use to be simple. Because a female need never accept sperm from a low-quality male (because of the large number of males from which to choose and her ability to reject them), she should accept sperm only from high-quality males. Therefore, sperm from different males in her spermathecae should have equivalent value to her. Under these circumstances, there should be no selective pressure for a female to engage in cryptic female choice (25, 26) or other manipulations of sperm within her reproductive tract. Thus, any complex mechanisms of sperm usage in this system should be a result of male-mediated strategies, which will be limited because of the lack of physical contact between males and females during sperm transfer. From this perspective, our observations are consistent with the idea that, because females can choose effectively among males before insemination, they need not evolve additional mechanisms, such as cryptic female choice, to circumvent at-

tempts by males to undermine female choice (22). Of course, we cannot definitively rule out a role for some small amount of cryptic female choice in generating some of the variance among females in P_2 , but no such complex mechanism is necessary to explain our data.

The pattern of sperm competition that we have documented in *T. granulosa* differs from the patterns described for other vertebrate taxa (3). Our study demonstrates a consistent first-male advantage in a vertebrate with long-term sperm storage. The closest vertebrate analog to the pattern we have observed in *T. granulosa* is the “passive sperm loss” (PSL) model that has been empirically described in birds (6). The PSL model is similar to our topping-off model in that both are passive and lack sperm stratification. However, the newt pattern of sperm storage differs in two important ways from the avian pattern. First, the PSL model typically produces last-male advantage (6), whereas our topping-off model produces first-male advantage. Second, in the PSL model, the timing of inseminations has a profound effect on the outcome of sperm competition (6). We found for newts, however, that timing of insemination had no effect on P_2 —

whether 4 days or 35 elapsed between inseminations, the first male still sired the majority of offspring (Table 1).

In summary, we have discovered a previously uncharacterized vertebrate mechanism of sperm competition, which we call topping off. This study is of general importance to research on sperm competition for several reasons. First, our technique of assaying offspring in temporal groups provides far more information regarding the mechanisms of sperm usage than simply measuring P_2 . Second, this study is consistent with the idea that sexual dialectics may explain an important aspect of the evolution of rough-skinned newt reproductive ecology. Finally, our study contributes to debates regarding the importance of cryptic female choice (26–31) by providing an example of a vertebrate system in which the effect of cryptic female choice is minimal relative to mate choice before insemination.

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