Female choice, male interference, and sperm precedence in the red-spotted newt

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Darwin first identified female choice and male–male competition as forms of sexual selection resulting in the evolution of conspicuous sexual dimorphism, but it has proven challenging to separate their effects. Their effects on sexual selection become even more complicated when sperm competition occurs because sperm precedence may be either a form of cryptic female choice or a form of male–male competition. We examined the effects of tail height on male–male competition and female choice using the sexually dimorphic red-spotted newt (*Notophthalmus viridescens viridescens*). Experiment 1 examined whether male tail height influenced male mating success. Males with deep tails were more successful at mating with females than those with shallow tails. Successful, deep-tailed males also were bigger (snout-vent length; SVL) than unsuccessful, shallow-tailed males, but they did not vary in tail length or body condition. Of these, only tail height and tail length are sexually dimorphic traits. Experiment 2 tested the hypothesis that the differential success of males with deeper tails was due to female choice by examining both simultaneous female preference for association and sequential female choice. We found no evidence of female choice. When males were not competing to mate with females, tail height did not influence male mating success. Successful males did not have different SVL and tail lengths than unsuccessful males. Thus, tail height in male red-spotted newts appears to be an intrasexually selected secondary sexual characteristic. Experiment 3 used paternity exclusion analyses based on molecular genetic markers to examine the effect of sperm precedence on sperm competition in doubly-mated females. Sperm precedence likely does not have a pervasive and consistent effect on fertilization success because we found evidence of first, last, and mixed sperm usage. *Key words:* female choice, male–male competition, molecular markers, newts, *Notophthalmus viridescens,* paternity exclusion analysis, sexual selection, sperm competition. *[Behav Ecol 11:115–124 (2000)]*

A primary goal of sexual selection theory is to explain the evolution of conspicuous sexual dimorphism (Andersson, 1994). Often female choice or male–male competition has been implicated in the evolution of secondary sexual dimorphism, and both can occur in nature (Andersson, 1994). However, separating the effects of female choice and male–male competition is difficult. Female choice and male–male competition might favor the same characters or select for different characters (Moore, 1990). For example, in swordtails, *Xiphophorus nigrensis,* male–male competition and female choice both contribute to the greater reproductive success of larger males (Morris et al., 1992). In contrast, in eastern tiger salamanders, *Ambystoma tigrinum tigrinum,* male–male competition favors increased male body length, while mate choice selects for greater male tail length (Howard et al., 1997). Therefore, the impact of male–male competition and female choice should be distinguished when examining the relative importance of each on the evolution of secondary sexual traits (Moore, 1990).

The interaction of male–male competition and female choice on sexual selection becomes more complicated and subtle when females mate multiple times, thus fostering sperm competition. Although the influence of sperm precedence on male reproductive success is not obvious, this form of cryptic female choice can be important in a mating system (Eberhard, 1996). Sperm precedence can be defined as a nonrandom likelihood of fertilization by sperm from a male that depends

on the order of matings by that male and other males with a particular female. The precedence effect, if present, can be positive or negative. According to Wade and Arnold (1980), the intensity of sexual selection on a male may be related to sperm precedence, the duration of sperm competition, and the number (or fraction) of sperm that are not used in fertilization. Wade and Arnold (1980) found that both cases of absolute advantage (either the first male or the second male always fathers all of the offspring) are equivalent in their effects on the intensity of sexual selection, whereas mixed paternity can reduce the variation among males in reproductive success but increase the intensity of sexual selection on males. In a multiply mating species, sperm precedence effects can influence sequential female choice. Halliday (1983) proposed that for species that mate multiply and have last-male sperm advantage, a female could ensure fertilization of her eggs by mating with the first male that she encounters; thereafter, she can maximize the quality of her progeny by sampling additional males and by mating only with males of higher quality than those with which she previously mated. Smooth newts, *Triturus vulgaris vulgaris,* use this type of mate choice (Gabor and Halliday, 1997). Thus, it is also important to examine the outcome of sperm competition in such systems. In the present study, we examined the impact of male traits on male–male competition, on female choice, and on paternity in the red-spotted newt, *Notophthalmus viridescens viridescens.*

The mating system of newts (Salamandridae) offers an opportunity to distinguish between hypotheses of female choice, male–male competition, and the subsequent influence of sperm competition. Males of the North American red-spotted newt (*N. v. viridescens*) develop deeply keeled tails during the courtship season. In addition to having deeper tails than females, male red-spotted newts also have longer tails ($n_{\text{males}} = 276$, mean $\pm$ SE $= 44.9 \pm 0.2$ mm; $n_{\text{females}} = 157$, mean $=$

42.7 ± 0.2 mm; $t' = 2.30$, df $= 342.5$, $p = .01$; pooled for 1995–1997). The impact of only one of these sexually dimorphic characters (tail depth) has been previously studied (Able, 1999). Able found that in the field, male tail depth was positively correlated with variation in male amplexus frequency. In the laboratory Able found that males with deeper tails had greater success at capturing females. The operational sex ratio (Emlen and Oring, 1977) in this species is usually male biased (1.75:1 male:female; Massey, 1990) because not all females are receptive at any given time (Halliday and Verrell, 1984). Males do not provide any known material resources, such as paternal care or oviposition sites (Verrell and McCabe, 1988), and female assessment of males is limited to aspects of male morphology, courtship vigor, and chemical cues (Halliday, 1977; Verrell, 1982). Female red-spotted newts mate two to three times in a season (Halliday and Verrell, 1984) and store viable sperm throughout the mating season but not between seasons (Sever et al., 1996). Sperm competition has not been examined in red-spotted newts and has rarely been studied in salamanders (but see Houck et al., 1985).

Courtship in red-spotted newts consists of either amplexus or a lateral display described as the ''hula'' (Verrell, 1982). Amplexus consists of a male clasping a female for an average of 3 h. Following amplexus, the male must dismount the female to deposit his spermatophores, thus providing the female an opportunity to acquire his gametes, thereby choosing to accept or reject him as a mate. Following dismount, if the female continues to follow the male and touches the base of his tail, he may deposit a spermatophore and then turn 90° while leading the female over the spermatophore (Verrell, 1982). Sperm transfer occurs when the sperm cap of the spermatophore attaches to the conically shaped cloaca of the female. Alternatively, when a female is initially responsive to a male's approach, amplexus may be bypassed (observed in the laboratory only) and replaced with the hula. The hula usually lasts for a few minutes and is followed by spermatophore deposition and perhaps transfer. Thus, the hula may be a low-cost display (energetically) compared to amplexus, but it is more susceptible to sperm transfer failure (Verrell, 1982). This led Verrell (1989) to predict that males of species with external sperm transfer but internal fertilization will be vulnerable to interference from other males and will adjust their mating strategies accordingly. He suggested that amplexus evolved as a form of sexual defense against sexual interference (as defined by Arnold, 1976) by rival males.

We examined the effect of tail height on female choice and male–male competition in *N. v. viridescens*. In addition, we examined the effects of tail length, snout-vent length (SVL), and body condition (1995 data only) on male mating success. In experiment 1, ''Male–male competition,'' we examined the hypothesis that male tail height influences male mating success. In this experiment, we conducted breeding encounters, each involving two males of varying tail depths and a female. These encounters were allowed to proceed fully to the time of insemination. In experiment 2, ''Female choice,'' we tested the hypothesis that the differential success of males with deeper tails was due to female choice. We performed two experiments to examine female choice. In experiment 2a, ''Simultaneous female preference,'' we examined the preference of females to associate with deep-tailed or shallow-tailed males. However, we did not allow the encounters to progress to insemination so as to separate the influence of female choice from the outcome of the male–male competition experiment. In experiment 2b, ''Sequential female choice,'' we examined female preference to mate in a sequence of encounters with individual males, with male tail height either differing in each subsequent encounter or remaining the same. A shortcoming inherent in many previous simultaneous female preference

experiments is that females were not allowed to mate with the males. We conducted our sequential female choice experiment (sperm transfer allowed) to correct for this problem and to test Halliday's (1983) prediction that females will become progressively more choosy.

Finally, in experiment 3, ''Sperm precedence,'' we performed paternity exclusion analyses based on molecular genetic markers to gain a more complete understanding of the red-spotted newt's mating system. Specifically, we tested for multiple paternity by assigning paternity for individual offspring within clutches of females. We examined whether sperm precedence depended on the length of time between mating with different males. This experiment was also designed to complement the sequential female choice experiment and to gather evidence that might be relevant to the supposition on which Halliday's (1983) prediction is based— namely, the existence of ''last-male advantage'' or at least the absence of ''first-male advantage.''

METHODS

Experiment 1: Male–male competition

We collected unmated female red-spotted newts, *N. v. viridescens,* from a drift fence that completely encircled the pond at Pond Ridge in George Washington National Forest, Rockingham County, Virginia, USA, 21–24 March and 11 April 1995. The use of a complete drift fence precluded the chance that females entered the pond, mated, left the pond, and then were captured. This assertion is supported by the fact that our unmated females did not lay fertile eggs (see experiment 3). Males and females were maintained temporarily in separate containers in the laboratory in Virginia on a 14:10 h light: dark photoperiod until they were brought to the laboratory in Louisiana. The newts were then separated by sex and maintained in aquaria ($50.8 \times 25.4 \times 30.5$ cm) on a 14:10 h photoperiod with 25 newts per aquarium at 18°C. The photoperiod was gradually changed to a 16:8 h light:dark cycle. We filled the tanks with aged tap water, lined the bottom with gravel, and placed two bricks in each tank to allow individuals to emerge from the water. We fed the newts earthworms (*Lumbricus terrestris*) and crickets (*Acheta domestica*) ad libitum. Water was partially changed weekly. We individually identified newts based on drawings of their spot patterns (Gill, 1978). Before testing, we measured the mass of each salamander using a Mettler PL-300 balance, SVL to the anterior end of the cloaca of each male and female, tail length from the anterior end of the cloaca to the tail tip, and the tail height of each male at the deepest section of the tail using a metric ruler. Body condition was determined for each male by using the residuals of the regression of the cube-root of mass against SVL (Baker, 1992; Howard et al., 1997).

We staged 30 encounters to examine male–male competition. In each encounter, a female ($n = 30$ females) was matched with a deep-tailed and a shallow-tailed male. Testing was performed 6–27 April 1995 in the laboratory at 16°–19°C. Test chambers ($61.0 \times 30.5 \times 30.5$ cm) were divided into three sections (left and right $= 15.5$ cm, center $= 30.0$ cm) by clear Plexiglas perforated with small holes that potentially permitted individuals to gain visual and chemical information but prevented matings. Test chambers were floored with gravel and were filled with aged tap water to a depth of 11 cm. We placed each female into the central compartment of a test chamber for a 15-min habituation period. A deep-tailed male (8.5–10 mm) and a shallow-tailed male (6.5–8 mm) were randomly placed on the right or left sides of the aquarium, one male per side. The difference in tail height between males within a pair was always ≥2 mm. Males were chosen based on

tail height, but otherwise random pairs (based on a random numbers table) were selected. Tail depth was within ±1 SD of the population mean. Some of the females used in this experiment had been previously used in experiment 2a. After 15 min of habituation, we carefully lifted the dividers and observed the behavioral interactions between the two males and between the males and the female. Often males began to court the female from behind the dividers. If no mating occurred within 90 min, we considered the encounter unsuccessful.

In each test, we recorded the form of courtship (amplexus or hula) used by a male, instances of sexual interference, and time to successful sperm transfer by a male. Sexual interference consists of a rival male: (1) wrestling and attempting to dislodge the courting male during amplexus, (2) displaying hula to the courted female, or (3) using female mimicry, which consists of the rival male moving between the courting male and female during the spermatophore deposition stage and eliciting spermatophore deposition by the courting male (Verrell, 1982).

We used a two-tailed binomial test to determine whether males with deep or shallow tail heights were more successful at mating with females and whether sexual interference affected sperm transfer, with $\alpha = 0.05$ (Siegel and Castellan, 1988). We subsequently used Student's t tests to examine the relationship between SVL, tail length, and body condition between successfully versus unsuccessfully mated males to examine the influence of other traits on male mating success.

Experiment 2a: Simultaneous female preference

Procedures were as in experiment 1. We examined female preference to associate with deep or shallow-tailed males to determine whether the results of the male–male competition experiment arose purely due to mate competition or whether mate choice by females was also important. We imposed three treatments with each female ($n = 55$) tested in all three treatments. Treatment orders were randomized for each female. In treatment 1 (control), females were matched with two males with shallow tails (6–7 mm). In treatment 2 (control), females were tested with two males with deep tails (8.5–11 mm). In treatment 3 (experimental), females were tested with one male with a shallow tail and one with a deep tail (≥ 2 mm difference between shallow and deep). Tail heights were within ±1 SD of the population mean. The control treatments were used as points of comparison for the experimental treatment.

In treatments 1–3, we used the same aquarium setup as in experiment 1. We placed the test female in the center chamber and one male in each outer compartment. Females and males were habituated in their separate compartments for 15 min. We placed a 12.7-cm mesh net over the females during habituation so that females could potentially observe both males. Females were then observed for 15 min while we recorded the amount of time spent on each side of the center line. We also observed courtship by males, but we did not use this in our analyses.

We used a Friedman two-way analysis of variance by ranks test to compare the time that females associated with the deep-tailed male in treatment 3 (experimental condition) with the time that they spent on the same side of the aquarium (randomly determined) when they were tested in treatment 1 (shallow-tailed males) and when they were tested in treatment 2 (deep-tailed males). For example, if in treatment 3 a female was tested with a deep-tailed male on the left and a shallow-tailed male on the right, then we compared the time she spent on the left side of the chamber among the three treatments.

Table 1

Experimental design and results of sequential female choice using red-spotted newts (*Notophthalmus v. viridescens*)

| Treatment | n | Initial pairing | | Second pairing | | |
		Mail tail depth	No. mated singly	Mail tail depth	No. mated singly	No. mated doubly
1	18	Shallow	2	Deep	4	10
2 (control)	20	Shallow	4	Shallow	3	11
3	20	Deep	6	Shallow	4	9
4 (control)	18	Deep	2	Deep	4	11

Each female was paired in the initial pairing with a deep- or a shallow-tailed male, and sperm transfer was recorded. Each female was re-paired in the second pairing with a male with the same tail height or with the opposite extreme in tail height from the male that she had met in the initial pairing. Number mated indicates the number of encounters in which successful sperm transfer occurred. For example, in treatment 1, 2 females mated only in the initial pairing, 4 females mated only in the second pairing, and 10 females mated in both the initial pairing and the second pairing out of the 18 females tested. Two females did not mate in either pairings.

Experiment 2b: Sequential female choice

We collected unmated males and females of *N. v. viridescens* from a drift fence around the same pond as in experiment 1 from 1–17 April 1996. The research was performed in Virginia so that the newts could be maintained outdoors in natural conditions, as newts remain in breeding condition for a longer period of time when maintained in natural conditions than when maintained in the laboratory (C. Gabor, personal observation; T. Halliday, personal communication). We maintained the newts in outdoor aquaria (50.8 × 25.4 × 30.5 cm) on a natural photoperiod and water temperature regime (0°–12°C). The tanks were filled with aged tap water and the bottom was covered with small, tan gravel. A perch was placed in each tank to provide shelter and a dry surface. We fed the newts live zooplankton, *Tubifex,* and minced earthworms ad libitum. We housed males and females in separate aquaria. Before the experiments, we recorded individual spot patterns and measured SVL, tail length, and the tail height of each male at the deepest section of the tail.

We examined female preference to mate in a sequence of encounters with individual males, with male tail height either differing in each subsequent encounter or remaining the same. The protocol for this experiment was essentially the same as used for *T. vulgaris vulgaris* in Gabor and Halliday (1997). A female was paired in the initial pairing with a shallow-tailed male (6.5–7.5 mm in treatments 1 and 2) or a deep-tailed male (9.0–10.5 mm in treatments 3 and 4; Table 1). Females ($n = 18$–20 females for each treatment) and males were habituated for 15 min on separate sides of an aquarium (as described in experiment 1) before mating was permitted. We lifted the Plexiglas divider after the habituation period and allowed courtship to occur. We permitted only one sperm transfer in the initial pairing by briefly placing a 12.7-cm net between pairs just before a male deposited a second spermatophore. After another 15-min habituation period, each female was then re-paired in the second pairing with a male bearing the opposite extreme in tail height (≥ 3 m difference) from the male with which she first had mated in either treatment 1 or 3 (experimental). In treatments 2 and 4 (controls), each female was re-paired in the second pairing with a male of the same tail height with which she first had been paired (Table 1). Females were re-paired in the second pairing regardless of whether they had mated in the initial pairing.

If sperm transfer did not occur in the second pairing, we attempted to determine whether the female was receptive by re-pairing her with an extremely deep-tailed male (10–13 mm, ≥2 mm deeper than the previous male) in the third pairing. However, so few matings occurred in the third pairing that these results are not presented. Encounters were considered unsuccessful if sperm transfer did not occur within 1 h. Male tail heights were within ±1 SD of the mean of the males collected.

We performed the experiments 15 March–4 May 1996, during the middle of the breeding season, in a laboratory (24°–27°C) at James Madison University, Virginia. All encounters within each treatment were randomized based on the assigned number for each male and female. We recorded time to sperm transfer for each pairing. We used a two-tailed Wilcoxon signed-ranks test to compare latency to sperm transfer within a treatment between the initial pairing and the second pairing. Comparisons within the initial pairings and the second pairings for treatments 1 and 3 (experimental treatments), and for all four treatments were analyzed using a 2 × 2 contingency test (Siegel and Castellan, 1988). We subsequently used Student's t tests to examine the relationship between SVL and tail length of successfully and unsuccessfully mated males to examine the influence of other traits on male mating success. We used a two-tailed Wilcoxon-Mann-Whitney test to compare the latency to sperm transfer between dyadic and triadic encounters to test the hypothesis that sexual interference increases time invested in courtship. All analyses were significant at α = 0.05.

Experiment 3: Sperm precedence

We collected unmated males and females of *N. v. viridescens* from the same ponds as in experiments 1 and 2 using the same complete drift fence to ensure that females were seasonally unmated virgins, 26–27 March 1997. We maintained newts in the same manner as in the procedures for experiment 2b.

We paired females ($n = 22$) with one male at a time using the same aquaria as in experiments 1 and 2. The order of testing females was randomly determined. Adult genotypes were determined before the matings and trios were selected such that each of two males would contribute unique alleles to offspring. Females were paired with one male and sperm transfer was observed. We used the individual spot patterns of the newts to identify each individual uniquely. We noted the chronological order of spermatophores deposited and which ones were successfully transferred to the females. After sperm transfer, the first-male was removed and a second male was immediately placed in the aquarium. Again we observed matings, but if no sperm transfer occurred within 3 h we left the pair together overnight and examined the aquarium within 15 h for spermatophores. If sperm caps were missing from spermatophores, we concluded that sperm were transferred during this time period. We measured the elapsed time between matings as the time between the first sperm transfer and the second sperm transfer. If the pair was left overnight, then we used the general term "overnight" to indicate an elapsed time greater than 3 h and less than 15 h between matings.

After females mated doubly ($n = 8$), they were individually housed in an egg-laying chamber ($27 \times 16 \times 11.5$ mm deep) with aged tap water, gravel, live zooplankton, and live plants. Females were also fed live worms (*Tubifex*) weekly. The chambers were placed at the base of a large open window with a natural photoperiod. We searched for eggs every 24 h, removed pieces of plants that contained eggs, and placed the pieces into separate containers uniquely identified for each female. As a control, to provide evidence that the experimen-

Table 2

Polymorphic loci used in paternity exclusion analysis

Locus	Allele designation[a]	Allele frequency[b]	Electrophoretic buffer
Ldh-1	132	0.03	Tris-Citrate pH 8.0
	100	0.97	
Ldh-2	100	0.96	Tris-Citrate pH 8.0
	43	0.04	
Mdh-1	120	0.13	Tris-Citrate pH 8.0
	100	0.87	
Mdh-2	145	0.06	Tris-Citrate pH 8.0
	100	0.92	
	60	0.02	
Pgi	355	0.07	Amino-Citrate pH 6.3
	291	0.37	
	180	0.05	
	100	0.57	

[a] Allele designations indicate relative electrophoretic mobility with a value of 100 assigned to the most common allele.

[b] Population estimates based on analysis of toe tissues from 130 adults captured at Pond Ridge, George Washington National Forest, Rockingham County, Virginia, USA. None of the loci exhibited any significant departures from Hardy-Weinberg equilibrium.

tal females were unmated before the experiment, we placed unmated females ($n = 6$) into chambers to determine whether the females would lay fertilized eggs. We collected hatched young from all females that laid eggs ($n = 6$ females) and at age 3 days, these were stored at -80°C.

We used genetic markers to determine paternity for offspring from females that were mated to two males. We determined the genotypes of each adult and offspring using standard horizontal starch-gel electrophoresis of proteins (Selander et al., 1971). Adults were genotyped from toes excised at capture and hatchlings were killed and genotyped whole. Four polymorphic loci that resolved consistently were used to exclude putative fathers from paternity: lactate dehydrogenase-2 (*Ldh-2*), malate dehydrogenase-1 and -2 (*Mdh-1, Mdh-2*), and phosphoglucose isomerase (*Pgi*; Table 2).

We used a chi-square test to determine, in cases of double paternity, whether paternity sharing was equal among the two sires. We used a two-tailed Wilcoxon-Mann-Whitney test to determine whether SVL and tail height were significantly different between males that successfully sired most of the offspring and those that did not.

RESULTS

Male–male competition

Eleven matings occurred in 30 trio encounters. Deep-tailed males ($n = 10$) were significantly more successful at mating with females than were shallow-tailed males ($n = 1$; two-tailed binomial test, $p = .012$). Significantly more mating attempts (amplexus or hula displays) were performed by deep-tailed males ($n = 19$) than were performed by shallow-tailed males ($n = 2$) in 30 total encounters (two-tailed, binomial test, $p < .01$). In the 11 successful encounters (two male pairs were used twice with different females), we found that successful males did not have significantly greater tail lengths than unsuccessful males (Table 3). Successfully mated males had significantly greater SVLs than unsuccessful males (Table 3). Males from successful encounters were not in significantly better condition than unsuccessful males (Table 3).

Sexual interference by the rival male occurred in 9 of the 11 successful encounters (82%), and 9 courting males used

Table 3
Summary of traits for successful males and unsuccessful males from experiment 1 (male–male competition) and experiment 2b (sequential female choice)

	Successful		Unsuccessful				
	n	Mean $\pm$ SE	n	Mean $\pm$ SE	df	t	p
Experiment 1							
Tail length	9	46.33 $\pm$ 0.83	9	44.00 $\pm$ 1.19	16	1.61	.13
SVL	9	46.80 $\pm$ 0.50	9	44.70 $\pm$ 0.60	16	2.80	.01
Condition	9	1.90 $\pm$ 1.40	9	-1.60 ± 1.30	16	1.80	.09
Experiment 2b—Initial pairing							
Tail length	55	45.73 $\pm$ 0.36	21	45.52 $\pm$ 0.54	74	-0.30	.76
SVL	55	45.40 $\pm$ 0.27	21	45.76 $\pm$ 0.25	74	-0.78	.44
Experiment 2b—Second pairing							
Tail length	56	45.40 $\pm$ 0.40	20	45.80 $\pm$ 0.50	74	-0.70	.51
SVL	56	45.64 $\pm$ 0.24	20	45.00 $\pm$ 0.38	74	1.41	.16

Sample sizes, mean, standard error, and two-tailed Student t test for differences between successfully mated males (sperm transfer) and unsuccessfully mated males (no sperm transfer). See text for explanation of how condition was measured. Tail length and SVL were measured in millimeters. Note that in experiment 1 two pairs of males were used twice and the same males were successful both times.

amplexus as opposed to hula in courtship. Four cases of sexual interference consisted of the rival male performing female mimicry, two of wrestling, two of displaying (hula), and one of approaching the courting pair. However, sexual interference did not significantly prevent successful sperm transfer by the courting male ($n_{\text{no transfer}} = 4$, $n_{\text{transfer}} = 7$, two-tailed binomial test $p = .27$). In 1 case out of 11 (9%), a shallow-tailed male successfully performed female mimicry by moving between the larger tailed courting male (the latter having just dismounted the female after amplexus) and the female and depositing a spermatophore that was transferred successfully

Figure 1
Time (seconds) that female red-spotted newts (*Notophthalmus v. viridescens*) associated with deep-tailed males in treatment 3 (trt. 3; experimental condition) as compared to the times that they spent on the same side of the aquaria (right or left) when they were tested in treatment 1 (trt. 1; shallow-tailed males) and when they were tested in treatment 2 (trt. 2; deep-tailed males). The upper and lower horizontal lines of the box on the box and whiskers plots (Sokal and Rohlf, 1995) represent the first and third quartiles and the middle horizontal line is the median. The mean is represented by X. The whiskers represent the range. The + represents outliers.

to the female. Subsequently the female picked up two more sperm caps from the deep-tailed male.

Simultaneous female preference

Females spent a similar amount of time with deep-tailed males (mean $\pm$ SE = 452.78 $\pm$ 23.08 s, $n = 55$) and shallow-tailed males (447.22 $\pm$ 23.08 s) in experimental treatment 3. Females demonstrated no significant difference in the time that they associated with deep-tailed males in the experimental condition (treatment 3) as compared with the time the same females spent with shallow-tailed males, treatment 1, and deep-tailed males, treatment 2, on the same side of the aquarium (Friedman test; $n = 55$, df = 2, $\chi^2 = 2.76$, $p = .25$; Figure 1).

Sequential female choice

Females demonstrated no significant preference to mate with deep or shallow-tailed males whether the females had mated once or twice. First, we examined female preference for deep-tailed or shallow-tailed males for all four treatments, and there was no significant difference in frequency of females mating or not mating with shallow-tailed males (treatments 1 and 2) versus with deep-tailed males (treatments 3 and 4) in the initial pairing (Table 4). There was also no significant difference in frequency of females mating or not mating with shallow-tailed males versus deep-tailed males for the second pairing (treatments 2 and 3 versus treatments 1 and 4; Table 4). Second, we examined this preference for only the experimental treatments, and there was no significant difference in the frequency of females mating or not mating with shallow-tailed males (treatment 1) versus deep-tailed males (treatment 3) in the initial pairing. There was also no significant difference between females mating and not mating with shallow versus deep-tailed males in the second pairing for the experimental treatments (treatment 3 versus treatment 1; Table 4). Third, we examined Halliday's (1983) prediction that females would become more choosy in subsequent matings. We found that for those females that mated twice, there was no significant difference in frequency of mating or not mating initially with shallow-tailed males and subsequently with deep-tailed males (treatment 1) versus with deep- to shallow-tailed males (treatment 3; Tables 1 and 4).
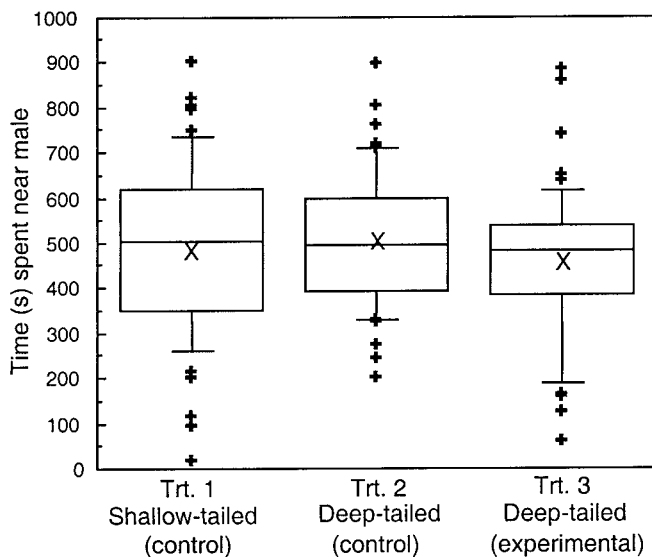
Table 4
Results of chi-square tests for sequential female choice using red-spotted newts

Male tail	Initial pair (1/2 vs. 3/4)		Second pair (2/3 vs. 1/4)		Initial pair (1 vs. 3)		Second pair (3 vs. 1)		Second pair (mate 2x)	
	F mating	F not mating	F mating	F not mating	F mating	F not mating	F mating	F not mating	F mating	F not mating
Shallow	27	11	27	3	12	6	13	7	10	8
Deep	28	10	29	7	15	5	14	4	9	11
	$\chi^2 = 0.07, p = .80$		$\chi^2 = 1.14, p = .29$		$\chi^2 = 0.32, p = .57$		$\chi^2 = 0.75, p = .39$		$\chi^2 = 0.42, p = .51$	

A summary of the number of females (F) that mated and did not mate with shallow- or deep-tailed males among treatments 1–4 in the initial and second pairing (df = 1 for all results). Second pair (mate 2×) refers to those females that mated both in the initial pairing and in the second pairing for the experimental treatments (1 and 3). The experimental treatments that is being compared in a given two-column group is described by the numbers in parentheses. For example, ''(1/2 vs 3/4)'' describes the comparison of treatments 1 and 2 combined versus treatments 3 and 4 combined.

There was no significant difference between the latency to sperm transfer (from the start of the test) in the initial pairing and the second pairing when females mated twice in the experimental treatments (treatment 1; two-tailed, Wilcoxon signed ranks, $n = 10$, $z = -.26$, $T^+ = 30$, $p = .80$: treatment 3; $n = 9$, $z = -0.54$, $T^+ = 18.0$, $p = .59$; Figure 2). In the control treatments, there was also no significant difference between the latency to sperm transfer (from the start of the test) in the initial pairing and in the second pairing (treatment 2; $n = 11$, $z = -1.92$, $T^+ = 54.5$, $p = .06$: treatment 4; $n = 11$, $z = -1.21$, $T^+ = 46.5$, $p = .23$; Figure 2).

There was no significant difference between the mean SVL of males that successfully (sperm transfer) mated with females and of unsuccessful (no sperm transfer) males in the initial pairing (Table 3) and in the second pairing (Table 3). There was also no significant difference between the tail lengths of males that successfully mated with females and of unsuccessful males in the initial pairing (Table 3) and in the second pairing (Table 3).

When the results of the trials from the male–male competition experiment (triadic) were compared with those from the sequential female choice experiment (dyadic), we found that there was a trend for the latency to sperm transfer to increase in the male–male competition trials, but this was not significant (triadic: $n = 11$; dyadic: $n = 54$; two-tailed Mann-Whitney test; $U' = 395.5$, $z' = -1.72$, $p = .08$; Figure 3).
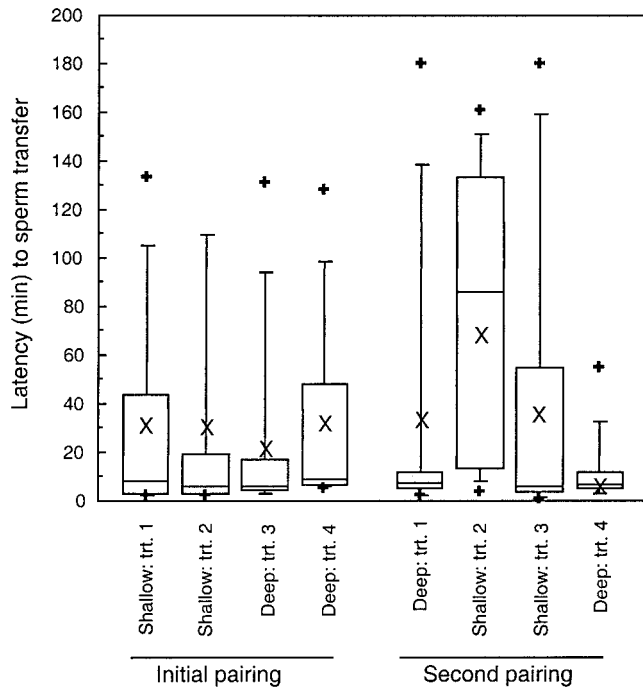
Sperm precedence

From the six doubly-mated females that laid eggs, five of the six clutches displayed multiple paternity (see Figure 4 for paternity determinations for the offspring of a doubly-mated fe-
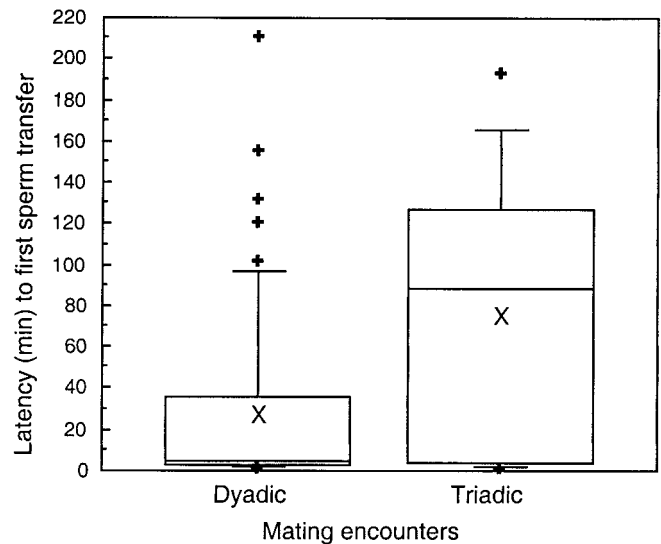
Figure 2
Comparison of latency (min) to sperm transfer in the ''initial pairing'' and ''second pairing'' when female red-spotted newts (*N. v. viridescens*) were paired with shallow- or deep-tailed males in treatments (trt) 1–4. The upper and lower horizontal lines of the box on the box and whiskers plots (Sokal and Rohlf, 1995) represent the first and third quartiles and the middle horizontal line is the median. The mean is represented by X. The whiskers represent the range. The + represents outliers.
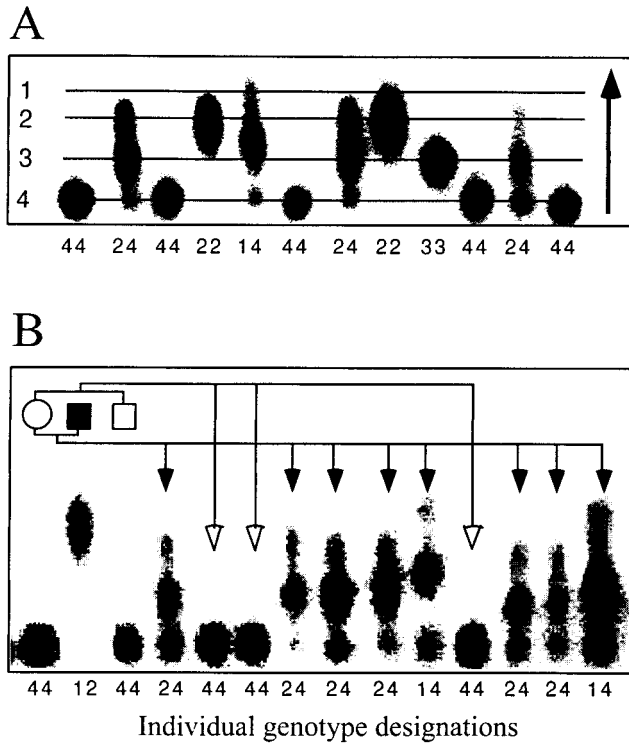
Figure 3
Comparison of latency (min) to sperm transfer when female red-spotted newts (*N. v. viridescens*) encountered one male (dyadic) or two males (triadic). The upper and lower horizontal lines of the box on the box and whiskers plots (Sokal and Rohlf, 1995) represent the first and third quartiles and the middle horizontal line is the median. The mean is represented by X. The whiskers represent the range. The + represents outliers.

A

B

Individual genotype designations

Figure 4
Visualization of phosphoglucose isomerase (*Pgi*) genotypes for *Notophthalmus viridescens* using starch-gel protein electrophoresis. (A) Genetic variability in a random sample of 12 adult males from the Pond Ridge, Virginia, population. The arrow indicates direction of movement of PGI proteins during electrophoresis. Four alleles (labeled 1–4) are present, and the population allelic frequencies are shown in Table 2. Because PGI is a dimeric enzyme, following translation, heterozygotes produce three types of PGI: one corresponding to each allele and an intermediate "heterodimer" product that migrates to a position between each allele during electrophoresis. Individual genotype designations are located below each lane in the gel. (B) Paternity assignments for the offspring of a doubly-mated female, with evidence of first-male advantage. The adult female is indicated by the circle, the fathers are indicated by a solid box (first male) and by an open box (second male).

male). We found statistically significant deviations from equal (shared) paternity in four of six clutches (Table 5). Overnight matings appeared to produce a second-male mating advantage, whereas those females that mated twice within a shorter time period (<3 h) had clutches exhibiting a first-male advantage. Thus, the time interval between mating and egg laying may have an effect on whether there is first- or second-male advantage (Table 5). These results were not confounded by the possibility that females were mated before being brought to the laboratory because none of the unmated females laid eggs ($n = 6$) in the laboratory.

There was no significant difference in the SVLs of males that successfully sired most of the offspring (mean ± SE, 47.3 ± 0.60 mm) as compared to those that did not (46.7 ± 0.40 mm; $n = 6$, two-tailed Mann Whitney test; $U = 24.5$, $z = -1.07$, $p = .28$). There was also no significant difference in the tail height of males that successfully sired most of the offspring (8.75 ± 0.44 mm) as compared to those that did not (9.42 ± 0.71 mm; $n = 6$, $U = 22.5$, $z = -0.70$, $p = .46$).

DISCUSSION

Male–male competition

Deep-tailed males were more successful at mating with females than shallower tailed males. These deep-tailed males had longer SVLs but were not necessarily in better body condition than shallow-tailed males. Tail length, the other sexually dimorphic trait, did not affect a male's success or lack of success in courtship encounters when another male was present.

Verrell (1986) found that bigger males (greater SVL) were more successful at preventing sexual interference during wrestling matches with an intruding rival male. We, too, found that bigger males with deep tails were significantly more successful at mating with females than were smaller males with shallow tails. Our results support the inference that male–male competition is a major factor in the mating system of red-spotted newts (Massey, 1988; Verrell, 1982, 1983, 1986). Eighty-two percent of the courtship attempts were affected by sexual interference at the spermatophore deposition stage. Sexual interference did appear to influence the form of courtship used by deep-tailed males, as courtship in most encounters consisted of amplexus instead of the more vulnerable hula display. This is consistent with prior results for red-spotted newts (Verrell, 1983). However, sexual interference did not prevent successful sperm transfer by the courting male. The ineffectiveness of sexual interference may be an incidental factor of test-

Table 5
Paternity identification for offspring of twice-mated *Notophthalmus viridescens* from Virginia

Female no.	Min elapsed between matings	Total no. offspring genotyped	No. offspring sired by 1st male	Proportion offspring sired by 1st male	No. offspring sired by 2nd male	Proportion offspring sired by 2nd male	No. offspring paternity ND
1	160	16	13	81 (<.02)[a]	3		0
2	113	30	19	100 (<.01)	0		11[b]
3	9	21	9	69 (.20)	4		8[c]
4	Overnight[d]	22	7		15	68 (.09)	0
5	Overnight	21	3		11	79 (<.05)	7[c]
6	Overnight	16	3		11	79 (<.05)	2

[a] *p* values generated by chi-square tests of statistical significance.

[b] The probability of detecting offspring paternity was 75% for the first-male and 50% for the second male. Thus, it is extremely likely that the second male sired no offspring in this clutch.

[c] The probability of detecting offspring paternity was equal for each putative sire. Thus the inability to assign paternity for some offspring did not bias the results.

[d] See text for explanation.

ing in the laboratory. In ponds, females usually swim away when two males are competing (Massey, 1988), but deep-tailed males may be more likely to follow and capture these females (Able, 1999).

Verrell (1989) hypothesized that competition between males for access to reproductive females provides a strong selective pressure in the evolution of male behavior. In red-spotted newts, male–male competition appears to be a strong selective force that has influenced the evolution of amplexus in the courtship of this species, as Verrell (1989) predicted. The greater amount of amplexus used versus hula by males in this experiment lends support to Verrell's (1989) hypothesis that amplexus is a form of sexual defense that has coevolved with sexual interference.

There was a trend for male–male competition and sexual interference to increase the length of time invested in courtship for males of *N. viridescens* that were in competitive encounters as compared to encounters with only one male and female, but it was not significant. This scenario may be similar to that found for the small-mouthed salamander, *Ambystoma texanum,* which significantly increased the total duration of courtship as more conspecific males were present (McWilliams, 1992).

Female choice

Females demonstrated no significant preference to associate with deep- or shallow-tailed males. They also did not demonstrate a significant preference to mate with shallow- or deep-tailed males in the first or second matings. When no male–male competition was involved, there was also no significant difference in the tail height, SVL, or tail length of successful or unsuccessful males. These results provide no evidence that sequential or simultaneous comparison processes affect mate choice of female red-spotted newts. These results are in contrast to other studies of salamanders where female choice has been shown (i.e., *Triturus vulgaris,* Gabor and Halliday, 1997; *Ambystoma tigrinum,* Howard et al., 1997; *Desmognathus ocrophaeus*; Houck and Reagan, 1990).

Choosing mates from sequential visits with males may be more difficult than simultaneous choice and requires the female's ability to remember previously visited males to make comparisons (Bakker and Milinski, 1991; Real, 1990). Although the mating system of smooth newts (*T. vulgaris*) supported Halliday's (1983) sequential choice hypothesis (Gabor and Halliday, 1997), our results for red-spotted newts did not.

Our results, when combined with the male–male competition experiment, indicate that tail height is an intrasexually selected characteristic. Within the scope of our study, these results suggest that tail length is not intra- or intersexually selected. Female choice does not appear to occur for these traits in red-spotted newts. Our results are further supported by Able (1999), who found that males of *N. viridescens* with deeper tails were more successful at capturing females in the pond and in laboratory aquariums. Our study also concurs with that of Howard et al. (1997) in which they found that male–male competition in *Ambystoma t. tigrinum* favored larger male body length. However, Howard et al. (1997) also found that female mate choice selected for greater male tail length; in our study, this was not evident for red-spotted newts.

Males of the European smooth newt (*T. v. vulgaris*) develop a dorsal crest during the courtship season, and females prefer males with larger crests (Gabor and Halliday, 1997; Hosie, 1992). Crest height has been demonstrated to be an indicator of a male's foraging success (Green, 1991). Gabor and Halliday (1997) found that in smooth newts, where there is no male amplexus of females during courtship, female choice of mates plays a major role during courtship (Halliday, 1990).

However, red-spotted newts, in which males amplex females during part of courtship, appear to have a sexual strategy that induces male–male competition for mates with little evidence of female choice playing a major role. Furthermore, male body condition does not seem to influence a male's mating success, so females may not benefit from being choosy.

Sperm precedence

While multiple paternity occurs in red-spotted newts in the laboratory (and it probably occurs in the field as well), the results from our sperm precedence and our sequential mate choice experiment suggest that cryptic female choice (Eberhard, 1996) through the selective use of stored sperm does not occur. In clutches of double paternity, significantly greater numbers of offspring were sired by the first male in some clutches and by the second male in others. In two clutches, no significant departure from shared paternity occurred. Because of the small number of clutches examined, we cannot infer that either first or last males will tend to have a fitness advantage. Our main result is that sperm precedence likely does not have a pervasive and consistent effect on fertilization success. However, to the extent that the range in latency of sperm transfer in our experiments may be unnatural, sperm precedence effects may be more consistent. For example, if double matings tend to occur within a few hours, then our observations suggest that first-male advantage may occur more frequently. Thus, females may show cryptic choice by controlling the magnitude of time between successive matings.

Our sperm precedence results, when combined with the sequential female-choice study, suggest that females are not controlling sperm usage and that they are not controlling matings based on sperm precedence patterns. This explains why our data did not support Halliday's (1983) prediction for sequential mate choice by females that capitalize on last-male advantage. Smooth newts, on the other hand, may have last-male sperm precedence (A. Pecio and J. Rafinski, unpublished data), hence their mating system supports Halliday's hypothesis (Gabor and Halliday, 1997).

Another study has found evidence for multiple paternity in salamanders, but little is known about possible manipulation of sperm by the female or about the mode of sperm competition among competing sperm populations. Houck et al. (1985), studying *Desmognathus ochrophaeus* (Plethodontidae), used sires from two different populations and females that may had been previously mated. They found evidence of mixed male paternity based on allozyme analyses, but they did not address the precise insemination process that was responsible for these results. We were able to examine both the number and rate of inseminations for female red-spotted newts, and it appears that second-male paternity was more likely to occur when there was >3 h between matings. In birds, last-male sperm precedence is achieved through passive stratification of stored sperm with the first male's sperm overlaid by sperm from subsequent matings. In birds if matings are <4 h apart, then sperm mixes; if matings are >4 h apart, then the last male's sperm overlays the first (Birkhead and Hunter, 1990), thus giving the last male an advantage. We did not find this pattern in red-spotted newts.

General inferences

In red-spotted newts, during the male–male competition experiment, males with greater SVL and deep tails were more successful at mating with females than those with smaller SVL and shallow tails. When males were not competing with each other to mate with females, tail height, SVL, and tail length did not appear to influence the success of mating with fe-

males. Moreover, there was no evidence for female choice in red-spotted newts (simultaneous female preference experiment). However, female choice may operate only when males are in overt contact, but this could not be differentiated from our data. Thus, male tail height in red-spotted newts appears to be an intrasexually selected secondary sexual characteristic. Although tail length is also a sexually dimorphic trait, it does not appear to be under either intra- or intersexual selection. Snout-vent length, on the other hand, is not a sexually dimorphic trait.

Within the power of our experiments, male–male competition appears to be the dominant force in the red-spotted newt's mating system. Although there was no evidence of female choice in our experiments, this cannot be ruled out entirely. For example, females may be controlling the timing between matings to control sperm precedence. Moreover, some other component of female choice, beyond the parameters of this study, may have selected for secondary sexual characters in males. If female mate choice occurs, it may be for some other characteristic than that measured here. For example, spermatophore deposition rate has been demonstrated as being important for female choice in smooth newts (Halliday and Houston, 1978). This, however, could not be measured in our experiment because we were also attempting to examine sperm precedence using the offspring from the sequential female choice experiment, so we had to limit the number of spermatophores that were deposited by males. If females are unable to judge differences among males, then they may be gaining indirect benefits from polyandry. For example, mating with multiple males may be a form of genetic bet-hedging. Watson (1991) suggested that in sierra dome spiders, *Linyphia litigiosa,* polyandry enables a female to distribute fertilizations among several mates, thus reducing the impact of occasional flawed evaluations of male quality. Thus, in newts, mating multiple times may reduce a female's risk of all her offspring being of low genetic quality because she was unable to differentiate between males of differing quality.

Two questions arise from the lack of evidence for female choice in our system. First, does amplexus impede female mate choice in newts? Second, how unusual is it for amplexus to impede female choice? In frogs, where amplexus also occurs, female choice does not appear to be limited. For example, Andersson (1994) reviewed 25 studies of frogs that examined sexual selection. In 10 studies, there was evidence of female choice, and in 12 studies, there was evidence of both female choice and male–male competition. In three other studies, there was only evidence of some form of male–male competition but no female choice. However, in two of these studies, the evidence was only observational and not experimental. Thus, amplexus in frogs generally does not limit female choice. However, the mating system of newts is different because there is internal fertilization and multiple mating, unlike most species of frogs. The lack of female choice in newts may be balanced by the potential for females to mate multiple times as a form of genetic bet-hedging.

One way to resolve the two questions above is to trace the phylogenetic history of courtship involving female capture (amplexus) within the newt family Salamandridae. This, however, is difficult because courtship behavior patterns of some important genera are lacking (Titus and Larson, 1995). Titus and Larson (1995) were unable to determine whether the absence of amplexus is the ancestral state. We propose that if amplexus were the ancestral state, then the absence of amplexus in *T. v. vulgaris* would be derived and a consequence of female choice precluding male capture of females. Alternatively, if the ancestral state were the lack of amplexus, then the presence of amplexus in *N. v. viridescens* is derived and is a consequence of female capture by males (and male–male

competition) impeding female choice. Alternatively, the evolution of amplexus to nonamplexus and of nonamplexus to amplexus may have evolved independently in each scenario (Halliday, 1990). We concur with Titus and Larson's (1995) suggestion that further examination is needed of the adaptive status of female capture in the selective regimes of newts.

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