

Differential Responses of Large and Small Male Red-spotted Newts, *Notophthalmus viridescens*, to Conspecific Chemical Cues

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Male red-spotted newts, *Notophthalmus viridescens*, use chemical cues to locate and select potential mates. To investigate whether large and small males respond differentially to chemical cues from conspecifics, we conducted a series of two-choice Y-maze tests with odorant stimuli from two large females (LF/LF), one large versus one small female (LF/SF), and one large female placed with four males versus one small female (LF+4M/SF). We found that males responded more rapidly in a LF/LF choice situation than in those involving LF/SF or LF+4M/SF. In addition, small males chose more quickly than did large males. These results suggest that both body size and odorant context affect the speed of odorant responses. By responding differentially to conspecific chemical cues, small males may arrive earlier at a courting area, increasing their chances of successfully guarding and mating with a female.

Mating competition among conspecific males is common, particularly when the average ratio of fertile females to sexually active males at any given time within a population (operational sex ratio, or OSR; Emlen and Oring, 1977) is male-biased. A male-biased OSR can increase the effects of differences in competitive ability (contestant asymmetry) among males (Maynard Smith and Parker, 1976). In amphibians, large males can often out-compete small males (Houck, 1988; Mathis, 1991; Park et al., 1996). In particular, large males are generally superior in fighting and in successfully guarding a female during courtship. Small males may try to circumvent low competitive ability by avoiding direct contests with large males (Wiltenmuth, 1996).

Chemosensory cues play a critical role in male-male competition as well as in courtship and mating in salamanders (Halliday, 1975; Arnold, 1976; Kikuyama et al., 1995; Rollmann et al., 1999). Differential responses to pheromones or other chemical cues that function at a distance, before direct contact, may be used by small males to compensate for their reduced ability to compete directly with large males. Such compensation has been implicated in studies of responses to chemical cues from different-sized male terrestrial red-backed salamanders, *Plethodon cinereus* (Mathis, 1990; Mathis and Simons,

1994). Male territorial intruders are aggressive when exposed to chemical cues from similar-sized individuals, but are more submissive when exposed to chemical cues from individuals that are larger than themselves, reducing the probability of asymmetric contests (Mathis, 1990). Given the frequency of asymmetric contests for mating opportunities (Houck, 1988; Green, 1991; Park and Park, 2000), it seems plausible that chemical cues may be used by some males to reduce direct competition with other males.

The red-spotted newt, *Notophthalmus viridescens*, is an ideal species in which to investigate the possibility that differential responses to chemical cues are used to compensate for low competitive ability before direct contact occurs. Male newts use chemical cues from females to locate and select potential mates (Verrell, 1982; Park and Park, 2002). During male-male mating competition, competitive ability among males is mainly dependent on body size (Verrell, 1986), although females do not show size-based preferences for males (Gabor et al., 2000). In the laboratory, large males have higher mating success when large and small males are simultaneously presented with a female (Verrell, 1986; Gabor et al., 2000). Nevertheless, in the field, male success in courting or engaging in amplexus with females is not associated with body size (Massey, 1988), suggesting that small males may use other strategies to compensate for low competitive ability. We hypothesized that small males may gain earlier access to females by

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responding more rapidly than large males to chemical cues from females.

To investigate whether large and small male red-spotted newts show different responses to chemical stimuli from males and females, we conducted a series of two-choice tests using a Y-maze. Specifically, we tested whether (1) large and small males differ with respect to the speed of their response to chemical stimuli from conspecifics and (2) different odorant stimuli differentially affect the speed of responses in large and small males.

Materials and Methods

Animals

Eastern red-spotted newts (*N. viridescens viridescens*) in breeding condition were purchased from a licensed supplier (Charles Sullivan Co., Nashville, TN). Upon arrival at the laboratory, males and females were kept in separate aquaria (80 cm length×40 cm width×50 cm height) containing approximately 25 liters of aged tap water at a density of no more than 20 individuals per tank. Half of the water was changed weekly. Water temperature was kept between 10–12°C and the animals were kept on a schedule of 16 h light: 8 h dark with lights on at 0600 h to mimic the spring breeding season. Hiding places were provided in the form of floating paper towels and pieces of broken pottery that were placed on the bottom of the aquaria. Animals were fed live *Tubifex* worms *ad libitum*.

All males used in the study were in full breeding condition, as evidenced by swollen cloacae, fully developed tailfins, and callosities on the hind legs (Petranka, 1998). The 45 largest and 45 smallest males were selected from a pool of more than 100 males to serve as test subjects. The snout-vent length (SVL: distance from the tip of the snout to the posterior of the cloacae) of 10 males randomly selected from each pool was measured and compared to verify that animals in the two groups were of different sizes. Mean±SD for the SVL of large males was 46.5±0.16 mm (range: 44.1–49.1 mm), and that of the small males was 41.5±0.16 mm (range: 39.1–43.7 mm). This difference is statistically significant ($t=7.06$, $df=18$, $P<0.01$). Given that a field study demonstrated that the mean SVL of male red-spotted newts from five different populations ranged from 41.2 to 45.9 mm, with a standard deviation of approximately 3.5 mm (Caetano and Leclair, 1996), an average 5 mm difference in the SVLs of our large and small males seems to reasonably reflect the variability in body size found in natural populations.

All experiments were conducted according to guidelines established by the U.S. Public Health Service and overseen by the Michigan State University All-University Committee on Animal Use and Care.

Preference tests

To investigate responses of male newts to chemical cues, we performed preference tests using a Y-maze, as described in a previous study (Park and Propper, 2001). In such mazes, the test animal begins the trial in a start box (8 cm long×4.5 cm wide×5 cm high) at the bottom of the stem of the “Y”. Stimuli, in this case other newts, are placed behind a perforated plastic mesh barrier at the ends of the two arms of the maze. The Y-maze was constructed of Plexiglas, and each arm of the maze measured 22 cm long×4.5 cm wide×5 cm high.

The two arms of the maze were continuously infused with aged tap water at a flow rate of 30 ml/min from a reservoir containing 500 ml aged tap water. At this flow rate, water flow from the two arms remained separate to the drain at the end of the Y-maze; nevertheless, test males could sample the water from both arms while behind the perforated starting gate. After each trial, mazes were washed using aged tap water. All experiments were conducted between 1000 and 1500 h.

After a 3-min adaptation period behind the starting gate, the gate was slowly raised, allowing the test animal to enter the maze. Three time parameters were measured to evaluate the speed of odorant responses to different chemical stimuli. (1) The latency time was defined as the time required for a test male to leave the starting area once the gate was opened, measured when the tip of the tail crossed the boundary of the starting gate. We measured using the tip of the tail instead of the snout because test males within the starting box often moved their snouts back and forth along the start line; in contrast, when test males began to move out of the starting area, they generally moved directly toward the source, and the tail tip crossed the line once. Thus, measuring the time until the tip of the tail crossed the starting line provided the most accurate measure of the time required for the test male to leave the starting area. Although this measure of latency may bias the data toward recording longer latencies for larger animals, the average size difference of less than 5 mm between large and small males is probably not sufficient to lead to significant latency differences between groups. Each animal was allowed 5 min for this response. An animal that failed to leave the starting area within 5 min was removed from the maze, and data for that animal were not included in the analysis for that trial. (2) The choice time was defined as the time required for the tip of the snout of a test male to travel more than half the length of one of the two arms of the Y-maze after leaving the starting area. Each animal was allowed 10 min for this response. An animal that failed to make a choice within 10 min was removed from the maze, and both latency and choice time data for that animal were not included in the analysis. (3) We also recorded the total maze time, the total of the latency and choice times. In addition, the

Table 1. Summary of results of choice tests

| | Choice tests | | | | | |
|-------------------------------------|----------------|----------------|----------------|----------------|------------------|-------------------|
| | LF / LF | | LF / SF | | LF+4M / SF | |
| Size of subject males | Small | Large | Small | Large | Small | Large |
| Number of subjects tested | 23 | 24 | 26 | 27 | 41 | 39 |
| Number leaving starting area | 19 | 22 | 25 | 21 | 27 | 30 |
| Number completing choice | 17 | 19 | 18 | 18 | 20 | 21 |
| Odorant stimulus chosen | LF=10 LF= 7 | LF=10 LF= 9 | LF=12 SF= 6 | LF=14 SF= 4 | LF+4M=7 SF=13 | LF+4M=12 SF= 9 |
| <i>P</i> value, binomial test | 0.63 | 1.00 | 0.24 | 0.03 | 0.26 | 0.66 |
| <i>P</i> value, Fisher's exact test | 0.25 | | 0.22 | | 0.09 | |

In the LF/LF test, one large female was placed at the end of each arm of a Y-maze as odorant stimulus; in the LF/SF test, a large female was placed at the end of one arm and a small female at the end of the other as odorant stimulus; and in the LF+4M/SF test, one large female and four males were placed at the end of one arm and a single small female was placed at the end of the other as odorant stimulus. A two-tailed binomial test was used to determine whether or not small and large males prefer one odorant stimulus to the other within each choice test. To analyze choice differences between small and large males, we used Fisher's exact test.

arm of the Y-maze chosen by each test male was recorded. Numbers of animals tested and of animals successfully leaving the starting area and making a choice are provided in Table 1.

The order in which animals from the large and small groups were used in Y-maze trials was decided by tossing a coin; therefore, small differences occurred in the sample sizes of large and small males used in each choice test (see Table 1). Each test animal was given each choice test only once.

Chemical stimuli used

Three different odorant choice tests were performed in Y-mazes, and subject animals were given three-day resting periods between the different odorant choice tests. In the first test, males were presented with a choice between two large females (the large female / large female, LF/LF test). In this test, the stimulus animals were 2 large females, selected from a pool of 20 large females and size-matched such that there was less than 0.5 g difference in their body masses. For each trial, a different combination of two size-matched females was selected from the pool. Our previous work indicates that males do not show any preferences when presented with two similarly-sized females (Park and Propper, 2001).

The second choice test was the large female / small female (LF/SF) test. For this test, groups of 10 large and 10 small females were used as pools from which stimulus animals were randomly selected at the beginning of each trial. Five animals from each pool were randomly selected and weighed: the mean \pm SD for the large females was 3.75 \pm 0.57 g (range: 2.91–4.39 g), and for the small females was 2.67 \pm 0.18 g (range: 2.41–2.86 g). For each trial, a different combination of females was selected from each pool. Previous results suggest that males faced with choice situations can discriminate female size using chemical cues, and prefer cues from large females to

those from small females (Verrell, 1985; Rowland et al., 1990).

In the third choice test, males were presented with stimuli from one large female placed with four males versus stimuli from one small female alone (the LF+4M/SF test). Large and small females were selected from the same pools used in the second experiment. In addition, 4 males selected from a pool of 20 were added to the side arm containing the large female. For each trial, different combinations of large and small females and four males were used. Before the beginning of each trial, the large female and four males were allowed to interact for 5 min. In this test, subjects may be faced with a complex choice because they are simultaneously exposed to chemical cues from potential competitors as well as from large and small females; subjects may face competition from other males if they select the large female (Park and Propper, 2001).

In all choice tests, stimulus animals or groups were assigned to a side of the Y-maze by tossing a coin. Some stimulus animals were used more than once, but not in successive trials.

Statistical analyses

For statistical analyses, we treated each experiment as independent of the others, as subjects were drawn at random from a larger pool for use in each experiment.

Chi-square (χ^2) tests were used to analyze frequency data, such as the number of large and small males completing the choice. Data from animals that did not complete a choice were not included in analyses. For each test, data concerning the choices made by small and large males were analyzed using a binomial test; differences in the choices made by small and large males were compared using Fisher's exact test.

Because the latency, choice, and total maze time data passed a normality test (one-sample Kolmogorov-Smirnov

test, $P > 0.05$) and a test for the homogeneity of variance (Levene's test of equality of error variance, $P > 0.05$) after a natural log (ln) or square root transformation, parametric statistical tests were used to analyze these data. A two-way analysis of variance (ANOVA) was used to analyze the main effects of body size (large and small) and odorant treatments (LF/LF, LF/SF, LF+4M/SF), as well as interaction effects for the latency and choice time data. Tukey's *post-hoc* tests were then used for pairwise two-point comparisons when the ANOVAs indicated significant effects of different odorants.

Because total maze time depends on the latency and

choice times, main and interaction effects of body size and odorant treatments were analyzed using a two-way ANOVA with a Bonferroni correction. The statistical program SPSS (v. 10) was used for all analyses.

Results

The percentage of subjects successfully making a choice did not differ across choice tests (χ^2 , $P = 0.36$): 76.6% of subjects (36 animals of 47 that initiated a trial) completed a choice in the LF/LF test; 67.9% (36 of 53) in the LF/SF test, and 51.3% (41 of 80) in the LF+4M/SF test. Large

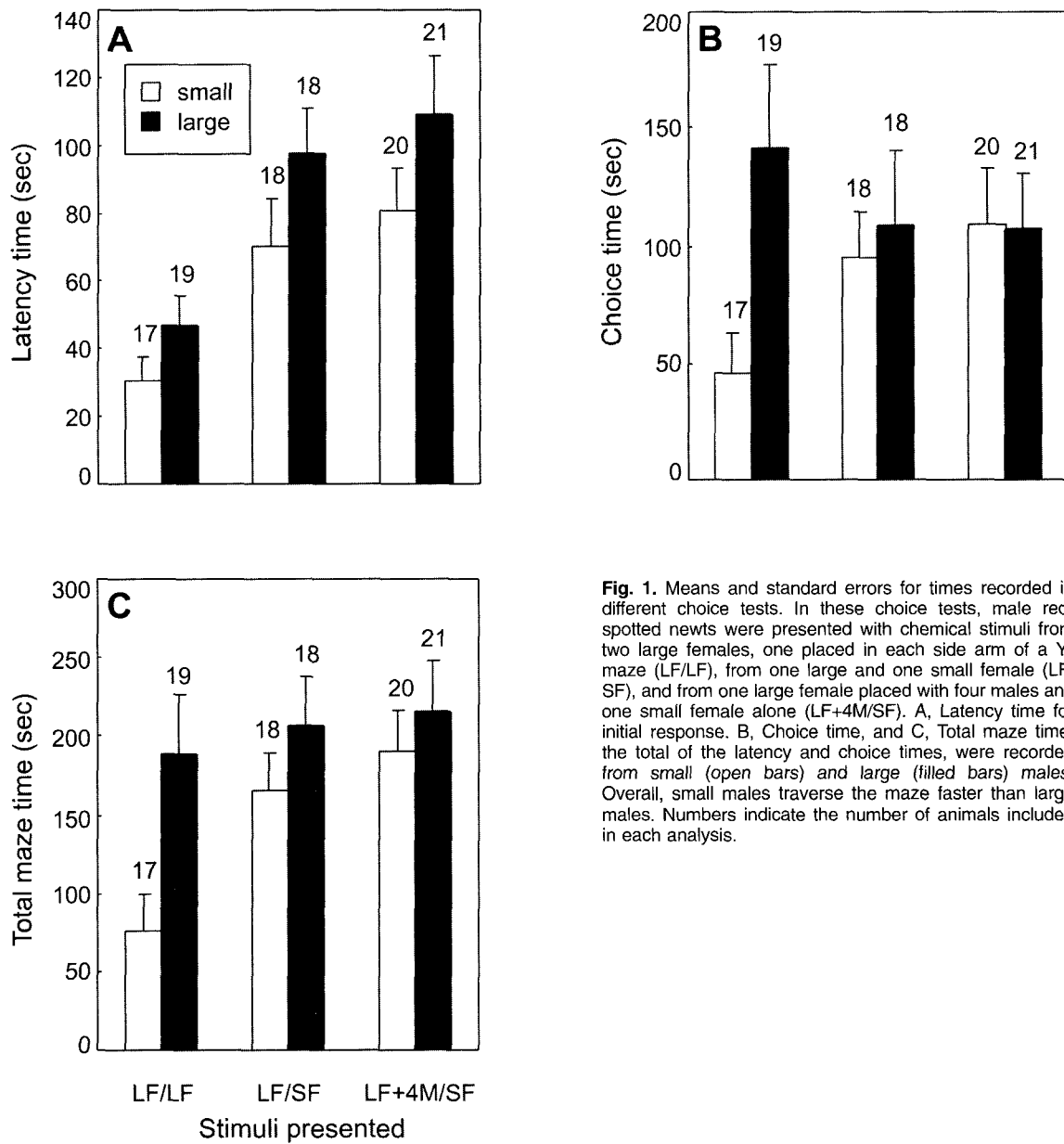


Fig. 1. Means and standard errors for times recorded in different choice tests. In these choice tests, male red-spotted newts were presented with chemical stimuli from two large females, one placed in each side arm of a Y-maze (LF/LF), from one large and one small female (LF/SF), and from one large female placed with four males and one small female alone (LF+4M/SF). A, Latency time for initial response. B, Choice time, and C, Total maze time, the total of the latency and choice times, were recorded from small (open bars) and large (filled bars) males. Overall, small males traverse the maze faster than large males. Numbers indicate the number of animals included in each analysis.

males displayed a significant preference for odorants from large females over odorants from small females (two-tailed binomial test, $P=0.03$), but small males did not ($P=0.24$). This difference in preference between small and large males was not statistically significant (Fisher's exact test, $P=0.22$). In other choice tests, no significant preference was displayed by either small or large males (Table 1).

Latency time ($F_{5, 112}=5.57$, $P<0.01$) and total maze time ($F_{5, 112}=4.56$, $P<0.01$) varied significantly with both body size and odorant stimuli. No significant interaction between body size and odorant stimuli was observed for latency time ($F_{2, 112}=0.04$, $P=0.96$) or total maze time ($F_{2, 112}=2.34$, $P=0.10$). Although large and small males tended to display different choice times in LF/LF experiment (Fig. 1B), overall, choice time did not vary with body size or among tests ($F_{5, 112}=1.57$, $P=0.17$), so we did not analyze the choice time data further.

Both body size ($F_{1, 112}=5.28$, $P=0.02$) and odorant stimuli ($F_{2, 112}=11.44$, $P<0.01$) emerged as main factors influencing latency time, the time to leave the starting area. As illustrated in Fig. 1A, the latency time was shorter for small males than for large males. *Post-hoc* tests indicate that the latency time in the LF/LF test was significantly shorter than in the LF/SF and LF+4M/SF tests (for both cases, $P<0.01$); the difference in latency times in the LF/SF and LF+4M/SF tests was not significant ($P=0.86$).

Both male body size ($F_{1, 112}=5.46$, $P=0.02$) and odorant stimuli ($F_{2, 112}=7.01$, $P<0.01$) significantly affected the total maze time. Fig. 1C illustrates these trends, in which smaller males tended to complete the maze more rapidly than did large males, and in which total maze time tended to increase with the complexity of the choice presented. *Post-hoc* tests demonstrate that the total maze time in the LF/LF test was significantly shorter than in the LF/SF ($P=0.01$) and LF+4M/SF ($P<0.01$) tests, but that the difference between the LF/SF and LF+4M/SF tests was not significant ($P=0.94$).

Discussion

Responses to chemical cues from potential mates may be affected by both the complexity of the stimulus and by the possibility of competition within the courting area. The complexity of an odorant is determined by the number of meaningful blends that subjects perceive within the stimulus and by the number of different odorant components (Jellinek and Köster, 1983). In humans, increasing the number of components in a stimulus increases overall response time (Livermore and Laing, 1996; 1998). In our study, we found that with the presentation of increasingly complicated choices, from the LF/LF test to the LF/SF test, the probability of successfully making a choice within the 15 min allotted decreased from 76.6% to 67.9% of total trials, and the

latency time and total maze time of animals making a choice significantly increased. In addition, adding male-male competition to the LF/SF test further decreased the probability of successful choice from 67.9% to 51.3%, although the latency and total maze time in the LF/SF choice test was not significantly different than in the LF+4M/SF test.

Large and small males may use different strategies to respond to information in conspecific chemical cues. In our tests, the latency time for small males was significantly shorter than that for large males. The latency to respond to an odorant is due to the time required for the odorant to reach detection threshold, for identification of the odorant, and for selecting the appropriate response (Livermore and Laing, 1998). We believe that the adaptation period of 3 min behind the starting gate is enough time to allow for threshold and identification of odorants in the stimuli presented, because we found that in trials in which no start gate was used, males left the starting area within 3 min (Park et al., unpublished data). Differences in latency between large and small males may therefore be due to differences in the time required to select an appropriate response. Although we cannot rule out the possibility that large and small males differ in their motivation to initiate responses, our results suggest that small males respond rapidly to chemical cues. Furthermore, the total maze time of small males to different odorant stimuli was also significantly shorter than that of large males, indicating that small males more rapidly complete their odorant choices. In the field, the consequences of these rapid odorant responses could be the earlier arrival at a courting area of small males than of large males.

Early arrival of males who have low competitive ability at courting areas or breeding sites may occur when the inferior males pay lower costs or gain higher benefits for arriving early as compared to arriving later. The reproductive consequences of differential arrival time at breeding sites have been studied in migratory birds (Møller, 1994; Kokko, 1999) and fishes (Candolin and Voigt, 2003). For example, in sticklebacks (*Casterosteus aculeatus*), inferior small males that arrive early gain the benefit of increased access to territories, but pay the cost of higher predation risk (Candolin and Voigt, 2003).

In our study we found that small males rapidly completed their choice, regardless of odorant stimuli, suggesting that in the field small males may quickly arrive at areas in which a female is alone or in which courtship between males and females is progressing but has not been completed. Female red-spotted newts do not show preferences for larger males (Gabor et al., 2000). Small males can mate successfully when no rivals are present, and the mating success of males that guard females is often higher than that of rivals (Verrell, 1986). Replacement of guarding males by smaller or similar-sized rival males does not occur in laboratory tests,

although large males can displace smaller guarding males in the field (Verrell, 1986). These results suggest that small males arriving earlier than large males may have a greater chance of courting and guarding a female, thus compensating for an apparent size disadvantage in direct male-male competition.

If earlier arrival at a courting area results in increased opportunities for a successful mating, why do large males not also rapidly respond to female chemical cues? There are several possible explanations. First, our finding that only large males chose large females significantly more than small females suggests that although small males choose more quickly, they may pay the cost of an increased rate of error in the choices made (see Table 1). The slower choice process in large males may result in fewer mistakes in evaluating a female's fecundity. Second, large males can out-compete small males during male-male interactions (Verrell, 1982; 1986), so large males arriving later may still have opportunities to mate with females. Third, later-arriving large males can replace courting small males engaged in amplexus with a female (Verrell, 1986). Finally, the reproductive success of courting (4.4%) and sexual interfering males (5.8%) is similar in the field (Massey, 1988). These results suggest that later-arriving large males may not have reduced probability of mating, because large males can successfully out-compete earlier-arriving small males. Taken together, the optimal arrival time of small individuals may depend on their competitive ability relative to large males and also on the tactics used by large males to conserve their reproductive fitness (Parker and Courtney, 1983).

Size-based differences in responses to chemical cues occur in other situations. In terrestrial salamanders (*P. cinereus*), territorial intruders modulate their behavior based on the perceived size of the territorial resident, reducing direct competition (Mathis, 1990; Mathis and Simons, 1994). In addition, sized-based differences in responses to chemical cues have been reported in studies of responses by amphibians and fishes to chemical cues from predators. In some cases, small individuals reduce their activity in response to chemical cues from predators, whereas large individuals increase their activity (Eklöv, 2000; Brown et al., 2001; Mathis et al., 2003). Presumably these differences are due to size-related abilities to escape versus defend against attacks from predators.

In conclusion, our data suggest that the complexity of odorants affect the speed of odorant responses in male newts. In addition, small males respond more rapidly to odorants and complete their choices more quickly than do large males. The early arrival of small males at a courting area where rival males are not yet present may increase their chances of successful mating by decreasing male-male competition and increasing priority of access to females by guarding. In contrast, large males may choose more slowly but may choose more fecund mates. Thus, differential responses to chemical cues by large

and small males before direct individual contact with prospective mates and rivals may play a significant role in male-male competition for mating in red-spotted newts.

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