THE PHEROMONAL REPELLING RESPONSE IN RED-SPOTTED NEWTS (NOTOPHTHALMUS VIRIDESCENS)

Daesik Park¹, ², ³, Heather L. Eisthen², and Catherine R. Propper³

1. INTRODUCTION

A potential courting site can be defined as an area in which at least one sexually receptive female is present, with or without males. Although many studies have examined sexual competition within courting sites, little is known about the process by which animals choose a specific courting site. To study the process of choosing a specific courting site, the concept of potential reproductive rate (PRR: the maximum number of independent offspring that parents produce per unit time; Clutton-Brock and Vincent, 1991) can be useful, as males would be expected to choose a courting site in which they can maximize their PRR.

When a male chooses a courting site, it should integrate information from different sensory systems to evaluate its expected PRR following the choice. Chemical cues may play a particularly important role such choices for aquatic animals, because visual signals are often limited in aquatic breeding habitats by plants and by turbid water (Dodson et al., 1994). For chemical cues to be useful in the evaluation of expected PRR, these cues should contain information about female fecundity, male-male competition, and courting progress within a courting site.

The use of chemical cues in the choice of courting sites has been suggested in insect species that display a pheromonal repelling response or pheromonal inhibitory response (Hirai et al., 1978; Bijpost et al., 1985). In wind tunnels, male tortricid moths display decreased sexual activity and an increase in lateral movement when courting males and females are placed upward (Hirai et al., 1978). These behaviors are interpreted as adaptive responses that increase the chance of the subject male finding another courting site at which his PRR may be higher.

A clear, explicit definition of a pheromonal repelling response has not yet been offered. In this paper, we define a pheromonal repelling response as the abandonment of a courting site in favor of others based on chemical cues from conspecífics. We were the first to describe a pheromonal repelling response in a vertebrate (Park and Propper, 2001), and to date this remains the only report of such a phenomenon. We are currently working to characterize the pheromonal repelling response in newts in greater detail.

Red-spotted newts, Notophthalmus viridescens, present an ideal model system for investigating the dynamics of a pheromonal repelling response in vertebrates. Red-spotted newts are subject to high levels of male-male competition during mating (Gill, 1978; Able, 1999), due to their highly male-biased operational sex ratio (OSR: the ratio of sexually active males to fertilizable females ready to mate; Emlen and Oring, 1977). Both males and females use chemical cues from two pheromone-releasing glands, the cloacal and genial glands, to discriminate conspecific sex (Pool and Dent, 1977; Dawley, 1984; Verrell, 1985) and male glandular secretions increase receptivity in females (Rogoff, 1927). These results suggest that male newts may use chemical cues from conspecifics to choose among courting sites at a distance.

Using a series of Y-maze olfactory choice tests, we found that male red-spotted newts show a pheromonal repelling response and that the occurrence of the repelling response depends on the number of males present, on male-female interactions, and on female fecundity. The purpose of the work presented here is to describe and elaborate on the main findings that we have reported in previous studies (Experiment I-IV, Park and Propper, 2001; Experiment V-VI, Park et al., submitted).

2. MATERIALS AND METHODS

2.1 Subjects and Maintenance

Eastern red-spotted newts (N. v. 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 (50 cm long x 26 cm wide x 30 cm high) containing approximately 25 L aged tap water at a density of no more than 20 individuals per tank. Half of the water was changed weekly. Water temperature was maintained at 10°C and the animals were maintained on a schedule of 16 h light: 8 h dark, with lights on at 0600 hr, to mimic the spring breeding season. Refuge 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). Stimulus and subject animals were randomly selected from a pool of more than 30 females and 50 males.

2.2 Y-maze Olfactory Choice Tests

To investigate responses of male newts to chemical cues, we performed choice tests using a Y-maze (Park and Propper, 2001). In such mazes, the test animal begins the trial in a start box (8 cm long x 4.5 cm wide x 5 cm high) at the bottom of the stem of the "Y". Stimuli, in this case other newts, are placed behind an opaque, 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 x 4.5 cm wide x 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 hr.

After a 3-min adaptation period behind the starting gate, the gate was slowly raised, allowing the animal to enter the maze. Each animal was allowed 5 min to leave the start box. If the animal performed this initial response, it was allowed another 10 min to complete its choice by traveling more than half length of a given side arm. Animals that failed to leave the start box within 5 min or to complete a choice within 10 min were removed from the maze, and data for those animals were not included in the analysis. The arm of the Y-maze chosen by each subject was recorded and analyzed using one-tailed binomial tests. Each subject animal was given each choice test only once. Stimulus animals were randomly assigned to one side of the maze for each trial by tossing a coin.

### 3. RESULTS

#### 3.1. Experiment I

We first verified that males have no preference when exposed to chemical cues from two same-sized females. An average, sexually-mature adult female newt has a mass of approximately 3 g. We used matched pairs of females that differed by less than 0.05 g in mass, placing one into each side arm of Y-maze. When subject males were allowed to choose between two same-size females, no preference was observed (Table 1, I; one-tailed binomial test, \( P = 0.37 \)).

#### 3.2. Experiment II

To determine whether a pheromonal repelling response occurs, we first placed one of two same-sized females into each arm of the Y-maze, and then added three males to one side to mimic a state of high male-male competition. In this experiment, the stimulus males and female were allowed to interact for 5 min in the side arm before each test begins. We found that subjects strongly preferred the arm containing a single female compared with that containing a female plus three males (Table 1, II; one-tailed binomial test, \( P = 0.017 \)).

#### 3.3. Experiment III

To determine whether chemical cues from three males alone are sufficient to repel conspecific males, we placed three males in one arm of the Y-maze and left the other arm empty. Subject males were then allowed to choose between the two arms. Subjects significantly preferred the arm containing three males to the arm containing nothing but plain water (Table 1, III; one-tailed binomial test, \( P = 0.003 \)).

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#### Table 1. Results of the Y-maze olfactory choice tests.

<table>
<thead>
<tr>
<th>Experiment (no. of subjects)</th>
<th>Sources of chemical cues used</th>
<th>No. of times chosen</th>
<th>One-tailed binomial test ( (P) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>One female</td>
<td>17</td>
<td>0.37</td>
</tr>
<tr>
<td>(37)</td>
<td>One female</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>One female + three males</td>
<td>5</td>
<td>0.017</td>
</tr>
<tr>
<td>(22)</td>
<td>One female</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Three males</td>
<td>21</td>
<td>0.003</td>
</tr>
<tr>
<td>(26)</td>
<td>Plain tap water</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>One female/ three males *</td>
<td>10</td>
<td>0.27</td>
</tr>
<tr>
<td>(24)</td>
<td>One female</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>One large female</td>
<td>21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(25)</td>
<td>One small female</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>One large female + four males</td>
<td>11</td>
<td>0.13</td>
</tr>
<tr>
<td>(29)</td>
<td>One small female</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

* No visual, tactile, or olfactory interactions were allowed between the female and the males.

#### 3.4. Experiment IV

In the next experiment, we determined whether behavioral interactions between the stimulus males and females are necessary to produce a repelling response. The set-up for this experiment was similar to that described for Experiment II, above, except that we inserted a solid barrier between the stimulus female and the stimulus three males that were placed in one arm of the maze. In this experiment, subject males displayed no preference between the two arms of the maze; that is, no repelling response occurred (Table 1, IV; one-tailed binomial test, \( P = 0.27 \)).

#### 3.5. Experiment V

To determine whether female fecundity affects male choice, we determined whether or not subject males display a preference when presented with large and small females. We placed one large (3.75 ± 0.57 g, \( n = 5 \)) and one small (2.67 ± 0.18 g, \( n = 5 \)) female into each arm of Y-maze and allowed subject males to choose between them. Males showed a strong preference for the side containing the large female (Table 1, V; one-tailed binomial test, \( P < 0.001 \)).

#### 3.6. Experiment VI

To determine whether female fecundity affects the repelling response, we placed one large and one small female (used in experiment V, above) into each arm of the Y-maze. We then added four males to the arm containing the large female and allowed the stimulus animals to interact for 5 min before the subject male was placed in the start box.
In this test, subject males did not show a preference for either side (Table 1, VI; one-tailed binomial test, $P = 0.13$).

4. DISCUSSION

To test whether red-spotted newts display a pheromonal repelling response and to further characterize the response, we conducted a series of Y-maze choice tests. Male red-spotted newts displayed a pheromonal repelling response by avoiding an arm containing one female plus three males that are interacting in favor of an arm containing one female alone. Our experiments demonstrate that the occurrence of a pheromonal repelling response depends on the number of stimulus males, on interactions between the female and male stimulus animals, and on female fecundity. These results support the existence of a pheromonal repelling response in vertebrates, and suggest that male red-spotted newts may use chemical cues from conspecifics to evaluate their expected PRRs before choosing a courting site.

Three different variables that affect the occurrence of a pheromonal repelling response emerged from our experiments. First, the number of males present within a courting site affects the probability that the subject will display a pheromonal repelling response. The expected PRR for a male at a courting site containing a single female will be determined by the female’s PRR and by the male’s ability to compete successfully with rivals. For example, if one male completely out-competes the others, his PRR should be the same as that of the female. Conversely, a high level of male-male competition should result in the reduction of each male’s PRR. In a previous study, we found that the minimal sex ratio that induces a significant pheromonal repelling response when compared with chemical cues from one female alone is 3 males: 1 female (Park and Propper, 2001). These results suggest that subject males prefer a courting site in which few or no other males are present, to maximize their PRR by avoiding male-male competition.

Second, behaviorial interactions between stimulus males and females are necessary for the production of a pheromonal repelling response. That is, although the number of males present at the courting site is an important factor in determining whether or not a pheromonal repelling response occurs, the response will only occur if the potential rivals are interacting with the female present at the courting site. The results of experiment IV therefore suggest that chemical cues from males and females that are interacting may contain information about the progress of courtship as well as the number of males within a courting site. This outcome is logical given that males that are not courting a female would probably not change the expected PRR of approaching males. Data from field studies support this inference, as latecomers have been shown to achieve lower general mating success than early-arriving animals (Candolin and Voigt, 2003).

Finally, female fecundity appears to affect the occurrence of the pheromonal repelling response: the result of experiment VI demonstrates that subject males do not display a preference when given a choice between one large female plus four males versus one small female. Because the expected PRR of an approaching male depends in part on the PRR of the female within the courting site, the high PRR expected for a male mating with a large female may be balanced by the low PRR expected when male-male competition is strong.

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Taken together, the results of our experiments suggest that males simultaneously analyze information about the probability and strength of male-male competition, the expected fecundity of different females, and the progress of courtship within a courting site to make a decision that will maximize the males’ expected PRR. The use of Y-mazes in our experiments demonstrates that chemical cues released by both male and female newts contain sufficient information about these different factors for males to be able to choose an appropriate courting site.

What are possible benefits of the pheromonal repelling response to the individuals involved? For approaching males, the benefits seem obvious: they can save their time and energy by avoiding a visit to a courting site that is not likely to result in successful mating, and can instead find another courting site where their expected PRR is higher. Males that have already chosen a courting site and may be interacting with a female can maximize their expected PRR by preventing an increase in male-male competition. For large females who are being courted, the benefit may be that they are more likely to mate successfully by avoiding sexual interference among rival males, which is the most common cause of mating failure in the field (Massey, 1988). Finally, as a by-product, small females that are not as attractive as large females may benefit from an increased probability of attracting males; males repelled from other courting sites may be attracted to a courting site in which small females are present without males.

In conclusion, the choice of a specific courting site should be a complex process, because individuals should consider many factors that affect their expected PRR. The details of the process of choosing a potential courting site are poorly understood. The existence of a pheromonal repelling response in red-spotted newts will allow us to investigate the relative importance of the factors involved. In future experiments, we plan to further explore the factors that contribute to the pheromonal repelling response, as well as the nature of the chemical cues involved. Finally, this paradoxical phenomenon, in which a mixture of two different odors is less attractive than either alone, provides a unique opportunity to examine the processing of pheromones and odorant mixtures in the olfactory system.

5. ACKNOWLEDGMENTS

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6. REFERENCES


