

Behavioral reduction of infection risk

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ABSTRACT Evolutionary biologists have long postulated that there should be fitness advantages to animals that are able to recognize and avoid conspecifics infected with contact-transmitted disease. This avoidance hypothesis is in direct conflict with much of epidemiological theory, which is founded on the assumptions that the likelihood of infection is equal among members of a population and constant over space. The inconsistency between epidemiological theory and the avoidance hypothesis has received relatively little attention because, to date, there has been no evidence that animals can recognize and reduce infection risk from conspecifics. We investigated the effects of *Candida humicola*, a pathogen that reduces growth rates and can cause death of tadpoles, on associations between infected and uninfected individuals. Here we demonstrate that bullfrog (*Rana catesbeiana*) tadpoles avoid infected conspecifics because proximity influences infection. This avoidance behavior is stimulated by chemical cues from infected individuals and thus does not require direct contact between individuals. Such facultative modulations of disease infection risk may have critical consequences for the population dynamics of disease organisms and their impact on host populations.

Biologists have long speculated that there should be fitness advantages to susceptible animals that can recognize and avoid infected conspecifics (1–8). If true, we would expect susceptible animals to recognize and avoid conspecifics that would be the source of potential infection. This avoidance hypothesis is in direct conflict with much of epidemiological theory, which is founded on the assumptions that the likelihood of infection is equal among members of a population and constant over space (9–10). However, despite long research traditions in both behavioral biology and epidemiology, we are aware of no empirical demonstrations of pathogen-mediated avoidance behavior. In the context of sexual-selection studies, it has been found that females of some species avoid breeding with diseased males; however, in these studies, the role of infection risk has not been explored directly. The existence of such responses would have critical implications for understanding patterns of disease transmission and the dynamics of host populations. In this paper, we document a disease-avoidance response among larvae of the bullfrog, *Rana catesbeiana*.

The yeast *Candida humicola* is an intestinal pathogen of larvae of several anuran species (11, 12, 16). *C. humicola* has a one-host life cycle and is naturally transmitted among tadpoles by ingestion of feces and water containing cells of the pathogen (12). Severe infections result from crowded conditions that promote coprophagy (11, 12). Intestinal pathogens of this nature are known to reduce growth rates, decrease competitive ability, impact the ability to detect and respond to predators, and result in mortality (11–16). We tested three predictions, in separate experiments, using larvae of the

bullfrog, *R. catesbeiana*: (i) uninfected larval bullfrogs recognize and avoid infected conspecifics (Response experiment); (ii) behavioral responses of larval bullfrogs are mediated by water-borne chemical cues (Cue experiment); and (iii) proximity to infected conspecifics leads to higher rates of infection of larval bullfrogs (Transmission experiment).

MATERIALS AND METHODS

We collected *R. catesbeiana* larvae from Wu pond, located in Cheshire, CT (New Haven County). All tests were conducted with Gosner stage 25 tadpoles (17). To generate infected individuals, we used two different methods. Tadpoles in nature may become infected with a variety of pathogens. To mimic this situation, we generated wild-infected tadpoles by exposing animals to feces and water of infected conspecifics. To isolate the effect of *C. humicola* on avoidance behavior of *R. catesbeiana*, we infected tadpoles with pure strains of *C. humicola*. Results of the response experiment revealed similar effects of wild- and culture-infected tadpoles; therefore, in subsequent experiments, we used culture-infected stimulus tadpoles exclusively. The presence or absence of infection was confirmed in tadpoles by postexperimental examination of intestinal tracts.

General Rearing Conditions. Animals were reared in the Greeley Laboratory Annex at Yale University at approximately 18°C on a 12:12 h light/dark cycle. Focal tadpoles were raised individually in plastic containers filled with 700 ml of dechlorinated tapwater. Stimulus animals were raised in groups of seven in 2-liter containers filled with dechlorinated tap water. All containers were cleaned every third day, and tadpoles were fed ad libitum a diet of alfalfa pellets and fish food. Animals were never used in more than one test, and all animals were size-matched within and between experiments.

Wild-Infected Protocol. We inoculated (daily for 13 days) stimulus animal containers with 30 ml of feces and water. Feces and water were obtained from a high-density (17 per liter) group of *R. catesbeiana*. Before removing feces and water from the high-density tadpole container, we stirred the water for 1 minute. The fraction removed from the container was approximately 50:50% water/feces. Uninfected stimulus animal containers were sham-inoculated with 30 ml of tap water. Focal animal containers also were infected by inoculating their containers with 10 ml of feces and water from the high-density groups of *R. catesbeiana*. Uninfected focal animals were also sham-inoculated with 10 ml of tap water daily.

Culture-Infected Protocol. *C. humicola* was obtained as a pure culture from the American Type Culture Collection and grown in standard media as required (12). Containers holding the infected tadpoles were inoculated with a suspension of *C. humicola* to produce a concentration of 10^3 cells per ml.

Postexperimental Gut Examination. After testing, we randomly selected trials ($n = 20$ trials for the response experiment; $n = 15$ trials for the cue experiment; all trials for the transmission experiment), and all tadpoles, both stimulus and

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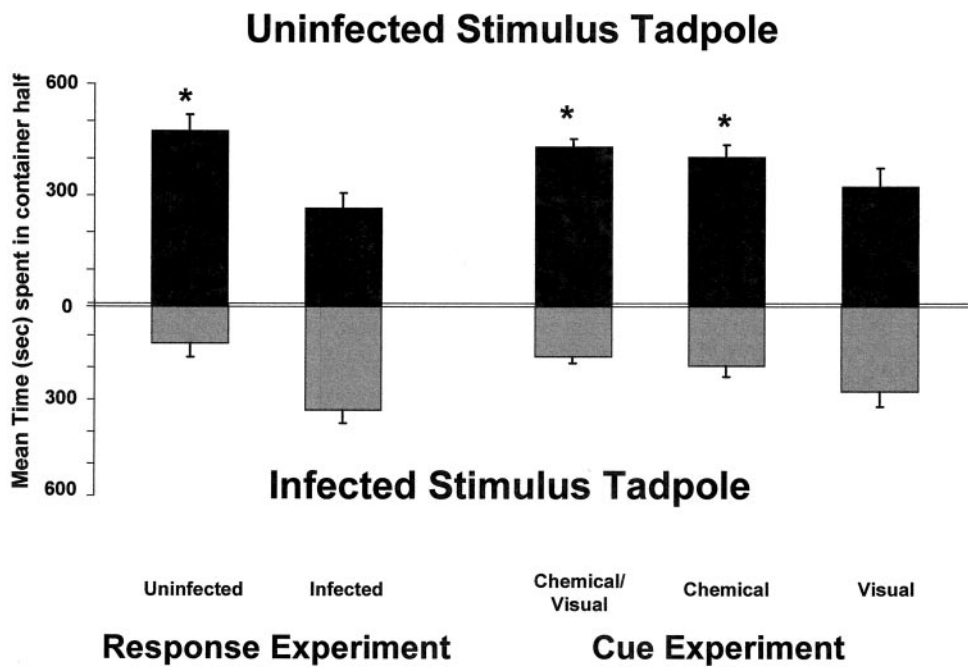


FIG. 1. Mean (\pm SE) time spent in either the infected tadpole (shaded bars) or uninfected tadpole (filled bars) portion of the testing chamber by focal animals in the response and cue experiments. Focal animals in the response experiment were either uninfected or infected. Infections in the response experiment were the result of exposure to feces of wild caught tadpoles or exposure to a pure culture of *C. humicola*. In the response experiment, behavioral responses to infected tadpoles were similar regardless of the mode of infection (wild-infected or culture-infected). For simplicity, we have only presented the results of culture-infected trials. Focal animals in the cue experiment were exposed to visual cues and/or chemical cues of stimulus animals. All focal animals were uninfected and infections in the stimulus animals were the result of exposure to a pure culture of *C. humicola*.

focal, were preserved in 70% EtOH. The intestinal tracts of preserved tadpoles were dissected from each animal, macerated with forceps, and diluted in 1 ml of water. The solution was then shaken to further dislodge intestinal contents, and a portion of the solution was transferred to a hemacytometer. The number of yeast cells in seven 0.04 mm² grids was scored, and the mean number of cells in three subsamples was used to estimate the relative number of cells in tadpole guts.

Response Experiment. During the response experiment (Oct. 14, 1997 to Oct. 18, 1997) we measured activity and microhabitat use of individual focal animals in response to uninfected and infected tadpoles. Uninfected ($n = 15$) and wild-infected ($n = 15$) focal animals were tested for their avoidance of wild-infected stimulus animals. In addition, uninfected ($n = 15$) and culture-infected ($n = 15$) focal animals were tested for their avoidance of culture-infected stimulus animals.

Testing took place in rectangular plastic arenas (14 \times 35 \times 50 cm) that were filled to a depth of 10 cm. During each test, an end cage contained an infected tadpole and an opposite end cage held an uninfected tadpole. Focal tadpoles were placed within the containers and given the choice of associating with either an infected or uninfected conspecific. One focal animal was placed in a central cage (10-cm diameter) and allowed to acclimate for 10 minutes. A test began after the 10-minute acclimation period, when the center cage was lifted, releasing the focal tadpole. A line divided the containers into widthwise halves, and each half was further divided with lines into four equal sections. Stimulus animals were housed in fiberglass screen cages (15 cm in diameter, 3 cm deep) that were placed vertically against the end walls of the container. Stimulus animals were placed in the end cages 20 minutes before the start of each test. Each test included a 10-minute period during which an observer recorded the location of the tadpole and each time it crossed from one container section to another. We estimated activity as the number of times the tadpole changed container sections during the 10-minute period. We randomly

altered the position of the infected tadpole from end to end among trials. Containers were cleaned between trials. All testing took place during daylight hours.

Cue Experiment. In the cue experiment (April 17, 1998), we examined the sensory modality used to detect infected conspecifics. Uninfected focal animals were assayed for their avoidance of culture-infected stimulus animals. Tests were identical to those described for the response experiment, except that stimulus animals were placed in cages that allowed transmission of chemical and visual cues, only chemical cues, or only visual cues.

Stimulus animal cages (10-cm diameter) used in both chemical/visual trials and chemical-only trials of the cue experiment were composed of opaque plastic. Cages in the chemical/visual trials had a 12.5 \times 8.0-cm portion of the cage removed and covered with fiberglass screen. Cages in the chemical-only trials had one-third of the cage perforated with small (300- μ m) holes. These holes were then oriented toward the wall of the enclosure, and we placed an airstone within both cages to facilitate the movement of chemical cues from stimulus to focal animals. Cages in the visual-only trials were composed of glass and allowed no movement of chemical cues.

Transmission Experiment. In the transmission experiment, we assessed the influence of proximity on disease transmission. The experiment was conducted in Nancy's pond, located in Woodbridge, CT (New Haven County) from April 22 to April 30, 1998. We used a factorial design with three isolation distances (0, 0.5, 1.0 m) from either infected or uninfected (control) conspecifics. The experiment was terminated after 7 days, at which time all animals were preserved in 70% EtOH. Infection level of focal animals was determined by estimating the relative number of yeast cells in tadpole guts.

Enclosures were placed in six arrays parallel to the east shoreline of the pond. At the center of each array was the stimulus animal enclosure with seven tadpoles that were either infected or uninfected. Focal-animal enclosures were then placed either at 0, 0.5, or 1.0 meters from the stimulus

Table 1. Estimated number of *C. humicola* cells in the intestines of *R. catesbeiana* larvae

Experiment	<i>n</i>	Mean	SE
Response			
Wild-infected tests			
Uninfected focal	5	33.8	13.9
Uninfected stimulus	10	26.3	6.7
Wild-infected focal	5	519.8	104.6
Wild-infected stimulus	10	456.3	57.1
Culture-infected tests			
Uninfected focal	5	38.2	16.3
Uninfected stimulus	10	31.2	6.7
Culture-infected focal	5	558.6	58.7
Culture-infected stimulus	10	614.1	46.0
Cue			
Uninfected focal animals		42.7	7.7
Infected stimulus animals		397.13	127.4
Uninfected stimulus animals		61.3	17.4

enclosures, each with one uninfected *R. catesbeiana*. Within each array, we randomized the position of each focal animal enclosure to one of four compass bearings (0°, 90°, 180°, 270°). Each treatment was replicated 3 times for a total of 18 focal animal enclosures. Enclosures (10-cm diameter) were composed of plastic hardware cloth (1-cm² mesh) that prevented tadpoles from leaving but allowed transmission of the pathogen. Enclosures were pressed into the mud of the pond; thus, each tadpole had exposure to natural substrate. Two meters separated each array. All enclosures within each array were matched for depth, and the depth of all enclosures ranged from 33.5 cm to 36.5 cm.

Statistical Analysis. The Wilcoxon signed rank test was used in the response experiment and the cue experiment to analyze total time spent by tadpoles in uninfected versus infected

halves of the tank. For each focal animal, we measured avoidance as the number of seconds spent away from the infected tadpole portion minus one-half of the trial duration (300 seconds). Three hundred seconds is the expected time spent in each half of the container under the null hypothesis of no avoidance. We compared activity of uninfected and infected tadpoles in the response experiment using a Student's *t* test. We compared the relative number of yeast cells per focal tadpole in the transmission experiment using a two-way ANOVA, with main effects, status (stimulus infected or uninfected), and proximity (0, 0.5, 1.0 m).

RESULTS

In the response experiment, behavioral responses to infected tadpoles were similar regardless of the mode of infection (wild-infected or culture-infected). When in the presence of an infected tadpole, uninfected focal animals spent 75.4% of their time away from the infected individual (wild-infected tests: 13/15 trials, $Z = 3.239$, $P = 0.001$; culture-infected tests: 13/15 trials, $Z = 2.79$, $P = 0.005$, Fig. 1). In contrast, infected focal individuals showed no tendency to associate with one side of the container over the other (wild-infected tests: 8/15 trials, $Z = 0.739$, $P = 0.460$; culture-infected tests: 7/15 trials, $Z = 0.966$, $P = 0.335$, Fig. 1). These patterns were not due to differences in activity because uninfected and infected tadpoles displayed similar levels of movement (wild-infected tests: $t = 0.527$, $P = 0.602$; culture-infected tests: $t = -1.671$, $P = 0.106$). Postexperimental gut examination confirmed that there were strong differences in estimated number of yeast cells per tadpole between uninfected and infected individuals (Table 1).

In the cue experiment, we found that when exposed to chemical and visual cues or only chemical cues of stimulus tadpoles, uninfected focal animals spent 72.2% and 67.2% of

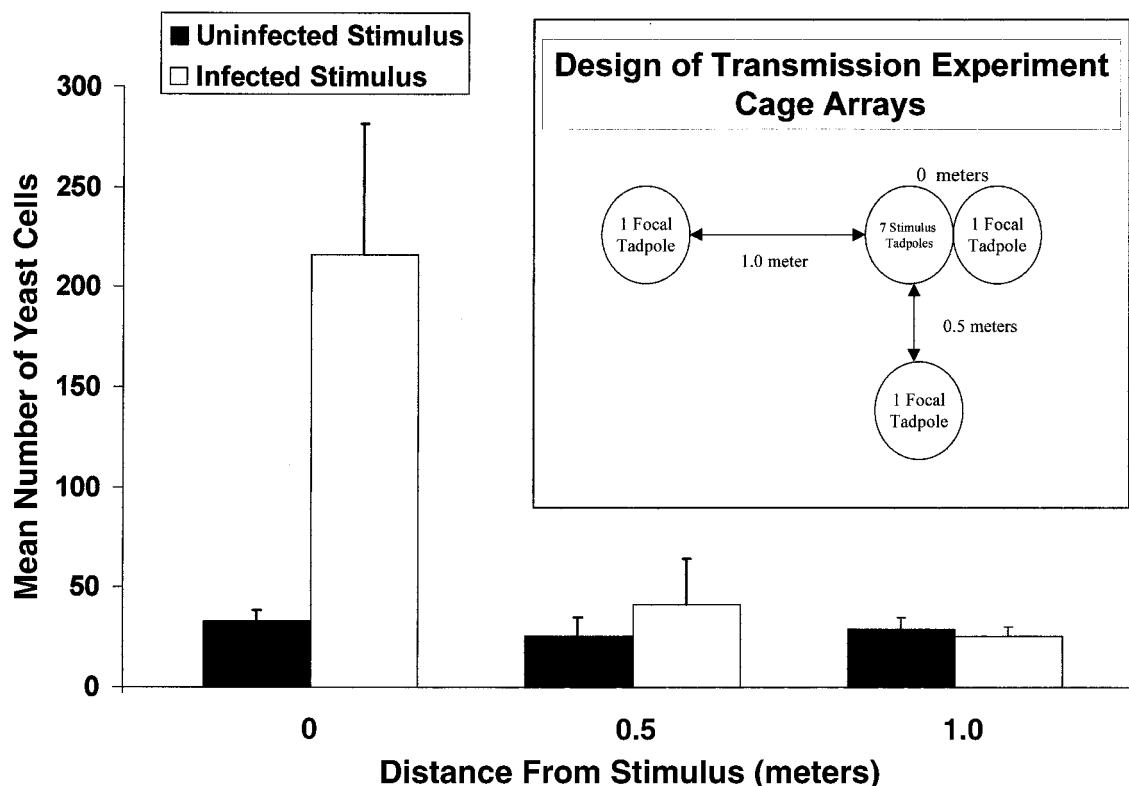


FIG. 2. Mean (\pm SE) number of yeast cells in the intestines of focal tadpoles in the transmission experiment. Focal tadpoles were placed at varying distances from stimulus animals. Stimulus animals were either uninfected or infected with a pure culture of *C. humicola*. Inset shows schematic of field cage arrays used in the transmission experiment.

their time away from the infected individuals (chemical + visual trials 14/15, $Z = 3.351$, $P = 0.001$; chemical-only trials 12/15, $Z = 2.556$, $P = 0.01$, Fig. 1). In contrast, when exposed only to visual cues of stimulus animals, there was no tendency to associate with one side of the container over the other (7/15 trials, $Z = 1.023$, $P = 0.307$).

In the transmission experiment, focal tadpoles confined immediately adjacent to infected stimulus tadpoles exhibited elevated counts of yeast cells in their intestines (Status: $F_{1,12} = 6.324$, $P = 0.017$; Proximity: $F_{1,12} = 7.122$, $P = 0.009$; Status \times Proximity: $F_{2,12} = 6.324$, $P = 0.013$, Fig. 2). However, focal tadpoles 0.5 and 1.0 m from infected-stimulus tadpoles exhibited low yeast-cell counts. All focal tadpoles exposed to control stimulus tadpoles had low numbers of yeast cells in their guts (Fig. 2).

DISCUSSION

Plasticity in behavior is known to modify interactions such as competition and predation. However, little is known about how individual behavior can shape disease transmission (1). Although there have been many demonstrations that tadpoles and other animals avoid proximity to other mortality risks, notably predators (18–21), this study shows that animals can recognize and respond to the potential costs associated with diseased conspecifics. Our results were observed in a context in which disease transmission occurs via ingestion of feces and water containing cells of the pathogen. Given the nature of transmission, avoiding areas containing infected individuals appears to lower the risk of infection.

In contrast, infected focal animals did not avoid infected conspecifics. Pathogenic infections often cause a physiological response to the infection that can alter behavior (22). In other systems, infected animals tend to avoid contact with other individuals, which may reduce the risk of contracting further disease (23). However, tadpoles infected with *C. humicola* are known to experience a decreased response to chemical cues (16). Thus, infected focal animals may not avoid infected conspecifics because of impaired recognition capabilities. Chemical cues are known to play important roles in predator recognition (24) and social interactions (25) of amphibians. The results of these experiments clearly show that it is not a change in the behavior of infected animals that induces avoidance but the detection of chemical cues that are required for infection mediated avoidance behaviors.

Until recently, it was widely believed that the impacts of predators on prey populations were mediated through mortality. However, it has become apparent that plastic responses by prey to the risk imposed by predators are found in a great variety of organisms can have important fitness consequences to prey and can impact the dynamics of predator–prey popu-

lations (26, 27). This body of research has led to a fundamental shift in the conception of predator–prey interactions. The responses we observed in this study have the potential to alter our understanding of the dynamics of infection. Where animals are able to recognize and modify their risk of infection, we should expect a comparable cascade of impacts on host and pathogen populations.

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