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David Bartholow
John Carroll University, dbartholow12@jcu.edu

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CHEMOTAXIS OF *BATRACHOCHYTRIUM DENDROBATIDIS* ZOOSPORES IN RESPONSE TO LIVE AMPHIBIANS

A Thesis Submitted to the

Office of Graduate Studies

College of Arts & Sciences of

John Carroll University

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

By

David S. Bartholow

2018

Abstract

Batrachochytrium dendrobatidis (*Bd*) is one of the primary drivers of amphibian decline, and has spread rapidly on a global scale, but the mechanisms of *Bd* movement on small spatial scales are poorly understood and may play a role in transmission and infection. The flagellated zoospores of this fungus exhibit chemotaxis in response to single chemical cues, towards potential nutrient sources and away from metabolites of anti-fungal bacteria (AFB) present on amphibian skin. Levels of cutaneous AFB were manipulated on *Eurycea bislineata* hosts (either by bathing in a culture of the AFB *Janthinobacterium lividum*, or bathing in antibiotics) to test the effects of differences in cutaneous microbiota on the chemotaxis of *Bd* zoospores. Chemotaxis was measured using a hemocytometer grid to track average movement of zoospores for 45 minutes. A stochastic model was implemented based on observed magnitudes of chemotaxis to estimate probability of zoospores reaching a host as a function of distance from host. Differences in net chemotaxis between treatments was non-significant, and overall levels of mean net chemotaxis were low with high variance. The model suggests that chemotaxis is not a strong driver of probability of *Bd* zoospores reaching a host relative to simple distance from the host. Results do not support chemotaxis as a strong driver of *Bd* transmission, but chemotaxis may play a role in the development of *Bd* infections.

Introduction

Batrachochytrium dendrobatidis (*Bd*) is the fungal causative agent of amphibian chytridiomycosis, a disease that is one of the primary drivers of recent amphibian population declines worldwide (Berger et al. 1998; Rosenblum et al. 2010). *Bd* has infected hundreds of species of amphibians in at least 56 countries (Olson et al. 2013) and is implicated in population declines of hundreds of species and some extinctions (Fisher et al. 2009). Chytridiomycosis spreads rapidly among and within populations (Lips et al. 2008; Olson et al. 2013). This rapid spread can at least partially explain the unusual ability of this pathogen to cause extirpations of entire populations and extinctions of species—in one case study, essentially all amphibians in a population were infected before widespread mortality began, such that density-dependent transmission effects did not come into play (Vredenburg et al. 2010). Across large spatial scales, *Bd* has been transported by humans (Fisher et al. 2012), amphibian hosts (Schloegel et al. 2012), and non-amphibian hosts (Garmyn et al. 2012; McMahon et al. 2013). On smaller spatial scales, the motile, flagellated zoospores likely play a part in the transmission of *Bd,* but the exact mechanisms for regulating movement of zoospores from zoosporangia on an infected host to a new individual are poorly understood (Piotrowski et al. 2004).

In addition to playing a part in spread of the disease, *Bd* zoospore movements may play a role in establishment and progress of infection. Infection begins when flagellated *Bd* zoospores attach to amphibian skin, where they encyst and then resorb their flagellum, and eventually develop into zoosporangia, which produce a new generation of zoospores by mitosis (Berger et al. 2005). Successful infections typically begin as a cluster of zoospores on the skin of a host, and single zoospores often fail to

develop in culture or on new hosts (Longcore et al. 1999). This may suggest that a mechanism exists to increase the likelihood that multiple zoospores reach a new host within a relatively short time span (Berger et al. 2005). Mature zoosporangia found within the epidermis tend to form discharge papillae in the direction of the skin surface, with released zoospores either dispersing to a new host or re-infecting the same host, thereby leading to exponential growth of infection and skin damage (Berger et al. 2005). This suggests that at least some zoospores may swim freely before re-infection.

Anti-fungal bacteria (AFB) found on amphibian skin inhibit *Bd* growth in culture (Austin 2000; Lauer et al. 2007; Flechas et al. 2012) and increase survivorship of infected amphibians (Becker et al. 2009; Becker and Harris 2010; Burkart et al. 2017), and AFB treatment has been used to reduce the severity of *Bd* infection symptoms in *Plethodon cinereus* (Harris et al. 2009a). Probiotic treatments such as this represent one of the few proposed methods for ameliorating outbreaks of *Bd* in wild amphibian populations (Woodhams et al. 2011; Bletz et al. 2013; Walke and Belden 2016). Anti-fungal bacteria have been found on all amphibian hosts that have been surveyed (Bletz et al. 2013), including a diverse variety of frogs (e.g., Walke et al. 2011; Flechas et al. 2012), newts (e.g., Bletz et al. 2017), and plethodontid salamanders, which have been the primary study system for amphibian cutaneous AFB (e.g., *Plethodon cinereus* [Lauer et al. 2007], *P. ventralis* [Austin 2000], and *Hemidactylium scutatum* [Lauer et al. 2008]), because they frequently occur in high abundance and the fact that large scale declines within this family have not been reported.

One possible explanation for the success of *Bd* zoospores in finding hosts is chemotaxis, the movement or orientation of unicellular organisms in response to

chemical gradients present in their environment. *Bd* zoospores exhibit positive chemotaxis in response to a variety of potential nutrient sources, including glucose, lactose, cysteine, and keratin (Moss et al. 2008), and negative chemotaxis in response to anti-fungal compounds (2,4-diacetylphloroglucinol and indole-3-carboxaldehyde) produced by two AFB, *Lysobacter gummosus* and *Janthinobacterium lividum,* respectively (Lam et al. 2011). Stochastic models based on observed chemotaxis translate an observed difference in chemotaxis into a probability of infection based on distance from host, and model outputs suggest that amphibian cutaneous AFB may both reduce the chances of infection by zoospores, and cause zoospores to disperse from an infected host rather than causing re-infection (Lam et al. 2011). Thus, chemotaxis of zoospores may be important in both the transmission and infection processes. The combined response to amphibian chemoattractants and AFB chemorepellents has yet to be studied, but this suggests a possible effect of amphibian microbiota on the movements of *Bd.*

In this study, the aquatic *Eurycea bislineata* (Northern Two-lined Salamander; Plethodontidae) was used as a stimulus for *Bd* zoospores to study chemotaxis in a realistic system with attractants and repellents. Levels of cutaneous AFB were manipulated on *E. bislineata* individuals to produce different levels of anti-fungal metabolites. I hypothesized that the strength of *Bd* zoospore chemotaxis would be highest in response to salamanders with reduced AFB, and lowest in response to salamanders with augmented AFB.

Methods

Bd **Cultures, AFB Cultures, and Amphibian Specimens.** The isolate of *Batrachochytrium dendrobatidis* used in this study was JEL660, which was isolated from a wild anuran in Ohio and cryopreserved at the University of Maine. *Bd* stock cultures were grown and maintained on 1% tryptone agar and broth according to standard methods (Boyle et al. 2003).

Adult *Eurycea bislineata* were collected from eight locations in Cuyahoga County, Geauga County, and Ashtabula County, Ohio (Table 1; Fig. 1), to minimize impacts on any single population. Salamanders were caught and handled by hand with nitrile gloves, and placed into clean 50mL centrifuge tubes, after which they were rinsed with Provosoli solution, a standardized artificial pondwater (Wyngaard and Chinnappa 1982), to remove transient bacteria and any possible chemical signatures from the water in which they were caught. Provosoli was used for maintenance of moisture of salamanders in captivity and for all instances in which water was needed in this study. To minimize changes in resident microbiota on salamanders as a consequence of maintenance in lab (e.g., feeding regimen or habitat differences), all trials were conducted within three weeks of collection. After the experiments, specimens were euthanized by immersion in chlorotone until a heartbeat was no longer observed, preserved in 75% ethanol (Simmons 2002), and deposited in the collection of the Cleveland Museum of Natural History.

For AFB, *Janthinobacterium lividum* was selected. This bacterium has been found on the skin of *Plethodon cinereus* and *Hemidactylium scutatum* (Lauer et al. 2008), produces anti-fungal metabolites (Woodhams et al. 2017), and has been shown to

increase survival of amphibians exposed to chytridiomycosis when augmented in soil or on amphibian skin (Brucker et al. 2008; Becker et al. 2009; Harris et al. 2009b; Muletz et al. 2012). *Janthinobacterium lividum* ATCC 12473™, the type strain of this species (isolated from soil in Michigan), was used in initial pilot studies for this project. During one of these pilot studies, *J. lividum* DSB001 was isolated via standard methods (Cappucino and Sherman 2013) from a dilution plate of an untreated *E. bislineata* from Duppy's Creek (Table 1). This new isolate was identified based on micro- and macro-morphology, particularly by the characteristic deep purple of mature colonies, which is caused by the pigment violacein, and which itself has been shown effective against *Bd* (Woodhams et al. 2017). This new, local, isolate was used in the primary chemotaxis assays.

To know when *J. lividum* cultures were actively growing for inoculation of salamanders, growth curves for both strains were created using two growth media (nutrient broth and 1% tryptone broth). Cultures were started from stocks using an inoculating loop and grown on a shaker at approximately 20°C for 48 hours. Serial dilutions of these cultures were used to achieve an approximate McFarland turbidity of 0.5. Controls (nutrient broth and 1% tryptone broth) and dilutions of *J. lividum* culture were pipetted into a 96 well plate and run through a Multiskan FC Microplate Photometer (Thermo Scientific) at 20°C for another 48 hours. Optical density readings at 620 nm were taken every 45 minutes for 1% tryptone and every 30 minutes for nutrient broth. Readings across twelve wells were averaged for each combination of strain and medium.

Biosecurity. All equipment was washed and disinfected with bleach solution (1% sodium hypochlorite) and rinsed with DI water between assays, both to ensure that no *Bd*

zoospores are released into the environment (Johnson et al. 2003) and to ensure that no chemical stimuli remain that might affect the chemotactic response of zoospores. Media and zoospore suspensions used in all studies were sterilized by autoclave or bleach solution before disposal. *Eurycea bislineata* used in *Bd* assays were kept separate from specimens not yet used, and all specimens for this study were kept separately from all other possible hosts at John Carroll University.

Treatment Categories. Four potential chemotactic stimuli comprised the treatments for the chemotaxis assays: a negative control of only Provosoli solution (Treatment W); *Eurycea bislineata* with augmented AFB (Treatment +B); *E. bislineata* with naturally occurring microbiota (Treatment C); and *E. bislineata* with reduced bacteria (Treatment -B). Salamander hosts for chemotactic stimulus were randomly assigned to the three treatment groups with salamanders (Treatments +B, C, -B). All salamanders were treated in 50mL centrifuge tubes and bathed in different liquids depending on treatment. Tubes were rolled along their long axis four times in all treatments to ensure all parts of salamanders were exposed. Treatment C salamanders bathed for 12 hours in 5 mL of Provosoli solution only. Treatment -B salamanders were bathed in 5 mL of Provosoli solution with 10 mg/L ciprofloxacin (an antibiotic which is ineffective for treatment of chytridiomycosis [Carpenter 2013]) for 12 hours. Treatment +B salamanders were treated according to the protocol of Harris et al. (2009a), in which: 1) *J. lividum* DSB001 was inoculated into 100 mL of 1% tryptone broth and grown for 60 hours at 20°C in a shaker; 2) 1 mL of this culture was centrifuged at 4500 G for 10 minutes; 3) the *J. lividum* pellet was resuspended in 1 mL of Provosoli solution; 4) this centrifuge procedure was repeated 3 additional times to remove all bacterial metabolites;

5) this cleaned *J. lividum* was then re-suspended in a total of 5 mL of Provosoli; 6) salamanders bathed in this solution for two hours; and 7) salamanders were rinsed with Provosoli and then bathed in 5 mL of Provosoli for 12 hours. After treatment, salamanders in all treatments were rinsed with Provosoli and bathed in 2 mL of Provosoli for 12 hours to capture their chemotactic stimulus for *Bd* zoospores. For Treatment W, Provosoli solution was left in centrifuge tubes for 12 hours before use in chemotaxis assays.

Chemotaxis Assays. To harvest zoospores, a petri dish of *B. dendrobatidis* on 1% tryptone agar grown for 5–7 days at 20°C was examined under the microscope to confirm the presence of actively-swimming zoospores, then flooded with 3 mL of Provosoli to trigger release of zoospores from sporangia (Boyle et al. 2003). After 30 minutes, another 1 mL of Provosoli was added to the plate, gently swirled, and the resulting zoospore suspension was removed with a micropipette. The zoospore suspension was pipetted to fill the counting chamber of a standard hemocytometer. A 6mm-diameter acid-free paper disk was soaked in 100 μL of the Provosoli with chemotactic stimulus and positioned on the hemocytometer such that the edge of the disk slightly overlapped the edge of the counting chamber (below the coverslip), to serve as potential chemotactic stimulus for the zoospores. Photographs were taken of the counting chamber at 40× magnification with an Olympus IX71 microscope and attached camera, Olympus DP70. Photographs were taken immediately (Time 0) and at 15, 30, and 45 minutes after adding zoospores and stimulus disk. Based on previous studies (Moss et al. 2008; Piotrowski et al. 2004), 45 minutes is sufficient time for *Bd* zoospores to exhibit a response at these spatial scales (1 mm across the central hemocytometer grid). Between photographs, the light on the

microscope was turned off to prevent heat from damaging zoospores or causing evaporation on the slide. The hemocytometer was not moved between photographs to prevent accidental movement of zoospores. The laboratory was maintained at 20°C during chemotaxis assays.

Zoospores were counted in the columns of the central counting grid nearest to, and farthest from, the stimulus disk. Microscope photographs of hemocytometers with zoospores were imported into Adobe Illustrator CC 2017 21.0.2 (Adobe Systems, San Jose, CA) and a mark was placed on each apparent zoospore to create a record of all counts. Zoospore marks for each column were saved in separate layers from the hemocytometer image, and all marks were counted automatically within Illustrator. All counts were performed by myself, but to ensure repeatability and objectivity of method, a randomly selected 16 photographs were counted by C. Sheil. Counts by the author and C. Sheil differed by an average of 10.34, which was not statistically significant (paired sample *t*-test, $t = 0.881$, $p = 0.222$) and were highly correlated ($R^2 = 0.9221$). To account for variation in density of zoospore suspensions, a proportional difference was calculated as the difference between the number of zoospores in the column nearest to the stimulus and the farthest column, divided by the total number of zoospores in the two columns:

$$proportional\ difference = \frac{\#\ of\ zoospores\ near\ -\ \#\ of\ zoospores\ far}{\#\ of\ zoospores\ near\ +\ \#\ of\ zoospores\ far}$$

For each time point, net chemotaxis was calculated as the change in proportional difference from time 0.

Experimental Design and Statistical Analyses. The level of replication was 16 for each treatment, two for each of the eight sites. Within each site, two zoospore

suspensions were used, to ensure zoospores remained active. Zoospore suspension was used as a blocking factor to account for heterogeneity of sites, density of zoospores, and level of activity of zoospores (i.e., proportion actively swimming). Comparisons of net chemotaxis among treatments used a randomized complete block design. Each block consists of an assay of each treatment made on the same day, with order of treatments randomized within block. Net chemotaxis was analyzed using repeated measures ANOVA, with treatment and block as factors. The Greenhouse-Geisser correction was used for all repeated measures factors and interactions, due to lack of sphericity of the data. All statistics were analyzed using SPSS Statistics 21 (IBM) with a significance level $\alpha = 0.05$.

Random Walk Model. Using the measure of mean net chemotaxis from this study, I adapted a model developed by Lam et al. (2011), which implements a stochastic one-dimensional random-walk model of zoospore movement to predict probability of reaching or dispersing away from a host as a function of relative distance from the potential host. This allows for more meaningful interpretation of the results of the chemotaxis assays by connecting net chemotaxis to a probability of infection. In this model, a zoospore iteratively moves a single step towards or away from the host until it either reaches the host or disperses by reaching an arbitrary threshold distance away from the host. The direction the zoospore moves at each step is determined randomly based on a key parameter of this model, λ, which is the ratio of the likelihood of moving towards a host to the likelihood of moving away from a host. The probability that a zoospore at a distance n (number of steps from the host) reaches the host before dispersing is:

$$\pi_n = \frac{\lambda^N - \lambda^n}{\lambda^N - 1}$$

where *N* is the total number of steps away from a host necessary to disperse. Net chemotaxis from the current study was used to estimate λ by assuming that a mean net chemotaxis of x% suggests that $\lambda = \frac{100+2x}{100}$. For modeling purposes, the highest magnitude of observed positive and negative mean chemotaxis from the chemotaxis assays was used, regardless of treatment or time point, to approximate the maximum possible effect of chemotaxis. The spatial scale over which chemotaxis was measured in this study (1 mm total across the hemocytometer central grid) is larger than the movements in Lam et al. (2011), but the model relies on an arbitrary number of steps rather than explicit spatial difference. Because net chemotaxis is used in this study, model results should be interpreted as average movements of aggregates of zoospores rather than as movements of a single zoospore.

Results

The growth curve for the two *Janthinobacterium lividum* strains after a single ten-fold dilution is shown in Figure 2. *Janthinobacterium lividum* DSB001 grew somewhat faster than *J. lividum* ATCC 12473, and cultures entered log phase growth around 48 hours, so cultures grown for 48 hours were used in the main assay, by which time a purple tinge associated with the production of violacein was visible.

The magnitude of net chemotaxis was small for all treatments (Fig. 3). The maximum mean net chemotaxis across all treatments was 2.84% (Treatment C at 45 minutes; SE = 2.06%; maximum = 12.62%; minimum = -3.81%). The minimum mean

net chemotaxis (i.e., movement away from the stimulus) was -2.93% (Treatment -B at 30 minutes; SE = 4.13%; maximum = 19.14%; minimum = -28.02%). There was no significant effect of treatment ($F = 0.476$; df = 3; $p = 0.716$), zoospore suspension block ($F = 0.766$; df = 14; $p = 0.685$) or time ($F = 0.274$; df = 2.218; $p = 0.787$) or interaction effects Treatment ✕ Block ($F = 0.824$; df = 42; $p = 0.681$); Time ✕ Treatment ($F = 0.510$; df = 6.653; $p = 0.800$); Time ✕ Block: ($F = 1.526$; df = 31.048; $p = 0.260$); Time ✕ Treatment ✕ Block ($F = 0.854$; df = 93.144; $p = 0.677$). Raw count data for the chemotaxis assays is presented in Appendix A.

The maximum positive net chemotaxis from Treatment C above yielded a λ of 1.0568, and highest magnitude negative net chemotaxis from Treatment -B yielded a λ of 0.9414. The resulting random walk model (Fig. 4) shows that, given values of λ this close to 1, the effect of chemotaxis on probability of reaching a host is fairly minor relative to the effect of distance from host. This is particularly true when N (the distance from host at which a zoospore is considered to have dispersed from the host) is small (Fig. 4 triangles). When N is large (Fig. 4 circles), chemotaxis has a greater effect, assuming that it continues to work equally regardless of distance from host.

Discussion

Chemotaxis Assays. Results of chemotaxis assays did not show significant differences in chemotaxis of *Bd* among treatments (Fig. 3). Despite lack of differences among treatments, these measurements give the best current estimate of magnitude of chemotaxis of *Bd* zoospores in a system with a multitude of positive and negative

chemotactic cues from living amphibians, and suggest that strong chemotaxis may not occur in natural systems. Net chemotaxis was less than 3% (positive or negative), whereas variance was relatively high, across all treatments. However, it can be difficult to interpret what a net chemotaxis of 3% means. Two approaches can shed light on what a net chemotaxis of approximately 3% means: the stochastic model of zoospore chemotaxis and comparisons to previous work on *Bd* chemotaxis.

Stochastic model. The random walk model attempts to connect the abstract net chemotaxis numbers to a more concrete probability of zoospores reaching a host from a given distance. The model employed herein used net chemotaxis values from this study, and gave probability curves that are much closer to linear than those found in Lam et al. (2011) (Fig. 4). The relatively small λ herein (Fig. 4 A, B), caused by the small values of mean net chemotaxis observed, causes the model to behave differently than that of Lam et al. (2011), who concluded that chemotaxis might have a strong influence on chance of reaching a host. On the contrary, our model output suggests that average chemotaxis of zoospores, at least in aggregate, is not a strong driver of probability of reaching a host, and distance from zoospores to host is the main driver of probability of reaching the host (when mean chemotaxis is as low as observed here). This holds true even when the arbitrary distance to be considered dispersed from the host is large (i.e., N is large), the scenario in which chemotaxis has the most opportunity to influence outcomes, although chemotaxis does play a stronger role in this case. However, it seems unlikely that the effect of chemotaxis is constant at various distances from the host, and it should be noted that this model assumes that probability of moving in either direction is independent of distance to host. If, instead, λ were to trend towards 1 as distance from potential host

increases (i.e., a weaker chemotactic effect at increasing distances from stimulus), then it would be expected that chemotaxis contributes little to overall chance of reaching host.

Given that chemotaxis was calculated in this study as a net movement of zoospores, and the fact that step size is different in our study than that of Lam et al. (2011), caution should be used in comparing output between the two models. Aggregate movements of zoospores may be as appropriate, or more appropriate, than single zoospore movements in this system, given that multiple zoospores are typically required to begin a successful infection (Long core et al. 1999). On the other hand, even when the model output suggests that likelihood of reaching the host is low for the aggregate, it may be that some few zoospores do reach the host, so probabilities may not be as low as they appear. As discussed below, the spatial scale of the two studies differs such that it may be more appropriate to compare their curve at $N = 50$ to the present studies curve at $N = 10$ (e.g., Fig. 3A triangles and Fig. 3C circles). Both studies suggest that chemotaxis is most likely to have an effect at very small spatial scales, perhaps less than one millimeter. On this scale chemotaxis might play a part in the re-infection of a single host but is unlikely to have a strong effect on transmission to new hosts.

Comparison to previous work on *Bd* chemotaxis. Two previous studies of *Bd* zoospore chemotaxis found evidence of stronger chemotaxis (Moss et al. 2008; Lam et al. 2011). To understand how my results compare to these studies, I will briefly summarize their methodologies and compare magnitude of chemotaxis, as well as approaches to spatial scale, temporal scale, chemotactic stimulus, and sample size. Table 2 summarizes the parameters and results from these studies.

Moss et al. (2008) showed positive chemotaxis using a similar approach to chemotaxis assays herein, with an attractant disk on one edge of a hemocytometer grid. Successful attractants were aqueous solutions of simple energy sources, such as glucose, lactose, cysteine, and keratin. Moss et al. (2008) quantified chemotaxis by counting zoospores on the hemocytometer grid at three time points (0, 45 minutes, and 90 minutes), and reported the percentage of zoospores counted at each time point out of the total of zoospores counted at all time points for that replicate (A.S. Moss, pers. comm.). Resulting differences in percentage over 90 minutes were between 25% and 40% depending on attractant, but it may be more comparable to my methodology to use percent change in absolute number of zoospores over 45 minutes, which would result in percent chemotaxis of over 200% for all treatments for which significant results were found.

Lam et al. (2011) showed negative chemotaxis using a very different approach: individual zoospores were photographed every 0.2 seconds over a 10 second period, and for each 0.2 second interval movement towards or away from the stimulus was recorded. Chemotaxis was thus measured as a ratio of movements towards or away from the stimulus (and modeled as a probability of movement towards or away). Lam et al. (2011) found a mean of approximately 15–25% more movements of zoospores away from single anti-fungal metabolites of bacteria, depending on the repellent (indole-3-carboxaldehyde or 2,4-diacetylphloroglucinol, respectively). It is worth noting that a tryptone and agar substrate was used for controls and for trials with the anti-fungal metabolites, and that tryptone has not been tested directly as a chemotactic stimulus for *Bd,* but because it is an effective nutrient for *Bd* growth it is reasonable to suggest that Lam et al. (2011)

presented a mixed positive- and negative-chemotactic stimulus—in their study, zoospores did show a propensity to move towards the control substrate in absence of the anti-fungal metabolites (Lam et al. 2011; Fig. 3).

Although none of the work on chemotaxis of *Bd* zoospores has used directly-comparable measures of chemotaxis, the magnitude of chemotaxis was much larger in the previous studies than in the current study (Table 2). Herein, failure to find a significant difference in treatments must be interpreted cautiously and might not mean that differences do not exist. However, when coupled with the extremely low values of mean net chemotaxis (as well as low maximum and minimum magnitudes), I suggest that this provides evidence that chemotaxis actually is weak to nonexistent in my study system. To understand why Moss et al. (2008) and Lam et al. (2011) found evidence of chemotaxis but I did not, it is necessary to explore some of the differences in methodology.

The spatial and temporal scales of the three studies differ greatly (Table 2). Because evidence for chemotaxis comes from one study at a larger spatial scale (3 mm; Moss et al. 2008) than the present study (1 mm) and one at a smaller spatial scale (100 μm; Lam et al. 2011), it is difficult to compare among the three. It is worth noting that there is some evidence of weaker chemotaxis at increasing distance from host in Lam et al. (2011; Fig. 3), as proportion of movements away from the AFB metabolites was lower at farther distances from the substrate. The biologically relevant scale for *Bd* zoospores is somewhat unclear: zoospores of *Bd* generally remain motile for less than one day and swim less than two centimeters in this time (Piotrowksi et al. 2004). The scale of movement needed for reinfection of an already infected host should be very small, on the order of a few micrometers, whereas the relevant scale of movement for successful

transmission is potentially much larger and limited only by zoospore dispersal capabilities and lifespan.

The appropriate temporal scale within which to study movements of zoospores is also somewhat unclear. A short time period such as that used by Lam et al. (2011; 10 s) might be most appropriate for understanding the possible effect of chemotaxis on the infection and reinfection process, while the longer time periods used in this study (45 m) and that of Moss et al. (2008; 90 m) might be more appropriate while thinking about dispersal. I chose 45 minutes for the temporal scale because Moss et al. (2008) found strong results over 45 minutes, with less change in the period from 45 minutes to 90 minutes. However, if the trend over time in Figure 3 were extrapolated out for another 15 minutes or more, it appears possible that a longer time scale may have yielded greater differentiation between treatments. On the other hand, longer movement times (and correspondingly longer distances) might make effective dispersal more difficult as hosts may move over time, and water currents or other outside factors might play a larger role.

The stimulus used to elicit a chemotactic response from zoospores is the factor that most distinguishes this study from the two previous papers on *Bd* chemotaxis and is the primary reason this study was undertaken. Previous studies used one simple chemical solution positive-cue only (Moss et al. 2008) or one positive- and one negative-cue in a single substrate (Lam et al. 2011), whereas this study attempted to use a complex suite of mixed cues that would be representative of actual amphibian hosts. The simple single-attractant of Moss et al. (2008) yielded very large chemotactic responses. The mixed attractant and repellant in Lam et al. (2011) gave a much smaller chemotactic response, but still significant at the small scales on which they observed movement. The study

herein used cues taken from living salamanders that would presumably contain many positive and negative chemotactic stimuli. This resulted in a low mean magnitude of net chemotaxis but with high variance, which suggests that conflicting stimuli canceled each other out, and that attempts to manipulate strength of stimuli may have been of limited efficacy (see Limitations, below). It is difficult to measure the combined chemotactic stimulus in any way other than the response of zoospores, as well as to say with certainty whether treatments were effective in terms of overall stimulus. It is also difficult to know how long chemical gradients are maintained on these spatial scales, given the variable and complex nature of the stimuli found in the salamander water. What can be said is that the relatively simple cues using known concentrations of chemoattractants and chemorepellents were successful in eliciting a significant and consistent chemotactic response from *Bd* zoospores, whereas the complex and variable chemical signature of *Eurycea bislineata* did not elicit a consistent response.

These results suggest that in natural situations, chemotactic stimuli are mixed and complicated and might not lead to strongly directional movement of zoospores. When considering natural water flow and currents, as well as movement of possible hosts, this level of chemotaxis may be unlikely to result in effective zoospore dispersal and infection of a new host. Instead, *Bd* may rely on natural water flow, host to host contact, and the production of very large numbers of zoospores for dispersal. It is a common reproductive strategy in Fungi to generate large numbers of (usually non-motile) spores, of which only a tiny fraction finds a suitable substrate by chance movement through air or water currents (Kendrick 1985).

Limitations. It is possible that the low levels of net chemotaxis and high variance observed herein are at least partially the result of the experimental methods. It may be that the treatment levels were insufficient to affect salamander microbiota strongly. There was no method to assess effectiveness of salamander treatments on skin microbial community in this study; one was attempted but was found to be unworkable. Pilot studies suggested that treatments were appropriate to augment or inhibit growth of AFB, but replication in pilots was low (data not shown). Treatment +B used a protocol from other studies that had success both in colonization of *Janthinobacterium lividum* on amphibian skin (Harris et al. 2009a), and in increasing survival when exposed to *B. dendrobatidis* (Harris et al. 2009b). The strain of *J. lividum* that was isolated in this study, DSB001, has not been directly tested for anti-fungal activity, but it did visibly produce violacein, which gives this bacterium its distinctive violet coloration and is known to have anti-fungal properties (Brucker et al. 2008; Becker et al. 2009). Treatment -B showed non-significant negative chemotaxis, which is opposite of the direction expected if AFB levels were reduced. One reasonable explanation might be that a residue of the antibiotic (ciprofloxacin) used to reduce bacteria remained on salamanders even after washing with Provosoli; if this is the case, the ciprofloxacin might act as a chemorepellent. However, other explanations cannot be ruled out based on this study due to lack of a ciprofloxacin control.

Several factors may contribute to high variance in this study. Although treatments attempted to influence amphibian microbiota, initial quantity and composition of cutaneous bacteria, microbiota can vary geographically (Muletz-Wolz et al. 2017), across seasons (Longo et al. 2015), based on duration in captivity (Kueneman et al. 2016), and

based on prior exposure to *Bd* (Jani and Briggs 2014). These sources of variation in microbiota might lead to variation in chemotactic stimulus and zoospore response. However, similar variation would be present in natural systems, and might present a challenge for chemotaxis to operate effectively and consistently for *Bd* zoospores. The blocking factor and replication used herein attempted to account for this variation, but may not have been adequate. It could be argued that, given that some non-significant differences in net chemotaxis are apparent, a higher level of replication might be needed to account for the high levels of variation. However, the high *p*-values in the ANOVA model (most *p* were greater than 0.6) suggest that differences were less extreme than would be expected by random chance; thus, higher replication might well result in similar results. Additionally, the sample size of 16 is already considerably larger than that used in previous *Bd* chemotaxis studies, which have found statistically significant results with sample sizes as low as 4 (Moss et al. 2008) or 5 (Lam et al. 2011), and so it seems reasonable to think that if substantial differences in net chemotaxis exist they would be detected by this study.

Density of zoospores in initial suspensions varied more than was expected based on pilot studies, which might have increased noise in the ANOVA model for chemotaxis assays. However, the zoospore suspension was used as a blocking factor in order to account for this variation as much as possible. Density could also directly affect *Bd* zoospore movements; *Bd* zoospores might give off their own chemotactic signal. This could conceivably be a positive signal due to the need for a threshold number of nearby zoospores to begin a successful infection (Longcore et al. 1999), as well as to overwhelm host defenses (Vredenburg et al. 2010). It could also be that zoospores act as

chemorepellents, if there is a density beyond which conspecific competition would reduce fitness due to limited resources on a given host. Thus, the variation in initial density might lead to different chemotactic responses or zoospores, or sporangia might give off different signals depending on maturity or contact with host.

Conclusions. The results of this study suggest that chemotaxis may not be a strong factor in the transmission of chytridiomycosis in natural systems with chemoattractants and chemorepellents. Although chemotaxis of *Bd* zoospores has been shown in simple lab systems, in this study using positive and negative realistic chemotactic cues from *Eurycea bislineata*, chemotaxis was inconsistent and overall magnitude of chemotaxis was low. When factoring in additional complexities of natural systems, it is difficult to imagine chemotaxis leading to effective transmission of zoospores from one host to another, except in cases where hosts are already very close together. Chemotaxis may still have some role in the infection and re-infection process on a single host, ensuring that zoospores released on the skin of a host find a new site on the same host rather than dispersing. Treatment with AFB is a promising option for dealing with *Bd* infections but does not appear to act through the mechanism of affecting *Bd* chemotaxis.

Acknowledgments

I would to thank Vin Trinh, Christy Baggett, Dr. Christopher Sheil, Dr. Carl Anthony and Dr. Cari Hickerson for help collecting salamanders in the field. Thanks to Dr. Matt Venesky for the culture of *Batrachochytrium dendrobatidis* JEL660 and training on working with *Bd* cultures. Thanks to Dr. Erin Johnson for help with isolation and

maintenance of *Janthinobacterium lividum* bacterial cultures. For help with statistical design and analysis I thank Dr. Tom Short. This project would not have been possible without permission from Ohio Department of Natural Resources, Cleveland Metroparks, the Geauga Parks District and the John Carroll University Intitutional Animal Care and Use Committee. Thanks to Cleveland Museum of Natural History for housing specimens. Finally, I would like to thank my committee, Dr. Carl Anthony, Dr. Erin Johnson, Dr. Matt Venesky and especially my advisor Dr. Christopher Sheil for help with planning, designing and executing the experiment, as well as writing and data analysis.

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Table 1. Locations and dates of collection of *Eurycea bislineata* in Cuyahoa, Geauga and Ashtabula Counties, Ohio. Six specimens were collected from each locale.

Location Name	#	Latitude (ºN)	Longitude (ºW)	Date
Sulphur Springs, South Chagrin Reservation	1	41.424	81.420	5/20/2015
Foster’s Run, North Chagrin Reservation	2	41.534	81.419	5/26/2015
Buttermilk Falls Creek, North Chagrin Res.	3	41.572	81.421	6/3/2015
Tinker’s Creek Tribs, Bedford Reservation	4	41.381	81.549	7/6/2015
Duppy’s Creek	5	41.755	80.909	7/15/2015
Squire Valleevue & Valley Ridge Farm	6	41.495	81.410	7/30/2015
Affelder House, West Woods	7	41.456	81.328	9/24/2015
American Society of Materials, West Woods	8	41.459	81.299	10/2/2015

Table 2. Comparison of methodologies and results of previous papers on chemotaxis of *Batrachochytrium dendrobatidis* zoospores and those present herein.

	Moss et al. 2008	**Lam et al. 2011**	**Present Study**
Chemotaxis	Positive	Negative	None
% chemotaxis	40	25	3
Spatial scale	3 mm	100 μm	1 mm
Temporal scale	45-90 min	10 sec	45 min
Stimulus	Single nutrient	Tryptone and single anti-fungal chemical	Live amphibians and their microbiota
Sample size	4-5	5	16

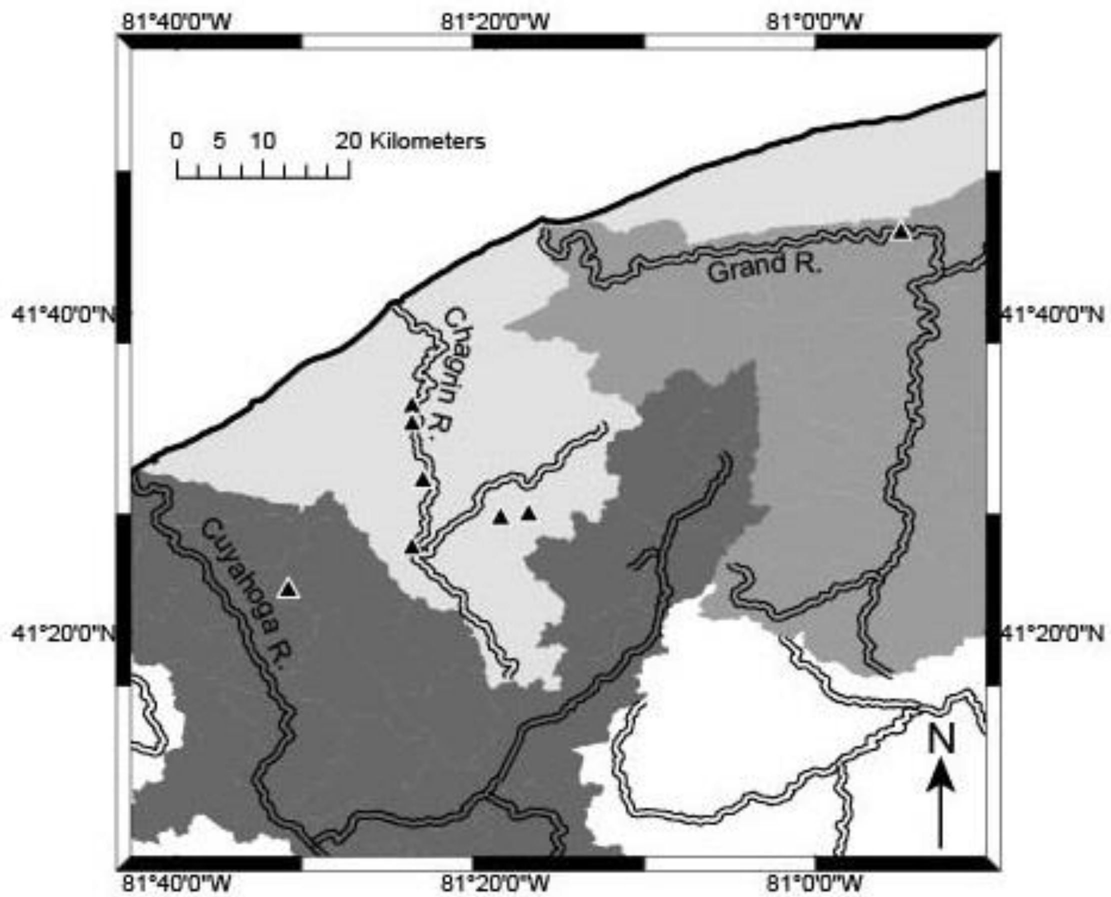

Figure 1. Map of collection locations for *Eurycea bislineata* hosts in northeastern Ohio. Shading represents the watersheds for labeled rivers; triangles represent collection locales. See also Table 1.

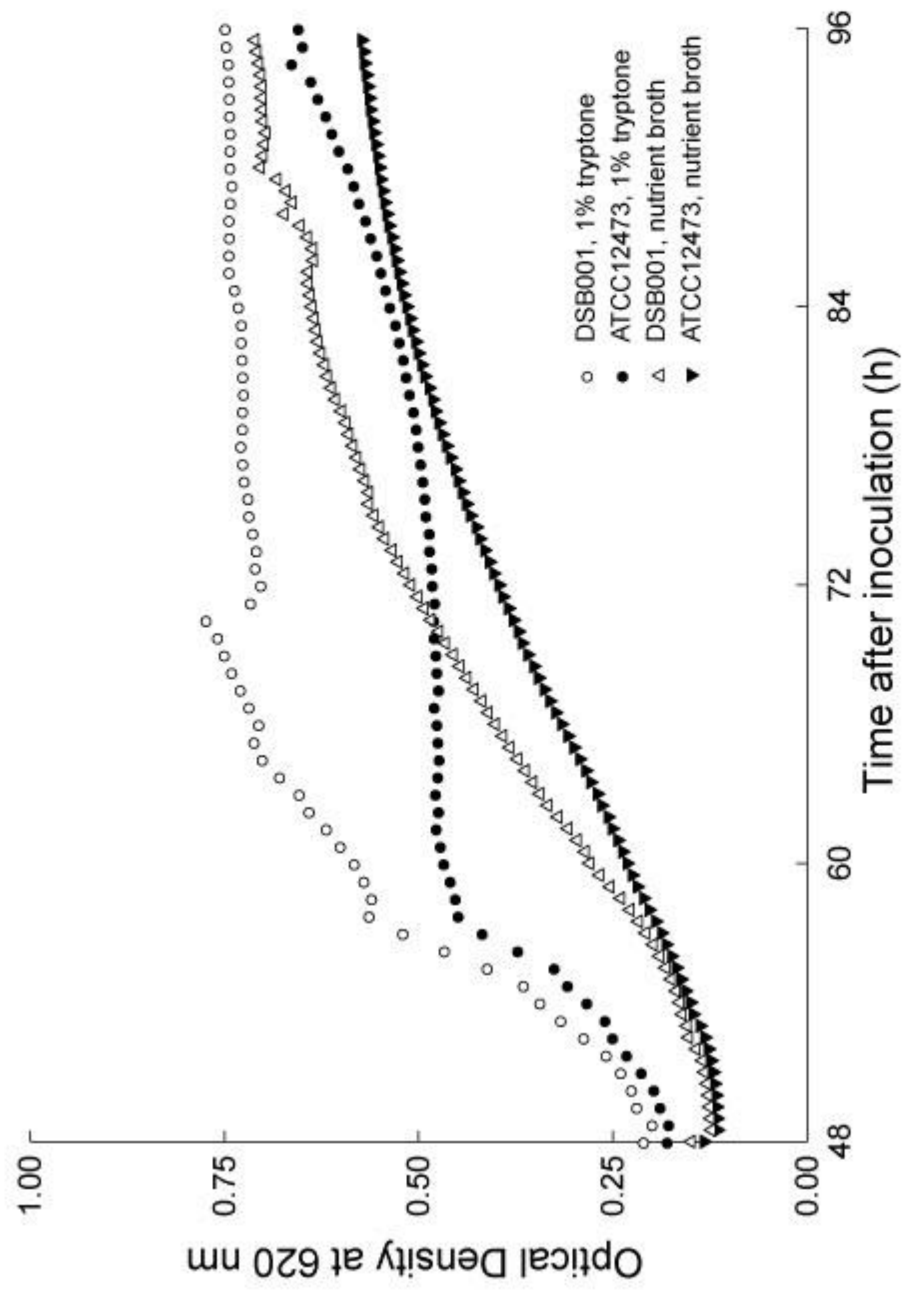
Optical Density at 620 nm
1.00
0.75
0.50
0.25
0.00
48
60
72
84
96
Time after inoculation (h)
DSB001, 1% tryptone
ATCC12473, 1% tryptone
DSB001, nutrient broth
ATCC12473, nutrient broth

Figure 2. Growth curve for two strains of *Janthinobacterium lividum* grown in different media. Strain ATCC 12473 is the type strain for this species and commercially available; strain DSB001 was isolated as part of this study (see Methods). The two strains were each grown on both 1% tryptone broth and nutrient broth at 20°C for a total of 96 hours, with optical density readings taken every 45 minutes. Values are averages of 12 wells for each combination of broth and strain.

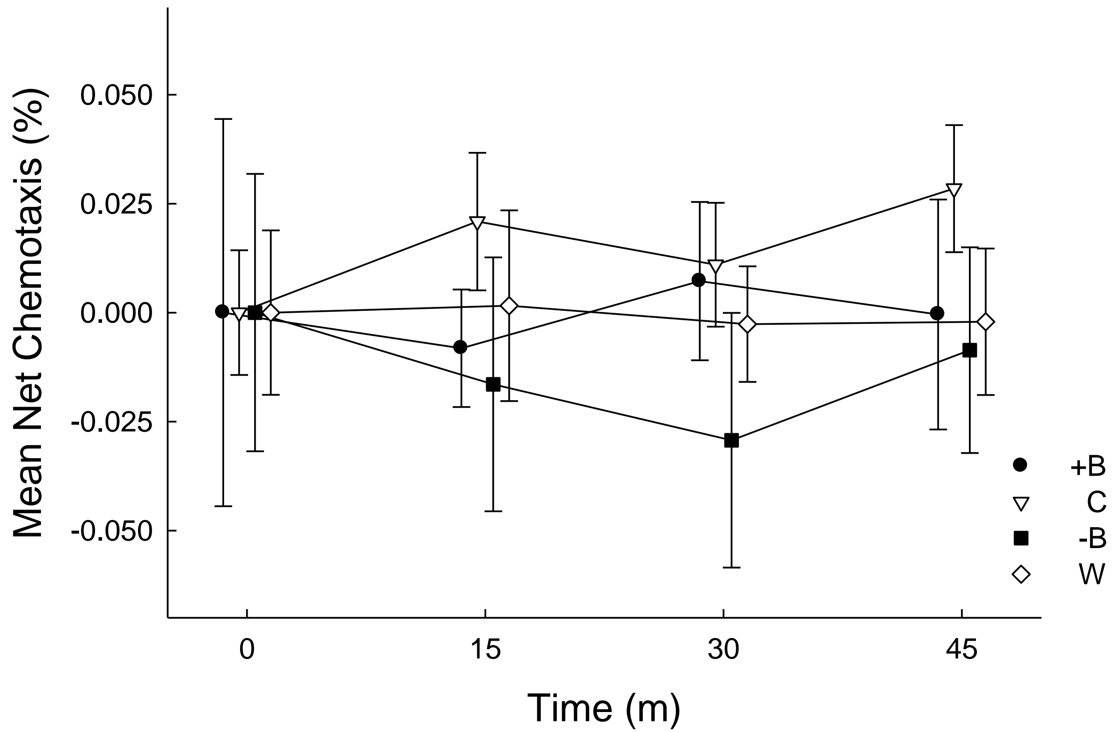

Figure 3. Net chemotaxictic movement of *Batrachochytrium dendrobatidis* zoospores over time in response to cues in bathwater from *Eurycea bislineata* treated with *Janthinobacterium lividum* (+B), untreated (C), treated with antibiotics (-B), and a negative control of artificial pondwater with no salamander (W). Net chemotaxis differences between treatments were not statistically significant (see text). Values are means ± standard error. Treatments are staggered to avoid overlap of error bars; all treatments were measured in 15 minute time increments.

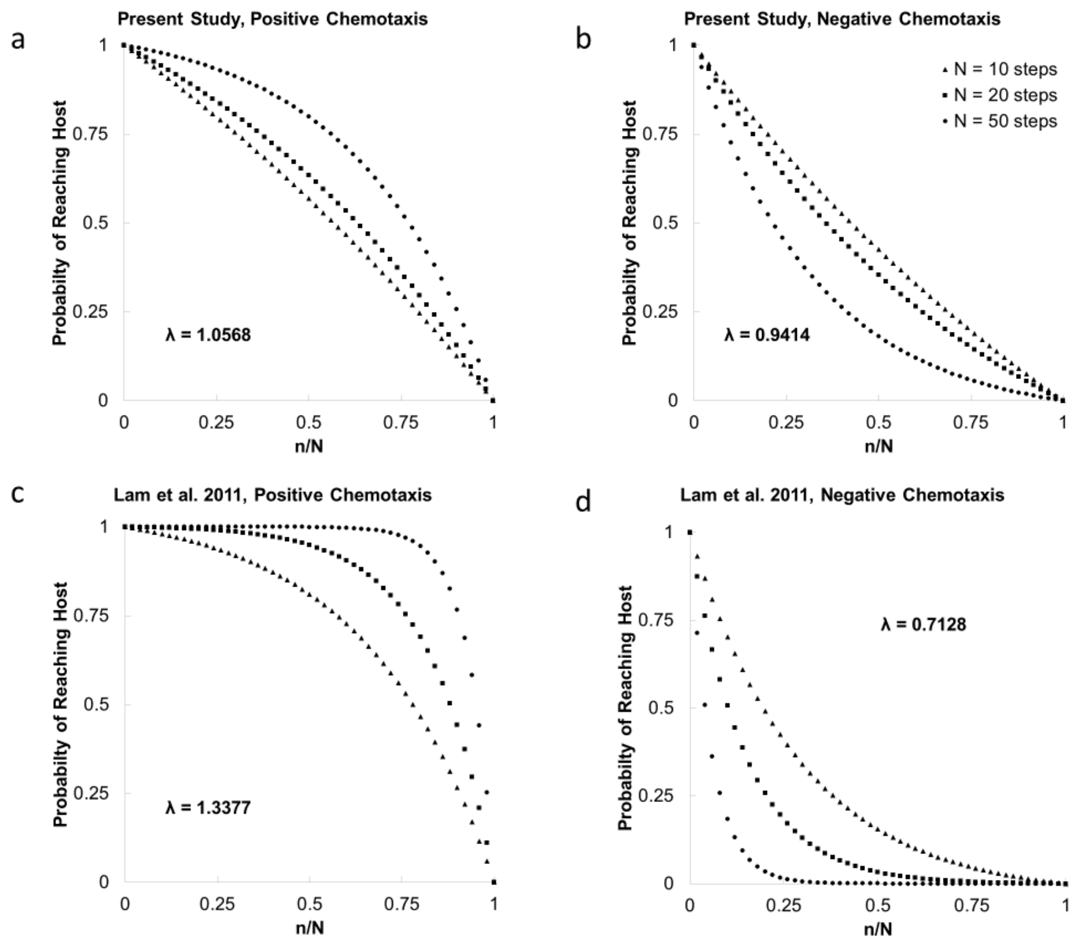
a
Present Study, Positive Chemotaxis
Probabilty of Reaching Host
λ = 1.0568
n/N
b
Present Study, Negative Chemotaxis
N = 10 steps
N = 20 steps
N = 50 steps
Probabilty of Reaching Host
λ = 0.9414
n/N
c
Lam et al. 2011, Positive Chemotaxis
Probabilty of Reaching Host
λ = 1.3377
n/N
d
Lam et al. 2011, Negative Chemotaxis
Probabilty of Reaching Host
λ = 0.7128
n/N

Figure 4. Probability of *Batrachochytrium dendrobatidis* zoospores reaching the host under either positive or negative chemotaxis based on a stochastic model developed in Lam et al. (2011) with mean net chemotaxis values from this study (top). The original model is provided for comparison (bottom, adapted from Lam et al. 2011). N is the arbitrary distance in number of steps from host at which a zoospore is considered dispersed (i.e., will not return to host), and n is the starting position of a zoospore in steps. The ratio of probability of moving towards or away from a host, λ, is the key model parameter.

Appendix A: Chemotaxis assay data

This appendix gives the raw counts of zoospores from microscope photographs. Sites correspond to site numbers in Table 1. Zoospore suspension is the blocking factor used; zoospores from the same Petri dish and collection were used for each block. Treatments are as defined in the methods. As explained in methods, the side of the hemocytometer on which the stimulus disk was placed was randomized, and counts were performed without knowledge of which side the stimulus was on. Therefore, the disk side column tells which side the stimulus was on (in other words, the “near side” for calculation purposes).

Replicate	Site	Zoospore Suspension	Treatment	Disk Side	Left				Right			
					0 min	15 min	30 min	45 min	0 min	15 min	30 min	45 min
1	1	1	+B	L	247	268	272	271	61	49	49	44
2	1	1	C	R	90	151	141	111	112	172	169	129
3	1	1	-B	R	48	53	58	65	115	90	90	120
4	1	1	W	L	56	70	79	73	52	97	71	59
5	1	2	+B	L	23	31	30	30	13	16	18	21
6	1	2	C	R	20	20	20	20	22	24	22	27
7	1	2	-B	R	18	14	17	12	23	25	19	18
8	1	2	W	R	20	19	21	24	13	16	14	15
9	2	3	+B	L	123	162	158	233	113	153	139	160
10	2	3	C	R	107	119	116	102	101	118	113	124
11	2	3	-B	R	156	158	161	146	152	139	144	155
12	2	3	W	L	173	170	166	150	156	165	179	178
13	2	4	+B	L	166	187	219	210	154	221	207	210
14	2	4	C	R	187	219	225	204	186	227	233	252
15	2	4	-B	R	188	223	238	237	164	223	228	259
16	2	4	W	R	164	225	218	231	160	213	211	259
17	3	5	+B	L	216	208	236	198	210	211	239	233
18	3	5	C	R	251	253	267	144	241	273	253	134
19	3	5	-B	R	327	335	321	339	283	390	284	276
20	3	5	W	L	212	240	219	194	191	182	192	224
21	3	6	+B	R	194	228	271	266	150	229	221	225
22	3	6	C	R	407	446	524	515	397	528	535	542
23	3	6	-B	R	324	329	338	384	269	352	412	400
24	3	6	W	R	321	331	353	325	309	387	372	300
25	4	7	+B	L	456	547	598	636	481	622	637	639
26	4	7	C	L	450	579	573	528	468	553	530	557
27	4	7	-B	R	413	597	577	527	396	496	503	509
28	4	7	W	R	399	467	472	518	457	501	514	642
29	4	8	+B	L	514	628	603	583	488	696	593	601
30	4	8	C	R	518	498	478	503	557	527	531	502
31	4	8	-B	R	406	499	473	471	419	496	496	520
32	4	8	W	L	598	670	588	604	551	738	578	562

Replicate	Site	Zoospore Suspension	Treatment	Disk Side	Left				Right			
					0 min	15 min	30 min	45 min	0 min	15 min	30 min	45 min
33	5	9	+B	R	39	66	64	78	58	73	72	72
34	5	9	C	R	51	56	54	52	47	57	54	53
35	5	9	-B	R	50	55	56	47	67	46	50	43
36	5	9	W	R	33	50	51	55	31	54	58	64
37	5	10	+B	L	392	481	480	522	383	472	470	494
38	5	10	C	L	273	377	341	418	255	365	392	421
39	5	10	-B	L	400	521	528	480	404	518	508	511
40	5	10	W	R	269	357	359	371	328	417	401	418
41	6	11	+B	R	315	365	314	335	317	395	369	393
42	6	11	C	R	330	401	424	387	327	468	446	459
43	6	11	-B	L	379	404	461	420	350	469	434	486
44	6	11	W	R	289	360	384	336	318	418	373	373
45	6	11	+B	L	310	387	350	355	291	407	392	418
46	6	11	C	L	188	210	225	204	225	193	197	215
47	6	11	-B	R	302	298	296	282	302	360	309	323
48	6	11	W	R	280	328	387	381	292	435	423	455
49	7	13	+B	R	259	314	296	312	234	298	275	279
50	7	13	C	L	219	296	283	293	194	268	273	261
51	7	13	-B	R	200	259	249	246	176	235	258	238
52	7	13	W	L	237	258	332	341	201	268	275	295
53	7	14	+B	L	80	76	78	79	82	87	76	72
54	7	14	C	L	73	96	95	80	80	102	93	77
55	7	14	-B	L	120	91	80	105	93	99	109	112
56	7	14	W	L	78	62	51	62	74	63	59	55
57	8	15	+B	L	234	360	275	308	309	366	249	289
58	8	15	C	L	361	443	443	444	366	457	481	463
59	8	15	-B	R	220	267	296	277	297	340	354	336
60	8	15	W	R	428	495	435	540	462	555	520	527
61	8	16	+B	R	244	384	304	354	252	359	301	346
62	8	16	C	R	241	318	249	262	312	310	300	314
63	8	16	-B	L	301	312	343	329	319	361	329	317
64	8	16	W	L	189	320	299	312	224	371	324	384