LYME DISEASE SPIROCHETE TRANSMISSION BETWEEN CO-FEEDING LARVAL AND NYMPHAL TICKS UNDER NATURAL CONDITIONS

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Abstract. Lyme disease is an emerging zoonotic disease in North America caused by the spirochete bacterium, *Borrelia burgdorferi*. The continued transmission and persistence of the bacteria depends on the timing of the complex life cycle of its vector, the black-legged tick, *Ixodes scapularis*. Most larval ticks are thought to be infected when they feed on infected hosts. However larvae may simultaneously co-feed on the same host as a nymph, which provides an alternate route of *B. burgdorferi* transmission. Previous studies in the laboratory have found that co-feeding is an effective mode of transmission of *B. burgdorferi*, at least under certain conditions. However no studies have looked at the importance of co-feeding in nature. In order to test whether co-feeding increases transmission of *B. burgdorferi* to *I. scapularis* larvae, I trapped and temporarily housed white-footed mice (*Peromyscus leucopus*) and eastern chipmunks (*Tamias striatus*), two important reservoirs of *B. burgdorferi*, from the Institute of Ecosystem Studies in Dutchess County, New York. I collected the ticks feeding upon them and then screened these ticks for *B. burgdorferi* infection using a direct immunofluorescent antibody assay. I then used a probabilistic model to test whether the probability that larval ticks become infected increased with increasing numbers of co-feeding nymphs. There was essentially no support for co-feeding increasing transmission of *B. burgdorferi* to larval ticks feeding on mice, and only moderate support in the case of chipmunks. Thus, co-feeding appears not to contribute to Lyme disease risk in this North American system.

Keywords**:** Lyme disease risk, transmission, *Borrelia burgdorferi, Ixodes Scapularis*

INTRODUCTION

Lyme disease is an emerging zoonotic disease that affects humans and family pets. In the northeastern and midwestern United States, Lyme disease is caused by the spirochete bacterium, *Borrelia burgdorferi,* and is vectored between hosts by the black legged tick, *Ixodes scapularis*. Hosts can become infected when fed on by infected ticks, generally nymphs, but potentially adults as well. The nymphal stage is the most likely to infect humans given their small size and the fact that their host-seeking activity peaks in early summer when humans spend a lot of time outdoors (Barbour and Fish 1993).

The timing and dynamics of *B. burgdorferi* transmission to and from *I. scapularis* is tightly coupled to the tick's life history (Figure 1). Larvae hatch from eggs free of *B. burgdorferi* (Piesman 1986, Patrican 1997A). In the late summer they quest for their first blood meal, generally a small vertebrate host such as a mouse, chipmunk, or ground nesting bird. After feeding for several days the larvae drop to the ground and molt into nymphs. These newly molted nymphs remain dormant until the following spring or early summer when they search for a second blood meal host, again, usually a small vertebrate. Fed nymphs then molt into adults, which are active later in the same year. Adult ticks tend to feed on larger mammals (e.g., deer) and so are less important for the cycle of *B. burgdorferi* transmission. The adults mate on the host and drop off. The females overwinter and lay their eggs in the spring. These eggs hatch into larvae later that summer, restarting the two year life cycle.

The fact that the nymphs feed earlier in the year than larvae is very important for the transmission of *B. burgdorferi*. Nymphal questing activity peaks in early summer (May-June), whereas, larval questing activity peaks in the late summer to early fall (August-September). Thus, the hosts that feed nymphs earlier in the year are also liable to feed larvae later on (Piesman and Happ 2001). If the host is infected by a nymph it can transmit

the bacteria to the uninfected larvae feeding on it, completing the transmission cycle. The prevailing view is that most transmission occurs this way, from nymph to host to larva. However, the biology is a bit more complex. Some larvae emerge and find hosts earlier in the year, in late May or early July (Daniels et al. 1996), the same time as the nymphal peak. During this time larvae and nymphs share blood-meal hosts, that is, they co-feed (Figure 1).

Co-feeding may provide an important alternative mode of *B. burgdorferi* transmission that does not require the host to become infected—tick-to-tick transmission as opposed to the tick-to-host-to-tick route. This is due to the fact that the spirochetes remain at the sight of inoculation for a few days before disseminating more widely throughout the host (Shih et al. 1992) allowing ticks to become infected from a localized infection before an animal gains a systemic infection. Even if the host is already infected, co-feeding may increase the probability that a feeding larva becomes infected because of a higher concentration of *B. burgdorferi* spirochetes in the skin where a tick is feeding. In either case, if co-feeding is an important route of *B. burgdorferi* transmission, then places or years in which larval and nymphal activity periods overlap to a greater extent, and thus support more co-feeding, would tend to have greater prevalence of infection in nymphs the following year and hence a greater risk of Lyme disease to humans.

In Europe co-feeding on sheep is important in the maintenance of Lyme disease. Sheep do not sustain an infection themselves, but ticks obtain the bacteria when they feed on the sheep next to other infected ticks (Ogden et al. 1997). Tick-to-tick transmission of *B. burgdorferi* also occurs on various small mammals in laboratory studies in Europe and North America. *B. burgdorferi* was transmitted between co-feeding *I. scapularis* nymphs on uninfected gerbils (Patrican 1997B), *I. ricinus* nymphs transferred the bacterium to larvae in laboratory mice (Gern and Rais 1996, Hu et al. 2003), and *B. afzelii* was transferred between *I. ricinus* ticks on uninfected white-footed mice (Richter et al. 2002). A study by Peisman and Happ (2001) found that co-feeding on white-footed mice was an effective mode of transmission of *B. burgdorferi*, but concluded that co-feeding would have no effect on the numbers of infected nymphs in North America due to the high densities of co-feeding nymphs needed for the transfer of bacteria. However, the attachment of nymphal and larval ticks in this study was simultaneous. Richter and colleagues (2002) found the highest rate of transfer occurred when the larval ticks attached three days after the nymphal ticks had begun to feed. These results suggest that the details of co-feeding matter. No studies, however, have looked at the importance of co-feeding on animals in nature in North America.

To test the importance of co-feeding for *B. burgdorferi* transmission under natural conditions I collected and temporarily housed white-footed mice and eastern chipmunks, two important reservoir hosts of *B. burgdorferi (*Donahue et al. 1987, Mather et al. 1989*)*. I screened the fed larval ticks for the presence of the bacterium and used probabilistic model of transmission to test whether co-feeding increased transmission of *B. burgdorferi* to larval ticks.

METHODS

Collecting ticks from small mammal hosts

Peromyscus leucopus and *Tamias striatus* were collected using Sherman traps on seven approximately 2.25 ha trapping grids on the grounds of the Institute of Ecosystem Studies (IES). Only males and juvenile females were collected to avoid separating nursing mothers from their offspring. If not already marked, animals were ear tagged for individual identification and to ensure no animal was used twice. Animals were immediately taken to the animal rearing facility at IES and housed in individual wire cages, each suspended over a shallow tub of water and kept on a 14h:10h light:dark photoperiod, mimicking natural light conditions. Animals were fed oats, sunflower seeds, rodent chow, and apple slices twice a day, and given water ad lib. Animals were kept for 72 h in order to let ticks feed to repletion and drop off, and were then returned to their exact location of capture on the trapping grid. Ticks were collected from the water every 24 hours, rinsed with DI water, and placed in glass vials with a layer of damp plaster of Paris and a mesh cap kept in desiccators at high humidity in an incubator at 25˚C.

Ticks from each animal were kept separately with no more than 9 nymphs or 11 larvae in a vial. In the cases where a tick died, the remaining live ticks were transferred to a clean vial.

Testing ticks for Borrelia burgdorferi infections

After the ticks molted they were tested for *B. burgdorferi* infection using direct immunofluorescent antibody (DFA) microscopy (LoGiudice et al. 2002). Ticks were placed in individual 1.5 ml centrifuge tubes washed by vortexing with 70% ethanol, and then rinsed twice by vortexing in distilled water. Each tick was then crushed in 100µl of phosphate buffer saline (PBS, pH 7.4), using a clean plastic pestle in order to expel the mid-gut content of the tick and then vortexed to suspend the contents. For each tick three 5µl samples of solution were then placed on MP Biomedical 15 well multitest slides, allowed to air dry, and then fixed in cold acetone for 10 minutes. Fixed slides were stored at -80˚C until they could be stained with 7µl rabbit anti-*Borrelia burgdorferi* FA conjugate antibody (ViroStat, Portland, Maine, USA) diluted 1:50 in PBS and incubated for 45 minutes at 37º C. Stained slides were washed twice in PBS for ten minutes and then with DI water for two minutes. After the slides had air-dried, cover slips were placed on them using a FA mounting medium specific for IES (ViroStat, Portland, Maine, USA).

Slides were read under with a fluorescent microscope at 400x magnification. A tick was considered positive if at least one bacterium was observed in at least one of the three wells.

Statistical Analysis

We analyzed the tick infection data using a modified version of the method of Brunner and colleagues (2008). In order for a larval tick to become infected, it must feed on an infected host, nearly all larvae hatch uninfected; (Piesman et al. 1986, Patrican 1997). The probability that a given host is infected is equal to the prevalence of infection in the host population (π; Figure. 2). Given that the host is infected, the host must then transmit the infection to the feeding larva, a probability termed "infectivity" (ϕ; Figure. 2). Assuming that these probabilities are constant across all individuals in a local population, and that there were no false positives, the probabilistic model of Brunner et al. (2008) can be used to find the values of prevalence and infectivity that maximize the likelihood of observing the data (i.e., the number of ticks that tested positive out of the total number of ticks tested for each individual).

This basic model does not account for co-feeding and so serves as a "null" model. If in fact co-feeding increases the probability that a feeding larva becomes infected, then infectivity of a given host should be a function of the number of co-feeding nymphs. I fit two models in which infectivity increased with the number of co-feeding nymphs. Because infectivity is a probability, it cannot exceed one, and so I constrained ϕ to be an increasing, saturating function of the number of co-feeding nymphs: $\phi = \beta_0 + (1- \beta_0)\times(\text{nymphs}/(\text{nymphs} + c))$ where β_0 is the infectivity with no co-feeding nymphs (and so $1-\beta_0$ is the maximum increase in infectivity due to co-feeding) and c is a constant that controls how quickly infectivity saturates. As c increases, infectivity changes less quickly with increasing numbers of nymphs; above values of approximately 200 the co-feeding and constant infectivity models are essentially identical. It is possible, however, that co-feeding does not increase infectivity when the host is infected, but allows for some transmission when it is not. Thus in the second model I allowed c to vary depending on whether the host was infected or not: $\phi_{Infected} = \beta_0 + (1- \beta_0)\times(\text{nymphs}/(\text{nymphs} + c_I))$ and $\phi_{Uninfected} = \text{nymphs}/(\text{nymphs} + c_U)$, where c_I and c_U are the constants for infected and uninfected hosts, respectively.

I then fit each of these three models to the tick infection data for mice and chipmunks individually and compared the support for each using Akaike Information Criterion, corrected for sample size (AIC_c). AIC_c measures the goodness of fit of a model while penalizing more complex models. Lower AIC_c values are better. The difference in AIC_c (ΔAIC_c) between models indicates the relative support in the data for each. A $\Delta AIC_c \leq 2$ between two alternative models suggests equivalent support for both, while differences > 5 suggest the model with a lower AIC_c, is substantially better supported. I also calculated AIC_c weights, which measure the proportion of the

evidentiary weight in a data set that supports each model. These weights sum to one and the higher the weight the greater the support for the model.

RESULTS

Data were collected between June 8 and July 6, 2007. A total of 127 animals were collected and returned to the laboratory, 72 mice and 55 chipmunks. The average burdens on mice were 1.1 (range: 0 – 6) nymphs and 2.3 (range: 0 – 11) larvae and on chipmunks 8.3 (range: 1 – 38) nymphs and 1.8 (range: 0 – 13) larvae. However, only animals with at least one larval tick could be used to estimate infectivity, which amounted to 44 mice and 24 chipmunks. Every chipmunk I collected had at least one nymph, so I could not estimate infectivity in the absence of co-feeding on chipmunks.

The prevalence of *B. burgdorferi*-infection in mice was 0.803 (Support Interval (SI): 0.643 – 0.929) across all three models (Table 1). For mice there was almost no change in infectivity as the number of nymphs increased (c = 9991 in the basic co-feeding model with a common infectivity term; c_I = 9974 and c_U = 9874 in the model with separate infectivity terms; Figure 3). The difference in AIC_c between the model with infectivity held constant and those in which infectivity increased with the number of nymphs was $\geq$ 2.3 in favor of the simpler, null model (Table 1). Also, the AICc weight was moderately in favor of a constant infectivity with three times as much evidentiary weight behind the null model (Table 1).

The estimated prevalence of *B. burgdorferi* infections in the chipmunks was 0.876 (SI: 0.613-1.0) in the model with infectivity held constant, but lower in the models in which infectivity increased with co-feeding nymphs (0.751 and 0.673; Table 1). Infectivity did increase with increasing numbers of nymphs in the two more complex models (c = 14.5 in the basic co-feeding model with one infectivity term, while c_I = 17.2 and c_U = 28.1 in the model with two; Figure. 4). These amount to moderate increases in infectivity with increasing co-feeding, but the difference in AIC_c between the basic co-feeding model and that in which infectivity was held constant was only 1.2, in favor of the simpler, null model, and the AIC_c weight for the null model was almost twice as high as for the co-feeding model (AIC_c w_i= 0.59 and 0.33, respectively).

DISCUSSION

The incidence of Lyme disease in humans is correlated with the number of questing infected nymphs in an area (Stafford et al. 1998). Nymphal infection prevalence is one of the factors that should be considered when assessing Lyme disease risk. To estimate risk it is important to understand the factors that increase the number or proportion of larvae that become infected nymphs. The two factors that determine the probability a larva is infected with *B. burgdorferi* are the prevalence of infection in the tick hosts and the host's infectivity to feeding larvae. When nymphal ticks co-feed with larvae, infectivity of a host may be amplified, thus increasing the number of infected nymphs the following year and Lyme disease risk to humans. Laboratory studies have found that co-feeding is an effective way to transmit *Borrelia spp*. from infected ticks to uninfected ticks. These previous studies have found that the distance between the larva and the infected feeding nymph, as well as the relative timing of attachment are important for tick-to-tick transmission of the bacteria (Richter et al. 2002, Piesman and Happ 2001). The key question, however, is whether this is important in nature. My results show that under natural conditions co-feeding does not substantially increase the transfer of *B. burgdorferi* to larval ticks North America.

In both chipmunks and mice there was greater support for the simpler null model in which infectivity is constant. There are at least two reasons why co-feeding may have had little effect on transmission in this study, but could elsewhere. First, estimated infectivity was high in both mice and chipmunks (infectivity = 0.804 and 0.640, respectively), meaning that infected hosts were very efficient at passing on the bacteria without having nymphal ticks attached. Co-feeding nymphs could increase transmission very little. Secondly, prevalence of infection in these two species was also high (prevalence = 0.803 in mice and 0.876 in chipmunks), as is common at IES

(Brunner et al. 2008, LoGiudice et al. 2003). Thus, there were relatively few opportunities to observe transmission via co-feeding on uninfected hosts. Put another way, tick-to-host-to-tick transmission is both very common and very efficient, and so co-feeding can play only a small part in transmission.

Prevalence and infectivity, or their product, "reservoir competence," vary between species and regions (Brunner et al. 2008; LoGiudice et al. 2003, Giardina et al. 2000). Co-feeding may play a more important role in North America in areas where the important blood-meal hosts have low infectivity and/or low prevalence. This would be similar to the role of sheep in Europe. The prevalence and infectivity of sheep is close to zero, yet co-feeding on sheep is able to sustain the *Borrelia* bacterium (Ogden et al. 1996). Future research on co-feeding should look at a wider range of vertebrate hosts, especially hosts with lower infectivity (e.g. eastern gray squirrels).

With high reservoir hosts like mice and chipmunks it appears that most larval ticks become infected while feeding on hosts with systemic infections. Although tick-to-tick transmission of the bacteria is possible, tick-to-host-to-tick transmission is so effective at transmitting the bacteria that co-feeding does not substantially increase the numbers of infected nymphs. This is consistent with the study by Piesman and Happ (2001) who found that the tick burdens on animals needed to be higher than what is generally found in nature for co-feeding to have an appreciable effect on the resulting numbers of infected nymphs. Co-feeding on hosts has little effect on the quantity or proportion of infected nymphs in natural conditions, at least in Dutchess County, New York.

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APPENDIX

TABLE 1. The evidentiary support and estimates of prevalence and infectivity terms for each of three models. The Null models hold infectivity constant and thus do not account for co-feeding, the Basic co-feeding model has infectivity increase as a saturating function of the number of co-feeding nymphs, and the third model includes separate terms relating nymphs to infectivity depending on whether the host is infected or not. Est is the parameter estimate, SI is 2 likelihood unit support interval.

					Prevalence		**Base-line Infectivity**	
	Model	AIC_c	ΔAIC_c	w_i	Est	SI	β_0	SI
Mice	Null	96.5	0	0.71	0.803	0.643 - 0.929	0.804	0.708 - 0.887
	Basic co-feeding	98.8	2.3	0.22	0.803	0.643 - 0.929	0.804	0.708 - 0.887
	Co-feeding different on infected & uninfected hosts	101.2	4.7	0.07	0.802	0.641 - 0.929	0.805	0.708 - 0.887
Chipmunks	Chippie Null	52.5	0	0.59	0.876	0.613 - 1	0.64	0.486 - 0.783
	Basic co-feeding model	53.7	1.2	0.33	0.751	0.413 - 1	0.481	0.231 - 0.719
	Infectivity different on infected & uninfected hosts	56.3	3.8	0.09	0.673	0.33 - 0.987	0.557	0.301 - 0.781

		Change in infectivity with co-feeding			
	Model	c or c_I	SI	c_U	SI
Mice	Null	-	-	-	-
	Basic co-feeding	9991	0 - 1000	-	-
	Co-feeding different on Infected & uninfected hosts	9974	0 - 1000	9874	0 - 1000
Chipmunks	Chippie Null	-	-	-	-
	Basic co-feeding model	14.5	4 - 80.9	-	-
	Infectivity different on infected & uninfected hosts	17.2	3.4 - 1000	28.1	3.7 - 1000

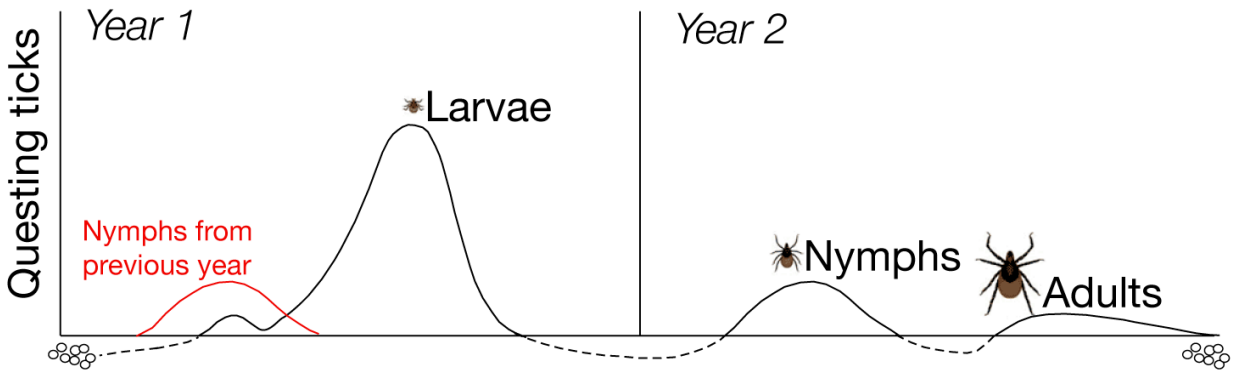

FIGURE 1. Life cycle and questing peaks of *Ixodes scapularis* ticks in the Northeast United States.

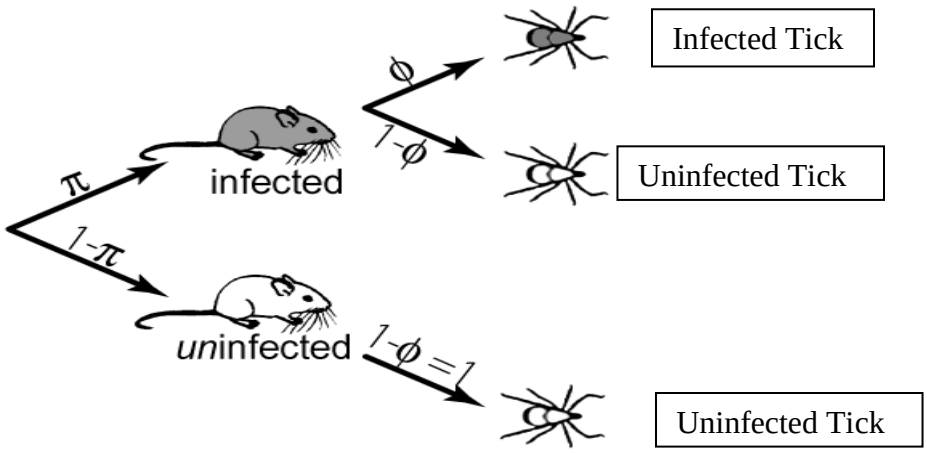

FIGURE 2. The two factors that determine whether a feeding larva is infected with *B. burgdorferi* are prevalence (π), which is the probability of the host being infected, and infectivity (ϕ), the probability of the tick becoming infected when feeding on an infected host.

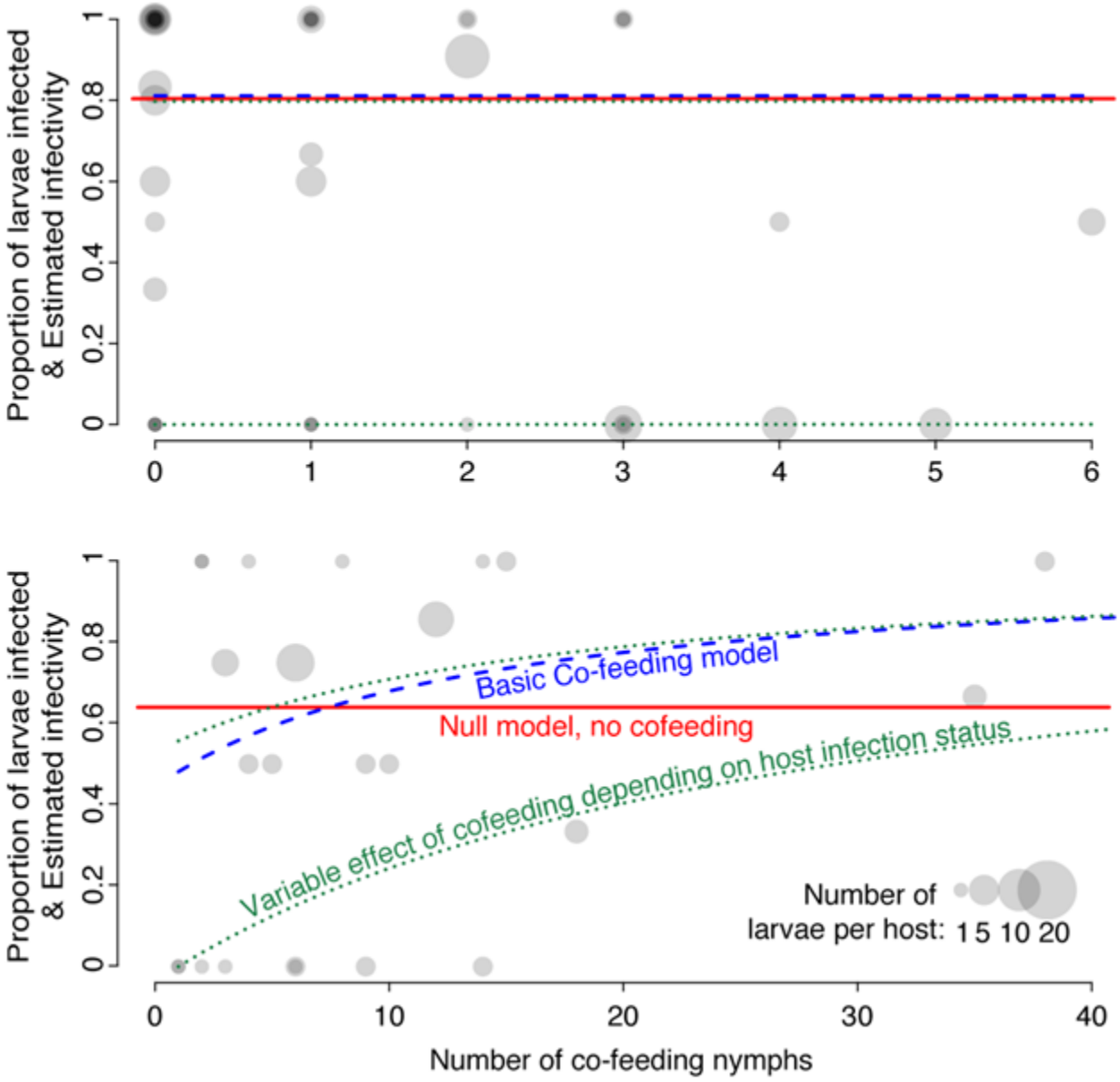

FIGURE 3. The proportion of larvae that are infected according to the number of co-feeding nymphs on mice (top) and chipmunks (bottom). The area of the points is proportional to the number of larvae that were tested on each individual host. Note that the points are translucent, so that individual hosts with the same proportion of larvae infected and numbers of nymphs overlap to create darker points. The solid red line corresponds to the predicted infectivity in the null model, where infectivity was held constant; the dashed blue line to the model where infectivity increases with increasing numbers of co-feeding nymphs, and the dotted green lines to the model where infectivity could increase with the number of co-feeding nymphs differently depending on whether the host was infected or not.