

# PATTERNS OF *MYCOBACTERIUM LEPRAE* INFECTION IN WILD NINE-BANDED ARMADILLOS (*DASYPUS NOVEMCTINCTUS*) IN MISSISSIPPI, USA

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**ABSTRACT:** The nine-banded armadillo (*Dasypus novemcinctus*) is the only known nonhuman reservoir of *Mycobacterium leprae*, the causative agent of Hansen's disease or leprosy. We conducted a 6-yr study on a wild population of armadillos in western Mississippi that was exposed to *M. leprae* to evaluate the importance of demographic and spatial risk factors on individual antibody status. We found that spatially derived covariates were not predictive of antibody status. Furthermore, analyses revealed no evidence of clustering by antibody-positive individuals. Lactating females and adult males had higher odds of being antibody positive than did nonlactating females. No juveniles or yearlings were antibody positive. Results of these analyses support the hypothesis that *M. leprae* infection patterns are spatially homogeneous within this armadillo population. Further research related to movement patterns, contact among individuals, antibody status, and environmental factors could help address hypotheses related to the role of environmental transmission on *M. leprae* infection and the mechanisms underlying the differential infection patterns among demographic groups.

**Key words:** Armadillo, *Dasypus novemcinctus*, leprosy, *Mycobacterium leprae*, network Ripley's K-function.

## INTRODUCTION

The nine-banded armadillo (*Dasypus novemcinctus*; hereafter, "armadillo") is the only free-ranging vertebrate other than humans known to exhibit naturally occurring infections with *Mycobacterium leprae*, the causative agent of leprosy (reviewed in Truman 2005, 2008). Mounting evidence indicates armadillos may be a public health concern because of potential transmission of leprosy to humans (Truman et al. 2011; Sharma et al. 2015). Laboratory investigations of leprosy in armadillos have been extensive (Truman 2008), but aside from prevalence surveys, details of infection patterns in wild populations remain scant. In particular, transmission mechanisms and resulting patterns of infection within wild populations are largely unknown. Infected animals are thought to transmit *M. leprae* via aerosol droplets (Truman 2005), through either direct contact with infectious individuals (e.g., during mating or

aggressive interactions) or indirect contact with contaminated soils (e.g., while foraging).

Understanding population-level transmission mechanisms associated with *M. leprae* requires knowledge of three key processes: 1) the rate of contact between individuals (or environmental reservoirs), 2) the probability that contact occurs with an infectious host (or contaminated surface), and 3) the probability that an infectious contact leads to transmission (Begon et al. 2002). With this study, we evaluate how location within the study area and spatial proximity among individuals contribute to the probability of *M. leprae* infection within a naturally occurring population of armadillos. We focus on infection patterns, rather than contact patterns, as a first step toward understanding population-level transmission mechanisms. Infection can be driven by heterogeneous processes, whereby infection probability varies according to location or demographic characteristics, or by homogeneous infection processes, whereby the probability of infection is relatively

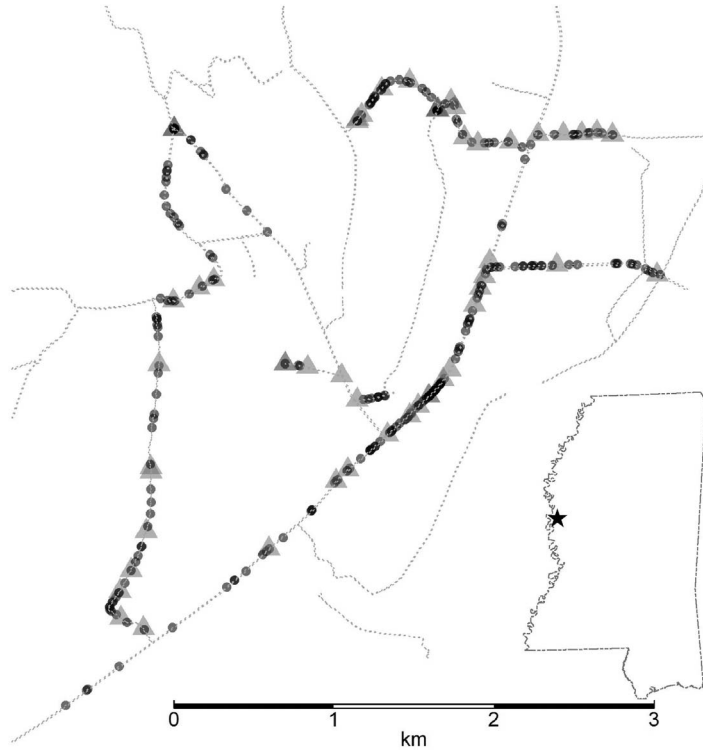


FIGURE 1. Map of the study site at Yazoo National Wildlife Refuge, Mississippi, USA, exhibiting the spatial locations of antibody-positive (triangles) and antibody-negative (circles) adult nine-banded armadillos (*Dasypus novemcinctus*) across all years of the study (2005–10). The star in the inset map of Mississippi provides the location of the study area, which is enlarged to display individual locations of captured armadillos. Points are the location of first capture for each armadillo sampled ( $n=466$ ).

constant for all individuals in the population without regard to location or demographic characteristics (Tompkins et al. 2011). Spatial structure influences transmission dynamics of various wildlife diseases (e.g., see Davis et al. 2015 and references therein). Thus, in the case of leprosy, spatial analyses are useful to evaluate the importance of localized infection patterns (and potential transmission) on underlying distribution of *M. leprae* prevalence within affected populations.

We applied spatial analyses and logistic regression to evaluate the importance of demographic and spatial risk factors on exposure to *M. leprae* within a population of armadillos in western Mississippi. We hypothesized that, if infection was spatially distributed as a heterogeneous process, we would observe significant clustering of *M. leprae* cases and significant associations among local

neighborhood properties and odds of exposure to *M. leprae*. Moreover, demographic characteristics would be significantly associated with odds of *M. leprae* exposure. Under the null assumption of homogeneously distributed infection, in which individuals were equally likely to encounter an infectious individual or contaminated surface, we would expect to find no such clustering or associations.

## MATERIALS AND METHODS

### Field work

Antibody status, morphometric, and spatial data were collected from 2005 to 2010 at the Yazoo National Wildlife Refuge in western Mississippi, US (33°05'N, 90°59'W). Most sampling was conducted within an approximately 750-ha area in the central part of the refuge, which contained an extensive network of roads and trails that facilitated capture and observation of armadillos

TABLE 1. Annual antibody prevalence of *Mycobacterium leprae* among armadillos (*Dasypus novemcinctus*) at Yazoo National Wildlife Refuge, stratified by age, sex, and lactation status, Mississippi, USA. Total numbers of individuals from respective subpopulations are listed within parentheses.

Category	2005	2007	2008	2009	2010
Juvenile	0 (3)	0 (13)	0 (39)	0 (41)	0 (57)
Yearling	0 (1)	0 (3)	0 (11)	0 (2)	0 (9)
Adult male	0 (35)	8.3 (60)	8.8 (57)	12.5 (80)	20.8 (96)
Adult female					
Not lactating	0 (14)	3.4 (29)	5.9 (34)	3 (33)	5.9 (17)
Probably lactating	0 (8)	20 (5)	42.9 (7)	28.6 (14)	41.7 (12)
Definitely lactating	7.1 (14)	14.3 (28)	35.5 (31)	9.4 (32)	20.8 (53)
Total	1.3 (75)	8.0 (138)	11.7 (179)	8.9 (202)	15.2 (244)

(Fig. 1). Although it is unlikely that individuals (and interactions between individuals) were confined to these areas, average home-range size was small (~6 ha; see Loughry and McDonough 2013) relative to the total sampling area. Thus, capture locations should reasonably represent an armadillo's relative location within the study area. Field work was conducted from mid May until late July each year (50–55 d in the field), except in 2005 and 2006, when sampling occurred for 2–3 wk in May.

Animal sampling methodology was approved by the Valdosta State University Institutional Animal Care and Use Committee (protocol 00013-2007). Armadillos were live-caught in dip nets during nightly censuses (for details, see Morgan and Loughry 2009). Upon initial capture, each animal was permanently marked by injecting a passive integrated transponder tag under the dorsal, front edge of the front carapace at its junction with the neck. Animals were also marked for temporary, long-range identification by gluing various shapes and colors of reflective tape to the carapace. After marking, animals were weighed and measured, the lactation status (not lactating, possibly lactating, definitely lactating) of females was classified by inspection of nipple size. A blood sample was collected onto Nobuto blood strips (Advantec, Dublin, California, USA) by clipping the end of one toenail to screen for exposure to *M. leprae*. Animals were recaptured frequently (2–6 times/yr) to reapply reflective tape, but blood samples were only collected on the first capture each year. Finally, the spatial position of each animal was obtained at the site of initial capture and at all subsequent sightings with a Trimble GeoExplorer 3 GPS unit (Trimble Navigation, Sunnyvale, California, USA). Positional error never exceeded 2 m with this unit.

Blood samples were tested for exposure to *M. leprae* at the Hansen's Disease Center (Baton Rouge, Louisiana, USA) using an enzyme-linked

immunosorbent assay to detect antibodies against the *M. leprae*-specific PGL-1 antigen (Truman et al. 1986). All samples were run at least twice to confirm consistency. A mean antibody titer of 0.70 optical density was the threshold for designating an animal as antibody positive.

#### Data analyses

Data from all age classes were used to describe spatial patterns in antibody status; however, only a subset of the data pertaining to adults ( $\geq 2$  yr old) were included in our disease clustering and regression analyses because no juveniles or yearlings had detectable antibodies (Table 1). Likewise, for statistical analyses based on partitions of the data by sample year, data from 2006 were not assessed because of relatively low sample size.

*Risk factors of M. leprae exposure:* We used logistic regression to evaluate the relationship between *M. leprae* antibody status and 1) sex, 2) lactation status of females (not lactating, possibly lactating, definitely lactating), 3) capture year (2005, 2007–10), and 4) local neighborhood antibody prevalence. Local neighborhood antibody prevalence for each year was defined as  $p_i = \sum_{j=1, j \neq i}^{n_i-1} I(l_j=1)/(n_i-1)$ , where  $n_i$  represents the number of adult armadillos within individual  $i$ 's neighborhood, and  $l_j$  indicates the antibody status of individual  $j$  ( $1 =$  antibody positive). Note that the antibody status of individual  $i$  is omitted from the calculation of local antibody prevalence.

We defined local neighborhoods based on spatial proximity. For animals that were captured or sighted more than once during the same year, a single point location was calculated by determining the centroid of all coordinates for that individual; centroids were not calculated for coordinates obtained in different years because

of the possibility of home range shifts over time. Individuals were spatially connected in a neighborhood if centroids were within the specified distance. We set our minimum neighborhood distance to 200 m because it encompassed the typical distance an armadillo moves between successive sightings (Loughry and McDonough 1998; Paige et al. 2002), and that distance was also used in a previous study (Morgan and Loughry 2009). The farthest distance travelled by individuals in the sample was 1,300 m, but 98.6% (900/912) of movements between sightings were <600 m. Thus, we set the upper-limit radius to 600 m. We defined a third distance class at 400 m based on equidistance between the 200 and 600 m classes. Supplementary Material S1 depicts spatial network graphs for the 200-, 400-, and 600-m neighborhood definitions across all sampling years.

Local neighborhood antibody prevalence was calculated for each spatial scale (200, 400, and 600 m), and logistic-regression models were fit to the data. All data, except those for 2006, were used in these analyses. Full models included terms for sex, lactation status, year, and local neighborhood antibody prevalence (which varied depending on the spatial scale used to define the neighborhoods). Final reduced models were defined based on a stepwise, backward variable selection procedure, using the minimum Akaike's Information Criterion (AIC) value as the selection criterion (Venables and Ripley 2002). Contrasts corresponding to sex and lactation status were also evaluated to determine whether estimated odds of being antibody positive differed among groups. Odds ratios and 95% confidence intervals (CIs) were calculated for corresponding regression coefficients, and a Hosmer and Lemeshow goodness-of-fit test was conducted on the final regression model using the *ResourceSelection* package in R software (Lele et al. 2014; R Foundation for Statistical Computing, Wien, Austria).

Model residuals represent unexplained variation in antibody status. We used standardized Pearson residuals from the final logistic-regression models to conduct a local form of the Moran's *I* test. The aim of this analysis was to identify locations in which unexplained variation in the outcome was similarly high among neighboring areas and could be related to unaccounted spatial factors. *P*-values from local Moran's *I* tests were adjusted using the false discovery-rate approach (Benjamini and Hochberg 1995).

**Disease clustering:** We conducted a linear *K*-function analysis (Okabe and Yamada 2001) to determine whether the capture locations of antibody-positive armadillos were clustered or (conversely) more regularly distributed than expected under the null hypothesis of a random

Poisson point process. Because armadillos were found by searching road verges, nature trails, and habitat edges, we treated the sampling space itself as the linear network. The linear network for the Yazoo study area was digitized in ArcGIS 10.2 software (ESRI, Redlands, California, USA), and capture locations were placed at the nearest perpendicular position along the linear network with the Geospatial Modeling Environment platform (Beyer 2010). A cluster of isolated capture locations near the northern end of the wildlife refuge ( $n=5$ ) were excluded from the analysis.

The linear *K*-function analysis was conducted with the *spatstat* package (Baddeley and Turner 2005) in R software, using the corrections for network geometry (Ang et al. 2012) and inhomogeneity (Baddeley et al. 2000). Inhomogeneity at each capture location was estimated by a kernel-smoothed intensity function with optimal smoothing bandwidth determined using Silverman's solution in GeoDaNet (ASU GeoDa Center 2015). We calculated a simultaneous 5% significance envelope via Monte Carlo simulation to test whether antibody-positive individuals were distributed at random with respect to the capture locations along the linear network. For each iteration ( $n=19$ , the minimum number required for a 5% simultaneous significance envelope), antibody status was randomly permuted over all capture locations, antibody-positive individuals were selected, and the linear *K*-function was recalculated each time with correction for inhomogeneity.

We conducted *K*-function analysis for the combined data set over all years and repeated it separately for each year between 2007 and 2010 (2005 and 2006 were excluded because of small sample sizes). For the combined data set over all sample years, we used the first location at which an individual was antibody positive or, for individuals that were never found positive, their first capture location. When the data set was partitioned by year, we considered all first-capture locations for that year, including recaptured armadillos that were antibody positive in a previous year. Plots corresponding to year-specific first-capture locations are provided in Supplementary Material S2.

## RESULTS

We obtained 1,382 GPS locations from 466 adult armadillos; 74 (15.9%) adults were antibody positive. The average ( $\pm$ SE) number of resightings per individual was  $3.92 \pm 0.12$ . First-capture locations for each animal across all years are shown in Figure 1; locations of

TABLE 2. Regression coefficients and standard errors from final logistic-regression models estimated using data from three spatial scales. Final models were identified according to backward, stepwise selection methods with minimum Akaike's Information Criterion (AIC) as the selection criterion. Across all scales, models that included a term for neighborhood prevalence of antibody to *Mycobacterium leprae* in armadillos (*Dasypus novemcinctus*) were within two AIC units of models that contained only terms for year, sex, and lactation status; however, in all cases, neighborhood antibody prevalence was not significantly associated with antibody status. Goodness-of-fit (GOF) tests indicated that final models adequately fit the data.

Spatial scale	Variable <sup>a,b</sup>	Estimate (SE)	Hosmer-Lemeshow GOF
200 m	Intercept	-5.5 (1.108)	$\chi^2_8=8.24, P=0.41$
	Male	1.04 (0.495)	
	Female (probably lactating)	2.22 (0.579)	
	Female (definitely lactating)	1.54 (0.509)	
	Local antibody prevalence	-1.53 (0.870)	
	2007	2.21 (1.063)	
	2008	2.97 (1.045)	
	2009	2.39 (1.043)	
	2010	3.16 (1.035)	
400 m and 600 m	Intercept	-5.48 (1.107)	$\chi^2_8=4.44, P=0.8154$
	Male	1.01 (0.494)	
	Female (probably lactating)	2.21 (0.577)	
	Female (definitely lactating)	1.49 (0.508)	
	2007	2.12 (1.061)	
	2008	2.81 (1.041)	
	2009	2.29 (1.042)	
	2010	2.94 (1.029)	

<sup>a</sup> Referent category: Nonlactating females.

<sup>b</sup> Referent year: 2005.

first captures for each year are provided in Supplementary Material S2. Centroid locations were quite similar and are available from the corresponding author. Overall, antibody prevalence was highest among lactating (probably and definitely lactating combined) females (Table 1). No juveniles or yearlings were antibody positive.

#### Risk factors of *M. leprae* exposure

Across all spatial scales, the final logistic-regression models included the effects of sex, lactation status, and year. For the 400- and 600-m spatial scales, models that included local neighborhood antibody prevalence, in addition to sex, lactation status, and year, were within two AIC units of the final model; however, the association between local antibody prevalence and antibody status was not statistically significant (Supplementary Material S8). The final model using data from 200-

m neighborhoods also included a term for local antibody prevalence; however, the corresponding coefficient estimate indicated a nonstatistically significant association with *M. leprae* exposure (Table 2). No global spatial autocorrelation among model residuals was detected using the 200-m neighborhood scale ( $I_{200}=0.009, P=0.204$ ), although with the 400- and 600-m, scale residuals exhibited a weakly positive spatial autocorrelation ( $I_{400}=0.0179, P=0.02$ ;  $I_{600}=0.0193, P=0.004$ ). Additionally, residuals from final models exhibited local patterns of positive spatial autocorrelation primarily in the northeastern section of the study area (Fig. 2).

Population antibody prevalence increased from 1% to 15% across years; however, results from two-sample tests of proportions (with continuity correction) demonstrated no significant differences in antibody prevalence between sequential years (Table 1). After controlling for sampling year, the odds of *M.*



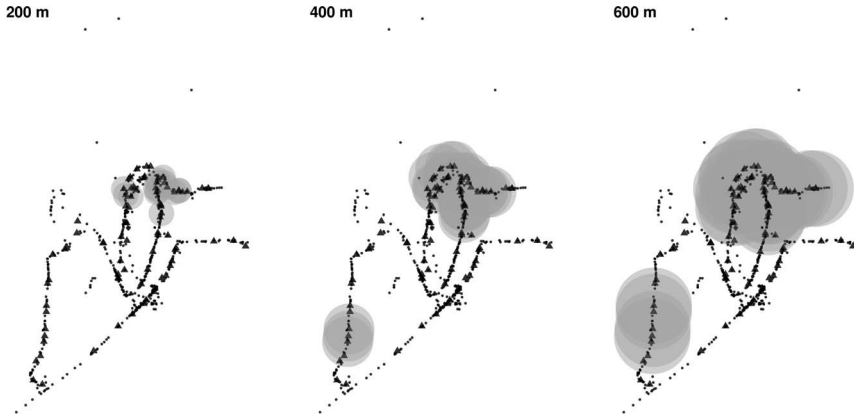


FIGURE 2. Capture locations of armadillos (*Dasypus novemcinctus*) at Yazoo National Wildlife Refuge, Mississippi, USA (2005–10) associated with significant positive spatial autocorrelation in standardized Pearson residuals from final logistic-regression models. At these locations, fitted probabilities are similarly underestimated (i.e., residuals are high), which could suggest that an underlying spatial process, unaccounted for by covariates included in the respective models, could be contributing to infection patterns among individuals located within the northeastern section of the study area.

*leprae* exposure for definitely lactating females were 4.5 times that of nonlactating females (95% CI: 1.6–12.0). Females suspected to be lactating had 9.1 times greater odds of being exposed to *M. leprae* than nonlactating females had (95% CI: 2.9–28.2). Males had 2.8 times greater odds of *M. leprae* exposure than nonlactating females had (95% CI: 1.0–7.3). The odds of seroconversion were higher for females suspected to be lactating, relative to males (odds ratio [OR]: 3.2; 95% CI: 1.4–6.7). Lactating females did not significantly differ from adult males with respect to odds of seroconversion (OR: 1.6; 95% CI: 0.9–2.7).

**Disease clustering**

For the combined data over all years, the linear *K*-function analysis supported the null hypothesis that capture locations of antibody-positive armadillos were randomly distributed (Fig. 3). The distribution of antibody-positive males over all years was also consistent with a random distribution (Supplementary Material S3), whereas the assumption of complete spatial randomness was rejected for antibody-positive females that were definitely lactating, probably lactating, and not lactating, with all three classes of females exhibiting regular distributions at intermediate to long

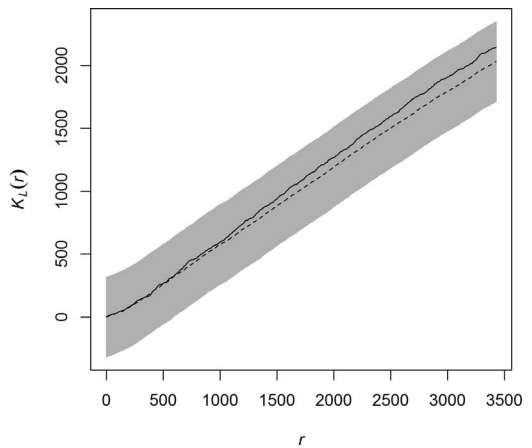


FIGURE 3. Inhomogeneous network Ripley’s *K*-function for all armadillos (*Dasypus novemcinctus*) at Yazoo National Wildlife Refuge, Mississippi, USA, that were found antibody positive for *Mycobacterium leprae*, 2005–10. The solid black line represents the value of the inhomogeneous network *K*-function over increasing distance (*r*) in meters. The gray area depicts the 5% simultaneous significance envelope for the inhomogeneous network *K*-function based on randomization (*n*=19 permutations) of the marks given the capture locations; the stippled line represents the average *K*-function for the randomized locations. The observed *K*-function never wanders outside the 5% significance envelope, indicating a close fit between the observed point pattern and the null model of complete spatial randomness.

distances (Supplementary Material S3). When the linear  $K$ -function for each sample year was evaluated separately, all  $K$ -functions indicated regular distribution of antibody-positive individuals at intermediate to long distances (Supplementary Material S4); deviation of the observed linear  $K$ -function from complete spatial randomness was most extreme in 2007 and 2009. After further stratifying data for each year by sex and lactation status, linear  $K$ -function results did not deviate from randomness for all years examined (Supplementary Material S5 and S6). Additional analyses of spatial patterns, using spatial network metrics and a join-count analysis, were consistent with the results from the linear  $K$ -function analyses (Supplementary Material S7). Spatial connectivity measures did not differ between antibody-positive and antibody-negative individuals, and after accounting for sex and lactation status, join-count analyses failed to detect significant spatial autocorrelation for antibody status across the three spatial scales (Supplementary Material S7).

## DISCUSSION

Our analyses represent a highly detailed examination of *M. leprae* infection patterns. We found observed heterogeneity in infection patterns was driven more by demographic than spatial factors. Specifically, antibody-positive individuals were not clustered within the sampling area, and spatial overlap with antibody-positive individuals was not significantly associated with antibody status. Despite theoretic differences among the methods, join-count, spatial network metrics, and linear  $K$ -function analyses all yielded similar results, with both the join-count analysis and the linear  $K$ -function analysis indicating that antibody-positive individuals were either randomly distributed or more regularly distributed than expected (at intermediate to long distances) within the sampling area. These results were further corroborated by the logistic-regression analysis and are consistent with findings from studies that compared local neighborhood antibody prevalence and near-

est neighbor distances between leprous and nonleprous animals (Paige et al. 2002; Morgan and Loughry 2009). Spatial analyses of model residuals, however, indicated the need to further investigate local processes contributing to infection in the northeastern portion of the study area. Because we failed to observe 1) significant clustering of antibody-positive individuals, and 2) significant associations between local neighborhood antibody prevalence and serologic status, results of this study support the hypothesis that *M. leprae* infection is distributed as a spatially homogeneous process among adult armadillos within this population.

A spatially homogeneous infection process may arise from a population in which individuals are equally likely to interact with infectious hosts or from a population living in a habitat with widespread contamination, such that individuals have equal risk of encountering viable pathogens from the environment (see Lavania et al. 2008; Truman and Fine 2010; Wheat et al. 2014). Both mechanisms are plausible, but further research is needed to distinguish between the relative importance of direct (i.e., host to host) and indirect (i.e., environmental) transmission of *M. leprae* in armadillo populations. In this study, spatial clustering of model residuals in the northeastern section of the sampling area could be related to unaccounted for variation in host-related factors (i.e., genetic predisposition, individual behaviors) or differences in microhabitat conditions that could influence environmental transmission of *M. leprae*. For example, differences in soil type and moisture content could influence the viability and persistence of *M. leprae* within the environment (Lavania et al. 2008). Additionally, based on histopathologic examinations of ear and nose tissue samples from wild armadillos, Job et al. (1986) suggested that thorn pricks could serve as a potential mode of transmission. Thus, plant composition (e.g., density of thorny bushes) could also affect transmission among armadillos occupying overlapping areas. Future work addressing the role of microhabitat conditions on *M. leprae* infection risk could involve longitudinal sampling of

soils throughout the study area or plant identification surveys, which could help elucidate potential mechanisms underlying environmental transmission.

One of our major findings was that antibody prevalence was much higher among lactating females (both probably and definitely lactating females). This replicates the result of Morgan and Loughry (2009; see also Truman et al. 1991) and most likely reflects an age effect in that leprosy is a slow-acting disease largely confined to older animals (Williams and Loughry 2012), and lactating females are typically older than nonlactating females (Truman et al. 1991; Morgan and Loughry 2009). Trade-offs between immune responses and reproduction have been observed in other animal systems (e.g., Hanssen 2006; Schwanz 2008). Chronic *M. leprae* infection in armadillos may similarly involve a fitness trade-off that supports the terminal investment hypothesis, whereby investment in current reproduction is maximized over investment in immune defenses (Clutton-Brock 1994; Perrin et al. 1996). Nonetheless, it remains surprising that lactating females can endure the physiologic costs associated with exposure to *M. leprae* (Steuber 2007; Truman 2008) on top of those associated with reproduction (Lengyel 2011). More broadly, the fitness costs of leprosy infection to all females, regardless of lactation status, remain poorly understood (Morgan and Loughry 2009).

Our failure to find any juveniles or yearlings positive for *M. leprae* exposure was surprising. Based on experimental transmission studies, a detectable immune response can take 10–12 mo to develop (Duthie et al. 2011). It is possible that infection in juveniles and yearlings went undetected; however, capture-mark-recapture data from this population also failed to detect seroconversion of juveniles and yearlings upon recapture 1 yr later (W.J.L. unpubl. data). Thus, the lag time associated with the development of a detectable immune response does not fully explain why juveniles or yearlings failed to seroconvert throughout the study period.

Several limitations were evident in our study. Firstly, the coarseness of spatiotempo-

ral resolution used to define local neighborhoods failed to capture individual movement that would have allowed for a more accurate depiction of spatial overlap. Secondly, we assumed that annual home ranges (i.e., neighborhood size) were relatively constant across sex, lactation classes, and seasons. Field studies of armadillos suggest this is a reasonable assumption (Loughry and McDonough 2013), but local neighborhood metrics may not be representative of true spatial overlap or contact among groups. Because of limited data on individual movement, we were unable to incorporate heterogeneities in neighborhood size, which could more accurately define local antibody prevalence. These limitations could be remedied with more frequent or continuous monitoring of armadillo movement patterns. However, obtaining detailed movement, behavioral, and contact data for a large number of individuals would be logistically challenging.

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#### SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at <http://dx.doi.org/10.7589/2015-03-066>.

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