Spatial Variation and Survival of *Salmonella enterica* Subspecies in a Population of Australian Sleepy Lizards (*Tiliqua rugosa*)

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The life cycles of many enteric bacterial species require a transition between two very distinct environments. Their primary habitat is the gastrointestinal tract of the host, while their secondary habitat, during transmission from one host to another, consists of environments external to the host, such as soil, water, and sediments. Consequently, both host and environmental factors shape the genetic structure of enteric bacterial populations. This study examined the distribution of four *Salmonella enterica* subspecies in a population of sleepy lizards, *Tiliqua rugosa*, in a semiarid region of South Australia. The lizards living within the 1,920-m by 720-m study site were radio tracked, and their enteric bacteria were sampled at regular intervals throughout their active seasons in the years 2001, 2002, and 2006. Four of the six subspecies of *S. enterica* were present in this population and were nonrandomly distributed among the lizards. In particular, *S. enterica* subsp. *diarizonae* was restricted to lizards living in the most shaded parts of the study site with an overstorey of *Casuarina* trees. Experiments undertaken to investigate the survival of *S. enterica* cells under seminatural conditions revealed that cell survival decreased with increased exposure to elevated temperatures and UV light. Among the three *S. enterica* subspecies tested, *S. enterica* subsp. *diarizonae* consistently had an average expected life span that was shorter than that observed for the other two subspecies. There was no indication in the data that there was any competitive dominance hierarchy among the *S. enterica* subspecies within individual hosts. Thus, the nonrandom distribution of *S. enterica* subspecies in this population of lizards appears to be driven by their different survival characteristics in the external environment.

Our study considers small-scale spatial variation in the occurrence of subspecies of *Salmonella enterica* inhabiting a large Australian lizard. The local distribution and abundance of a species may be influenced by a number of factors, including competition (both within and between species), predation, disease, physical barriers to movement, and the physiological barriers imposed by climate or microclimate. The varying influences of these factors have been a central focus in studies of the ecology of plants and animals for over a century. Bacterial ecology, in contrast, has focused on defining and describing the diversity of taxa and classifying them into ecotypes (1, 2). However, many studies have shown associations between particular bacterial communities and characteristics of the physical environment they are found in, for instance, soil (3, 4) and freshwater (5) habitats, and of the seasonal dynamics of the bacterial community structure (6).

In bacteria, as in eukaryotes, different species have different ecological niches and life history characteristics. Many bacterial species also exhibit extensive genetic substructures, and different genetic subgroups within a species may also have distinct ecological niches and life history characteristics (7). The life cycle of enteric bacterial species exposes them to two distinct habitats, the primary habitat of the gastrointestinal tract of the host and the secondary habitat of the external environment during host-to-host transmission (8). Thus, there are two habitat components that each might contribute to the distribution of enteric bacterial species.

One component is the range of different host species that the bacteria infect, and this is likely to be influenced by biological interactions between host and bacteria and to reflect host-related adaptations in the bacterial species or their subspecific strains. For example, among native animals in Australia, *Salmonella enterica* is primarily an inhabitant of reptiles (9, 10), while *Escherichia coli* is

an inhabitant of mammals. Gordon and Cowling (11) found that for *E. coli*, the different genetic subgroups are nonrandomly distributed among different Australian mammal species. Bacterial species or strains of a species may compete with each other within the host environment, and such competition may also influence the range of host species infected. This competition among bacterial species or strains has the potential to influence within-host bacterial diversity. For example, at the species level, it has been shown that mammalian hosts harboring *E. coli* are less likely to harbor related species of bacteria (12). Competition can also occur among strains of the same species. For example, particular genetic subgroups of *E. coli* can competitively limit the total number of *E. coli* genotypes observed within a host individual (13, 14).

The second component that influences the distribution of bacteria is differences in the range of external environmental conditions that particular bacterial subgroups can tolerate. For example, *S. enterica* survives desiccating conditions in the external

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environment better than does *E. coli* (15). This explains how *S. enterica* can persist in reptiles inhabiting the arid and semiarid regions of Australia (9) and why *E. coli* is more likely to be isolated from Australian mammals inhabiting temperate regions than from those in arid regions (11). Not only do these nonrandom distribution patterns occur at the climate region scale, they also occur at much smaller scales, as described by Koeppel et al. (2), who showed that different genetic subgroups of *Bacillus simplex* had distinct ecological niches in the external environment in that they were isolated nonrandomly in relation to the microhabitat where they were collected. Small-scale spatial heterogeneity in the external environment experienced by bacteria might, in turn, lead to variation in the distribution of bacteria among hosts in a host population that occupies habitats with varying microclimatic conditions.

S. enterica is divided into six subspecies: *S. enterica* subsp. *enterica* (*S. enterica* subsp. I), *S. enterica* subsp. *salamae* (*S. enterica* subsp. II), *S. enterica* subsp. *arizonae* (*S. enterica* subsp. IIIa), *S. enterica* subsp. *diarizonae* (*S. enterica* subsp. IIIb), *S. enterica* subsp. *houtenae* (*S. enterica* subsp. IV), and *S. enterica* subsp. *indica* (*S. enterica* subsp. VI). Although *S. enterica* is better known as a human pathogen, all six subspecies of *S. enterica* have been isolated from reptiles (16–18). No evidence suggests pathogenic effects in reptiles, but little is known of their ecology in reptile hosts. The six subspecies differ in their phenotypic characteristics and in the range of host species they have been reported from (19, 20). However, despite some apparent host specificity, preliminary data have shown that in some host species, different subspecies can be isolated from different host individuals in the same population or even coexisting in the same host individual.

The scincoid lizard *Tiliqua rugosa* (the sleepy lizard) is a common inhabitant of the semiarid regions of southern and eastern Australia and is known to host at least four of the six subspecies of *S. enterica*. We studied the distribution of those four subspecies of *S. enterica* in one small subsection of a population of the sleepy lizard in a semiarid region in South Australia over the spring and summer period, when they are active, in 3 years, 2001, 2002, and 2006. Individual lizards were radio tracked and sampled for *S. enterica* at regular intervals throughout each active period. Experiments were also conducted to investigate the survival characteristics of each subspecies of *S. enterica* in the external environment of the study plot. For enteric bacteria with a fecal-oral transmission pathway among individual hosts, the pattern of fecal deposition by the host is an important ecological consideration. Sleepy lizards appear to scat haphazardly as they move around their home ranges (21). Our aim was to describe any spatial patterning and subspecies diversity of *S. enterica* within individual sleepy lizards and to determine whether any spatial patterns were driven primarily by the environment outside the host or by competition among subspecies within the host.

MATERIALS AND METHODS

Host species. The sleepy lizard, *T. rugosa*, a large viviparous skink, is distributed widely in southern Australia and occupies a range of mesic to arid habitats (22, 23). Adult sleepy lizards are long-lived (>20 years) (23) and mainly herbivorous (24, 25). They are predominantly active from spring to early summer (late August to December) (26, 27). They form monogamous partnerships for 6 to 8 weeks before mating in early November, and these partnerships re-form over successive years (28–30). They maintain long-term, stable home ranges of approximately 4 ha (31, 32), which overlap extensively (27). Within the home range, there are

between two and four areas of core activity, each usually centered around a refuge site (32), although individual lizards use multiple refuge sites over their active season (33).

Study site. The study was conducted in the spring and early summer (usually September to December) of the years 2001, 2002, and 2006. This is the period of highest lizard activity each year (25, 33). The study site was an area of 1,920 m by 715 m located approximately 4 km west of Bunday Bore Station (33°55'S, 139°21'E) within the Mt. Mary study area described by Bull et al. (34) and Petney and Bull (35). The vegetation of the study site is chenopod shrubland, predominantly bluebush (*Mariana sedifolia*), with patches of *Casuarina cristata* woodland largely in the center of the study site. The site shows small topographic variation (altitude differences of a few meters) but heterogeneity in the degree of shading (see Fig. S1 in the supplemental material). Both of these factors might influence local soil moisture and ground temperature, providing a range of microhabitats that bacteria outside their host lizards could be exposed to. During spring, open areas support a covering of herbaceous annual plants. The area has cool, wet winters and hot, dry summers, with daily maximum summer temperatures often exceeding 30°C (27). The average annual rainfall at Bunday Bore (1970 to 1997) was 287 mm (27), but the average was only 192 mm during the years 2000 to 2006 due to two exceptionally dry years, 2002 (87 mm) and 2006 (110 mm).

Sampling. Lizards were captured within the study site and fitted with 3.6-g radio transmitters (Sirtrack, New Zealand) at the start of each active season. The transmitters were attached to the lateral surface of the tail using adhesive tape (Leukoplast). After release, the lizards could be regularly relocated using a receiver (Teletronics, AZ, USA) with a directional antenna (Sirtrack, New Zealand). Individual lizards could be distinguished by the unique signal produced by each transmitter, and their positions were recorded using a Garmin (GPS 76) global positioning system (GPS).

The lizards were treated using procedures formally approved by the Flinders University Animal Welfare Committee (approval no. E204) in compliance with the Australian Code of Practice for the Use of Animals for Scientific Purposes, and the study was conducted under a Department of Environment and Heritage Permit to Undertake Scientific Research (permit no. A23436).

The lizards were sampled for their gut microbiota between early spring and early summer of each year (26 September 2001 to 17 January 2002, 23 August to 4 December 2002, and 17 September to 14 December 2006). In 2001, 179 cloacal samples from 51 individuals (25 males and 26 females) were collected on six separate sampling occasions (3 weeks apart). In 2002, 151 cloacal samples were collected from 38 individuals (20 males and 18 females) on seven separate sampling occasions (3 weeks apart). In 2006, 372 cloacal samples were collected from 29 sleepy lizards (18 males and 11 females) sampled up to three times per fortnight. Not all lizards could be located on every sampling occasion.

Bacterial isolation and identification. Cloacal samples were collected using a sterile swab/transport tube system containing Aimes transport agar (36). Samples from the first 2 years were stored at 5°C until they were shipped on ice to the Australian National University for processing and were processed within a week of collection, and members of the *Enterobacteriaceae* were isolated as described by Gordon and FitzGibbon (12). Isolates positive for hydrogen sulfide (H_2S) production and negative for indole production were preliminarily identified as *S. enterica* (19).

Cloacal samples from 2006 were processed as described above on the day of collection at the Mt. Mary field site. In addition, the swabs were vortexed in 3 ml of Rappaport-Vassiliadis broth (Merck), a medium designed to enrich for *S. enterica* (37). The broth culture was incubated overnight at 37°C. The following day, 20 μ l of the inoculated Rappaport-Vassiliadis culture was dilution streaked onto a MacConkey agar plate and incubated at 37°C. The next day, up to 10 presumptive *S. enterica* colonies (selected based on colony morphology) were transferred onto H_2S agar plates and incubated overnight at 37°C. Colonies that were H_2S positive

TABLE 1 RFLP and biochemical characteristics used to distinguish each of the four subspecies of *S. enterica* isolated from the study site

Subspecies	No. of isolates	No. of bands produced with:		% of strains positive for:	
		HindIII	HaeIII	β -Glucuronidase	Dulcitol
<i>S. enterica</i> subsp. <i>enterica</i>	64	1	1	88	97
<i>S. enterica</i> subsp. <i>diarizonae</i>	48	2	2	100	10
<i>S. enterica</i> subsp. <i>salamae</i>	52	2	1	29	100
<i>S. enterica</i> subsp. <i>houtenae</i>	4	1	1	0	0

were toothpicked onto a Luria broth agar plate, incubated overnight at 37°C, and stored at 4°C for further characterization.

Subspecies identification. Four subspecies of *S. enterica* were recognized among the samples (*S. enterica* subsp. *enterica*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *salamae*, and *S. enterica* subsp. *houtenae*), and the results of two methods were combined and used to assign each isolate to a subspecies in each year of the study. The first method was based on a restriction fragment length polymorphism (RFLP) analysis of a fragment of the *mdh* gene, and the second was based on the partial biochemical profile of an isolate.

RFLP method for subspecies discrimination. The *mdh* sequences available for *S. enterica* were downloaded from GenBank (accession numbers U04761.1 to U04769.1, U04771.1 to U04776.1, and U04778.1 to U04784.1). An *in silico* analysis using Sequencher V3 (Gene Codes Corporation) was used to determine which restriction enzymes or combination of enzymes would distinguish the subspecies. HindIII and HaeIII were found to be the most suitable enzymes (Table 1). PCR primers targeting conserved regions of the *mdh* gene and which encompassed the discriminatory region of the gene were chosen: *mdh*-f (5'-CACGCTGGA TATCATCCGCT-3') and *mdh*-r (5'-CCTTCCACATAGGCGCATTC-3'), yielding a 327-bp fragment (38). This region of *mdh* was sequenced in 168 *S. enterica* isolates collected from the Mt. Mary sleepy lizard population. The sequence data revealed that the 168 isolates clustered into four distinct clades with very high bootstrap support and corresponded to 64 isolates of *S. enterica* subsp. *enterica*, 48 of *S. enterica* subsp. *salamae*, 52 of *S. enterica* subsp. *diarizonae*, and 4 of *S. enterica* subsp. *houtenae*.

The restriction digestions were carried out as follows. Isolates were grown on lysogeny broth agar plates at 37°C overnight. The following day, a single colony was placed into a microcentrifuge tube containing 50 μ l of ultrapure water and vortexed to suspend the cells. The microcentrifuge tubes were heated at 105°C for 15 min and then centrifuged at 13,000 rpm for 1 min. A 5- μ l aliquot of the supernatant was used as a DNA template. The 18- μ l reaction mixture consisted of 5 μ l of template DNA, 0.14 μ l of Platinum *Taq* (1.25 U), 2 μ l each of both forward and reverse *mdh* primers (10 pmol ml⁻¹), 3.4 μ l 25 mM MgCl₂, 3.4 μ l 5 $\times$ buffer [67 mM Tris-HCl, 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg gelatin ml⁻¹, and 0.2 mM deoxynucleoside triphosphates (dNTPs)] (Fisher Biotec), and 1.06 μ l ultrapure water (Fisher Biotec). The PCR amplification conditions were as follows: 1 cycle at 95°C for 12 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 3 min; and 1 cycle at 72°C for 10 min. The amplified product was divided in half. To each half, a restriction enzyme mixture of either HindIII or HaeIII was added. The 1.75- μ l restriction enzyme mixtures consisted of 0.15 μ l ultrapure water (Fisher Biotec), 1 μ l 10 $\times$ restriction buffer (Promega), 0.1 μ l bovine serum albumen (BSA), and 0.5 μ l restriction enzyme (Promega). The restriction digestion mixture was incubated at 37°C for between 1 and 4 h. Electrophoresis was carried out using 2% agarose gels stained with 2 μ l ethidium bromide. The gels were visualized and photographed using a UV transilluminator.

Biochemical characterization for subspecies discrimination. The ability of an isolate to utilize dulcitol as a growth substrate and to produce β -glucuronidase can be used to distinguish among the subspecies of *S. enterica* found at the study site (19). The 168 isolates identified based on nucleotide sequence data for the 323-bp *mdh* fragment were screened for the ability to utilize dulcitol and to produce β -glucuronidase (Table 1). The ability to exploit dulcitol was determined by inoculating cells into

96-well plates (Nunc) containing 200 μ l of phenol red broth base (BBL) and dulcitol (5 mg/ml). The ability of an isolate to produce β -glucuronidase was determined using Rainbow agar O157 (Biolog).

While the RFLP analysis could not distinguish between *S. enterica* subsp. *enterica* and *S. enterica* subsp. *houtenae* isolates, *S. enterica* subsp. *houtenae* is β -glucuronidase and dulcitol negative, while the majority of *S. enterica* subsp. *enterica* isolates are β -glucuronidase positive and almost always dulcitol positive (Table 1). Further, *S. enterica* subsp. *houtenae* represented less than 2% of the isolates recovered from this population of sleepy lizards.

***S. enterica* subspecies survival.** In the spring and summer of 2006, we performed an experiment examining the survival of *S. enterica* cells under different shade conditions within the study site. From the isolates collected from sleepy lizards at the study site in 2001, 39 strains (13 each of *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, and *S. enterica* subsp. *diarizonae*) were selected for use in the experiment. The survival characteristics of strains of *S. enterica* subsp. *houtenae* were not determined. Given the rarity of the subspecies, the decision was made that the substantial additional workload involved in including it was unwarranted.

Each of the 39 strains was grown in 10 ml Rappaport-Vassiliadis broth and shaken for 24 h at ambient temperature (18 to 25°C). Two 50- μ l aliquots of the overnight culture of each of the 39 strains were transferred into 2 of 78 randomly selected wells in a 96-well microtiter plate (Nunc). An additional nine randomly selected wells were filled with 50 μ l of uninoculated Rappaport-Vassiliadis broth. Then, 50 replicate survival blocks were prepared, each block consisting of 87 cotton buds with their stalks inserted into a grid of holes in a block of wood positioned so that the cotton buds corresponded to the wells of the microtiter plate. The block of wood was inverted, and each cotton tip was dipped into the medium contained in each well of the microtiter plate. This procedure transferred about 1.0×10^8 cells to the cotton bud. The nine cotton tips with uninoculated Rappaport-Vassiliadis broth were controls to detect, after environmental exposure, the incidence of any field-based contamination with *S. enterica* strains.

Ten replicates of the survival blocks were each exposed to one of five shade treatments—0%, 30%, 50%, 70%, and 80% shade—using four strengths of shade cloth. All 10 replicates for each of the four shade treatments were placed 3 cm below a single 86- by 62-cm piece of shade cloth. The treatments were located within the study site in a 3-m by 3-m patch of open bluebush habitat exposed to full sunlight.

The experiment was repeated three times between October and December 2006, when the lizards were active, using the same shade cloth locations. Each experiment ran for 3 to 5 days, and Hobo data loggers (OneTemp Pty Ltd, Australia) were used to record the temperature in each treatment for the duration of each experiment. Rain shortened the duration of the second experiment.

Each experiment began after sunset on the first day (20:30). Then, one survival block was removed from each treatment every 4 to 12 h (depending on the treatment) until all 10 blocks had been sampled. Each block removed was inverted and dipped three times into the wells of a plate that contained 200 μ l of Rappaport-Vassiliadis broth. A further nine wells on each plate had no cotton tips inserted, and these were used as controls to detect any laboratory contamination. Each plate was incubated overnight at 37°C and then visually scored for turbidity indicating the presence of bacterial growth.

FIG 1 Changes in the proportions of sleepy lizards in which *S. enterica* was detected over the course of the 2006 field season at Mt. Mary, South Australia.

A Weibull model of survivorship and the software JMP 7.01 (SAS Institute) were used to compare the survival rates of the three *S. enterica* subspecies across the five shade treatments. The input data were the number of cotton tips from a subspecies in a shade treatment where growth was observed on each sampling occasion. Wilcoxon tests were used to determine the significance of any differences among subspecies or among treatments.

RESULTS

For 2006, the year with the most detailed temporal data, the proportion of lizards in which *S. enterica* was detected increased from spring to summer (Fig. 1). Across the 3 years of the study, *S. enterica* subsp. *houtenae* was rarely observed in this population of sleepy lizards (<1% of isolates). Of the other three subspecies, *S. enterica* subsp. *salamae* was the most common and represented 43% of the 419 isolates recovered; 36% of the isolates were *S. enterica* subsp. *enterica*, and 21% were *S. enterica* subsp. *diarizonae*. There was little difference in the relative abundances of the three subspecies between 2001 and 2002 (likelihood ratio test; $P > 0.09$), but there was a significant shift in the relative abundances of the subspecies between the periods 2001–2002 and 2006 (likelihood ratio test; $P < 0.0001$). In 2006, there was an increase in the frequency of *S. enterica* subsp. *salamae* isolates and a concomitant decline in the frequency of *S. enterica* subsp. *diarizonae* isolates (Table 2).

Temporal dynamics of *S. enterica* subspecies within a year.

In 2006, if a sleepy lizard harbored *S. enterica*, then on 85% of the occasions on which a lizard was sampled only a single subspecies was detected in that lizard, on 13% of the sampling occasions two subspecies were detected, and 2% of the time three subspecies were detected simultaneously inhabiting the host. There was no significant change in the average number of subspecies harbored by a lizard over the 2006 activity season (linear regression; R^2 , 13.7%; $P = 0.174$) (Fig. 2). Although at any given time a lizard

FIG 2 *S. enterica* subspecies carriage in the sleepy lizard population. ▲, average number of subspecies present per lizard; ●, cumulative number of lizards, out of a total of 28 lizards, that harbored two or more subspecies over the season.

typically hosted only a single subspecies of *S. enterica*, by the end of the 2006 activity season, 21 of 28 (75%) lizards had harbored two or more subspecies of *S. enterica* over the season (Fig. 2). The temporal trends of subspecies occurrence in individual lizards are shown in Fig. 3.

Although the data were too sparse for rigorous analysis, there was no evidence of any dominance hierarchy among the subspecies of *S. enterica*. In lizards that harbored multiple subspecies, it appeared that they could cooccur within a host for extended periods (e.g., lizards 76 and 10606 [Fig. 3]). There was no indication that one of the subspecies was more likely to replace another subspecies in a lizard.

Spatial structure of *S. enterica* subspecies within a sleepy lizard population. The geographic location of a lizard was determined each time it was sampled. To determine if the subspecies of *S. enterica* were nonrandomly distributed in the environment, an analysis was conducted in which the year of sampling (2001, 2002, or 2006) and the lizard's location in an east-west and a north-south direction at each sampling time were used as predictor variables in a nominal logistic regression model. The year, as well as the lizard's location, were found to predict the subspecies most likely to be observed in a lizard (Table 3) (nominal logistic regression; full model; $P < 0.001$). However, the significant interaction terms indicate that the spatial distribution of the subspecies differed among years.

In 2001 and 2002, lizards sampled in the western part of the study site were more likely to harbor *S. enterica* subsp. *enterica* than lizards living in the eastern portion of the study site, while *S. enterica* subsp. *salamae* was distributed throughout the study site (Fig. 4). The pattern observed in 2006 indicated that lizards sampled in the northern end of the study site were more likely to harbor *S. enterica* subsp. *salamae*, while lizards in the eastern portion were more likely to harbor *S. enterica* subsp. *enterica* (Fig. 4).

Although the spatial patterns observed in 2006 varied from those observed in 2001 and 2002, in all years, *S. enterica* subsp. *diarizonae* appeared to be mapping onto the central region of the study site, where there was an extensive stand of *Casuarina* trees. In order to determine if subspecies distributions were associated with the stand of *Casuarina* trees, all surveyed locations of each

TABLE 2 Relative abundances of three subspecies of *S. enterica* isolated from *T. rugosa* individuals at Mt. Mary, South Australia

Yr	No. of isolates	% of isolates		
		<i>S. enterica</i> subsp. <i>enterica</i>	<i>S. enterica</i> subsp. <i>diarizonae</i>	<i>S. enterica</i> subsp. <i>salamae</i>
2001	83	35	29	36
2002	68	47	32	21
2006	268	34	16	50

FIG 3 Temporal changes in the *S. enterica* subspecies detected in each *T. rugosa* individual over the active season of 2006. On the left are the lizard numbers, while columns 1 to 15 represent the sampling occasions in chronological order from left to right. Each colored box represents a sample. White, no *S. enterica*; yellow, *S. enterica* subsp. *enterica*; blue, *S. enterica* subsp. *diarizonae*; red, *S. enterica* subsp. *salamae*; black, *S. enterica* subsp. *houtenae*; gray, lizard not sampled. The amounts of color in each sampling box represent the relative frequency of each of the subspecies found in the sample.

lizard in 2006 were plotted onto a vegetation map of the study site. If a lizard was located even once within the *Casuarina* stand in that year, that lizard was defined as having a home range that included the *Casuarina* stand. Each lizard was also scored as the host of a *Salmonella* subspecies if the subspecies was detected in the lizard in at least one sampling survey. *S. enterica* subsp. *diarizonae* was detected in 52% of the lizards whose home range included the *Casuarina* stand but in only 13% of the lizards never observed

within the *Casuarina* stand (contingency table analysis; likelihood ratio; $\chi^2 = 6.395$; $P = 0.011$). Similarly, *S. enterica* subsp. *salamae* was significantly more likely to be detected in lizards whose home range included the *Casuarina* stand (74% of the lizards) than in the lizards whose home range did not (40%) (contingency table analysis; likelihood ratio; $\chi^2 = 4.391$; $P = 0.036$). The presence of *S. enterica* subsp. *enterica* in a lizard was not significantly affected by the presence or absence of trees in the lizard's home range (74% versus 80%, respectively) (contingency table analysis; likelihood ratio; $\chi^2 = 0.189$; $P = 0.664$).

***S. enterica* subspecies survival.** Of the 1,080 swabs that served as controls to detect field contamination, 10 (0.93%) became contaminated, but none of the uninoculated wells designed to detect contamination in the laboratory became contaminated. As a result, it was considered that contamination either in the field or in the laboratory did not significantly influence the results of the survival experiments.

The ambient climatic conditions varied among the three experiments. The mean, minimum, and maximum temperatures were all higher in the third experiment (data not presented).

TABLE 3 Likelihood ratio test statistics to determine the factors influencing the distribution of *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, and *S. enterica* subsp. *diarizonae*

Source of variation	df	χ^2	$P > \chi^2$
Yr	4	12.38	0.0147
Easting	2	16.95	0.0002
Yr $\times$ easting	4	26.87	<0.0001
Northing	2	11.35	0.0034
Yr $\times$ northing	4	9.79	0.0441
Easting $\times$ northing	2	2.00	0.3678
Yr $\times$ easting $\times$ northing	4	2.33	0.6758

FIG 4 Distribution of *S. enterica* subspecies in the Mt. Mary study site in the years 2001, 2002, and 2006. In each diagram, each point corresponds to the location of a lizard harboring a particular subspecies when it was sampled. The spatial locations of each subspecies detected in a lizard were fitted using a nonparametric bivariate density algorithm. This analysis identifies the spatial locations corresponding to the region where a particular subspecies was most likely to be detected in a lizard. The red and yellow contours correspond to a high probability of detecting a particular subspecies, while the green and blue contours correspond to the areas where a particular subspecies was less likely to be detected in a lizard. D, *S. enterica* subsp. *diarizonae*; E, *S. enterica* subsp. *enterica*; S, *S. enterica* subsp. *salamae*.

Within each experiment, the five shade treatments produced different ambient temperatures. Although there were no replicate locations for the treatments to allow statistical comparison, the 80% shade treatment resulted in ambient temperatures that were consistently 3 to 4°C cooler than the 0% shade treatment.

In the first experiment, survival time increased significantly with increasing shade levels for each of the *S. enterica* subspecies (Table 4). Survival time also varied significantly among the *S. enterica* subspecies, with *S. enterica* subsp. *diarizonae* cells exhibiting the shortest survival times at all shade levels (Table 4). The same patterns were observed in the 2nd and 3rd repeats of the survival experiment (data not presented).

DISCUSSION

This study has demonstrated that the three subspecies of *S. enterica* that are commonly found in this population of sleepy lizards showed a nonrandom spatial distribution among hosts within the study site. This nonrandom spatial distribution was most likely a consequence of the location of a lizard's home range within the study site rather than of host competition among *S. enterica* subspecies. Sample processing varied between the years 2001 and 2002 compared to 2006 in terms of the time between sample collection and processing and the media used to isolate the *Salmonella* bacteria. These differences are unlikely to have affected our

TABLE 4 Average predicted times at which 50% of swabs failed to yield viable *S. enterica* cells in the first repeat of the survival experiment

% shade treatment	Avg temp (minimum–maximum) (°C) ^a	Avg predicted time (h) ($\bar{x} \pm \text{SE}$) ^c			Subspecies effect ^b
		<i>S. enterica</i> subsp. <i>enterica</i>	<i>S. enterica</i> subsp. <i>diarizonae</i>	<i>S. enterica</i> subsp. <i>salamae</i>	
0	22 (4–57)	69.9 $\pm$ 2.20	67.1 $\pm$ 2.24	69.5 $\pm$ 2.21	0.5219
30	21 (5–50)	72.9 $\pm$ 2.21	66.8 $\pm$ 2.24	71.2 $\pm$ 2.18	0.0768
50	21 (5–49)	74.3 $\pm$ 2.16	71.1 $\pm$ 2.19	75.1 $\pm$ 2.20	0.3297
70	19 (5–36)	79.4 $\pm$ 2.25	70.6 $\pm$ 2.21	76.8 $\pm$ 2.26	0.0088
80	18 (5–37)	84.5 $\pm$ 2.15	75.2 $\pm$ 2.17	84.1 $\pm$ 2.22	0.0016

^a Temperatures were recorded at 15-min intervals for the duration of the experiment.

^b Wilcoxon tests were used to determine differences in survival in subspecies ($P > \chi^2$).

^c Shade effects were as follows: *S. enterica* subsp. *enterica*, <0.0001 ; *S. enterica* subsp. *diarizonae*, 0.0105; *S. enterica* subsp. *salamae*, <0.0001 . Wilcoxon tests were used to determine differences in survival in shade treatments ($P > \chi^2$).

conclusions to a significant extent. *S. enterica* is one of the most common species in this population of lizards, and the 2006 sampling showed that in the great majority of samples, a single subspecies of *Salmonella* was detected. Further, the distribution of the *S. enterica* subspecies was nonrandom in each year of the study, and *S. enterica* subsp. *diarizonae* was consistently associated with lizards living in proximity to the *Casuarina* stand.

The fraction of lizards in which *S. enterica* could be detected typically increased over the course of the active season. Lizards are inactive and generally do not feed between January and August, and this likely explains the lower prevalence of *S. enterica* in the lizards in early spring, when they emerge from their overwintering retreats and when they were first sampled. We suggest that subsequent rises in prevalence reflect increasing exposure for feeding lizards. Further, at any time during the active season, if a lizard harbored *S. enterica*, then it typically harbored a single subspecies. In 17 of the 28 lizards, the dominant subspecies in the first 2006 sample remained the dominant subspecies in the last 2006 sample. These outcomes may suggest competition by precedence, rather than any hierarchical competitive dominance structure. Further, when subspecies replacement did occur, there was no clear pattern of replacement, suggesting that no subspecies is competitively dominant. This conclusion was supported by observations of some cases of prolonged coexistence of subspecies within a lizard. Thus, within-host competitive interactions are unlikely to explain the nonrandom distribution of the subspecies among lizards.

The nonrandom spatial distribution of the subspecies is also unlikely to have occurred simply as a result of some past chance event that has been maintained as a result of the restricted movement patterns of the lizards. Although the activity of a lizard is largely confined to its home range, each home range extensively overlaps those of its neighbors (32). Therefore, over time, any nonrandom pattern resulting from a chance event would eventually be lost. This is illustrated by the significant “location $\times$ year” interactions in the analyses of the spatial location of each subspecies; their distributions changed over the course of the study.

The most likely explanation for the nonrandom distribution of *S. enterica* subspecies among lizards is that the subspecies found in a lizard is a consequence of where the lizard lives within the study site. This was most apparent for lizards living in the part of the study site where there was a significant *Casuarina* overstory. *S. enterica* subsp. *diarizonae* was significantly less likely to be detected in lizards whose home range did not include the *Casuarina* stand, and a similar pattern was observed for *S. enterica* subsp. *salamae*. These patterns could be observed, because sleepy lizards occupy, over several years, small, stable home ranges of approximately 4 ha (31, 32). Thus, the scale of home ranges is small relative to the scale at which the vegetation changes within the study site.

Although the survival experiments likely underestimate *Salmonella* survival in naturally deposited feces, the results of these experiments support the conclusion that it is where the lizard lives in the study site that is responsible for the nonrandom spatial distribution of subspecies among the lizards. The survival experiments demonstrate that, for all *S. enterica* subspecies, the length of time they survived, and presumably remained infectious, depended on their degree of exposure to elevated temperature and sunlight. However, of the three *S. enterica* subspecies examined, *S. enterica* subsp. *diarizonae* exhibited the poorest survival. The

shade provided by *Casuarina* is likely to provide a bacterium with cooler soil temperatures, lower levels of UV, and reduced temperature fluctuations compared to other areas within the study site. For *S. enterica* subsp. *enterica*, survival was marginally better than for the other subspecies, and this was the subspecies whose occurrence in lizards was independent of whether a lizard's home range included the *Casuarina* stand. The prevalence of *S. enterica* subsp. *diarizonae* in the host population—the species with the poorest survival in the lizard population—declined substantially from 2001 to 2006, and during this period, there were 2 years with drought conditions. The results of the study indicate that the spatial distribution of *Salmonella* subspecies in the study area is shaped, in part, by events occurring after fecal deposition has occurred. The distribution of *E. coli* genotypes (phylogroups) has also been shown to be shaped by environmental patterns after fecal deposition (39).

In the current study, there was little evidence that among subspecies competition plays a significant role in determining the diversity of *S. enterica* strains present in this population of sleepy lizards. However, it does appear that the subspecies vary in their capacities to persist in the external environment, with *S. enterica* subsp. *diarizonae* having the poorest survival of the three subspecies. Thus, all else being equal, one would predict that *S. enterica* subsp. *diarizonae* would not persist in this population, as it should eventually be replaced by strains belonging to the other two subspecies, both of which are predicted to survive better than *S. enterica* subsp. *diarizonae*. The manner in which *S. enterica* subsp. *diarizonae* persists in this population of lizards is unknown.

Host social interactions have been shown to influence the transmission dynamics of *S. enterica* (40), as well as other enteric bacteria (41). Host population dynamics can also play an important role in bacterial strain coexistence (42). However, the impact of sleepy lizard dynamics and behavior on *S. enterica* subspecies coexistence is difficult to predict. Sleepy lizards occur at relatively high densities, but the rate of population turnover is low (23). Thus, at one level, the rate of new hosts entering the population per year is low. However, the very extended nonfeeding period (7 to 8 months) seen in sleepy lizards may result in most lizards apparently losing their *S. enterica* populations. Consequently, for *S. enterica*, the host population may effectively turn over every year.

Although this research has left many unanswered questions, it represents a substantial contribution to the underexplored area of the ecology of bacterial infections of wildlife. Further research should be directed toward gaining a better understanding of the separate key factors that limit *S. enterica* survival in the external environment, such as high temperatures, exposure to UV light, and desiccating conditions. Second, an understanding of the genetic differences between the subspecies that influence their differential survival in the external environment may help provide an understanding of the survival and transmission of pathogenic strains of *S. enterica*.

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