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PREVALENCE OF *SALMONELLA* IN INTESTINAL MUCOSAL SAMPLES FROM FREE-RANGING RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*) IN ILLINOIS

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***Abstract.*—Turtles can be carriers of bacteria of the genus *Salmonella,* and numerous studies have documented *Salmonella* shedding in both captive and free-living populations. Because turtles may shed *Salmonella* intermittently, however, data based on fecal samples may underestimate the true prevalence of the bacterium in turtles. We examined intestinal mucosal scrapings using molecular methods to identify *Salmonella* in free-ranging Red-eared Sliders (*Trachemys scripta elegans)* to determine carriage rates. *Salmonella* was detected in 11% (8/73) of turtles. Prevalence of infection did not vary among the nine ecologically varied ponds sampled. The prevalence of *Salmonella* infection in this study was higher than documented in a previous study that was conducted on conspecifics in the same area and during the same year but using fecal samples, suggesting that free-ranging Red-eared Sliders can harbor *Salmonella* but not shed the bacterium in feces.**

Key Words.—bacteria; infections; Red-eared Sliders; *Salmonella*; *Trachemys scripta*; turtles

INTRODUCTION

Reptiles are generally asymptomatic carriers of bacteria in the genus *Salmonella* (Chiodini and Sundberg 1981) and have been identified as a source of infection in human cases and outbreaks of salmonellosis (Chiodini and Sundberg 1981; Mermin et al. 2004; Harris et al. 2009). The association between salmonellosis and pet turtles motivated a 1975 federal ban on the sale of turtles with a carapace of < 10.2 cm (4 in) in the United States (Cohen et al. 1980). This ban prevented an estimated 100,000 cases of turtle-associated salmonellosis in children between 1970 and 1976 alone (Cohen et al. 1980). These public health concerns have also prompted studies to identify the prevalence of *Salmonella* in captive turtles (Otis and Behler 1973; Siebeling et al. 1984; Shane et al. 1990; Abalem de Sá and Solari 2001). More recently, studies of free-ranging turtles have reported prevalence of shed *Salmonella* as low as 0% (Richards et al. 2004; Saelinger et al. 2006; Readel et al. 2008) and as high as 50–100% (Chambers and Hulse 2006; Hahn et al. 2007; Gaertner et al. 2008a), highlighting the extreme variability in shedding rates characteristic of these bacteria.

In a recent study, we failed to isolate *Salmonella* from cloacal samples collected from Red-eared Sliders (*Trachemys scripta elegans)* living in multiple southern Illinois ponds that varied in pond type (natural vs. manmade), size, and biotic communities (Readel et al. 2008). Studies of cloacal shedding rates may provide an incomplete picture of the prevalence of *Salmonella* in free-ranging turtles because *Salmonella* shedding might be intermittent and stress-related (DuPonte et al. 1978; Burnham et al. 1998). Therefore, the prevalence of cloacal shedding could underestimate the true infection rate of turtles. Nevertheless, few studies have attempted to quantify carriage rates of *Salmonella* in free-ranging turtles (for an exception, see Saelinger et al. 2006), which limits not only accurate assessments of public health risk, but also our ability to examine the influence of host and environmental characteristics on rates of infection.

The aim of this study was to determine the prevalence of *Salmonella* carriage rates by examining gastrointestinal mucosal samples from free-ranging Red-eared Sliders in southern Illinois. Red-eared Sliders are common in the pet trade, notorious as a globally invasive species, and inhabit a variety of aquatic habitats. Numerous studies have already quantified *Salmonella* shedding rates in this species in captivity (McCoy and Seidler 1973; Otis and Behler 1973; Chassis et al. 1986; Abalem de Sá and Solari 2001; Pasmans et al. 2002a); our study provides complementary information on rates of carriage independent of shedding. Additionally, because environmental and host life-history characteristics may influence *Salmonella* shedding in

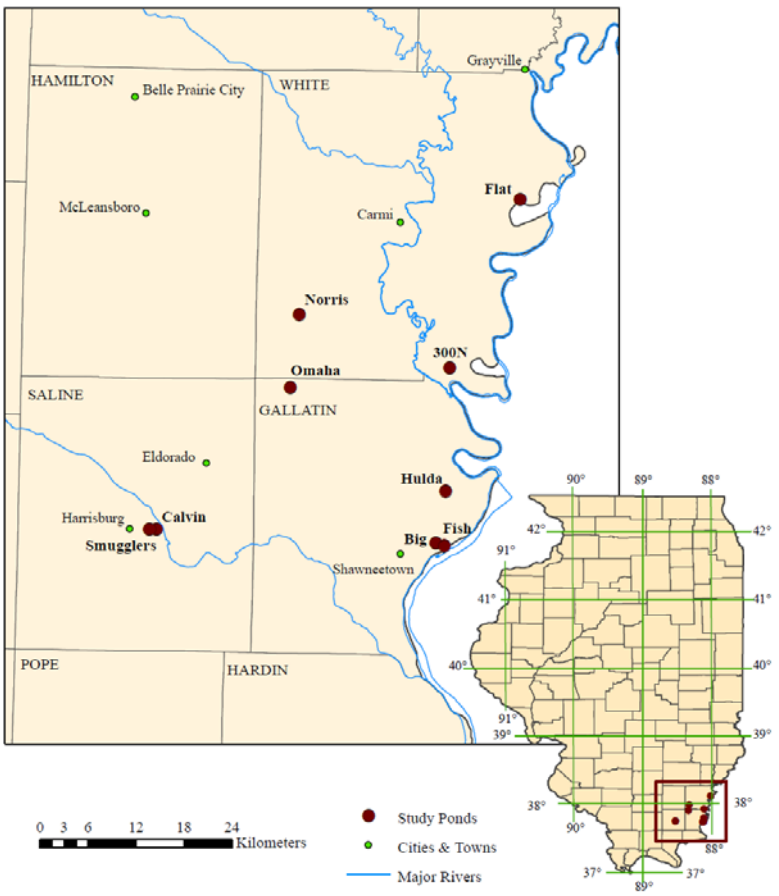

FIGURE 1. The location of the study ponds where we captured Red-eared Sliders (*Trachemys scripta elegans*) in southeastern Illinois.

free-ranging turtles (Hidalgo-Vila et al. 2006; Gaertner et al. 2008a, b; Readel et al. 2008), we also examined the influence of host characteristics (body size, and body condition) and pond location on *Salmonella* infection rates.

MATERIALS AND METHODS

We captured turtles using baited hoop traps from nine water bodies in southern Illinois from 2-25 August 2006 (Fig. 1; Table 1), a region characterized primarily by a mosaic landscape of forest, grassland, and intensive row crop agriculture. Ponds varied in size (2–50 hectares), and in the percentage of intensive row crop agriculture (2–50%) and forest (0–54%) surrounding each pond edge within a 125 m buffer. Half of the ponds were natural oxbow lakes along the Ohio River floodplain that supported a high diversity of fishes as well as diverse turtle assemblages (Dreslik et al. 2005). The others were manmade ponds created in or before the 1970's as city reservoirs or from highway borrow pits, contained only common lentic turtle species, and some have been stocked with game fish. All ponds were used for recreational fishing. Turtle abundance, defined as the number of individual turtles of any species captured per trap hour, was also determined for each pond.

We collected only adult male turtles for this study. This was done to minimize potential life history differences among individuals and because this study was conducted as part of a larger investigation that required only males. We handled turtles using individual bleach-decontaminated plastic tubs and instruments, and we used sterile procedures throughout the study to prevent cross-contamination. Upon return to the laboratory, we measured turtles for plastron length (PL) to the nearest mm and we killed them by overexposure to halothane vapors for two hours. Halothane induces anesthesia rapidly and is the most effective and preferred inhalant anesthetic for euthanasia of small animals (American Veterinary Medical Association. 2007. AVMA Guidelines on Euthanasia. Available from http://www.avma.org/resources/euthanasia.pdf [Accessed 2 July 2010]). Death was confirmed by prolonged (in excess of 30 min) lack of

TABLE 1. Prevalence of *Salmonella* in male Red-eared Sliders (*Trachemys scripta elegans)* from nine ecologically varied ponds in Illinois. Confidence intervals (95%) for the prevalence of *Salmonella* in turtles were calculated using the modified Wald method (Agresti and Coull, 1998).

Pond	Location	No. Turtles Sampled	Prevalence of Salmonella (%)	95% Confidence Interval
300N	37.9229N, 88.0992W	8	12.5	0.1 – 49.2
Big	37.7258N, 88.1162W	8	12.5	0.1 – 49.3
Calvin	37.7371N, 88.5118W	7	28.6	7.5 – 64.8
Fish	37.7228N, 88.1043W	8	12.5	0.1 – 49.3
Flat	38.1136N, 88.0022W	10	0.0	0.0 – 32.1
Hulda	37.7841N, 88.1034W	7	14.3	0.5 – 53.4
Norris	37.9810N, 88.3139W	10	0.0	0.0 – 32.1
Omaha	37.8987N, 88.3253W	8	12.5	0.1 – 49.3
Smugglers	37.7367N, 88.5215W	7	14.3	0.5 – 53.4
Total		**73**	**11.0**	**5.4 – 20.4**

righting response. Turtles were then transferred to a -80°C freezer until dissection in 2008. Turtles were dissected, and a mucosal scrape was obtained from the small and large intestine of each turtle. Scrapes were collected by scraping the length of the mucosal lining with the blunt end of a pair of sterile forceps after large food particles and parasites had been removed. The amount of mucus collected from each scrape varied between < 50 µl and 2 mL. Material from scrapes was placed in 2 mL microcentrifuge tubes and stored at -80°C. Additionally, all body fat was removed from each turtle and weighed to the nearest 0.01 g on an electronic balance (Denver Instrument Company, Model XE-100A, Denver, Colorado, USA) for a direct estimate of body condition (fat weight / turtle PL).

We subjected mucosal samples to a diagnostic PCR to identify *Salmonella*. We extracted DNA from 50–100 µl of intestinal mucus using the ZR-96 Genomic DNA Kit (Zymo Research, Orange, California, USA) eluted in a 50 µl volume, according to the manufacturer's protocol. The *Salmonella* specific primer pair *invA*-1 (5´-ACAGTGCTCGTTTACGACC TGAAT) and *invA*-2 (5´- AGACGACTGGTACTGAT CGATAAT) was used to amplify a 243 bp segment of the *Salmonella invA* gene, which has been shown to be both sensitive and specific for this bacterial genus (Chiu and Ou 1996). As an internal control for DNA extraction and amplification, we used another published PCR primer pair of 16S-F (5´- AGACTGCTACGGGAG GCAGCAGT) and 16S-R (5´- GTTGCGCTCGTTGC GGGACTTAA) to amplify a 755 bp fragment of the 16S ribosomal RNA subunit gene, which is present in all bacteria (Villalobo and Torres 1998). PCR was conducted in a 25 µl volume using the FailSafe™ PCR System (Epicentre Biotechnologies, Madison, Wisconsin, USA) containing 12.5 µl FailSafe™ PCR PreMix Buffer B, 0.6 µM each of primer, 0.5 µl FailSafe™ Enzyme Mix, and 1 µl Template DNA. Reactions were cycled in an i-Cycler thermocycler (BioRad Inc, Hercules, California, USA) at 94°C for 2 min, then for 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, followed by a final 72°C 4 min extension step and an indefinite 4°C soak. We used a sample of wild-type *Salmonella enterica* serotype Typhimurium DNA from the University of Illinois Veterinary Diagnostic Laboratory as a positive control, and we used sterile distilled water as a negative control. Amplicons were visualized by electrophoresis in 1.5% agarose /Tris-acetate-EDTA (TAE) buffer followed by ethidium bromide staining and digital imaging under ultraviolet light. We considered samples positive only if PCR produced both the 243 bp *invA* band and the 755 bp internal control 16S rRNA band.

To determine the sensitivity of our PCR, we used a NanoDrop™ spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) to identify the concentration (ng/µl) of DNA in our positive control. The number of genome equivalents per sample was then determined from the concentration of DNA in this sample and the known size of the *Salmonella* genome. We then subjected serial dilutions from 10^5 - 10^0 genome equivalents per reaction to our PCR protocol, and we determined the sensitivity of the PCR as the lowest concentration at which both the 243 bp *invA* band and the 755 bp internal control 16S rRNA band could be detected.

We fitted a generalized linear model to a binomial distribution to examine the effect of host size and body condition on prevalence of *Salmonella* in turtles. To determine the differences in *Salmonella* prevalence among ponds, we used a Fisher's Exact Test. Confidence intervals (95%) for the prevalence of *Salmonella* spp. in turtles were calculated using the modified Wald method (Agresti and Coull 1998). We used $\alpha = 0.05$ to test for significance in all cases. Statistics were performed using the computer program SAS (SAS Institute, Cary, North Carolina, USA).

RESULTS

We captured 73 free-ranging male Red-eared Sliders that were between 123–210 mm PL with body

conditions between 0.00–0.20 g fat/mm PL from 2-25 August 2006 (Table 1). We dissected all turtles and then collected 146 mucosal scrapes from the 73 turtles. However, the small or large intestine sample was < 50 µl for five turtles. For those turtles, we pooled the small and large intestine mucosal scrapes to obtain a sufficient quantity of mucus for molecular analyses. Therefore, we analyzed 141 mucosal samples and eight individuals tested positive for *Salmonella* (two individuals tested positive for both mucosal scrapes, six individuals tested positive from the large intestine sample only). The prevalence of *Salmonella* spp. in the male turtles sampled was therefore 11% (8/73) with a 95% confidence limit of 5.4–20.4% for the entire population of 73 turtles (Table 1). Among the nine ponds sampled, the prevalence of *Salmonella* spp. in male turtles ranged between 0% and 28.6% (Table 1). Our PCR protocol resulted in a sensitivity of detection of 10^2 to 10^3 genome equivalents per reaction. Because we did not isolate *Salmonella*, we were unable to determine bacterial serotypes. The prevalence of *Salmonella* spp. in turtles did not vary significantly with turtle size ($\chi^2 = 2.13$, df = 1, $P = 0.145$), turtle body condition ($\chi^2 = 0.81$, df = 1, $P = 0.368$), or pond ($P = 0.699$, Fisher's Exact Test).

DISCUSSION

The prevalence of *Salmonella* in the male turtles in our study was 11% overall (CI: 5.4–20.4%) and ranged from 0% to 28.6% among the nine ponds sampled. To our knowledge, our study is the first to examine gastrointestinal mucosal samples from free-ranging turtles to determine *Salmonella* carrying rates. One other study examined 16 gastrointestinal mucosal samples from free-ranging turtles submitted to a clinic, but did not find *Salmonella* (Saelinger et al. 2006). Information on *Salmonella* carriage rates is important because *Salmonella* shedding in reptiles may be intermittent and stress-induced (DuPonte et al. 1978; Burnham et al. 1998), such that studies of *Salmonella* shedding could underestimate the true prevalence of infection. The results of our study indirectly support this possibility because the prevalence of *Salmonella* in male Red-eared Sliders was higher than that reported in a similar study of Red-eared Slider shedding rates (Readel et al. 2008). This study was conducted in the same region and during the same year as our study, which did not detect *Salmonella* in any of the 100 *T. scripta* sampled using cloacal swabs, which were analyzed using a combination of culture and molecular techniques (95% confidence interval of *Salmonella* prevalence was 0–4.4%, Readel et al. 2008). The fact that different methods were used between the studies makes comparisons of prevalence difficult, however, because detection methods can vary in their sensitivity (Mitchell et al. 2000). If the methods used to identify *Salmonella* from cloacal swabs were less sensitive than those used on gastrointestinal mucosal samples, they may have failed to detect low-level infections and intermittent shedding, and may have accounted for the differences in prevalence between the two studies.

Variation in the sensitivity of different detection methods may also partially explain the wide range of *Salmonella* prevalence reported in other studies (Gaertner et al. 2008a; see below). Although our results were consistent with many other studies that have reported a moderate-low prevalence of *Salmonella* in free-ranging chelonians (8%, Jackson et al. 1969; 5%, Harwood et al. 1999; 0%, Richards et al. 2004; 0%, Saelinger et al. 2006; 5%, Lockhart et al. 2008; 0%, Readel et al. 2008; 10%, Charles-Smith et al. 2009), they were considerably lower than those of many other studies (32%, Briones et al. 2004; 100%, Chambers and Hulse 2006; 50%, Hahn et al. 2007; 51%, Gaertner et al. 2008a; 46%, Gaertner et al. 2008b), including other data on Red-eared Sliders (48%, Gaertner et al. 2008b). The relatively low prevalence of *Salmonella* in the male turtles in this study could be due to the sensitivity of our methods. Because the density of *Salmonella* organisms may be low in free-ranging turtles, highly sensitive procedures may be required to identify infections (Gaertner et al. 2008a). In other studies, a pre-enrichment and/or enrichment step were used to detect *Salmonella* from cloacal, carapace, and environmental samples (Hahn et al. 2007; Gaertner et al. 2008a; Charles-Smith et al. 2009), and may have been useful for identifying turtles with low levels of infection in this study. Nevertheless, we directly sampled portions of the gastrointestinal tract where *Salmonella* is known to reside (Pasmans et al. 2002b), we performed duplicate reactions that would have increased our probability of detection, we optimized our PCR for sensitivity, and we were able to isolate *Salmonella*.

Environmental characteristics may also increase *Salmonella* exposure or shedding rates in free-ranging turtles. *Salmonella* survives for long periods of time in the aquatic environment (Morse and Duncan 1974; Chiodini and Sundberg 1981) and ingestion of fecal material or contaminated water is a likely mode of infection (Chiodini and Sundberg 1981; Polo et al. 1999; Baudart et al. 2000). Therefore, a high number of turtles in closed systems, and consequent reinoculation of *Salmonella* through feces, is believed to be partially responsible for high levels of *Salmonella* in captive turtles (Kaufman et al. 1972; Chiodini and Sundberg 1981). The lower prevalence of *Salmonella* in the male turtles in our study suggests that environmental contamination of our ponds with *Salmonella* may be low, and possibly that turtle abundance in our ponds might not have represented overcrowding.

Additionally, we found no differences in *Salmonella* infection prevalence among ponds, despite a range of

pond characteristics that could have enhanced *Salmonella* infection or transmission. For instance, intensive row crop agriculture adjacent to ponds might have been expected to result in higher nutrient content in the pond water, promoting the growth of coliform bacteria, or forests adjacent to ponds might have had higher densities of animal reservoirs of *Salmonella*. The lack of an association between *Salmonella* prevalence and environmental characteristics is, however, consistent with the results of other studies. In those studies, the prevalence of *Salmonella* in free-ranging turtles was similar across seven sites along one river (range: 40–75%, Gaertner et al. 2008a), and between spring (53%, Hahn et al. 2007) and slough (50%, Gaertner et al. 2008b) headwater sections of a river. These results suggest that *Salmonella* infection rates in free-ranging aquatic turtles are stable across microhabitats within ecosystems.

Although our study did not find associations among host and environmental characteristics and *Salmonella* infection levels, our study was cross-sectional and did not examine temporal variability in infection rates. Furthermore, we only sampled male turtles; however, this is unlikely to have had a large impact on the *Salmonella* prevalence reported in this study because other studies have not found *Salmonella* prevalence to differ between male and female turtles (Gaertner et al. 2008a; Lockhart et al. 2008). Turtles were also only sampled from southeastern Illinois, so the generality of our findings to wild turtle populations across the state and elsewhere may be limited. Additional longitudinal sampling, however, might shed light on the underlying factors that explain *Salmonella* infection rates in free-ranging turtles. Finally, our results emphasize that people working with or interacting with wild turtles need to be aware of the possibility of *Salmonella* infection and should use precautions when handling turtles.

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