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SURVEILLANCE FOR UPPER RESPIRATORY TRACT DISEASE AND *MYCOPLASMA* IN FREE-RANGING GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*) IN GEORGIA, USA

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ABSTRACT: Upper respiratory tract disease (URTD) in the gopher tortoise (*Gopherus polyphemus*) is highly contagious and has been implicated in the reduction of populations throughout the range. With the exception of a few limited studies, the prevalence of URTD in Georgia, USA tortoise populations is poorly known. We found that exposure to *Mycoplasma agassizii* and *Mycoplasma testudineum*, associated with URTD, varied geographically among 11 Georgia tortoise populations. The prevalence of antibodies to *M. agassizii* in individual populations was either very low (0–3%, $n=7$ populations) or very high (96–100%, $n=4$ populations), whereas there was variation in the prevalence of antibodies to *M. testudineum* among populations (20–61%, $n=10$) with only one site being negative. Five sites had tortoises with antibodies to both pathogens, and these were the only sites where we observed tortoises with clinical signs consistent with URTD. We did not find tortoises with clinical signs of URTD at sites with tortoises with antibodies only to *M. testudineum*, which provides evidence that this organism may be of limited pathogenicity for gopher tortoises. Collectively, these data indicate that both *M. agassizii* and *M. testudineum* are present in Georgia populations of gopher tortoises and that clinical disease is apparent in populations where both pathogens are present. Additional research is needed to better understand the role of these two pathogens, and other potential pathogens, in the overall health of tortoise populations, especially if future conservation efforts involve translocation of tortoises.

Key words: Georgia, gopher tortoise, *Mycoplasma agassizii*, *Mycoplasma testudineum*, upper respiratory tract disease.

INTRODUCTION

Gopher tortoise (*Gopherus polyphemus*) populations have declined throughout much of their historic range in the southeastern US due to habitat loss and fragmentation, human activity, and disease (Auffenberg and Franz 1982; Diemer-Berish et al. 2010). In 1987, the gopher tortoise was federally listed as threatened in the western portion of its range (west of the Mobile and Tombigbee Rivers in Alabama, Louisiana, and Mississippi; USFWS 1987). The tortoise is state-listed in Georgia and is currently a candidate for federal listing in the eastern portion of its range (USFWS 2011).

Upper respiratory tract disease (URTD) in gopher tortoises is caused by several contagious pathogens including *Mycoplasma agassizii* and *Mycoplasma testudineum* (Jacobson et al. 2014), herpesvirus (Jacobson et al. 2012), and ranavirus (Westhouse et al. 1996; Johnson et al. 2010), with the former two being the most-commonly associated with URTD clinical signs. Upper respiratory tract disease has been implicated in the reduction of some tortoise populations (e.g., McLaughlin 1997; Gates et al. 2002; Diemer-Berish

et al. 2010). However, there is debate about the long-term impacts of URTD in free-ranging tortoise populations (e.g., Seigel et al. 2003; McCoy et al. 2007; Sandmeier 2009). Upper respiratory tract disease is characterized by mild to severe nasal and ocular discharge, conjunctivitis, and swelling of the eyes and nares. The factors that predispose a tortoise to develop clinical disease are unknown. Some gopher tortoise populations in Florida have a high prevalence of clinical URTD, while other populations with a high prevalence of *Mycoplasma* antibodies have low prevalence of clinical URTD (Diemer-Berish et al. 2010). Because *Mycoplasma* can cause persistent infections, it is possible that stressors such as habitat disturbance, translocation, or coinfections with other pathogens could cause infected but clinically normal tortoises to develop clinical signs. Also, because multiple species of *Mycoplasma* infect gopher tortoises, these species, or strains of a species, may vary in pathogenicity (Wendland 2007).

Few investigators have studied the prevalence of *Mycoplasma* in gopher tortoise populations outside of Florida, USA. In Georgia, USA, studies have been restricted to three isolated populations (Kahn 2006; Tuberville et al. 2008; Hernandez et al. 2010). In a study conducted in 1994, 93 tortoises from Bullock County, Georgia were translocated to St. Catherines Island in Liberty County, Georgia (Tuberville et al. 2008). At the time of translocation, 80% of the relocated tortoises had detectable antibodies to *M. agassizii* (Tuberville et al. 2008). Currently, over 100 adult tortoises in this population have been tested and are antibody positive, yet clinical disease is rare (T. Norton unpubl. data). Screening of tortoises at Fort Benning, in Muscogee County, Georgia, for *Mycoplasma* antibodies revealed seasonal variation in exposure rates with only 27% of tortoises ($n=50$) tested in the summer being positive and 44% of tortoises tested in the spring being positive (Kahn 2006). Finally, in a 2001 translocation of tortoises from McIntosh County, Georgia to the Savannah River Site in Aiken County, South Carolina, USA, one of 14 tortoises tested was positive for antibodies to *M. agassizii* and an additional four were suspect (Hernandez et al. 2010). Importantly, none of these studies evaluated the prevalence of antibodies to *M. testudineum*. Molecular or culture evidence of *Mycoplasma* infections in Georgia is very limited, with *M. agassizii* being identified from 10 of 21 tortoises from St. Catherines Island (none of which had clinical signs; Tuberville et al. 2008) and *M. agassizii* being cultured from a single, moribund tortoise from the Joseph W. Jones Ecological Research Center (JERC) in Baker County (Wendland 2007).

Our purpose was to acquire knowledge of the distribution of *Mycoplasma* and URTD in gopher tortoise populations. The specific goal was to determine the prevalence of URTD through health assessments and through antibody and PCR testing for *M. agassizii* and *M. testudineum* on tortoises from selected populations in Georgia.

MATERIALS AND METHODS

Study sites

Samples were collected from tortoises from 11 sites in Georgia between 1995 and 2013 (Fig. 1 and Table 1). Sites had varied land uses including public use ($n=2$), private lands ($n=7$), and military installations ($n=2$). All properties were reported by land managers to have >30 tortoises, and tortoise population densities were available for five sites: JERC, Reed Bingham State Park (RBSP), Effingham County (EFCO), Orianne Indigo Snake Preserve (OISP), and Fort Gordon Air Force Base (FGAB). The JERC had approximately 7,600 ha of suitable habitat with a tortoise population density of 0.71 ± 0.08 tortoises/ha (L.L.S. unpubl. data). Orianne Indigo Snake Preserve had 95.4 ha of habitat and an estimated density of 1.23 ± 0.21 tortoises/ha (Ballou 2013). Reed Bingham State Park has a high tortoise density of 3.08 ± 0.67 tortoises/ha in 66 ha of habitat (Ballou 2013). Fort Gordon Airforce Base is at the northern extent of the range of the gopher tortoise and has approximately

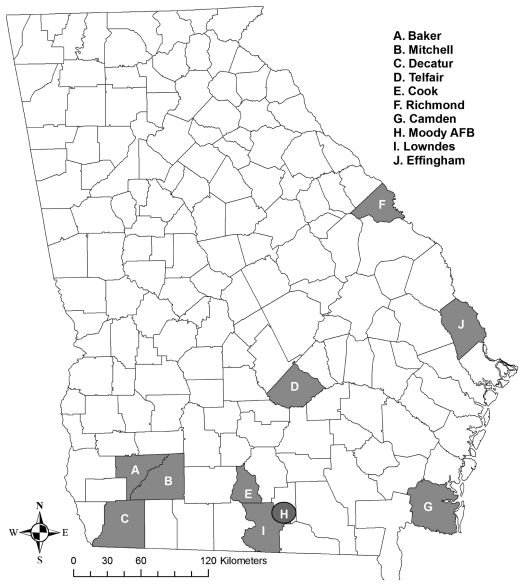

FIGURE 1. Counties and one air force base (AFB) in Georgia, USA where gopher tortoises (*Gopherus polyphemus*) were sampled for upper respiratory tract disease. Table 1 provides details about each site.

13,000 ha of suitable habitat but a low tortoise density (0.02±0.004 tortoises/ha; J. Stober unpubl. data). Specific locality data is withheld due to the status of the species and requests of private landowners.

Tortoise capture and sampling

Tortoises were collected using a combination of methods that included opportunistic hand-capture, trapping, and excavation of burrows in an attempt to capture at least 30 adults (see Gonynor 2013) for details about trapping). Juvenile tortoises were trapped at one site (JERC; Ballou 2013). Upon capture, animals were transported to a shaded area or to a laboratory for sample collection. Each tortoise was physically examined and measured (straight-line carapace length, plastron length, gular length, anal notch, and anal fork) and weighed (McRae et al. 1981). At sites with long-term monitoring objectives, tortoises were permanently marked by notching the marginal scutes (Cagle 1939) with a rotary tool (Dremel™, Racine, California, USA). Tortoises were examined for clinical signs suggestive of URTD (e.g., nasal exudates, conjunctivitis, swollen eyes, lethargy, labored/wheezy breathing) and lesions suggestive of chronic URTD (e.g., nasal scarring and asymmetric nares); we recorded whether each of these clinical signs were either present or absent. Gender was determined based on external morphology such as gular length, plastral concavity, and the ratio of anal notch to anal fork width (McRae et al. 1981; Eubanks et al. 2003). Tortoises whose gender could not be confirmed because of ambiguous characters were grouped as "unknown." Due to difficulty in differentiating between males and females based on shell morphology alone, tortoises less than 230 mm CL were categorized collectively as "juveniles" (McRae et al. 1981).

Between 0.5–4.0 mL of blood (<1% of body weight) were collected from either the caudal vein, brachial vein, or the subcarapacial venous sinus (Wendland 2007). Blood was added to heparinized tubes and centrifuged. Plasma was removed and immediately frozen at −80 C.

In 2010 and 2011, nasal exudates were collected from tortoises exhibiting clinical signs of URTD (i.e., nasal discharge) using sterile rayon swabs (Puritan Medical Products Company LLC, Guilford, Maine, USA; Microbrush International, Grafton, Wisconsin, USA). After collection, swabs were placed in tubes and frozen at −80 C until testing. Tortoises with eroded nares were considered to have chronic URTD with recurring clinical signs.

Tortoises were hydrated in warm water for 15–20 min (Wendland et al. 2009) prior to release at the site of capture. All tortoises were sampled and released within 48 hr of capture. Equipment was disinfected with a 10% bleach solution between tortoises. All methods were reviewed and approved by the University of Georgia's Animal Care and Use Committee (A2010 11-563).

Pathogen testing

Plasma samples were submitted to the *Mycoplasma* research laboratory at the University of Florida College of Veterinary Medicine (Gainesville, Florida, USA) for enzyme-linked immunosorbent assay (ELISA) testing. All samples were tested for antibodies to *M. agassizii* and a subset was tested for antibodies to *M. testudineum*. Results for both *M. agasizzi* and *M. testudineum* were grouped into one of three classes based on antibody titers; positive (titer≥64), negative (titer<32), and suspect (titer=32–63) (Wendland et al. 2007).

To detect shedding of *Mycoplasma* spp. in nasal exudates, PCR testing for the 16S rRNA gene of *Mycoplasma* spp. was conducted. Genomic DNA was extracted from the swabs according to the manufacturer's protocols (Qiagen DNA purification Kit, Germantown,

TABLE 1. Characteristics of sites throughout Georgia, USA in which gopher tortoises (*Gopherus polyphemus*) were sampled to measure prevalence of infection with *Mycoplasma* spp.

Site	Abbreviation	County[a]	Ownership and land use	Approximate size and site characteristics
Jones Ecological Research Center, Baker County	JERC	A	Private, mixed research/hunting	11,700-ha reserve, of which ~6,880 ha is second-growth longleaf pine/wiregrass forest and 2,020 ha of wildlife food plots. Surrounded by large-scale agriculture.
Forest Lodge Farms, Mitchell County	FLF	B	Private, silviculture	607-ha pine plantation interspersed with hardwood stands and wildlife food plots.
Cedars Farm Plantation, Decatur County	CFP	C	Private, hunting	400-ha primarily longleaf pine/wiregrass forest. Surrounded by large-scale agriculture.
Orianne Indigo Snake Preserve, Telfair County	OISP	D	Private, mixed research/hunting	400-ha indigo snake preserve. Sandhills with mixed loblolly pine/hardwood. Longleaf pine restoration efforts underway. Surrounded by state-owned wildlife management areas.
Telfair County site B	TFB	D	Private, agriculture	200-ha mixed purpose agricultural land surrounded pine plantation and hardwoods. Tortoises were later relocated from this site.
Reed Bingham State Park, Cook County	RBSP	E	Public, state park	650-ha recreational area with a campground, 152-ha lake, and trails throughout primary tortoise habitat.
Ft. Gordon, Richmond County	FGAB	F	Public, Department of Defense, military training	13,118-ha open canopy pine forest with active artillery ranges/artillery impact areas.
St. Mary's Airport, Camden County	SMAP	G	Public, airport	40-ha open grass habitat surrounded by dense pine/mixed hardwoods, wetlands, and industrial development.
Moody Air Force Base, Lowndes and Lanier counties	MAFB	H	Public, Department of Defense, military training	4,420 ha of which ~1,050 ha are mixed upland pine/hardwood forest and ~2,225 ha are bottomland forest.
Lowndes County	LCO	I	Private, agricultural	200-ha open field, mixed pine, and mixed hardwoods. Previously a waif site for rescued tortoises. Tortoises were later relocated from this site.
Effingham County	EFCO	J	Private, timber	590-ha pine plantation with mixed hardwoods.

[a] Letters correspond to the county in which the sites are located on Figure 1.

Maryland, USA) and each sample was tested in duplicate using primers GMF-1 (5′-ACACCATGGGAGCTGGTAAT-3′) and GMR-1 (5′-CCTCATCGACTTTCAGACCCAAGGCAT-3′) as described in Lauerman (1998) and Diemer-Berish et al. (2010).

Fisher's exact test was used to analyze differences in antibody prevalence and PCR results among sites and among age classes and between sexes. Linear regression was used to assess the relationship between carapace length and pathogen prevalence.

RESULTS

Samples from at least 30 tortoises were obtained at six sites: JERC, OISP, Cedars

TABLE 2. Demographic data for gopher tortoises (*Gopherus polyphemus*) included in *Mycoplasma* surveillance in Georgia, USA.

Site[a]	Total	Males	Females	Unknown adults	Juvenile <230 mm
SMAP	7	4	2	0	1
CFP	30	16	12	0	2
EFCO	31	7	15	0	9
FGAB	9	5	4	0	0
JERC 2009–12[b]	191	94	73	13	11
LCO	11	5	6	0	0
FLP	7	2	5	0	0
MAFB	157	86	71	0	0
OISP	35	10	21	3	1
RBSP	35	13	15	6	1
TFB	27	15	11	1	0
Total	540	257	235	23	25

[a] See Table 1 for site descriptions.

[b] Data from JERC in 1997 are not included.

Farm Plantation, Decatur County (CFP), EFCO, Telfair County site B (TFB), and Moody Air Force Base, Lowndes and Lanier counties (MAFB) (see Table 1). We captured <30 tortoises at each of the other five sites (already described) because population densities were low. Serum samples were collected from 540 tortoises at the 11 sites (Table 2). Additional tortoises were captured and examined for signs of URTD at JERC; however, serum samples were not collected from these individuals.

Prevalence of antibodies to *M. agassizii* and *M. testudineum* varied among sites (Table 3). The prevalence of antibodies to *M. agassizii* was either very low (≤3%) or very high (92–100%), whereas prevalence of *M. testudineum* varied from 20–61%. A low prevalence of antibodies (≤3%) to *M. agassizii* was detected at seven sites (MAFB, OISP, EFCO, TFB, St. Mary's Airport, Camden County [SMAP], Lowndes County [LCO], and Ft. Gordon, Richmond County [FGAB]) and a high prevalence (92–100%) was detected at the remaining four sites (JERC, Forest Lodge Farms, Mitchell County [FLF], Cedars Farm Plantation, Decatur County [CFP], and RBSP). Tortoises (n=239) at 10 sites were tested for both *M. agassizii* and *M. testudineum*. Tortoises with antibodies to both *M. testudineum* and *M. agassizii* were detected at five sites: JERC (n=25/70), FLF (n=2/5), CFP (n=5/25), OSIP (n=1/30), and RBSP (n=19/33). Tortoises with acute or chronic clinical signs of URTD were only documented at sites with both *M. agassizii* and *M. testudineum* in the population (Table 3). We found no relationship between detection of *M. testudineum* and clinical signs (χ^2=3.190; P=0.203). Only two sites where *M. agassizii* antibodies were not detected had evidence of chronic URTD (tortoises with asymmetric nares).

Nasal swabs were collected from 136 tortoises at three sites (JERC, n=129; FLF, n=1; and CFP, n=6) and of these, 50 (37%) swabs were PCR positive for *Mycoplasma* spp. (Table 3). Based on ELISA results, all three sites also had a high prevalence of *M. agassizii* antibodies (92–100%). Among the PCR-positive tortoises (n=50), 39 (29%) had nasal discharge and 38 (28%) had chronic lesions on the nares suggestive of URTD. Of the six CFP swabs tested, two (33%) were PCR positive, both of which were antibody positive to both *M. a*gassizii and *M. testudineum*. Both PCR-positive tortoises from CFP were antibody-positive to *M. agassizii* and *M. testudineum*. The FLF swab was PCR negative, but the tortoise was antibody positive for *M. agassizii*.

TABLE 3. Serology and PCR (nasal exudates) results for *Mycoplasma* and prevalence of upper respiratory tract disease (URTD) in 11 gopher tortoise (*Gopherus polyphemus)* populations in Georgia, USA.

Site[a]	Map[b]	Year	No. antibody positive/no. tested (%)		*Mycoplasma* no. PCR positive/no. tested (%)[c]	No. with clinical signs of URTD/no. examined (%)[c,d]	No. with chronic URTD lesions/ no. examined (%)[c,e]
			M. agassizii	*M. testudineum*[c]			
JERC	A	1997	174/182 (96)	NT	NT	NT	NT
		2009–12	125/136 (92)	28/70 (40)	48/129 (37)	44/191 (23)	101/366 (28)
MAFB	H	2000–04	1/100 (1)	NT	NT	2/100 (2%)	NT
FLF	B	2010	7/7 (100)	2/5 (40)	0/1	1/7 (14)	2/7 (29)
CFP	C	2010	29/30 (97)	5/25 (20)	2/6 (33)	6/30 (20)	4/30 (13)
OISP	Da	2010	1/35 (3)	14/30 (47)	NT	0	3/36 (8)
TFB	Db	2011	0/27	0/20	NT	0	0
RBSP	E	2010	34/35 (97)	20/33 (61)	NT	3/35 (8)	8/35 (23)
FGAB	F	2010	0/9	3/8 (38)	NT	0	2/9 (22)
SMAP	G	2010	0/7	4/7 (57)	NT	0	0
LCO	I	2012	0/11	5/11 (45)	NT	0	2/11 (18)
EFCO	J	2013	0/30	0/30	NT	0	0
TOTAL			371/609 (61)	81/239 (34)	50/173 (29)	56/192 (30)	122/524 (23)

[a] See Table 1 for site descriptions.

[b] Letters correspond to the county in which the sites are located on Figure 1.

[c] NT = not tested.

[d] Clinical signs suggestive of URTD; however, no etiologic agent was determined as a cause for the clinical signs. Only JERC animals with at least one test, enzyme-linked immunosorbent assay and/or PCR, were included.

[e] Chronic URTD lesions were either nasal scarring or nares abnormalities; again, no etiologic agent was identified for abnormalities. All JERC captures are included but may not have been tested for *Mycoplasma*.

TABLE 4. Serologic (enzyme-linked immunosorbent assay [ELISA]) and molecular testing (PCR) results, number tested/number positive (%), and evidence for upper respiratory tract disease caused by two *Mycoplasma* spp. in juvenile (carapace length<23 cm) gopher tortoises (*Gopherus polyphemus*) at four sites in Georgia, USA.

Site[a]	*M. agassizii* ELISA	*M. testudineum* ELISA[b]	*Mycoplasma* PCR[b]	Clinical signs	Scarring/lesions
JERC	0/10	2/9 (22)	1/2 (67)	1/11	2/11 (18)
SMAP	0/1	0/1	NT	0/1	0/1
RBSP	1/1	NT	NT	0/1	0/1
CFP	1/2	0/2	NT	0/2	1/2 (50)
TOTAL	2/14 (14)	2/12 (17)	2/3 (67)	1/15 (7)	3/15 (20)

[a] See Table 1 for site descriptions.

[b] NT = not tested.

Both PCR and serology data are available for 39 tortoises across the three sites (JERC, CFP, OISP); 23 (59%) tortoises had antibody to both *M. agassizii* and *M. testudineum* and 13 (33%) were PCR positive. Twenty-nine samples were bidirectionally sequenced at the Georgia Genomics Facility (Athens, Georgia, USA). All samples had >97% identity to *M. agassizii* (GenBank AY80802).

Prevalence of antibodies to *M. agassizii* among the 14 juvenile tortoises was significantly lower than among adults (Fisher's exact test, $P<0.001$), but there was no difference in prevalence between adults and juveniles for *M. testudineum* (Table 4). A single juvenile from JERC was PCR positive and sequenced as *M. agassizii*. This tortoise (CL 20.4 cm) was antibody positive to *M. testudineum* but not to *M. agassizii* (Table 4). A PCR-negative juvenile (CL 21.2 cm) was suspect for *M. agassizii* and positive for *M. testudineum*. One tortoise of unknown gender with a CL of 23 cm was antibody positive to *M. agassizii*, suspect for *M. testudineum*, and PCR positive. When data from all sites were combined, there was no difference in prevalence for *M. agassizii* between males and females (Fisher's exact test, $P=0.148$), but the prevalence of *M. testudineum* was significantly higher for females (43/88) compared with males (33/95) (Fisher's exact test, $P=0.006$) (Tables 5 and 6).

Recapture data were available for two sites, MAFB and JERC. Thirty-seven tortoises from MAFB were recaptured and tested between 2000 and 2004, and 21 tortoises at JERC were tested in 1997 (Kahn 2006) and again between 2009 and 2011. At MAFB, serostatus changes were documented for three tortoises. One tortoise was suspect for *M. agassizii* antibodies in August 2002 and August 2003, positive (titer 64) in September 2003, and was suspect again in September 2004. A second tortoise was antibody negative to *M. agassizii* in August 2000, suspect in September 2001, and was negative in July 2002 and June 2003. The third tortoise was negative for antibodies to *M. agassizii* in August 2000, suspect in September 2001, and negative in July 2003. The remaining 34 resampled tortoises at MAFB were negative at each sampling point. At JERC, all 21 resampled tortoises were antibody positive in 1997 and remained positive for *M. agassizii* in 2009–11. No differences were noted in seroprevalence between sampling years at either site.

DISCUSSION

Among the gopher tortoise populations tested in Georgia, the prevalence of *M. agassizii* antibodies was either very high or very low in a population (nearly all or none), with seven of 11 sites (64%) having antibody-positive tortoises. In contrast, antibodies to *M. testudineum* were detected at all but two sites (82%) and prevalence within the populations varied.

TABLE 5. Serologic (enzyme-linked immunosorbent assay [ELISA]) and molecular testing (PCR) results, number positive/number tested (%), for gopher tortoises (*Gopherus polyphemus*) sampled at 10 sites in Georgia, USA.

	M. agassizii ELISA serology		*M. testudineum* ELISA serology		*Mycoplasma* spp. PCR[b]	
Site[a]	Male	Female	Male	Female	Male	Female
JERC	79/80 (98)	59/60 (98)	12/33 (36)	12/25 (48)	22/66 (33)	21/49 (43)
FLF	5/5	2/2	2/3 (67)	0/2	0/1	—
CFP	16/16	12/12	2/14 (14)	3/9 (33)	2/3 (67)	0/3
OISP	0/10	0/21	3/9 (33)	10/17 (59)	—	—
TFB	0/15	0/11	0/10	0/9	—	—
RBSP	13/13	14/15 (93)	8/13 (62)	10/14 (71)	—	—
FGAB	0/4	0/4	1/4	1/4	—	—
SMAP	0/4	0/2	2/4	2/2	—	—
LCO	0/5	0/6	1/5	4/6	—	—
EFCO	0/6	0/15	0/6	0/15	—	—
TOTAL	113/158 (72)	87/148 (59)	31/101 (31)	42/103 (41)	24/70 (35)	21/52 (40)

[a] See Table 1 for site descriptions.

[b] Dashes (—) indicate data not available.

Approximately half of the sites had tortoises with antibodies to *M. agassizii* whereas a higher percentage (73%) of sites had tortoises with antibodies to *M. testudineum*. Five sites (45%) had tortoises with antibodies to both pathogens and four of these sites had tortoises with clinical signs of URTD. *Mycoplasma testudineum* can cause clinical disease in the absence of *M. agassizii*; however, there is some evidence that *M. testudineum* might be less pathogenic in desert tortoises (Jacobson and Berry 2012). In a Florida study, *M. testudineum* was documented at three of 11 field sites (27%), all located in the northeastern part of the state (Wendland 2007). To our knowledge, ours is the first report of *M. testudineum* in Georgia. In contrast to the Florida study, we detected *M. testudineum* on a greater proportion of sites than *M. agassizii*. We also observed significantly more female tortoises with antibodies to *M. testudineum*. Additionally, we only observed clinical signs of URTD in tortoises with antibodies to *M. agassizii* alone or at sites with tortoises with antibodies to both *M. testudineum* and *M. agassizii*. The same

TABLE 6. Presence of clinical signs or chronic lesions of upper respiratory tract disease for gopher tortoises (*Gopherus polyphemus*) sampled at nine sites in Georgia; number with condition/number observed (%).

	Clinical signs of acute disease		Scars/lesions suggestive of chronic disease	
Site[a]	Male	Female	Male	Female
JERC	22/93 (24)	19/72 (26)	27/93 (29)	23/72 (32)
FLF	0/5	0/0	0/5	0/0
CFP	0/16	3/12 (25)	3/16 (19)	0/12
OISP	0/10	0/21	0/10	1/21 (5)
TFB	0/15	0/11	1/15 (7)	1/11 (9)
RBSP	0/13	3/15 (20)	3/13 (23)	2/15 (13)
FGAB	0/5	0/4	1/5 (20)	1/4 (25)
SMAP	0/4	0/2	0/4	0/2
LCO	0/5	0/6	0/5	0/6
TOTAL	22/166 (5)	25/143 (17)	35/166 (21)	28/143 (20)

[a] See Table 1 for site descriptions.

study also observed clinical signs (nasal discharge) in tortoises infected with *M. testudineum* (PCR-positive nasal discharge), but it is unclear how many of those tortoises were coinfected with *M. agassizii*. Other studies also documented clinical signs at sites with a high prevalence of antibodies to *M. agassizii* (Wendland 2007).

Contrary to other reports of fluctuating antibody prevalence within tortoise populations over time (Kahn 2006; Diemer-Berish et al. 2010), prevalence at two of our sites, MAFB and JERC, remained stable over the 4-yr and between the 15-yr periods, respectively. *Mycoplasma testudineum* testing has not been done at MAFB; we do not know if the pathogen is present. Wendland et al. (2007) warned that one positive tortoise detected in a population with low antibody prevalence should be interpreted with caution, as there are occasional false-positive ELISA results. We recommend that land managers perform repeated health assessments to look for clinical signs as well as pathogen surveillance in concert with population monitoring. Additionally, PCR should be utilized when possible to determine current infection because ELISA results only indicate exposure. Interestingly, the positive tortoise from MAFB only developed a low titer and was "suspect" at two other testing times; therefore, the true infection status of this tortoise is unknown. Antibody prevalences to *M. agassizii* at JERC were similar between two sampling periods (92% in 2009–12 vs. 96% in 1997) even though ELISA sensitivity and specificity had been improved between the sampling periods (Wendland et al. 2010a).

Despite the high prevalence of antibodies to *M. agassizii* at JERC, the prevalence of current clinical signs and chronic URTD lesions was <30%, suggesting that not all tortoises shed the bacteria and develop clinical signs or that past infections were mild and did not result in visible chronic lesions. Future studies should be conducted to determine if antibody-positive tortoises develop recrudescence of clinical signs.

Several juvenile tortoises from different sites had antibodies or were PCR positive for *Mycoplasma* spp. Previous investigators have shown a correlation between body size (CL) and antibody prevalence, with tortoises <23 cm CL being antibody-negative (Beyer 1993; Wendland 2007). In our study, the smallest tortoise with detectable antibodies to *M. agassizii* was from RBSP (CL 13.1 cm), and the smallest tortoise with antibodies to *M. testudineum* was from JERC (CL 20.4 cm). In previous studies, juvenile tortoises antibody positive to *Mycoplasma* were from populations undergoing epizootic events (Wendland 2010b); otherwise, most juveniles were negative for both *M. agassizii* and *M. testudineum* antibodies (Wendland 2007). The PCR-positive (sequenced as *M. agassizii*) juvenile tortoise from JERC was interesting in that it was antibody negative for *M. agassizii* and positive for *M. testudineum*. It is possible that this tortoise was recently exposed to *M. agassizii* and had not mounted an immune response. At CFP the only juvenile (CL 20.0 cm) with nares abnormalities was antibody positive for *M. agassizii* but negative for *M. testudineum*. The PCR was utilized on samples collected from tortoises with clinical signs. However, nasal flushes (McGuire et al. 2014) would have been helpful on sites where there were no tortoises with clinical signs. Sequencing in concert with serology would be optimal but was not practical on most sites.

We observed lower than expected numbers of tortoises at several sites and, as a result, were unable to sample our target of 30 individuals per population. Despite low sample sizes we detected evidence of both *Mycoplasma* and chronic URTD at FLF. Similarly at SMAP and FGAB, we detected tortoises with antibodies to *M. testudineum*. Two tortoises at FGAB had lesions consistent with previous URTD, yet no antibodies to *M. agassizzi* were detected in the population. These nasal lesions could have been due

to *M. testudineum* or another pathogen. Data from this site highlight the fact that the presence of URTD signs does not indicate that the tortoise or population is infected with *M. agassizii*. Three of our study populations were subsequently translocated following testing (EFCO, LCO, and TFB). Tortoises at two of these sites were negative for antibodies to *M. agassizii* but positive for *M. testudineum* antibodies. Ideally, no *Mycoplasma*-positive tortoises should be relocated; however, at one site a few native or waif tortoises (tortoises of unknown origin turned in to authorities such as state agencies) of unknown *Mycoplasma* status were already present. This is concerning because waif tortoises could be at increased risk of infection or be a source of infection to naïve tortoises (McLaughlin 2000).

The gopher tortoise is one of the most commonly translocated animals in North America (Tuberville et al. 2011); therefore, it is important to understand the distribution of pathogens in populations. Multiple strains of *M. agassizii* exist (Wendland et al. 2010a) and geographic variation in virulence may occur among strains (Seigel et al. 2003). Our study highlights the importance of continued monitoring of tortoise health because our results differed from previous studies in several important ways. In Florida, antibodies to *M. agassizii* were widespread and prevalences varied considerably (Diemer-Berish et al. 2000; Wendland 2007), whereas in Georgia prevalences of antibodies to *M. agassizii* were either very high or very low/absent. *Mycoplasma testudineum* is also a confirmed etiologic agent for URTD (Brown et al. 2004). However, it has been hypothesized that *M. testudineum* could be less pathogenic than *M. agassizii* in desert tortoises because tortoises infected with *M. testudineum* had less-severe lesions than those infected with *M. agassizii* (Jacobson and Berry 2012). We did not observe clinical signs at sites with tortoises that were antibody positive only to *M. testudineum.* The relationship between the two pathogens is unclear. However, we observed that sites with both pathogens had tortoises that exhibited signs of clinical disease. Further surveillance for *Mycoplasma* spp., in addition to other pathogens, in more tortoise populations throughout the range is warranted. Long-term studies need to be initiated, particularly in populations of management concern, to understand the consequences of disease and to fill in knowledge gaps concerning behavior and reproduction.

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