

Clinical *Mycoplasma* sp. Infections in Free-living Three-toed Box Turtles (*Terrapene carolina triunguis*) in Missouri, USA

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ABSTRACT: *Mycoplasma* species, which can cause upper respiratory tract disease (URTD), are significant pathogens of birds, mammals, fish, and reptiles. Mycoplasmosis is of high concern for chelonian conservation, with the most well-documented cases in gopher and desert tortoises. *Mycoplasma* sp. infections have been reported in captive and free-living box turtles (*Terrapene* spp.). We documented URTD associated with *Mycoplasma* sp. in two free-living, three-toed box turtles (*Terrapene carolina triunguis*) in Missouri, US. Both turtles were *Mycoplasma* sp. positive by PCR and had URTD-like clinical signs, including nasal and ocular discharge, palpebral edema, lethargy, and weight loss, during a 6–8-wk period between June and September 2014.

Key words: Box turtle, chelonian conservation, *Mycoplasma* sp., *Terrapene carolina triunguis*.

Mycoplasma spp. infections have raised conservation concern in captive and free-living chelonians, with population declines associated with *Mycoplasma agassizii* documented in *Gopherus* species (Berry 1997; McLaughlin 1997). Both *M. agassizii* and *Mycoplasma testudineum* infect gopher and desert tortoises in the US (Jacobson et al. 2014). An unnamed species of *Mycoplasma* has also been documented in two species of box turtles, eastern (*Terrapene carolina carolina*) and ornate (*Terrapene ornata ornata*; Feldman et al. 2006; Farkas and Gal 2009). Coinfections with other agents, such as ranaviruses, herpesviruses, and adenoviruses, may exacerbate *Mycoplasma* spp. morbidity and mortality in reptiles, as demonstrated in mammal models (Prysljak et al. 2011). Additionally, environmental and anthropogenic

disturbances, including extreme weather and turtle relocations, may play a role in *Mycoplasma* disease (Jacobson et al. 1991; Sandmeier et al. 2009).

As part of a larger ecologic and health study of free-living box turtles in Missouri, US, we encountered two clinical cases of *Mycoplasma* sp.-associated disease. During June–September 2014, two free-living, three-toed box turtles developed upper respiratory tract disease (URTD) clinical signs, including nasal and ocular discharge, palpebral edema, lethargy, and progressive weight loss.

Our study site was Forest Park (38°38'N, 90°16'W), a 556-ha urban park in St. Louis, Missouri. We collected location information using very high-frequency (VHF) telemetry tags (Holohil Systems, Ltd., Carp, Ontario, Canada) and handheld GPS units (Garmin International, Inc., Olathe, Kansas, USA). Body weights were collected and visual health exams were performed weekly from March to November in 2013 and 2014. Additionally, in May–June 2013 and 2014, we collected blood from the subcarapacial sinus for serologic testing for *M. agassizii* antibodies (Hernandez-Divers and Cooper 2006; Wendland et al. 2007), and separate choanal and cloacal swabs (Puritan Sterile Cotton-tipped Applicators, Puritan Medical Products, Guilford, Maine, USA) were obtained from all VHF-tagged turtles. Samples were stored in cryotubes (Nunc™ Thermo Scientific™, Waltham, Massachusetts, USA) in a cooler on ice for up to 4 h in the field and then at –80 C. DNA was extracted from the swabs using a Maxwell 16

TABLE 1. *Mycoplasma* sp. identification by pan-*Mycoplasma* consensus PCR with sequencing and serology and clinical signs during the period of infection for two three-toed box turtle (*Terrapene carolina triunguis*) cases in Missouri, USA.

Case and date	Swab type	<i>Mycoplasma agassizii</i> / <i>Mycoplasma testudineum</i> antigen serology ^a	<i>Mycoplasma</i> PCR results ^{a,b}	Clinical signs
Case 1				
20 May 2013	Choanal	<32/<32	Positive	None present
14 June 2013	Cloacal	n/a	Positive	None present
2 June 2014	Choanal	<32/<32	Positive	None present
21 June 2014	Cloacal	n/a	Positive	Mild ocular and nasal discharge, palpebral edema, lethargy
23 June 2014	Choanal and nasal	n/a	Positive	Ocular and nasal discharge, palpebral edema, lethargy
25 June 2014	Choanal	<32/<32	Negative	Ocular and nasal discharge, palpebral edema, lethargy
9 July 2014	Cloacal and nasal	n/a	Positive	Ocular and nasal discharge, palpebral edema, lethargy
21 July 2014	Choanal	n/a	Positive	Ocular and nasal discharge, palpebral edema, lethargy
5 June 2015	Choanal	n/a	Positive	None present
Case 2				
24 May 2013	Choanal	n/a	Negative	None present
3 June 2014	n/a	<32/32	n/a	None present
10 June 2014	Choanal	n/a	Negative	None present
18 September 2014	Nasal	n/a	Positive	Ocular and nasal discharge, palpebral edema, lethargy
23 September 2014	Choanal	n/a	Positive	Mild nasal discharge and palpebral edema
1 May 2015	Nasal	n/a	Positive	None present

^a n/a = no sample collected at this time.

^b Unnamed *Mycoplasma* sp., same as previously identified in box turtles (GenBank accession no. FJ159564). All samples were negative for other viruses of concern (ranaviruses, herpesviruses, adenoviruses) in 2013–14.

automated extractor (Promega, Madison, Wisconsin, USA). We tested cloacal swabs for adenovirus using a pan-adenoviral consensus PCR/sequencing assay (Wellehan et al. 2004). We tested choanal swabs for *Terrapene* herpesvirus 1 using a probe-hybridization quantitative PCR (Hausmann et al. 2015), for *Ranavirus* using a probe-hybridization quantitative PCR (N. Steckler et al. unpubl.), and for *Mycoplasma* using a pan-*Mycoplasma* consensus PCR/sequencing assay (Shahhosseiny et al. 2010). Products were electrophoresed on agarose gels, and bands of the appropriate size were cut and Sanger sequenced. Sequences were submitted to Gen-

Bank under accession numbers KT238890 and KT238891.

Case 1, in June 2014, an adult, female three-toed box turtle had severe UR TD, with nasal and ocular discharge, full body edema, and lethargy. The turtle lost 115 g (714.8 g to 599.8 g; 16% of body weight) over the 6-wk clinical course. Within 2 d of clinical signs, a blood sample and cloacal and choanal swabs were collected, and another six swabs were collected opportunistically over the 6-wk period (Table 1). Swab samples were analyzed by PCR for *Mycoplasma* spp., and plasma samples were submitted for serology analysis for *M. agassizii* (Wendland et al. 2007; Shahhosseiny et al. 2010). The turtle moved <5 m

during this period and was often observed basking in direct sunlight, despite hot ambient temperatures (maximum 38 C). In 2013 and in early June 2014, prior to clinical URTD, choanal and cloacal samples were PCR-positive for a *Mycoplasma* sp. that had been reported in other box turtle species (Feldman et al. 2006; Farkas and Gal 2009; Table 1). In June and July 2014, when URTD signs were present, cloacal, choanal, and nasal discharge swabs were PCR-positive for the same box turtle *Mycoplasma* sp. One choanal swab on 25 June 2014 was PCR-negative for *Mycoplasma*; this was most likely a false negative. The turtle was PCR-negative for ranaviruses, adenoviruses, and herpesviruses. By early August 2014, clinical signs had resolved, weight increased 65 g (664.8 g), and the turtle's movement increased. In October 2014, after clinical signs had cleared, case 1 moved >300 m from its previous home range and across two paved roads into an unconnected forest fragment (Fig. 1). When tested again in June 2015, the turtle was PCR-positive for the same *Mycoplasma* sp. as reported in 2013–14, although no URTD signs were present (Table 1).

Case 2, in August 2014, a second adult, female three-toed box turtle was reported with severe URTD, including muco-purulent nasal and ocular discharge, palpebral edema, and lethargy. In early July 2014, prior to clinical signs, the turtle traveled outside of its documented home range and into an unconnected forest fragment. Then in mid-August, it traveled back into its home range (Fig. 1). On 18 August, URTD clinical signs were documented. The turtle did not move from this location until late September 2014, when the URTD was less severe. On 6 October 2014, no clinical signs were evident. However, 10 d later, the clinical URTD was again noted. Over this 8-wk period, body weight decreased 75 g (675.5 g to 600.5 g; 11% of body weight). Prior to clinical URTD, PCR was negative for all four infectious agents tested (Table 1). In September 2014, choanal and nasal discharge swabs were PCR-positive for the same box turtle *Mycoplasma* sp. as in case 1 and PCR-negative for the other three agents (Table 1).

By mid-November 2014, the turtle had moved 400 m to the hibernaculum site from the previous winter. The URTD signs were absent when the turtle emerged from its hibernaculum in March 2015. However, on 1 May 2015, the turtle was PCR-positive for the same *Mycoplasma* sp. as in 2014 samples (Table 1).

Gopher tortoises with severe mycoplasmal URTD may move further than asymptomatic tortoises or those with mild mycoplasmal URTD signs (McGuire et al. 2014). However, in our study, neither turtle with clinical URTD moved significant distances. The limited movement of sick turtles may control disease transmission for this highly contagious pathogen. In case 1, the URTD signs may have been a flare-up caused by a chronic infection, but PCR results for case 2 suggest a newly acquired *Mycoplasma* infection, and the URTD signs likely indicate an acute response to new infection.

The turtle in case 1 moved farther after clinical signs cleared than had been recorded for the 2 yr prior to disease onset (Fig. 1). It is possible that a stressor precipitated immunosuppression, leading to clinical disease and abandonment of its home territory. In case 2, the turtle traveled outside of its home range just prior to detection of URTD clinical signs. The movements of both turtles before (case 2) and immediately after (case 1) *Mycoplasma* infections were larger than home ranges for clinically healthy turtles in Forest Park (S.B., S.L.D., J.L.P. unpubl.).

The box turtle *Mycoplasma* sp. identified in this study, and previous studies, is related to *M. agassizii* but divergent at a level that places it clearly as a separate species (Feldman et al. 2006; Farkas and Gal 2009). The possibility for cross-reactivity of this unnamed box turtle *Mycoplasma* sp. with *M. agassizii* antibodies illustrates an important confounding factor in infectious disease diagnostics. The potential for unknown immunologically cross-reactive agents of differing clinical relevance is significant, underscoring the importance of sequence-based identification of understudied pathogens in understudied species. Understanding of the host range and significance of different chelonian *Mycoplasma* species is still

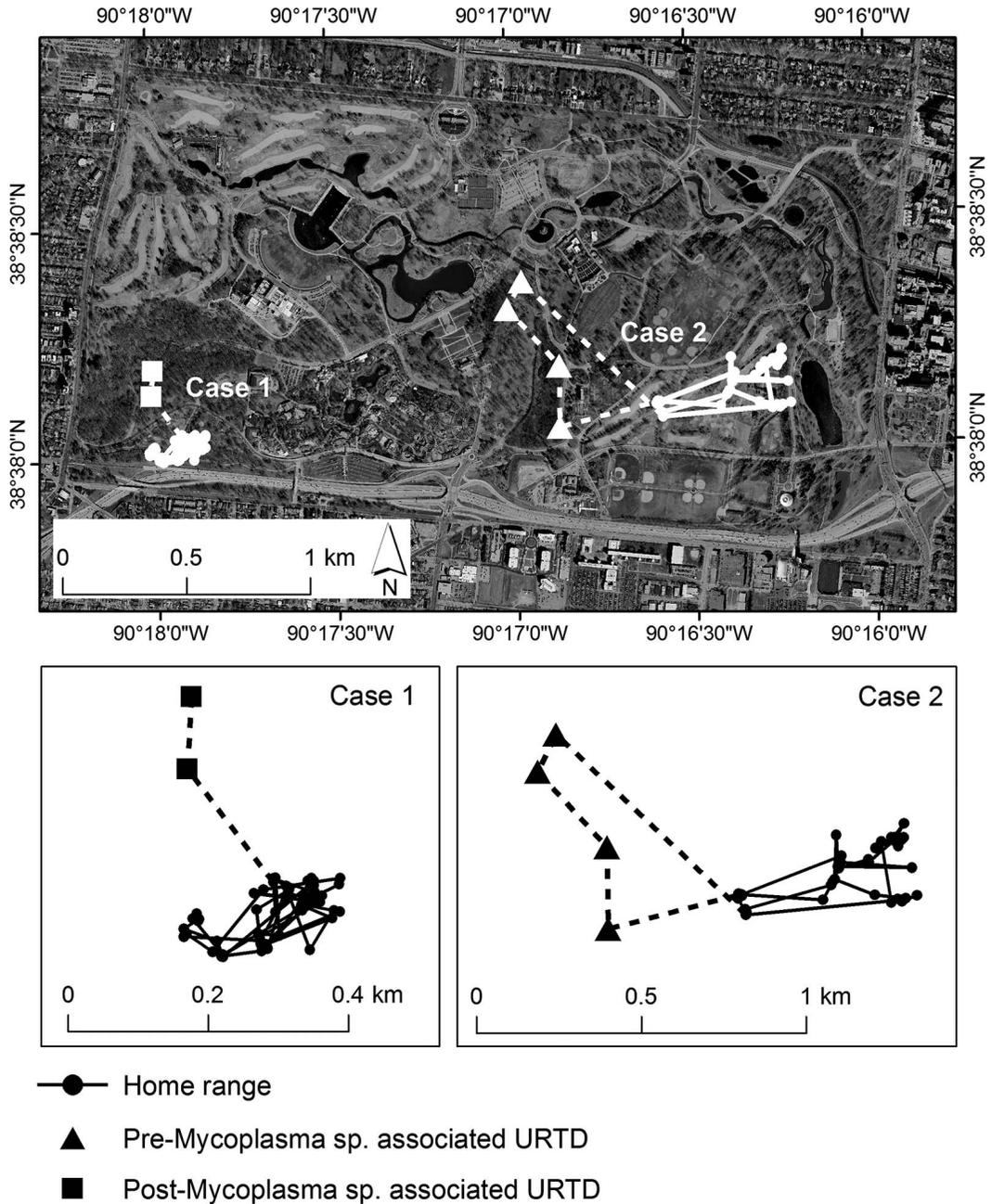


FIGURE 1. Map of movements for the three-toed box turtles (*Terrapene carolina triunguis*) with *Mycoplasma* sp.-associated upper respiratory tract disease (URTD) in Forest Park, St. Louis, Missouri, USA, 2013–14.

preliminary. In addition to the previous box turtle reports, *Mycoplasma* species have been sequenced from other Emydid hosts, including bog turtles (*Glyptemys mühlenbergii*) and spotted turtles (*Clemmys guttata*) in the northeastern US (Ossiboff et al. 2015). While

distinct single-nucleotide polymorphisms were used to segregate these isolates into genotypes, they do not appear to be sufficiently divergent to be a distinct species from that in the previous box turtle reports (Feldman et al. 2006; Farkas and Gal 2009).

Further study of the diversity of *Mycoplasma* species among turtle hosts is indicated.

To our knowledge, these are the first reported clinical cases of *Mycoplasma*-associated disease in free-living turtles in Missouri. The long-term effects of mycoplasmal URTD in the population dynamics of box turtles are unknown. We recommend continued monitoring to understand the epidemiology of mycoplasmosis in box turtles at the individual and population levels.

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LITERATURE CITED

- Berry KH. 1997. Demographic consequences of disease in two desert tortoise populations in California, USA. In: *Proceedings: Conservation, restoration, and management of tortoises and turtles—An international conference*, Purchase, New York, July 1993, Van Abbema J, editor. New York Turtle and Tortoise Society, New York, New York, pp. 91–97.
- Farkas SL, Gal J. 2009. Adenovirus and mycoplasma infection in an ornate box turtle (*Terrapene ornata ornata*) in Hungary. *Vet Microbiol* 138:169–173.
- Feldman SH, Wimsatt J, Marchang RE, Johnson AJ, Brown W, Mitchell JC, Sleeman JM. 2006. A novel *Mycoplasma* detected in association with upper respiratory disease syndrome in free-ranging eastern box turtles (*Terrapene carolina carolina*) in Virginia. *J Wildl Dis* 42:279–289.
- Hausmann JC, Wack AN, Allender MC, Cranfield MR, Murphy KJ, Barrett K, Romero JL, Wellehan JFX, Blum SA, Zink MC, et al. 2015. Experimental challenge study of FV3-like ranavirus infected eastern box turtles (*Terrapene carolina carolina*) to assess infection and survival. *J Zoo and Wildl Med* 46:732–746.
- Hernandez-Divers SJ, Cooper JE. 2006. Diagnostic techniques. In: *Reptile medicine and surgery*, 2nd Ed., Mader DR, editor. Saunders, St. Louis, Missouri, pp. 490–532.
- Jacobson ER, Brown MB, Wendland LD, Brown DR, Klein PA, Christopher MM, Berry KH. 2014. Mycoplasmosis and upper respiratory tract disease of tortoises: A review and update. *Vet J* 201:257–264.
- Jacobson ER, Gaskin JM, Brown MB, Harris RK, Gardiner CH, LaPointe JL, Adams HP, Reggiardo C. 1991. Chronic upper respiratory tract disease in free-ranging desert tortoises (*Xerobates agassizii*). *J Wildl Dis* 27:296–316.
- McGuire JL, Smith LL, Guyer C, Yabsley MJ. 2014. Effects of mycoplasmal upper-respiratory tract disease on movement and thermoregulatory behavior of gopher tortoises (*Gopherus polyphemus*) in Georgia, USA. *J Wildl Dis* 50:745–756.
- McLaughlin GS. 1997. *Upper respiratory tract disease in gopher tortoises, Gopherus polyphemus: Pathology, immune responses, transmission, and implications for conservation and management*. PhD Dissertation, University of Florida, Gainesville, Florida, 110 pp.
- Ossiboff RJ, Raphael BL, Ammazalorso AD, Seimon TA, Niederriter H, Zarate B, Newton AL, McAloose D. 2015. A *Mycoplasma* species of Emydidae turtles in the northeastern USA. *J Wildl Dis* 51:466–470.
- Pryslak T, van der Merve J, Lawman Z, Wilson D, Townsend H, van Drunen Littel-van den Hurk S, Perez-Casal J. 2011. Respiratory disease caused by *Mycoplasma bovis* is enhanced by exposure to bovine herpes virus 1 (BHV-1) but not to bovine viral diarrhea virus (BVDV) type 2. *Can Vet J* 52:1195–1202.
- Sandmeier FC, Tracy CR, duPre S, Hunter K. 2009. Upper respiratory tract disease (URTD) as a threat to desert tortoise populations: A reevaluation. *Biol Conserv* 142:1255–1268.
- Shahhosseiny MH, Hosseiny Z, Khoramkhorshid HR, Azari S, Shokrgozar MA. 2010. Rapid and sensitive detection of Mollicutes in cell culture by polymerase chain reaction. *J Basic Microbiol* 50:171–178.
- Wellehan JFX, Johnson AJ, Harrach B, Benkő M, Johnson C, Pessier A, Garner MM, Jacobson ER. 2004. Detection and analysis of six lizard adenoviruses by consensus primer PCR provides further evidence of a reptilian origin for the atadenoviruses. *J Virol* 78:13366–13369.
- Wendland LD, Zacher LA, Klein PA, Brown DR, Demcovitz D, Littell R, Brown MB. 2007. Improved enzyme-linked immunosorbent assay to reveal *Mycoplasma agassizii* exposure: A valuable tool in the management of environmentally sensitive tortoise populations. *Clin Vaccine Immunol* 14:1190–1195.

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