Review

Mycoplasmosis and upper respiratory tract disease of tortoises: A review and update

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ABSTRACT

Tortoise mycoplasmosis is one of the most extensively characterized infectious diseases of chelonians. A 1989 outbreak of upper respiratory tract disease (URTD) in free-ranging Agassiz's desert tortoises (Gopherus agassizi) brought together an investigative team of researchers, diagnosticians, pathologists, immunologists and clinicians from multiple institutions and agencies. Electron microscopic studies of affected tortoises revealed a microorganism in close association with the nasal mucosa that subsequently was identified as a new species, Mycoplasma agassizi. Over the next 24 years, a second causative agent, Mycoplasma testudineum, was discovered, the geographic distribution and host range of tortoise mycoplasmosis were expanded, diagnostic tests were developed and refined for antibody and pathogen detection, transmission studies confirmed the pathogenicity of the original M. agassizi isolate, clinical (and subclinical) disease and laboratory abnormalities were characterized, many extrinsic and predisposing factors were found to play a role in morbidity and mortality associated with mycoplasmal infection, and social behavior was implicated in disease transmission.

The translation of scientific research into management decisions has sometimes led to undesirable outcomes, such as euthanasia of clinically healthy tortoises. In this article, we review and assess current research on tortoise mycoplasmosis, arguably the most important chronic infectious disease of wild and captive North American and European tortoises, and update the implications for management and conservation of tortoises in the wild.

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Introduction

Respiratory infection of tortoises was first reported in California, USA, in the 1970s in confiscated Agassiz's desert tortoises (Gopherus agassizi) with nasal exudates (Fowler, 1980a), and in the UK in the 1980s in captive Greek (Testudo graeca) and Hermann's (Testudo hermanni) tortoises with rhinitis (Lawrence and Needham, 1985). Viruses (Jackson and Needham, 1983), Mycoplasma spp. (Fowler, 1980b; Lawrence and Needham, 1985) and Pasteurella testudinis (Snipes and Biberstein, 1982; Snipes et al., 1995) were hypothesized as possible causes.

In the 1980s, major declines in desert tortoise populations in the Mojave Desert of California, USA (Berry and Medica, 1995), and an associated upper respiratory tract disease (URTD; Jacobson et al., 1991), led to desert tortoises in the Mojave Desert north and west of the Colorado River being declared threatened (US Fish and Wildlife Service, 1990). A similar disease was seen in both captive (Beyer, 1993) and wild (McLaughlin, 1990; Beyer, 1993) gopher tortoises (Coppericus polyphemos) in Florida, USA. A microbial and pathological study (Jacobson et al., 1991) resulted in the identification of a new mycoplasma, Mycoplasma agassizi (Brown et al., 1995) and the confirmation of its causal relationship with URTD in desert (Brown et al., 1994) and gopher tortoises (Brown et al., 1999b).

Tortoise mycoplasmosis has since become one of the most extensively characterized infectious diseases of chelonians. Seminal research studies include: (1) a description of the anatomy and histology of the upper respiratory tract of healthy and affected tortoises (Jacobson et al., 1991); (2) identification and characterization of two new Mycoplasma spp. (Brown et al., 1995, 2001, 2004); (3) fulfillment of Koch's postulates, establishing that M. agassizi is a causative agent of URTD (Brown et al., 1994, 1999b); (4) development (Schumacher et al., 1993) and refinement (Wendland et al., 2007)
of an ELISA to determine exposure of tortoises to M. agassizii (Brown et al., 1999a and b); (5) development of a conventional PCR (Brown et al., 1995, 2004) and a quantitative PCR (qPCR; DuPré et al., 2011) to detect M. agassizii and Mycoplasma testudineum DNA; and (6) correlation of specific antibodies against M. agassizii and M. testudineum with upper respiratory tract lesions in infected tortoises (Homer et al., 1998; McLaughlin et al., 2000; Jacobson and Berry, 2012).

In this article, we review these (and other) key studies and assess new research to update the current state of knowledge on mycoplasmal URTD in tortoises and its implications for management and conservation of tortoises in the wild.

Species of Mycoplasma in tortoises

Two mycoplasmas have been isolated from desert and gopher tortoises, and characterized: M. agassizii, originally isolated from a desert tortoise with URTD (2001), and M. testudineum, a genetically distinct organism (Brown et al., 2004). Both organisms cause similar lesions in the nasal cavities of tortoises, with those caused by M. testudineum possibly being less severe (Jacobson and Berry, 2012). A third mycoplasma, Mycoplasma testudinis, was isolated from the cloaca of a healthy pet Greek tortoise in England (Hill, 1985) and has not been associated with URTD. Recently, a novel, unnamed, Mycoplasma sp. was identified by genomic sequencing in a sample obtained from the phallus of a wild desert tortoise (Wellehan et al., 2014).

Hosts and geographic distribution of mycoplasmas of tortoises

Evidence of infection with M. agassizii in many species of wild and captive tortoises across the world has been determined using serology, PCR and/or culture. Most information for wild tortoises pertains to gopher tortoises (Beyer, 1993; Berish et al., 2000, 2010; McLaughlin et al., 2000; Wendland, 2007) in South-Eastern USA, both Agassiz’s (Jacobson et al., 1991, 1995; Lederle et al., 1997; Christopher et al., 2003; Dickinson et al., 2005; Johnson et al., 2006) and Morafka’s (Gopherus morafkai, formerly G. agassizii; Dickinson et al., 2005; Jones, 2008; Murphy et al., 2011) desert tortoises in South-Western USA, and the Texas tortoise (Gopherus berlandieri; Guthrie et al., 2013) in Texas, USA.

In Europe, mycoplasmas have been identified in wild spur-thighed tortoises (Testudo graeca graeca) in Morocco, wild Hermann’s tortoises in France (Mathes et al., 2001; Mathes, 2003), captive Hermann’s and spur-thighed tortoises in France (Mathes et al., 2001; Mathes, 2003), wild spur-thighed, Hermann’s and marginated (Testudo marginata) tortoises in Italy (Lecis et al., 2011), captive spur-thighed and Russian (Testudo, formerly Agrionemys, horsfieldii) tortoises in Spain (Salinas et al., 2011), and captive spur-thighed, Hermann’s, Russian and leopard (Stigmochelys, formerly Geochelone, pardalis) tortoises in the UK (McArthur et al., 2002; Soares et al., 2004). Mycoplasmas have also been identified in many captive non-native pet tortoises in the USA (Brown et al., 2002; Wendland et al., 2006).

M. testudineum was originally isolated from the nasal cavity of a clinically ill desert tortoise from the Mojave Desert, USA (Brown et al., 2004). This organism was subsequently identified in three wild gopher tortoise populations in North-Eastern Florida (Wendland, 2007).

Mycoplasma spp. also have been identified in other cheloniens, including free-ranging Eastern box turtles (Terrapene carolina carolina) with URTD in Virginia, USA (Feldman et al., 2006) and a captive ornate box turtle (Terrapene ornata ornata) in Hungary (Tarkas and Gál, 2009). M. agassizii has also been identified by PCR in the lungs of red-eared sliders (Trachemys scripta elegans) with pneumonia from Louisiana, USA (J. Roberts and E. Jacobson, unpublished data).

Clinical disease and pathology

Clinical vs. subclinical infection

Clinical signs of mycoplamosis in tortoises include palpebral edema, conjunctivitis, and nasal and ocular discharges (Jacobson et al., 1991; McLaughlin et al., 2000; Mathes, 2003; Jacobson and Berry, 2012). However, subclinical infection with Mycoplasma spp. also occurs (Jacobson et al., 1995). Cycles of convalescence and recrudescence of clinical signs have been observed in captive and free-ranging desert and gopher tortoises (Brown et al., 1999a and b).

Histopathology

Mycoplamosis in tortoises is typically seen as an URTD, primarily affecting the nasal cavity (Jacobson et al., 1991, 1995). Pneumonia is occasionally seen. Histologically, normal nasal cavities of tortoises consist of a ventral, mucous and ciliated, epithelial mucosa, and a dorsal, multilayered, olfactory epithelium. In tortoises with mycoplamosis due to M. agassizii, lesions in the nasal cavity may be focal to diffuse, minimal to severe and may include basal cell hyperplasia in the mucosa, infiltrates of heterophils and histiocytes, and lymphoid hyperplasia in the submucosa. Depending on the epithelial changes and the extent of the inflammatory response, the following categories have been used to classify lesions: (1) mild inflammation; (2) moderate inflammation, and (3) severe inflammation (Jacobson et al., 1995).

In a group of desert tortoises that were serologically positive for M. testudineum, lesions in the nasal cavities were less diffuse and severe than in desert tortoises infected with M. agassizii (Jacobson and Berry, 2012). This could indicate that M. testudineum is less pathogenic than M. agassizii, or that the desert tortoises were more recently infected.

Serology

Serological assays

An ELISA was developed to detect antibodies against M. agassizii in plasma and serum using a monoclonal antibody (MAB HL673) against the light chain of desert tortoise immunoglobulins IgY and IgM (Schumacher et al., 1993). The antigen used in the ELISA was derived from M. agassizii PS6, the type strain from a desert tortoise with URTD. The reactivity of MAB HL673 was validated by Western blot analysis (Schumacher et al., 1993) and reference polyclonal T. horsfieldii IgY and IgM antiseras were obtained from H. Ambrosius, Leipzig, Germany (Ambrosius, 1976). The MAB HL673-based ELISA was further validated using experimental transmission studies in desert (Brown et al., 1994) and gopher tortoises (Brown et al., 1999b). In these studies, reference standards that were independent of the mycoplasmal diagnostic tests were presence of clinical signs and histological lesions (Schumacher et al., 1997; Brown et al., 2002).

An ELISA was also developed to determine exposure of gopher and desert tortoises to M. testudineum using M. testudineum CB57 as the antigen (Jacobson and Berry, 2012). In studies with >1000 tortoises (M. Brown, unpublished data), relatively few serum samples reacted with both M. agassizii and M. testudineum, and those samples that reacted with both Mycoplasma spp. were from tortoises in populations with documented presence of both pathogens. As new species of mycoplasmas are isolated from tortoises, validation and standardization of serological assays will be required.

Whereas the original ELISA results were reported as an enzyme immunoassay (EIA) ratio (Schumacher et al., 1993), the reporting system was eventually converted to end-point titers (Wendland et al., 2007). Results for ~6000 independent desert and gopher tortoises
were used to develop a distribution curve of absorbance values. A subset of samples (n = 90) that were randomly distributed over the spectrum of absorbance values was then used to determine endpoint titers and construct a standard curve. Test refinements substantially improved assay performance (sensitivity 0.98, specificity 0.99, J = 0.98), and test reliability. The authors considered this to be a clinically more meaningful and reliable diagnostic test than the original test based on EIA values.

**Natural antibodies and interpretation of serological results**

Natural antibodies are a function of innate immunity and can react with epitopes on multiple unrelated antigens of potentially pathogenic microbes (Marchalonis et al., 2002). The antigen binding specificities of some natural antibodies have been characterized (Grönwall et al., 2012). Hunter et al. (2008) reported that desert tortoises have natural antibodies (predominantly IgM) that could confound ELISA results for anti-mycoplasmal antibodies. However, natural antibodies are generally irrelevant in immunological tests, since sera are usually diluted sufficiently to avoid interference from so-called ‘nonspecific background’ (Ochsenbein and Zinkernagel, 2000).

Hunter et al. (2008) used Western blot analysis in an attempt to distinguish between natural and acquired anti-\textit{M. agassizii} antibodies, and concluded that banding patterns obtained using a single strain of \textit{M. agassizii} could distinguish between uninfected tortoises with natural antibodies and exposed tortoises with acquired antibodies. However, most mycoplasmas, even within an individual animal with a defined isolate, exhibit extensive intraspecies genotypic and phenotypic variability that is manifested as antigenic variation (Simmons and Dybvig, 2007). The ability to vary their antigenic patterns not only allows mycoplasmas to evade immune surveillance, but also to confound analysis of mycoplasmal immunogen recognition when only a single isolate is used as the source of antigen, especially on Western blot analysis (Kittelberger et al., 2006). The need for multiple strains in Western blot analysis, but not in ELISA, are consistent with findings for other mycoplasmal species (Tola et al., 1996; Kittelberger et al., 2006).

Using sera from culture positive gopher tortoises with URTD confirmed by histopathological examination, Wendland et al. (2010a) demonstrated that mycoplasmal strain variation was responsible for the differences in observed Western blot binding patterns. Several URTD-positive gopher tortoises had binding patterns similar to those reported by Hunter et al. (2008) for plasma samples from URTD-negative desert tortoises. Western blot analysis using a single antigen (PS6) failed to detect gopher tortoises known to have URTD in approximately 25% of cases, whereas an ELISA using the same strain as an antigen reliably detected all infected tortoises (Wendland et al., 2010a).

**Maternal antibodies and antibody persistence**

Female desert tortoises transfer\textit{Mycoplasma}-specific antibodies (IgG class) to their offspring through the egg and these antibodies are still detectable at 1 year of age (Schumacher et al., 1999). Antibody titers were substantially lower in offspring than in paired maternal serum, but higher in the offspring of sick female tortoises than healthy female tortoises. Importantly, hatchlings from \textit{Mycoplasma} antibody positive tortoises were not infected with \textit{Mycoplasma} spp. (Schumacher et al., 1999). Residual maternal antibody potentially can confound interpretation of ELISA tests and result in misdiagnosis of \textit{M. agassizii} infection in juveniles if ELISA tests are not appropriately validated. The current \textit{M. agassizii} ELISA has eliminated this as a problem by appropriate dilution of sera (Wendland et al., 2007). Similar to the long-term persistence of maternal antibodies in desert tortoises, Sandmeier et al. (2012) found that acquired, experimentally induced, anti-ovalbumin antibody titers in desert tortoises could persist for over a year.

**Pathogen detection**

**Culture**

\textit{M. agassizii} and \textit{M. testudineum} are fastidios organisms that grow slowly (2–6 weeks) at 30 °C in SP4 broth or agar (Brown et al., 1995, 2004). The organism ferments glucose under aerobic conditions, resulting in an acid pH shift in the medium. The most common sample cultured is nasal lavage fluid, obtained using 0.5–5.0 mL sterile (-phosphate buffered) saline or 0.5–1.0 mL sterile SP4 broth. Since bacteria and fungi are normal microbiota of the upper respiratory tract of tortoises, penicillin, polymyxin B and amphotericin B are usually added to SP4 medium to inhibit undesirable growth. To further minimize microbial contamination, a portion of each sample should be passed through a 0.45 μm filter prior to culture.

**PCR**

Testing using PCR has many advantages for the diagnosis of mycoplasmosis in tortoises, including high specificity and rapid detection. A positive PCR provides direct proof of the presence of mycoplasmal genomic material at the time of sampling. Whereas the PCR product can be used to identify the \textit{Mycoplasma} sp. accurately, it does not provide information about the viability of the organism at the time of testing. Since primers used in both conventional PCR and qPCR generally target specific gene sequences of a specific organism, they may not detect closely related species that differ in genomic content; therefore, specificity should be assessed stringently. Conventional PCR with restriction fragment length polymorphism (RFLP) analysis and full 16S sequencing remains an important testing option, particularly in cases where a new species may be involved. Important considerations for diagnostic tests targeting the presence of the pathogen are that the microbial load may be decreased when clinical signs are absent and that inadequate sampling of the upper respiratory passages may lead to false negative results. Finally, molecular techniques do not provide clinical isolates for further testing, including determination of antimicrobial sensitivity.

A conventional PCR was developed to detect tortoise mycoplasmas in culture medium and nasal lavage samples (Brown et al., 1995; Wendland et al., 2007). If a band of the correct size was present, then restriction enzyme digests using \textit{Agel} and \textit{Nci} were used for speciation. These two restriction enzymes give unique patterns for mycoplasmal 16S rRNA. In the event that an aberrant pattern is observed, complete sequencing of both strands of the 16S rRNA gene using a minimum coverage of reads obtained from two primers is recommended. Using conventional PCR, 23/146 (15.8%) samples from captive spur-thighed, Hermann's, Russian and leopard tortoises in the UK were positive for \textit{M. agassizii} (Soares et al., 2004). Russian tortoises were more frequently infected than other species tested. In this study, 8.2% of the samples tested were also positive for chelonian herpesvirus (ChHV). Co-infection of \textit{M. agassizii} and ChHV was also found in Mediterranean and one Russian tortoise in Spain (Salinas et al., 2011). These pathogens may work synergistically, causing more severe clinical signs when present as co-infections, or one agent may predispose tortoises to infection and disease associated with the other agent. Of oral swabs obtained from 30 free-living spur-thighed, Hermann's and margined tortoises temporarily housed in a wildlife center in Sardinia, Italy, 11 (36.7%) were mycoplasma PCR positive, with the amplified sequences having close similarity to \textit{M. agassizii} (Lecis et al., 2011). Three PCR positive tortoises exhibited signs of respiratory disease.
A qPCR using primers specific for *M. agassizii* and *M. testudineum* was developed to quantify mycoplasmas in samples and to correlate microbial burdens with clinical signs (DuPré et al., 2011). *M. agassizii* DNA (6–72,962 pg/μL) was detected by PCR in 100% of 20 captive desert tortoises tested. When tested by Western blot analysis, only 16/20 (80%) qPCR positive tortoises were seropositive. This was interpreted to mean that tortoises were colonized but not infected (DuPré et al., 2011). However, this study did not include ELISA results available for these tortoises, in which 19/20 (95%) tortoises were seropositive. A false negative rate of 25% has been observed when the Western blot analysis does not include the homologous *M. agassizii* strain as a source of antigen (Wendland et al., 2010a).

Although not a diagnostic assay, a method for labeling and quantifying viable *M. agassizii* using a membrane dye that is converted to a fluorescent signal by viable cells and then quantified by flow cytometry has been described (Mohammadpour et al., 2011). This methodology can be used to determine the number of viable mycoplasmal cells in a broth culture and may be applicable to experimental studies and antimicrobial testing where the number of microbes is of interest.

**Transmission and host response**

**Experimental transmission**

Experimental challenge studies in adult tortoises have demonstrated that *M. agassizii* is a causative agent of URTD in desert and gopher tortoises (Brown et al., 1994, 1999b). Experimentally challenged tortoises had lesions in the nasal cavity consistent with those seen in naturally infected tortoises. Preliminary observations indicated that seronegative hatchlings are at least as susceptible to infection as adults and that the disease progresses more rapidly in younger tortoises, with high morbidity in the first 6 weeks post-infection (McLaughlin, 1997).

Under experimental conditions, the onset of clinical signs in desert and gopher tortoises is as early as 2 weeks post-inoculation (PI) with *M. agassizii* (Brown et al., 2002). Seroconversion lagged behind clinical signs, with reliable detection of antibodies by 8 weeks PI. In an experimental challenge study involving gopher tortoises, the clinical sign scores of challenged tortoises (previously exposed to *M. agassizii*) at 2 weeks PI were higher than those of naïve animals (McLaughlin, 1997). ELISA values were also greater for challenged than naïve tortoises at each time point PI. A significant increase in serum ELISA values of challenged tortoises was observed at 4 weeks PI.

**Natural transmission**

Based on the behavioral inventory of the desert tortoise (Ruby and Niblick, 1994), we believe that horizontal transmission by direct contact (combat or courtship) is the most likely route of transmission of *Mycoplasma* spp. between tortoises. Whereas transmission is more likely to occur when the infected tortoise exhibits clinical signs, tortoises with subclinical infections may be able to transmit *Mycoplasma* spp. under appropriate conditions (Jacobson et al., 1995). Although aerosol transmission is possible, control gopher tortoises housed in pens adjacent to clinically affected tortoises did not become clinically diseased or seroconvert, suggesting that *M. agassizii* did not travel even relatively short distances over low (0.7 m) barriers (McLaughlin, 1997).

In the study by McLaughlin (1997), there was no evidence to support vertical transmission of *M. agassizii* in hatchlings derived from female gopher tortoises that were seropositive for exposure to *M. agassizii*. However, due to the small sample size, vertical transmission cannot be ruled out (Brown et al., 2002). Since experimental transmission of *Mycoplasma gallisepticum* by fomites has been reported in American house finches (*Carpodacus mexicanus;* Dhondt et al., 2007), fomite transmission may be possible in tortoises, but has not been demonstrated.

In a 4-year study of dynamics of URTD caused by *M. agassizii* in wild populations of gopher tortoises, the force of infection (FOI; probability per year of a susceptible tortoise becoming infected) and the effect of URTD on survival in free-ranging tortoise populations were followed in 10 populations in central Florida (Ogzul et al., 2009). Sites with high (≥25%) seroprevalence had higher FOI than sites with low (<25%) seroprevalence. These findings provided the first quantitative evidence that the rate of transmission of *M. agassizii* is directly related to seroprevalence.

Age (size) differences in exposure to *M. agassizii* in gopher tortoises may affect the spread of URTD in wild populations. In one study, adult gopher tortoises had a higher rate of exposure to *M. agassizii* than subadults (Karlin, 2008). In a 5-year study of mycoplasmal URTD, free-ranging adult gopher tortoises were 11 times more likely to be seropositive than immature tortoises (Wendland et al., 2010b), suggesting that direct or prolonged interaction between immature and adult tortoises was minimal.

In a study conducted at the Kennedy Space Center, Florida, from 1995 to 2000, to monitor the impact of URTD on gopher tortoises, there was an increase in the number of tortoises showing signs of URTD and, starting in 1998, a sudden increase in numbers of dead tortoises at the site (Seigel et al., 2003). Sex ratios and body sizes of dead tortoises were not distinguishable from living tortoises, indicating that mortality was not confined to a single sex or age class. However, no results of postmortem examinations, histopathology or PCR for detection of *Mycoplasma* spp. were reported for any of the tortoises showing signs of illness. Therefore, a causal relationship between mycoplasmosis and deaths of tortoises at this site was not established. Gopher and other tortoises are also susceptible to infection and death from other pathogens such as *Ranavirus*, which can result in clinical signs that overlap with URTD (Johnson et al., 2008).

McCoy et al. (2007) did not demonstrate any correlation between the presence of anti-*M. agassizii* antibodies and a decrease in the numbers of tortoises at several study sites. Furthermore, the proportions of tortoises that were seropositive or had intermediate antibody levels were positively correlated with the number of tortoises tested at each site. This is in agreement with well-established epidemiological principles for determining the number of animals in a population to sample in order to detect the presence of a disease. Valid population sampling will depend on the population size, the true prevalence of infection, and the sensitivity and specificity of each test (Brown et al., 2002). Sampling inadequate numbers of animals can lead to inaccurate conclusions regarding the status of a disease in a population.

McCoy (2008) compared the mean size of male and female tortoises found dead (based on the presence of shells) with the mean size of those found alive at a site (McCoy et al., 2007). The mean size of males found dead did not differ from the mean size of live males, but the mean size of dead females was lower than the mean size of live females. Their findings differed from those of Seigel et al. (2003), who did not detect differences in the mean sizes of dead and live tortoises for either sex. The difference in these findings may be related to differences in size distribution of live animals between different populations (McCoy, 2008). Although, it was assumed that URTD was the cause of death of tortoises at both sites, no post-mortem findings were reported, so the possibility of other causes of death, such as Ranavirus infection, could not be excluded.

**Host response**

In many mycoplasmal diseases, the host adaptive immune response is dysregulated, often providing limited or no protection
(Szczepanek and Silbart, 2014). In tortoises with mycoplasmosis, pathological studies have revealed an over-exuberant host response to Mycoplasma spp., resulting in dysplastic changes to the nasal mucosa (Jacobson et al., 1991, 1995; McLaughlin et al., 2000). The response of clinically healthy, seropositive, adult gopher tortoises in experimental challenge studies with M. agassizii was more rapid and severe than in naïve tortoises, suggesting that previous exposure to the organism may exacerbate disease (McLaughlin, 1997). In contrast, several clinically healthy desert tortoises, which were culture positive and positive for serum antibodies by ELISA, had normal nasal cavities (Jacobson et al., 1995). Thus, not all tortoises responded to M. agassizii with a severe inflammatory response, suggesting that multiple strains of M. agassizii may exist with variable pathogenicity or that different responses are related to different genotypes.

We suspect that mycoplasmosis in tortoises is characterized by initial high mortality, followed by low mortality and high morbidity. We have seen several infected tortoises survive in captivity for many years, with clinical signs varying over time. Since tortoises use olfaction for finding and selecting food, the histological changes seen in the nasal cavities of tortoises with URTD suggest that their ability to locate food and feed would be impaired. To determine the impact of nasal discharge on a tortoise’s ability to locate food, Germano et al. (2014) designed a study to determine the responses of Agassiz’s tortoises with or without nasal discharge, and positive or negative for M. agassizii antibodies, to a visually hidden olfactory food stimulus and an empty control. The presence of nasal discharge was associated with a reduced ability to locate food. This study also showed that moderate chronic nasal discharge in the absence of other clinical signs did not affect appetite in desert tortoises. We have seen tortoises with experimentally induced URTD and captive tortoises with URTD continue to feed even with a nasal discharge.

Impact of mycoplasmosis on tortoises
Factors contributing to mycoplasmal disease in tortoises

Factors that appear to contribute to outbreaks of URTD include environmental stress, human impacts, exposure to heavy metals and other toxicants, and the escape or release of captive tortoises (Jacobson et al., 1991; Brown et al., 2002; Sandmeier et al., 2009, 2013). In the Desert Tortoise Natural Area (DTNA), Kern County, California, where mycoplasmosis was first identified in wild desert tortoises, mercury (Hg) concentrations in the livers (0.326 parts per million, ppm) of affected tortoises were significantly higher than those of controls (0.0287 ppm) (Jacobson et al., 1991). Mercury can have a variety of toxicological effects, including cellular, cardiovascular, hematological, pulmonary, renal, immunological, neurological, endocrine, reproductive and embryonic effects (Rice et al., 2014). Altered levels of thyroid hormones have been detected in western pond turtles (Emys marmorata) with elevated concentrations of Hg (Meyer et al., 2014). Further work on the physiological impact of Hg on desert tortoises is needed.

Environmental perturbations and annual fluctuations in temperature, rainfall, and forage availability may result in activation of a subclinical infection to a clinical level. Although drought is a natural part of the desert tortoise’s environment (Henen et al., 1998), it can contribute to morbidity and mortality if combined with disease or habitat loss (Peterson, 1996). Clinical signs of URTD and heteropenia were noted at the time of emergence of desert tortoises from hibernation in years that followed periods of intense drought (Christopher et al., 2003), suggesting that tortoises entering hibernation in a drought year may be physiologically compromised. Sandmeier et al. (2013) suggested that cold winters could enhance conditions for the growth of M. agassizii. However, in a study in Las Vegas Valley, although Mycoplasma spp. and other bacteria were isolated during the warmer months, few aerobic bacteria and no Mycoplasma spp. were isolated from the nasal cavities of tortoises in January, the coldest month of the year (Jacobson et al., 1995). In addition, since the optimal growing temperature of M. agassizii is 30 °C (Brown et al., 1995), it is unlikely that colder temperatures would enhance growth of the microbe. However, cold winters could have an impact on the host immune system at the time of emergence of tortoises from their burrows in the spring, making them more susceptible to infection and lowering the infectious dose required to establish infection.

Human impacts on tortoises and their habitats, whether through disruption of normal behavior patterns, degradation of habitats through agriculture, silviculture, mining, land development or pollution, may cause sufficient physiological stress to trigger outbreaks of mycoplasmal disease. Wild tortoises in remote areas of the central Mojave Desert, distant from human beings and paved roads, were significantly less likely to be seropositive for M. agassizii than those in close proximity to human developments (Berry et al., 2006). The capture, manipulation and transport of tortoises during research projects, as well as relocation, restocking and repatriation efforts, also may be sources of stress that result in overt disease (Berry et al., 2002).

The escape or release of captive tortoises in urban and remote areas may be a significant factor accounting for URTD in wild populations. Thousands of captive desert tortoises were released into wild lands prior to their federal listing as a threatened species in 1990, and releases have continued in recent years (Berry et al., 1986; Ginn, 1990; Jennings, 1991; Connor and Kaur, 2004; Field et al., 2007; Murphy et al., 2007; Nussear et al., 2012). The outcome of a survey of 179 captive desert tortoises around Barstow, California revealed anti-mycoplasmal antibodies in 148 (82.7%) (Johnson et al., 2006). A statistically significant positive association was found between severity of clinical signs and serum antibody ELISA status. Furthermore, adult desert tortoises were more likely to have a positive serum antibody ELISA result than sub-adults or young adults of undetermined sex. These findings suggest that captive tortoises can be a reservoir of infection for wild desert tortoises.

Similarly, Morafka’s desert tortoises (G. morafkai; Murphy et al., 2011) from suburban Tucson, Arizona, were 2.3 times more likely to test seropositive for antibodies against M. agassizii than tortoises from remote locations (Jones, 2008). In addition, captive tortoises were 1.8 times more likely to test seropositive for Mycoplasma spp. than free-ranging tortoises, even in Arizona counties with high visitor use. Epizootics of URTD occurred on Sanibel Island, Florida, following the release of gopher tortoises collected in northern Florida and southern Georgia for use in tortoise races (Dietlein and Smith, 1979; Beyer, 1993; McLaughlin et al., 2000).

Effects of mycoplasmosis on tortoise populations

The effects of mycoplasmosis on mortality, morbidity and the long-term health and viability of tortoise populations are poorly understood. Mortality events could be due to an acute outbreak or the end result of long term physiological stress combined with an exacerbating extrinsic stressor. In the acute outbreak on Sanibel Island, up to 50% of adult gopher tortoises at one site died with signs of URTD (McLaughlin, 1990). A similar acute mortality event occurred at the Desert Tortoise Natural Area, Kern County, California (Jacobson et al., 1991). At this site, URTD evolved from an acute, episodic disease with high mortality to a chronic endemic disease with variable morbidity, low mortality and a high seroconversion rate for antibodies against M. agassizii (Brown et al., 1999a). In a study of URTD in gopher tortoises, Ozgul et al. (2009) theorized that seropositive tortoises were those that had survived initial infection and developed chronic disease.
While mortality events are easily documented and attract considerable attention, morbidity can be more subtle and difficult to assess. Abnormal hormone profiles observed in desert tortoises with mycoplasmosis (Rostal et al., 1996; Homer et al., 1998) could lead to alterations in foraging and reproductive behavior and decreased reproductive potential. Chronic inflammation in nasal and olfactory tissues of affected tortoises (Jacobson et al., 1991; Homer et al., 1998) could disrupt olfactory function and affect foraging and reproductive behavior. Soluble proteins in shell scutes also may be affected by mycoplasmosis (Homer et al., 2001). Further monitoring with follow-up pathological evaluation is needed to assess the long-term consequences of mycoplasmosis on tortoise morbidity.

Management implications for wild tortoise populations

Reliance on ELISA results to support management decisions

Antibody ELISA testing has been used to manage gopher and desert tortoises in parts of their range. Jacobson et al. (1995) discussed several scenarios for the disposition of seropositive desert tortoises. Although these authors did not recommend euthanasia of clinically healthy tortoises that were antibody ELISA positive for M. agassizii, such a policy was adopted in the state of Nevada. This policy was terminated in 2007, in part (R. Averill-Murray, personal communication) based on the following statement in Brown et al. (2002): ‘There are inadequate scientific data to provide definitive guidelines for the disposition of seropositive tortoises’.

Euthanasia of seropositive tortoises results in elimination of animals that might otherwise provide valuable reproductive and genetic contributions to wild populations and is not recommended. However, relocation of seropositive tortoises could result in spread of mycoplasmosis to susceptible animals, with detrimental impacts on recipient populations. Likewise, healthy tortoises that have not been exposed to Mycoplasma spp. should not be relocated to populations with extensive clinical disease or those undergoing increased mortality events (Brown et al., 2002; Sandmeier et al., 2009).

While antibody ELISA testing remains an important tool for making management decisions, it is critical to first establish clear goals for the tortoise population of interest. Importantly, antibody ELISA testing should not be used as the sole means of evaluating the health of an individual animal; rather, it is only one tool among many for comprehensive health assessment (Brown et al., 2004; McCoy et al., 2007; Sandmeier et al., 2009).

Modeling population dynamics

Understanding disease transmission dynamics in the context of tortoise social behavior is an important consideration for the success of future conservation programs. The finding that adult gopher tortoises were more likely to be seropositive than immature tortoises (Karlin, 2008; Wendland et al., 2010b) has broad implications for disease modeling. During mortality events caused by pathogens having minimal environmental transmission, such as M. agassizii, immature size classes may be spared, providing a pool of tortoises for later recruitment. However, a significant limitation of this hypothesis is that immature size classes constitute a small proportion of the overall population and usually are inadequate to sustain a population. Managing habitat to increase the successful recruitment of juvenile tortoises would be a valuable strategy in these circumstances. Alternatively, land managers could target smaller size classes for augmentation or restocking of depleted populations, thereby reducing the risk of pathogen introduction.

Population modeling techniques hold promise for understanding the impact of mycoplasmosis on wild populations of infected tortoises. Mycoplasmal URTD in free-ranging gopher tortoise populations was used as a model system for studying the effects of chronic recurring disease epizootics on host population dynamics and persistence (Perez-Heydrich et al., 2012). The findings indicate that the impact of disease on host population dynamics appears to depend primarily on how often a population experiences an epizootic, rather than on how long the epizootic persists. Models such as this will have more value once validated and tested.

Conclusions

Mycoplasmosis is a complex, multifactorial upper respiratory tract disease, of captive and wild tortoises. M. agassizii and M. testudineum are proven etiological agents of URTD. Extrinsic factors that most likely contribute to outbreaks of URTD include environmental stress, human impacts, exposure to heavy metals and other toxics, and the escape or release of captive ill tortoises. Because M. agassizii has been isolated from multiple species of tortoises in North America and Europe, all tortoises should be considered potentially susceptible. Like most respiratory mycoplasmoses, URTD is a chronic and often subclinical disease. Clinical signs may vary in onset, duration, and severity. Both subclinically and clinically affected animals have damage to the respiratory and olfactory epithelial surfaces, which affects their ability to identify food. Direct contact (combat or courtship) between tortoises is the most likely route of transmission, and transmission rates are directly related to overt clinical signs and seroprevalence. Several diagnostic tests are available to determine the exposure (serology to determine antibodies) and infection (direct culture and 16S rRNA PCR) status of individuals and populations of tortoises. Specific antibodies against Mycoplasma spp. do not appear to provide protective immunity, and the host’s inflammatory response may contribute to the severity of nasal lesions. Translocation as a management tool should include the health status of translocated tortoises and those at the recipient site, as well as long-term monitoring of effects on translocated and recipient populations.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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