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# Dynamics of Partial Recovery from Upper Respiratory Tract Disease (URTD) in Mojave Desert Tortoises

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***Abstract.*—Nine Mojave Desert Tortoises (*Gopherus agassizii*) had previously been experimentally infected with the type strain of *Mycoplasma agassizii* (PS6$^T$). Eight of the nine tortoises only developed signs of disease roughly two or more years after infection. All tortoises were diagnosed as positive for mycoplasmal URTD via visual signs of nasal exudate, presence of *M. agassizii* via quantitative PCR (qPCR), and/or seroconversion. Here, we quantified tortoise responses to a two-week long treatment that included local and systemic antibiotic and local dexamethasone treatments. We used qPCR to quantify loads of *M. agassizii* and loads of two putative virulence genes known to occur in PS6. We also quantified loads of a ubiquitous, largely commensal bacteria, *Pasteurella testudinis*. We monitored changes in circulating white blood cells via blood smears and differential white blood cell counts. To account for natural variations in both microbe loads and circulating white blood cells, we also compared these measures to those of a captive, *M. agassizii*-naïve population, held under identical husbandry conditions. We found that the treatment reduced loads of *M. agassizii* and the more prevalent virulence factor only in the short term. *Pasteurella testudinis* did not recover to levels found in healthy tortoises after 4 mo, however, suggesting that commensal gram-negative bacteria may be negatively affected by this antibiotic treatment. Finally, we found that treated tortoises showed long-term changes in circulating white blood cells over 4 mo, with increased levels of heterophils and decreased levels of lymphocytes, compared to healthy animals.**

*Key Words*.—antibiotic; dexamethasone; differential white blood cells; *Gopherus agassizii*; immunopathology; *Mycoplasma agassizii*; *Pasteurella testudinis*; virulence factors

## Introduction

The bacterium *Mycoplasma agassizii* can cause an upper respiratory tract disease (URTD) in Mojave Desert Tortoises (*Gopherus agassizii*), especially at high loads of the microbe (Aiello et al. 2016; Sandmeier et al. 2018). Much heterogeneity occurs in the development of disease across individuals, however, possibly due to properties of the microbe, the physiology of the host, and the environment (Sandmeier et al. 2009, 2018; Bauschlicher et al. 2023). Mixed-strain infections of *M. agassizii* may be one cause of differences in clinical disease across Mojave Desert Tortoise populations (Bauschlicher et al. 2023). In addition, two other microbes have been implicated in potentially affecting URTD. *Pasteurella testudinis* has primarily been linked to higher loads of *M. agassizii*, but not directly to disease in wild Mojave Desert Tortoises and is likely a mostly commensal bacterium (Sandmeier et al. 2022, 2023). *Mycoplasma testudineum* has been isolated from tortoises with URTD (Brown et al. 2004) but has an unknown pathogenicity in wild populations of Mojave Desert Tortoises (Braun et al. 2014; Weitzman et al. 2017). Stressors, including temperature fluctuations, crowding of animals (that may also increase infection pressure), and other adverse conditions such as low food quality, lack of sufficient hydration, frequent handling, etc. all may contribute to increasing levels of disease (Aiello et al. 2016; Drake et al. 2016; Sandmeier et al. 2017a).

Furthermore, two exo-alpha sialidase genes have been identified as potential virulence factors in the type strain PS6$^T$ of *M. agassizii*, and they were present in mixed-strain *M. agassizii* infections in wild tortoises, particularly in southern Nevada and in eastern California populations (Bauschlicher et al. 2023). Exo-alpha sialidases are enzymes that can

exacerbate pathogenic infections by cleaving large sialic acid molecules that are present in the mucosa of deuterostomes (Corfield 1992; Lewis and Lewis 2012). This action liberates nutrient sources for bacterial growth through the cleaved carbohydrates, may facilitate biofilm formation, and often exposes new sites for bacterial adhesion in the host mucosal membrane (Vimr et al. 2004; Severi et al. 2007; Hardy et al. 2017). Bauschlicher et al. (2023) found that one sialidase gene (528, GenBank accession number PAF55528.1) appears to increase loads of *M. agassizii* through changes in growth rates of the bacterium. A second sialidase gene (905, GenBank accession number PAF54905.1) is associated with increased risk of URTD through mechanisms that are not linked with the load of *M. agassizii* (Bauschlicher et al. 2023). Interestingly, many natural infections in wild tortoises did not contain these virulence genes or only very low levels, which may show selection in favor of lower virulence (Bauschlicher et al. 2023).

Antibiotic treatments to combat URTD have been used in captive tortoises, and two therapies were developed around the time of our study by the San Diego Zoo Wildlife Alliance at the request of the Desert Tortoise Recovery Office of the U.S. Fish and Wildlife Service specifically as a therapy for chronic *M. agassizii* infections in Mojave Desert Tortoises (Nadine Lambreski, unpubl. report). It is similar to other treatments, in which the goal of treatment is to reduce signs of URTD, using enrofloxacin or clarithromycin (Kinney et al. 2014; Caron et al. 2025), even though side effects at injection sites may occur and the efficacy of clearing subclinical infections has not been demonstrated *in vivo* (Prezant et al. 1994; Wimsatt et al. 1999; Wimsatt et al. 2008). The preferred therapy for tortoises that can be handled repeatedly included a broad-spectrum antibiotic (enrofloxacin) injected intramuscularly and a combination of the same antibiotic and dexamethasone washed through the nares (Caron et al. 2025; Nadine Lambreski, unpubl. report). Dexamethasone is an anti-inflammatory agent and is used to reduce quickly mucosal exudate (Aharon et al. 2017), which has been linked to some immunopathology, especially in desert tortoises (Sandmeier et al. 2017a, 2018; Caron et al. 2025). For example, even mild signs of nasal exudate are associated with long-term recrudescence of URTD in Mojave Desert Tortoises (Sandmeier et al. 2017a) and are also associated with higher levels of heterophils in the peripheral blood (Sandmeier et al. 2018). While other treatments are being investigated (e.g., Kinney et al. 2014; Moeller et al. 2025), this was the treatment recommended in 2011 (Nadine Lambreski, unpubl. report) and approved by the Institutional Animal Care and Use Committee (IACUC; protocols 00245 and 0555) and the U.S. Fish and Wildlife Service (Permit # TE07670-7/8), for the treatment of our captive animals.

We used nine tortoises that had been inoculated with one strain of *M. agassizii* PS6$^{T}$ (ATCC® 700616T) and treated them with this antibiotic protocol 3.5–6.5 y post-exposure to reduce signs of disease. Experimental infections had taken place in 2004 (three juvenile tortoises) and 2008 (six adult tortoises; Table 1). Of the nine animals, only one tortoise developed acute URTD and seroconverted (created adaptive antibodies) within 2 mo (Mohammadpour 2011). The other eight tortoises developed URTD very slowly; on the order of two or more years developing similar chronic infections as tortoises in natural and semi-natural conditions in the

**Table 1**. Timeline of infections, treatments, and sampling dates of treated Mojave Desert Tortoises (*Gopherus agassizii*) including which diagnostic tests were run pre- and post-treatment. Information before 2011 from Mohammadpour et al. (2009) and Mohammadpour (2011).

| Date | Experimental timeline | Tortoises | Treatment & Diagnostic Tests |
|---|---|---|---|
| March 2004 | | three juveniles | Infection with live *M. agassizii* (PS6) |
| May 2008 | | six adults | Infection with live *M. agassizii* (PS6) |
| November 2011 | Pre-treatment | six adults | Antibody test |
| 10 December 2011 | Pre-treatment | All | Differential white blood cell count, qPCR (pathogens/virulence factors) |
| 15–29 December 2011 | Treatment | All | Two-week course of treatment (antibiotic + dexamethasone) |
| 30 December 2011 | 24 h post-treatment | All | Differential white blood cell count, qPCR (pathogens/virulence factors) |
| 2 March 2012 | 2 mo post-treatment | All | Differential white blood cell count, qPCR (pathogens/virulence factors) |
| 4 June 2012 | 4 mo post-treatment | All | Differential white blood cell count, qPCR (pathogens/virulence factors) |

Mojave Desert (Mohammadpour 2011; Sandmeier et al. 2017a; Sandmeier et al. 2019). In comparison, the only other published experimental infection in which tortoises were inoculated with a different strain of *M. agassizii* caused visible URTD and seroconversion within 1–3 mo (Brown et al. 1994). Thus, we focused this study on evaluating the effect of the antibiotic treatment over 4 mo on these chronic infections, on numerous measures of microbial presence (*M. agassizii*, *M. testudineum*, and *P. testudinis*), putative virulence genes specific to *M. agassizii* PS6$^T$, and white blood cell profiles. Given that both microbial loads and white blood cell profiles change seasonally in these animals, we also used blood and nasal lavage samples of healthy, *M. agassizii*-naïve, captive animals sampled across seasons in the same year and housed in a separate room in the same facility (Sandmeier et al. 2012).

We hypothesized that antibiotic treatment would affect microbial loads in Mojave Desert Tortoises. We predicted that loads of all three microbes, with virulence factors, would be reduced over the short term. We also predicted that virulence factors would rebound if *M. agassizii* returned, especially because gene 528 may facilitate growth of *M. agassizii* (Bauschlicher et al. 2023). Further, we predicted that the commensal, ubiquitous species, *P. testudinis*, would return over time, as suggested by ecological studies (Sandmeier et al. 2023). We also hypothesized that white blood cell profiles would change over time. We predicted that local dexamethasone treatment might increase circulating inflammatory cells in the short term if these cells moved from the mucosa into the peripheral blood. Subsequently, a longer-term decrease in nasal pathogens might shift lymphocytes from the mucosa and/or spleen into the peripheral blood. If infections were chronic over time, despite treatment, we predicted that white blood cell ratios would differ from those of healthy animals, possibly with increased heterophil levels due to chronic inflammation (Sandmeier et al. 2018).

## Materials and Methods

***Animal husbandry and experimental infections***.—All Mojave Desert Tortoises were kept in an animal care facility at the University of Nevada, Reno. Tortoises included a group of 30 healthy animals (approximately 21 y old in 2008) that had been reared from eggs at the former Desert Tortoise Conservation Center (Las Vegas, Nevada, USA) and had been housed in captivity and unexposed to *M. agassizii*. Our facility had a separate room to quarantine and raise any infected tortoises in isolation with strict cleaning protocols to avoid contamination of un-exposed animals. All tortoises were similarly housed in large cattle tanks in this indoor animal care facility, with a covered burrow and a basking site under a heat lamp. Healthy animals were kept 1–2 per tank, while infected animals were kept in individual tanks. The facility had additional artificial lights on a natural light cycle, which changed with the seasons. All were fed a mixed diet of soaked alfalfa and timothy grass pellets, supplemented with fresh greens (primarily lettuce mixes) and weekly additions of calcium and vitamins. Tortoises were also soaked weekly to ensure adequate hydration.

In 2004, three juvenile tortoises, raised from eggs at the former Desert Tortoise Conservation Center, were transported to the same animal care facility within 1 y of their hatch date (unpubl. data). They were infected with a thawed culture of *M. agassizii* PS6$^T$ (0.5 ml of SP4 broth with $10^8$ cells of *M. agassizii*) in March 2004 (*sensu* Sandmeier et al. 2017a; Table 1). Tortoises did not develop overt signs of URTD and were kept separated from other captive tortoises. In May 2008, six additional adult tortoises (approximately 21 y old), reared from eggs at another *M. agassizii*-naïve research colony at University of Nevada, Las Vegas were infected with a separate, cultured sample of PS6$^T$ containing $3.5 \times 10^8$ viable cells in 0.5 ml of SP4 broth (Mohammadpour 2011). The infection protocol matching the past protocol but used a newly developed method of quantifying live mycoplasma via flow cytometry (Mohamadpour et al. 2009; Mohammadpour 2011). Tortoises were tested for seroconversion and adaptive antibodies to *M. agassizii* over 6 mo via Western Blot (Hunter et al. 2008; Table 1). Approximately 3.5 y later (November 2011; Table 1), all of the tortoises consistently showed intermittent signs of moderate URTD with visible exudate. At this point they were tested again for adaptive antibodies to *M. agassizii* via Western blots (Hunter et al. 2008; Table 1). Nasal lavages from all adult tortoises, both the healthy/unexposed animals and the six infected adults, were verified to be *M. agassizii*-negative via qPCR (see below). No nasal lavages were available for the juvenile tortoises prior to infection, but they were egg-reared in controlled conditions, which we assume prevented prior exposure to *M. agassizii*.

***Monitoring and antibiotic treatment of infected tortoises***.—We followed one of the two antibiotic

treatments that were established for the Desert Tortoise Conservation Center by the San Diego Zoo Wildlife Alliance (Nadine Lambreski, unpubl. report). This treatment was thought to be the most successful at reducing URTD if animals can be handled multiple times over two weeks (Nadine Lambreski, unpubl. report). We gave intramuscular injections of enrofloxacin (Baytril® 2.27%) at 5 mg/kg in alternate forelimbs once daily for 14 d (15–29 December 2011). Tortoises were also given a nasal rinse of a combination of 1.2 ml Enrofloxacin (22.7 mg/ml), 0.4 ml Dexamethasone (4 mg/ml), and 6.4 ml saline for 5 d (15–20 December 2011), followed by the same rinse once every other day for another 6 d (16–22 December 2011).

Immediately before antibiotic treatment in (10 December 2011), we took a nasal lavage sample by gently rinsing nares of each tortoise with 2 ml of sterile saline to be tested by quantitative PCR (qPCR; *sensu* Braun et al. 2014; Sandmeier et al. 2018; Bauschlicher et al. 2023; Table 1). We also took blood samples (< 500 µl) via subcarapacial venipuncture (Hernandez-Divers et al. 2011) and made blood smears that were stained with Wright-Giemsa reagents (Sandmeier et al. 2018; Table 1). We immediately preserved nasal lavage samples in RNAlater (Qiagen, Germantown, Maryland, USA) and frozen at -80° C. We took similar samples 24 h after a 14-d course of antibiotics, described below, and at 2 and 4 mo post antibiotic treatment (Table 1). We examined tortoises weekly throughout the entire 5.5 mo to monitor signs of disease, focusing on the presence of nasal exudate, occluded nares, new signs of damaged scales around the nares from past exudate, and swollen eyes (Sandmeier et al. 2017b). We also evaluated skin for any damage to tissue at the site of injection on their forelimbs and for any changes in behavior.

***Sampling of healthy control animals***.—To compare blood cell and *P. testudinis* values to those of healthy animals, we used similar samples that had been collected from the 30 unexposed, captive animals, housed in the same facility as part of parallel study (Sandmeier et al. 2016). We took blood smears from these tortoises December, March, and June of the same year (December 2011 - June 2012), albeit during different weeks, from a study by Sandmeier et al. (2016; n = 18–29 based on seasonal availability). Also, we collected nasal lavages at these same time points from the same animals and preserved them in RNA later and frozen, but these samples had not previously been tested or included in analyses presented in Sandmeier et al. (2016).

***Laboratory analyses***.—We conducted differential white blood cell counts based on 100 cells per slide, excluding thrombocytes. We extracted DNA with Qiagen DNEasy kits (Qiagen) from all nasal lavages (infected/treated and healthy groups). We then used probe-based qPCR assays to quantify loads of the microbes *M. agassizii*, *M. testudineum*, and *P. testudinis* (Braun et al. 2014; Sandmeier et al. 2019). We used similar qPCR assays to test for loads of the two sialidase genes, 528 and 905 (Bauschlicher et al. 2023). All five assays were run separately, and samples were run in triplicate. Each plate included a negative control as well as a full dilution curve of the double-stranded target sequences (gBlocks, Integrated DNA Technologies, San Diego, California, USA). These were used to convert Ct values to number of copies of DNA for each target sequence (Sandmeier et al. 2019; Bauschlicher et al. 2023). We considered Ct values of 40 or greater negative for all assays, and we only considered samples positive if two of three of the replicates tested positive.

***Statistical analyses***.—We used the mean numbers of copies in statistical analyses as Ct values of different assays should not be compared directly due to different detection limits and sensitivity of assays (Bustin et al. 2009). We transformed white blood cell counts to normalize data using centered log transformations, as is appropriate with compositional data (Sandmeier et al. 2019). We log-transformed copy numbers of DNA (loads) from all qPCR assays for normality to not violate assumptions of our statistical analyses. We used Mixed Model Regressions, including tortoise identity as a random effect and juvenile/adult as a covariate, to test for the effect of time after treatment (pre-antibiotics, 24 h post-antibiotics, 2 mo post-antibiotics, and 4 mo post-antibiotics) on white blood-cell counts. We ran separate analyses for lymphocytes, heterophils, eosinophils, monocytes/macrophages, and basophils. We conducted similar regression analyses to test for the effects of treatment on number of copies of DNA of each microbe (*M. agassizii*, *M. testudineum,* and *P. testudinis*) and of the *M. agassizii*-specific sialidase genes (528 and 905). Because we conducted 10 separate analyses, we calculated a reduced a for this set of regressions (Keppel 1991):

$$a_{FW} = (\text{degrees of freedom})(0.05)$$

$a_{individual\ comparison} = a_{FW}$ /(number of comparisons)

We used an $a_{FW}$ (familywise a) of 0.15 (four time points in the treatment) and an a of 0.015 for individual comparisons (10 comparisons across leukocyte types and microbe/gene loads).

We used *t*-tests to compare white blood cell profiles and quantities of *P. testudinis* between sick/treated and healthy animals from samples taken in December 2011, March 2012, and June 2022. Each set of analyses were run per season to directly compare values between treated and healthy groups. Because we treated tortoises in December of 2011 (Table 1), we ran Analysis of Variance (ANOVA) tests for differences among untreated sick, treated sick, and healthy tortoises. When ANOVAs were significant, we ran *post-hoc* Tukey's HSD tests for pairwise analyses. Through these analyses, we were able to tease apart the effects of time since treatment and natural seasonal changes that occur in desert tortoises (Sandmeier et al. 2012). Due to multiple comparisons across types of leukocytes and *P. testudinis* levels, we used the same formula as above to calculate an $a_{FW}$ (familywise a) of 0.05 (treated vs healthy animals) and an a of 0.002 for individual comparisons (18 comparisons). All analyses were conducted using JMP® 16 (SAS Institute, Cary, North Carolina, USA).

## Results

Only one adult tortoise produced adaptive antibodies to the inoculation with *M. agassizii* within 6 mo (Fig. 1); however, all six adult tortoises inoculated in 2008 showed seroconversion 928 d post-inoculation (Fig. 1). All but one of the nine tortoises had a positive qPCR test for *M. agassizii* immediately prior to treatment (Appendix Table). The tortoise (L1) with the negative qPCR test, did, however, seroconvert to *M. agassizii* prior to treatment (Fig. 1). All tortoises had visible exudate and signs of URTD prior to treatment. Signs of URTD gradually decreased over the next months, with no tortoise showing discharge of exudate 4 mo post-treatment. We never observed any sign of tissue damage around the sites of antibiotic injection on the forelimbs and did not observe any other adverse reactions to the antibiotic treatment (e.g., changes in behavior, eating, physical characteristics). All un-exposed animals had negative qPCRs for *M. agassizii*, one individual had one sample positive for *M. testudineum*, and all had some samples positive for *P. testudinis*, throughout this experiment. No animal in the un-exposed group showed any signs of URTD throughout this experiment.

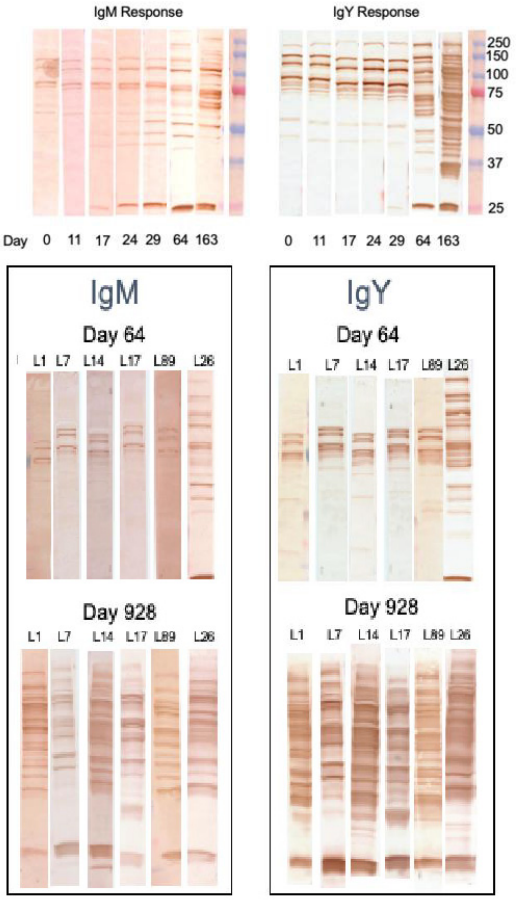


**Figure 1**. Western blots depicting antibody production, specific to *Mycoplasma agassizii*, in Mojave Desert Tortoises (*Gopherus agassizii*). Western blots show a progression from a small number of background, natural antibodies that bind *M. agassizii*, to seroconversion and production of a wide range of adaptive. (Top) One tortoise (L26) seroconverted within a few weeks of inoculation with *M. agassizii*, as shown by the production of adaptive antibodies (IgM followed by IgY isotypes). (Bottom) The other five tortoises showed no seroconversion for more than 2 mo, but all tortoises showed sustained production of adaptive antibody responses roughly 2.5 y later.

In regression analyses modeling effects of time after treatment on leukocyte metrics, only heterophils showed significant changes due to time after treatment ($F_{3,24} = 9.690$, $P < 0.001$; Fig. 2). Heterophil numbers were higher 24 h after the two-week long antibiotic treatment (Fig. 2; Appendix Table). Lymphocytes, basophils, monocytes/macrophages, and eosinophils all showed no effect of treatment ($F_{3,24} = 1.490$, $P = 0.242$; $F_{3,24} = 1.619$, $P = 0.211$; $F_{3,24} = 1.171$, $P = 0.190$; $F_{3,24} = 1.200$, $P = 0.331$, respectively). Loads of *M. agassizii* were significantly affected by time

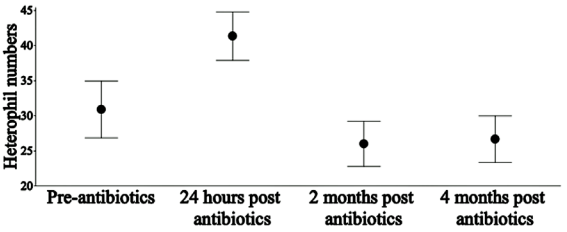


**Figure 2**. Number of heterophils across time and treatments in treated animals. In the treated animals, only heterophils showed significant changes across time (mean ± standard error; number of heterophils in a count of 100 leukocytes). Heterophils showed a peak increase 24 h after the full antibiotic and anti-inflammatory treatment was completed. This short-lived peak likely occurred as a direct result of the Dexamethasone treatment.

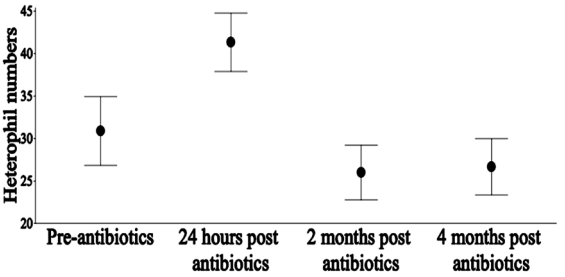


**Figure 3**. Levels of microbes and microbe-specific genes in treated tortoises. Microbe and microbe-specific genes were quantified via qPCR (mean ± standard error). Levels of *P. testudinis* generally decreased post antibiotic treatment and remained depressed, while *M. agassizii* only decreased initially and began to rebound after multiple months. The putative virulence gene 528 followed the same pattern, and gene 905 was not affected by treatment and persisted at low levels across all time points.

after treatment ($F_{3,24}$ = 6.453, $P$ = 0.002), but levels were only significantly lower 24 h and 2 mo post treatment (Fig. 3; Appendix Table). The regression models for loads of *M. agassizii* gene 528 showed a significant effect of time after treatment ($F_{3,24}$ = 4.489, $P$ = 0.012). Conversely, loads of gene 905 were not predicted by time after treatment ($F_{3,24}$= 2.627, $P$ = 0.074), although an early reduction in load was also observed (Fig. 3; Appendix Table). Only three samples tested positive for *M. testudineum*, and time after treatment was not significant ($F_{3,24}$ = 2.061, $P$ = 0.132). The regression model predicting loads of *P. testudinis* had a significant effect of time after treatment ($F_{3,24}$ = 15.776, $P$ < 0.001) and all longer time points (2 mo, and 4 mo post-antibiotics) showed reduced loads (Fig. 3; Appendix Table).

When comparing ill/treated animals to healthy captive animals in December, March, and June, we observed a somewhat consistent pattern of reduced levels of *P. testudinis* (Fig. 4), increased levels of heterophils, and decreased levels of lymphocytes with variation across time (Fig. 5). We found no significant differences across the other classes of leukocytes (Fig. 5). In December, *P. testudinis* load across untreated (n = 9), treated (n = 9), and healthy animals (n = 29) showed no significant differences among groups ($F_{2,31}$ = 17.56, $P$ = 0.032; Fig. 4). Both pre-antibiotic and post-antibiotic groups had significantly higher levels of heterophils ($F_{2,44}$ = 17.56, $P$ < 0.001) than healthy animals (Tukey HSD, $P$ = 0.002, $P$ < 0.001, respectively; Fig. 5). Lymphocytes ($F_{2,44}$ = 27.12, $P$ < 0.001), showed lower levels of lymphocytes in both pre- and post-antibiotic animals (Tukey HSD, $P$ < 0.001 for both; Fig. 5). In March, compared to 29 healthy animals, nine treated animals had significantly lower loads of *P. testudinis* ($t$ = 3.806, df = 37, $P$ = 0.002; Fig. 4), and no difference in levels of heterophils ($t$ = -3.113, df = 37, $P$ = 0.006) or lymphocytes ($t$ = 2.62, df = 26, $P$ = 0.014; Fig. 5). In June, compared to 18 healthy, the nine treated animals had lower loads of *P. testudinis* ($t$ = 4.686, df = 26, $P$ < 0.001; Fig. 4), higher levels of heterophils ($t$ = -4.087, df = 26, $P$ < 0.001), and lower levels of lymphocytes ($t$ = 3.878, df = 26, $P$ < 0.001; Fig. 5).

## Discussion

Our hypothesis that microbes would be reduced due to treatment was largely supported, but to a greater degree in *P. testudinis* than in *M. agassizii*. We also found support for our hypothesis that URTD and treatment would change white blood cell profiles in tortoises compared to healthy controls, with the largest changes occurring in relative numbers of heterophils. Mycoplasma *agassizii* saw a temporary reduction in detectable presence in some animals and load in all animals after treatment of antibiotics, but

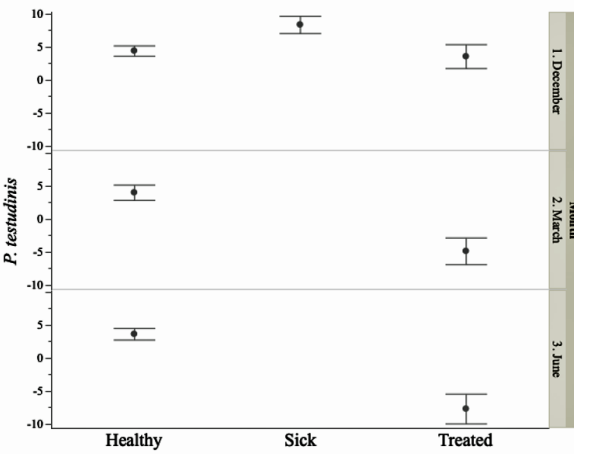


**Figure 4**. *Pasteurella testudinis* in healthy, sick, and treated tortoises. Levels were not lower in treated animals than infected animals in December. In both other seasons (March and June), they were lower than in healthy animals. *Pasteurella testudinis* levels are depicted as log-transformed copies of DNA.

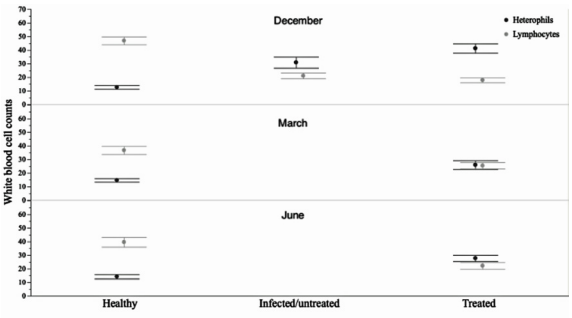


**Figure 5**. Differential numbers of heterophils and lymphocytes in infected/treated and healthy tortoises. Comparing healthy animals to infected/treated animals accounts for natural, seasonal variation of differential lymphocyte and heterophil counts per 100 total white blood cells (mean ± standard error). Panels show levels in December (with infected animal numbers shown pre and post treatment), March, and June.

the non-pathogenic *P. testudinis* showed a greater and longer-term reduction. Enrofloxacin is broadly effective against both gram-positive and gram-negative bacteria (Mitchell 2006), and a potential side effect of this treatment could be a long-term reduction in the microbiome. Because healthy microbiomes can contribute to the reduction of commonly transmitted or chronic infections (e.g., Woodhams et al. 2014; Knutie et al. 2017; Weitzman et al. 2018), this reduction in other microbes could contribute to the cycles of disease-recrudescence that have been quantified in another long-term study of URTD in this species (Sandmeier et al. 2017a). Gene 528, which likely acts as a virulence factor in wild tortoises (Bauschlicher et al. 2023), decreased and increased in tandem with loads of *M. agassizii* and did not seem to be affected in the long-term by treatment.

Similar to a recent study of the effects of a similar treatment of enrofloxacin/dexamethasone on *M. agassizii*-induced URTD in tortoise species (Caron et al. 2025), we did see a short-term reduction in loads of *M. agassizii*. Caron et al. (2025) used higher flush volumes in the nares and sedated tortoises to confirm flushes of the choanae and a higher dosage of injected enrofloxacin; however, their treatment included fewer flushes (five instead of our eight nasal flushes) and fewer intramuscular infections (five instead of our eight injections). The specifics of these treatments did not seem to matter in disease-level results, and all 15 of their treated mixed species of tortoises resolved signs of URTD and *M. agassizii* in 1 mo (Caron et al. 2025). Because these were pet tortoises, only six were available for follow-up exams, and three of six were genetically positive for *M. agassizii* 4–10 mo after treatment (Caron et al. 2025). Thus, our results match their conclusions that tortoises had short-term recoveries and no adverse effects of treatment (Caron et al. 2025). In our tortoises, levels of *M. agassizii* only increased by 4 mo after treatment, which could give animals time to deal with negative physiological impacts of the disease. Indeed, no tortoise showed signs of URTD via active exudate over the course of 4 mo, possibly allowing wild or semi-wild tortoises to regain increased success in foraging and reduction of water loss via mucosal exudate (Germano et al. 2014; Sandmeier et al. 2023).

Conversely, other recent treatments tested on Texas Tortoises (*Gopherus berlandieri*) showed extreme negative effects of danofloxacin and ineffective resolution of URTD with two other, separate treatments with tulathromycin and oxytetracycline (Moeller et al. 2025). Moeller et al. (2025), however, we were not able to reliably test for the presence of *M. agassizii* in nasal flushes and the two latter treatments could have led to reductions in loads of *M. agassizii*. Overall, we recommend protocols of enrofloxacin and dexamethasone, as they were non-harmful and show a better resolution of signs of URTD across Mojave Desert Tortoises, Gopher Tortoises (*Gopherus polyphemus*), Leopard Tortoises (*Stigmochelys pardalis*), and African Spurred Tortoises (*Centrochelys sulcata*; Caron et al. 2025). This treatment, however, did not result in long-term clearance of *M. agassizii* in all treated individuals (Caron et al. 2025).

An important caveat of our study is that tortoises received higher doses of *M. agassizii* than we expect would occur from most natural transmission events (Aiello et al. 2016). Thus, unlike many wild animals with subclinical infections and low loads of *M. agassizii*, all tortoises in this study eventually produced adaptive antibodies to this pathogen, which has been posited as an immunopathological response that leads to chronic, intermittent external signs of inflammation, especially in Mojave Desert Tortoises (Jacobson et al. 2014; Sandmeier et al. 2017a, 2018). At the time of treatment, these animals also had more severe signs of disease than those commonly observed in wild animals (Sandmeier et al. 2013, 2017b, 2018). Unlike wild infections, we did not expect these animals to have multi-strain infections, and levels of gene 528 roughly matched levels of *M. agassizii*. Given that this gene may encourage growth rates and evasion of immune responses (Bauschlicher et al. 2023), our results reinforce the idea that reductions in the load of *M. agassizii* may not reduce virulence, allowing for recrudescence (Sandmeier et al. 2017a).

Copies of gene 905 were consistently lower than copies of *M. agassizii* throughout the study. One possible mechanism for this pattern is that *M. agassizii* is grown in liquid culture (ATCC protocol: 700616T), and therefore individual cells cannot be selected to grow as a single-strain solution during the growing procedure. This would allow for mutations and/or gene loss or gain even within a type culture. Whether some cells have lost or mutated gene 905 in culture or in the tortoise is not possible to ascertain from our study procedure.

Compared to healthy animals, infected animals had higher levels of heterophils and lower levels of lymphocytes across time. The short-lived increase in heterophils immediately after treatment is likely due to a shift of heterophils from the nasal and oral mucosa, where Dexamethasone was administered, to the peripheral blood stream. Dexamethasone is an exogenous corticosteroid, usually acts as a local anti-inflammatory agent, and similar results have been found in other animals (Anderson et al. 1999; Abraham et al. 2009). Both intramuscular and topical treatment can also cause an increase in circulating glucocorticoids, and an increase in the heterophil:lymphocyte ratio (Davis et al. 2008; Abraham et al. 2009); however, over time, this effect should be mitigated by the relatively quick metabolism of the drug (Abraham et al. 2009; Aharon et al. 2017), and the sustained increase in heterophils in infected animals is likely due to an increase in the production of heterophils to combat lingering *M. agassizii* infections. Chronic stress can also increase heterophil:lymphocyte ratios across vertebrate animals (Davis et al. 2008). Stressors in wild tortoises, such as translocations, suboptimal food and water availability, and/or access to ideal thermal conditions, may exacerbate this pattern and lead to slower recovery times or increased rates of recrudescence (Drake et al. 2012, 2016; Sandmeier et al. 2017a).

Chronic, low levels of lymphocytes in the infected group can be explained by two potential mechanisms. Adaptive antibody responses in tortoises tend to be sustained over a year or more (Sandmeier et al. 2012). We suspect that B2 lymphocytes may be producing these antibodies largely in the spleen, accounting for a reduced lymphocyte population in the peripheral blood (Origgi 2007; Zimmerman et al. 2010; Sandmeier et al. 2012). B1 lymphocytes in Mojave Desert Tortoises have recently been shown to account for at least 40–60% of circulating lymphocytes, and these cells do not respond to infection with an adaptive response but have constitutive functions of natural antibody production and phagocytosis (Baumgarth et al. 2005; Li et al. 2006; Slama et al. 2022). Currently, it is not known whether the lifespan of these cells is affected by phagocytosis of pathogens or whether these cells may migrate into areas with active infections such as the nasal mucosa. Both mechanisms could contribute to a reduction of lymphocytes in the peripheral blood.

While heterophils and lymphocytes, and possibly other components of the immune system, seem to be combatting signs of disease over months, it is worrying that *M. agassizii* loads appeared to rebound. Our study supports the idea that URTD in Mojave Desert Tortoises is not well controlled by adaptive antibody responses and inflammation and involves immunopathology, which was also evident in altered white blood cell profiles (Sandmeier et al. 2017a). In wild, largely healthy populations, a disconnect has been observed between signs of URTD and presence of *M. agassizii* (Sandmeier et al. 2013; Sandmeier et al. 2018). In populations with higher average lymphocyte numbers in the peripheral blood, loads of *M. agassizii* were lower, while increased levels of heterophils and increased seroprevalence were associated with higher levels of URTD (Sandmeier et al. 2013, 2018). Thus, it seems as if there is a tipping point at which constitutive responses, possibly mediated by anti-inflammatory B1 lymphocytes (Baumgarth et al. 2005; Slama et al. 2022), can no longer keep levels of *M. agassizii* in check and long-term inflammatory and adaptive antibody responses lead to intermittent signs of URTD (Sandmeier et al. 2017a). Mutations and differential gene expression could also lead to changes in *M. agassizii* that may help it evade the immune system (Jacobson et al. 2014; Bauschlicher et al. 2023).

Given these patterns of potential long-term immunopathology, we suggest that additional steps may be necessary to augment antibiotic treatment in captive or semi-captive animals to include potential microbiome augmentation and husbandry changes such as providing supplemental food, water, and basking sites. Semi-captive tortoises could include animals being reared as part of head-starting projects, animals being temporarily contained in pens prior to translocation, and/or animals used in experiments to inform conservation strategies (e.g., Drake et al. 2012; McGovern et al. 2020). While the possibility exists that a more targeted antibiotic against *M. agassizii* exists that would preserve more of the microbiome, slow-growing mycoplasmas are notoriously difficult

to target with antibiotics (Razin et al. 1998; Baseman et al. 2004). Augmenting the microbiome with that of healthy tortoises via nasal lavages may be an option and may also combat the known association between URTD and reduced microbial diversity in this species (Weitzman et al. 2018). Nutritional considerations may also improve the ability of the tortoise to make lymphocytes, as healthy captive populations with *ad libitum* food tend to have higher levels of lymphocytes than some natural populations (Sandmeier et al. 2019). Juvenile tortoises fed only invasive grasses have been shown to exhibit reduced immune function versus tortoises fed a diet that included forbs, which have a higher protein content (Drake et al. 2016). Thus, supplementing captive tortoise food with high levels of forbs (alfalfa, dandelions, native forbs) may be beneficial for animals with URTD. Similarly, maintaining tortoise habitat to favor native forbs over introduced annual grasses may also help wild populations experience reduced morbidity due to *M. agassizii* (Drake et al. 2016). Finally, *M. agassizii* does not survive well at high temperatures (> 40° C), making it imperative that sick tortoises have access to safe basking sites in the wild or heat lamps in captive treatment conditions (Mohammadpour et al. 2009). How frequent basking elevates metabolic rates and nutritional requirements (Beaupre 1996; Deen and Hutchison 2001) has not been well-studied in this species but may also be an important consideration in treating tortoises with chronic URTD.

Finally, this study highlights the importance of using healthy animals kept in similar conditions and across similar seasons as sick animals to provide researchers with true baseline health values, especially because white blood cell profiles and microbe levels are known to change across seasons (Sandmeier et al. 2016, 2019). While baseline white cell profiles have been published for Mojave Desert Tortoises, most of these were based off of both wild and captive animals from the western portion of the range of the species in California and southeastern Nevada (Rosskopf 1982; Christopher et al. 1999). Studies of both captive and wild tortoises, however, have shown significant differences in differential white blood cell counts across seasons, populations, and climatic conditions (Sandmeier et al. 2016, 2018, 2019). Without our captive control population, we would have had less power to detect true differences among healthy and treated animals.

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**Appendix Table**. White blood cell (numbers/100 total white blood cells) and microbe values (DNA copy number quantified by qPCR) across treatments for all nine experimentally infected Mojave Desert Tortoises (*Gopherus agassizii*). The abbreviations TAAT = time after antibiotic treatment and RAI = relative age category at infection.

| Tortoise ID | Date sampled | TAAT | RAI | Copies of *M. agassizii* DNA | Copies of *M. testudineum* DNA | Copies of *P. testudinis* DNA | Copies of gene 528 | Copies of gene 905 | Heterophils | Basophils | Lymphocytes | Monocytes | Eosinophils |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 89 | 10 December 2011 | Pre | Adult | 8 | 0 | 37 | 31 | 3 | 21 | 40 | 16 | 14 | 9 |
| L1 | 10 December 2011 | Pre | Adult | 369 | 0 | 1067 | 264 | 37 | 25 | 36 | 19 | 6 | 22 |
| L14 | 10 December 2011 | Pre | Adult | 289 | 59 | 1844 | 1797 | 131 | 16 | 17 | 24 | 29 | 14 |
| L17 | 10 December 2011 | Pre | Adult | 193 | 3 | 48 | 191 | 6 | 47 | 15 | 19 | 7 | 11 |
| L26 | 10 December 2011 | Pre | Adult | 0 | 17 | 10148 | 0 | 0 | 36 | 24 | 24 | 11 | 5 |
| L7 | 10 December 2011 | Pre | Adult | 868 | 0 | 77 | 0 | 0 | 14 | 20 | 36 | 20 | 10 |
| 10055 | 10 December 2011 | Pre | Juvenile | 42 | 0 | 14051 | 29 | 5 | 41 | 8 | 20 | 22 | 9 |
| 10072 | 10 December 2011 | Pre | Juvenile | 9 | 0 | 5 | 48 | 3 | 42 | 13 | 16 | 14 | 15 |
| 10154 | 10 December 2011 | Pre | Juvenile | 4 | 3 | 186 | 14 | 0 | 36 | 13 | 16 | 15 | 20 |
| 89 | 30 December 2011 | 24 h | Adult | 0 | 0 | 339 | 0 | 0 | 45 | 17 | 15 | 11 | 12 |
| L1 | December 2011 | 25 h | Adult | 0 | 0 | 139 | 0 | 0 | 48 | 24 | 13 | 8 | 7 |
| L14 | December 2011 | 26 h | Adult | 26 | 6 | 61 | 21 | 7 | 29 | 26 | 11 | 13 | 20 |
| L17 | December 2011 | 27 h | Adult | 0 | 0 | 14 | 0 | 0 | 52 | 15 | 19 | 11 | 3 |

**Appendix Table**, continued

| Tortoise ID | Date sampled | TAAT | RAI | Copies of *M. agassizii* DNA | Copies of *M. testudineum* DNA | Copies of *P. testudinis* DNA | Copies of gene 528 | Copies of gene 905 | Heterophils | Basophils | Lymphocytes | Monocytes | Eosinophils |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L26 | December 2011 | 28 h | Adult | 0 | 4 | 0 | 0 | 0 | 42 | 18 | 17 | 13 | 10 |
| L7 | December 2011 | 29 h | Adult | 0 | 0 | 5 | 0 | 0 | 21 | 21 | 28 | 14 | 11 |
| 10055 | December 2011 | 30 h | Juvenile | 5 | 0 | 8 | 0 | 0 | 48 | 10 | 17 | 21 | 9 |
| 10072 | December 2011 | 31 h | Juvenile | 16 | 0 | 39 | 0 | 0 | 49 | 11 | 17 | 21 | 2 |
| 10154 | December 2011 | 32 h | Juvenile | 0 | 0 | 55 | 0 | 0 | 38 | 14 | 24 | 10 | 14 |
| 89 | 2 March 2012 | 2 mo | Adult | 0 | 0 | 0 | 0 | 0 | 24 | 35 | 21 | 9 | 11 |
| L1 | 2 March 2012 | 3 mo | Adult | 181 | 0 | 0 | 121 | 35 | 18 | 33 | 22 | 11 | 17 |
| L14 | 2 March 2012 | 4 mo | Adult | 0 | 0 | 0 | 0 | 0 | 21 | 28 | 40 | 4 | 7 |
| L17 | 2 March 2012 | 5 mo | Adult | 0 | 0 | 7 | 0 | 0 | 27 | 20 | 22 | 11 | 22 |
| L26 | 2 March 2012 | 6 mo | Adult | 0 | 0 | 0 | 0 | 0 | 42 | 22 | 21 | 8 | 8 |
| L7 | 2 March 2012 | 7 mo | Adult | 0 | 0 | 0 | 0 | 0 | 10 | 27 | 35 | 21 | 7 |
| 10055 | 2 March 2012 | 8 mo | Juvenile | 11 | 0 | 2 | 8 | 2 | 27 | 15 | 22 | 10 | 26 |
| 10072 | 2 March 2012 | 9 mo | Juvenile | 11 | 0 | 2 | 8 | 2 | 38 | 12 | 24 | 17 | 9 |
| 10154 | 2 March 2012 | 10 mo | Juvenile | 0 | 0 | 2 | 0 | 0 | 27 | 15 | 22 | 10 | 26 |

**Appendix Table**, continued

| Tortoise ID | Date sampled | TAAT | RAI | Copies of *M. agassizii* DNA | Copies of *M. testudineum* DNA | Copies of *P. testudinis* DNA | Copies of gene 528 | Copies of gene 905 | Heterophils | Basophils | Lymphocytes | Monocytes | Eosinophils |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 89 | 4 June 2012 | 4 mo | Adult | 0 | 0 | 0 | 0 | 0 | 35 | 31 | 26 | 19 | 6 |
| L1 | 4 June 2012 | 5 mo | Adult | 69 | 0 | 0 | 116 | 14 | 23 | 34 | 13 | 5 | 25 |
| L14 | 4 June 2012 | 6 mo | Adult | 7 | 0 | 0 | 0 | 0 | 14 | 22 | 39 | 16 | 9 |
| L17 | 4 June 2012 | 7 mo | Adult | 8698 | 0 | 1264 | 8994 | 1091 | 31 | 21 | 16 | 17 | 14 |
| L26 | 4 June 2012 | 8 mo | Adult | 0 | 3 | 0 | 0 | 0 | 34 | 19 | 21 | 20 | 6 |
| L7 | 4 June 2012 | 9 mo | Adult | 6 | 0 | 0 | 0 | 0 | 23 | 26 | 19 | 17 | 16 |
| 10055 | 4 June 2012 | 10 mo | Juvenile | 3 | 0 | 0 | 0 | 0 | 28 | 13 | 22 | 19 | 10 |
| 10072 | 4 June 2012 | 11 mo | Juvenile | 267 | 244 | 0 | 260 | 55 | 33 | 16 | 26 | 15 | 10 |
| 10154 | 4 June 2012 | 12 mo | Juvenile | 6 | 0 | 0 | 0 | 0 | 29 | 12 | 18 | 17 | 24 |