

University of Nevada, Reno

**Transmission of Mycoplasmal Upper Respiratory Tract  
Disease in the Desert Tortoise (*Gopherus agassizii*)**

A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in  
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by

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## Transmission of Mycoplasmal Upper Respiratory Tract Disease in the Desert Tortoise (*Gopherus agassizii*)

### Abstract

*Mycoplasma agassizii* has been identified as a cause of disease, and a possible cause of declines in wild populations of desert tortoises (*Gopherus agassizii*) (USFWS 1994). In Clark County in Nevada, more than 3,230 federally-protected desert tortoises were euthanized over the course of 17 years, as a means to reduce transmission of upper respiratory tract disease (URTD) to a wild population. Immunological tools have been developed to diagnose an immune response to the pathogen. However, there remains a paucity of information on the conditions required to transmit the disease, and how the immune response influences transmissibility. This study is the first large-scale, longitudinal investigation of transmission of pathogen in this host/pathogen complex. Reported here are the results of a three-year, semi-natural field experiment in which serological tests and clinical signs are used to determine transmission of *M. agassizii* among desert tortoises. We found that appearance of disease does not require exposure to sick tortoises. The appearance of clinical signs does not predict a tortoise seroconverting. There are no differences in disease between females and males. Tortoises emerge from brumation immunologically challenged, and for most tortoises, there is a lag period of eighteen months between clinical signs and seroconversion.

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## Introduction

The desert tortoise (*Gopherus agassizii*) was listed as a threatened species under the U.S. Endangered Species Act due to reports of widespread population declines in the Mojave Desert (Department of Interior, 1990). The Desert Tortoise Recovery Plan (USFWS 1994) outlined a need for epidemiological studies of respiratory disease in natural populations of the desert tortoise. Instead of focusing on epidemiology, veterinarians conducted research to establish that *Mycoplasma agassizii* is a causal agent for upper respiratory tract disease (URTD) in the desert tortoise (Schumacher et al., 1993; Brown et al., 1994).

Our goal here has been to understand the progression of general immune responses in desert tortoises, and the interaction of these immune responses in *M. agassizii*. We hypothesize that rates of transmission of pathogens, and responses to pathogens will follow patterns indicated in published literature on seasonal variation and within season sex differences. Here, we analyze the relationship between clinical signs and immunological seroconversion. We also analyze differences in serological patterns within individuals directly after and immediately prior to brumation. By correlating serological tests to clinical signs, we analyze differences between sex and among seasons.

In order to create efficacious management policy concerning conservation of desert tortoises, managers must grapple with a number of questions about the transmissibility of pathogens causing URTD, including *M. agassizii*. A primary goal of

this experiment was to assemble new knowledge that can inform such conservation decisions. For example, if a conservation action necessitates translocating an animal testing positive for *M. agassizii* antibodies, or showing signs of URTD, what is the best action for this individual, or the uninfected animals that it might interact with? If the animal were to be quarantined for six months in optimal conditions, could the animal recover? If the sick animal were relocated to an area with other negative animals, would this pose a threat to the other non-infected animals?

Our experiment analyses the progression of disease and clinical signs from *M. agassizii* among seropositive, seronegative, and experimentally infected tortoises.

### ***Description of basic immune response***

With the exception of a few differences, reptilian immune components and responses are similar to those found in mammals (Zimmerman et al., 2010). The immune system is comprised of molecules, cells, tissues, and organs that protect organisms in their defense against pathogens. Mammalian and reptilian immune systems are divided into two major parts: the innate response and the adaptive response (Zimmerman et al., 2010).

Innate immunity is the first line of defense used when an individual is colonized by a pathogen (Janeway et al., 2005). This network acts to prevent infection, is rapid in response, and is not specific to particular pathogens. Molecules and cells in innate

immunity include non-specific leukocytes, lysozymes, and peptides that activate a complement pathway and/or induce an inflammatory response. Three different complement pathways that ultimately produce the same results are found in mammals. The three pathways include: complement, lectin, and alternative pathways. They defend against pathogens by first attaching to and tagging, proteins to the surface of invading pathogens. The attached complement proteins release chemoattractants, cytokines and chemokines, which attract phagocytes that engulf the pathogen. Additionally, the complement proteins can produce a membrane-attack complex that result in destructive pores in the pathogens' outer protective membrane. While the complement cascade response does not rely on the presence of induced antibodies to be activated, this innate immune response can be initiated by natural antibodies (Janeway et al., 2005).

Reptiles share only two of the three complement pathways, classical and alternative (Zimmerman et al., 2010). Two other differences between mammalian and reptilian immune systems also have been documented. For example, in the reptilian inflammatory response, once heterophils accumulate, they persist longer in mammals than in reptiles (Zimmerman et al., 2010). The second difference is that while mammals can physiologically induce a fever, ectothermic reptiles rely upon behavioral manipulation of their body temperatures, which can be highly effective (Zimmerman et al., 2010).

Natural antibodies (NAbs) can be thought of as “a bridge” between innate and adaptive immunity. They circulate in plasma of pathogen-free individuals, and the production of these proteins is not dependent upon pathogen exposure (Baumgarth et al., 2005). NAbs have lower binding affinity than isotype-specific antibodies, are found predominantly in the IgM isotype class, and are believed to trigger an innate complement response (Baumgarth et al., 2005).

In contrast to the innate immune response, an adaptive immune response is much slower, and requires previous exposure to a specific pathogen (Janeway et al., 2005). Adaptive immune responses use both cell-mediated and humoral responses. T-cells, one type of lymphocyte, release cytokines that activate humoral activity. In cell-mediated immunity, one function of T-cells is essentially to tag foreign pathogens for elimination (Janeway et al., 2005). While one role for T-cells is to regulate antibody production, a role for B-cells is to produce antibodies. The purpose of antibody production from B-cells is to tag pathogens for neutralization or elimination (Janeway et al., 2005).

Antibody structure in mammals and reptiles is essentially the same (Zimmerman et al., 2010). Each antibody protein has two identical heavy chains, and two identical light chains with variable and constant regions. Antibodies are divided into classes based upon their structure and function. Mammals have five classes of antibodies: IgM, IgA, IgG, IgD, and IgE (Janeway et al., 2005). In reptiles, two main isotypes have

been identified, IgM and IgY, and a third sub-type is a truncated version of IgY, known as IgY( $\Delta$ )Fc (Zimmerman et al., 2010). In reptiles, the antibody class IgM is produced first in an immune response. Isotype switching occurs as the B-cell population becomes predominantly IgY-producing and as antibody-affinity towards the pathogen increases dramatically (Zimmerman et al., 2010). Isotype switching in reptiles has been shown to aid in reptilian humoral immunity (Zimmerman et al., 2010). Chelonian natural and acquired antibodies are confirmed to exist, (Benedict and Pollard, 1972; Chartrand et al., 1971; Coe, 1972; Herbst and Klein, 1995; Hunter et al., 2008) as well as isotype switching in desert tortoises (Hunter et al., 2008).

A primary response occurs when a host is first infected with a pathogen and responds via an induced-immune response. A secondary response, or a memory response, occurs after a host is re-exposed to that same pathogen. This immunological memory process has been reported to exist in some reptiles (Zimmerman et al., 2010). Immunological memory protects the host by reacting more quickly and by the rapid production of high levels of high-affinity antibodies (predominantly IgG in mammals and IgY in reptiles). Thus, increased binding-affinity to pathogens is a key characteristic both of isotype-switching in a primary response and of secondary, memory responses (Janeway et al., 2005).

There are several important distinctions between mammalian and reptilian induced immune responses. In reptiles, humoral immunity is slower and less robust, and a

secondary response does not always increase the amount and kind of antibodies in some reptiles and in others, isotype switching may be absent or delayed (Zimmerman et al., 2010). In mammals, exposure to a new pathogen typically will produce an immunological response within days, antibody titers will peak within several weeks, and titers will fall thereafter (Janeway et al., 2005). In reptiles, a primary immune response will often require weeks to months to occur, isotype switching is delayed, but antibodies remain detectable long periods after the response is initiated (Zimmerman et al., 2010).

An ELISA (enzyme-linked immunosorbent assay) was developed and used to infer infected which tortoises were infected with *M. agassizii*, through the proxy of measuring an immune response in individuals (rather than actually measuring the presence of the pathogen directly) (Schumacher et al., 1993). From this assay, captive desert tortoises targeted for translocation to new habitat in Nevada were tested for the presence of antibodies to *M. agassizii*. A positive test result from the ELISA for antibodies to *M. agassizii* (high antibody titers) was deemed a reason to euthanize the tortoises collected from development sites in Clark County, while animals with negative ELISAs (low titers) were translocated to a large fenced area called the Large Scale Translocation Site (Tracy et al., 2004). More than 3,230 federally listed and protected tortoises were euthanized between 1990 and 2007 (Sandmeier et al., 2009). This policy of euthanasia has since ceased, though

controversy regarding disease ecology of *M. agassizii* continues (Sandmeier et al., 2009).

Table 1. Terms and definitions used throughout the text regarding assays and classifications of tortoises.

Seronegative	Acquired antibodies are not detectable in blood
Seropositive	Acquired antibodies are detectable in blood
Seroconvert	Phase in infection when acquired antibodies against <i>M. agassizii</i> are first detectable in the blood
Infected tortoises	Tortoise that initially tested negative for acquired antibodies and was experimentally infected with <i>M. agassizii</i>
Positive tortoises	Tortoise that initially tested positive for acquired antibodies to <i>M. agassizii</i>
Negative tortoises	Tortoise that initially tested negative for acquired antibodies to <i>M. agassizii</i>
High WB band patterns	Band pattern indicating acquired and innate antibody production
Low WB band patterns	Band pattern indicating innate antibody production

There are a number of problems in using an ELISA test as the main criterion for determining the disease status of a tortoise infected with *Mycoplasma*. For example, the ELISA test used does not distinguish between acquired antibodies (indicating current or previous infection) and innate, natural antibodies (not indicating an infection) (Hunter et al., 2008; Sandmeier et al., 2009). In the past, animals testing high in antibody titers were deemed to be both infected and infectious. In reality,

animals with high ELISA titers (and thus ones doomed to be euthanized) could very well have been animals that had prior exposure to this pathogen, and had built up a strong immune response (high ELISA titers). These animals could also have been ones with very high levels of natural antibodies. In neither instance would these animals have necessarily been infected with *M. agassizii* at the time their blood was sampled for the presence of antibodies. In other words, these animals could have been the most resistant to mycoplasmal URTD, because they had high ELISA titers containing natural and acquired antibodies (Sandmeier et al., 2009).

In contrast to the only existing ELISA test being used to serologically diagnose tortoises for URTD (Schumacher et al., 1993; USFWS, 1994), the Western blot (WB) assay aims to increase accuracy in diagnosing animals (Hunter et al., 2008). The WB was developed to detect the number of different antibodies that a tortoise makes against *M. agassizii* proteins (Hunter et al., 2008). The Western blot, thus, extends the ELISA, which tests only for the total quantity of antibody that a tortoise is producing. In other words, the Western blot and ELISA, together, are able to reveal the diversity and quantity of antibodies involved in a tortoise's immune defense.

*Pertinent hypotheses and questions concerning epizootic URTD in desert tortoise.*

***Does transmission of disease occur?***

If infection with *M. agassizii* is readily transmissible, seronegative tortoises in close contact with seropositive tortoises should show clinical signs of URTD and seroconvert at a higher rate than will seronegative tortoises in contact only with other seronegative tortoises.

***Does seroconversion correlate with signs of disease in groups of animals?***

Tortoises with signs of URTD are more likely to seroconvert than those without signs of URTD.

While presence of antibodies to *M. agassizii* has been used to identify tortoises with URTD, particular clinical signs indicating upper respiratory tract disease (nasal discharge, wheezing breath, ocular discharge, exaggerated lethargy) have also been used to evaluate the health status of a tortoise (Jacobson et al., 1991). Complicating diagnosis, clinical signs of URTD are not unique to an infection solely caused by *M. agassizii*, but might also occur in response to infections with *M. testudinum*, *Pasteurella testudinis*, and various viruses (Jacobson et al., 1991).

Earlier studies (Brown et al., 1999a; Jacobson et al., 1991; Schumacher et al., 1997) examined the infectivity of URTD, but these studies are difficult to apply to patterns of URTD in wild populations, mainly due to small sample sizes, short study-times, and complexities of the tortoise immune system. Brown et al., (1999a) infected nine

gopher tortoises (*Gopherus polyphemus*) intranasally with *M. agassizii*. Seven of the nine infected tortoises made an adaptive antibody response after four weeks, and all nine infected animals made a response after eight weeks. At week four, only seven of the nine animals showed clinical signs, while at week eight only one tortoise failed to show clinical signs of URTD. These animals were euthanized and necropsied after sixteen weeks.

In a field study (Brown et al., 1999b), samples from a wild population of desert tortoises were tested for antibodies to *M. agassizii*. Over the course of four sampling seasons, ten radio-tracked tortoises were sampled, and ELISAs showed that there was no significant effect of sex or season on antibody production. In that study, clinical signs of URTD correlated with positive ELISA titers (an indicator of infection), with correlated variation of titers and clinical signs ranging from 5% to 42%. In one season, only 19% of the animals lacked clinical signs, and those had negative antibody titers, while in another season 52% of the population had both positive antibody titers and clinical signs. Therefore, there appeared to be a certain amount of unpredictability in the manifestation of URTD in terms of clinical signs and ELISA antibody titers.

***Does season influence antibody responses?***

If brumation (hibernation-behavior in reptiles) suppresses immunocompetence, then frequencies of seroconversion and appearances of clinical URTD will be greater immediately after brumation than will be the case at other times in the activity season.

Literature on ecological immunology in reptiles indicates that season and environmental temperature can influence immune function (El Ridi et al., 1998; Saad et al., 1990; Zimmerman et al., 2010). Because reptiles are ectothermic, their immunological responses to disease may be influenced by behavior and thermal biology more than in endothermic vertebrates (Zimmerman et al., 2010).

Alterations in ambient temperature, food availability, and activity are some consequences of the process of brumation, and it seems likely that these differences could influence both immunocompetence and disease dynamics.

Vitally important to their survival and optimal performance, ectotherms choose thermal environments to avoid achieving extreme body temperatures as they select environments that are as close to optimal as the environment provides (Huey and Kingsolver, 1989). Studies of the relationship between body temperature and immune response in reptiles indicate that there is often an optimal body temperature range for immunological function (Deeb et al., 1980; Green and Cohen, 1977; Zimmerman et al., 2010). Merchant et al. (2003) indicated that optimal immune responses in alligators (*Alligator mississippiensis*) to bacteria were between

5 °C and 40 °C, and they found that below 15 °C and above 40 °C the immune competence was depressed. In hibernating leopard frogs (*Lithobates pipiens*), individuals experienced decreased complement activity compared to their non-hibernating counterparts, with their activity returning to normal levels after they emerged from hibernation (Green and Cohen, 1977). Thus, evidence of an optimal body temperature range for immune function in ectotherms suggests that seasonal fluctuations in thermal environments, or resulting body temperatures, may be important for immunocompetence and disease ecology.

***Are there differences in transmission rates and clinical signs between females and males?***

If testosterone suppresses immunocompetence, then males will seroconvert more frequently than will females, and show more signs of clinical disease compared to females.

While most organisms do not share similar life history strategies, one could argue that choosing between reproduction and immune function is a fundamental trade-offs made within many or all life history strategies. Theories of sex influencing immune function have been around a long time, are widely shared among species, and affect many taxa of vertebrates (Klein, 2004; Martin et al., 2008; Zimmerman et al., 2010; Zuk and McKean, 1996). Sex Gender also appears to influence the immunological system in reptiles, likely mediated through fluctuations in androgens,

estrogen, glucocorticoids, and other hormones (Martin et al., 2008) and energy budgets, which can be more limiting in gravid females.

The “handicap principle”, first proposed by Zahavi (1975), was later refined to the “immunocompetence handicap” by Folstad and Karter (1992). The hypothesis states that male vertebrates, which have higher amounts of testosterone than females, should have higher reproductive success due to exaggerated sexual characteristics, but additionally they should face increased parasitic infection, compared to their male competitors who have lower testosterone levels (Folstad and Karter, 1992). Male immunocompetence might also be explained by differential exposure and differential susceptibility to pathogens. In particular, increased testosterone levels are linked to aggressive behavior and dispersal, both of which increase successful mating but also increase exposure to pathogens (Zuk, 1996).

Rostal et al. (1994) describe hibernation and mating patterns in desert tortoises. Desert tortoises typically emerge from hibernation in April, and remain active until October, when they enter seasonal dormancy from November to March. Although tortoises may mate throughout the year, there are two major mating seasons. A brief period occurs in April and a longer period occurs in August and September. Desert tortoise hormone profiles correspond closely with their seasonal reproductive cycles (Lance et al., 2001), and suggest that the “immunocompetence handicap” might be applicable to this species.

## Methods

### *Experimental design*

Twenty-three outdoor pens were built at the Desert Tortoise Conservation Center in Clark County, NV, using 1.75 cm hardware cloth for the walls of the pens. Each pen was 80 x 90 meters, with a 2-m alley between each pen to ensure no contact among tortoises from pen-to-pen. The pens were built with sensitivity not to harm the enclosed vegetation so that the conditions inside the pens could be regarded as semi-natural. Each pen was equipped with twelve artificial burrows, and each pen was populated with twelve tortoises according to an experimental protocol outlined below. Tortoises remained in the pens for a total of 24 months.

Table 2.

<b>Tortoise group:</b>	<b>Refers to:</b>
Negative	All 132 tortoises, ELISA seronegative
Infected	All 72 tortoises, ELISA seronegative, experimentally infected
Positive	All 72 tortoises, ELISA seropositive
<b>Group treatment:</b>	
Neg/Neg (N/N)	Control group, all Negative, 5 pens
Neg/Pos (N/P)	Negative & Positive paired, 6 pens
Neg/Inf (N/I)	Negative & Infected paired, 6 pens
Pos/Inf (P/I)	Positive & Infected paired, 6 pens

Common terms, with definitions, used throughout the study.

This experiment was designed as a repeated-measures, block design. Each pen contained two groups of six tortoises (12 tortoises per pen summing to 276 tortoises over all). Every pen contained 8 females and 4 males, and any manipulative treatment was divided equally between the sexes. All females used in the experiment were at least 200 mm maximum carapace length (MCL) and males chosen for the experiment were between 220 and 280 mm MCL to ensure that animals were mature adults. The size of tortoises picked for each pen was ordered so that each pen had very similar-sized tortoises.

Before being assigned to a pen, each of the 276 wild-caught tortoises (collected by the Clark County Desert Conservation Program) was tested twice with ELISA for an immune response to *M. agassizii* by scientists at the University of Florida, Department of Infectious Diseases and Pathology (see Brown et al., 1994; Brown et al., 1999b). Tortoises were assigned to one of three treatments. The first treatment

group, hereafter referred to as Negative, was a “control group” consisting of tortoises all testing negative by ELISA twice, and all showed no clinical signs of URTD. The second tortoise group, hereafter referred to as Positive, tested ELISA positive twice, yet showed no outward clinical signs of URTD. The third tortoise group, hereafter referred to as Infected, tested ELISA negative twice, showed no clinical signs of disease, and then had their nares experimentally flushed with a solution of 0.5 ml  $10^8$  cells of *M. agassizii* in SP4 broth prior to release into the research pens (late September 2003). Tortoises in groups Negative and Positive had their nares flushed with 0.5 ml of SP4 broth as a control against the act of flushing nares. Five of the pens were assigned to be controls, i.e. contained two groups of Negative tortoises (N,N). Six pens consisted of six Negative and six Positive tortoises (N,P). Six pens consisted of six Negative and six Infected tortoises (N,I) and the remaining six pens consisted of six Infected and six Positive tortoises (I,P).

The initial ELISA used to assign animals to treatments (the University of Florida ELISA) differs slightly from the ELISA used in the rest of this study (the University of Nevada ELISA). Therefore, a random subset of animals from each treatment was double-checked to insure that the ELISAs gave the same result by using a Western blot on blood samples collected before the experiment began (Hunter et al. 2008). The Western blot is the most thorough test available to assess the production of induced antibodies to *M. agassizii* in desert tortoise plasma samples (Hunter et al. 2008).

For the rest of the experiment, all samples were first tested with a polyclonal ELISA (U. of Nevada ELISA) to assess amount of baseline antibodies. A seroconversion was deemed to have occurred when ELISA titers increased three-fold or more above the baseline value in any given tortoise (Origgi et al. 2007). Tortoises that appeared to have seroconverted using this ELISA were then re-tested with isotype-specific ELISAs to IgM and IgY. These two tests were used to confirm seroconversion in the tortoise through either elevated production of IgY isotypes (indicative of a mature induced antibody response) or a three-fold increase in IgM antibodies (indicative of an early induced antibody response).

### ***Blood sample collection***

Tortoises in all pens were sampled for clinical signs of URTD (Schumacher et al., 1997), and samples of their blood were taken every four weeks for 22 consecutive months (except during months of brumation). Tortoises were handled with examination gloves, and all sampling equipment was sterilized to minimize the risk of disease transmission due to sampling. Blood samples (0.5 - 2 ml) were obtained by brachial venipuncture using a 25 gauge, 2 cm needle with a 3 ml syringe (Becton-Dickenson). The blood was transferred to a Microtainer plasma separator tube using lithium heparin, and samples were stored on ice until they were processed in the lab at the end of the day (Jacobson et al., 1992). Each sample was centrifuged for 5 min, and the plasma was removed and frozen in 2.0 ml vials (Sarstedt) at -30°C.

### ***Serological assays***

*Western Blot (used to confirm antibody-status)*

Protein from 20  $\mu$ l *M. agassizii* antigen (25  $\mu$ g/ml) was separated by gel electrophoresis using 10% tris-SDS for 53 min at 180 volts (Criterion precast gel, Bio-Rad, Hercules, CA). Proteins were transferred from gel to nitrocellulose paper (Bio-Rad, Hercules, CA) for 40 min at 80 volts. Nitrocellulose paper was then blocked in a solution of TBS and 5% Carnation non-fat dry milk for 60 min at room temperature on an agitator plate. Using 50 ml plastic screw-top tubes, 70  $\mu$ l of tortoise serum sample was added to a 7 ml solution of 5% non-fat milk in 5% Tween-TBS for a 1:100 dilution. Nitrocellulose paper was transferred to tubes containing tortoise samples and incubated overnight at 4 °C on an agitator plate. Nitrocellulose paper was washed four times in TBST, and placed on agitator plates between each wash for 5 min. Each tube received a 10 ml solution of rabbit anti-tortoise Ig, at a dilution of 1:5,000 in TBST and 5% non-fat dry milk, and incubated at room temperature for one hour on an agitator plate. Nitrocellulose paper was again washed four times, as described above, goat anti-rabbit IgG whole molecule horseradish peroxidase conjugated (Zymed, San Francisco, CA) was added to each tube at a 1:5,000 dilution in TBST and 5% non-fat dry milk, and incubated for 1h in the dark on an agitator plate at room temperature. Nitrocellulose paper was washed four times, as described above, and a 2 ml solution of metal enhanced DAB (Thermo Scientific, Rockford, IL) was used to develop protein bands for 5 min.

*ELISA (to assess levels of all tortoise antibodies)*

We predominantly used a polyclonal ELISA that measured total antibody production ("University of Nevada ELISA"; described in detail in Hunter et al. 2008). Briefly, 50  $\mu$ l of antigen containing 10  $\mu$ g/ml *M. agassizii* strain PS6 (Rockville, MD: ATCC 700616) in PBS was used to coat 96-well plates (Immulon NUNC; Fisher Scientific, Fairlawn, NJ). Plates were sealed and incubated overnight at 4 °C. Plates were then washed four times with PBS and blocked with 200  $\mu$ l per well of 5% Carnation non-fat milk in PBS for two hours at 4 °C. Plates were then washed four times with PBS-Tween (0.05% Tween 20). Tortoise plasma samples were serially diluted in PBS-Tween at concentrations from 1:100 to 1:100,000. 50  $\mu$ l of tortoise sample was added to each well and incubated overnight at 4 °C. Plates were washed four times with PBS-Tween, 50  $\mu$ l of diluted (1:10,000) polyclonal rabbit anti-tortoise antibody was added and incubated at room temperature for one hour. Plates were once again washed four times with PBS-Tween, 50  $\mu$ l of diluted (1:5,000) goat anti-rabbit IgG conjugated to whole molecule horseradish peroxidase (Zymed, San Francisco, CA) was added to each well and incubated in dark at room temperature for one hour. Plates were washed four times with PBS-Tween and 50  $\mu$ l TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) was added to each well to develop. Plates were incubated for 22 min at room temperature, 50  $\mu$ l of 1N hydrochloric acid was added to each well, and plates were immediately read at 450 nm on a Spectra-Max micro-ELISA reader (Molecular Devices, Hercules, CA) to determine optical density. Each tortoise sample was run in duplicate. Negative control samples were used from pooled sera of sixteen desert tortoises housed at UNR. These tortoises have never been exposed to natural populations, thus have never been exposed to mycoplasmas.

To account for plate-variation, each plate was equally adjusted to the average absorbance of each serially-diluted, negative control sample. The dilution vs. the absorbance for each experimental sample was plotted on a  $\log_{10}$  scale, and a best-fit line was used to approximate the linear portion of each curve. End-point titers were determined by using cut-off values of 0.5, and titers were  $\log_{10}$ -transformed to approximate a normal distribution.

#### *Isotype-specific ELISAs*

Polyclonal ELISAs specific for IgM or IgY are described in detail in Mohammadpour (PhD dissertation, 2011). All methods were similar to those for the total-antibody ELISA (U. of Nevada ELISA), with the exception that the secondary antibody was isotype specific.

*Experimental Design* – The overall experiment was set up as a three-level, nested hierarchical linear model looking at rates of seroconversion, clinical signs of URTD, and mortality at four treatment levels, with six pens (fixed) per treatment.

Individual tortoises (random effect) in each pen were sampled at six different times during the two-year experiment, where the tortoise was nested within each treatment level.

We analyzed end-point ELISA titers to answer questions regarding disease transmission, as well as long-term serological status. We assessed any signs of URTD

(e.g., nasal discharge (good/clear, wet, purulent exudate), eyes (normal appearance or ocular discharge), and breathing (wheezing or smooth), and these signs were analyzed in relation to serological status of each tortoise.

### ***Statistical Analysis***

Frequencies of seroconversion and clinical signs were evaluated as binomial data (positive/negative). All binomial data were analyzed with contingency tables, and means of measures (e.g., titers) were arcsine-square root transformed and analyzed by ANOVA. P values of  $\leq 0.05$  were considered significant. All statistical analyses were performed using Aabel (ver. 3.0.2; Gigawiz Ltd.).

Scores from the binomial response variables, positive or negative for clinical signs or seroconversion, were treated summed for each tortoise, and by pen treatment. Contingency tables were used to analyze differences among treatments. To assess differences in the means of clinical signs and seroconversion from each individual pen, data were arcsine-square root transformed and analyzed by analysis of variance. To assess the extent to which clinical signs predict seroconversion, each tortoise was evaluated as positive or negative for clinical signs, and positive or negative for seroconversion. Contingency tables were used to assess similarity in these two measures. To assess effects of gender on clinical signs and seroconversion, a contingency table was used to compare response variables for both genders. To evaluate effects of hibernation on clinical signs and seroconversion rates, the response variables were summed and analyzed with contingency tables.

## Results

### Effects of treatment

**Clinical Signs of URTD:** When Negative tortoises were paired with Positive or Infected tortoises, versus with other Negatives, there was a significant effect on Negatives ( $\chi^2 = 8.61$ ,  $df = 2$ ,  $p = 0.014$ ; Table 3). The greatest increase in clinical signs of URTD occurred when Negatives were paired with Positives (Table 3). When Infected tortoises were paired with Negatives or Positives versus Negatives, there was a significant effect on Infecteds ( $\chi^2 = 9.42$ ,  $df = 1$ ,  $P = 0.002$ ; Table 3). Again, the largest increase in clinical signs of URTD occurred when Infecteds were paired with Positives (Table 3). When Positives were paired with Negatives and Infecteds, there was no significant effect on Positives ( $\chi^2 = 2.42$ ,  $df = 1$ ,  $P = 0.12$ ; Table 3). There were differences in frequencies of clinical signs among the treatment groups (NN, NP, NI, and IP:  $\chi^2 = 32.53$ ,  $df = 3$ ,  $P < 0.0001$ ), and significant effects occurred among tortoise groups (N, I, and P:  $\chi^2 = 12.08$ ,  $df = 2$ ,  $P = 0.002$ ; Table 4).

Table 3. Group treatment effects on clinical signs (%) divided by tortoise groups.

	Grouping Treatment	Number of Tortoises	% with signs
<b>Negative</b>	<b>NN</b>	60	43
	<b>NI</b>	36	33
	<b>NP</b>	36	67
<b>Infected</b>	<b>NI</b>	36	28
	<b>IP</b>	36	67
<b>Positive</b>	<b>NP</b>	36	61
	<b>IP</b>	36	81

Table 4. Difference of frequencies of clinical sign (%) among tortoise groups was significant.

Tortoise group	Number of Tortoises	% signs
<b>Negative</b>	132	47
<b>Infected</b>	72	47
<b>Positive</b>	72	71

**Seroconversion:** There was no significant difference among treatment groups on frequencies of seroconversion. Negative tortoises seroconverted at the same frequency in all three group treatments (NN, NP, NI:  $\chi^2 = 0.02$ ,  $df = 2$ ,  $P = 0.99$ ; Table 5), as did Infecteds in their two group treatments (NI, PI:  $\chi^2 = 0$ ,  $df = 1$ ,  $P = 1.0$ ). Seroconversions between Negatives and Infecteds were not different ( $\chi^2 = 0.13$ ,  $df = 1$ ,  $P = 0.718$ ). In general, seroconversion was roughly 20% in all groups.

Table 5. Seroconversion (%) in treatment groups.

Tortoise Group	Grouping Treatment	Number of Tortoises	% Converting
Negative	NN	60	23
	NI	36	22
	NP	36	22
Infected	NI	36	19
	NP	36	19

### Clinical signs of URTD as predictors of seroconversion.

The appearance of clinical signs neither predicts seroconversion in Negative tortoises ( $\chi^2 = 3.6$ ,  $df = 1$ ,  $P = 0.058$ ; Figure 1) nor in Infected tortoises ( $\chi^2 = 0.34$ ,  $df = 1$ ,  $P = 0.56$ ; Figure 2)

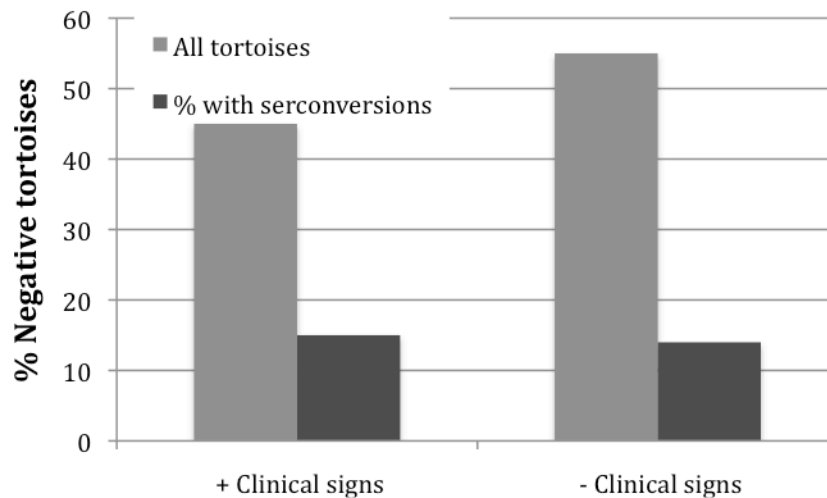


Figure 1. The frequency (%) of Negative tortoises that either showed or lacked clinical signs, and the frequency of seroconversion.

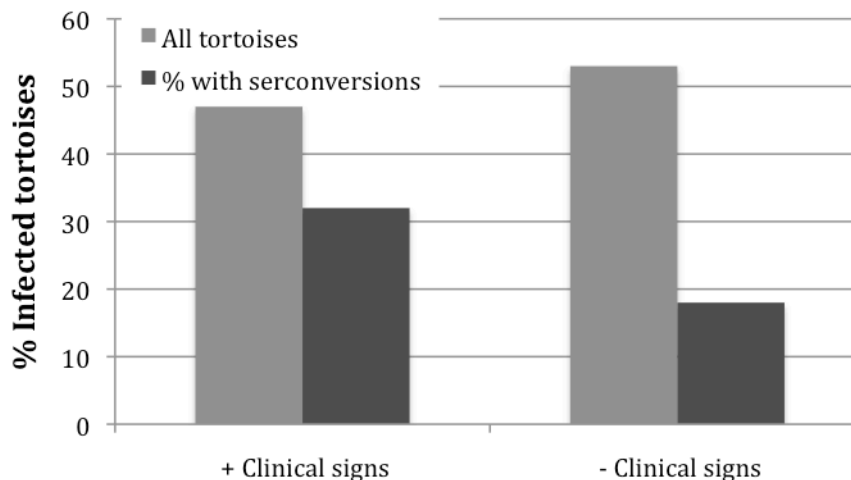


Figure 2. The frequency (%) of Infected tortoises that either showed or lacked clinical signs, and the frequency of seroconversion.

**Progression of disease:** There is a peak appearance of new clinical signs in the spring following brumation in all groups of tortoises (Figure 3), but the peak appearance of seroconversions does not occur until a year later (Figure 4). Total clinical signs, including new and persisting/reoccurring signs, are shown in Figure 5.

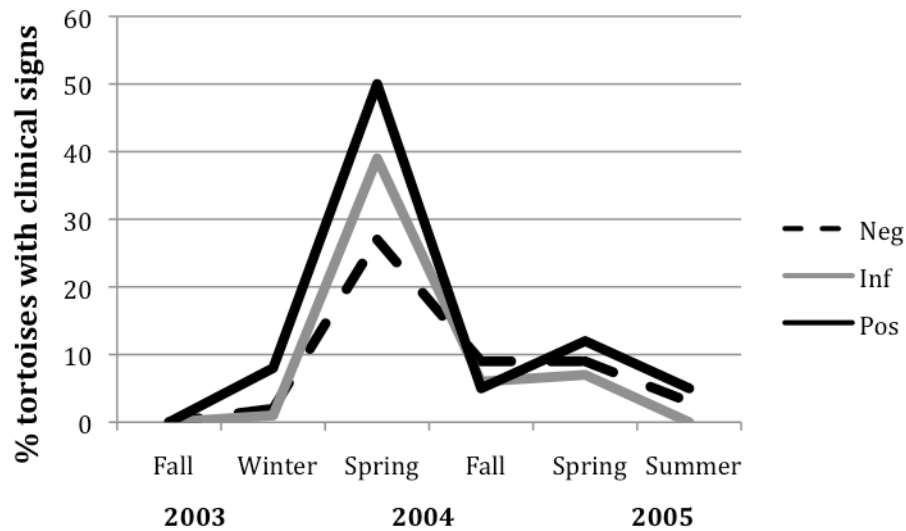


Figure 3. First appearance of clinical signs by season in tortoises in the three different groups.

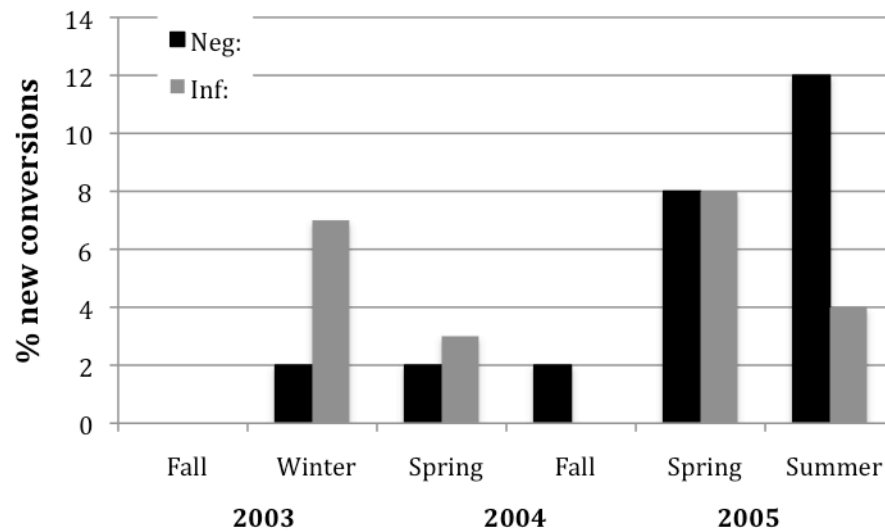


Figure 4. New seroconversions by season for the two tortoise groups that were initially seronegative.

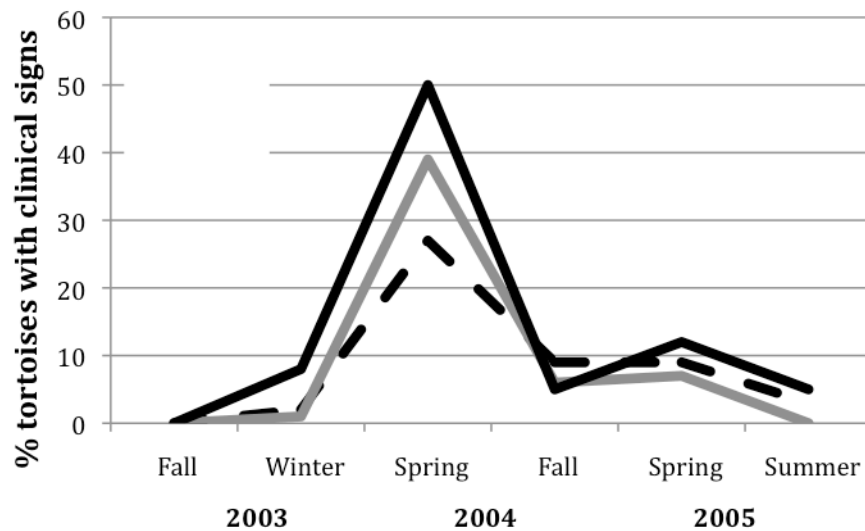


Figure 5. Frequency (%) of clinical signs of URTD by season in three groups of tortoises (signs of URTD include new, re-occurring, and persistent cases).

### Effects of brumation

**Clinical signs:** There was a statistically greater appearance of clinical signs of URTD among Negative tortoises in spring 2005 compared to fall 2004 ( $\chi^2 = 4.46$ ,  $df = 1$ ,  $P = 0.035$ ; Figure 6, Table 6), and these frequencies of clinical signs were not related to group treatment (NN, NP, and NI:  $\chi^2 = 3.76$ ,  $df = 2$ ,  $P = 0.153$ ; Figure 6). Figures 7 and 8 show the frequency of clinical signs of URTD before and after brumation for Positive and Infected tortoises.

Table 6. Seasonal pattern of the appearance of signs and seroconversion.

<i>New &amp; reoccurring Clinical signs</i>	<b>NEG n (%)</b>	<b>INF n (%)</b>	<b>POS n (%)</b>
<b>spring 04</b>	40 (31%)	27 (44%)	28 (42%)
<b>fall 04</b>	27 (23%)	24 (37%)	25 (39%)
<b>spring 05</b>	41 (35%)	30 (52%)	30 (47%)
<i>First Seroconversion</i>	<b>NEG n %</b>	<b>INF n %</b>	
<b>spring 04</b>	3 (3%)	2 (8%)	
<b>fall 04</b>	2 (2%)	0 (0%)	
<b>spring 05</b>	9 (8%)	5 (8%)	

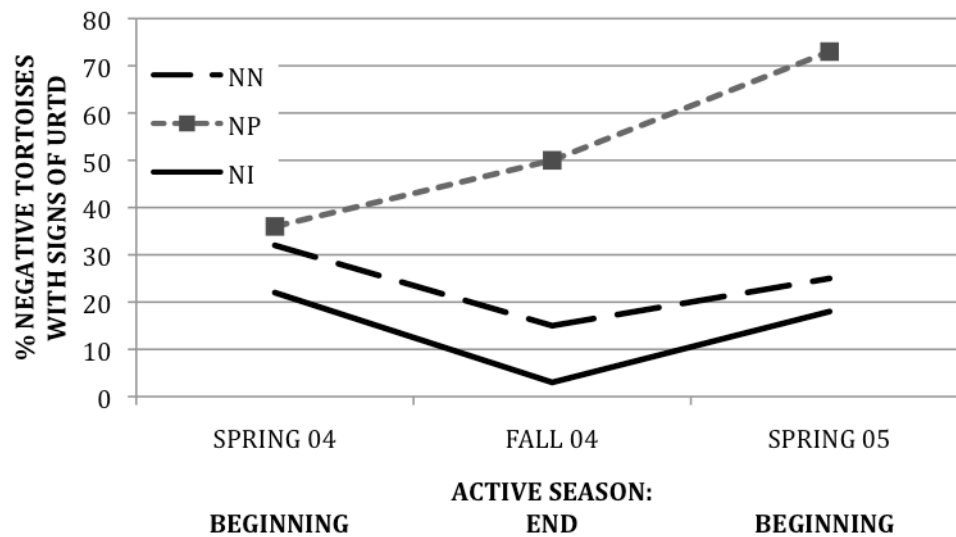


Figure 6. Frequency (%) of clinical signs (new and persistent) by season (before and after brumation) in Negative tortoises in the three group treatments.

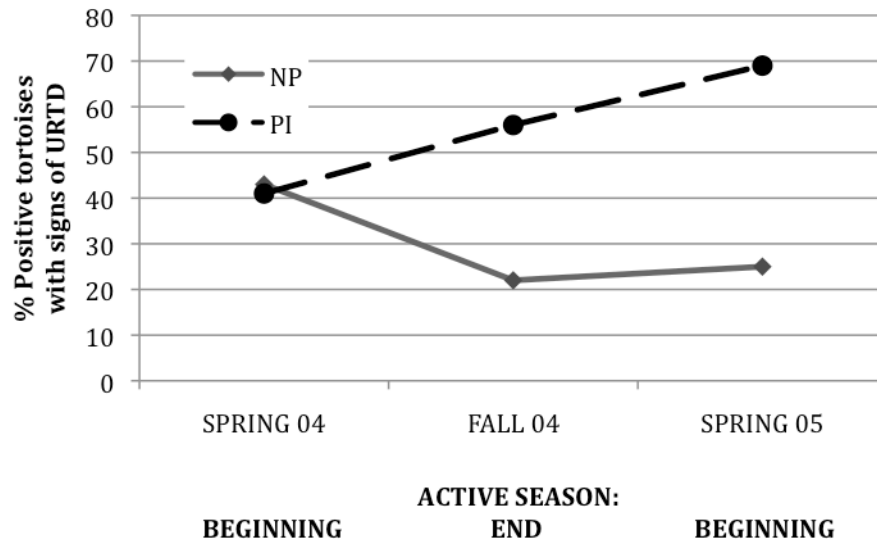


Figure 7. Frequency (%) of clinical signs before and after brumation in Positive tortoises in the two group treatments.

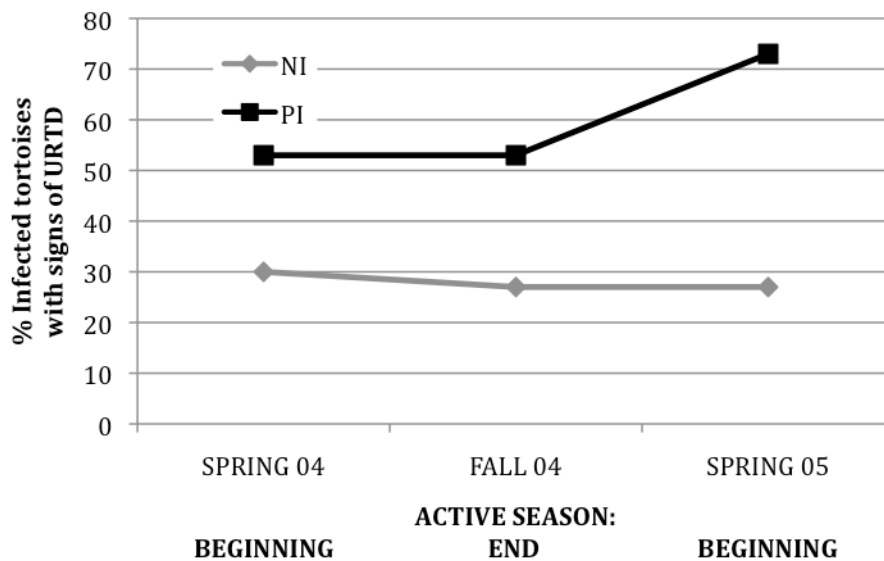


Figure 8. Frequency (%) of clinical signs by season (before and after brumation) in Infected tortoises in the two group treatments.

**Seroconversion:** Negative tortoises more frequently seroconverted for the first time in spring compared to fall ( $\chi^2 = 5$ ,  $df = 1$ ,  $P = 0.025$ ; Figure 4; Figure 9).

Seroconversion rates before/after brumation were not affected by group treatment in Negative tortoises (NN, NP, NI:  $\chi^2 = 1.67$   $df = 2$   $P=0.434$ ; Figure 9).

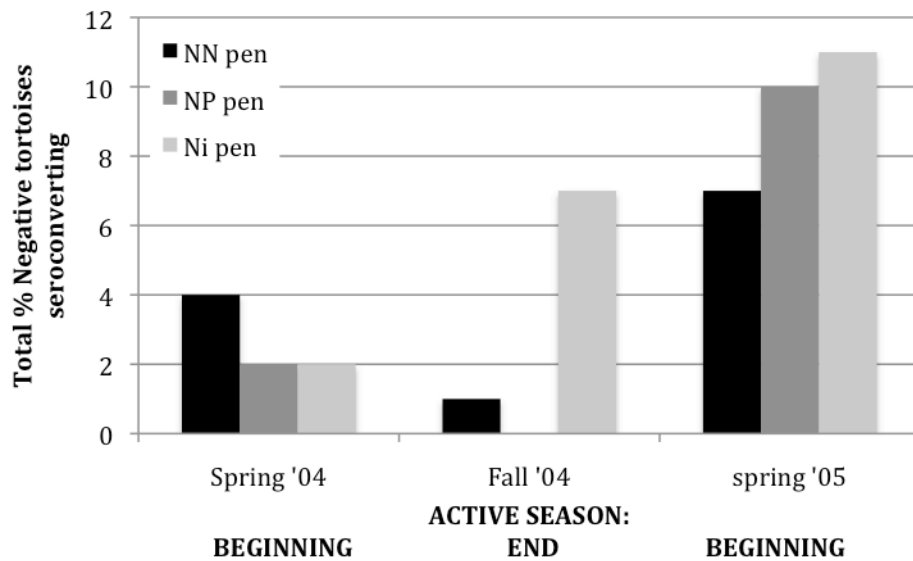


Figure 9. Frequency (%) of seroconversion by season (before and after brumation) in Negative tortoises in the three group treatments.

**Season and sex:** There were no differences between Negative males and females in their frequencies of clinical sign in spring and fall regardless of tortoise group ( $\chi^2 = 0.1$ ,  $df = 1$ ,  $P = 0.752$ ). Similarly, there was no difference in seroconversion rates between Negative males and females ( $\chi^2 = 0.15$ ,  $df = 1$ ,  $P = 0.699$ ; Table 7).

Table 7. Frequency (%) of seroconversion and clinical signs in spring and fall seasons for all tortoises, separated by sex and treatment.

	Spring 04	Fall 04	Spring 05	Spring 04	Fall 04	Spring 05
<b><i>Females</i></b>	<b>Seroconversion</b>			<b>Clinical Signs</b>		
<b>NEG</b>	2 (5%)	1 (3%)	7 (19%)	23 (27%)	15 (19%)	25 (33%)
<b>INF</b>	2 (5%)	0 (0%)	2 (5%)	16 (37%)	15 (35%)	20 (48%)
<b>POS</b>				15 (33%)	14 (31%)	17 (38%)
<b><i>Males</i></b>	<b>Seroconversion</b>			<b>Clinical Signs</b>		
<b>NEG</b>	1 (5%)	1 (6%)	2 (13%)	17 (37%)	12 (31%)	16 (51%)
<b>INF</b>	0 (0%)	0 (0%)	3 (17%)	11 (52%)	9 (43%)	10 (56%)
<b>POS</b>				13 (62%)	11 (58%)	13 (68%)

### Effects of sex

**Clinical signs:** The overall proportion of females and males displaying clinical signs was not different ( $\chi^2 = 1.98$ ,  $df = 1$ ,  $P = 0.16$ ; Table 8). There was also no significant difference between the sexes displaying clinical signs when tortoises were divided into their groups: Negative (female 45%, male 53%), Infected (female 44%, male 54%), Positive (female 67%, male 79%) (Figure 10). For Negative females and males, there was also no effect of group treatment on rates of clinical signs (Females in NN, NP, NI vs Males in NN, NP, NI:  $\chi^2 = 0.27$ ,  $df = 2$ ,  $P = 0.874$ ).

Table 8. Frequency (%) of clinical signs between females and males.

Sex and Tortoise group	Number of Tortoises	% Clinical Signs
<b>Females</b> (all groups)	<b>184</b>	<b>50</b>
NEG	88	45
INF	48	44
POS	48	67
<b>Males</b> (all groups)	<b>92</b>	<b>60</b>
NEG	44	53
INF	24	54
POS	24	79

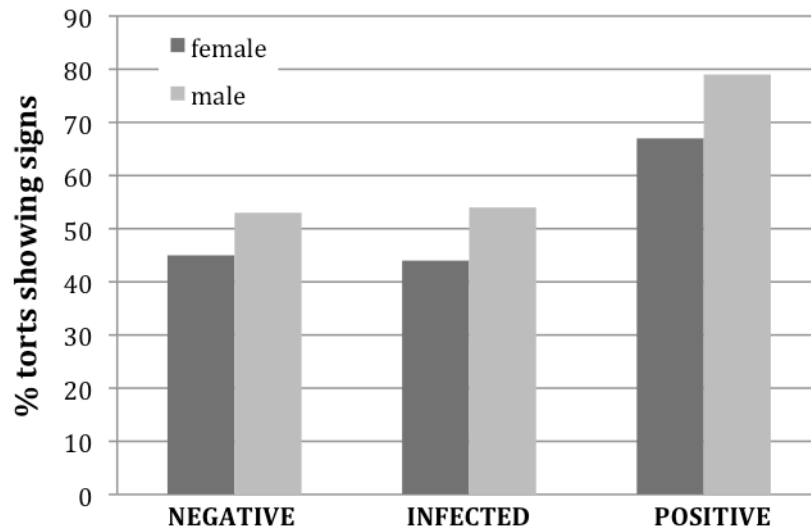


Figure 10. Frequency (%) of clinical signs in female and male desert tortoises.

**Seroconversion:** Within all tortoises, frequencies of seroconversion were not different for females and males ( $\chi^2 = 0.01$ ,  $df = 1$ ,  $P = 0.920$ ; Table 9). Similarly, Negative females and males did not have different seroconversion rates (21% vs 27%,  $\chi^2 = 0.44$ ,  $df = 1$ ,  $P = 0.51$ ), and frequency of seroconversion for the sexes in each of the three group treatments were not different (Females in NN, NP, NI vs Males in NN, NP, NI:  $\chi^2 = 4.36$ ,  $df = 2$ ,  $P = 0.119$ ).

Table 9. Frequency (%) seroconversion between females and males.

<b>Tortoise group</b>	<b># of deaths</b>	<b>(%) of group</b>
NEG	8 OF 132	(6%)
POS	5 OF 72	(7%)
INF	12 OF 72	(17%)
<b>Sex</b>		
MALES	14 OF 92	(16%)
FEMALES	11 OF 184	(6%)

### Tortoise survival

The appearance of clinical signs did not predict survival of tortoises ( $\chi^2 = 0.04$ ,  $df = 1$ ,  $P = 0.842$ ; Table 10). By far, the majority of tortoises that died did so in the first spring after the brumation season (Figure 11). Infected tortoises died more frequently than did Negative and Positive tortoises ( $\chi^2 = 6.89$ ,  $df = 2$ ,  $P = 0.032$ ; Table 11) and males died more frequently than females ( $\chi^2 = 5.28$ ,  $df = 1$ ,  $P = 0.022$ ; Table 11).

Table 10. The overall numbers of tortoises with/without clinical signs compared to numbers of deaths.

	Dead	Alive
Signs	14	130
No Signs	11	121

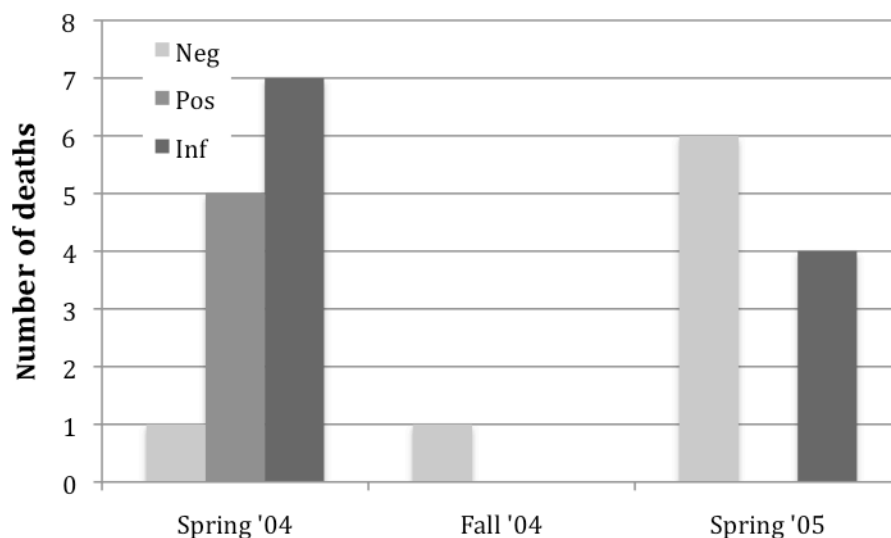


Figure 11. Number of deaths before and after brumation in Negative, Positive, and Infected tortoises.

Table 11. Frequency (%) of deaths among tortoise groups, shown for both sexes.

<b>Tortoise group</b>	<b># of deaths</b>	<b>(%) of group</b>
NEG	8 OF 132	(6%)
INF	12 OF 72	(17%)
POS	5 OF 72	(7%)
<b>Sex</b>		
MALES	14 OF 92	(16%)
FEMALES	11 OF 184	(6%)

## Discussion

### *Patterns of appearance of URTD in experimental tortoises (ELISA-negative tortoises)*

Two hundred and seventy six tortoises were used in this experiment. All tortoises were held for observation for any clinical signs of URTD, and they were tested twice (by ELISA) for antibodies to *M. agassizii* 3-4 months prior to inclusion into this study. These tortoises were deemed to be free of all clinical disease signs and free of any serological evidence of an immune response to *M. agassizii*, so we referred to these tortoises as “Negative”. Of these 132 Negative tortoises, 60 were divided between five pens, creating our control group. Over the two years of this study, 43% of these tortoises showed clinical signs of URTD and 23% seroconverted, indicating that they had an immune response to an infection with *M. agassizii*. From what source did these tortoises obtain *Mycoplasma*? The most parsimonious explanation is that they, the Negative tortoises used as the control group, possessed the pathogen from the beginning regardless of all reasonable indications that they were free of *Mycoplasma* at the outset of the experiment. Thus, this experiment indicates that contracting mycoplasmosis does not require that a tortoise come into contact with a serologically positive (infected) tortoise.

An alternative explanation for the high rates of seroconversion and clinical signs in the Negative tortoises penned with Negative tortoises is in the inherent inability of the initial ELISA used to determine that tortoises were unexposed to the pathogen-

free. ELISAs, including both those that test total antibody levels, and those designed to assess isotype-specific antibody levels, are incapable of discerning between an infected animal and a previously infected animal and non-infected animal (Brown et al., 1995; Hunter et al., 2008; Lederle et al., 1997; Sandmeier et al., 2009). In fact, in this system, ELISAs can also be very ineffective at detecting the presence of the pathogen, as animals frequently appear to be colonized with *M. agassizii* in the absence of an immune response (DuPre et al., 2011).

Sandmeier et al. (2009) have clarified definitions of mycoplasmal infected, colonized, and naïve animals. A mycoplasmal naïve desert tortoise does not house the pathogen, nor would it show clinical signs, and truly naïve individuals may be rare in nature. A colonized animal would have the pathogen on a mucosal surface, but it may not be immunologically reactive, so low antibody titers (consisting only of Nabs) would indicate no previous or current “infection”. Thus, the “colonist” microbe exists as a commensal rather than a pathogen. An infected tortoise is one in which the pathogen has sufficiently invaded an epithelial barrier to cause an induced immune response, and therefore, the tortoise would exhibit clinical and serological changes. However, immune responses, including antibody production and clinical signs, are related to the stage of infection or immunological capacity of the host (Schmid-Hempel, 2003; Zimmerman et al., 2010). If an animal is tested too early in an infection, or if the tortoise is immunocompromised, the absence of clinical signs or antibodies could mislead one to assume that the tortoise should be labeled “Negative”. Therefore, it is possible that some of the “Negative” tortoises in

our study, which were housed with other Negative tortoises, were colonized with *M. agassizii*, but the progression of infection and subsequent immune response had yet to occur.

Similar to a tortoise appearing to be naïve vis-à-vis antibodies to the pathogen when tested too early in an infection, a recovering tortoise can appear to be infected well after the tortoise has ceased to be infectious (Hunter et al. unpublished, Sandmeier et al., unpublished). As stated earlier in describing reptilian immune responses, antibody titers do not rapidly decrease, but instead, these titers can remain elevated for prolonged periods (Zimmerman et al., 2010). Thus, the initial ELISA assays could have misled categorizing tortoises as Negative or Positive, and this could have affected results in the experiments reported upon here.

Still another possible explanation for the high rates of infection and URTD signs among our Negative tortoises is that the design of the experiment could have produced exaggerated results. The highest density of desert tortoises in the Mojave reportedly could be as high as about 85 tortoises per km<sup>2</sup> (Tracy et al. 2006). In contrast, the density of tortoises in the pens in this study was 3,840 tortoises per km<sup>2</sup>. Higher host population densities can directly increase incidence of disease (Collinge and Ray, 2006). Additionally, the high density of tortoises in pens, and the frequent handling of tortoises, could have contributed to the high stress levels and high incidence of disease. For example, tortoises were evaluated during the active season for clinical signs, and blood samples and measurements for growth were

taken. Increased stress may have contributed to reduced immunocompetence (Nelson and Demas, 1996), and thus, affected disease dynamics among Negative tortoises.

*Patterns of Appearance of URTD in Positive tortoises (initially ELISA-positive tortoises)*

At the outset of the experiment, 72 tortoises had no clinical signs of URTD, yet tested positive for high levels of antibodies by ELISA for an immune response to *Mycoplasma*; we called these tortoises “Positive”. Thirty-six Negative tortoises were placed in six pens with the 36 Positive tortoises. Sixty-seven percent of these Negative tortoises developed signs of URTD, which was a statistically greater percent of tortoises developing signs of URTD than was the case when Negative tortoises were paired with other Negative tortoises. Thus, contact with tortoises that have evidence of an infection with *Mycoplasma*, increases the probability that uninfected tortoises will show signs of URTD.

From this same group of 36 Negative and 36 Positive tortoises, 22% percent of Negative tortoises in contact with Positive tortoises seroconverted, indicating an immune response that is concordant with an infection with *Mycoplasma*.

Interestingly, this was not a statistically higher seroconversion frequency than that among control tortoises (Negative tortoises paired with Negative tortoises: 23%).

This result is antithetical to the result with clinical signs, but this may be explained

by the unusual dynamics of the reptilian immune response. Specifically, the appearance of clinical signs of URTD was greatest after the first brumation in spring 2004. However, the first peak occurrence of seroconversion was in spring 2005, which was nearly two years after the beginning of the experiment. There is no way to know if still more tortoises could have seroconverted after the termination of the experiment. Had it been known that the onset of an immune response could take more than 1.5 years, the experiment could have been extended beyond the two years of our study.

Dynamic changes in signs and seroconversion in different treatment groups including Positives is of interest (Figures 7, 8, 9). For example, Negative and Infected tortoises paired with each other experience a seasonal decrease in clinical signs. However, the frequencies of clinical signs experienced by either of these groups do not decrease when these tortoises are paired with Positives. For Negatives paired with Positives, clinical signs monotonically increase both before and after brumation. Signs in Infecteds paired with Positives similarly monotonically increase both before and after brumation. Interestingly, Positive tortoises paired with Negatives show a decrease in clinical signs prior to brumation, and emerge from brumation with no significant increase in signs. This is simply a mystery as there is no obvious reason why Positives would show reduced signs when paired with Negatives or Infecteds. It seems that the presence of Positive with Negatives lessens the frequency of clinical signs in Positives, but Positives with Infecteds increases frequency.

*Patterns of Appearance of URTD in Infected tortoises (initially ELISA-negative, experimentally infected tortoises)*

Seventy-two tortoises that neither showed clinical signs of URTD, nor antibodies to *M. agassizii*, were intra-nasally inoculated with 360,000,000 cells of *M. agassizii* using the methods of Brown et al. (1994). These tortoises were called “Infected”. However, the percentage of these tortoises that subsequently showed clinical signs of URTD was not statistically different from the percentage exhibiting signs among the Negative tortoises (controls) in our study, and the progression of disease in this group also was not different from that in Negative (control) tortoises. This is enough for us to suspect that our methods for inoculating pathogen cells could have been flawed, and indeed a newer method for inoculating *Mycoplasma* into tortoises appeared after our study (Johnson et al., 2006; Mohammadpour, PhD dissertation, 2011). It is harder to explain the increased mortality rates in the Infected tortoises. Specifically, a statistically higher percentage of our Infected tortoises died compared to Negative and Positive tortoises (17% compared to 6% and 7% in Negative and Positive tortoises respectively;  $P = 0.032$ ). We have no explanation for this mysterious result.

An interesting dynamic between an immediate or delayed infection, in this study and in other studies, should be noted. The progression of URTD in experimentally infected tortoises has been documented in desert and gopher tortoises (Brown et al.,

1994; Brown et al., 1999a), and those tortoises showed clinical signs of URTD within four weeks, and most tortoises seroconverted within two months. In our study, we saw two peaks in URTD signs during the progression of disease. Keeping in mind that the tortoises were introduced into pens with no signs of disease, there is a remarkable rise in disease within 3 months. Of the 26 Negative tortoises that seroconverted, 8% occurred within 3 months. Of the 22 Infected tortoises that seroconverted, 32% occurred within 3 months. The second wave of seroconversions in both groups occurred 19-22 months post introduction: 77% of seroconversions in Negatives, and 54% of seroconversions in Infecteds. Furthermore, in an unpublished pilot study six tortoises were experimentally inoculated in the laboratory (Hunter et al., unpublished). The progression of disease in one tortoise in this study was similar to that in Brown et al. (1994) and Brown et al. (1999a), and occurred within three months (Hunter et al., unpublished). The remaining five tortoises showed clinical signs of disease and seroconverted more than two years post-infection (Hunter et al., unpublished).

These differences in pattern of disease progression (immediate versus delayed) remain unexplained. Both characteristics of the tortoise and of the pathogen possibly could explain these patterns of the progression of disease. Importantly, a delayed immunological response is an expected characteristic in ectotherm and reptilian immunology (Zimmerman et al., 2010). At initial infection, disease from the pathogen could occur because the strain is sufficiently virulent to cause disease immediately. Alternatively, if the pathogen strain is insufficiently virulent, or a

tortoises' strong innate response is sufficient to keep the microbe "in check", disease could be ablated or delayed. In this latter scenario, a tortoise might show clinical signs upon being colonized, but would not seroconvert. In our experiment, two years into the experiment, sufficient evolution may have occurred in the pathogen that a more virulent strain emerged to cause disease, which would then result in seroconversion. This current study, in addition to previous studies, show the need for more data that includes sampling for the presence of the microbes in the nasal passages, as a means to more fully understand of the biology of mycoplasmosis.

*Does seroconversion correlate with signs of disease in groups of animals?*

Previous research on the relationship between clinical signs of URTD and seroconversion is not consistent. For example, Schumacher et al. (1997) evaluated the clinical signs and associated antibody titers to *Mycoplasma* of 144 free-ranging desert tortoises from Nevada. They found 50% of the tortoises tested seropositive, yet only 53% of these seropositive tortoises showed clinical signs. Additionally, Lederle et al. (1997) tested wild populations of desert tortoises in the Mojave Desert for three consecutive years and found no relationship between appearance of clinical signs of URTD (<1% of the sample) and evidence of an adaptive immune response to *Mycoplasma* (19% of the sample). Twenty-four wild-caught gopher tortoises were evaluated for clinical signs, and for an immune response to *M. agassizii* (McLaughlin et al., 2000). Fourteen of the 24 tortoises showed no signs of URTD, and eight of those tested ELISA negative for antibodies, and the remaining six

(lacking clinical signs) tested positive for antibodies. Of the ten animals showing clinical signs, all but one were ELISA positive (McLaughlin et al., 2000). Hence, the literature indicates that immunological status and clinical signs are not consistently associated with each other in desert and gopher tortoises. Our research supports this prior research in that clinical signs did not predict seroconversion in our study either for Negatives or for Infecteds.

*Does brumation influence immunological responses and/or disease?*

Clinical signs in Negative tortoises peaked in both 2004 and 2005 soon after tortoises emerged from brumation, but we did not see a consistent seasonal effect on seroconversion. The lack of a clear relationship between brumation and seroconversion is likely due to the ponderous dynamics in the progression of diseases as seroconversion peaked one year after the first appearance of signs. While many post-brumation tortoises emerge with clinical signs, and thus, seem more likely to seroconvert, the frequency of clinical signs often declined by the end of the activity season.

Seasonal variation in immunity has been seen across many classes of vertebrates including mammals (Nelson and Demas 1996), birds (Martin et al., 2008), and many amphibians and reptiles (Hussein et al., 1978; Zapata et al., 1992). During winter months, desert tortoises appear to show a decrease in immune function and antibody production (Christopher et al., 1999). Physical changes to reptilian

immune systems have been shown in ocellated skinks (*Chalcides ocellatus*) (El Ridi et al., 1988), and in the red-eared slider (*Trachemys scripta*) (Zimmerman et al., 2010), and this seems to help explain observations of seasonal immunocompetence.

Glucocorticoids, including corticosteroids and cortisol, appear to suppress humoral, cell-mediated, and innate immune functions in numerous species of mammals (Klein, 2004; Nelson and Demas, 1996), birds (Martin et al., 2008), and reptiles (Saad, 1988). Similar to sex hormones, stress hormones elevate and decrease in cyclic patterns coinciding with seasonal changes and mating periods. Glucocorticoid levels respond to changes in ambient temperature, low food availability, and high population density (Nelson and Demas, 1996). These factors commonly co-occur during brumation in desert tortoises (Nussear et al., 2007), and may bear on the mycoplasma disease patterns in tortoises.

In addition, cell proliferation and death of *M. agassizii* is expected to vary with temperature. Specifically, *M. agassizii* cells are killed at moderately high temperatures. *In vitro* studies (Hunter et al., unpublished) have shown that approximately 10% of cells die when subjected to a temperature of 39°C for one hour. Even greater kill rates occur at just slightly higher temperatures, and we do not know precisely how prolonged exposure to such temperature will effect pathogen populations. For example, if one hour at 39°C causes 10% of cells to die, will three hours at 39°C cause a 30% kill rate? The growth rate of *M. agassizii* is exceedingly slow, growing at 0-50% per day *in vitro* under ideal growth conditions.

It is possible that the decreased body temperatures that tortoises experience during brumation could promote mycoplasma growth.

*Are there differences in transmission rates and clinical signs by females and males?*

Based on previous research in other animal taxa (Klein, 2004; Martin et al., 2008; Zimmerman et al., 2010; Zuk and McKean, 1996), we expected to see a difference in frequencies of clinical signs and seroconversions between males and females.

However, the only significant difference between females and males was that a significantly higher proportion of males died during the experiment compared to females ( $P = 0.022$ ). A lack of interaction between sex and season in signs and seroconversion are concordant with several earlier studies (Lederle et al., 1997; Sandmeier, 2009). Thus, despite literature that would otherwise suggest inherent physiological differences that would cause immunological differences between females and males, there appears to be none in this system.

Most research on how sex status causes differences in immunology focuses on males and testosterone. Numerous studies on increasing and decreasing testosterone (through either gonadectomizing or hormone supplementation) in insects, mammals, birds, and reptilian males indicate deleterious effects of androgens on humoral and cell-mediated immunity (Klein, 2004). Testosterone is the primary biologically active androgen in males; it appears to suppress the immune system, as well as alter gene expression and behavior, all of which contribute to increased rates

of disease in males (Klein, 2000). Increased levels of male sex hormones in males appear to suppress immune function, contrasting the effects of female sex hormones in females. Female sex hormones, including estradiol and progesterone, are thought to increase both cell mediated and humoral immune responses, thus increasing overall immune resistance to disease (Klein, 2004). Increased estrogen levels during pregnancy appear to suppress cell mediated immune functions while promoting humoral immune responses (McMurray, 2001).

One potential explanation for why we didn't see differences in levels of disease in females and males may be explained by the rise in corticosteroid levels in females prior to, and post brumation. In a study of tortoises in semi-natural enclosures, Lance et al. (2001) showed that when female tortoises emerge from brumation in April corticosteroid plasma levels peak in May, as estradiol decreases during ovulation and egg laying. This peak is followed by a dramatic decrease in corticosteroid until June. After a seasonal low of estradiol in June, levels rise to a seasonal high in August, with corticosteroid levels increasing until September. In females as well as in males, testosterone and corticosteroid levels show a close association with one another. The increase in corticosteroid levels in females at the end of the active season might explain the results of lack of immune differences between the sexes. The increased stress hormones in females might have been sufficient enough to negate the immunological enhancement properties from estradiols.

Because the ELISA (University of Florida) used to initially diagnose the animals in this study did not differentiate between acquired or innate antibodies, previous thought and policy indicated seropositive animals were infected and infectious. With the development of isotype-specific ELISAs and Western blots, which allow discrimination between quantitative and qualitative differences in antibodies, these new assays now allow differentiation between innate and acquired antibodies. However, isotype-specific antibodies still do not tell us whether a tortoise is colonized with the microbe commensally, or whether it is infected by the microbe as a pathogen.

To determine an accurate rate of infection of *M. agassizii*, correct diagnostic tests must be used. Previous studies correlating PCR amplicons (as an index of the presence of pathogen) and clinical sign as a means to diagnose mycoplasmal infections are few, and the results are varied. Dickinson et al. (2005) sampled desert tortoises from the Mojave and Sonoran deserts. In the Mojave population, 32% were ELISA seropositive and 32% showed clinical signs, although no microbiological tests were used to confirm the presence of *M. agassizii*. In the Sonoran population, less than 2% were ELISA seropositive, no animals showed clinical signs, and 6% were PCR positive indicating the presence of the microbe.

Other studies on rates of URTD and mycoplasmosis in gopher tortoises and box turtles (*Terrapene carolina*) show inconsistent correlation. McLaughlin et al. (2000) tested the serological status and frequencies of clinical sign in 24 gopher tortoises:

63% tested ELISA positive, 58% PCR positive and 42% showed clinical signs. In contrast, Berish et al. (2010) conducted a four-year field study with 205 tortoises. They found 88% showed clinical signs, 5% PCR positive, and 49% were ELISA seropositive. A new *Mycoplasma* sp. was found by Feldman et al. (2006) in eastern box turtles. Of the twenty-three turtles samples, 26% showed symptoms of URTD and 30% tested PCR positive to *Mycoplasma* sp. These lines of evidence indicate a greater need for reliable diagnostic tests of mycoplasmosis.

While not currently cost effective, the only definitive assay to test for the presence of *M. agassizii* is to assess the nasal discharge from tortoises for the microbe by looking for genetic markers of *Mycoplasma* in the nasal exudate. Evidence of rates of morbidity and mortality caused by mycoplasmal URTD have currently not been quantified for wild populations (Sandmeier et al., 2009). In light of the research presented here, use of ELISAs as the main test for *M. agassizii* infection unquestionably needs to be re-considered. Previous studies of URTD often focus on disease ecology (population level), or specific immunological responses (cell/individual level). Future work involving epidemiology in the desert tortoise needs to explore the variation of immunology of individuals and immunological systems within and among populations as well as assessing differences in the distribution of host strains and pathogen strains at a landscape level, and the implications of various combinations of hosts and pathogens.

Management policy and procedures involving “*Mycoplasma*-positive” or “*Mycoplasma*-negative” tortoises have been applied to entire populations, but consideration for differences between immunological function within populations needs to be addressed. *M. agassizii* and *M. testudinum* are currently considered as important disease-threat to desert tortoises, however the need for understanding other diseases remains largely unexplored (USFWS, 2011).

## Conclusions and New Hypotheses

*Overarching aim:* To understand the frequency of transmission of *M. agassizii* and the progression of respiratory disease caused by *M. agassizii* in the desert tortoise.

*Review of significant findings:*

- Contracting mycoplasmosis does not require that a tortoise come into contact with an infected tortoise.
- Contact with tortoises that appear to be infected with *Mycoplasma* increases the probability that uninfected tortoises will develop signs of URTD.
- For many tortoises, the lag period between first appearance of clinical signs of URTD and seroconversion was one year. These dynamics are different from those chronicled in the literature, and suggests differences in strains of pathogen, different environmental conditions in different studies, or different levels of health/immunocompetence in wild tortoises.
- Appearance of clinical signs and seroconversion rates in Negative tortoises appear predominantly after brumation. We hypothesize that the physiological stresses tortoises experience during brumation lead to decreased immunocompetence that result in signs of disease as tortoises emerge from brumation. An alternative, but not mutually exclusive, hypothesis is that when brumation occurs at body temperatures not low enough to ablate cell proliferation of the pathogen, then the host will awaken from brumation with a large population of pathogen cells that may overwhelm the host in spring and cause disease.

- Positive tortoises did not show seasonal variation in appearance of clinical signs. The density of individuals in pens, as well as the increased, and prolonged, stresses the tortoises experienced, might explain the high occurrence of clinical signs.
- While males died at higher rates than females in this experiment, they did not differ in rates of seroconversion or clinical signs.

Variation of immunological response among individual tortoises needs to be considered. For example, a seropositive titer for a tortoise might indicate a robust immune response in a healthy tortoise, and a seronegative titer could indicate reduced immunocompetence in another tortoise. This points to limitations in the conclusions that can be drawn from immunological assays. Directly testing for pathogens is critical to understanding how tortoises respond to microbes. There is a strong need to understand the role of genetic strains and strain virulence in the patterns of infection in desert tortoises. Understanding variability in immunological responses among individual tortoises will help explain our observed levels of variation in the manifestation of disease in response to exposure to *M. agassizii* (due to experimental infections or contact with a diseased individual). Understanding variation in pathogen virulence, tortoise immunocompetence, and prevalence of disease at population levels will advance our knowledge of the epidemiology of this disease and will greatly contribute to the formulation of effective conservation strategies. In addition, this system may serve as a case study of a complex disease in which host immunocompetence and physiological stress, relative pathogen

virulence, transmission rates, and environmental conditions (such as ambient temperature) may all effect epidemiology and, hence, population-level effects of disease.

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