

# Final Report: Epidemiology of Desert Tortoise (*Gopherus agassizii*).

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## EXECUTIVE SUMMARY

We conducted a comprehensive program of research to understand the mechanisms by which disease stress causes population processes not concordant with the goal of population recovery. We discovered (to our amazement) that *G. agassizii* had ELISA antibody titers to this organism that overlapped with titers obtained from some *M. agassizii*-infected tortoises. These natural antibodies were predominantly of the IgM class. Western blots of plasma from these non-infected tortoises produced a characteristic banding pattern against *M. agassizii* antigens. Desert tortoises were tested by ELISA and showed antibody titers significantly higher than non-infected tortoises, but there was considerable overlap at the lower titer levels. Western blot analysis revealed distinct banding patterns that could readily distinguish between the non-infected tortoises and tortoises with acquired antibodies, regardless of ELISA antibody titers. We conclude that desert tortoises have natural antibodies to *M. agassizii* that can compromise the determination of infection status by ELISA. However, the Western blot technique can distinguish between natural and acquired antibody patterns and can be used to confirm the diagnosis of *M. agassizii* infections in the desert tortoise.

The policy of euthanizing tortoises based upon ELISA scores was a mistake that has created a population of tortoises at the LSTS that are deficient in natural antibodies. This population may be dangerous for conservation in Clark County, so the policy of euthanasia should stop.

Using ELISA and Western Blots is still inadequate to assess disease status of individuals and populations because (a) ELISA scores can indicate high natural antibodies and/or an infection, (b) Western Blot positive scores can indicate an infection and/or a past infection that has past in the last year. We suggest that microbiological assessment of the presence of individuals of *Mycoplasma* are critically important to tell if the pathogen is present. The assessment of *Mycoplasma* in the noses of tortoises likely can be accomplished by looking for RNA from nasal lavages and quantified using qPCR techniques. Nasal lavage technology needs to extend beyond *Mycoplasma* to assess other pathogens that also may be responsible for URTD.

Environmental disturbance can affect immunity of tortoises and potentiate devastating URTD outbreaks. Thus, managing habitat to be of utmost quality for desert tortoise is very important to protect against URTD.

Ecological metrics (i.e., variance in the levels of natural antibodies among individuals in the population) seem to be the best predictors of *Mycoplasmosis*. Thus, large tracks of habitat are needed to maintain populations that have the potential to have complex disease dynamics in which clusters of individuals in the population contract and recover from *Mycoplasmosis* at the same time as

other clusters in the population are out of synchrony with the similar dynamics. This metapopulation of host-pathogen relationships requires protecting large patches of desert tortoise habitat to allow several clusters within populations to have host-pathogen dynamics that run out of phase as a means to protect the whole population.

## INTRODUCTION

Epizootiology studies of *M. agassizii* have suffered from the difficulty in culturing mycoplasmas from nasal discharge fluid or nasal lavages of the upper respiratory tract of desert tortoises. These organisms are notoriously slow growing and it often takes six weeks to obtain culture results (Brown et al. 1994). Because of the need to do protracted culturing, there is also a high incidence of culture contamination from bacteria resident in the upper respiratory tract. Although a sensitive and specific PCR method has been developed (Brown et al. 1999), this technique is expensive, time-consuming, and not readily field-adaptable. We have used our new tools to increase our knowledge of the natural relationship between *Mycoplasma agassizii*, the bacterium causing URTD (Brown 1994), and the desert tortoise, *Gopherus agassizii*, throughout the Mojave desert. In particular, we examined a number of environmental variables that can be stressors for tortoises -including yearly and seasonal rainfall, geomorphology, prevalence of invasive plants, and anthropomorphic disturbance -all are suspected as having impacts on the general health, and more specifically on the health of the immune system, of the populations of desert tortoises. The data from our surveys have been used in GIS analysis of the correlations among URTD, immunological competence, interactions between illness and immune competence, mortality, and population processes. Our study yields MANY new ideas and recommendations for conservation of the desert tortoise in Clark County.

## MATERIALS AND METHODS

### STUDY AREA

The Mojave desert tortoise is distributed within the Mojave and Colorado Deserts in California, southern Nevada, the southwest corner of Utah, and the northwest corner of Arizona (Fig 1). The Mojave and Colorado deserts (> 115,000 km<sup>2</sup>) are heterogeneous in climate, geology, and topography (Berry et al. 2006), and vegetational associations (Rowlands et al. 1982). The range of geography and physiognomy of the desert tortoise distribution includes the lower reaches of the Colorado Plateau in Utah to physiographic Great Basin in Southern Nevada and California. Each physiographic area has distinctive landforms and geological structure. A majority of the tortoise's distribution is encompassed by the larger Basin and Range Province (Hunt 1974, Trimble 1989). Although plains and alluvial fans cover 65% of the Mojave Desert, imposing mountain ranges, such as the Spring Mountains (3652 m) and the Providence Mountains (2148 m), provide commanding relief (Rowlands et al. 1982). Variation in elevation, slope, and soil type may be extremely important for habitat selection of this species (Andersen et al. 2000).

Abundance and seasonality of precipitation within the Mojave Desert is highly variable within and among years, but there is a consistent pattern of variation along a west-east gradient (Rowlands et al. 1982). Winter precipitation dominates in the Western Mojave, with greater than 75% of precipitation occurring between November and March, and less than 10% of precipitation occurring during the summer months of June - August (Germano et al. 1994). The percentage of summer and fall rainfall increases dramatically in the Eastern Mojave Desert (Germano et al. 1994). The phenology of annual vegetation, and the composition of the grass and forb flora is related to these differences. The majority of annual plants in the Western Mojave germinate during Fall and Winter months. Rainfall becomes more predictable in the southern portion of the Colorado Desert, which receives monsoonal precipitation typical of the Sonoran Desert (Burk 1977). Temperatures vary along a north-south gradient with the number of days below freezing, varying with both latitude and elevation. The number of freezing days decreases along a transect from southwestern Utah to the southern tip of the Colorado Desert in California (USFWS 1994).

Five major biotic regions occur in the Mojave Desert (Rowlands et al. 1982), and three regions occur in the Colorado Desert (USFWS 1994; Rowlands unpublished data). Vegetation is different in the Mojave and the Colorado Deserts. While many plant species overlap between these two deserts, the Colorado Desert contains some arboreal species that are sensitive to freezing (Burk 1977, Lovich and Bainbridge 1999). In many regions of the Mojave, creosote bush scrub, which is largely dominated by *Larrea tridentata* and *Ambrosia dumosa* covers up to 70% of the landscape (Germano et al. 1994, Rowlands et al. 1982, USFWS 1994). This association occurs below 1500 m on alluvial fans and bajadas. On the upper slopes, a succulent scrub association dominated by stem succulent species, including *Cactaceae* and *Yucca*, can be common (USFWS 1994). Different combinations of plant associations occur in each desert region and some unique plant communities occur in localized areas. For example, the Mojave saltbush – Allscale scrub community (dominated by *Atriplex spinifera* and *A. polycarpa*) only occurs in the Western Mojave Desert near Fremont Peak and Kramer Junction, CA (Rowlands et al. 1982, USFWS 1994). The Northern Mojave Desert is a transitional vegetation zone with a combination of plants common to the Mojave Desert and the Great Basin Desert (Rowlands et al. 1982). The Colorado Desert contains a unique combination of Sonoran Desert and Mojave Desert flora (Burk 1997).

#### SAMPLING DESIGN

Sampling design differed based on land ownership and density of tortoises; however, efforts were made to sample evenly from all putative populations and to collect at least 20 samples from each geographic location where possible. Approximately half of blood samples used in this study were collected along randomly-placed transects during routine population monitoring conducted by the U.S. Fish and Wildlife Service (USFWS 2006). A small percentage of individuals (1.5%) were sampled opportunistically while technicians were en route to a transect. The remaining samples were collected from efforts not associated with population monitoring between 2004 and 2006. Some of these samples were collected along

random transects within the Piute and Eldorado Valleys and from animals tracked with radio transmitters in those valleys, and other samples were collected from transects (4-12km) placed systematically to cover poorly sampled areas of the range. Many of these sampling transects were located outside of desert tortoise critical habitat to determine more effectively the locations of genetic boundaries for populations, and to sample any populations not located within currently-delineated critical habitat.

Due to permitting restrictions, blood was collected from toenail clips in 2004, from toenail clips (NV sites) and via brachial venipuncture (CA sites) in 2005, and via subcarapacial venipuncture (Hernandez-Divers et al. 2002) in 2006. Methods of blood sampling did not appear to effect serological results, quantitatively nor qualitatively (unpublished data). All blood samples were centrifuged as soon as possible and stored frozen at -30° F for long-term storage at the University of NV, Reno. At the time a blood sample was taken, geographic data (UTM coordinates and elevation) and tortoise-specific data, including physical measurements and clinical symptoms of disease, were recorded.

Overall, whole blood was collected from more than 600 desert tortoises throughout the Mojave and Colorado Deserts between 2004 and 2006 (Table 1). These samples were grouped subjectively into 24 sampling locations that were considered to be a specific geographic area, often constituting one or two valleys, and reflecting geography and political boundaries (Table 1 provides a brief description of locations).

Table 1. Sampling locations based on geography (including the state and abbreviation for the site), the number of individuals from each location, and how samples were collected (STS = systematic transect sampling; LDS = Line distance sampling (random)). Each site is associated with a desert tortoise Recovery Unit.

Recovery Unit	Sampling location	Abr.	State	Sample collection
U.Virgin River	Red Cliffs Desert Reserve	RC	UT	STS
Northeastern	Beaver Dam Slope	BD	UT, NV	LDS, STS
Mojave	Mormon Mesa	MM	NV	LDS, STS
	Gold Butte-Pakoon Basin	GB	NV, AZ	LDS, STS
	Coyote Springs	CS	NV	LDS, STS
	Muddy Mountains	MD	NV	STS
	Northeast Las Vegas Valley	NEL	NV	STS
	Northwest Las Vegas Valley	NWL	NV	STS
NA	Pahrump Valley	PA	NV	STS
NA	Amargosa Desert, Oasis Valley, Greenwater Valley	AM	NV, CA	STS
	Southwest Las Vegas Valley	SWL	NV	STS
	South I-15 Corridor (Goodsprings, Jean Dry Lake, Sloan)	SI	NV	STS
	Southeast Las Vegas Valley (River Mountains)	SEL	NV	STS
	Eldorado Valley	EL	NV	LDS, STS
Eastern Mojave	Piute Valley	PI	NV	LDS, STS
	Ivanpah Valley	IV	CA	LDS, STS
	Shadow Valley	SV	CA	STS

	East Providence Mountains	EP	CA	LDS, STS
	West Providence Mountains	WP	CA	LDS, STS
No Colorado	Chemehuevi DWMA	CM	CA	LDS
East Colorado	Chuckwalla DWMA	CK	CA	LDS
East Colorado	Pinto Mountains DWMA/Joshua	PM	CA	LDS
West Mojave	Tree NP			
Western	Ord-Rodman DWMA	OR	CA	LDS
Mojave	Superior-Cronese DWMA	SC	CA	LDS, STS
	Fremont-Kramer DWMA	FK	CA	LDS

#### *M. AGASSIZII CULTURE AND ANTIGEN PREPARATION*

The PS6 strain of *M. agassizii* was obtained from the American Type Culture Collection (Rockville, MD; ATCC 700616). Originally isolated from a desert tortoise with URTD, these organisms were propagated in spiroplasma SP4 medium as previously described (Brown et al., 1994), except that 2 mM L-glutamine was added to the medium (HyClone, Logan, UT). Mycoplasma cells were harvested at mid-log phase and washed four times by centrifugation to remove media contaminants. Antigen for ELISA was prepared by three consecutive freeze–thaw cycles of organisms, and protein content of the freeze–thaw antigen was determined by the Bio-Rad Protein assay (Bio-Rad, Hercules, CA) modified from the method of Bradford (1976).

#### *PREPARATION OF A RABBIT ANTI-TORTOISE IMMUNOGLOBULIN (Ig) REAGENT*

Chelonians have three major class of immunoglobulin; IgM, IgY, and IgY( $\Delta$ )Fc (Benedict and Pollard, 1972). Since it is not known whether tortoise Ig classes have a common light chain, we decided to prepare a polyclonal reagent in rabbits that would detect both IgM and IgY. It is likely that this reagent would detect IgY( $\Delta$ )Fc as it is an IgY missing the last constant domain. Ig from 30 mL of pooled desert tortoise serum was obtained by ammonium sulfate precipitation at 33% saturation. After dialysis into 0.1 M phosphate buffered saline (PBS) pH 7.4, the crude Ig fraction was subjected to gel filtration chromatography on a precalibrated 1.5°–75 cm Sephacryl-A300 column in PBS buffer. One milliliter fractions were collected at a flow rate of 1.5 mL/min, and the optical density at 280 nm was determined by spectrophotometry. A single void volume peak was obtained, along with a second broad peak (Fig. 1A). Samples pooled from the peak fractions, and a sample of the starting total Ig, were diluted to 1 mg/mL in Tris running buffer with bromophenol blue dye and run by non-denaturing PAGE using 4–20% linear gradient gels. Two predominant bands were observed with expected size distribution (Fig. 1B). New Zealand white rabbits were immunized intradermally with 1 mg of the pooled IgM and IgY-rich fractions emulsified in complete Freund's adjuvant, then boosted one month later with the same material in incomplete Freund's adjuvant. The rabbit polyclonal antibodies reacted against total tortoise Ig, and both the IgM-rich and IgY-rich fractions in a standard ELISA (data not shown). An IgG enriched fraction of this rabbit antiserum was obtained by anion exchange chromatography, and this reagent is referred to as rabbit anti-tortoise Ig.

*ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)*

ELISA was performed using NUNC 96-well MaxiSorp polystyrene plates (Fisher Scientific, Fairlawn, NJ). To establish the optimal dilutions of *M. agassizii* antigen and tortoise plasma, a checkerboard titration was performed (data not shown). The optimal concentration of *M. agassizii* antigen used to coat the 96-well plates was determined to be 10 µg/mL PBS. Each well of the plates was coated with 50 µL of diluted antigen. After overnight incubation at 4 °C, coated plates were washed three times with PBS. Wells of each plate were blocked by adding 200 µL of PBS with 5% Carnation nonfat dry milk and incubated for 1 to 2 h at 4 °C. Plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). All subsequent washes were done in the same manner, and dilutions of tortoise plasma and all subsequently added reagents were made in PBS-T. Dilutions of tortoise plasma were added to the washed plates (50 µL to each well) and incubated for 2 h or overnight at 4 °C. After washing, a previously determined optimal dilution of rabbit anti-tortoise Ig reagent was added and incubated for 1 h at 4 °C. Following another wash, 50 µL of goat anti-rabbit IgG conjugated with horseradish peroxidase (Zymed, San Francisco, CA) were added at a 1:5000 dilution to each well and incubated at 4 °C for 1 h. After a final wash, 50 µL of freshly prepared TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) were added to each well and the plates incubated for 30 min at room temperature. Fifty microliters of 1 N hydrochloric acid were added to each well and the optical density was determined at 450 nm using a Spectra-Max micro-ELISA reader (Molecular Devices, Hercules, CA). Plots of mean optical density versus log plasma dilution were made in Microsoft Excel, and a logarithmic curve fit was determined for the linear portions of each line [ $y = a \ln(x) + b$ ]. The y value was chosen as an optical density in the mid portion of the linear curves (e.g., 1.0 optical density units), and titer was defined as the reciprocal of the serum dilution (x value) corresponding to the y value.

*INTERPRETATION OF ELISAS*

Plasma samples run on separate ELISA plates were calibrated to a standard sample. This standard consisted of pooled serum, collected in August/September 2007 from a group of 21 captive desert tortoises, housed at the University of Nevada, Reno. For each sample, absorbances were plotted (dilution vs. absorbance) on a  $\log_{10}$  scale, and best-fit line was used to approximate the linear portion of each resultant curve. Because all tortoise samples had relatively large *M. agassizii*-specific ELISA titers, we did not have a “negative” sample to use as a null absorbance. To calculate antibody titers, we instead chose a “cut-off” absorbance of 0.5, a low value that intersected each best-fit line. Titers are ( $\log_{10}^{-1}$ -transformed) dilution values at which the corresponding absorbance, on the best-fit line, equals 0.5. Titer values were  $\log_{10}$ -transformed to approximate a normal distribution.

*SDS-PAGE AND WESTERN BLOTTING*

*M. agassizii* antigen (25 µg/mL protein) in PBS and protein loading buffer was heated at 95 °C for 10 min, then proteins were separated by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% tris-glycine gel

(Invitrogen, Carlsbad, CA). Gels were run under a constant 20 mA current until the bromophenol dye indicator reached the bottom. Some gels were washed and stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL), others were used for Western blotting (Towbin et al., 1979). Molecular masses were calculated using Precision Plus Protein Standards (Bio-Rad, Hercules, CA) run in parallel. For Western blots, proteins separated by SDS-PAGE as described above were transferred to nitrocellulose paper using a Bio-Rad Criterion Blotter (Bio-Rad, Hercules, CA). The papers were blocked overnight with 5% Carnation nonfat milk in PBS, washed in PBS-T, and then 1:100 dilutions of tortoise plasma samples diluted in PBS-T were added for overnight incubation at 4 °C. After washing with PBS-T, a 1:5000 dilution of rabbit anti-tortoise Ig reagent was added and incubated for 1–2 h at room temperature. Following another wash, an optimal dilution of 1:5000 of goat anti-rabbit IgG conjugated with horseradish peroxidase (Zymed, San Francisco, CA) was added and incubated for 2 h at room temperature. After a final wash with PBS-T, the blots were developed with metal enhanced DAB (Pierce, Rockford, IL). Images were made using a GelDoc imager and molecular weights calculated using image analysis software (Bio-Rad, Hercules, CA).

#### *INTERPRETATION OF WESTERN BLOT*

The same standard sample was used to calibrate each set of Western blots. Banding patterns of antibodies from the plasma samples were compared to the natural antibody pattern of the standard, negative sample. Completed Western blots were photographed with a digital camera. We used Canvas to calibrate the exposure of all photographs. Photographs were then used to count bands and score the Western blot by one researcher (FS). Tortoise samples were assigned to one of five categories, according to the following method: 0 = same number of bands as the natural antibody pattern of the negative standard; 1 = one-three additional bands; 2 = five-seven additional bands, or more than seven, very weak, additional bands; 3 = more than 8 additional, strong bands; 4 = many additional bands that form a “smear” and are not possible to count accurately. Categories 0 and 1 were considered to be “negative”; category 2 was considered to be “suspect”, and categories 3 and 4 were considered to be “positive”.

## **RESULTS AND EVIDENCE OF THE RESULTS**

It took quite a long time for us to understand that the immune system of tortoises is different from mammals, but the Western Blots of naïve tortoises with respect to *Mycoplasma* are dramatically different from those of ill tortoises (Fig. 1), and the ability to run Western blots makes it possible to know which tortoises have an infection and which definitely do not.

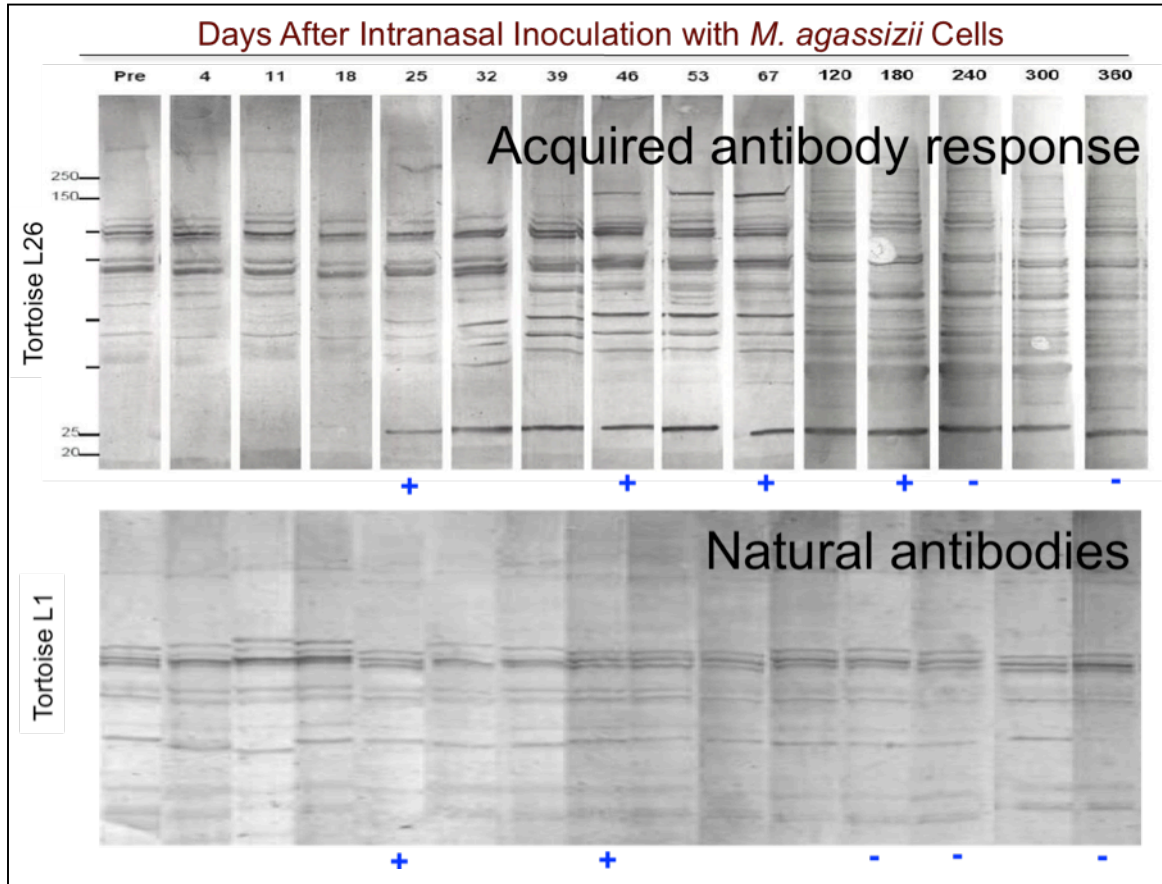


Fig.1. (A) Western blots of plasma from an infected desert tortoise as a function of time showing a characteristic natural antibody banding pattern against *M. agassizii* antigens which develops over time, and begins to diminish after the pathogen is no longer present in the nose of the tortoise. (B) Western blots of plasma from a desert tortoise with only natural antibody proteins throughout the year after which the tortoise was given *Mycoplasma*. The pluses and minus signs below each panel indicate the presence or absence of actual pathogens in the nose of the tortoise.

Discovering the presence of natural antibodies has opened new concepts of what tortoises do to defend themselves against pathogens, and how novel these defense mechanisms are relative to those in mammals and birds. Unlike conventional antibodies, natural antibodies are coded for in the genome (Baccala *et al.* 1989; Casali & Notkins 1989; Kantor & Herzenberg 1993; Baumgarth *et al.* 2005), are mainly of the IgM isotype (Casali & Schettino 1996; Baumgarth *et al.* 2005), and are thought to be an important component of innate immunity against infectious agents (Briles *et al.* 1981; Szu *et al.* 1983; Ochsenbein *et al.* 1999; Baumgarth *et al.* 2000, 2005). The existence of such antibodies reactive with the non-infectious OVA probably results from their inherent polyspecificity (Gonzalez *et al.* 1988; Flajnik & Rumfelt 2000; Baumgarth *et al.* 2005). Natural antibodies to a wide variety of antigens (including OVA) are present in non-mammalian vertebrates (e.g. Gonzalez *et al.* 1988; Flajnik & Rumfelt 2000; Sinyakov *et al.* 2002, 2006;



Hangalapura *et al.* 2003; Parmentier *et al.* 2004; Madsen *et al.* 2007). These kinds of natural antibodies have been seen in other species such as galapagos sharks (Marchalonis *et al.* 1993), carp (Kachamakova *et al.* 2006), chickens (Hangalapura *et al.* 2006, Parmentier *et al.* 2004, Van Loon *et al.* 2004), water python (*Liasis fuscus*) (Madsen *et al.* 2007).

The relationship between ELISA and Western Blots is complex. Thus, ELISAs can be very low or very high, and ELISAs alone do not tell you whether an infection has occurred that elicits an immune response. Of 660 tortoises in which we collected both ELISA and Western Blots, it is clear that many tortoises with very high ELISA scores do not necessarily have positive Western Blots that indicate the presence of an infection (Fig. 2). On the other hand, the highest ELISA score was for a tortoise with a negative Western Blot. This suggests that very high levels of natural antibodies can falsely suggest an infection when actually it only indicates natural protection from infection.

Fig.2. ELISA titer as a function of Western Blot scores. A score of 0 indicates no infection, and a score of 2 indicates a clear positive immune response (a score of 1 is intermediate).

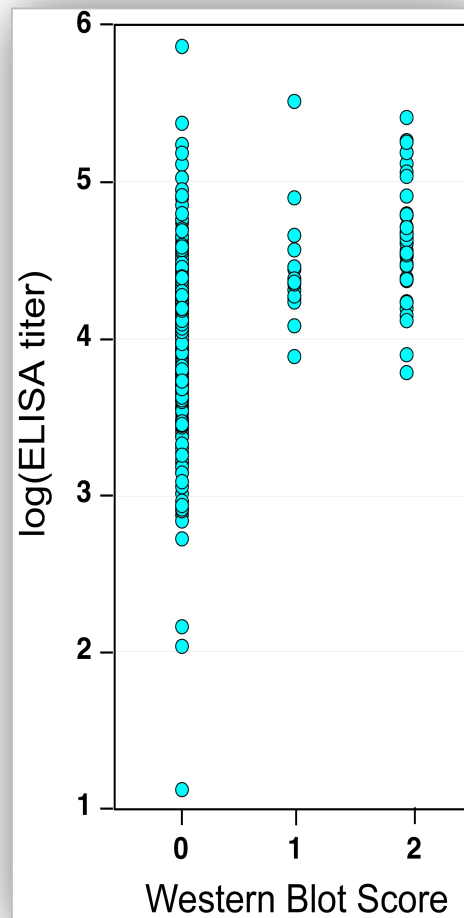
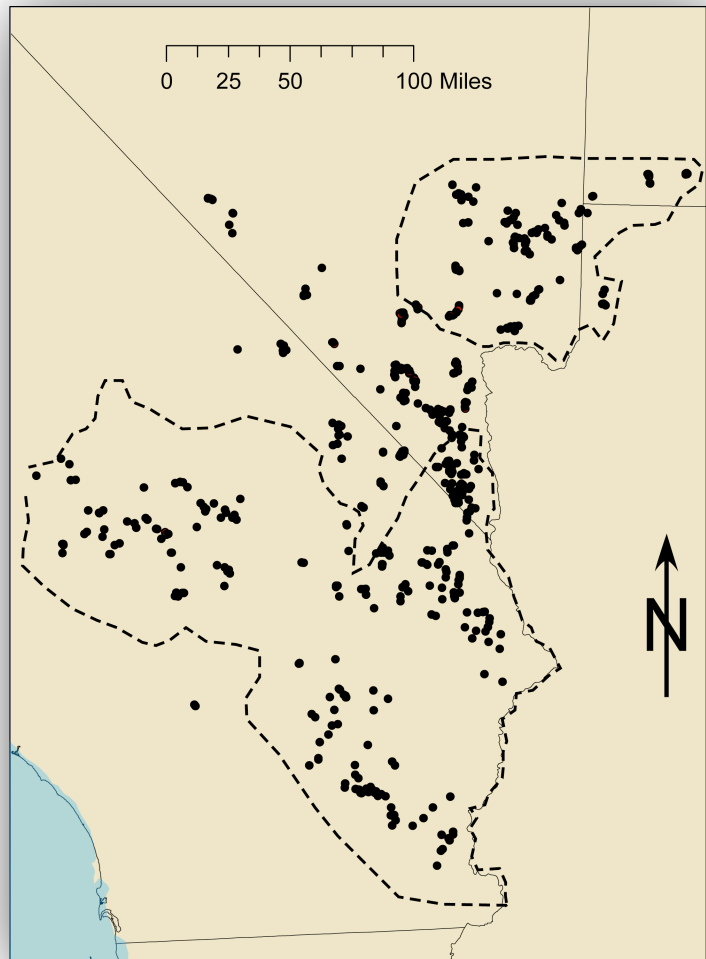


Fig.3. Sampling sites for 660 blood samples in three genetically different areas.



Blood samples were taken in a pattern that covers the entire range of the desert tortoise, in a way that blankets the known distribution without over, or under, sampling in areas. Most samples were taken in Desert Wildlife Management Areas, but sampling also occurred outside of critical habitat in order to fill in the entire range (Fig. 3).

From those samples, we assessed the natural antibody scores by taking all ELISA results for tortoises with negative Western Blots. Those showed a statistically significant pattern of distribution (Fig. 4). In particular, the California genetic cluster had significantly lower ELISA scores than all other parts of the range.

Natural antibody titers also differed by genetic cluster (Kruskal Wallis:  $x^2 = 5.991$ ,  $p < 0.001$ ). The CA cluster had significantly lower natural antibody titers than the Las Vegas and the North Mojave cluster (Mann-Whitney U: Las Vegas:  $z = -4.717$ ,  $p$  (two-tailed)  $< 0.001$ ; North Mojave:  $z = -5.503$ ,  $p < 0.001$ ). The Las Vegas and North Mojave cluster did not differ from each other in natural antibody titers (Mann-Whitney U:  $z = -1.222$ ,  $p = 0.222$ ).

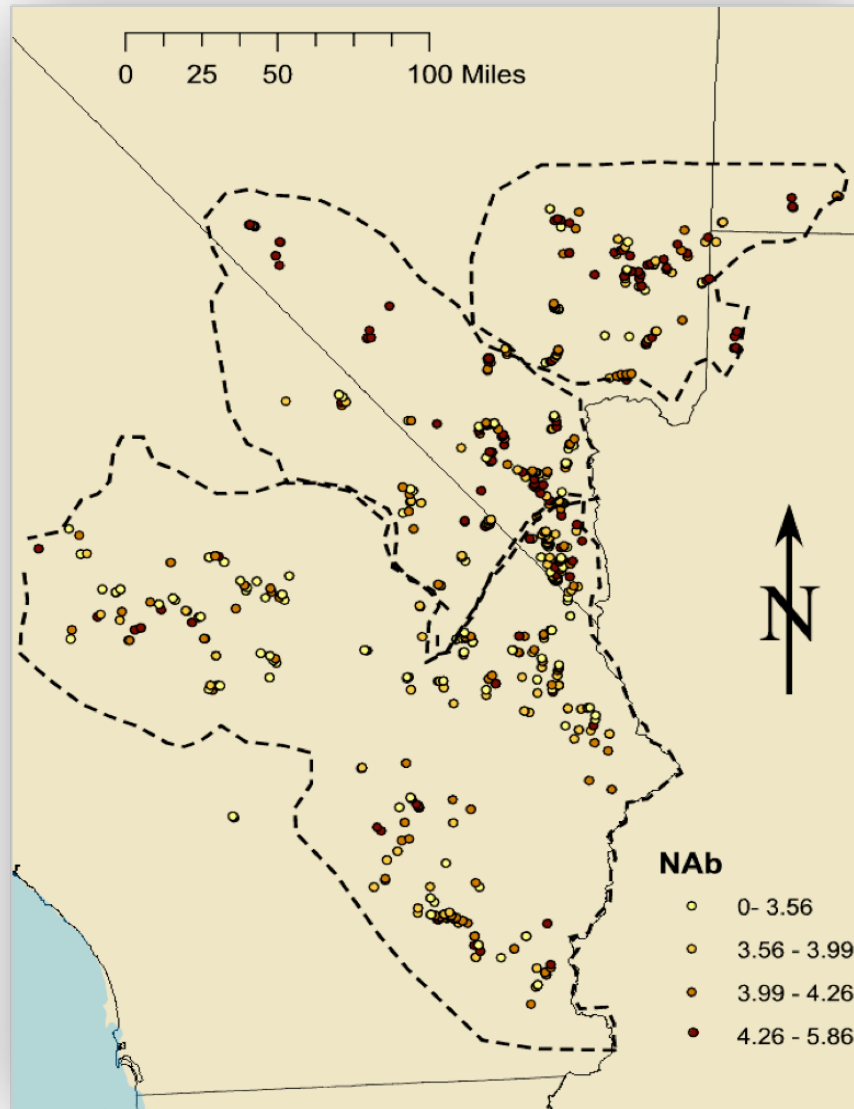


Fig.4. Natural antibody titers across the range for 660 tortoises. Darker icons indicate higher levels of titers

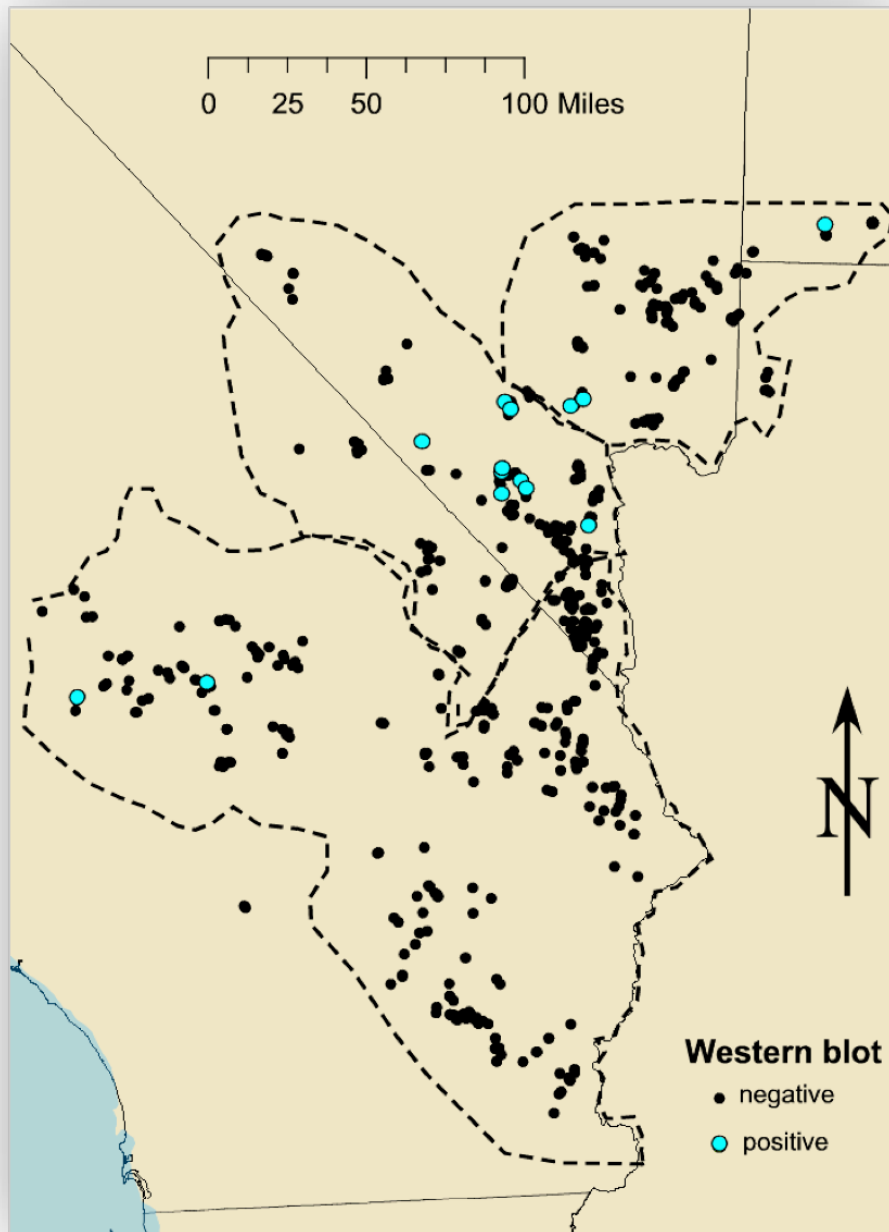


Fig.4. Western Blot Scores from the 660 samples taken. Positives are indicated in blue. The proportion of WB positives in Nevada is significantly greater than that in California.

The proportion of Western Blot positives in the Las Vegas and Northern Mojave genetic clusters are significantly greater than the proportion in California ( $p < 0.001$ ). Additionally, the main determinants of higher Western Blots include one environmental variable (days below freezing), and one population variable (variance in natural antibodies in the entire population, which accounts for 67% of the explanation for higher proportions of Western Blot positive tortoises across the range of the species). This is extremely important as it shows that a population-level variable is important as well as an environmental variable in predicting vulnerability to disease. This represents the first time that anybody has looked past the level of individuals to predict vulnerability and this has enormous conservation implications.

Finally, we discovered that the ELISA titers of tortoises in the LSTS are not significantly different from the scores of tortoises in California ( $p < 0.5$ ), but that they are significantly different from the scores of tortoises from the Las Vegas genetic cluster ( $p = 0.006$ ) and the Northern Mojave genetic cluster ( $p = 0.001$ ) even though these are the areas from which these tortoises came. Thus, the euthanasia experiment in Clark County has produced a population of tortoises in the LSTS with lowered natural immunity (like in California) and this could have implications for conservation in the future. In particular, it may be the case that the LSTS is a bastion of tortoises that could have greater vulnerability to disease than tortoises elsewhere, and that it stands as a bit of a time bomb for the general region as a result.

## EVALUATION/DISCUSSION OF RESULTS

The terms “URTD”, disease, “mycoplasmosis”, and infection often have not been clearly defined. This may be due to the current technical inability to differentiate between all possible health states, which in the medical literature are often referred to as “naïve”, “colonized”, “infected”, and “diseased” (sensu American Heritage Medical Dictionary, 2007; Blood et al., 2007). Within this framework of terminology, a naïve animal is unexposed to a particular micro-organism. A colonized animal’s tissues have been invaded by the pathogen, but there is no detectable local or systemic damage. An infected animal’s tissues have been invaded, and the pathogen has caused either local or systemic physiological response. A diseased animal is an infected animal with observable symptomatic disease. The state of infection usually induces a clear adaptive immune response in vertebrates, except in severely immunocompromised individuals. A colonized animal may or may not produce a detectable adaptive immune response. These are general terms used most often in the human medical literature. However, when applied to wildlife disease, “infection” is not always defined as necessarily causing local or systemic damage. For example, Wobeser (2006) defines infections simply as “the invasion and replication of an agent within a host animal.” This may be due to Wobeser’s emphasis on the importance of host-pathogen-environment interactions in determining the progression and severity of disease (Wobeser, 2006).

It is also important to note that “infection” is used slightly differently in

epidemiological models within the ecological literature. In that literature, “infected” individuals include all those that may transmit the disease to others, regardless of whether they are colonized or infected and regardless of clinical or sub-clinical symptomatic disease. Most diseases that are commonly modeled are highly infectious and pathogenic, and therefore a clear distinction between colonized and infected individuals may be unnecessary. At most, models of some diseases include a “lag time” to account for the time it takes for an exposed individual to become infectious to others.

Opportunistic infections are defined as infections by an organism that do not ordinarily cause disease, but becomes pathogenic under certain circumstances, such as under impaired immune function (e.g. Blood et al., 2007). Two of the most commonly recognized causes of impaired immune function include inadequate nutrition and concurrent infections with other pathogens (e.g. Wobeser, 2006). Therefore, opportunistic pathogens may colonize hosts under certain conditions without causing harm, yet they may cause harmful infectious disease under other conditions. Measurements of the presence of *M. agassizii* in desert tortoises have relied largely on indirect measurements, such as tortoise antibody production. However, relationships among adaptive antibody production, the load (or number of individuals) of *M. agassizii* present, and the degree of local or systemic damage caused by *M. agassizii* remains unknown. Clear distinctions between naïve, colonized, and infected desert tortoises are currently not possible.

Tortoises can be described as “URTD-positive”, or “diseased” if they show symptoms of URTD regardless of the cause. “Mycoplasmosis” is used refer to tortoises mounting an adaptive antibody response to *M. agassizii* and/or related *Mycoplasma* spp. Although the term “mycoplasmosis” implies infection, it is important to recognize the current inability to assess the degree of local or systemic harm associated with the presence of mycoplasmas in the desert tortoise respiratory tract of live animals. Similarly, no adequately sensitive and accurate technique has been used in the literature to determine whether tortoises without an adaptive immune response may be “colonized” with small numbers of essentially commensal mycoplasmas.

Due to its widespread application, it is important to recognize the inherent limitations of the currently used monoclonal ELISA (Brown et al., 2002). Briefly, an ELISA measures antibody levels through a number of procedures that culminate in an enzymatic reaction. The enzymatic reaction is detected as a color change and measured as a light absorbance in optical density units (OD). The serum samples may be serially diluted until the optical density is not detectable as different from “background” absorbance. Thus, results of blood serum samples tested via ELISA are usually reported as either end-point titer values (the dilution at background absorbance) or as absorbances at a single, selected dilution. The particular monoclonal ELISA currently in use for detecting mycoplasmosis measures absorbances at two dilutions to estimate the end point titer from calibration curves using full dilution curves taken to the end-point titer values (Brown et al., 2002).

An ELISA is an indirect measurement of disease, and only has the ability to detect the current or recent production of antibodies in the peripheral blood instead of assaying the actual presence and/or load of pathogen at a discrete point in time (Brown et al., 2002). In addition, any assay has imperfect specificity and sensitivity, and it includes a subjective “cut-off” value separating positive and negative health diagnoses. The test’s specificity (accuracy in correctly identifying infected subjects) and sensitivity (accuracy in correctly identifying uninfected, healthy subjects) ultimately determine the magnitude of inherent inaccuracies (Brown et al., 2002; Loong, 2003).

While a certain amount of subjectivity is inherent in any clinical assay, the greatest, and least emphasized, problem of this particular ELISA test is that no true “gold standard” was used to determine the test’s sensitivity and specificity, and to test its population-specific positive and negative predictive values (Brown et al., 2002; Loong, 2003). A “gold standard” is an independent standard used in determining whether an individual is truly positive or negative, and can therefore be used in the validation of a separate assay (Loong, 2003). The current ELISA reportedly has a sensitivity >90%, a specificity >85%, mean positive and mean negative predictive values of about 88%, and rates of false positives between 0-27% (Brown et al., 2002). In human biomedicine, western blots are often routinely used as a gold standard to designate true positive and true negative subjects. For example, western blot positives and negatives have been used in the validation of an ELISA to detect antibodies to HIV (Gürtler, 1996; Mas et al., 1997; Kleinman et al., 1998; Kassler et al., 1995; CDC, 1989, 1992). Although Schumacher et al. (1993) presented western blots of three individual desert tortoises, western blots were not subsequently used as an independent gold standard in ELISA validation. Schumacher et al. (1993) recognized the problem of not having true positive and negative control animals in their assessment of the ELISA, and they used pathologic and histologic evaluations of necropsy specimens to determine true health status. In other studies, the presence of clinical signs, and/or histopathological lesions, have been used to approximate positive and negative predictive value of the ELISA (Brown et al., 1994, 1999b, 2002; Schumacher et al., 1997). Therefore, determinations of truly infected animals have not been consistent among studies.

Given the great reliance on the current monoclonal ELISA, and somewhat incomplete knowledge of chelonian populations of antibody molecules (Benedict and Pollard, 1972; Coe, 1972; Ambrosius, 1976; Herbst and Klein, 1995; Turchin and Hsu, 1996), it is also surprising that there has been no discussion in the desert tortoise literature, of the different strengths of monoclonal ELISAs versus polyclonal ELISAs, both of which have been used in studies in mammalian immunology (Janeway, 2005). In particular, there is no comparison between these two types of ELISAs in measuring *M. agassizii*-specific tortoise antibodies. The current monoclonal ELISA was created to recognize a light chain of desert tortoise antibody molecule IgY (Schumacher et al., 1993). This monoclonal antibody is reported to recognize all *M. agassizii*-specific IgM, IgY, and IgΔFc, because these antibody molecules are expected to be made up of different heavy chains, but equivalent light chains (Schumacher et al., 1993; Brown et al., 2002).

However, many vertebrates have more than one type of light chain, and the proportion of these light chains in antibodies varies greatly from species-to-species (Pilström et al., 1998). Research is needed to quantify the number, and relative proportion, of light chains in the desert tortoise to interpret data correctly that is obtained through the use of the monoclonal ELISA described in Schumacher et al. (1993). A polyclonal ELISAs recognize all heavy and light chains of specific antibodies. Polyclonal ELISAs should be used to assess the monoclonal ELISA (Schumacher et al., 1993) to verify the assumption that the monoclonal ELISA is truly measuring all *M. agassizii*-specific tortoise antibodies.

Current cutoff values of the *M. agassizii*-specific monoclonal ELISA reportedly were chosen conservatively for the purpose of minimizing false negative results (Brown et al., 2002). These values were considered “conservative” for the purpose of reducing the chance of falsely declaring a tortoise free of mycoplasmosis, therefore reducing the chance of translocating a potentially sick tortoise into a new tortoise population. Conversely, this assay bias increases the chance of making false positive errors, thereby increasing the chance of declaring an uninfected tortoise ill. In Clark County, NV, this bias increased the chance of euthanizing uninfected animals, and even a false positive error of only 10% could have resulted in more than 300 tortoises being euthanized even though they were healthy.

During the ELISA's use by researchers and managers, the University of Florida lab conducting ELISA tests changed the cut-off values used in differentiating between positive and negative animals while improving the assay (Lederle et al., 1997). This serves as an example of the possible subjectivity in ELISA diagnoses. Lederle et al. (1997) calculated that this change in assay interpretation actually decreased the proportion of seropositive animals detected in a their from a putative 43% (using the “old” cut-off values) to only 19% (Lederle et al., 1997). Such levels of change in interpretation of ELISA results can affect the comparability of studies carried out before and after the change in cut-off values.

Although several possible functions of natural antibodies have been hypothesized within the literature (e.g. Avrameas, 1991; Flajnik and Rumfelt, 2000), natural antibodies have been shown to be protective, or involved in protective immunity, in a wide range of vertebrate species, including humans (Ben-Aissa-Fennira et al., 1998; suggested in Kohler et al., 2003), mice (Briles et al., 1981; Szu et al., 1983; Ochsenbein et al., 1999; Baumgarth et al., 2000, 2005), bony fish (Sinyakov et al., 2002; Magnadóttir, 2006), and sharks (suggested in Marchalonis et al., 1993; Flajnik and Rumfelt, 2000). Natural antibodies have also been shown to augment adaptive immune responses (Ehrenstein et al., 1998; Boes, 2000; Ochsenbein and Zinkernagel, 2000), and are considered to be a link between innate and adaptive immune responses (Ochsenbein and Zinkernagel, 2000).

*M. agassizii*-specific antibodies have been described in previous studies (Brown et al., 1994; Schumacher et al., 1993). Specifically, Schumacher et al.'s (1993) western blot data showed that both a negative control tortoise and a pre-inoculation tortoise (subsequently infected with *M. agassizii*), produced multiple



types of antibody specific to *M. agassizii* proteins. Furthermore, Schumacher et al. (1993) found that negative animals had relatively high “background” antibody levels to other species of *Mycoplasma*, especially to *M. testudinis* and *M. gallisepticum*. Surprisingly, the background antibody levels to these two species of *Mycoplasma* were equal to, or higher than, antibody levels to *M. agassizii*. However, this demonstration of relatively high levels of antibodies reactant to multiple pathogens is consistent with the general polyreactivity of natural antibodies (Guilbert et al., 1982; Gonzalez et al., 1988; Marchalonis et al., 1993; Boes, 2000; Flajnik and Rumfelt, 2000; Paramentier et al., 2004; Baumgarth et al., 2005). Desert tortoise natural antibodies may bind to antigens conserved across of mycoplasmas.

Green sea turtles (*Chelonia mydas*) also appear to have high levels of “background” IgM antibodies that bind to pathogens and/or protein antigens. One of two individuals of *C. mydas* immunized with a common experimental antigen (DNP-BSA, or 2,4-dinitrophenylated bovine serum albumin) had relatively high titers of IgM, but not IgY, prior to immunization (Herbst and Klein, 1995). Natural antibody levels to DNP in *C. mydas* had also been noted in an earlier study of turtle antibodies (Benedict and Pollard, 1972). In still another experiment, individuals of *C. mydas* with FPHV-specific antibodies (fibropapillomatosis-associated herpes virus) did not test ELISA positive for IgY specific for another virus, LETV (lung-eye-trachea disease-associated herpes virus) (Coberly et al., 2001). However, the same turtles tested positive for antibody (possibly both IgY and IgM) that reacted with LETV-infected cultured cells via an immunohistochemistry assay (Coberly et al., 2001). This observation suggests the possible presence of polyreactive IgM (either natural or induced) that binds multiple types of virus.

Because of the uncertainty in the interpretation of chelonian antibody levels as indicators of current (or recent past) infection, research on mycoplasmosis in the desert tortoise warrants reinterpretation. Past infection studies have selected tortoises with the lowest “background” levels of antibodies as the “best” negative control specimens (Schumacher et al., 1993; Brown et al., 1994), which may have introduced bias into the research design. Therefore, no distinction has been made between the ability of *M. agassizii* to cause disease in all experimental desert tortoises, and the ability of *M. agassizii* to cause disease in the tortoises with the low levels of natural antibodies. Tortoises with low levels of natural antibodies may have low innate resistance towards *M. agassizii*.

Consistent with the hypothesis that low levels of natural antibody to *M. agassizii* may increase a tortoise’s susceptibility to infection, Brown et al. (1994) found that two of their experimentally infected tortoises with relatively high pre-inoculation levels of antibody failed to show an adaptive immune response in the form of an increase in antibody titer. This result suggests that tortoises with relatively low innate levels of *M. agassizii*-specific antibody mount relatively large adaptive humoral immune responses to experimental infection with *M. agassizii*.

Previously overlooked, high levels of tortoise natural antibodies specific to *M. agassizii* may be viewed as an example of immunology’s general

preoccupation with the mammalian adaptive immune system and a failure to consider innate immune mechanisms as biologically important defense strategies (Turner, 1994b; Janeway et al., 2005). Different components of adaptive and innate immunity may have varying importance among taxa, and the relative importance of these immune mechanisms may vary with such traits as metabolic strategies (e.g., homeothermy vs. ectothermy) or life-history traits (e.g. Turner, 1994a; Hsu et al., 1998; Norris and Evans, 2000; Bayne and Gerwick, 2001; Hangalapura et al., 2003). Ectotherms may rely, to a greater degree than do mammals, on innate immune mechanisms (ectotherms generally: Avtalion et al., 1976; fish: Manning, 1994; Bayne and Gerwick, 2001; Magnadóttir, 2006; amphibians: Horton, 1994; reptiles: Jurd, 1994). In particular, large proportions of natural antibodies have been documented in sharks (up to 40 or 50% of total blood serum protein) (Marchalonis et al., 1993; Flajnik and Rumfelt, 2000), bony fish (Gonzalez et al., 1988; Morrison et al., 2005), and one reptile (the water python *Liasis fuscus*) (Madsen et al., 2007). High “background” antibody levels to a wide variety of natural pathogens, but not to artificial, injected protein antigens, have long been recognized as a relatively common phenomena in fish immunology (Avtalion et al., 1976; Sinyakov et al., 2002).

Since approximately the time of the official listing of the Mojave population of the desert tortoises, the practice of euthanasia of displaced, “diseased” animals has been supported in both peer-reviewed and non-peer-reviewed publications. In a 1989 conference on URTD, cited in the Recovery Plan (FWS, 1994), the idea of removing sick tortoises from natural populations was first discussed (Berry and Slone, 1989). As the monoclonal ELISA test (Schumacher et al., 1993) became essentially the only measure of diagnosing URTD, seropositive animals were viewed as potential carriers of disease regardless of whether they exhibited symptoms of URTD. The treatment of large numbers of seropositive, displaced tortoises became a complicated management problem. Jacobson et al. (1995) wrote “*The Desert Tortoise (Mojave Population) Recovery Plan . . . fails to recommend what to do with ill or subclinically affected tortoises. Because there is no known drug therapy for long-term improvement of tortoises with URTD, euthanasia rather than relocation should be considered for such tortoises. Healthy appearing, sero-positive tortoises from populations in which URTD has been seen should be considered infectious, and should not be released into areas where URTD has not been observed.*” Managers took that advice and euthanasia became common in some areas of the Mojave (perhaps cite a Clark County publication on this – such as the original tortoise HCP).

The ethical and social concerns associated with euthanasia of domestic and wild animal species are intrinsically hard to quantify, because objections to culling are largely based on economics, individual values pertaining to animal welfare, the value of wildlife, and biodiversity (McCallum and Hocking, 2005; Kitching et al., 2006). Explicit cost-benefit analyses are often used to justify culling programs of agricultural and wildlife species (McCallum and Hocking, 2005; Hasonova and Pavlik, 2006). The “costs” of culling should logically be less than the “costs” of the disease in any particular population. Cost and benefit

considerations of disease management via culling in species of conservation concern require a longer time scale than calculating immediate economic returns of an agricultural animal population. The cost of reducing biodiversity due to the culling is considered to be much greater for a threatened species than for a livestock population, but there are no established ways to “calculate” the worth, and the loss, of this type of biodiversity (Sturner and Smith, 2006). Brown et al. (2002) emphasized the trade-off between the potential spread of disease, and the loss in reproduction and genetic diversity in the desert tortoise. We propose that, in a threatened species such as the desert tortoise, increases in mortality and decreases in potential reproduction due to culling normally will be less than the population-wide increase in mortality and/or decrease in reproduction and recruitment associated with the disease. Importantly, no such data exist for the population-level effect of mycoplasmosis or URTD in the desert tortoise.

The euthanasia policy in desert tortoises assumed that managers could accurately distinguish between infected and susceptible individuals. This assumption is largely undermined by the recognition of high natural antibody levels in desert tortoises coupled with the current reliance on an ELISA to diagnose tortoise health status. Subclinical *M. agassizii* infections in tortoises may be common (Brown et al., 2002). Similar to subclinical mycoplasmal infections in various vertebrate species, may be difficult to detect, even when using a combination of sensitive diagnostic techniques, such as culture, PCR, and/or ELISA (Rottem, 2003; Baseman et al., 2004; Waites and Talkington, 2004). A euthanasia policy assumes that the native tortoise populations at translocation sites are either naïve to mycoplasmosis, or have a significantly lower proportion of infected animals than the population of translocated animals. However, desert tortoises with URTD have been observed at the current Clark Co. translocation site (LSTS) (pers. observation), and no data have ever been published on either serological (ELISA) data or on other measures of mycoplasmosis in the native tortoise population at LSTS.

Regarding the possibility of future translocations of desert tortoises at various locations in the Mojave desert, some evidence exists of study areas with few URTD symptomatic (e.g. Lederle, 1997; Dickinson et al., 2005) and/or ELISA-positive tortoises (e.g. Berry et al., 2006). There are no published accounts of an entire Mojave desert tortoise population or discrete subpopulation that appear to be naïve to mycoplasmosis. Therefore, our current knowledge of mycoplasmosis in natural desert tortoise populations should not necessarily preclude the translocation of ELISA-positive animals into certain populations. However, the possible introduction of different strains and/or species of *Mycoplasma* with intentional or accidental translocations is a serious concern that should become a focus of scientific research and possibly, management policy (see Table 2). While establishing “disease free” herds or breeding stocks is sometimes stated as a management objective in poultry and livestock (Stipkovits and Kempf, 1996; Collins and Socket, 1993), the feasibility and desirability of this objective in wild populations is questionable, and currently this management strategy does not seem to be amenable to the desert tortoise-*Mycoplasma* spp system (e.g. Read and Taylor, 2001; Wobeser, 2006).

There is a current dearth of data and analyses concerning the individual- and population- level deleterious effects of *M. agassizii* infection and prevalence of *M. agassizii*, which is exacerbated by concerns regarding the ethics and potential loss of biodiversity due to culling a threatened species. A policy to euthanize displaced desert tortoises testing ELISA-positive to *M. agassizii* quite simply cannot be justifiable.

To manage both displaced and resident tortoise populations optimally, information on the health status of resident and displaced tortoise populations should ideally include information beyond prevalence of mycoplasmosis, to include a range of other potentially important pathogens and possibly other indices of stress, body condition, reproduction, and estimates of longevity.

## CONCLUSIONS

Desert tortoises have a remarkable system of defending themselves from Mycoplasmosis that includes as a front line of defense, natural antibodies. The variance among individuals in populations with regard to natural antibody titers ultimately predicts better than any other variable, the vulnerability of individuals in the population to infection from *Mycoplasma*. Understanding disease dynamics in desert tortoise falls to the missing parts of the story to date. Specifically, we understand now that desert tortoises can become ill due to *Mycoplasma*, but we don't know the population consequences of those illnesses. We know that tortoises defend themselves from *Mycoplasma* using natural antibodies, but we don't know how effective that defense is and how much an adaptive immune response is needed to complete the defense. We do know that the prevalence of an adaptive immune response in the population is related to the variance in natural immune competence among individuals in the population, and this is a very new and important finding. However, we don't know the population consequences of infections among individuals in the population, and very importantly, all that we know about disease dynamics comes from serological evidence, and not from direct assessments of pathogens. Thus, to understand the biological and ecological relationship between *Mycoplasma* and tortoises in generating disease dynamics, it is critically important that we include microbiology as a critical supplement to serology in assessing disease status.

## RECOMMENDATIONS

1. To test for immune responses from desert tortoises, it is critically important NOT to depend on ELISA results alone, but to use Western Blot as a way to ascertain the presence of an adaptive immune response typically indicating an infection, but importantly, it also could indicate a past infection that is still resulting in production of proteins for protecting the tortoise from *Mycoplasma*.

2. The policy of euthanizing tortoises based upon ELISA scores is a terrible idea that has actually created a population of tortoises at the LSTS that are deficient in natural antibodies. This population may be a ticking bomb for conservation in Clark County, so the policy of euthanasia should stop and never restart.
3. Using ELISA and Western Blots is still inadequate to assess disease status of individuals and populations because (a) ELISA scores can indicate high natural antibodies and/or an infection, (b) Western Blot positive scores can indicate an infection and/or a past infection that has past in the last year. Thus, microbiological assessment of the presence of individuals of *Mycoplasma* are critically important to tell if the pathogen is present. This has not been done properly ever, so this requires new research. The assessment of *Mycoplasma* in the noses of tortoises likely can be assessed by looking for RNA from nasal lavages properly managed against deterioration and later quantified using qPCR techniques, which need to be verified.
4. Nasal lavage technology needs to extend beyond *Mycoplasma* as our review of the literature has suggested that other pathogens may be responsible for URTD in many tortoises. Thus, as we move towards microbiological assessments, we should include all potential pathogens. This will not represent a much greater investment of resources as the ability to screen for additional pathogens can be made easy through some up-front research.
5. It seems clear that environmental disturbance can affect immunity of tortoises and set up populations for potentially devastating URTD outbreaks. Thus, managing habitat is terrifically important to protect against URTD.
6. Ecological metrics seem to be the best predictors of Mycoplasmosis. Thus, it seems that, as suggested in the original Recovery Plan, large tracks of habitat are needed to maintain populations in the large tracks that have the potential to have complex disease dynamics in which clusters of individuals in the population can contract and recover from Mycoplasmosis at the same time as other clusters of individuals are out of synchrony with the same dynamics. This theory of how host-pathogen relationships occur would explain all of the statistical results on acquired immune responses in the Las Vegas and Northern Mojave genetic groupings. If that theory is valid, then it is critically important to protect patches of desert tortoise habitat that are big enough to allow several clusters within populations to have host-pathogen dynamics that run out of phase, and still be entirely viable as a whole population.

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