



An ecoimmunological approach to disease in tortoises reveals the importance of lymphocytes

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Citation: Sandmeier, F. C., C. L. Weitzman, and C. R. Tracy. 2018. An ecoimmunological approach to disease in tortoises reveals the importance of lymphocytes. *Ecosphere* 9(9):e02427. 10.1002/ecs2.2427

Abstract. We quantified the severity of upper respiratory tract disease (URTD) and immunological metrics (differential white blood cell counts and bacteria-killing ability of blood plasma) in relation to climatic variables in 20 populations of Mojave desert tortoise (*Gopherus agassizii*). Prevalence and infection intensity of *Mycoplasma agassizii*, an etiological agent of URTD, have previously been quantified for these populations (Weitzman et al. 2017). Immunological variables were reduced by principal component analyses and separated into cells involved in inflammation (PC1) and cellular functions mediated by lymphocytes and basophils (PC2). In population-level models, the mean number of lymphocytes per individual was associated with mean annual number of days below freezing. Lymphocytes were also positively associated with mean infection intensity of *M. agassizii*. Additionally, prevalence of URTD was closely associated with PC1 (cells associated with inflammation). This suggests that at least two immunological strategies are involved in responding to *M. agassizii*, one that involves primarily lymphocytes and one that involves inflammatory mechanisms. Recent studies on immunology in Testudines suggest that a large proportion of lymphocytes in this taxon are similar to B-1 lymphocytes of mammals and have phagocytic properties. Controlled experiments are needed to understand the disease mitigation of these lymphocytes in desert tortoises.

Key words: chronic disease; ectotherm; inflammation; phagocytosis; reptile; temperature.

Received 30 March 2018; revised 29 June 2018; accepted 14 August 2018. Corresponding Editor: Andrew R. Wargo.

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INTRODUCTION

Ecoimmunology is a field that has grown tremendously over the last 20 yr, but gaps in knowledge and techniques remain obstacles to developing generalities that apply across species (Martin et al. 2011). For example, many ecoimmunological studies use techniques that have been validated to measure immune function among species, but that may not be ecologically relevant in terms of defense against actual pathogens affecting populations (Martin et al. 2011). In addition, many of the commonly used techniques in ecoimmunology were developed to measure immune function in short-lived endothermic vertebrates such as

songbirds and small mammals (reviewed in Demas and Nelson 2012). It has been argued that long-lived ectothermic vertebrates likely have evolved different strategies of immune defense, probably mediated by environmental constraints on thermoregulation, temperature-dependent metabolic rates, and repeated exposure to common pathogens (Sandmeier and Tracy 2014).

Here, we focus on measuring immune function in Mojave desert tortoise (*Gopherus agassizii*) populations, using the common techniques of quantifying bacteria-killing ability (BKA) of blood plasma and differential white blood cell counts. The sampled populations have known levels of the pathogen (*Mycoplasma agassizii*), an

etiological agent of what is thought to be an ecologically relevant disease, upper respiratory tract disease (URTD; Weitzman et al. 2017). Upper respiratory tract disease is a chronic, persistent disease with relatively stable prevalences of 15–20% across the species range during most years, yet low morbidity (i.e., mild, intermittent signs of disease; Sandmeier et al. 2009, 2013, Weitzman et al. 2017). The term URTD is not pathogen-specific and simply describes visible signs of an inflammatory respiratory disease, and we use this term as such throughout (Sandmeier et al. 2009, Jacobson et al. 2014). A survey of tortoise populations conducted in 2004–2006 revealed prevalences of URTD and seroprevalences to *M. agassizii* (i.e., prevalence of induced antibodies to the microbe) were associated with each other and both increased in populations found in cooler thermal regimes (Sandmeier et al. 2013). Genetic diversity in Mojave desert tortoises is primarily determined by isolation by distance, and three distinct genotypes are recognized,

commonly referred to as California, Las Vegas, and Northeast Mojave genotypic groups (Fig. 1; Hagerty et al. 2011). Thermal environments vary along an eastwest and northsouth gradient, similar to the three genotypes, and patterns of disease also vary by genotype (Sandmeier et al. 2013).

Seroprevalence is a poor indicator of prevalence of the microbe *M. agassizii*, but quantitative PCR (qPCR) can detect very low levels of the pathogen within tortoises (Braun et al. 2014, Sandmeier et al. 2017a). The 20 populations of tortoises evaluated in this study had a range of prevalence of *M. agassizii* (0–90% of animals tested positive per population), low infection intensities, low genetic diversity of *M. agassizii*, and low prevalence of URTD (Weitzman et al. 2017). Within individuals, the quantity of *M. agassizii* (the inverse of Ct values by qPCR), but not the presence/absence of *M. agassizii*, was a significant predictor of URTD (Weitzman et al. 2017). There exists some support for the idea that induced antibody responses to *M. agassizii* incur

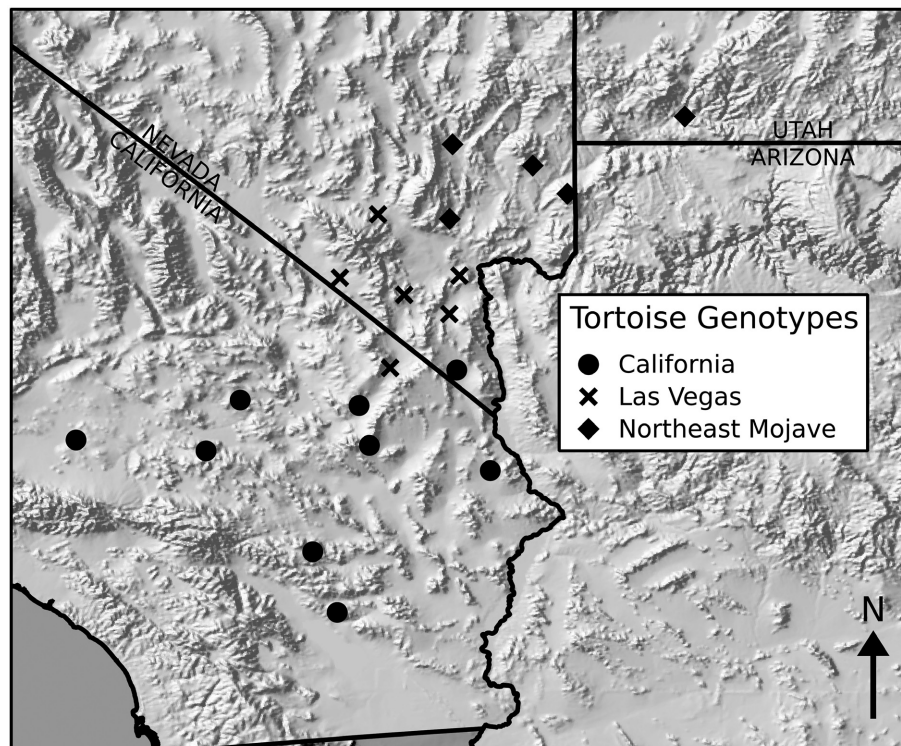


Fig. 1. Local populations of tortoises sampled in this study, labeled according to which genotypic population they assign to, sensu Hagerty et al. (2011).

immunopathology, as they do in some other host-mycoplasma systems (Simecka et al. 1992, Hurtado 2012, Sandmeier et al. 2017b). Therefore, tortoises may use multiple mechanisms to keep *M. agassizii* at levels low enough not to cause visible signs of inflammation referred to as URTD. We tested a number of hypotheses about such immunological mechanisms animals may have for reducing loads of *M. agassizii*.

First, we quantified general patterns among tortoise genotypes in *M. agassizii* prevalence, visible disease, and immunological measures. We also quantified the severity of URTD (Sandmeier et al. 2017a), and we compared that to infection intensity of *M. agassizii*. We then quantified general relationships among differential quantities of white blood cells and BKA. Bacteria-killing ability is a functional assay that measures the effects of constitutive natural antibodies, complement proteins, and other components such as acute phase proteins in limiting bacterial growth and survival (Murphy 2012). Given past data (Sandmeier et al. 2013), we predicted that measures of URTD and associated immune functions would be higher in the Northeast Mojave tortoise genotype.

We then tested four specific hypotheses: (1) Animals with URTD will have changes in immune measures related to inflammatory processes. (2) The severity of URTD and measures of immune function will be associated with levels of *M. agassizii*, indicating some strategies of defense against disease. We predict that some changes in immune function should be present in animals with *M. agassizii* and that these changes should depend on infection intensity. If no immune changes are observed, this could be evidence for the possibility that *M. agassizii* is sometimes non-pathogenic and/or another pathogen influences disease. (3) Among populations, there will be an association between immunological measures and both the infection intensity of *M. agassizii* and prevalence of URTD. Due to the intermittent nature of signs of disease within individuals, we predict that patterns between infection intensity of *M. agassizii*, prevalence of URTD, and immunological measures will be stronger when comparing populations versus individuals. (4) Climatic conditions that vary regionally, as well as conditions at the time of sampling, should predict measures of disease

and immune function in tortoise populations. We predicted that colder climatic conditions (e.g., colder winters and cooler monthly temperature during the active season) would be associated with increased levels of disease and decreased levels of immune function.

METHODS

Data collection

From April to June in 2010–2012, 419 wild Mojave desert tortoises were handled and evaluated for signs of URTD in an attempt to evenly sample 20 populations, representing the entire range of the species (previously described in Weitzman et al. 2017; Fig. 1). We rinsed the nares with 3 mL of sterile saline, and we collected a 0.5 mL blood sample from the subcarapacial sinus (Hernandez-Divers et al. 2002). One drop of blood was used to make a smear, which was stained with Wright-Giemsa. The remainder was added to heparinized vials and kept on ice. Plasma was separated and frozen at -20°C within six hours of collection. Severity of URTD was scored according to Sandmeier et al. (2017a): 0, no signs of disease; 1, no occlusion of the nares, but damaged/missing scales near the nares and/or wheezing; 2, partially or fully occluded nares; 3, visible, serous exudate; 4, visible, opaque exudate; 5, visible exudate with deeply eroded/disfigured nares; and 6, visible exudate and a poor body condition index. Animals with scores of 2–6 were considered positive for URTD (Sandmeier et al. 2017a).

Climate data were calculated for each population to quantify fine-scale differences in climate only during the month that a tortoise was sampled and also to reflect broad-scale regional differences in annual climate experienced over 15 yr by tortoise populations. PRISM data at a 4-km resolution (<http://www.prism.oregonstate.edu>) were used to calculate climate values during the month a tortoise was sampled (April, May, or June) at the center of each population. Because tortoises have relatively large home ranges and our study sites were relatively small, we assumed that this method approximated climatic conditions an animal experienced while moving across the local landscape. Data calculated included mean temperature, minimum temperature, maximum temperature, and mean

rainfall. We calculated mean annual climate conditions experienced by tortoises over the 15 yr preceding this study from NOAA weather station data (<http://www.ncdc.gov/cdo-web/>; sensu Sandmeier et al. 2013). These variables included mean annual days above 32°C, mean annual days below freezing, extreme maximum temperature, extreme minimum temperature, mean annual rainfall, mean winter rainfall (October–March), and mean summer rainfall (April–September).

Laboratory analyses

Quantitative PCR was performed on all nasal lavages, according to the protocol of Braun et al. (2014), and described in detail in Weitzman et al. (2017). Samples were run in triplicate, and all animals with at least two positive replicates (Ct scores below 40) were considered positive for *Mycoplasma agassizii* (Weitzman et al. 2017). Mean Ct values per sample were used to indicate infection intensity. Ct values are the inverse of infection intensity, as they refer to the number of PCR cycles necessary to amplify the DNA in a sample.

Bacteria-killing ability and white blood counts were conducted as described in Sandmeier et al. (2012). Briefly, for BKA analyses, 10 µL plasma was added to 10 µL of 500–600 colony-forming units of *Escherichia coli* (ATCC #8739) and 180 µL sterile broth and incubated for 30 min at 37°C, prior to plating on sterile nutrient agar. Samples were run in duplicate, with one positive control (blood plasma from captive animals) and one negative control (no blood plasma) for each set of samples assayed. Bacteria-killing ability was calculated as the mean proportion of cells killed per sample relative to the negative control. Blood smears were used to count relative numbers of heterophils, lymphocytes, eosinophils, basophils, and monocytes per 100 white blood cells, excluding thrombocytes (Alleman et al. 1992).

Statistical analyses

Individual-level analyses.—Pearson’s chi-square statistics were used to test for an effect of genotype on the frequency of animals that tested qPCR-positive for *M. agassizii* and URTD. ANOVA was used to test for a difference in load of *M. agassizii* by genotype, followed by Tukey’s pairwise comparisons. A generalized linear model with a Poisson distribution and a logit link

was used to assess the effect of *M. agassizii* Ct value on URTD score.

Relationships among immunological values were evaluated by correlation. The effects of URTD and tortoise genotype on immune variables were evaluated by ANOVAs, with a modified Bonferroni correction for multiple comparisons for each family, or group, of tests, according to the following formulas (Keppel 1991).

$$\alpha_{\text{family-wise}} = (\text{degrees of freedom}) * (0.05)$$

$$\alpha_{\text{individual comparisons}} = \frac{\alpha_{\text{family-wise}}}{(\text{number of comparisons})}$$

We used $\alpha = 0.025$ for individual comparisons in family-wise tests for the effect of genotype and $\alpha = 0.0167$ for individual comparisons in family-wise tests for the effect of URTD. Tukey’s HSD tests were used for pairwise comparisons when ANOVAs were significant. All immunological values were normally distributed, except for eosinophils and BKA. These were fourth root-transformed for normality.

A principal component analysis (PCA) was used to reduce related immunological variables, including relative lymphocyte, basophil, monocyte, eosinophil, and heterophil counts, and BKA (Table 1). PC1, PC2, BKA, lymphocytes, basophils, monocytes, eosinophils, and heterophils were evaluated as possible explanatory variables for *M. agassizii* in linear regression models. Correlated explanatory variables were not included in the same statistical models. Due to relatively weak associations among immunological values and levels of *M. agassizii* (see *Results* section), model selection was not performed on this dataset.

Population-level analyses.—Prevalence of URTD and *M. agassizii*, as well as means for all other variables, was calculated for the 20 tortoise populations. Associations between prevalence of URTD and both mean Ct values of *M. agassizii* and prevalence of *M. agassizii* were analyzed by linear regressions. To inform model selection (below), relationships among potential explanatory variables (weather, immune function, and genotype) were tested by linear regression and ANOVA. A PCA was used to reduce mean immunological values. Model selection based on corrected Akaike information criteria scores (AIC_c) was used to select the best linear regression

models of prevalence of *M. agassizii*, mean Ct of *M. agassizii*, and prevalence of URTD. We attempted to be as exhaustive as possible in evaluating models, and we analyzed all possible univariate models, models containing all combinations of variables that generated the best univariate models, and models generated by forward and backward selection within all possible sets of non-correlated x -variables. We used the following variables as possible predictors: all climatic variables, PC1, PC2, leukocyte counts, BKA, and genotype. Correlated variables were not included in the same models (e.g., models did not include PCs and individual measures of immune function, days below freezing and lymphocytes, or other correlated weather variables). All statistical analyses were run in JMP, version 12.2.0 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Out of 419 animals, no animal had severe URTD (highest severity score found was 4 on a

scale of 0–6). One animal exhibited purulent (opaque) discharge, 10 had serous discharge from the nares, and the other 50 animals with clinical signs had some partial occlusion of the nares. A total of 14.5% of animals were considered mildly positive for signs of URTD.

Individual-level analyses

There was a significant difference among genotypes in the frequency of animals that tested positive for *Mycoplasma agassizii* ($\chi^2 = 14.57$; $P < 0.001$), with 58%, 53.6%, and 36.2% of animals testing positive for *M. agassizii* in California, Las Vegas, and Northwest Mojave genotypes, respectively. There was not a significant difference in the frequency positive for URTD ($\chi^2 < 0.01$; $P > 0.999$), with 14.3%, 14.4%, and 14.3% testing positive for URTD in California, Las Vegas, and Northeast Mojave genotypes, respectively. There was a significant difference among genotypes in Ct values ($F_{2, 395} = 4.04$, $P = 0.018$), with the Northeast Mojave genotype having higher Ct values (lower infection intensity) than

Table 1. Correlation matrix of white blood cell numbers and BKA within individuals.

Immune variables	Lymphocytes	Basophils	Monocytes	Heterophils	Eosinophils	BKA
Lymphocytes	1.00	−0.12	−0.28	−0.46	−0.37	0.12
Basophils	−0.12	1.00	−0.19	0.01	0.09	−0.15
Monocytes	−0.28	−0.19	1.00	−0.02	0.00	−0.11
Heterophils	−0.46	0.01	−0.02	1.00	0.21	−0.20
Eosinophils	−0.37	0.09	0.00	0.21	1.00	−0.16
BKA	0.12	−0.15	−0.11	−0.20	−0.16	1.00

Notes: Bold values indicate $P < 0.05$. There was a relatively strong inverse relationship between lymphocytes and monocytes/eosinophils/heterophils, with a strong positive correlation between eosinophils and heterophils. Bacteria-killing ability (BKA) was only positively associated with lymphocytes.

Table 2. Descriptive statistics (means and standard deviations [SD]) of immunological values measured in desert tortoises and results of ANOVAs and Tukey's pairwise tests comparing values among the three genotypic populations (California, Las Vegas, and Northeast Mojave).

Variable	Mean	SD	ANOVA	Tukey's pairwise comparisons
Lymphocytes	30.9	15.7	$F_{2, 295} = 5.63$; $P = 0.004$	Las Vegas ($P = 0.014$) and NE Mojave ($P = 0.013$) higher than California
Basophils	18.1	10.0	$F_{2, 295} = 6.36$; $P = 0.002$	California higher than NE Mojave ($P = 0.002$)
Monocytes	16.8	12.4	$F_{2, 295} = 11.21$; $P < 0.001$	NE Mojave higher than California ($P = 0.003$) and Las Vegas ($P < 0.001$)
Heterophils	17.1	12.1	$F_{2, 295} = 4.44$; $P = 0.013$	California higher than Las Vegas ($P = 0.009$)
Eosinophils	9.5	9.2	$F_{2, 295} = 6.41$; $P = 0.002$	California higher than NE Mojave ($P = 0.003$)
Bacteria-killing ability	9.3	14.1	$F_{2, 306} = 3.56$; $P = 0.030$	

Note: A Bonferroni correction for multiple comparisons considers each test significant at a P -value of 0.025 or less.

the California genotype ($P = 0.013$). *Mycoplasma agassizii* Ct values were associated with the degree of URTD exhibited by a tortoise (GLM with a Poisson-logit link: $\chi^2 = 30.76$; $P < 0.001$), with lower Ct values (higher infection intensity) positively associated with increased severity of URTD.

When evaluating immune measures within tortoises, we found significant correlations among levels of various white blood cells and BKA within individual tortoises (Table 1). Lymphocytes were negatively correlated with all other cell types and positively correlated with BKA. Table 2 summarizes differences in leukocyte counts and BKA across genotypes of tortoises. Similar analyses (ANOVA) comparing differences between tortoises with and without URTD in these measures of immune function were all insignificant ($P > 0.017$).

PC1 (32.1%) was largely influenced by measures of cell-based inflammation (Fig. 2a). Heterophil and eosinophil numbers loaded strongly positive on PC1, while lymphocytes and BKA loaded strongly negative on PC1. PC2 (20.8%) was largely influenced by parasite-based and/or specific immunity. Basophils loaded strongly positive and monocytes loaded strongly negative on PC2 (Fig. 2a). Of all possible immunological variables, only PC1 and the number of lymphocytes were significantly, but weakly, associated with loads of *M. agassizii* in linear regressions ($R^2 = 0.04$, $P = 0.002$; and $R^2 = 0.06$, $P < 0.001$, respectively). Inclusion of genotype in the models did not greatly change predictive power (genotype and PC1: $R^2 = 0.06$, $P < 0.001$; genotype and lymphocytes: $R^2 = 0.09$, $P < 0.001$).

Population-level analyses

A mean of 19 animals was sampled in each of 20 populations, and no population had <6 animals sampled. Per population, prevalence of URTD was negatively associated with a population's mean Ct value for *M. agassizii* ($R^2 = 0.25$, $P = 0.023$), but prevalence of URTD was not associated with the prevalence of *M. agassizii* ($P = 0.32$). PC1 (47.9%) was positively associated with cells involved in inflammatory processes, including heterophils, eosinophils, and basophils, and negatively associated with lymphocytes and BKA (Fig. 2b). PC2 (21.9%) was

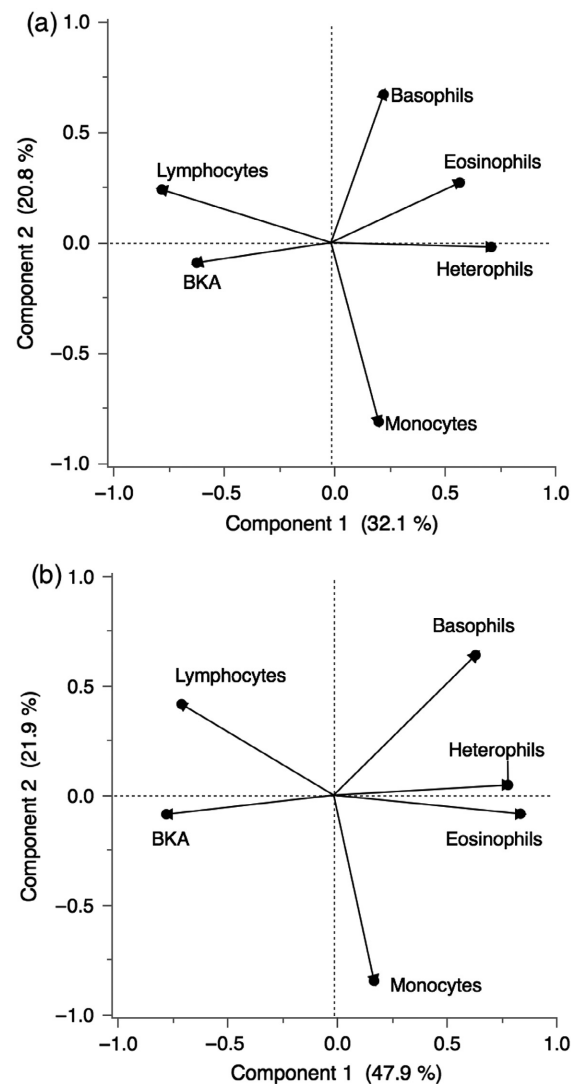


Fig. 2. Principal component analyses (PCA) of immunological measures are shown as vectors loading onto the PC1 and PC2 within (a) individuals and (b) populations. Population-level immunological measures are means of the individuals in that population.

positively influenced by lymphocytes and basophils and negatively influenced by monocytes.

The four best models for predicting prevalence of *M. agassizii*-positive animals, mean infection intensity of *M. agassizii* (inverse of mean Ct values), and prevalence of URTD are presented in Table 3. No correlated variables were included within the same model. The only significant association between an immunological and a weather

Table 3. The best four models (by AIC_c) for the following population-level predictor variables of disease: prevalence of *Mycoplasma agassizii*, the mean Ct value for *M. agassizii* per population (inversely related to infection intensity), and prevalence of upper respiratory tract disease (URTD).

Response variable	Explanatory variable(s)	AIC _c	Akaike weight	R ²	P
Prevalence of <i>M. agassizii</i> -positive animals					
1	Days below freezing	1.55	0.54	0.25	0.034
2	Days below freezing + PC2	2.83	0.28	0.33	0.196
3	Days below freezing + lymphocytes	3.73	0.18	0.30	0.330
4	Lymphocytes	4.46	0.13	0.13	0.132
Mean Ct value of <i>M. agassizii</i>					
1	Mean number of lymphocytes	46.52	0.59	0.55	0.004
2	Mean number of lymphocytes + days > 32C	48.15	0.26	0.59	0.246
3	Lymphocytes + days > 32C + Bacteria-killing ability	50.51	0.08	0.63	0.279
4	PC2 + days below freezing	50.91	0.07	0.52	0.033
Prevalence of URTD					
1	PC1	-42.79	0.33	0.28	0.026
2	<i>M. agassizii</i> Ct	-42.75	0.32	0.27	0.026
3	PC1 + <i>M. agassizii</i>	-42.38	0.27	0.30	0.123
4	PC1 + days below freezing	-40.05	0.08	0.30	0.480

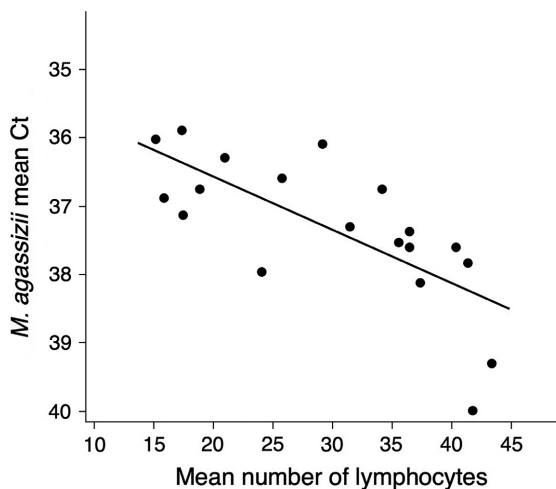


Fig. 3. Mean infection intensity of *M. agassizii* in tortoise populations decreases with increasing numbers of lymphocytes (higher Ct values indicate lower quantities of *M. agassizii*; $R^2 = 0.55$, $P = 0.004$).

variable was between mean lymphocyte numbers and mean number of days below freezing ($R^2 = 0.26$, $P = 0.026$; Fig. 3). The best model for predicting prevalence of *M. agassizii* included mean annual days below freezing (Table 3). The best models for predicting mean Ct values of *M. agassizii* included mean numbers of lymphocytes

(Table 3; Fig. 4). Days below freezing was significantly associated with mean *M. agassizii* Ct ($R^2 = 0.27$, $P = 0.026$), but was not included in the five best models for predicting Ct values (AIC_c = 55.18, Table 3). The best three models for predicting the proportion of each population with URTD had very similar AIC_c scores and included PC1, mean *M. agassizii* Ct, and PC1 plus mean load of *M. agassizii* Ct (Table 3, Fig. 5).

DISCUSSION

Summary of general patterns and tests of hypotheses

In contrast to a previous disease survey (Sandmeier et al. 2013), levels of URTD signs in tortoises did not differ among genotypic groups, despite the frequency of *Mycoplasma agassizii* being lower in the NE Mojave genotype. Thus, patterns of elevated URTD in the northern part of the range of *Gopherus agassizii* are not temporarily stable, and overall, prevalence of URTD was lower across the range in 2010–2012 compared to the previous survey in 2004–2006 (Sandmeier et al. 2013). Hypothesis 1: Animals with URTD did not exhibit significant differences in immunological measures. Thus, neither differential white blood cell counts, nor BKA, can be used as a diagnostic measure of disease in wild animals with

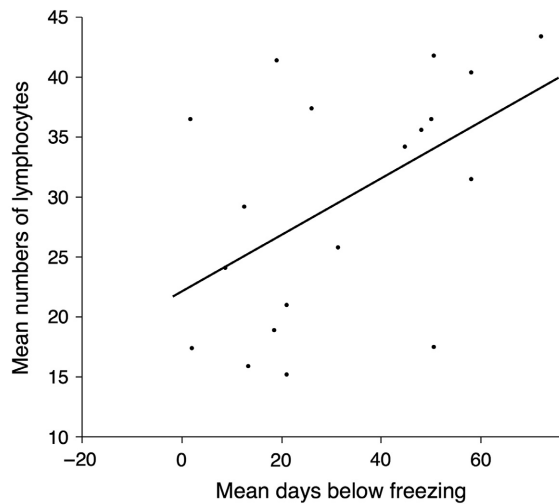


Fig. 4. Mean number of lymphocytes was the only immune measure strongly associated with climate (mean annual days below freezing; $R^2 = 0.26$, $P = 0.026$) among tortoise populations.

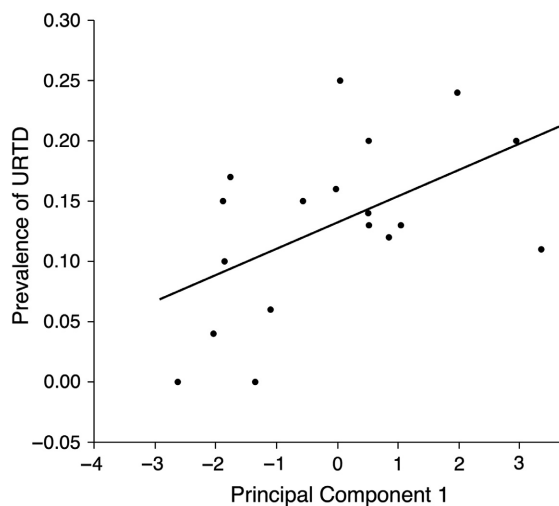


Fig. 5. Prevalence of upper respiratory tract disease in tortoise populations was associated with mean values of PC1 (representing cells involved in inflammation; $R^2 = 0.28$, $P = 0.026$).

mild signs of URTD. Hypothesis 2: Intensity of *M. agassizii* infection was related to increased severity of URTD, showing that disease is more closely associated with infection intensity versus the simple presence of the pathogen. There were significant, but weak associations among disease

and immune measures, largely driven by a positive relationship between lymphocytes and *M. agassizii* levels. Hypothesis 3: As predicted, these patterns of association were much stronger at the population level, though qualitatively similar to individual-level analyses. Relationships were mostly driven by lymphocytes being closely associated with mean levels of *M. agassizii*, and signs of URTD were associated with cells involved in inflammatory responses. This finding suggests two immunological responses to mycoplasmal infection—one that results in URTD and one that does not. In particular, lymphocytes appear important in reducing pathogen loads in the absence of inflammation. Hypothesis 4: The most significant climatic measure associated with measures of disease and immune function (primarily lymphocytes) was mean number of days below freezing, which roughly dictates the minimum length of winter dormancy in tortoises (Nussear et al. 2007). In contrast to our prediction, lymphocytes increased in areas with cooler winters. A lack of importance of climatic variables across the range during the active season was surprising, but may be due to behavioral temperature regulation during this time and available microclimates at a given site.

Disease and immune function in individuals

The lack of association between immune measures and URTD was likely influenced by the majority of URTD-positive tortoises showing very weak signs of disease in contrast to animals with experimental infections (Brown et al. 1994, Sandmeier et al. 2017b). In addition, even healthy animals show a large degree of individual variation in both differential white blood cell counts and BKA under controlled laboratory conditions (Sandmeier et al. 2016). Such individual variation may obscure patterns of changes that occur within a tortoise in response to mild infection. We did find a relationship between increased levels of *M. agassizii* and increased severity of URTD. Animals may only react with inflammatory responses, visible as external exudate, when experiencing high levels of pathogen.

Evident in all analyses with genotype as a factor, the California genotype had the highest prevalence of *M. agassizii*, and high levels of cells involved in inflammation (Table 1). The

Northeast Mojave genotype had lower levels of PC2, mostly driven by reduced levels of basophils (Table 1, Fig. 2a). Although the functional role of basophils is not entirely understood in reptiles, basophils have been shown to be elevated in Testudines with inflammatory diseases, suggesting similar functions across vertebrate species (Rosskopf 2000, Wilkinson 2004). Because prevalence of URTD was similar across genotypes, this suggests that the action of inflammatory cells (heterophils and eosinophils, contributing strongly to PC1) and more specific cell-based immunity (lymphocytes and basophils, contributing strongly to PC2) varies across tortoise genotypes and that inflammatory responses are associated with increased prevalence of URTD.

Disease and immune function in populations

As expected, patterns of association among disease and immune measures were stronger on a population level (Table 3, Figs. 3, 4, 5). In part, this can be explained by the intermittent nature of signs of URTD and the slow immunological response of desert tortoises (Sandmeier et al. 2012, 2017b). Importantly, mean levels of *M. agassizii* and URTD were explained by different variables (Table 3). Both infection intensity of *M. agassizii* and inflammatory responses (PC1) predicted URTD (Table 3, Fig. 5). Conversely, lymphocytes were associated with decreased mean infection intensity of *M. agassizii*. If lymphocytes reduced mycoplasma numbers, they did so without concomitant increases in inflammatory cells (PC1; Table 3, Fig. 4). Because lymphocytes were not associated with URTD and seroprevalence has been associated with URTD in past studies (Brown et al. 1994, Sandmeier et al. 2013, 2017b), it is unlikely that the sole function of lymphocytes is the production of induced antibodies. We hypothesize that B-1 lymphocytes form a large portion of the lymphocytes quantified in these tortoises and that they function both in the production of natural antibodies and phagocytosis. Similar hypotheses have recently been proposed to explain immunological responses in other species of Testudines, described below (Zimmerman et al. 2009, 2013, Goessling et al. 2017).

Lymphocytes in all vertebrates are made up of diverse cell types, including the broad categories of helper cells, T cells, B-1 cells, and B-2 cells

(Murphy 2012). B-1 and B-2 lymphocytes produce natural and induced antibodies, respectively (Murphy 2012). In mammals, B-2 populations of cells are much larger than B-1 populations of lymphocytes, but recent findings question this pattern in ectothermic vertebrates (Li et al. 2006, Murphy 2012, Goessling et al. 2017). A relatively large portion of B cells in red-eared sliders (*Trachemys scripta*) was shown to have phagocytic properties (Zimmerman et al. 2009), and phagocytic ability of lymphocytes has been quantified in species of fishes and an amphibian (*Xenopus*; Li et al. 2006). These cells are hypothesized to be equivalent or similar to mammalian B-1 cells (Li et al. 2006). Subsequently, phagocytic B-1 cells were also discovered in mice (Gao et al. 2012, Parra et al. 2012). Interestingly, the tortoise populations with higher levels of lymphocytes also showed higher levels of natural antibodies to *M. agassizii* in a past study (Sandmeier et al. 2013). Goessling et al. (2017) hypothesized that gopher tortoises also have large populations of B-1 lymphocytes.

If lymphocytes in desert tortoises are phagocytic against *M. agassizii*, then the negative relationship between lymphocytes and other phagocytic cells (heterophils, eosinophils, and monocytes) could be explained as a functional trade-off (Table 2, Fig. 2). In that case, two types of immune responses may result from infection with *M. agassizii*: one directed through B-1 lymphocytes capable of phagocytosis without stimulation of the rest of the immune system, and one directed through inflammatory phagocytic cells (heterophils, eosinophils, and monocytes), which also stimulate B-2 cells to produce induced antibodies (Murphy 2012). We further hypothesize that an induced antibody response only occurs as *M. agassizii* infection intensity increases (sensu Brown et al. 1994, Aiello et al. 2016, Sandmeier et al. 2017b). We suggest that induced antibodies, produced by B-2 cells and known to be recognized by macrophages, prolong inflammatory responses, causing the immunopathology witnessed in some, but not all, tortoises exposed to *M. agassizii* (reviewed in Sandmeier et al. 2009, Jacobson et al. 2014).

Furthermore, lymphocytes seem to be produced at higher quantities in cooler thermal regimes. In a captive desert tortoise population, lymphocyte numbers increased in fall and winter, again suggesting an immune strategy

acclimated to cooler temperatures (Sandmeier et al. 2016). Together, these data imply that lymphocyte function may acclimate well to cooler temperatures. Similarly, antibody production by lymphocytes in gopher tortoises increased during cooler seasons, and lymphocytes showed acclimation to seasonal temperatures in both summer and winter (Goessling et al. 2017). If desert tortoise populations in cooler areas produce more lymphocytes, then the question is why tortoises in the other areas did not have higher levels of URTD. We hypothesize that tortoises in warmer thermal regimes have more opportunities to bask on an annual basis, and partially rely on this mechanism to lower levels of *M. agassizii*, which are killed above 16°C (Mohammadpour et al. 2009).

Next steps in testing our hypotheses include *in vitro* phagocytic assays of isolated lymphocytes in the presence of *M. agassizii*. The creation of IgM- and IgY-specific reagents would further allow for different sub-populations of lymphocytes to be labeled, as currently the only reagents available are not isotype-specific (Schumacher et al. 1993, Hunter et al. 2008).

CONCLUSIONS

The relationship between *Mycoplasma agassizii* and morbidity has been enigmatic in wild tortoises. Here, we show that lymphocytes are strongly associated with reduced levels of the pathogen, *M. agassizii*, within individuals and lymphocytes also vary with climatic variation across the natural range of the host species. The hypothesis that lymphocytes control *M. agassizii* via phagocytosis, without incurring pathology to the host, needs to be tested via experimentation, but such experimentation would provide wildlife managers with additional tools to quantify resistance to disease in wild populations.

ACKNOWLEDGMENTS

All applicable institutional and/or national guidelines for the care and use of animals were followed. Permits included the University of Nevada, Reno IACUC (00465 and 0555), U.S. Fish and Wildlife Service (TE076710-7/8), California Fish and Game (SC-007374), Nevada Department of Natural Resources (S-33080 and S-355714), Utah Division of Wildlife (5COLL8886),

and National Park Service (MOJA-2011-SCI-0022, MOJA-2012-SCI-009; ZION-2012-SCI-0006, and JOTR-2011-SCI-0021). Funding was provided by the U.S. Fish and Wildlife Service grant 1320-114-23DZ. We would like to thank multiple student laboratory and field technicians including Kendra Leonard, Chris Cannon, Quoc (Steve) Nguyen, Makis Gomez, Lindsay Parton, Kristen Pietrzyk, John Prescott, Brett Davis, Jenny Todd, Stephanie Siemeck, Martyn Drabik-Hamshare, Tim Ichien, and Lauren Schumacher. The datasets generated during the current study are available as supporting information Appendix S1 (summarized disease, immune measures, and climate data for populations of Mojave desert tortoises). Additional information is available from the corresponding author on request.

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