Tools to understand seasonality in health: quantification of microbe loads and analyses of compositional ecoimmunological data reveal complex patterns in tortoise populations

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Tools to understand seasonality in health: quantification of microbe loads and analyses of compositional ecoimmunological data reveal complex patterns in tortoise populations

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Tools to understand seasonality in health: quantification of microbe loads and analyses of compositional ecoimmunologcal data reveal complex patterns in tortoise populations


Abstract

Using data from six wild Mojave desert tortoise (Gopherus agassizii, Cooper 1861) populations, we quantified seasonal differences in immune system measurements and microbial load in the respiratory tract, pertinent to this species’ susceptibility to upper respiratory tract disease. We quantified bacteria-killing activity of blood plasma and differential leukocyte counts to detect trends in temporal variation in immune function. We used centered log-ratio (clr) transformations of leukocyte counts and stress that such transformations are necessary for compositional data. We tested animals for the potential pathogen, Pasteurella testudinis with a newly created quantitative PCR assay, as well as for the known respiratory pathogens Mycoplasma agassizii and M. testudineum. We found very little disease and suggest that P. testudinis is a prevalent, commensal microbe in these tortoise populations, and its quantification may be tool to study natural fluctuations in microbe levels in tortoise respiratory tracts. Our analyses showed that both the potential for inflammatory responses and microbe levels are highest in the spring for healthy tortoises, when lymphocyte levels are lowest. The genetic and statistical tools we used are easily applicable to other wildlife systems and provide the necessary data.
to quantify species-wide trends in health and test hypotheses pertinent to host-microbe dynamics.

**Key words:** *Gopherus agassizii;* Mojave desert tortoise; ecoimmunology; ectotherm; temporal variation;
Introduction

Quantifying risk of disease outbreaks in species of conservation-concern is increasingly important to wildlife management (USFWS 2011). Accurately predicting disease dynamics, depends on accurately quantifying variation in host immunological parameters and pathogen loads across seasonal changes (Altizer et al. 2006; Wobeser 2006). Quantification of seasonal fluctuations in the normal microbiome, or non-pathogenic microbes, may also influence physiological health of the host and is becoming a more common tool to assess disease-risk in wild populations (Woodhams et al. 2014). In ectothermic hosts both immune function and microbe growth rates are expected to fluctuate with changes in seasonal temperature, and seasonal changes are likely to affect epidemiological patterns (Martin et al. 2008; Goessling et al. 2017).

Upper respiratory tract disease (URTD) is a chronic disease of concern to conservation-efforts that occurs in populations of threatened Mojave desert tortoises (*Gopherus agassizii*, Cooper 1861) (USFWS 2011). Populations are subject to increasingly aggressive management strategies, such as translocations and population augmentation, largely due to continued growth of urban areas and the solar energy industry (Lovich and Ennen 2011). While URTD persists at low prevalence in populations without dangerous epizootic outbreaks, the extrinsic or intrinsic triggers for these outbreaks are not well understood (Sandmeier et al. 2009). The prevalence of URTD and its predominant etiological agent, *Mycoplasma agassizii*, are correlated with regional variations in temperature across the range of the tortoise and URTD prevalence can increase in cooler climates (Sandmeier et al.
Thus, it is suspected that seasonal fluctuations in temperature may also influence effects of pathogens and immune mechanisms. Desert tortoises show some seasonal differences in levels of leukocytes (white blood cells) and the bacteria-killing activity (BKA) of blood plasma, even under constant thermal laboratory conditions (Sandmeier et al. 2016).

Here, we quantified seasonal infection intensity of three microbes, which are known or suspected to be associated with URTD in a load-dependent fashion (Weitzman et al. 2017). *Mycoplasma agassizii* and *M. testudineum* are known etiological agents of URTD in *Gopherus* tortoises (Brown et al. 1994, 2004), and can be quantified by qPCR (Braun et al. 2014). *Pasteurella testudinis* does not confer URTD directly, as shown in experimental infections (Brown et al. 1994), but it is widespread in tortoises and has been hypothesized to exacerbate disease in the presence of *M. agassizii* under natural conditions (Snipes and Biberstein 1982; Snipes et al. 1995). In other host-mycoplasma systems, infections often are exacerbated when they occur with species belonging to the Pasteurellaceae (e.g., Brogden et al. 1998; Gustafson et al. 1998). Here, we created a qPCR to quantify *P. testudinis*, which can be used in conjunction with the existing qPCR assays for *M. agassizii* and *M. testudineum* (Braun et al. 2014).

We also quantified two aspects of the innate immune-function – BKA of blood plasma and circulating leukocytes. Both these mechanisms are constitutively present, can be quantified in healthy and diseased animals, and seasonal fluctuations in these measures have previously been quantified in captive tortoises (Sandmeier et al. 2016). These measures of the immune system do not provide a
complete view of "immunocompetence", but if shifted or depressed, indicate differences in how the body will react to infections (Martin et al. 2008). The BKA assay measures the efficacy of innate levels of proteins in the blood plasma (e.g., natural antibodies, complement, and acute phase proteins) to limit growth or kill extracellular bacteria, using a standard strain of *Escherichia coli* (ATTC 8739) (Millet et al. 2007). These proteins can directly lyse bacteria or "tag" the bacteria for destruction by phagocytic cells (Murphy 2012). Circulating leukocytes are a major component of immune responses and depressions of particular cell types can be indicative of susceptibility to disease (Jacobson et al. 2007). Here we refine analyses of differential leukocyte counts, taking into account that these data are ratios, or compositional. Problems in analyzing compositional data as raw counts have long been recognized by geologists and medical researchers, and recently have been highlighted in analyses of microbiome data (Aitchison 1983; Muriithi 2015; Gloor et al. 2017). Ecoimmunologists have analyzed these data as raw counts (Davis et al. 2008), which is statistically inappropriate for compositional datasets (Aitchison 1983). Here, we used the centered log-ratio (clr) transformation, and suggest the widespread adoption of this technique.

Our analyses are based on six, free-ranging tortoise populations representing all three genetic populations in the Mojave Desert (Fig. 1; Hagerty and Tracy 2010). Animals were sampled repeatedly in spring, summer, and fall. Because we detected very little disease, we examined a number of hypotheses about natural seasonal fluctuations in immune measures and microbial loads. First, we tested whether clr transformation of leukocyte counts changes relationships among variables. We then
quantified the influence of both site and season on immune function – by testing the hypotheses that expensive inflammatory responses should be maximized in summer and lymphocyte levels should be highest toward in fall and winter (Sandmeier et al. 2016; Goessling et al. 2017). We then compared seasonal changes in wild populations to those previously reported for a captive population, to test the hypothesis whether true “baseline” physiological measures for a species are informative or heavily influenced by environmental conditions. Finally, we used model selection to identify factors that appear important in influencing microbial loads (*P. testudinis*), such as the measured variables of immune function, season, broad differences in regional climate, and immediate climatic conditions at time of sampling. We hypothesized that warmer temperatures and/or reduced immune function would increase levels of bacteria in the respiratory tracts of tortoises.

**Materials and Methods**

**Field collection**

Tortoises were tracked by radio-telemetery in six populations across the year (Holohil Systems LTd, Carp, Ontario, Canada) and health assessments were conducted on as many animals as available at the beginng of each season, except winter, in 2012 (Fig. 1). Because tortoises hibernate in deep burrows, we were not able to sample them in winter. Animals were all uniquely marked, and we attempted to collect samples from each animal three times, within approximately two weeks of each equinox and solstice. We only handled animals that were in the open or within reach in their burrows during the day, when air temperatures were between 20 and 35°C. Therefore, during some seasons we were unable to sample animals in deep
burrows. We sampled a mean of 16 animals per population, although animal numbers per populations ranged from 7 to 29.

URTD was scored according to Sandmeier et al. (2017): 0 (no disease), 1-3 (some damage or occlusion of the nares), 4-6 (visible exudate), with scores of 2 or higher considered indicative of URTD. Three ml of sterile saline was gently washed through the nares of each tortoise, and immediately added to RNAlater (Qiagen) at a concentration of 500 µl of sample to 200 µl of RNAlater (Weitzman et al. 2017). We collected a maximum of 0.5 ml of blood from the subcarapacial sinus of each tortoise within 15 minutes of capture (Hernandez-Divers et al. 2002). A thin blood smear was made in the field and stained with Wright-Giemsa stain within the week. Whole blood was kept on ice for a maximum of eight hours, before plasma was separated and frozen at -20°C. All procedures were approved by the Institutional Animal Care and Use Committee (A06/07-49).

Laboratory analyses

A hydrolysis probe (Taqman) qPCR for Pasteurella testudinis (Table 1) was developed from the rpoB sequence deposited on GenBank (AY362972) using Primer Express software 3.0.1. We used BLAST (Blast.ncbi.nlm.nih.gov/Blast.cgi) to test potential target sequences for specificity to P. testudinis.

The qPCR reaction mixture (25 µl volume) consisted of 12.5 µL Taqman Environmental Master Mix 2.0, 0.2 µL forward primer, 0.2 µL reverse primer, 0.025 µL probe, 9.58 µL DNA free water, and 2.5 µL of DNA. Plates were analyzed using a QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). The thermal profile included 2 minutes at 50°C, 10 minutes at 95°C, and 45 cycles of 15 seconds at 95°C
and 1 minute at 58.8°C. In vitro specificity to *P. testudinis* (ATCC 33688) was tested against two other species within the Pasteurellaceae and other microbes likely to be in the respiratory track: *Pasteurella multocida* (ATCC 43137), *Phocoenobacter uteri* (ATCC 700972), *Escherichia coli* (ATCC 87446), *M. agassizii* (ATCC 700616), and *M. testudineum* (ATCC 700618) (Dewhirst et al. 1993; Gregersen et al. 2009). DNA was extracted directly from pellets obtained through ATCC (American Type Culture Collection; https://www.atcc.org) using a Qiagen DNeasy Blood and Tissue kit, and quantified on a nanodrop (ND1000, Thermofisher Scientific). To convert Ct value to copy number, we used dilution curves of plasmids (Integrated DNA technologies), each containing the target-sequence of the qPCR assays designed for *M. agassizii*, *M. testudineum* (Braun et al. 2014), or *P. testudinis*. Three dilution curves (5x10^9-5 copy numbers), run in triplicate, were used to calculate inter- and intra-plate variation and detection-limit for *P. testudinis* (Fig. 2).

For each nasal lavage, 500 μl were extracted using a Qiagen DNeasy Blood and Tissue Kit and the protocol for gram-negative bacteria. Each sample was tested for *P. testudinis* using the protocol described above, as well as for *M. agassizii* and *M. testudineum* (Braun et al. 2014). Samples were run in triplicate and two or more Ct values less than 40 were considered positive (Braun et al. 2014). The average Ct of a sample was converted into number of copies of DNA, according to plasmid dilution curves.

All desert tortoise plasma samples were tested via BKA within less than one year of collection. We used the protocol previously described for desert tortoises in Sandmeier et al. (2016). Briefly, 10 μl of plasma was added to 190 μl of nutrient
broth, containing approximately 500 colony forming units of *E. coli* (ATCC 8739). Cultures were incubated for 30 minutes at 37°C and plated onto sterile nutrient agar to quantify colonies after incubation overnight. Samples were run in duplicate, with one set of negative controls (no tortoise plasma) and one positive control (pooled sample from captive tortoises). Mean percent of bacteria killed by each tortoise plasma sample was calculated in relation to the negative control.

Differential leukocyte counts were conducted manually to quantify heterophils, lymphocytes, eosinophils, basophils, and monocytes/azurophils per 100 leukocytes, excluding thrombocytes (Alleman et al. 1992; Sandmeier et al. 2016).

**Quantification of weather variables**

We calculated two different types of weather data. One set of data quantified long-term differences in regional climate and was averaged over 15 years preceding this study. Values were averaged directly from two to four NOAA weather stations (ww.ncdc.noaa.gov/data-access/land-based-station-data) nearest to each site (Sandmeier et al. 2013). These data included mean, minimum, and maximum annual temperatures, precipitation (annual, summer (April-September), and winter (October-March)), mean number of days above 32.2°C, and mean number of days below 0°C. The second set of data quantified monthly mean weather during the month each tortoise was sampled for specific locations, using the PRISM database (http://www.cefa.dri.edu/Westmap/Westmap_home.php?page=Prism101.php). Data included monthly mean, minimum, and maximum temperatures and mean precipitation for the spatial center of each sampling location.
Statistical analyses

Copy numbers of microbe DNA were log$_2$-transformed for normality. Due to uneven and non-normal distribution of residuals in regression models, transformed values were also used as independent variables in analyses. We used logistic regressions, with individuals included as a random effect, to test whether infection intensity of either *M. agassizii* and/or *P. testudinis* was predictive of URTD within individuals.

Prevalence of URTD and microbes were calculated per population and were transformed for normality, with a modified arcsin-squareroot transformation, as recommended for data in the form of proportions and containing values of zero (Zar 1999):

\[
p' = \frac{1}{2} \left[ \text{arcsin}(\sqrt{x/(n+1)}) + \text{arcsin}(\sqrt{((x+1)/(n+1))}) \right]
\]

We used regression models, with site included as a random effect, to test for associations among a population’s proportion of animals positive for *M. agassizii* and/or *P. testudinis* and the proportion positive for URTD.

Leukocyte counts were clr transformed with CoDaPack, a free software package (http://www.compositionaldata.com/codapack.php; Muriithi 2015). We compared Principal Component Analyses (PCA) conducted on clr-transformed data and data treated as counts. Count data was normalized through square-root transformations (all leukocyte counts except basophils). BKA was fourth-root transformed for normality.

Regression models, with tortoise included as a random effect to account for repeated-measures, were used to compare seasonal differences among individual
measures of immune function (clr transformed leukocyte values and fourth-root transformed BKA). Because site was significant in all models, it was included as a covariate. In these analyses, $\alpha$ was adjusted to 0.017, according to the following formulas, to correct for multiple comparisons (Keppel 1991):

$$\alpha_{\text{family-wise comparisons}} = (\text{degrees of freedom})(0.05) = 0.10$$

$$\alpha_{\text{individual comparisons}} = \frac{\alpha_{\text{FW}}}{(\text{number of comparisons})} = 0.017$$

We used similar models to test for seasonal effects on principal component 1 (PC1) and principal component 2 (PC2) (both with normal distributions), using $\alpha=0.05$.

We also compared the seasonal fluctuations in leukocytes and BKA in wild populations to previously-published changes in leukocyte counts and BKA of a captive population (data was obtained from Sandmeier et al. 2016). These animals were tested within the same year, and included 30 captive-reared individuals, kept under controlled laboratory conditions and fed $ad$ $libitum$. Raw data was clr-transformed and the same analyses as above were preformed, including “captive” as a seventh site. When models were significant, we conducted post-hoc, Tukey’s pairwise comparisons on site as well as season.

We used corrected Akaike Information Criteria (AICc) scores and weights to evaluate a number of regression models, including individual as a random effect, to predict infection intensity of $P. testudinis$ within tortoises (Burnham and Anderson 2002). Independent variables included all measures of immune function, all climatic
variables, genetic population (Hagerty and Tracy 2010), season, and site. A correlation matrix was used to evaluate relationships among variables, and correlated variables were not included in the same models.

**Results**

Field sampling

We sampled most tortoises two to three times across the seasons. We performed 282 health evaluations, with samples collected from 116 unique animals. A total of 246 nasal lavages were tested for *M. agassizii*, *M. testudineum*, and *P. testudinis*. BKA assays were performed on 229 blood plasma samples and differential leukocyte counts were performed on 220 blood smears – 202 samples were evaluated by both techniques and could be evaluated by PCA. No animal had more than one diagnosis of being positive for URTD, and signs of the disease were only observed in 18 tortoises. Signs of disease were weak, with 16 URTD scores of “2”, and only two tortoises displayed serous or purulent discharge of mucous once.

Development of *P. testudinis* qPCR

The qPCR assay for *P. testudinis* did not amplify any of the microbes it was tested against. Intraplate variation ranged from 0.16-0.24 Ct values, and interplate variation was ±0.52 Ct values (Table 1, Fig. 2). The detection limit of the assay was 50 copies, with a replication efficiency of 96% (Fig. 2).

Patterns of microbe prevalence and statistical analyses

Sixteen samples tested positive for *M. agassizii*, none tested positive for *M. testudineum*, and 100 tested positive for *P. testudinis*. On a population-level, the
mean prevalence per season of URTD, \textit{M. agassizii}, and \textit{P. testudinis} were 8.0%, 8.3%, and 43.4%, respectively.

Infection intensities of \textit{M. agassizii} or \textit{P. testudinis} were not predictive of URTD in logistic regressions ($\chi^2=0.642$, $p=0.42$; $\chi^2=2.352$, $p=0.125$, respectively). Neither a population’s prevalence of \textit{M. agassizii}, nor \textit{P. testudinis}, was associated with the prevalence URTD ($p=0.2186$; $p=0.5144$, respectively). Prevalence of URTD did not change by season ($p=0.1229$). Prevalence of \textit{M. agassizii} also did not vary by season ($p=0.1994$).

PC1 and PC2 are depicted in Figure 3. Compared to untransformed values, clr transformations changed some of the relationships among variables, primarily increasing the influence of eosinophils and heterophils on PC1 and reducing their effect on PC2. There was some seasonal differentiation, along with overlap, among data points (Fig. 3c). PC1 was positively influenced by cells involved in early inflammation (heterophils and eosinophils) and was negatively influenced by lymphocytes (Fig. 2b). PC2 was positively influenced by monocytes (Fig. 2b).

All analyses examining seasonal variation in immune measures included tortoise as a random variable and site as a covariate, and site was significant in these analyses (Table 2, Fig. 4). Seasonal variations were significant for all immune measures, except for BKA (Table 2, Fig. 4). Measures associated with inflammation (PC1, heterophils) were highest in spring and fall and lowest in summer, and the inverse was true for lymphocytes (Fig. 4).

When the captive population was included as an additional “site”, all significant seasonal changes remained the same, except for decreased variation
across seasons in heterophils and PC2 (Table 2). While at least one population differed from the captive population in all models, numbers of lymphocytes per season were lower than the captive population in five out of six populations (Table 2).

Model selection indicated that a number of models predicted levels of *P. testudinis* (Table 3). Models with the lowest AICc values included combinations of PC1, PC2, and mean monthly temperature. The highest levels of *P. testudinis* occurred in spring. Decreases in *P. testudinis* occurred with warmer monthly mean temperatures, and with increased levels of lymphocytes and basophils. Increases in *P. testudinis* occurred with increased levels of heterophils, eosinophils, PC1, and PC2.

**Discussion**

*Low prevalence of disease*

We observed very little disease in our six populations in 2012 and likely did not have the statistical power to detect associations between microbial load and URTD. However, the creation of a sensitive qPCR assay allowed us to show that free-ranging, healthy tortoises have a high prevalence of *P. testudinis*. We interpreted the presence of *P. testudinis* as a commensal microbe that is typically part of the normal flora in the upper respiratory tract. Similarly, a recent study of the respiratory microbiome in various species of *Gopherus* found high levels of *Pasteurella* in many healthy individuals (Weitzman 2017). However, our data could not address whether or not *P. testudinis* could act as an opportunistic pathogen when it is present with *M. agassizii*. 

https://mc06.manuscriptcentral.com/cjz-pubs
**Clr-transformation of differential leukocyte counts**

When examining relationships among leukocyte counts, clr transformations changed relationships quantitatively more than qualitatively (Fig. 3). However, appropriate transformations of compositional data are especially important if those measures are then used in further statistical. Specifically, clr transformations retain the relationships among variables and normalize all variables – thus allowing for both clear interpretation and statistical analyses that assume normality in error variances (Aitchison 1983). Leukocyte counts are very informative and relatively easy to perform across taxa (Davis et al. 2008), but we voice caution in interpreting these studies. Analyses can easily be duplicated with transformed data, which can readily be calculated with easy-to-use, free software packages.

**Variation in immune measures**

Site was always significant in models of seasonality of immune measures (Table 2). Unlike past studies focusing on desert and gopher tortoises, our study included a greater geographic extent across the range of the species, and we found a much greater influence of site (Christopher et al. 1999; Dickenson et al. 2002). These results highlight the importance of encompassing the genetic and environmental variation experienced by a species. Establishing general trends in hematological values for species may be more useful to conservation than establishing “baseline values” that vary across conditions (Christopher et al. 1999). For example, the captive population varied from natural populations largely due to elevated lymphocyte levels (Table 2), which may be attributed to lymphocytes possibly increasing with *ad libitum* water and forage availability. A similar pattern
has been noted for Mojave desert tortoise populations in wet years and for Sonoran desert tortoise populations that experience higher levels of rainfall than Mojave populations (Christopher et al. 1999; Dickenson et al. 2002).

As predicted, all leukocytes showed significant seasonal variation, and lymphocytes levels were lowest in spring (Fig. 4). We interpret PC1 as the potential for short-term inflammation due to the positive influence of heterophils and eosinophils and PC2 as the potential for long-term inflammation/maintaining immune responses due the positive influence of monocytes (Fig. 3; Murphy 2012). Both were highest in the spring, in opposition to lymphocyte numbers (Table 2; Fig. 4; Murphy 2012). Recently, large proportions of lymphocytes in ectothermic vertebrates have been shown to be innately phagocytic and similar to B-1 lymphocytes in mammals (Li et al. 2006; Zimmerman et al. 2009). B-1 lymphocytes often down-regulate inflammatory processes, in contrast to the other phagocytic cells (Murphy 2012; Parra et al. 2012). This suggests that there might be a seasonal trade-off in phagocytosis in tortoises – with inflammation more likely to occur in springtime. This trend is important to understanding the epidemiology of URTD in tortoises, as pathology is associated with chronic inflammation while the numbers of circulating lymphocytes are associated with reduced loads of *M. agassizii* (Sandmeier et al. 2018).

*Variations in* *P. testudinis* *load*

While site, season, and monthly temperature were all predictive of load of *P. testudinis*, the best models included immunological variables, namely PC1 and PC2 (Table 3; Fig. 3). Because PC1 and PC2 also varied by season and site, these
measures likely integrate environmental conditions and their effects on tortoise physiology (Table 2). Unlike models of *M. agassizii* load (Sandmeier et al. 2018), regional climatic differences were not important and *P. testudinis* was only associated with climate during the month of sampling. In contrast to our predictions, levels of *P. testudinis* were highest in the spring, at times when inflammatory-potential (PC1 and PC2) was highest, and at cooler temperatures. Thus, we found that both inflammatory responses and bacterial load were highest in the spring. This likely means that tortoises may be especially susceptible to showing signs of URTD in the spring, in years with higher rates of *M. agassizii* prevalence.

Immune function, especially at warmer, optimal body temperatures in summer and fall (sensu Zimmerman et al. 1994) and/or dominated by lymphocytes may be important in regulating not just pathogens, but also normal flora in tortoises – here represented by *P. testudinis*. How immune function regulates the normal microbiome is currently not fully understood but is broadly important to understanding the process of symbionts acting as opportunistic pathogens under certain circumstances and associated declines in health of the host (Rózsa et al. 2015). Our *P. testudinis*-specific qPCR assay could be an important tool for detecting potential load-dependent shifts from a commensal to a pathogenic relationship in tortoise hosts (Rózsa et al. 2015).

Alternatively, if further studies confirm that *P. testudinis* is a true commensal bacterium then the qPCR assay can be used to answer current questions in host-microbe ecology. For example, how microbiomes are shaped by environmental and host factors and how those change seasonally are not well understood across taxa.
(McKenzie et al. 2012; Longo et al. 2015). Such questions are best answered by identifying a core microbiome in a host species, but a recent study of Gopherus species did not identify a core respiratory microbiome in tortoises (Weitzman 2017). However, within each species roughly 2/3 or more of individuals did test positive for P. testudinis (Weitzman 2017). Given the cost of measuring microbiome data across seasons for many individuals, as well as difficulties in accurately measuring bacterial loads with universal 16s rRNA primers (Horz et al. 2005), the use of qPCR specific for common commensal microbes represents a cost-efficient way to understand general trends in in vivo microbe growth rates and can be applied across a diversity of host-microbe systems.

**Acknowledgements**

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References


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probiotic effectiveness. PLOS One, 9(4): e96375. doi:
10.1371/journal.pone.0096375


Table 1. Assay parameters and information specific to the target sequence for the newly developed qPCR specific for *P. testudinis*.

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Table 2. Repeated-measures regression models, including site as a covariate, of the variation of different immunological measures by season. Due to multiple comparisons, \( p \)-values for all models, except PC1 and PC2, were considered significant at \( \alpha=0.017 \). Different letters depict significant differences among seasons by post-hoc Tukey's pairwise comparisons (also depicted in Fig. 4). The first set of models does not include the captive population (see text) for comparison, while the second set of models includes a captive population as an additional “site”. When site was significant, post-hoc Tukey’s pairwise comparisons were used to indicate which sites were different from the captive populations, listed in the last column.

Leukocytes were clr-transformed (see text) and BKA was fourth-root transformed for normality.

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<tr>
<td>Heterophils</td>
</tr>
<tr>
<td>Eosinophils</td>
</tr>
<tr>
<td>BKA</td>
</tr>
<tr>
<td>PC1</td>
</tr>
<tr>
<td>PC2</td>
</tr>
</tbody>
</table>
Table 3. Regression models of the infection intensity of *P. testudinis* in individual tortoises, with tortoise included as a random effect to account for repeated measures. Partial p-values only refer to the fixed effects in each model. Models are presented in order of the best, or lowest, AICc score to the highest.

<table>
<thead>
<tr>
<th>X variable(s)</th>
<th>Partial p of fixed effects</th>
<th>AICc</th>
<th>Akaike weights</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-correlated variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1+PC2</td>
<td>0.48</td>
<td>&lt;.0001/.0098</td>
<td>1118.500</td>
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<tr>
<td>PC1</td>
<td>0.47</td>
<td>0.0004</td>
<td>1123.050</td>
</tr>
<tr>
<td>PC2+Mean mo temp</td>
<td>0.52</td>
<td>0.0473/0.0039</td>
<td>1129.050</td>
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<tr>
<td>PC2</td>
<td>0.47</td>
<td>0.0400</td>
<td>1131.126</td>
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<td>Lymphocytes</td>
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<td>0.0290</td>
<td>1202.590</td>
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<tr>
<td>Heterophils</td>
<td>0.36</td>
<td>0.0005</td>
<td>1205.590</td>
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<td>Basophils</td>
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<td>0.0036</td>
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<tr>
<td>Eosinophils</td>
<td>0.30</td>
<td>0.0019</td>
<td>1213.040</td>
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<td>&lt;.0001</td>
<td>1562.847</td>
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<td>Site</td>
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<td>&lt;.0001</td>
<td>1566.106</td>
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<tr>
<td>Monthly mean temp</td>
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<td>&lt;.0001</td>
<td>1593.546</td>
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<tr>
<td>Monthly min temp</td>
<td>0.44</td>
<td>&lt;.0001</td>
<td>1593.909</td>
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<tr>
<td>Monthly max temp</td>
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<td>&lt;.0001</td>
<td>1596.745</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Six populations of tortoises (*Gopherus agassizii*) were sampled three times in spring, summer, and fall of 2012 in California and Nevada. From north to south they are commonly referred to as (1) Halfway Wash, (2) Coyote Springs, (3) McCullough Pass, (4) the Large-Scale Translocation Site (LSTS), (5) Piute Valley, and (6) the Fort Irwin Study Site (FISS). This map was created using ArcGIS® software by Esri.

Figure 2. An example dilution curve of copy numbers of *P. testudinis* DNA (manufactured within a plasmid), showing corresponding cycle threshold (Ct) values when analyzed with the qPCR assay described herein (see Methods for details). These data were generated from three dilution curves run in triplicate, on three plates. DNA copy number was log-transformed to generate the linear regression equation: *P. testudinis* copy number = 10^(Ct-39.39)/(-3.42). The regression equation was used to calculate an average replication efficiency of 96%.

Figure 3. A principal components analysis (PCA) was used to reduce immunological measurements from individual tortoise samples to PC1 and PC2. (a) PCA performed on raw data counts, with non-normal values square-root transformed (numbers of lymphocytes, heterophils, eosinophils, and monocytes) or fourth-root transformed (BKA) to approximate normality. (b) PCA performed on data more appropriately
transformed by a centered log-ratio (clr) transformation and BKA (fourth-root transformed), due to the compositional nature of differential leukocyte counts. (c) Values of (b) are highlighted by season (spring=upright triangle, summer=down-turned triangle, fall=circle) to show seasonal shifts in immune function, with considerable overlap.

Figure 4. Seasonal variation in immune values in the six free-ranging tortoise populations. For multiple comparisons an adjusted \( \alpha \) of 0.017 was used, and all statistical models included individual as a random effect and site as a covariate. Different letters (A, B, C) signify statistical differences among seasons. Seasonal differences in relative numbers of leukocytes, with center-log ratio (clr) transformations: (a) lymphocytes \( (p<0.0001) \), (b) basophils \( (p<0.0001) \), (c) monocytes \( (p<0.0001) \), (d) heterophils \( (p<0.0001) \), (e) eosinophils \( (p=0.0003) \), (f) PC1 \( (p<0.0001) \), and (g) PC2 \( (p<0.0001) \). See Figure 3 for a description of the PC1 and PC2.