Herpetological Conservation and Biology 14(3):568–577.
Submitted: 10 September 2017; Accepted: 9 September 2019; Published 16 December 2019.

Mycoplasma Prevalence in a Reintroduced Gopher Tortoise (*Gopherus polyphemus*) Population

Jean P. Elbers*[1] *and Sabrina S. Taylor

School of Renewable Natural Resources, 227 RNR Bldg., Louisiana State University and AgCenter, Baton Rouge, Louisiana, 70803, USA
[1]Corresponding author, e-mail: jean.elbers@gmail.com

Abstract.—**Season can affect many aspects of reptile biology not only because of hormonal changes, but also because of the close association between environmental and reptile internal body temperatures. Despite its importance to disease susceptibility and resistance, we know very little about the effects of seasonality on reptile immune responses. Here, we performed a pilot study to test the effects of season, sex, and immune gene variation on immunocompetence in a species of conservation concern, the Gopher Tortoise (*Gopherus polyphemus*). We examined free-ranging Gopher Tortoises from a reintroduced Louisiana population in June and September for presence/absence of *Mycoplasma* spp., organisms associated with upper respiratory tract disease in Gopher Tortoises, and production of *Mycoplasma* antibodies, an indication of exposure to *Mycoplasma*. We also sequenced part of an immune gene in the major histocompatibility complex (MHC), and related MHC haplotype, season, sex, and the sex by season interaction to immunocompetence as estimated by microbiocidal killing assays. This pilot research provides an important first step in estimating variability of the parameters examined as well as identifying an appropriate sample size for future research. Our results suggest MHC haplotype and season may influence microbiocidal killing, but our analyses are likely underpowered, especially given the small (partial eta^2 = 0.036) and miniscule effect sizes (partial eta^2 = 4.0e-^{7}) of parameters predicting *C. albicans* and *E. coli* killing, respectively. *Mycoplasma* was not detected in nasal flushes, and only one tortoise was positive for *Mycoplasma agassizii* antibodies. These results indicate a reintroduced tortoise population that is relatively free of *Mycoplasma*.**

Key Words.—bacterial killing assay; Gopher Tortoise; immunocompetence; MHC variation; microbiocidal killing assay

Introduction

The ability of an organism to mount an effective response to pathogens depends on a properly functioning immune system. Individual components of the immune system are influenced by developmental, genetic, and physiological factors, which mediate the strength and consistency of the immune response (reviewed in Ardia et al. 2011). Some of these factors may vary depending on age, sex, or time of year (Love et al. 2008; Martin et al. 2008). For example, physiological factors vary throughout the year coinciding with seasonal changes that influence resource availability (Love et al. 2008). Seasonality thus results in tradeoffs in the allocation of limited resources for growth, reproduction, and defense, including immune responses, which can influence the dynamics of infectious disease (Altizer et al. 2006; Martin et al. 2008).

In reptiles, we know little about the effects of seasonality on immune response, even though season is directly related to temperature, and reptile internal temperature typically correlates with environmental temperature. Although prior research suggests that seasonal differences in immunity of many reptiles correlate with changes in temperature and/or hormone levels (reviewed in Zapata et al. 1992; Origgi 2007; Zimmerman et al. 2010), it is not clear how different components of the reptile immune system vary in response to changing seasons. For example, constitutive innate immune function is lowest in winter in Desert Tortoises (*Gopherus agassizii*), but lymphocyte counts (i.e., B cell, T cell, natural killer cells), which influence cellular, humoral, and innate immunity, are highest in winter (Sandmeier et al. 2016).

Understanding the effects of seasonality on immune responses may benefit management of Gopher Tortoises (*Gopherus polyphemus*), a species of conservation concern in southeastern USA. Gopher Tortoises produce extensive burrows and are surface active from spring to autumn, spending most if not all of winter within their burrows (Douglass and Layne 1978; Ashton and Ashton 2008). These tortoises are able to regulate their body temperatures behaviorally through basking or seeking refuge in shade or by retreating to their burrows (Douglass and Layne 1978; Goessling et al. 2017). Little is known regarding seasonal changes in Gopher Tortoise immune responses, but there are seasonal changes in key components of the Gopher Tortoise immune system. White blood cell counts (i.e., the number of white blood cells in a blood sample) are higher in spring than autumn

in Gopher Tortoises in Florida and Louisiana (Taylor and Jacobson 1982; Diaz-Figueroa 2005). Heterophils (i.e., the reptilian equivalent of neutrophils, which fight microbial invasions and participate in inflammation), are higher in spring than autumn for Gopher Tortoises in Louisiana but not in Florida and Alabama (Taylor and Jacobson 1982; Diaz-Figueroa 2005; Goessling et al. 2016). Monocytes (i.e., phagocytic white blood cells that mature into macrophages) are higher in spring than autumn in Gopher Tortoises in Florida and Alabama, but not in Louisiana (Taylor and Jacobson 1982; Diaz-Figueroa 2005; Goessling et al. 2016). Interestingly, lymphocyte counts are not different between spring and autumn in Gopher Tortoises from Alabama, Florida, or Louisiana (Taylor and Jacobson 1982; Diaz-Figueroa 2005; Goessling et al. 2016). Goessling et al. (2016) also found that constitutive innate immunity in Gopher Tortoises was higher in summer than in autumn. These findings suggest that Gopher Tortoises may have reduced or compromised cellular, humoral, and innate immune responses in autumn relative to spring, and autumn innate immune responses may be lower than in summer.

Gopher Tortoises are also susceptible to the infectious and occassionally fatal upper respiratory tract disease (URTD; Brown et al. 1999). URTD in gopher tortoises is characterized by nasal discharge and tearing and is associated with lethargy and loss of appetite, which can eventually lead to dehydration, emaciation, and mortality (Brown et al. 1999). While several pathogenic agents have been associated with the clinical signs of URTD in tortoises (Brown et al. 1999; McLaughlin et al. 2000; Brown et al. 2004; Johnson et al. 2006; Johnson et al. 2008), inoculation of Gopher Tortoises with *Mycoplasma agassizii* and *M. testudineum* induces URTD clinical signs (Brown et al. 1999; Brown et al. 2004), so most URTD studies focus on *Mycoplasma* species (e.g., Weitzman et al. 2017). Gopher Tortoises exposed to *Mycoplasma* are expected to have pathogen DNA in nasal swab or flush samples as detected through culture and/or PCR, and are also expected to have specific antibody production through an enzyme-linked immunosorbent assay (ELISA; Smith et al. 1998; McLaughlin et al. 2000; Diemer Berish et al. 2010; Wendland et al. 2010; Braun et al. 2014). Adult Gopher Tortoises are more likely to be found with antibodies against *Mycoplasma* than immature individuals, and males are more likely to be found with *Mycoplasma* antibodies than females (Wendland et al. 2010), possibly because males have larger home ranges (McRae et al. 1981) and are more likely to come into contact with pathogens through more frequent intra-specific interactions than females (Auffenberg 1969).

Although seasonality may influence the immune responses of Gopher Tortoises, so may genetic background. In particular, variation in genes of the major histocompatibility complex (MHC) is associated with resistance to certain pathogens in a variety of vertebrates (e.g., Paterson et al. 1998; Langefors et al. 2001; Bonneaud et al. 2006; Meyer-Lucht and Sommer 2009). Thus, Gopher Tortoise MHC haplotype may influence immune responses.

Here, we estimated the presence of *Mycoplasma* in a reintroduced population of Gopher Tortoises by testing for *Mycoplasma* antibodies and the presence/absence of these bacteria in nasal flushes as measured with qPCR. We then performed a pilot to study to examine whether season, sex, and MHC haplotype predicted *Mycoplasma* presence (both measures) and immunocompetence as measured by microbiocidal killing assays. We hypothesize that season and sex affects the presence of antibodies and *Mycoplasma* in Gopher Tortoises. We predict that tortoises will be more likely to test positive for antibodies and *Mycoplasma* in nasal flushes and have stronger immunocompetence in June compared to September given previous research findings (Taylor and Jacobson 1982; Diaz-Figueroa 2005; Goessling et al. 2016). We also predict that males will be more likely to test positive for antibody production and presence of *Mycoplasma* than females, as males have larger home ranges (Eubanks et al. 2003; Castellón et al. 2018), and thus may come into contact with a greater number of individuals, potentially increasing their exposure to *Mycoplasma*. Finally, we predict that specific MHC haplotypes will predict immunocompetence.

Materials and Methods

Study site.—We studied Gopher Tortoises at the Sandy Hollow Wildlife Management Area (30°50’23.1”N, 90°25’07.1”W) in southeastern Louisiana, USA. Sandy Hollow (1,732 ha) is comprised of upland rolling hills covered in a young Longleaf/Loblolly Pine mix with hardwoods in lower, wetter areas. It is currently managed for wildlife such as Northern Bobwhite (*Colinus virginianus*) and Gopher Tortoises and is divided into a larger northern and a smaller southern tract by Louisiana Highway 10. We sampled Gopher Tortoises in the northern tract, which is within the range of the species, but does not have a historic Gopher Tortoise population, in contrast to the southern tract. The Louisiana Department of Wildlife and Fisheries (LDWF) uses the northern tract to harbor waif animals (individuals of unknown origin) released as part of a Gopher Tortoise reintroduction program started in 1989. Between 1989–2002, 79 Gopher Tortoises were released into the northern tract (Maxit 2002), and in the years leading up to the present, dozens of additional Gopher Tortoises have been released (Keri Landry, pers. comm.).

Sampling.—We conducted fieldwork during two sampling sessions, 10–28 June and 16–25 September 2013. We captured adult Gopher Tortoises (carapace length of males ≥ 180 mm and females ≥ 220 mm; Diemer and Moore 1994) with Havahart # 1089 live traps (Woodstream Corporation, St. Lititz, Pennsylvania, USA). We covered live traps in burlap cloth and placed them at the entrances of burrows.

Mycoplasma antibody production: plasma ELISA assays.—After capture, we immediately collected about 1 mL blood from each tortoise using venipuncture of the subcarapacial venous sinus. We stored half of the blood sample in a 1.5-mL tube containing 1.0 mL Queen's lysis buffer (Seutin et al. 1991) for MHC haplotype analysis (see below) and the other half in a BD plasma microtainer # 365971 (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Blood in microtainers was inverted 10 times, immediately centrifuged for 90 s to separate blood cells from plasma, and placed on ice until return to the laboratory, where it was aliquoted and then stored at -20º C. We sent 200 µL plasma for ELISA analysis (Wendland et al. 2007) to the Tortoise Mycoplasma Lab of the University of Florida to determine if tortoises had developed antibodies against *M. agassizii* and *M. testudineum*. We used the remaining frozen plasma aliquots to measure immune responses *in vitro* with microbiocidal killing assays (see below).

Presence/absence of Mycoplasma: nasal flush qPCR assays.—Following venipuncture, we performed a nasal flush. Briefly, we positioned the tortoise at a 45° angle with the head down and cleaned the outside of the nares and mouth of the tortoise with water and then alcohol swabs. Next, we opened its mouth and held it open with a clean BD microtainer. Then, we instilled 5 mL 0.9% sterile saline solution using a 10-mL syringe fitted with a 22 gauge 2.54 cm (1 in) IV catheter into the left naris and collected the exudate from the open mouth into a 120-mL sterile specimen container. We repeated this procedure for the right naris, closed the specimen container, and placed it on dry ice. We stored nasal flushes in the lab at -80º C. After thawing, we added a 1.0 mL flush to 200 µL SP4 Glucose broth # R20057 (Thermo Scientific, Pittsburgh, Pennsylvania, USA); we refroze aliquots and submitted them for qPCR analysis to detect presence of *M. agassizii* and *M. testudineum* (Braun et al. 2014) by the Amphibian Disease Laboratory/Wildlife Disease Laboratory of the San Diego Zoo Institute for Conservation Research.

Immunocompetence: microbiocidal killing assays.—To quantify immunocompetence, we used a functional measure of pathogen control, which tests the antimicrobial capacity of whole blood or plasma *in vitro* (Millet et al. 2007). In this procedure, whole blood or plasma is extracted, mixed with known amounts of a particular microorganism, and incubated to allow blood components to interact with the microorganisms. The microbiocidal capacity of the blood or plasma is then determined by the difference between the final and initial number of microorganisms, which is measured by the number of colony-forming units (CFUs) on agar plates. Thus, this protocol gives an overall measure of constitutive innate immune function. We performed microbiocidal killing assays using plasma stored at -20º C for less than 4 mo (storage for 4 mo did not affect microbiocidal killing percent: data not shown) following methods from Millet et al. (2007). Briefly, we used two lyophilized Epower™ microorganism preparations from Microbiologics Inc. (St. Cloud, Minnesota, USA): *Escherichia coli* ATCC # 8739 and *Candida albicans* ATCC # 10231. We resuspended lyophilized microorganism cultures following manufacturer instructions, then diluted cultures such that there were ~200 CFUs in 5 uL for *E. coli* or ~100 CFUs in 20 uL for *C. albicans* (hereafter, working solution). Each microbiocidal killing assay sample consisted of 10 µL plasma, 90 µL PBS (phosphate buffered saline), and 5 µL of the working solution of *E. coli* or 20 uL of the working solution of *C. albicans*. We incubated *E. coli* samples for 0.5 h and *C. albicans* for 3 h, both at 34º C. Next, we plated 50 µL of each sample on replicate tryptic soy agar plates, inverted the plates, and incubated them for 12 h (*E. coli*) or 24 h (*C. albicans*) at 34º C. Controls consisted of four agar plates in total with 100 µL PBS and 5 µL of the working solution of *E. coli* or 20 uL of the working solution of *C. albicans* plated immediately after adding microorganisms. After the end of incubation (i.e., 12 or 24 h), we counted CFUs on plates, and we calculated microbiocidal killing percentage as: (mean number of colonies of controls – mean number of colonies of sample)/mean number of colonies of controls*100 (Zysling et al. 2009).

Genetic analyses.—We analyzed a 188bp fragment of GopoDAB exon 2 (a MHC class II locus of Gopher Tortoises) using primers GopoMhcIIβex2SF and GopoMhcIIβex2SR (Elbers et al. 2017) to assess the influence of MHC haplotype on *Mycoplasma* antibody, *Mycoplasma* presence/absence, and microbiocidal killing assays. The 188bp fragment analyzed here is shorter than the 199bp fragment reported in Elbers et al. (2017; GenBank accession numbers KU949620-KU949732). Some additional variation may exist in the unamplified region (11 bp) that would distinguish sequences obtained here from sequences obtained previously. We phased heterozygous individuals using the Phase algorithm implemented in DnaSP 5.10.01

(Librado and Rozas 2009). We then constructed maximum likelihood trees using the phased alleles for the tortoises in MEGA 5.1 (Tamura et al. 2011) using 500 bootstraps to cluster the MHC alleles. We defined clusters as branches having greater than 75% nodal support. Haplotypes were assigned to clusters of alleles.

Statistical analyses.— To test if MHC haplotype, season (June versus September sampling period), sex, or the sex by season interaction (dependent variables) predicted *Mycoplasma* antibody, *E. coli* killing, or *C. albicans* killing (independent variables), we used the glmer function in the R package lme4 (Bates et al. 2015) to perform Generalized Linear Mixed Effect Models (GLMMs) in R 3.6.0 (R Core Team 2019). For all GLMMs, tortoise identity was a random effect to account for repeated measures, and we used the dredge function in the MuMIn package (Barton 2019) to rank candidate models by AICc, which is preferred to AIC (Akaike 1974) when sample sizes are small relative to the number of variables (Burnham and Anderson 2002). For *Mycoplasma* antibody GLMMs, we used a Poisson distribution, and for microbiocidal assay GLMMs, we used a Gaussian distribution. If models were within delta AICc of 2 or less, we performed conditional model averaging with the model.avg function in the MuMIn package. We included a sex by season interaction as hormones can affect immunity (reviewed in Zimmerman et al. 2010), and hormones can vary between the sexes at different times of the year (Ott et al. 2000).

We estimated the number of tortoises required to reach a power of 0.80 at an alpha level of 0.05 using the lmSupport R package (Curtin 2017). First, we simplified our GLMMs to Generalized Linear Models (GLMs) by using data from only the first time a tortoise was sampled (i.e., no random effect), then we excluded the sex by season interaction term. Using the glm R function, we modeled whether *Mycoplasma* antibody titers or microbiocidal assays would be predicted by sex+season+MHC haplotype. For *Mycoplasma* antibody GLMs, we used a Poisson distribution, and for microbiocidal assay GLMs, we used a Gaussian distribution. We first used the modelEffectSizes function in the lmSupport package to estimate partial eta^2 for each independent variable in a GLM. Partial eta^2 (i.e., the effect size) can be defined as the proportion of the total variance in a dependent variable explained by the examined independent variable with effects of other independent variables removed (Richardson 2011). Then, we used the modelPower function in the lmSupport package to estimate the required sample size to reach a power of 0.80 at an alpha level of 0.05 for each independent variable given its degrees of freedom and partial eta^2. We extracted average daily temperature data for the Baton Rouge Ryan AP weather station for June (http://www.losc.lsu.edu/products/climate/btr/jun_2013.html) and September (http://www.losc.lsu.edu/products/climate/btr/sep_2013.html). Because the data were not normal according to the Shapiro-Wilk test implemented with the shapiro.test R function, we compared mean daily average temperature between June and September sampling dates with a Wilcoxon rank sum test implemented in R ($\alpha = 0.05$).

Results

We collected antibody (ELISA), *Mycoplasma* presence/absence (qPCR), and immune response (microbiocidal killing assay) data from 12 Gopher Tortoises (six females, six males; Table 1). We sampled seven animals once between the two seasons, and we sampled five animals during both sampling sessions for a total of 17 observations

Table 1. Summary of Gopher Tortoise (*Gopherus polyphemus*) production of *Mycoplasma* antibodies and immune response as measured by microbiocidal killing assays (*E. colli* = *Escherichia coli*, *C. albicans* = *Candida albicans*). Negative microbiocidal killing percentage values indicate microorganisms grew better with plasma than without. Presence/absence data of *Mycoplasma* bacteria in tortoise nasal flushes are not shown because all assays were suspect (n = 1) or negative (n = 16).

					Antibody production interpretation (titer level)		Microbiocidal killing percentage	
	Identity	MHC Haplotype	Sex	Season	*M. agassizii*	*M. testudineum*	*E. coli*	*C. albicans*
1	SH002	5	Male	June	Suspect (32)	Suspect (64)	1.070	43.60
2	SH014	2	Male	June	Suspect (32)	Suspect (32)	42.11	33.95
3	SH071	4	Female	June	Suspect (32)	Negative (< 32)	27.11	33.21
4	SH22+9	1	Male	June	Positive (64)	Suspect (32)	66.00	33.21
5	SH228	1	Female	June	Negative (< 32)	Suspect (32)	-1.990	20.59
6	SH005	1	Male	June	Negative (< 32)	Negative (< 32)	50.38	36.92
7	SH270	1	Female	June	Negative (< 32)	Suspect (32)	59.88	28.76
8	SH018	6	Female	September	Negative (< 32)	Negative (< 32)	-14.24	30.24
9	SH228	1	Female	September	Negative (< 32)	Suspect (32)	33.84	40.63
10	SH071	4	Female	September	Suspect (32)	Suspect (32)	37.21	30.98

Table 1 (continued). Summary of Gopher Tortoise (*Gopherus polyphemus*) production of *Mycoplasma* antibodies and immune response as measured by microbiocidal killing assays (*E. colli* = *Escherichia coli*, *C. albicans* = *Candida albicans*). Negative microbiocidal killing percentage values indicate microorganisms grew better with plasma than without. Presence/absence data of *Mycoplasma* bacteria in tortoise nasal flushes are not shown because all assays were suspect (n = 1) or negative (n = 16).

					Antibody production interpretation (titer level)		Microbiocidal killing percentage	
	Identity	MHC Haplotype	Sex	Season	*M. agassizii*	*M. testudineum*	*E. coli*	*C. albicans*
11	SH031	3	Male	September	Negative (< 32)	Suspect (32)	61.41	59.18
12	SH079	6	Male	September	Negative (< 32)	Suspect (64)	4.130	30.24
13	SH005	1	Male	September	Negative (< 32)	Negative (< 32)	60.18	16.14
14	SH002	5	Male	September	Negative (< 32)	Suspect (32)	26.19	21.34
15	SH204	1	Female	September	Negative (< 32)	Negative (< 32)	29.86	36.92
16	SH014	2	Male	September	Negative (< 32)	Suspect (32)	70.90	69.57
17	SH240	NA	Female	September	Negative (< 32)	Suspect (32)	95.41	76.25

(Table 1). In June, Gopher Tortoise SH22+9 had an antibody titer level of 64 and was considered positive for *M. agassizii*. The remaining tortoises were either suspect (antibody titers of 32) or negative (titers < 32) for *M. agassizii*. We tested five Gopher Tortoises twice, once in June and again in September. *Mycoplasma agassizii* titer levels did not change in tortoises SH005, SH228, and SH071, but did change from 32 to < 32 in SH002 and SH014. There were no Gopher Tortoises considered positive for *M. testudineum* (antibody titers ≥ 128); they were either suspect (antibody titers of 64 or 32) or negative (antibody titers < 32). Of the five tortoises we tested twice, once in June and again in September, *M. testudineum* titer levels did not change for SH005, SH228, and SH014, but did change from < 32 to 32 for SH071 and from 64 to 32 for SH002.

Table 2. Model statistics for *Mycoplasma agassizii* and *M. testudineum* titer levels. A plus sign indicates the inclusion of a parameter in the model. The abbreviations MHC = major histocompatibility complex; df = degrees of freedom; AICc = corrected Akaike Information Criterion.

Model	MHC Haplotype	Season	Sex	Season×Sex	df	Log Likelihood	AICc	Delta AICc	Weight
Mycoplasma agassizii									
1					2	-52.301	109.526	0.000	0.564
5			+		3	-51.851	111.701	2.175	0.190
3		+			3	-51.865	111.729	2.203	0.188
7		+	+		4	-51.379	114.393	4.868	0.049
15		+	+	+	5	-50.976	117.951	8.425	0.008
2	+				7	-51.601	131.201	21.675	< 0.001
6	+		+		8	-50.140	136.851	27.325	< 0.001
4	+	+			8	-51.297	139.165	29.639	< 0.001
8	+	+	+		9	-49.873	147.746	38.221	< 0.001
16	+	+	+	+	10	-49.401	162.802	53.276	< 0.001
Mycoplasma testudineum									
1					2	-59.363	123.649	0.000	0.454
5			+		3	-58.309	124.618	0.968	0.280
3		+			3	-58.750	125.501	1.852	0.180
7		+	+		4	-57.818	127.271	3.622	0.074
15		+	+	+	5	-57.481	130.962	7.312	0.012
2	+				7	-54.035	136.069	12.420	0.001
6	+		+		8	-52.420	141.411	17.762	< 0.001
4	+	+			8	-52.875	142.322	18.673	< 0.001
8	+	+	+		9	-51.503	151.007	27.358	< 0.001
16	+	+	+	+	10	-51.503	167.007	43.357	< 0.001

Table 3. Model statistics for *Escherichia coli* and *Candida albicans* killing. A plus sign indicates the inclusion of a parameter in the model. The abbreviations MHC = major histocompatibility complex; df = degrees of freedom; AICc = corrected Akaike Information Criterion.

Model	MHC Haplotype	Season	Sex	Season×Sex	df	Log Likelihood	AICc	Delta AICc	Weight
Escherichia coli									
2	+				8	-46.442	129.456	0.000	0.409
4	+	+			9	-40.842	129.683	0.227	0.365
6	+		+		9	-41.429	130.858	1.402	0.203
8	+	+	+		10	-35.950	135.900	6.444	0.016
15		+	+	+	6	-58.655	138.644	9.188	0.004
7		+	+		5	-62.019	140.039	10.583	0.002
3		+			4	-66.354	144.344	14.888	< 0.001
5			+		4	-66.885	145.406	15.950	< 0.001
1					3	-71.222	150.445	20.989	< 0.001
16	+	+	+	+	11	-32.629	153.258	23.802	< 0.001
Candida albicans									
2	+				8	-41.201	118.973	0.000	0.819
6	+		+		9	-38.150	124.301	5.327	0.057
4	+	+			9	-38.311	124.623	5.649	0.049
15		+	+	+	6	-51.743	124.820	5.846	0.044
7		+	+		5	-55.381	126.762	7.789	0.017
5			+		4	-58.442	128.520	9.547	0.007
3		+			4	-58.703	129.042	10.068	0.005
1					3	-61.752	131.504	12.530	0.002
8	+	+	+		10	-35.218	134.437	15.463	< 0.001
16	+	+	+	+	11	-31.419	150.838	31.865	< 0.001

Null models (i.e., models not including MHC haplotype, season, sex, or the sex by season interaction) were the top ranked models for *M. agassizii* and *M. testudineum* (Table 2) antibody titer levels, although models including sex and season alone as single predictors were within 2 AICc points in both analyses. No Gopher Tortoises were positive for the presence of either *Mycoplasma* species. There were three top ranking models for *E. coli* killing (Table 3). After conditional averaging of these three models, killing of *E. coli* was not predicted by sex (95% confidence intervals [CI] included 0; Table 4), but was predicted by MHC haplotype and season (95% CI excluded 0; Table 4). In *C. albicans*, the top-ranking model included MHC haplotype (Table 3), which did not predict *C. albicans* killing ($F_{5,15} = 1.971$, $P = 0.142$).

The number of tortoises required to adequately predict a given dependent variable varied greatly (Table 5). For example, as few as 12 tortoises would have been needed to predict *M. testudineum* antibody titers given the large effect size for sex (partial $eta^2 = 0.42$) in that GLM, but tens of thousands to millions of tortoises would have been needed to predict *E. coli* killing given the miniscule effect sizes for sex, season, or MHC haplotype (partial $eta^2 < 7.0e^{-4}$). These small effect sizes are probably not biologically meaningful. Data available from Baton Rouge suggest that air temperature was similar in June (mean temperature for June sampling session = 28.1 ± [SD] 0.2º C) and September (26.7 ± 0.7º C) at the Sandy Hollow study site ($W = 117$, df = 27, $P = 0.118$).

Table 4. Conditional parameter averages from models 2,4,6 (delta AICc [corrected Akaike Information Criterion] < 2) for *Escherichia coli* microbiocidal percentage. The abbreviation SE = standard error; LCL = lower 95% confidence limit, and UCL = upper 95% confidence limit.

	Estimate	SE	Adjusted SE	LCL	UCL
(Intercept)	39.076	11.274	13.146	13.310	64.842
MHCHAP2	8.361	24.030	28.538	-47.572	64.295
MHCHAP3	9.467	26.406	31.258	-51.798	70.731
MHCHAP4	-10.716	23.961	28.435	-66.448	45.016
MHCHAP5	-34.514	24.030	28.538	-90.447	21.420
MHCHAP6	-54.364	20.262	23.869	-101.15	-7.582
Season	20.337	5.154	6.218	8.149	32.525
Sex	25.362	13.047	15.741	-5.489	56.213

Table 5. Required sample size to reach power = 0.80 and alpha = 0.05 for *Mycoplasma* antibody titers and microbiocidal assays. Power analyses are based on data from the first time a tortoise was sampled and the model: response~ sex+season+MHC haplotype. *Mycoplasma* antibody titers were modeled with a generalized linear model (GLM) with Poisson distribution, and microbiocidal assays were modeled with a GLM with Gaussian distribution. The partial eta^2 for MHC haplotype was chosen as the lowest partial eta^2 for sex and season for each GLM. The abbreviation MHC = major histocompatibility complex; df = degrees of freedom.

	df	Partial eta^2	Required sample size
Mycoplasma agassizii antibody titer			
Sex	1	0.27	22
Season	1	0.0086	903
MHC Haplotype	5	0.0086	1,475
Mycoplasma testudineum antibody titer			
Sex	1	0.42	12
Season	1	0.076	96
MHC Haplotype	5	0.076	156
Escherichia coli killing			
Sex	1	$7.0e^{-4}$	11,446
Season	1	$4.0e^{-7}$	1,981,8558
MHC Haplotype	5	$4.0e^{-7}$	32,390,006
Candida albicans killing			
Sex	1	0.036	212
Season	1	0.039	195
MHC Haplotype	5	0.036	345

Discussion

Mycoplasma in the reintroduced population.—Tortoise exposure to *Mycoplasma* was estimated using two approaches, which suggested that very few waif animals at the northern tract of Sandy Hollow were exposed. Specifically, only one individual was positive for antibody production against *M. agassizii,* and no individuals had detectable levels of *Mycoplasma* in their nasal flushes as estimated by qPCR. A single antibody-positive *Mycoplasma* assay may indicate that the reintroduced population at the northern tract of Sandy Hollow is largely free of Mycoplasmal URTD, which is important for ongoing conservation efforts. Although the other animals were either *Mycoplasma* suspect or negative, this does not mean these animals were never exposed to these pathogens. When Gopher Tortoises were first released at the northern tract of Sandy Hollow in 1989, they were not screened using a *Mycoplasma* ELISA because the test was not developed until 1993 (Schumacher et al. 1993; later refined by Wendland et al. 2007). Before release at the northern tract of Sandy Hollow, Gopher Tortoises were examined physically and only released if not presenting Mycoplasmal URTD clinical signs. Given the chronic nature of *Mycoplasma* infections (Brown et al. 1999), it is possible that some animals could have been infected with *Mycoplasma* but were not presenting clinical signs when examined. This is especially so if the nasal passage is obstructed internally, and the nasal flush cannot reach deep enough to dislodge *Mycoplasma* cells (e.g., McLaughlin et al. 2000). Further, antibody production against *Mycoplasma* is not always consistent with Mycoplasmal URTD clinical signs or detection of the pathogen (Brown et al. 1999; McLaughlin et al. 2000).

The lack of individuals positive for *Mycoplasma* in nasal flushes also suggests a *Mycoplasma*-free population at the northern tract of Sandy Hollow, an unsurprising result as no animal presented clinical signs of Mycoplasmal URTD when captured. A negative nasal flush, however, does not guarantee that an animal is free of *Mycoplasma*. First, only an aliquot of the entire nasal flush was tested in this study using qPCR following the methods of Braun et al. (2014), but Brown et al. (1999) found that PCR following culture for 48 h of nasal flush fluid in SP4 broth had higher sensitivity than an initial PCR of nasal flushes without a culturing step. Second, McLaughlin et al. (2000) found that nasal flushes could lead to false negatives because the flush fluid did not reach far enough into the upper respiratory tract to dislodge *Mycoplasma* cells and/or some animals had a caseous exudate blocking part of the upper respiratory tract, preventing nasal flushes from dislodging *Mycoplasma* cells.

Immunocompetence.—This pilot research provides an important first step in estimating the variability of the parameters examined as well as identifying an appropriate sample size for future research. Our results suggest that MHC haplotype and season may influence microbiocidal killing. Our analyses are likely underpowered; however, the effect sizes of parameters predicting *C. albicans* and *E. coli* killing are so small that they probably are not biologically meaningful.

Conclusions.—Negative results for both *Mycoplasma* antibody production and *Mycoplasma* presence indicate that the tortoise population at the northern tract of Sandy Hollow is relatively free of *Mycoplasma*. This is a positive result for the reintroduction program, because it means that any tortoises dispersing from the northern to the neighboring southern tract are probably free of *Mycoplasma* and are unlikely to spread this bacterial disease. Future work would involve sampling more tortoises during the spring, summer, autumn, and perhaps winter to better understand if titer levels, presence/absence of *Mycoplasma*, and immunocompetence vary across other seasons. Further, culturing nasal flush samples in SP4 media before qPCR

analysis could improve detection of *Mycoplasma*. Other improvements would include adding immune response assays to estimate function of the adaptive branch of the immune system.

Acknowledgments.—We wish to thank the Louisiana Department of Wildlife and Fisheries (LDFW), especially Gopher Tortoise biologist Keri Landry. We also wish to thank Drs. Javier Nevarez, Josephine Braun, Christopher Austin, Austin Allen, Philip Stouffer, and Susannah French. Dr. Christine Bergeon-Burns, Maria Bianco, Lisa Delahoussaye, Anna Evans, Caitlyn Glymph, Kaitlin Kuylen, Jaclyn Shanley, Charleston Shirley, and Bonnie Slaton were instrumental during fieldwork. This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, McIntire Stennis project LAB04066 and LAB94169. Funding for JPE was provided by the Lucius Gilbert Foundation. All research was conducted with the approval of the Institutional Animal Care and Use Committee of the Louisiana State University Agcenter (A2013-01) and under LDWF permit LNHP-12-097.

Literature Cited

Akaike, H. 1974. A new look at the statistical model identification. IEEE Transactions on Automatic Control 19:716–723.

Altizer, S., A. Dobson, P. Hosseini, P. Hudson, M. Pascual, and P. Rohani. 2006. Seasonality and the dynamics of infectious diseases. Ecology Letters 9:467–484.

Ardia, D.R., H.K. Parmentier, and L.A. Vogel. 2011. The role of constraints and limitation in driving individual variation in immune response. Functional Ecology 25:61–73.

Ashton, R.E., and P.S. Ashton. 2008. The Natural History and Management of the Gopher Tortoise. Krieger Publishing Company, Malabar, Florida, USA.

Auffenberg, W. 1969. Tortoise Behavior and Survival. Rand McNally and Company, Chicago, Illinois, USA.

Barton, K. 2019. MuMIn: Multi-model inference. R package version 1.43.6. https://CRAN.R-project.org/package=MuMIn.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistcal Software 67:1–48.

Bonneaud, C., J. Pérez-Tris, P. Federici, O. Chastel, and G. Sorci. 2006. Major histocompatibility alleles associated with local resistance to malaria in a passerine. Evolution 60:383–389.

Braun, J., M. Schrenzel, C. Witte, L. Gokool, J. Burchell, and B.A. Rideout. 2014. Molecular methods to detect *Mycoplasma* spp. and Testudinid herpesvirus 2 in Desert Tortoises (*Gopherus agassizii*) and implications for disease management. Journal of Wildlife Diseases 50:757–766.

Brown, D.R., J.L. Merritt, E.R. Jacobson, P.A. Klein, J.G. Tully, and M.B. Brown. 2004. *Mycoplasma testudineum* sp. nov., from a Desert Tortoise (*Gopherus agassizii*) with upper respiratory tract disease. International Journal of Systematic and Evolutionary Microbiology 54:1527–1529.

Brown, M.B., G.S. McLaughlin, P.A. Klein, B.C. Crenshaw, I.M. Schumacher, D.R. Brown, and E.R. Jacobson. 1999. Upper respiratory tract disease in the Gopher Tortoise is caused by *Mycoplasma agassizii*. Journal of Clinical Microbiology 37:2262–2269.

Burnham, K.P., and D.R. Anderson. 2002. Model Selection and Multimodel Inference: A Practical Information-theoretic Approach. 2nd Edition. Springer-Verlag, New York, New York, USA.

Castellón, T.D., B.B. Rothermel, and J.M. Bauder. 2018. Gopher tortoise burrow use, home range, seasonality, and habitat fidelity in scrub and mesic flatwoods of southern Florida. Herpetologica 74:8–21.

Curtin, J. 2017. lmSupport: support for linear models. R package version 2.9.8. https://CRAN.R-project.org/package=lmSupport.

Diaz-Figueroa, O. 2005. Characterizing the health status of the Louisiana Gopher Tortoise (*Gopherus polyphemus*). M.Sc. Thesis, Louisiana State University, Baton Rouge, Louisianna, USA. 119 p.

Diemer, J.E., and C.T. Moore. 1994. Reproduction of Gopher Tortoises in north-central Florida. Pp. 129–137 In Biology of North American Tortoises. Bury, R.B., and D.J. Germano (Eds.). U.S. Department of the Interior, National Biological Survey, Washington, D.C., USA.

Diemer Berish, J.E., L.D. Wendland, R.A. Kiltie, E.P. Garrison, and C.A. Gates. 2010. Effects of mycoplasmal upper respiratory tract disease on morbidity and mortality of Gopher Tortoises in northern and central Florida. Journal of Wildlife Diseases 46:695–705.

Douglass, J.F., and J.N. Layne. 1978. Activity and thermoregulation of the Gopher Tortoise (*Gopherus polyphemus*) in southern Florida. Herpetologica:359–374.

Elbers, J.P., R.W. Clostio, and S.S. Taylor. 2017. Neutral genetic processes influence MHC evolution in threatened Gopher Tortoises (*Gopherus polyphemus*). Journal of Heredity 108:515–523.

Eubanks, J.O., W. Michener, and C. Guyer. 2003. Patterns of movement and burrow use in a population of Gopher Tortoises (*Gopherus polyphemus*). Herpetologica 59:311–321.

Goessling, J.M., C. Guyer, and M.T. Mendonça. 2016. Seasonal acclimation of constitutive immunity in

Gopher Tortoises *Gopherus polyphemus*. Physiological and Biochemical Zoology 89:487–497.

Goessling, J.M., C. Guyer, and M.T. Mendonça. 2017. More than fever: thermoregulatory responses to immunological stimulation and consequences of thermoregulatory strategy on innate immunity in Gopher Tortoises (*Gopherus polyphemus*). Physiological and Biochemical Zoology 90:484–493.

Johnson, A.J., D.J. Morafka, and E.R. Jacobson. 2006. Seroprevalence of *Mycoplasma agassizii* and tortoise herpesvirus in captive Desert Tortoises (*Gopherus agassizii*) from the Greater Barstow Area, Mojave Desert, California. Journal of Arid Environments 67:192–201.

Johnson, A.J., A.P. Pessier, J.F.X. Wellehan, A. Childress, T.M. Norton, N.L. Stedman, D.C. Bloom, W. Belzer, V.R. Titus, R. Wagner, et al. 2008. Ranavirus infection of free-ranging and captive box turtles and tortoises in the United States. Journal of Wildlife Diseases 44:851–863.

Langefors, A., J. Lohm, M. Grahn, O. Andersen, and T. von Schantz. 2001. Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic Salmon. Proceedings of the Royal Society of London B 268:479–485.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Love, O.P., K.G. Salvante, J. Dale, and T.D. Williams. 2008. Sex-specific variability in the immune system across life-history stages. American Naturalist 172:E99–E112.

Martin, L.B., Z.M. Weil, and R.J. Nelson. 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. Philosophical Transactions of the Royal Society B: Biological Sciences 363:321–339.

Maxit, I. 2002. State Reports - Louisiana. The Tortoise Burrow: Newsletter of the Gopher Tortoise Council 22:3.

McLaughlin, G.S., E.R. Jacobson, D.R. Brown, C.E. McKenna, I.M. Schumacher, H.P. Adams, M.B. Brown, and P.A. Klein. 2000. Pathology of upper respiratory tract disease of Gopher Tortoises in Florida. Journal of Wildlife Diseases 36:272–283.

McRae, W.A., J.L. Landers, and J.A. Garner. 1981. Movement patterns and home range of the Gopher Tortoise. American Midland Naturalist 106:165–179

Meyer-Lucht, Y., and S. Sommer. 2009. Number of MHC alleles is related to parasite loads in natural populations of Yellow Necked Mice, *Apodemus flavicollis*. Evolutionary Ecology Research 11:1085–1097.

Millet, S., J. Bennett, K.A. Lee, M. Hau, and K.C. Klasing. 2007. Quantifying and comparing constitutive immunity across avian species. Developmental and Comparative Immunology 31:188–201.

Origgi, F.C. 2007. Reptile immunology. Pp. 131–166 *In* Infectious Diseases and Pathology of Reptiles: Color Atlas and Text. Jacobson, E.R. (Ed.). Taylor & Francis, Boca Raton, Florida, USA.

Ott, J.A., M.T. Mendonça, C. Guyer, and W.K. Michener. 2000. Seasonal changes in sex and adrenal steroid hormones of Gopher Tortoises (*Gopherus polyphemus*). General and Comparative Endocrinology 117:299–312.

Paterson, S., K. Wilson, and J.M. Pemberton. 1998. Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries* L.). Proceedings of the National Academy of Sciences of the United States of America 95:3714–3719.

R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Richardson, J.T.E. 2011. Eta squared and partial squared as measures of effect size in educational research. Educational Research Review 6:135–147.

Sandmeier, F.C., K.R. Horn, and C.R. Tracy. 2016. Temperature-independent, seasonal fluctuations in immune function of the Mojave Desert Tortoise (*Gopherus agassizii*). Canadian Journal of Zoology 94:583–590.

Schumacher, I.M., M.B. Brown, E.R. Jacobson, B.R. Collins, and P.A. Klein. 1993. Detection of antibodies to a pathogenic mycoplasma in Desert Tortoises (*Gopherus agassizii*) with upper respiratory tract disease. Journal of Clinical Microbiology 31:1454–1460.

Seutin, G., B.N. White, and P.T. Boag. 1991. Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology 69:82–90.

Smith, R. B., R.A. Seigel, and K.R. Smith. 1998. Occurrence of upper respiratory tract disease in Gopher Tortoise populations in Florida and Mississippi. Journal of Herpetology 32:426–430.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731–2739.

Taylor, R.W., and E.R. Jacobson. 1982. Hematology and serum chemistry of the Gopher Tortoise, *Gopherus polyphemus*. Comparative Biochemistry and Physiology Part A: Physiology 72:425–428.

Weitzman, C.L., R. Gov, F.C. Sandmeier, S.J. Snyder, and C.R. Tracy. 2017. Co-infection does not predict disease signs in *Gopherus* tortoises. Royal Society

Open Science 4:171003.

Wendland, L.D., J. Wooding, C.L. White, D. Demcovitz, R. Littell, J.E.D. Berish, A. Ozgul, M.K. Oli, P.A. Klein, M.C. Christman, and M.B. Brown. 2010. Social behavior drives the dynamics of respiratory disease in threatened tortoises. Ecology 91:1257–1262.

Wendland, L.D., L.A. Zacher, P.A. Klein, D.R. Brown, D. Demcovitz, R. Littell, and M.B. Brown. 2007. Improved enzyme-linked immunosorbent assay to reveal *Mycoplasma agassizii* exposure: a valuable tool in the management of environmentally sensitive tortoise populations. Clinical and Vaccine Immunology 14:1190–1195.

Zapata, A.G., A. Varas, and M. Torroba. 1992. Seasonal variations in the immune system of lower vertebrates. Immunology Today 13:142–147.

Zimmerman, L.M., L.A. Vogel, and R.M. Bowden. 2010. Understanding the vertebrate immune system: insights from the reptilian perspective. Journal of Experimental Biology 213:661–671.

Zysling, D.A., A.D. Garst, and G.E. Demas. 2009. Photoperiod and food restriction differentially affect reproductive and immune responses in Siberian Hamsters *Phodopus sungorus*. Functional Ecology 23:979–988.

Jean P. Elbers is a Postdoctoral Researcher for the Research Institute of Wildlife Ecology at the University of Veterinary Medicine, Vienna, Austria. He received his Ph.D. in Renewable Natural Resources from Louisiana State University (2016) in Baton Rouge, USA, his M.Sc. in Biology from Missouri State University (2010) in Springfield, USA, and his B.S. in Biology from Southeastern Louisiana University (2008) in Hammond, USA. His research interests include conservation genetics and genomics, evolutionary ecology, immunogenetics, and comparative genomics. (Photographed by Heidi Elbers).

Sabrina S. Taylor is an Associate Professor in the School of Renewable Natural Resources, Louisiana State University in Baton Rouge, USA. She obtained her PhD from the University of Otago, New Zealand (2006), her MSc from Dalhousie University, Canada (2000), and her BSc from the University of Victoria, Canada (1995). Her research interests include conservation genetics, immunogenetics, historic DNA analysis, and genetic aspects of behavioral ecology. (Photographed by William Boyd Lee II).