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Research article

Coupling gene-based and classic veterinary diagnostics improves interpretation of health and immune function in the Agassiz's desert tortoise (*Gopherus agassizii*)

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The analysis of blood constituents is a widely used tool to aid in monitoring of animal health and disease. However, classic blood diagnostics (i.e. hematologic and plasma biochemical values) often do not provide sufficient information to determine the state of an animal's health. Field studies on wild tortoises and other reptiles have had limited success in drawing significant inferences between blood diagnostics and physiological and immunological condition. However, recent research using gene transcription profiling in the threatened Mojave desert tortoise (*Gopherus agassizii*) has proved useful in identifying immune or physiologic responses and overall health. To improve our understanding of health and immune function in tortoises, we evaluated both standard blood diagnostic (body condition, hematologic, plasma biochemistry values, trace elements, plasma proteins, vitamin A levels) and gene transcription profiles in 21 adult tortoises (11 clinically abnormal; 10 clinically normal) from Clark County, NV, USA. Necropsy and histology evaluations from clinically abnormal tortoises revealed multiple physiological complications, with moderate to severe rhinitis or pneumonia being the primary cause of morbidity in all but one of the examined animals. Clinically abnormal tortoises had increased transcription for four genes (SOD, MyD88, CL and Lep), increased lymphocyte production, biochemical enzymes and organics, trace elements of copper, and decreased numbers of leukocytes. We found significant positive correlations between increased transcription for SOD and increased trace elements for copper, as well as genes MyD88 and Lep with increased inflammation and microbial insults. Improved methods for health assessments are an important element of monitoring tortoise population recovery and can support the development of more robust diagnostic measures for ill animals, or individuals directly impacted by disturbance.

Key words: blood biochemistry, clinical status, desert tortoise, gene transcription, health, hematology, Mojave Desert, molecular, morphology, necropsy, reptile

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Introduction

Animal health plays an important role in the management and conservation of sensitive wildlife (Chabanet *et al.*, 2016; Desforges *et al.*, 2016; Rodriguez-Jorguera *et al.*, 2016; Schoeman, 2016). Although a number of survey practices (i.e. physical examinations, hematological and biochemical blood panels, serology tests, and pathogen and toxicology screens) are used to assess wildlife health, these practices have had limited success in accurately tracking health status, particularly in free-ranging reptiles (Christopher *et al.*, 2003; Madliger *et al.*, 2016). Wildlife declines are generally the result of a combination of both anthropogenic (e.g. overharvest, habitat destruction, introductions of exotic species, contaminants) and natural (e.g. disease epidemics, drought, flood) stressors and rarely due to one single factor operating in isolation (Irwin and Irwin, 2006; Micheli *et al.*, 2016). The response of an individual or population to environmental stressors often differs according to individual variability and the cumulative effects of the stressors present in their environment (Patyk *et al.*, 2015). As a result, understanding animal health and the effects of environmental change requires the monitoring and assessments of these complex interactions, ideally using a range of innovative ecological, biomedical and behavioral indicators (Lloyd *et al.*, 2016).

In reptiles, robust health assessments for disease status or physiological problems are further complicated by lack of research, challenging logistics of individual capture for repeated assessments, and their metabolic characteristics (Christopher *et al.*, 2003; Keller *et al.*, 2006; Allender *et al.*, 2016; Buckley and Huey, 2016). As ectotherms, reptiles undergo strong seasonal shifts in behavior, physiology and metabolism (Porter and Gates, 1969; Huey, 1982; Zimmerman *et al.*, 2010; Vitt, 2016), which can directly influence how reptiles partition resources to self-maintenance activities, including the immune system and other physiological functions (Zimmerman *et al.*, 2010; Sandmeier and Tracy, 2014). Consequently, their physiological responses are highly variable and change with age, sex, nutritional, individual, seasonal and environmental conditions (Wright and Cooper, 1981; Zapata *et al.*, 1992; Dickinson *et al.*, 2002; Moore and Jessop, 2003, MacDonald *et al.*, 2007; Nechaeva, 2011; Vitt, 2016).

Despite these drawbacks, standard wildlife diagnostic blood panels (e.g. hematology, biochemistry, serology and cytology) and physical examinations are still routinely used on reptiles to investigate conditions that may affect blood cells or cause a change in blood cell composition including anemia, inflammation, hematopoietic disorders and parasitemia (Christopher *et al.*, 1999; Christopher, 1999; Jacobson and Origgi, 2002; Nardini *et al.*, 2013; Sheldon *et al.*, 2016). Comprehensive panels often require more blood or plasma than can be safely collected from wild or smaller reptiles, and the interpretation of these data is challenging because of the low number of studies and the lack of reference values for certain species or wild populations (Nardini *et al.*, 2013; Lloyd *et al.*, 2016). Furthermore, these laboratory tests are not designed for or capable of identifying specific intrinsic or extrinsic stressors impacting animal health.

Gene transcription profiling has been experimentally validated to improve the interpretation of health status and response of reptiles to environmental stressors (Krivoruchko and Storey, 2013, 2015; Drake *et al.*, 2016). Using a small amount of tissue (e.g. 0.1 ml blood), ecologists and microbiologists can identify specific genes responding to perceived environmental stressors and measure their response by quantifying the amount of messenger RNA (mRNA) that is transcribed for targeted genes (Burcyznski *et al.*, 2000; Bartosiewicz *et al.*, 2001; Sitt *et al.*, 2008; Bowen *et al.*, 2012; Miles *et al.*, 2012). This approach improves the assessment of physiological status by detecting the earliest observable signs of changes in health at the cellular level (Acevedo-Whitehouse and Duffus, 2009). For chelonians, this technology has proven useful for understanding the systemic health effects that occur with dietary changes and malnutrition such as the down-regulation of immune function and reductions in growth, calcium metabolism and shell calcification (Drake *et al.*, 2016) as well as changes that occur in stressed or physiologically perturbed animals (Krivoruchko and Storey, 2013, 2015; Bowen *et al.*, 2015). As reference genomes and primer sequences for targeted genes are made available for a wider range of non-mammalian species, our ability to understand molecular reactions to intrinsic and extrinsic stressors as well as assessments of animal and ecosystem health will improve.

Here, we provide an integrative health assessment approach for the Agassiz's desert tortoise (*Gopherus agassizii*) by pairing molecular biomarkers with traditional blood panel and physical assessments. This threatened species is subject to a wide variety of environmental and human stressors (USFWS, 1994, 2011, 2016), and considerable attention has been focused on understanding aspects of health, nutrition, disease and general survivorship in this species (Brown *et al.*, 1994; Christopher *et al.* 2003; Longshore *et al.* 2003; Tracy *et al.* 2006; Sandmeier *et al.* 2009; Esque *et al.* 2014). Most studies and field experiments have found it challenging

to track, monitor and measure health in tortoises and other ectotherms (Christopher *et al.*, 1999; Tracy *et al.*, 2004; Hunter *et al.*, 2008; Sandmeier *et al.*, 2013, 2016).

Due to the reduced metabolic state and activity level of poikilothermic organisms, clinical signs of health or disease conditions may be slow to emerge (Christopher *et al.*, 2003; McArthur, 2004; Allender *et al.*, 2016). The use of diagnostic assays largely designed for mammals often lead to difficulties in interpretation (Llyod *et al.*, 2016), as tortoises exert less control over their homeostatic mechanisms than birds and mammals and their 'normal' ranges are often wider and in many species subject to marked seasonal variation (Lillywhite, 1987; Peterson *et al.*, 1993; Wilkinson, 2004). Furthermore, humoral immune reactions such as targeted antibody responses can be highly delayed (>18 months) in Agassiz's desert tortoises following the appearance of clinical abnormalities (Maloney, 2011; Aiello *et al.*, 2016; our unpublished work).

We evaluated both standard (hematologic and plasma biochemistry values, trace element screens, proteins, vitamin A levels) and gene-based (transcript profiling) diagnostic blood panels from clinically abnormal and normal adult tortoises from Clark County, NV, USA. In addition, we investigated aspects of both constitutive (e.g. white blood cell and leukocyte counts, gene transcription) and humoral (e.g. antibody production) immune responses to known pathogens (*Mycoplasma agassizii* and *Testudinid* herpesvirus 2; Brown *et al.*, 2002; Wendland *et al.*, 2007; Jacobson *et al.*, 2012; Braun *et al.*, 2014). Necropsy and histological evaluation were performed on clinically abnormal tortoises with chronic illness to provide additional evidence of health condition relative to blood panels. We investigated whether transcript profiles for genes involved in immune responses to pathogenic microbes (SAA, ATF, CD9, MX1, Myd88; Kibenge *et al.*, 2005; Tumpey *et al.*, 2007; Zhou *et al.*, 2008, 2011; Li *et al.*, 2011) and genes often correlated with malnutrition (Lep; Otero *et al.*, 2005) and cellular stress superoxide dismutase (SOD; Walsh *et al.*, 2010) would be higher in clinically abnormal tortoises suspected of having bacteria-related infection and disease (Bowen *et al.*, 2015; Drake *et al.*, 2016). By pairing molecular biomarkers with traditional hematologic and biochemical blood panels, and comparing data from necropsy and histological evaluation, our efforts consider how new screening methods can improve the diagnostic capacity of blood-based health assessments in reptiles.

Materials and Methods

Study animals

Captive adult tortoises (n = 11; 5 Female:6 Male) from the Desert Tortoise Conservation Center in Clark County, NV, USA that were classified as 'clinically abnormal' based on long-term health evaluations by specialized veterinarians were used in this study. Each tortoise in this category presented multiple clinical signs of potential illnesses associated with long-term weight loss and reduced or under-conditioned body condition. Due to their poor overall health, these tortoises were euthanized, and immediately necropsied to evaluate tissue conditions morphologically and histologically. 'Clinically normal' tortoises were selected from an *in situ* wild population that had been monitored since 2006 (n = 10; 6 Female:4 Male) in Hidden Valley, Clark County, NV, USA (Drake *et al.*, 2015) to act as a healthy control for comparison. These tortoises were deemed clinically normal based on visual examination by specialized veterinarians and tortoise biologists and each tortoise had been evaluated and assessed as clinically normal for nine consecutive years (Drake *et al.*, 2015). Clinically normal, wild tortoises were not euthanized. All tortoises were assessed and sampled in July between 06:00 and 08:00 h to minimize circadian influences on measured blood analytes. All handling and experiments using animals were conducted according to Institutional Animal Care and Use Committee guidelines (US Geological Survey WERC 2012-03, University of California-Davis WERC-2007-02) and covered under state (Nevada Division of Wildlife Permit #S33762) and federal (US Fish and Wildlife Service TE-030 659) permits.

Animal condition

All tortoises were assessed to characterize their general health and body condition. Assessments included an examination of the animal's general posture, respiration, face (with specific attention to the eyes, periocular tissue, nares, mouth, tongue and oral mucosa), skin and shell for any clinical signs of disease, abnormalities, damage or discoloration (USFWS, 2016). We looked for discharge from the cloaca, eyes, nares and mouth and examined the skin for evidence of ulceration, erythema, swelling or discharge. We also palpated the coelomic cavity to confirm masses (e.g. urolith and egg) present during evaluation (USFWS, 2016). Clinical condition was quantified for each tortoise by summing the number of signs of disease, abnormalities, damage or discoloration present (Table 1).

Numerical body condition scores (BCSs) were used to assess overall muscle condition and fat stores with respect to skeletal features of the head and limbs (USFWS, 2016). BCS scores were first categorized as 'under,' 'adequate' or 'over' condition, and then numerical values were assigned to provide a more precise and repeatable measurement (i.e. under: 1–3, adequate: 4–6, over: 7–9) (USFWS, 2016). The shell length for each tortoise was recorded to the nearest 1.0 mm using digital calipers.

Animal necropsy and histology

The clinically abnormal tortoises were in poor condition and were euthanized, necropsied, and evaluated for morphological and microscopic conditions at the Wildlife Disease

Table 1: The clinical condition of adult captive tortoises (A1–A11; 6 F:5 M) and wild tortoises (N1–N10; 6 F:4 M) in Clark County, NV, USA

ID	Sex	MCL (mm)	Mass (g)	Assessed status	Eyes	Nares	Oral cavity	Skin	Shell	Other	BCS
A1	F	250	2411	Abnormal	E	A, DS	–	–	–	–	4
A2	F	274	2862	Abnormal	E, R	A, DM, Er	–	–	–	–	4
A3	M	270	3914	Abnormal	E, R	A, DM, Er	L	–	–	–	4
A4	M	na	2507	Abnormal	E, R	DS, Er	–	–	–	–	4
A5	M	274	2165	Abnormal	–	A, DS, Er	–	–	–	CM	3
A6	F	283	4329	Abnormal	DS, CR, E, R	A, DS, Er	–	–	–	–	4
A7	F	260	3037	Abnormal	E, DM	A, DM, Er, O	–	–	–	LR	4
A8	F	246	2430	Abnormal	E, DS	A, DM, Er, O	–	–	–	–	4
A9	M	213	1248	Abnormal	E, R	–	–	L	–	–	3
A10	M	171	856	Abnormal	–	A, DS, Er, O	–	–	–	–	4
A11	F	259	2764	Abnormal	E, R	A, DS, Er	–	–	–	CM	4
N1	F	240	2415	Normal	R	–	–	–	–	–	4
N2	M	285	4200	Normal	R	–	–	–	–	–	5
N3	M	294	5035	Normal	R	–	–	–	–	–	4
N4	F	244	2710	Normal	R	–	–	–	–	–	4
N5	M	309	4305	Normal	R	–	–	–	–	–	4
N6	F	224	2095	Normal	R	–	–	–	–	–	4
N7	F	261	3370	Normal	R	–	CP	–	–	–	4
N8	F	267	3498	Normal	R	–	CP	–	–	–	4
N9	M	317	5525	Normal	R	–	–	–	–	–	4
N10	F	256	3200	Normal	R	–	–	–	–	–	4

Tortoises were evaluated mid-summer (July) immediately before sampling of blood. The following codes indicate clinical anomalies observed during evaluation: A, asymmetrical; CM, coelomic mass; CP, coloration pale; CR, coloration red; DS, discharge serous; DM, discharge mucoid; E, edema; Er, eroded; L, lesions present; LR, labored respiration; O, occluded; R, recessed; '–', clinically normal; MCL, maximum Carapace Length.

Laboratories, San Diego Zoo Institute for Conservation (San Diego, CA, USA). All major organs and tissues including the eyes, palpebra, nasal cavity, trachea, lungs, oral cavity and tongue, esophagus, stomach, small intestine, large intestine, pancreas, liver, spleen, heart, kidney, bladder, adrenal glands, gonads, cloaca, brain, skeletal muscles and shell/skeletal structure were evaluated.

Blood collection

Blood (~3 ml) was extracted from all tortoises via jugular venipuncture (Jacobson *et al.*, 1992) using a 1.91-cm, 25-gage needle-IV infusion set and 3 ml syringe (Fig. 1). Blood was collected from clinically abnormal tortoises prior to euthanasia. Aliquots of whole blood were placed immediately into an RNeasy® Animal Protect collection tube (0.5 ml blood for gene transcription analyses; Qiagen, Valencia, CA, USA) and BD Microtainer® tubes with lithium heparin (~2.5 ml blood for complete blood counts, hematological evaluations, chemistry panel screens, trace element screens and vitamin A analyses; Becton Dickinson and Company, Franklin Lakes, NJ, USA). Samples were stored on ice for no >2 h. A small droplet of blood (0.01 ml) was smeared onto a microscope slide for hematological evaluations. Aliquots of whole blood were shipped overnight for analysis of hematology. Plasma was separated from the remaining sample using centrifugation with a force of 1318×*g* and stored in an ultra-cold freezer (−70°C) until analysis. Aliquots of plasma (0.01 ml) were screened for antibodies to *M. agassizii* using an enzyme-linked immunosorbent assay (ELISA; Wendland *et al.*, 2007). Additionally, sloughed epithelial cells were collected using sterile oral swabs, and screened for Testudinid herpesvirus 2 using polymerase chain reaction (PCR) (Jacobson *et al.*, 2012; Braun *et al.*, 2014).

Gene transcription

RNA extractions and cDNA synthesis were performed as described in Bowen *et al.* (2015) on each sample collected for gene transcription. PCR primers developed for *G. agassizii* were used to amplify 11 genes of interest and 1 ribosomal housekeeping gene within each sample (Supplementary Table 1,

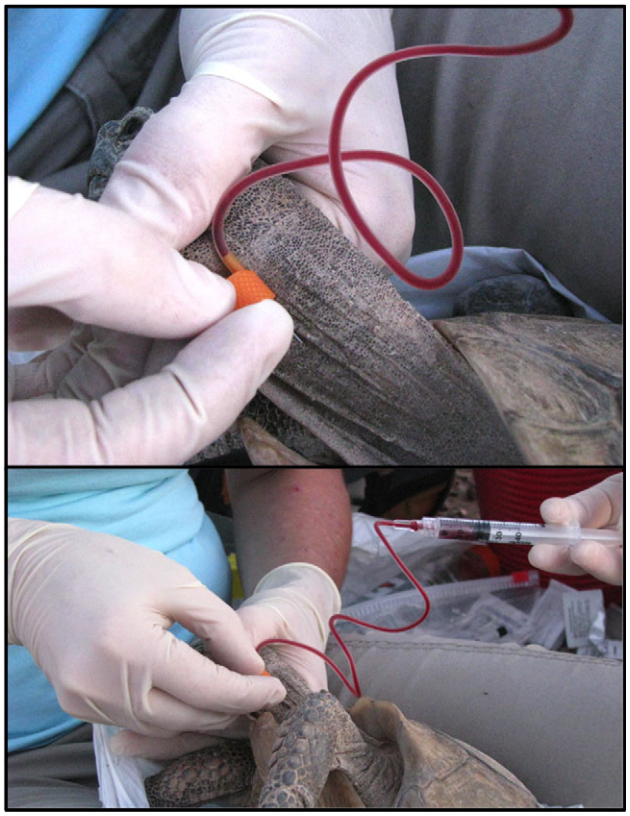

Figure 1: Photograph of blood (~3 ml) being extracted from an adult Agassiz desert tortoise (*G. agassizii*) in Clark County, NV, USA. Blood was collected via jugular venipuncture using a 1.91-cm, 25-gage needle-IV infusion set and 3 ml syringe. Photographs taken by D. Johnson.

Bowen *et al.*, 2015, Drake *et al.*, 2016). Gene transcription cycle threshold values (C_T) were measured for the housekeeping gene (18 S) and the genes of interest: CaM-Calmodulin, AHR-Arylhydrocarbon Receptor, Mx1, HSP70-Heat Shock Protein 70, SAA-Serum Amyloid A, MyD88-Myeloid Differentiation Factor 88, CD9, SOD, ATF, CL-Cathepsin L and Lep-Leptin (Supplementary Table 1) from each sample in duplicate using quantitative PCR. Amplifications were conducted on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Hanover Park, IL, USA). Gene transcription measures were normalized by subtracting the average 18 S housekeeping ribosomal gene C_T value from the gene of interest C_T for each tortoise.

We analyzed the qPCR gene transcript data using normalized C_T values. These values are inversely proportional to the amount of subject mRNA in the sample such that the lower the normalized value, the more transcripts are present. A change in normalized value of two is approximately equivalent to a 4-fold change in the amount of the transcript. We evaluated normalized gene transcript profiles for each gene of interest to examine potential differences in immune function between animal sex and clinical status (Supplementary Table 1).

Blood analytes

Blood samples were evaluated for complete blood counts, leukocyte morphology, biochemical panels, trace elements and vitamin A levels at the San Diego Zoo Global Clinical Laboratory (Escondido, CA, USA) and the University of California, Davis William R. Pritchard Veterinary Medical Teaching Hospital, Clinical Diagnostic Laboratory (Davis, CA, USA).

'Hematology'—Blood erythrocyte parameters (hematocrit, red blood cell (RBC) morphology), leukocyte morphology (white blood cell-WBC; WBC differentials including lymphocytes, heterophils, azurophils, eosinophils, basophils, heterophil to lymphocyte ratio) and other parameters (platelets, plasma protein, plasma fibrinogen, protein to fibrinogen ratio and icterus) (Table 2). Packed cell volume was determined by centrifugation of blood at 10 000 ×*g* in a Sorvall Legend Micro 17 Micro-centrifuge (Thermo Scientific Corp, Waltham MA, USA). Two hundred cell differential and leukocyte counts were done manually by certified clinical laboratory scientists on Wright-stained blood smears stained with an automatic stainer (Wescor Inc., Logan, UT, USA). Fibrinogen was measured by the standard micro heat-precipitation.

'Biochemical Panels'—Plasma parameters were evaluated for biochemical panels including: 'enzymes' (alkaline phosphatase—ALK, aspartate aminotransferase—AST, creatine kinase—CK, glutamate dehydrogenase—GLUD and lactic dehydrogenase—LDH), 'organics' (blood urea nitrogen-BUN, cholesterol-Chol, glucose-Gluc, triglycerides-Trig, total protein-TP and uric acid-UA), 'proteins' (prealbumin, albumin-ALB, α_1 globulins, α_2 globulins, β-globulins, ϒ-globulins, albumin to globulin ratio (A:G) and total protein-TP), 'minerals' (calcium-Ca, phosphorus-P), and 'electrolytes' (anion gap, chloride-CL, sodium-Na, potassium-K, total carbon dioxide-CO2) (Table 3; Taylor and Jacobson, 1982; O'Connor *et al.*, 1994; Christopher *et al.*, 1999; Christopher *et al.*, 2003). Frozen plasma samples were thawed at room temperature and analyzed using a Roche cobas c501 system (Roche Diagnostics, Indianapolis, IN, USA). Analytes were measured using commercially available kits for albumin (ALB, Roche-ALB2), ALP (Roche-ALP2), anion gap (calculated), AST (AST, Roche-AST-L), bicarbonate (Roche-CO2-L), calcium (CA-Roche-CA2), cholesterol (CHOL-Roche = CHOL2), CK (Roche-CKL), electrolytes-sodium (Na), potassium (K) and calcium (Ca, Roche-ISE indirect Gen 2), globulin (Glob, calculated), glucose (GLU, Roche-GLUC3), glutamate dehydrogenase (Randox-GL441), inorganic phosphate (P, Roche-PHOS2), total protein (TP, Roche-TP2), urea nitrogen (BUN, Roche-UREAL) and uric acid (UA, Roche-UA2) (Table 3).

Table 2: Geometric mean normalized C_T transcription values for 11 genes of interest for 21 adult Mojave desert tortoises (*G. agassizii*) that were assessed as clinically normal (5 F:5 M) and clinically abnormal (6 F:4 M)

Gene	Range	All torts	Clinically normal	Clinically abnormal
SAA	13.80–20.17	15.82	16.04	15.63
HSP70	11.41–14.14	12.86	12.57	13.13
MX1	14.15–21.00	17.45	17.87	17.08
CD9	10.92–14.38	12.52	12.81	12.27
SOD*	9.35–14.23	11.25	12.29	10.37
AHR	12.34–16.47	14.90	15.20	14.64
MyD88*	14.37–18.05	16.11	16.93	15.40
CaM	8.75–11.91	10.42	10.28	10.55
ATF	7.58–16.05	11.12	11.87	10.48
CL*	13.59–22.17	16.15	16.72	15.65
Lep*	11.35–15.44	13.43	14.14	12.82

The smaller the mean value, the higher the level of transcript for the 11 genes. '*' Indicates significant difference ($P \leq 0.05$) between clinical groups.

Table 3. Blood hematology values for 21 adult Mojave desert tortoises (*G. agassizii*) that were assessed as captive clinically abnormal (5 F:6 M) and wild clinically normal (6 F:4 M) in July at Clark County, NV, USA

Parameters	PS	Abnormal range	Normal range	Reference range	Abnormal geometric mean	Normal geometric mean
RBC (%)	All	19–26	13–43.5	19.5–37.1	23	25
WBC (/μl)*	All	3500–21 700	6900–10 000	1496–10 924	6421	8321
Heterophils (%)*	All	9–68	20–58	NA	21	36
Heterophils (/μl)*	All	520–4556	1500–5320	719–7159	1326	2900
Lymphocytes (%)*	All	14–67	12–47	NA	47	23
Lymphocytes (/μl)	All	938–14 322	962–3700	63–2746	3027	1878
Hetero:Lympho*	All	0.14–4.86	0.43–4.67	NA	0.44	1.5
Azurophils (%)*	All	0–5	3–13	NA	1	7
Azurophils (/μl)*	All	0–335	258–949	0–557	22	570
Eosinophils (%)	All	0–26	4–23	NA	6	12
Eosinophils (/μl)*	All	0–1312	300–1980	0–950	292	1005
Basophils (%)	All	6–22	6–27	NA	10	15
Basophils (/μl)*	All	228–2387	438–2300	62–3574	625	1258

Reference values were taken from published research wild adult Mojave desert tortoises in the summer season (Christopher *et al.*, 1999). Population Specifications (PS): All, all tortoises. '*' indicates significant difference ($P \leq 0.05$) between clinical groups. Hetero: Lympho, heterophils: lymphocytes ratio.

'Trace Element Screens'—Plasma was evaluated for trace elements of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), potassium (K), sodium (Na) and zinc (Zn). A 0.5 ml sample of plasma was placed into a test tube with 4 ml of protein precipitating internal standard solution. The sample was mixed and centrifuged to produce a clear supernatant free of proteins. The sample was then analyzed for elements by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Thermo iCAP 6500 Radial instrument). Each element was reported in parts per million (ppm), except Na and K which were reported as milli-equivalents per liter (mEq/l; Table 3).

'Vitamin A Analysis'—Vitamin A (retinol) was extracted from 0.5 ml plasma with petroleum ether after precipitation of proteins with ethanol. Each sample was mixed and centrifuged to produce a clear supernatant free of protein. After

concentration to dryness, samples were exchanged into methanol and filtered through a 0.45 µm syringe into an auto-sampler vial. Quantitation was performed by high-performance liquid chromatography (Agilent 1200 series) using fluorescence detection (Waters model 2475 fluorescence detector).

Statistical analyses

Most blood analytes were not normally distributed even after log transformations; therefore, we used non-parametric tests for analyses using R statistical software (R Development Core Team, 2016). The geometric means and 95% confidence intervals were calculated for all blood analytes in each clinical group. We used conventional mean responses per clinical group (clinically abnormal or normal) and animal sex (male or female) with data assessed for statistical significance between classification ranks using Wilcoxon Signed Rank Tests (Hollander *et al.*, 2014; R package stats v3.2.2). Analyte responses between clinical groups and animal sex were compared using a non-parametric Multivariate Analysis of Variance (permutation MANOVA; R package vegan v2.3–1). We also performed a nonmetric, multivariate, multidimensional scaling (NMDS; R package vegan v2.3–1; Oksanen *et al.*, 2011) ordination with the Bray–Curtis similarity measure in conjunction with cluster analysis for statistical and graphical representation of individual tortoises clustered by similarity in transcription, hematology, biochemistry and trace element screens and not by pre-defined groups such as clinical status. Statistical significance was based on P-values ≤ 0.05. Spearman's rank-order correlation was used to measure the strength and direction of association between variables in our data set, using a statistical significance based on P-values ≤ 0.01.

Results

Physical condition

All clinically normal tortoises had optimal ranged BCSs (range 4–5), indicating adequate muscle and fat deposits relative to skeletal features such as the sagittal crest. Recessed eyes were consistently observed in clinically normal tortoises, which is a typical finding for desert tortoises during the dry summer season and likely indicates a period of temporary dehydration (Table 1). Two clinically normal tortoises were found to have pale oral and tongue muscosa. Each tortoise classified as clinically abnormal was found to have multiple significant physical anomalies. Anomalies included periocular edema, conjunctival edema and hyperemia due to inflammation, recession of periocular tissue, ocular and nasal discharge (both serous and mucoid), occluded and eroded nares, labored respiration, pale and reddened oral muscoa and tongue, coelomic masses indicative of urolithiasis, skin lesions and associated lethargy (Table 1). General signs of upper respiratory tract disease (URTD) (e.g. nasal discharge, occluded and eroded nares, periocular edema) were observed in all abnormal tortoises. Two out of 11 clinically abnormal tortoises had BCSs classified as a '3' indicating a palpable or visible sagittal crest and atrophied forelimbs (Table 1).

Necropsy

Necropsy and histological evaluation was performed on 9 of the 11 clinically abnormal tortoises to provide more comprehensive information on physical condition and health. The primary findings were moderate to severe rhinitis in seven of the tortoises and moderate to severe pneumonia in the other two tortoises. Additional significant findings were renal gout in one tortoise and a chronic liver hematoma in another. Secondary inflammatory findings were mild glossitis, mild to moderate tracheitis, mild gastritis and mild enteritis in several of the animals. Two tortoises also showed degenerative changes in the kidney including glomerular atrophy. Incidental findings were skeletal muscle sarcocysts in one tortoise and urolithiasis in three tortoises. These impairments likely contributed to the observed poor health and body condition each animal exhibited (Supplementary Table 2).

Pathogens

ELISA test results for antibodies specific to *M. agassizii* for all clinically normal tortoises were negative. In contrast, ELISA test results were positive for all clinically abnormal tortoises, potentially indicating prior or current mycoplasmid infections. PCR test results for *Testudinid* herpesvirus 2 were negative for all tortoises.

Gene transcription

Gene transcript (C_T) profiles indicative of immune and physiological function were statistically different between clinically abnormal and normal individuals (perMANOVA $F_{1,20} = 4.95$, $P < 0.01$). When analyzed without *a priori* structure of clinical status, tortoises separated into two well-defined groups as depicted by NMDS and cluster analysis (Fig. 2). Transcription levels of most genes were higher in clinically abnormal than normal tortoises (Table 2). When grouping tortoises by clinical status, we found significant differences in the genes SOD ($W = 12.0$, $P < 0.01$), MyD88 ($W = 5.0$, $P < 0.01$), CL ($W = 27.0$, $P = 0.05$) and Lep ($W = 12.5$, $P < 0.01$) (Table 2). These genes were indicative of molecular reactions for enzymatic protection from superoxide radicals (SOD; Walsh *et al.*, 2010), signaling of innate immunity against microbial infection (Myd88; Li *et al.*, 2011), protein synthesis (CL; Zhou *et al.*, 2008), and overall nutritional condition and neuroendocrine and immune functions (Lep; Otero *et al.*, 2005). Transcript levels for SOD and MyD88 in abnormal tortoises represented a fourfold increase in transcription compared to normal animals. Other genes important for innate and adaptive immune defenses against bacterial and microbial infection and inflammation (i.e. SAA, MX1, CD9 and ATF), environmental toxicants

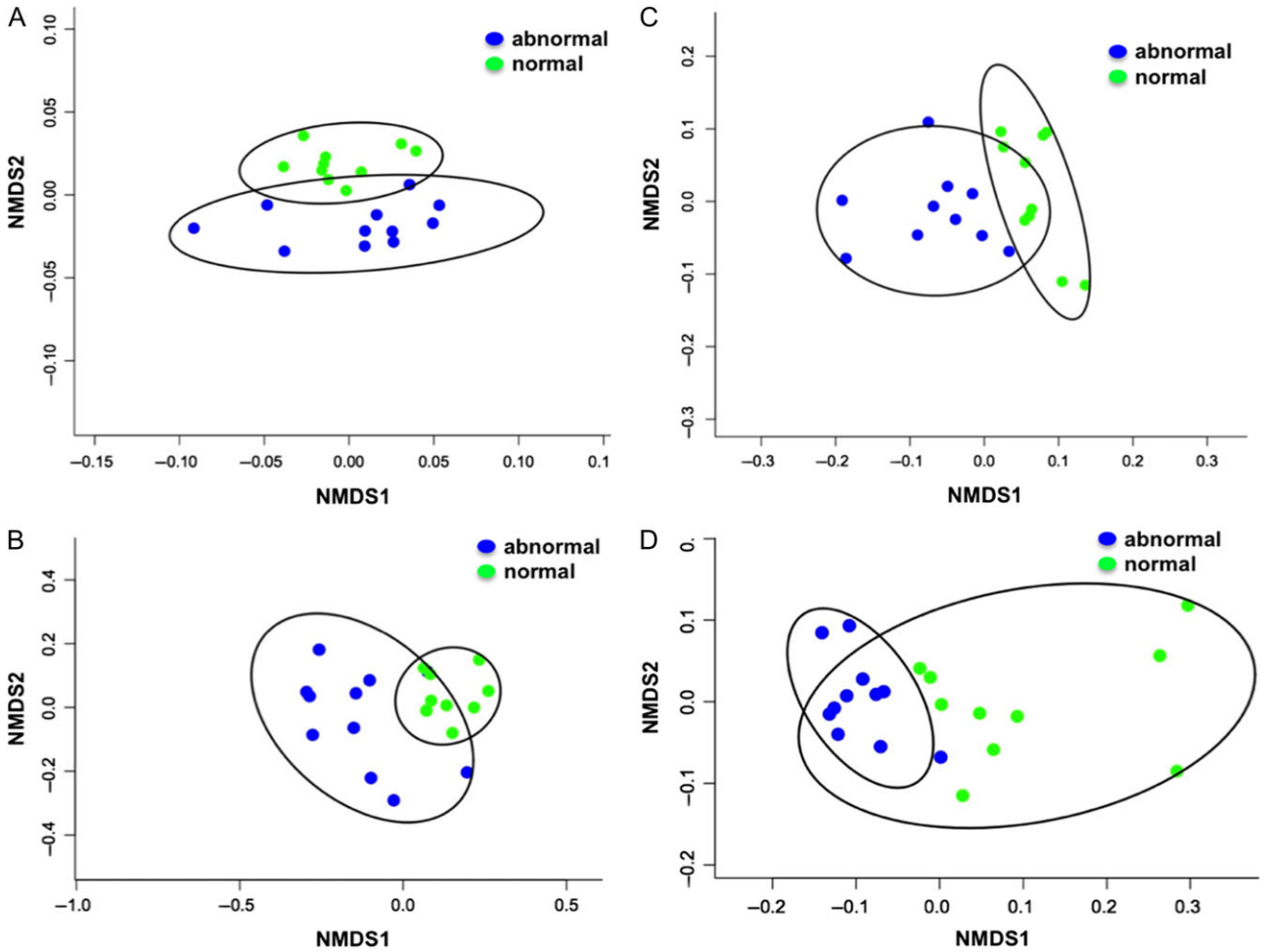

Figure 2: Multivariate, nonmetric multidimensional scaling (NMDS) 2D plots for (**A**) normalized cycle threshold values for 11 genes of interest, (**B**) counts and percentages for 13 blood hematology analytes, (**C**) 19 plasma biochemical analytes and (**D**) seven trace element analytes from collected blood samples. Adult Agassiz's desert tortoises (*G. agassizii*; $n = 21$) were sampled and categorized based on clinical condition (11 abnormal and 10 normal) in July.

(AHR), detoxification (HSP70), calcium metabolism and cellular regulation (CaM) did not differ between normal and abnormal tortoises (Table 2). We found no evidence of differences in gene transcript profiles between males and females (perMANOVA $F_{1,20} = 1.51$, $P = 0.20$).

Hematology

Hematological blood parameters were also statistically different between individuals assessed as clinically abnormal or normal tortoises (perMANOVA $F_{1,20} = 4.40$, $P < 0.01$). As with the gene transcription, we found no statistical differences by sex (perMANOVA $F_{1,20} = 1.02$, $P = 0.37$). Multivariate analyses (nMDS and cluster analysis) of hematological data revealed two distinct groups of tortoises (Fig. 2). Most hematological values including counts of white blood cells, heterophils, azurophils, eosionphils and basophils as well as percentages of hematocrit, heterophils, azurophils, eosinophils and basophils were lower in abnormal tortoises (Table 3). Direct counts and percentages of lymphocytes were markedly higher in abnormal tortoises, indicating increased humoral immune reactions (antibody production; Table 3). Statistical analyses using *a priori* clinical groupings indicated significant differences in WBCs (#/ul, $W = 18$, $P = 0.01$), heterophils (%, $W = 24.5$, $P = 0.03$; #/µl, $W = 22.5$, $P = 0.02$), lymphocytes (%, $W = 97$, $P < 0.01$), azurophils (%, $W = 4$, $P < 0.01$; #/µl, $W = 2$, $P < 0.01$), eosinophils (#/µl, $W = 22$, $P = 0.02$) and basophils (#/µl, $W = 27$, $P = 0.05$) (Table 3). Counts of WBCs were lower in clinically abnormal tortoises, despite noted tissue inflammation and infection in clinically abnormal tortoises (Supplementary Table 2). Heterophil:lymphocyte ratios were higher in clinically normal tortoises than clinically abnormal tortoises ($W = 18$, $P < 0.01$; Table 3).

Plasma biochemical panels

We found no significant overall differences in plasma biochemical values between clinically normal or abnormal individuals (perMANOVA $F_{1,19} = 1.24$ $P = 0.26$). However, differences were found between animal sex (perMANOVA $F_{1,19} = 3.74$, $P = 0.04$), as levels of calcium ($W = 99$, $P < 0.01$) and cholesterol ($W = 84$, $P = 0.03$) were higher in female than male tortoises (Table 4). Most biochemical values including enzymes (AST, CPK), organics (Alb, BUN, Chol, Globulins, Gluc, Fibrogen, total protein and UA), minerals (Ca, P) and electrolytes (Cl, Na) were increased but not statistically different in abnormal tortoises compared to clinically normal tortoises (Table 4). Measurements of the enzyme ALK, electrolyes K and C02, and vitamin A were higher in clinically normal tortoises. We found statistical differences in ALK ($W = 17.5$, $P < 0.01$), AST ($W = 95.5$, $P < 0.02$), Alb ($W = 92$, $P < 0.01$), BUN ($W = 94$, $P < 0.01$), Chol ($W = 90$, $P = 0.01$), Globulins ($W = 98$, $P < 0.01$), Total Protein ($W = 87$, $P = 0.03$) and UA ($W = 91.5$, $P = 0.01$) (Table 4) between clinically abnormal and normal tortoises.

Trace element screens

We found no significant overall differences in trace elements between clinically abnormal and normal individuals (perMANOVA $F_{1,20} = 1.07$, $P < 0.31$) or by sex (perMANOVA $F_{1,20} = 1.63$, $P = 0.20$), although quantitatively levels of copper (cu), phosphorus (p) and sodium (na) were numerically higher in clinically abnormal tortoises, and calcium (ca), magnesium (mg) and zinc (zn) were higher in clinically normal tortoises (Table 4). Statistical analyses using *a priori* clinical groupings indicated significant differences only in trace elements of cu ($W = 110$, $P < 0.01$; Table 4).

Correlations

We found 74 significant ($P \leq 0.01$) spearman's rank variable correlations within evaluated blood variables including gene transcript, hematological, plasma biochemical and trace element panels (Supplementary Table 3).

Discussion

Here, we present the first comprehensive comparative study investigating molecular, constitutive and humoral aspects of the immune system along with other physiological and histological characteristics in tortoises. We found Agassiz's desert tortoises with long-term illness (classified as clinically abnormal) had general signs of URTD (Brown *et al.*, 1994; Berry and Christopher, 2001; Sandmeier *et al.*, 2009; Jacobson *et al.*, 2014) whereas all clinically normal animals exhibited recessed eyes, a potential sign of dehydration (USFWS, 2016). Two tortoises previously determined to be clinically normal were found to have pale oral and tongue muscosa, which may indicate anemia associated with limited food availability during the sampling period. URTD was confirmed in clinically abnormal tortoises by necropsy and lesions in other tissues, particularly in the lung, kidney and liver. Notable differences in molecular (gene transcript) and hematological blood profiles were found between animals assessed as clinically abnormal or normal; yet, profiles for most plasma biochemistry, trace element screens and vitamin A levels were similar for all tortoises evaluated. Most variables evaluated did not differ between male and female tortoises, except for plasma biochemical panels, as females had higher levels of calcium and cholesterol (Christopher *et al.*, 1999). Transcription levels were increased in clinically ill tortoises including genes responding to defenses against microbial pathogens (MyD88; Li *et al.*, 2011), cellular and oxidative stress (SOD; Walsh *et al.*, 2010; Sarma and Sharma, 2016), protein synthesis (CL; Zhou *et al.*, 2008) and malnutrition (Lep; Otero *et al.*, 2005). Ill tortoises also showed increases in lymphocyte production, antibodies to pathogenic bacteria (*M. agassizii*), biochemical enzymes (AST), organics (BUN, chol, globulins, total protein and UA), and trace elements of copper. Hematological biomarkers (e.g. white blood cells, heterophils, azurophils, eosinophils, basophils and heterophil to lymphocyte ratios) routinely used to evaluate infection and inflammation in reptiles (Aguirre *et al.*, 1995; Christopher *et al.*, 1999, 2003; Keller *et al.*, 2006; Zimmerman *et al.*, 2010; Eshar *et al.*, 2014; Sandmeier *et al.*, 2016) were similar or lower in clinically abnormal animals than tortoises presumed healthy in our study and within reference ranges for wild Agassiz's desert tortoises during the summer season (Christopher *et al.*, 1994, 1999, 2003). Collectively, these findings emphasize the complexities involved in assessing, diagnosing, and interpreting fitness, immune function, and general health in tortoises.

Assessment of Tortoise Health

Hematological and plasma biochemical patterns, clinical disease and laboratory abnormalities in adult Agassiz's desert tortoises have been well studied (Nagy and Medica, 1986; Peterson *et al.*, 1993; Christopher *et al.*, 1994, 1999, 2003; O'Connor *et al.*, 1994; Peterson, 1996a, b; Homer *et al.*, 1998; Christopher, 1999; Berry and Christopher, 2001; Sandmeier *et al.*, 2016). However, many blood analytes in these studies were likely strongly influenced by sampling time, season and year, animal condition such as age, sex, hydration state and nutritional condition, environmental variables such as rainfall and plant food availability, and the geographic location of the populations evaluated, making it difficult to compare those diagnostic findings with current and future health studies. In addition, puncture site and levels of blood and lymph mixtures can strongly affect counts and concentrations of blood solutes (Bonnet *et al.*, 2016), making it important to compare results from precise sampling techniques. To avoid some of these pitfalls, we sampled tortoises during discrete time periods (e.g. within 1 week between 06:00 and 08:00 h) to capture similar environmental conditions and used jugular venipuncture to minimize lymph contamination.

Table 4. Plasma biochemical results for adult Mojave desert tortoises (*G. agassizii*) that were assessed as captive clinically abnormal (5 F:6 M) and wild clinically normal (5 F:5 M) in July at Clark County, NV, USA

Biochemical parameters	Abbreviations	PS	Abnormal range	Normal range	Reference range	Abnormal geometric mean	Normal geometric mean
Enzymes							
Alkaline phosphatase (U/l)	ALK*	All	19–116	26–134	25–114	32.86	65.34
Aspartate Animotransferase (U/l)	AST*	All	25–369	19–46	NA	58.46	29.97
	AST	M	–	–	24–123	–	–
	AST	F	–	–	15–78	–	–
Creatine kinase (U/l)	CK	All	604–108 520	955–2774	NA	2081.74	1343.67
Glutamate dehydrogenase (U/L)	GLDH	All	NM	0–4	NA	NM	0.80
Lactic dehydrogenase (U/l)	LDH	All	159–4270	NM	25–250	315.89	NM
Organics							
Albumin (g/dl)	Alb*	All	1.2–2.0	0.4–1.6	0.08–1.9	1.61	1.01
PreAlbumin	PreAlb	All	0.54–1.16	NM	0.01–0.19	0.79	NM
Albumin:Globulins	Alb:Glob	All	0.27–0.56	0.31–0.64	0.41–0.97	0.38	0.49
Blood urea nitrogen (mg/dl)	BUN*	All	3–45.0	0.9–6.0	1–37	8.32	2.42
Cholesterol (mg/dl)	Chol*	All	79–361	23–164	NA	162.67	76.70
	Chol*	M	23–56	–	60–381	–	–
	Chol*	F	94–164	–	33–217	–	–
Globulins (g/dl)	Glob*	All	1.91–4.46	0.9–3.0	1.33–3.9	3.20	2.06
α_1 Globulins	α_1 Glob	All	0.1–0.26	NM	0.03–1.22	0.18	NM
α_2 Globulins	α_2 Glob	All	0.38–0.89	NM	0.03–1.15	0.62	NM
β Globulins	β Glob	All	0.92–2.76	NM	0.05–2.82	1.79	NM
Υ Globulins	Υ Glob	All	0.34–0.73	NM	0.02–0.50	0.57	NM
Glucose (mg/dl)	Gluc	All	67–182	27–85	65–186	90.08	63.26
Plasma fibrinogen (mg/dl)	Fib	All	3.5–6.0	2.6–5.4	NA	4.7	3.9
Plasma protein (mg/dl)	Pro	All	NM	100–300	NA	NM	120
Triglycerides (mg/dl)	Trig	All	5.0–546	NM	NA	52.16	NM
	Trig	M	–	–	7–32	–	–
	Trig	F	–	–	14–603	–	–
Total Protein (g/dl)	TP*	All	3–6.0	1.4–4.6	2.3–5.3	4.45	3.09
Uric acid (mg/dl)	UA*	All	1.9–12.5	0.9–5.4	1.7–9.2	4.90	2.41
Minerals							
Calcium (mg/dl)	Ca	All	9.2–20.4	0–20.6	NA	12.48	9.30
	Ca*	M		8.1–11.0	8.6–12.5	10.24	9.18
	Ca*	F		0.0–20.6	11.3–23.9	14.66	9.38
Phosphorus (mg/dl)	P	All	12–70	14–52	NA	29.71	26.85
	P	M	–	–	1.1–3.3	–	–
	P	F	–	–	2.0–6.5	–	–

(Continued)

Table 4. continued

Biochemical parameters	Abbreviations	PS	Abnormal range	Normal range	Reference range	Abnormal geometric mean	Normal geometric mean
Electrolytes							
Anion gap (mmol/l)	AnionG	All	NA	5–18	2–29	NM	9.22
Chloride (mmol/l)	Cl	All	107–161	103–145	101–138	124.44	118.78
Sodium (mmol/l)	Na	All	139–190	136–172	127–176	153.50	146.33
Potassium (mmol/l)	K	All	3–5.5	3–6.9	3.7–7.5	4.21	4.64
Total CO_2 (mmol/l)	CO_2	All	15–27	17–26	14–32	21.22	22.24
Other							
Vitamin A (ppm)	Vit A	All	0–0.23	0.06–0.28	NA	0.10	0.16
Trace Elements							
Calcium (ppm)	Ca	All	52–230	66–170	NA	106.56	116.15
	Ca	M	–	–	NA	–	–
	Ca	F	–	–	NA	–	–
Copper (ppm)	Cu*	All	0.4–0.8	0.0–0.3	–	0.58	0.16
	Cu	M	–	–	0.5–0.7	–	–
	Cu	F	–	–	0.3–0.7	–	–
Iron (ppm)	Fe	All	NA	0.2–2.5	NA	NM	0.69
	Fe	M	–	–	NA	–	–
	Fe	F	–	–	NA	–	–
Magnesium (ppm)	Mg	All	19–51	29–62	NA	32.38	37.56
	Mg	M	–	–	NA	–	–
	Mg	F	–	–	NA	–	–
Phosphorus (ppm)	P	All	12–70	14–52	NA	29.71	26.85
	P	M	–	–	NA	–	–
	P	F	–	–	NA	–	–
Potassium (mEq/l)	K	All	2.1–5.0	NM	NA	3.5	NV
	K	M	–	–	3.5–4.7	–	–
	K	F	–	–	3.7–4.7	–	–
Sodium (mEq/l)	Na	All	77–160	110–130	NA	124.13	113.82
	Na	M	–	–	122.4–136	–	–
	Na	F	–	–	122.3–138.5	–	–
Zinc (ppm)	Zn	All	1.0–3.5	0.6–2.4	NA	1.68	1.70
	Zn	M	–	–	0.4–3.4	–	–
	Zn	F	–	–	0.7–3.7	–	–

Reference values were taken from published research wild adult Mojave desert tortoises (Christopher *et al.*,1994; Christopher *et al.*,1999; Christopher *et al.*, 2003). NM = not measured, NA = information not available, and '–' = information not calculated. '*' indicates significant difference ($P \leq 0.05$) between clinical abnormal and normal tortoises (All) or animal sex (M, F). Population specifications (PSs): All, all tortoises, M, male, F, female.

Findings from the literature suggest that comparisons among individuals with and without clinical abnormalities were helpful to identify potential disease cues or immune responses within populations, but typically do not indicate direct associations between laboratory data and specific disorders (Christopher *et al.*, 2003). For example, previous research on plasma biochemistry in clinically abnormal tortoises documented marked azotemia (BUN > 100 mg/dl), mild hyperuricemia, moderate cholesterolemia, hypophosphatemia and increased AST activity (Christopher *et al.*, 1999, 2003; Christopher, 1999). Although Christopher *et al.* (2003) found that tortoises thought to be responding to inflammation and infection had marked increases in heterophilia, leukocytosis and lymphocytosis, we found no evidence of this in our data.

Transcript Profiling

By analyzing transcript profiles in tortoises with clinical and pathophysiological impairments, our study expands the diagnostic tool kit being applied to domestic and wild tortoise populations. Gene transcript profiling has the ability to expedite and expand the detection of physiological changes and immune reactions at the cellular level, often before other changes in hematology, biochemistry or clinical conditions have occurred (McLoughlin *et al.*, 2006; Acevedo-Whitehouse and Duffus, 2009; Miles *et al.*, 2012). Previous studies in ectothermic organisms used transcript profiling with small suites of genes to identify specific molecular signatures to chemical contaminants (Connon *et al.*, 2012; Hirakawa *et al.*, 2012), malnutrition (Drake *et al.*, 2016), cellular and environmental stressors (Ju *et al.*, 2002; Connon *et al.*, 2012; Bowen *et al.*, 2015), and other physiological perturbations (Costanzo and Lee, 2013; Krivoruchko and Storey, 2013, 2015; Sujiwattanarat *et al.*, 2016), highlighting environmental and anthropogenic stressors negatively impacting animal health and survival (McLoughlin *et al.*, 2006; Acevedo-Whitehouse and Duffus, 2009; Connon *et al.*, 2012).

We investigated if transcription for genes responding to innate and adaptive immune defenses against microbial pathogens (e.g. SAA, ATF, CD9, CL, MX1, MyD88; Supplementary Table 1) would increase in tortoises with lethargy, clinical abnormalities and presumed disease. URTD in tortoises is caused by exposure to bacterial pathogens (*M. agassizii* and *M. testidenum*; Brown *et al.*, 1994) and subsequent infection, and is arguably one of the more important chronic infectious diseases of wild and captive North American and European tortoises (Jacobson *et al.*, 2014). Infection and inflammation associated with *Mycoplasma* spp. and other pathogenic microbes such as *Testudinid* herpesvirus 2 are often subclinical and hide in tissues for years within the host without showing clinical signs (e.g. nasal exudate, periocular edema) or physical impairment (Jacobson *et al.*, 2014). Therefore, we assumed that most clinically ill tortoises likely had multiple morphological, histological and physiological impairments in various organs, and that transcripts responding to cellular and oxidative stress (SOD), antigen processing and protein synthesis (CL), and malnutrition (Lep) would also be increased in ill animals.

We found significant differences in transcript profiles between tortoises classified as clinically abnormal or normal. These genetic differences likely reflect long-term suboptimal nutritional and environmental conditions endured prior to this study as well as repeated bouts with microbial infection, inflammation, disease and improper organ function. Clinically, abnormal tortoises in our study had access to food and water for two years prior to the experiment; however, their nutritional and disease conditions may reflect prior years in captivity or physiological and behavioral conditions that prevented them from foraging or processing important nutrients. Genes transcribed at significantly higher levels (SOD, Myd88, CL and Lep) in clinically abnormal tortoises may indicate a response to increased environmental toxicants, oxidative stress, microbial and bacterial infections, and malnutrition respectively. Increased transcription for SOD and Lep were previously described in malnourished juvenile tortoises used in a controlled food trial (Drake *et al.*, 2016) and other wild adult tortoises presumed to be environmentally stressed (Bowen *et al.*, 2015).

Oxidative Stress

Micro-molecules such as reactive oxygen species (ROS) are released by phagocytic cells during pathogen attack, and are generically cytotoxic because they do not differentiate between cells and tissues of the host and infective agent (Cherry and Silverman, 2006; Sorci and Faivre, 2009). Oxidative stress resulting from ROS is regularly cited as a major cause of immunopathology in humans and non-human animals (Costantini, 2008; Pursall and Rolff, 2012). Previous studies found elevated levels of copper and oxidative stress or superoxide (O_2-) radicals in blood were linked to diseases such as cancer, diabetes, premature aging and hyptertension (Strausak *et al.*, 2001). In our study, both trace elements of copper and transcription for the gene regulating oxidative stress (SOD; Walsh *et al.*, 2010; Sarma and Sharma, 2016) were elevated and highly correlated in clinically ill tortoises. Copper is an essential element for the activity of physiological important enzymes and is also a cofactor of the enzyme Cu/Zn-SOD, which plays a key role in the cellular response to oxidative stress by scavenging ROS (Harman, 1956, 1994; Fridovich, 1989; Strausak *et al.*, 2001). Free radicals such as superoxide are a type of ROS that can strip electrons from proteins, lipids or nucleic acids, thereby destroying their function and resulting in cell dysfunction or death. In chelonians, two SOD enzymes have been described (Willmore and Storey, 1997; Sarma and Sharma, 2016), with SOD1 (Cu/Zn-SOD) occurring in the

cytoplasm and outer mitochondrial space protecting the cells against any lethal effects of radiation, drugs or toxicity of ROS (Epstein *et al.*, 1987) and SOD2 (Mn SOD) being found in the inner mitochondrial space, promoting cellular differentiation, apoptosis, tumorgenesis and hypoxia induced pulmonary disease (Scott *et al.*, 1989; Wispe *et al.*, 1992; St. Clair *et al.*, 1994).

Conservation Implications

Improving our understanding of wildlife health is essential to inform management and policy decisions. However, 'health' remains a difficult concept to define and assess (Deem *et al.*, 2008; Nordenfelt, 2011; Hanisch *et al.*, 2012; Stephen and Karesh, 2014; Stephen, 2014). Research on tortoises and other wildlife health is limited, largely reactive and disease-centric, with an emphasis on responding to existing or imminent animal health events instead of understanding environmental and physiological conditions in the context of vulnerability and resilience (Stephens, 2014). Most wildlife and ecosystems face an assault of threats from continued habitat disturbance or loss (Hughes *et al.*, 2003; Cox *et al.*, 2006; Irwin and Irwin, 2006; Patz *et al.*, 2008; Jarvis *et al.*, 2009; Gioria and Pysek, 2016) and chemical contamination and pollution (Hughes *et al.*, 2003; Keller *et al.*, 2004) from increased human use. Long-lived species such as the desert tortoise may be at particular risk from the effects of degraded habitats and accumulated contaminants (Tracy *et al.*, 2004; Gardner, 2006; Keller *et al.*, 2006). Conservation efforts are especially challenged by a paucity of basic knowledge on how reptiles respond, tolerate or adapt to environmental stressors (Anderson *et al.*, 1997; Gibbons *et al.*, 2000; Hayes *et al.*, 2016; Masters *et al.*, 2016; Wise *et al.*, 2016). In addition, abnormalities and subclinical infection (e.g. recent exposure to bacterial pathogens) often go undetected in tortoises and other reptiles (Aiello *et al.* 2016), potentially impacting accurate assessments of health. Incorporating new technologies and studying targeted physiological changes may be key to understanding how species cope with environmental variability and increasing anthropogenic stressors.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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