EcoHealth
DOI: 10.1007/s10393-014-0998-8

ECOHEALTH

Original Contribution

Integrating Gene Transcription-Based Biomarkers to Understand Desert Tortoise and Ecosystem Health

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Abstract: Tortoises are susceptible to a wide variety of environmental stressors, and the influence of human disturbances on health and survival of tortoises is difficult to detect. As an addition to current diagnostic methods for desert tortoises, we have developed the first leukocyte gene transcription biomarker panel for the desert tortoise (*Gopherus agassizii*), enhancing the ability to identify specific environmental conditions potentially linked to declining animal health. Blood leukocyte transcript profiles have the potential to identify physiologically stressed animals in lieu of clinical signs. For desert tortoises, the gene transcript profile included a combination of immune or detoxification response genes with the potential to be modified by biological or physical injury and consequently provide information on the type and magnitude of stressors present in the animal's habitat. Blood from 64 wild adult tortoises at three sites in Clark County, NV, and San Bernardino, CA, and from 19 captive tortoises in Clark County, NV, was collected and evaluated for genes indicative of physiological status. Statistical analysis using a priori groupings indicated significant differences among groups for several genes, while multidimensional scaling and cluster analyses of transcription C_T values indicated strong differentiation of a large cluster and multiple outlying individual tortoises or small clusters in multi-dimensional space. These analyses highlight the effectiveness of the gene panel at detecting environmental perturbations as well as providing guidance in determining the health of the desert tortoise.

Keywords: *Gopherus agassizii*, mRNA, immune function, physiological health, reptile

Introduction

Desert tortoises (*Gopherus agassizii*) occur throughout the Mojave Desert and are subjected to a myriad of ecological and environmental stressors. Increased human development has led to habitat loss, increased predation, contaminant and pathogen exposure, and altered wildfire regimes. These stressors have been suggested as synergistic threats to desert tortoise populations, attributed frequently to population declines (Luckenbach 1982; Christopher et al. 2003; Tracy et al. 2004; Esque et al. 2010; USFWS 2011a). Desert tortoises are slow to respond to environmental stressors and seldom display clinical signs of disease, which is typical of other long-lived reptiles (Christopher et al. 2003). Understanding how tortoises respond to these environmental stressors is key to supporting conservation and management of habitat needed for this threatened species.

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Published online: 06 January 2015

Tortoises are susceptible to a wide variety of environmental stressors, and the influence of human disturbances on health and survival of tortoises is difficult to detect. In addition to current diagnostic methods for desert tortoises (e.g., Brown et al. 2002; Ritchie 2006), we propose a genetic biomarker panel, enhancing the ability to identify specific environmental conditions potentially linked to declining animal health. Despite the urgent need for ecological studies on reptile health and physiology, the development of molecular genetic tools for research in reptiles has been slow, and most are adaptations from studies in other taxa. Applying contemporary gene transcript analysis to identify genomic response to environmental stress or disease has the potential to transform studies of reptile ecology (Burczynski et al. 2000; Bartosiewicz et al. 2001). Advanced technologies, based upon and developed from biomedical models of human physiology and disease, aid researchers with cutting-edge diagnostic tools for both domestic and wildlife veterinary applications (Burczynski et al. 2000; Bartosiewicz et al. 2001; Bowen et al. 2007, 2012; Miles et al. 2012; Sitt et al. 2008). Gene-based analyses of desert tortoises afford the opportunity for minimally invasive assessments of physiologic state in response to intrinsic and extrinsic factors, not only in individuals or populations, but potentially at landscape scales (Acevedo-Whitehouse and Duffus 2009).

Gene transcript analysis may improve the assessment of desert tortoises by detecting the earliest observable signs of physiological perturbation, as gene transcripts are typically evident prior to clinical manifestations to environmental stressors (McLoughlin et al. 2006). Consequently, application of quantitative gene transcript analysis technology will likely provide an invaluable addition to current approaches for monitoring potential physiological impairment (McLoughlin et al. 2006). Herein, we describe the development of quantitative real-time polymerase chain reaction (qPCR) assays to measure differential transcript levels of multiple genes in the desert tortoise. The genes described are fundamental to immune function (Cray et al. 2009; Zhou et al. 2008, 2011; Li et al. 2011), responses to pathogens (Kibenge et al. 2005; Zhou et al. 2008), nutritional and thermal stress (Otero et al. 2005; Iwama et al. 1999; Tsan and Gao 2004), shell formation (Chen et al. 2012), xenobiotic metabolism (Walsh et al. 2010; Oesch-Bartlomowicz and Oesch 2005), and tumorigenesis (Zhou et al. 2008) (Table 1). Measurement of differential transcription of a selected suite of genes potentially can provide an early warning of compromised health and related environmental stressors in free-ranging animals.

Methods

Study Animals

Health evaluations were conducted on wild desert tortoises (n = 64, 22F:42M) between May and October 2011 on Bureau of Land Management public lands at Hidden Valley and Piute Valley in Clark County, Nevada and at Ft. Irwin in San Bernardino, CA, USA. Habitat at all sites was dominated by Mojave desertscrub vegetation (Turner and Brown 1982) consisting of creosote bush/white bursage [*Larrea tridentata/Ambrosia dumosa*] plant association with many other shrubs, grasses, and annual plants present. We also evaluated 19 captive adult tortoises (11F:8M) at the Desert Tortoise Conservation Center in Clark County, NV, USA. These animals were deemed unhealthy based on visual examination by specialized veterinarians. Each captive tortoise presented multiple clinical signs of potential illnesses associated with long-term weight loss and reduced or under-conditioned body scoring. These tortoises were identified by veterinarians for euthanasia and as such were not screened serologically at the time of our sampling for known bacterial or viral infections. These tortoises were neither under our care nor management, and we could not control decisions about their care or disease screening. Their inclusion in the data set was merely exploratory to determine how these ill captives compare to the wild populations.

Tissue Collection

Blood samples (1.0 mL) were collected from each tortoise via subcarapacial venipuncture (Hernandez-Divers et al. 2002) using a 3.8-cm, 23-gage needle and 3 cc syringe coated with sodium heparin. An aliquot (0.5 mL) of whole blood was placed immediately into an RNeasy Animal Protect^{TM} collection tube (Qiagen, Valencia, CA) and frozen at −20°C. The remaining blood (0.5 mL) was transferred to lithium heparin microtainers and stored on ice for no longer than 4 h. Plasma was separated using centrifugation with a centrifugal force of 1318×g. All tissue samples were stored at –70°C. Aliquots of plasma (0.01 mL) were screened for antibody presence to *Mycoplasma agassizii* and *M. testudineum* using an enzyme-linked immunoassay

Table 1. Documented Function of 11 Genes Identified in Free-Ranging Desert Tortoises

Gene	Gene function
CaM	Calmodulin (CaM) is a small acidic Ca^{2+}-binding protein, with a structure and function that is highly conserved in all eukaryotes. CaM activates various Ca^{2+}-dependent enzyme reactions, thereby modulating a wide range of cellular events, including metabolism control, muscle contraction, exocytosis of hormones and neurotransmitters, and cell division and differentiation (Chen et al. 2012). CaM has also been reported to be a pivotal calcium metabolism regulator in the shell formation (Li et al. 2004)
AHR	The arylhydrocarbon receptor (AHR) responds to classes of environmental toxicants including polycyclic aromatic hydrocarbons, polyhalogenated hydrocarbons, dibenzofurans, and dioxin (Oesch-Bartlomowicz and Oesch 2005). Depending upon the ligand, AHR signaling can modulate T-regulatory (T_{REG}) (immune-suppressive) or T-helper type 17 (T_H17) (pro-inflammatory) immunologic activity (Quintana et al. 2008; Veldhoen et al. 2008)
Mx1	The Mx1 gene responds to viral infection (Tumpey et al. 2007). Vertebrates have an early strong innate immune response against viral infection, characterized by the induction and secretion of cytokines that mediate an antiviral state, leading to the upregulation of the MX-1 gene (Kibenge et al. 2005)
HSP 70	The heat shock protein 70 is produced in response to thermal or other stress (Iwama et al. 1999; Tsan and Gao 2004). In addition to being expressed in response to a wide array of stressors (including hyperthermia, oxygen radicals, heavy metals, and ethanol), heat shock proteins act as molecular chaperones (De Maio 1999). For example, heat shock proteins aid the transport of the AHR/toxin complex in the initiation of detoxification (Tanabe et al. 1994)
SAA	Serum Amyloid A (SAA), an acute phase protein, serves as a core part of the innate immunity involving physical and molecular barriers and responses (Cray et al. 2009). Upon infection and inflammation or tissue damage and stress, SAA is induced by pro-inflammatory signals, and is a major indicator of bacterial infection, especially at early stage, in reptiles (Zhou et al. 2008, 2011)
MyD 88	Myeloid differentiation factor 88 (MyD88) is one of the key adaptor proteins to signal transduction that triggers downstream cascades involved in innate immunity. MyD88 might possess an important role in defense against microbial infection in Chinese soft-shelled turtles similar to that in mammals (Li et al. 2011)
CD9	CD9 is a molecular facilitator, provides co-stimulation to naïve T lymphocytes, regulates the aggregation of MHC-II molecules, and triggers antigen presentation. Upregulation of turtle CD9 was shown in response to bacterial infection (Zhou et al. 2008)
SOD	Superoxide dismutase (SOD)—Superoxide dismutases are a class of enzymes that catalyze dismutation of superoxide into oxygen and hydrogen peroxide and function as important antioxidant defense molecules (Walsh et al. 2010). Oxidative stress itself can lead to or result from certain inflammatory conditions (Walsh et al. 2010)
ATF	Similar to inflammatory responses in mammals, ATF can mediate inflammatory responses in reptiles. The upregulation of ATF in reptiles indicates involvement in bacterial infection (Zhou et al. 2008)
CL	Cathepsin L, an acute phase protein, plays a major role in antigen processing, tumor invasion and metastasis, bone resorption, and turnover of intracellular and secreted proteins involved in growth regulation (Zhou et al. 2008). Turtle cathepsin L may be involved in anti-bacterial immune response (Zhou et al. 2008)
LEP	Leptin links nutritional status with neuroendocrine and immune functions. Initially thought to be a satiety factor that regulates body weight by inhibiting food intake and stimulating energy expenditure, leptin is a hormone whose multiple effects include regulation of endocrine function, reproduction, and immunity (Otero et al. 2005)

(ELISA; Wendland et al. 2007). Plasma samples (n = 24 wild tortoises) were randomly selected and evaluated for additional clinicopathologic variables such as serum amyloid A (SAA; Belgrave et al. 2013), haptoglobin (Cray and Belgrave 2013), and electrophoresis (using Helena electrophoresis system; Cray et al. 2011). Protein electrophoresis includes fraction values for prealbumin, albumin, α_1 globulins, α_2 globulins, β-globulins, γ-globulins, albumin to globulin ratio (A:G), and total protein. Additionally, sloughed oral epithelial cells were collected using sterile oral swabs, and screened for Testudinid herpesvirus 2 using polymerase chain reaction (PCR) (Jacobson et al. 2012).

RNA Extraction

Blood samples were placed directly into RNeasy Protect Animal Blood Tubes (Qiagen, Valencia, CA) and then frozen at −20°C until extraction of RNA (Bowen et al.

2012). Rapid RNA degradation and induced transcription of certain genes after blood draws have led to the development of methodologies for preserving the RNA transcription profile immediately after blood is drawn. The RNeasy Protect Animal Blood Tube contains a blend of RNA stabilizing reagents that protect RNA molecules from degradation by RNases and prevents further induction of gene transcription. The RNA from blood in RNeasy Protect Animal Blood Tubes was isolated according to manufacturer's standard protocols except each sample was initially split in two to account for the nucleated red blood cells. The extracted RNA was stored at −80°C until analysis. All RNA was checked for quality on a nanodrop 2000 and achieved A260/A280 ratios of approximately 2.0 and A260/A230 ratios of less than 1.0.

cDNA Synthesis

A standard cDNA synthesis was performed on 2 μg of RNA template from each animal. Reaction conditions included 4 U reverse transcriptase (Omniscript, Qiagen, Valencia, CA), 1 μM random hexamers, 0.5 mM each dNTP, and 10 U RNase inhibitor, in RT buffer (Qiagen, Valencia, CA). Reactions were incubated for 60 min at 37°C, followed by an enzyme inactivation step of 5 min at 93°C, and then stored at −20°C until further analysis.

PCR Primers

Degenerate primers were designed based upon multi-species alignments (GenBank) as previously described by Bowen et al. (2007). Briefly, degenerate primer pairs were developed for the desert tortoise and were used on cDNA from three randomly selected tortoise samples. Degenerate primer pairs were designed to amplify 11 genes of interest and one ribosomal housekeeping gene (Bowen et al. 2007). PCR amplifications using these primers were performed on 20 ng of each cDNA sample in 50 μL volumes containing 20–60 pmol of each primer, 40 mM Tris–KOH (pH 8.3), 15 mM KOAc, 3.5 mM Mg $(OAc)_2$, 3.75 μg/mL bovine serum albumin (BSA), 0.005% Tween-20, 0.005% Nonidet-P40, 200 μM each dNTP, and 5U of Advantage® 2 *Taq* polymerase (Clontech, Palo Alto, CA). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and consisted of 1 cycle at 94°C for 3 min, and then 40 cycles at 94°C for 30 s, at 60°C for 30 s, and 72°C for 2 min, with a final extension step of 72°C for 10 min. The products of these reactions were electrophoresed on 1.5% agarose gels and resulting bands visualized using ethidium bromide staining. Definitive bands representing PCR products of a predicted base pair size of the targeted gene were excised from the gel, and extracted and purified using a commercially available nucleic acid-binding resin (Qiaex II Gel extraction kit, Qiagen, Valencia, CA).

Nucleotide sequences of both strands were determined by dideoxy nucleotide methods using an automated sequencer (Model 373; Applied Biosystems, Foster City, CA). Nucleotide sequences of the PCR products were analyzed using Align™ and Contig™ sequence alignment software programs (Vector NTI™; Informax Inc, North Bethesda, MD) and compared to known sequences using the NCBI BLAST program (Altschul et al. 1990), and the IMGT/HLA database (Robinson et al. 2001). Primer pairs appropriate for real-time PCR were designed based on the elucidated desert tortoise sequences for each gene (Table 2).

Real-Time PCR

Real-time PCR systems for the individual, tortoise-specific housekeeping gene (18S), and genes of interest (Table 1) were run in separate wells. Briefly, 1 μL of cDNA was added to a mix containing 12.5 μL of QuantiTect SYBR Green® Master Mix [5 mM Mg^{2+}] (Qiagen, Valencia, CA), 0.5 μL each of forward and reverse sequence-specific primers, 0.5 μL of uracil-*N*-glycosylase (Invitrogen, Carlsbad, CA), and 10.0 μL of RNase-free water; total reaction mixture was 25 μL. The reaction mixture cDNA samples for each gene of interest and 18S were loaded into 96-well plates in duplicate and sealed with optical sealing tape (Applied Biosystems, Foster City, CA). Reaction mixtures containing water, but no cDNA, were used as negative controls; thus approximately three to four individual tortoise samples were run per plate.

Amplifications were conducted on a 7300 Real-time Thermal Cycler (Applied Biosystems, Foster City, CA). Reaction conditions were as follows: 50°C for 2 min, 95°C for 15 min, 45 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 31 s, and an extended elongation phase at 72°C for 10 min. Reaction specificity was monitored by melting curve analysis using a final data acquisition phase of 60 cycles of 65°C for 30 s and verified by direct sequencing of randomly selected amplicons (Bowen et al. 2007). Cycle

Table 2. Desert Tortoise-Specific Quantitative Real-Time Polymerase Chain Reaction Primers Used in the Analysis of Free-Ranging Tortoises

Gene	Forward primer	Reverse primer	Amplicon length (bp)
AHR	atgcttaccgaagacagcac	ctcctcatcctgctgaatac	119
CAL	agacagcgaggaggagatc	tcatcatctgtcagcttctca	122
CD9	gccattgaagtagctgttgc	gctggctgattcctcttatc	106
MDF88	gtccaagagatgatcagaga	catcttcctacaccgtctct	134
MX-1	tggacactaaggttcagagt	cacgtggcaccacttctg	128
HSP 70	gccttcacsgacaccgag	aggcgcttggcatcgaaga	87
SAA	gacatgcgggaagcgaattata	gccaggccctctttgg	83
Leptin	caccaactaaagattcagcac	gtggagtagtcattgatgtgt	145
CL	gtcagtgtggatcttgttnggca	tcatntccttttgggatgtccac	127
SOD	tcaatcctaatggcaaaaacca	cagcaatcacattgccaagatc	79
18S	actcaacacgggaaacctca	aaccagacaaatcgctccac	124
ATF	agtgttggcaaaggtgaaggga	gcacataacttctactctgactacc	70

See Table 1 for interpretation of gene abbreviations.

threshold (C_T) crossing values, the measure of gene transcription for the genes of interest, were normalized to the 18S housekeeping gene.

Clinical Health Assessments

Health evaluations were performed using recommended clinical procedures (USFWS 2011b; Lamberski et al. 2012). Evaluations included the animal's general posture, respiration, and detailed visual inspection of the face (with specific attention to the eyes, periocular tissue, nares, mouth, tongue, and oral mucosa), skin, and shell for any clinical or physical abnormality in association with the animal's weight, behavior, activity, and body condition (USFWS 2011b). Clinical signs associated with physiological changes in desert tortoises often include periocular or conjunctival swelling or sunken eyes, mucous and serous ocular and nasal discharge, occluded eroded or asymmetrical nares, abnormal respiration, and weight loss. Abnormal respiration includes shortness of breath, uncomfortable awareness of breathing effort, and wheezing. For this study, tortoises categorized as having *only* "sunken eyes" (often an indicator of dehydration or emaciation) or "asymmetrical nares" (which may be a developmental anomaly or indicator of previous respiratory diseases) were not considered clinically compromised.

The body condition of each tortoise was scored using a value ranging from 1 to 9 to provide a relative index of the muscle mass, size, and attitude or behavior (USFWS 2011b; Lamberski et al. 2012). Body condition scoring (BCS) is commonly used for domestic animals and wildlife to estimate body condition in animal herds by quantifying the muscle mass and fat deposits in relation to skeletal features. In particular, BCS indicators for tortoises include the amount of muscle tissue and fat deposits around the sagittal crest and limbs, in addition to the relative amount of muscle and fat tissue around the limbs and inguinal areas of exposed body tissue. For desert tortoises, BCSs are generally ranked in three categories: under-conditioned (1–3), acceptable or good condition (4–6), and over-conditioned (7–9).

Statistical Analyses

We analyzed qPCR data using normalized values (housekeeping gene threshold crossing subtracted from the gene of interest threshold crossing); the lower the normalized value, the more the transcripts present. A change in normalized value of 2 is approximately equivalent to a fourfold change in the amount of the transcript. Commonly used parametric tests to distinguish differential gene transcription among populations were based partly on the assumption that the values being compared were sampled from normally distributed populations. We tested the assumption that transcript levels of our genes of interest were log-normally distributed, in order to use such tests for detection of disease or environmental stressors (McLoughlin et al. 2006). Geometric means and 95% upper and lower confidence limits were computed, and Anderson–Darling and Shapiro–Wilk tests for normality were performed for all genes (NCSS, Statistical and Power Analysis Software, UT, USA). We used

nonparametric statistical analyses because C_T measures of gene transcription provided by qPCR were not normally distributed for 10 of the 11 genes of interest. First, we used conventional mean responses per classification group (Piute Valley, Hidden Valley, Ft. Irwin, and captive-impaired) with data assessed for statistical significance between classification ranks using Kruskal–Wallis with Dunns' Multiple Comparison Tests (NCSS, 2007, Kaysville, Utah). Then we conducted multivariate, multidimensional scaling analysis (MDS) in conjunction with cluster analysis for statistical and graphical representation of individual desert tortoises clustered by similarity in transcription and not by pre-defined groups such as location (Primer v6 software, Plymouth, UK). Statistical comparisons of individuals grouped by clusters were made using SIMPROF (Primer v6), which is a similarity profile permutation test for significance among a priori, unstructured clusters of samples. Transcription responses of sex by sites were compared using ANOSIM (Primer v6), a nonparametric analog to a 2-way ANOVA. Statistical significance was based on P values ≤ 0.05.

Results

Clinical Conditions

Of the 64 free-ranging animals sampled, 11 (17.2%) were found to have clinically significant anomalies, with 7 (10.9%) having more than one anomaly. Anomalies included periocular edema, conjunctival edema and hyperemia due to inflammation, recession of periocular tissue, ocular and nasal discharge (both serous and mucoid), and occluded and eroded nares with associated reduction in activity. All wild tortoises had middle ranged BCSs (range 4–6), indicating adequate muscle and fat deposits relative to skeletal features such as the sagittal crest. ELISA test results for *M. agassizii* were 9 of 64 positive (14.0%) and 12 positive for *M. testudenium* (18.8%). PCR test results for Testudinid Herpesvirus 2 were negative for all tortoises.

Means and ranges, respectively, of additional laboratory analyses for 24 desert tortoises were as follows: total protein (g/dL): 3.18, 0.2–6.0; A/G: 0.30, 0.16–0.49; prealbumin (gpe): 0.09, 0.01–0.19; albumin (g/dL): 0.57, 0.03–0.95; α_1-globulins: 0.48, 0.03–1.22; α_2-globulins: 0.55, 0.03–1.15; β-globulins: 1.20, 0.05–2.82; γ-globulins: 0.27, 0.02–0.50; SAA (mg/L): 23.23, 0.17–77.01. Electrophoresis results were non-remarkable; however, standard reference ranges for this species do not exist for most variables.

Gene Transcription Profiling

Desert tortoise-specific qPCR primers for the 11 genes of interest and housekeeping gene are defined in Table 2. All genes had unimodal distributions, but only one gene, serum amyloid A (SAA), had transcript values following a normal distribution ($P = 0.24$). Additionally, the 95% confidence intervals were independent of the mean normalized values, indicating that the dynamic ranges did not depend upon a gene's transcript level, as all primer efficiencies were approximately equal.

Statistical analysis using a priori groupings (i.e., Piute Valley, Hidden Valley, Ft. Irwin, and captive-impaired) indicated significant differences among groups for several genes (Table 3). Three genes were transcribed at significantly higher levels in the captive-impaired tortoises than in other groups (MX1, CD9, and MyD88). Two genes were transcribed at significantly lower levels in captive-impaired tortoises than in other groups (ATF and leptin). Significant differences in gene transcription were also identified among free-ranging tortoise groups; transcription patterns consistent with a stronger anti-inflammatory and anti-bacterial response existed in tortoises from Fort Irwin in comparison with tortoises from Hidden Valley. Transcription patterns consistent with an increased bacterial response in tortoises from Piute Valley were evidenced in comparison with tortoises from Hidden Valley (Table 3).

MDS and cluster analyses of transcription C_T values indicated strong differentiation of a large cluster and multiple outlying individual tortoises or small clusters in multidimensional space (Fig. 1). There was little to no indication of separation or clusters by sites (Fig. 1). In addition, transcription responses did not differ significantly between sexes ($P > 0.69$) across all sites or among sites by sex ($P > 0.19$). Cluster analysis and SIMPROF identified primarily significant differences ($P < 0.001$–0.024 and one at $P < 0.05$) among 18 distinct clusters, 17 of those consisting of 1–9 individual tortoises, and one cluster consisting of 26 individuals. We used the geometric mean C_T of the largest cluster as the benchmark to qualitatively compare the remaining clusters, with a conservative focus on genes in clusters that were 3 or more C_T difference from genes in the benchmark cluster (Table 4). The 64 free-ranging tortoises had normal BCS, and only 17.2% had visual signs of clinically significant anomalies; thus it stands to reason that the largest grouping would represent animals generally in good health. Examining transcription by individual genes (Table 1), the data were not highly variable. For convenience, we arrange the clusters into

Table 3. Geometric Mean Normalized (to the 18S Housekeeping Gene in Each Animal) Cycle Threshold (C_T) Transcription Values for Targeted Genes (see Table 1) in Desert Tortoises Sampled at Piute Valley, Hidden Valley, Ft. Irwin and Captive Desert Tortoises

	Location			
	Captive-impaired	Piute valley	Hidden valley	Ft. Irwin
n:	19	17	8	39
SAA	16.58 (15.58, 17.63)	16.14 (15.05, 17.31)	15.98 (12.60, 20.27)	16.76 (15.94, 17.63)
HSP70	11.93 (11.00, 12.94)	12.20 (11.16, 13.34)	12.21 (11.20, 13.31)	12.48 (11.99, 12.99)
MX1	15.58[a] (14.47, 16.77)	18.35[b] (17.52, 19.23)	16.06 (12.54, 20.58)	16.82[b] (15.75, 17.96)
CD9	12.64[a] (11.97, 13.35)	11.31[a] (9.39, 13.62)	14.35[b] (12.85, 16.03)	13.02 (12.10, 14.02)
SOD	9.56 (8.86, 10.31)	9.49 (8.41, 10.70)	10.43 (9.40, 11.58)	10.50 (9.75, 11.31)
AHR	13.95 (12.88, 15.10)	14.54 (13.92, 15.19)	14.55 (11.50, 18.41)	14.17 (13.71, 14.66)
MyDA88	15.43[a] (14.75, 16.14)	15.56 (14.94, 16.20)	16.91[b] (15.19, 18.81)	15.61 (15.19, 16.03)
CaM	9.56 (9.18, 9.95)	9.55 (8.78, 10.40)	10.20 (8.85, 11.74)	9.53 (9.00, 10.10)
ATF	11.17[a] (9.88, 12.62)	10.28 (8.81, 12.00)	12.98[a] (9.46, 17.82)	8.74[b] (7.70, 9.91)
CL	16.68 (15.33, 18.16)	15.96 (14.81, 17.21)	19.38[a] (14.45, 26.00)	15.00[b] (14.25, 15.79)
Leptin	14.15[a] (12.90, 15.51)	12.97 (11.71, 14.36)	13.71 (11.31, 16.62)	12.37[b] (11.71, 13.06)

Note that the *smaller* the mean value, the *higher* the level of transcription. Unique letters indicate significant difference. An absence of letters indicates no significant difference

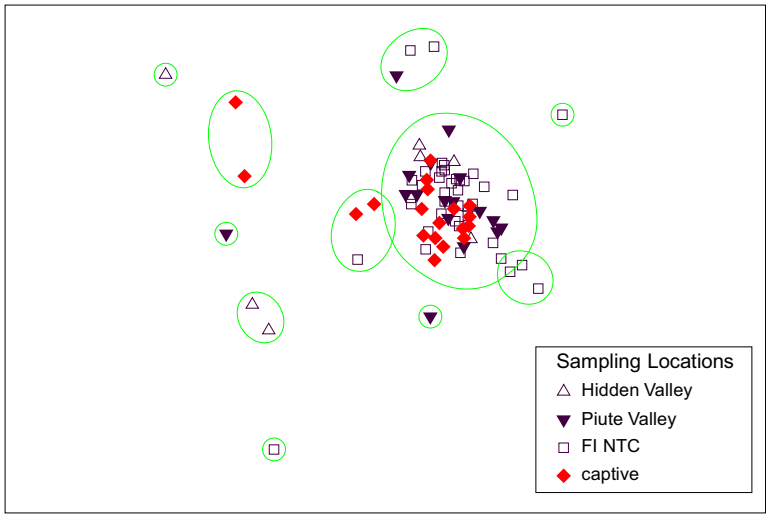

Figure 1. Multivariate, nonparametric, multidimensional scaling of gene transcription profiles (see Table 1) of free-ranging desert tortoises. Interpretive cluster analysis and SIMPROF (similarity profile permutation test, Primer v6) indicated significant ($P < 0.001$–0.007 for 16 clusters and $P < 0.05$ for one cluster) separation among all clusters depicted by *circles*. Shown is two-dimensional (stress = 0.09), but three-dimensional (stress = 0.04) representation shows less overlap of *circles* among clusters.

three groups (A, B, and C) based on similarity of transcript profile.

Group A: Transcripts in clusters 2 and 3 were <3 C_T difference from those in the benchmark, cluster 1. These two clusters differed because overall transcription was slightly higher (0.3–2.4 C_T difference) across all genes compared to the benchmark (Table 4).

Group B and C: Transcription of the other 42 tortoises separated among 15 clusters was variable, but within group patterns were evident. Group B (14 tortoises) comprised 7 clusters or individual outliers (4–10 in Table 4) and had 4–7 genes with 3–11 C_T difference from the benchmark. Transcription patterns between individual tortoises and among clusters within this group were highly variable. For example, an individual tortoise identified in Cluster #4 (Table 4) had elevated transcription for all genes evaluated, whereas the individual tortoise identified in cluster # 5 had elevated transcription for SAA, HSP70, CD9, SOD, AHR, MyD88, and CaM, but had downregulated the transcription for MX1, ATF, CL, and Leptin.

Table 4. Geometric Mean C_T Values for Each Cluster

Cluster	*n*	saa	HSP70	MX1	CD9	SOD	AHR	MDF88	CaM	ATF	CL	Leptin
Group A	41											
1 (dataset standard)	26	18.02	12.82	18.87	13.89	10.80	15.08	15.95	10.29	10.88	15.55	13.69
2	9	17.51	11.96	17.43	11.97	9.21	13.78	14.90	8.61	8.68	14.39	12.70
3	6	15.71	12.68	16.50	12.69	9.88	13.75	15.20	9.60	10.53	15.49	12.33
Group B	14											
4	1	15.75	9.40	13.09	7.02	6.79	11.42	13.44	6.52	3.69	12.08	11.27
5	1	14.18	8.00	20.59	5.36	6.14	13.27	13.46	6.92	12.41	16.86	14.71
6	1	9.06	11.03	8.18	24.00	24.00	10.06	19.06	19.06	15.61	18.88	11.17
7	1	20.70	15.40	20.87	16.59	13.78	14.56	16.00	10.02	2.61	10.60	5.85
8	5	14.31	11.34	15.53	10.93	7.99	13.24	14.12	7.63	7.65	13.27	11.37
9	3	22.24	17.36	21.97	18.87	16.67	17.68	18.67	10.93	11.40	18.17	13.37
10	2	10.38	10.82	10.31	14.85	11.46	10.58	19.08	11.27	18.19	28.69	12.23
Group C	28											
11	6	16.16	12.60	14.85	12.92	9.58	12.02	14.99	9.14	7.75	14.99	13.05
12	2	15.91	11.36	19.96	9.56	7.80	14.41	14.94	8.46	6.66	13.73	13.11
13	3	14.96	12.41	14.51	11.47	9.99	15.26	16.54	9.89	13.21	21.58	14.70
14	4	17.08	9.50	18.21	11.76	7.81	17.95	18.17	10.86	21.31	23.90	22.05
15	2	15.38	11.50	12.94	7.87	8.51	12.63	14.50	8.09	3.98	13.77	11.97
16	3	16.06	12.40	17.09	12.55	9.78	13.76	14.94	9.79	7.70	15.30	8.61
17	7	14.87	11.98	13.38	12.27	10.12	13.96	14.84	8.92	11.11	16.17	12.83
18	1	19.87	15.67	18.20	14.80	13.41	16.33	17.54	12.24	9.18	16.70	10.01

Statistical analyses of transcription C_T values indicated strong differentiation of a large cluster and multiple smaller clusters in multidimensional space (Fig. 1). Cluster analysis and SIMPROF identified primarily significant difference ($P < 0.001$–0.024 and one at $P < 0.05$) among 18 distinct clusters, 17 of those consisting of 1–9 individual tortoises, and the largest cluster consisting of 26 individuals. We used the geometric mean C_T values of the largest cluster (Cluster 1) as a benchmark or dataset standard to qualitatively compare the remaining clusters, with focus on genes that had 3 or more C_T value difference from genes in this cluster.

The 28 tortoises in group C separated across 8 clusters (11–17 in Table 4). Fifteen tortoises had transcription values of 3 genes that expressed a 3–4 C_T difference, and 13 tortoises had just 1–2 significantly different genes but most of these were 5–6 C_T different from the benchmark. The 15 tortoises also had elevated transcription of MX1, AHR, and ATF (6 tortoises), elevated CD9, SOD, and ATF (2 tortoises), and elevated SAA, MX1, but low (6 C_T value difference) CL (3 tortoises), or elevated HSP70 and low transcription of ATF, CL, and leptin (4 tortoises). The other 13 tortoises clustered by elevated MX1 and CD9 (2 tortoises), ATF and leptin (3), SAA and MX1 (7), or only elevated leptin (1).

Transcription of MX1 was elevated (i.e., 3–11 C_T value difference from the benchmark) in more (8 out of 18) clusters than any other gene. Transcription of SAA was elevated in 6 clusters (and low in another cluster) and was most closely associated with elevated C_T of MX1 in those 6 clusters. Transcription of ATF was high in 6 clusters and low in 3 other clusters. Transcriptions of the genes CD9 and SOD were elevated in 4 clusters, with CD9 elevated in an additional cluster. The gene AHR was elevated in 4 clusters, and the remaining genes (HSP70, CaM, CL, leptin, MyD88) were elevated in 3 or fewer clusters with no apparent association of increased (or decreased) transcription with other genes.

Clinical health matrices analyzed by clusters did not correlate with gene transcription profiles. The 19 captive adult tortoises deemed unhealthy generally did not cluster by gene transcription pattern when considering all genes combined. However, two-thirds of these animals were associated with clusters other than the benchmark or the 2 clusters with pattern similar to the benchmark. Proportionately, fewer (11%) were associated with the benchmark than any other cluster where found (17–71%) (Table 4).

Discussion

We describe the first leukocyte gene transcript data for desert tortoises. Acquisition of a blood leukocyte transcript profile has the potential to identify stressed or diseased animals prior to the onset of clinical signs (Bowen et al. 2012; Stott and McBain 2012). This gene transcript profile included a combination of immune response or physiological defense-associated genes with the potential to be modified by biological or physical injury and consequently provide information on the type and magnitude of stressor present in the animal's environment. Variable transcript patterns may be indicative of the presence of environmental or immunological stressors. Our findings suggest increased biotic or abiotic stimuli in these tortoises' environments that triggered increased immune or cellular detoxification responses. The pattern across genes and among geographic groups of animals indicates that some of these environmental effects were location specific. These patterns probably reflect site-specific environmental perturbations. Additionally, gene transcript profiles support clinical diagnoses for captive tortoises.

Significant transcript profile differences existed among a priori groupings (i.e., Piute Valley, Hidden Valley, Ft. Irwin, and captive-impaired). This is likely reflective of different environmental conditions in these locations. The three genes transcribed at significantly higher levels in the captive-impaired tortoises than in other groups (MX1, CD9, and MyD88) may be indicative of response to viral, bacterial, and microbial infection, respectively. These animals were held in captivity and were selected for euthanasia due to their poor clinical conditions. The increased transcription of these three genes could simply be associated with animals in poor condition or it could potentially be associated with microbial spread in captive conditions. The two genes transcribed at significantly lower levels in captive-impaired tortoises than in other groups (ATF and leptin) are indicative of an anti-inflammatory response and poor nutrition, respectively. Significant differences in gene transcription were also identified among free-ranging tortoise groups, indicating differential environmental pressures present in each area.

A key requirement for translation of gene transcript biomarker technology to clinical applications is the establishment of a normal or "reference" range of values, distinguishing between natural variation in gene transcript levels among healthy subjects and changes among those with compromised health (McLoughlin et al. 2006). In desert tortoises, determining a normal baseline for clinically healthy tortoises proved challenging. The transcript results taken as a whole weakly correlated with standard clinical assessments of health and may be more sensitive indicators of physiological perturbations (McLoughlin et al. 2006). Standard clinical assessments of health, limited pathogen diagnostics, and generalized definitions and terminology associated with both may be inadequate to accurately reflect the health status of such wildlife species (Christopher et al. 2003). Due to the reduced metabolic state and activity level of poikilothermic organisms, clinical signs of health or disease conditions may be slow to emerge (Lykakis and Cox 1968; Christopher et al. 2003) even when transcriptional responses have begun.

The earliest detectable signs of physiologic imbalance are altered levels of gene transcripts, and those are often associated with immune function (McLoughlin et al. 2006). Measurements of the functional immune states as elucidated by gene transcription could potentially add valuable information to current health assessment matrices. To analyze our transcript results, we used the largest cluster of tortoises identified by transcript analysis, a subset of those deemed clinically healthy, as our benchmark with which to compare other tortoises varying transcript patterns and clinical health conditions.

Of particular note, in order of prevalence, transcription of MX1, SAA, ATF, and to a lesser extent CD9, SOD, and AHR was elevated in 4 or more clusters of tortoises. The association of MX1 with SAA, and also ATF and SOD has been linked with bacterial or viral infections in other species (Wiens and Vallejo 2010; Zou et al. 2013). These findings correlate well with observations of potential pathogen-related health issues in desert tortoises across their range (Jacobson et al. 1995; Sandmeier et al. 2013). Elevated AHR has been linked to organic contamination, and though not as prevalent as the pathogen indicator genes, this finding was noteworthy in typically remote natural areas. One possible explanation is that Ft. Irwin and the surrounding areas are largely military bases with active artillery and bombing ranges. Our findings of elevated transcription did not signify that these tortoises were experiencing ill health and could be more indicative of a properly functioning immune response rather than an immune-compromised system. However, the potential for elevated environmental stressors in the habitats of these tortoises requires investigation. Costs associated with

mounting an immune or detoxification response create a delicate balance between a protective immune phenotype (transcription pattern in this case) and a potential misallocation of resources. Trade-offs among components of the vertebrate immune defenses themselves are common and occur when one portion of an organism's immune system is upregulated while another portion is downregulated (Pedersen and Babayan 2011). Failure to acknowledge or measure these trade-offs may lead to erroneous conclusions.

There is a dearth of knowledge regarding protective transcription phenotypes of non-model organisms. Identification of the ideal protective immune phenotype can only be measured through long-term sampling or experimental manipulations (Pedersen and Babayan 2011). Thus, continued development of gene transcription research is essential for the development of future diagnostic capabilities and improved understanding of environmental effects on sensitive wildlife species such as the desert tortoise. To date, a full clinical, morphological, and transcript workup on tortoises has not been permitted as this species is protected under the Endangered Species Act; however, information gleaned from known healthy and unhealthy tortoises would help create a baseline from which to understand transcriptional responses.

Fourteen known wild tortoises were identified in this study that appeared most indicative of environmental stressors that taxed their immune or detoxification systems. Health assessments of these and also other animals in the study would further refine identification of conditions that may link directly to elevated transcription of multiple but specific genes. Study focused on the habitats of these animals to determine factors that could stimulate transcription would also be useful. Finally, our study was by no means a comprehensive evaluation of gene transcription as a useful tool for assessing desert tortoises' state of health. Future studies are necessary to identify gene responses specific to known injurious stimuli, and to increase the efficacy of the diagnostic capability by inclusion of additional immune or detoxification genes.

Acknowledgments

This research was supported by A. Modlin, R. Lamkin, F. Chen, V. Van Zerr, A. Berger, P. Emblidge, C. Aiello, R. Saulino, J. Lopez, Z. Cava, S. Lauricella, C. Everly, R. Averill-Murray, J. Johnson, N. Lamberski, R. Foster, and numerous others in assisting with sample collection, logistics, and expertise. This project was conducted under permits TE-030659, TE-102235, SCP-011076, and SCP-33762, and was supported by the US Department of Defense, Ft. Irwin, California, San Diego Zoo, and the Desert Tortoise Conservation Center. Any use of trade, product, or firm names in this publication is for descriptive purposes and does not imply endorsement by the US government.

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