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# ELDORADO VALLEY PRE- AND POST-TRANSLOCATION SURVEYS: 2014, 2015, AND 2016

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*Introduction*

Starting in 2014, the Desert Tortoise Recovery Office (DTRO) focused on completion of projects to use all suitable captive tortoises at the DTCC to begin implementation of the population augmentation strategy for recovery (USFWS 2011). To prepare for these projects, we worked with the Bureau of Land Management (BLM) and Clark County to approve recipient sites in addition to the Large Scale Translocation Site. Among a set of potential recipient sites we selected Eldorado Valley to promote translocations (Averill-Murray et al. 2013). We coordinated with the San Diego Zoo (ZSSD) to release 310 tortoises at Eldorado Valley in 2014 (serology for *Mycoplasma agassizii*: 59.0% positive, 24.8% negative, 3.9% suspect, 12.3% less than 100 g and too small to test), increasing the density by 1 adult tortoise per square kilometer.

To describe population abundance and trends in areas affected by translocation and nearby control sites, Clark County's Desert Conservation Program (DCP) contracted with survey crews from Great Basin Institute (GBI) to complete three surveys, one in 2014 just before the translocation and two in each of the years following the translocation. DTRO cooperated in these operations by identifying the survey plots, providing survey training, coordinating surveys, providing independent review of weekly data, and analyzing the survey results. DTRO also coordinated the translocation planning with the Zoological Society of San Diego (ZSSD), which prepared and released captive tortoises from the DTCC. We hypothesized that with the tortoise exclusion fencing in place since 2002, the ability of the area to support more tortoises would not be limited by habitat quality.

*Methods*

DCP contracted surveyors from GBI to survey the translocation site, marking any residents they encountered and temporarily attaching transmitters so these residents could be located for health assessments. Translocated animals were assessed and marked before release by the ZSSD in spring 2013 and fall 2014.

After 2 days of refresher training on handling tortoises and specialized training on attaching temporary transmitters and hydrating tortoises, surveyors walked 5-km transects running east-west through 4 25-sq km plots. Two plots were in areas where the translocation would occur, while the other 2 were separated from the release locations by a fenced road or a distance of more than 6.5 km. Tortoises at least 180 mm midline carapace length (MCL; "adults") were given an epoxied paper tag, and a transmitter was attached. Health assessments require a minimum of 30 days since the start of the spring active period, so health assessments were conducted at a later date by using the transmitters to relocate tortoises. The transmitters were then removed.

Among the translocatees were 21 juveniles (<100 mm MCL) radio-tagged and released in spring 2014 as part of a multi-site research project by ZSSD that includes these two objectives: 1.

Identify habitat characteristics that associated with decreased dispersal and increased survival in translocated juvenile desert tortoises; and 2. Improve understanding of ecological and habitat factors driving growth, fecundity, and survival by desert tortoises. Final details of this study will be reported under recovery permit #TE-08592A-2.

Additionally, heavy metal exposure was screened through aliquots of blood samples collected during the health assessments. A full report on the heavy metals exposure screening is attached (Cohn et al. 2014).

*Results*

Analysis of the pre-translocation survey data indicated that the translocation plots both had higher densities that the average for the Eldorado Valley TCA, so this is not a depauperate area that would normally have been selected for population augmentation. Densities in the control plots were lower, but still higher than average for this TCA.

| Plot | Area ($km^2$) | Transect length (km) | # Tortoises | Density (tortoises /$km^2$) | Abundance (N) | Target N | # Released |
|---|---|---|---|---|---|---|---|
| Release 1 | 25 | 500 | 26 | 7.9 | 1148 | 1073 | 185 |
| Release 2 | 25 | 538 | 14 | 4.5 | | | |
| Control 3 | 25 | 501 | 16 | 4.4 | | | |
| Control 4 | 25 | 501 | 10 | 3.5 | | | |

After the 2014 surveys but before completion of the above analysis, 185 adult tortoises and 125 juvenile (<180 mm MCL) tortoises were released in fall 2014 to the 185-sq km translocation area. ZSSD released another 2 juveniles in 2015 to replace 2 individuals that died. So far, little change has been detected between the release and control sites, but annual confidence limits are relatively wide.

Results of 2015 surveys in 2 release and 2 control plots.

| Plot | Area ($km^2$) | Transect length (km) | # Tortoises | Density (tortoises /$km^2$) | 95% CI Lower Limit | 95% CI Upper Limit |
|---|---|---|---|---|---|---|
| Release 1 | 25 | 497 | 22 | 7.4 | 2.10 | 25.85 |
| Release 2 | 25 | 505 | 23 | 7.5 | 2.17 | 25.82 |
| Control 3 | 25 | 502 | 8 | 2.9 | 0.78 | 10.83 |
| Control 4 | 25 | 503 | 9 | 3.9 | 1.05 | 14.63 |

Results of 2016 surveys in 2 release and 2 control plots.

| Plot | Area ($km^2$) | Transect length (km) | # Tortoises | Density (tortoises /$km^2$) | 95% CI Lower Limit | 95% CI Upper Limit |
|---|---|---|---|---|---|---|
| Release 1 | 25 | 528 | 12 | 3.5 | 0.95 | 13.13 |
| Release 2 | 25 | 501 | 30 | 8.8 | 2.52 | 30.41 |
| Control 3 | 25 | 501 | 12 | 3.8 | 0.95 | 15.04 |
| Control 4 | 25 | 501 | 18 | 5.3 | 1.43 | 19.63 |

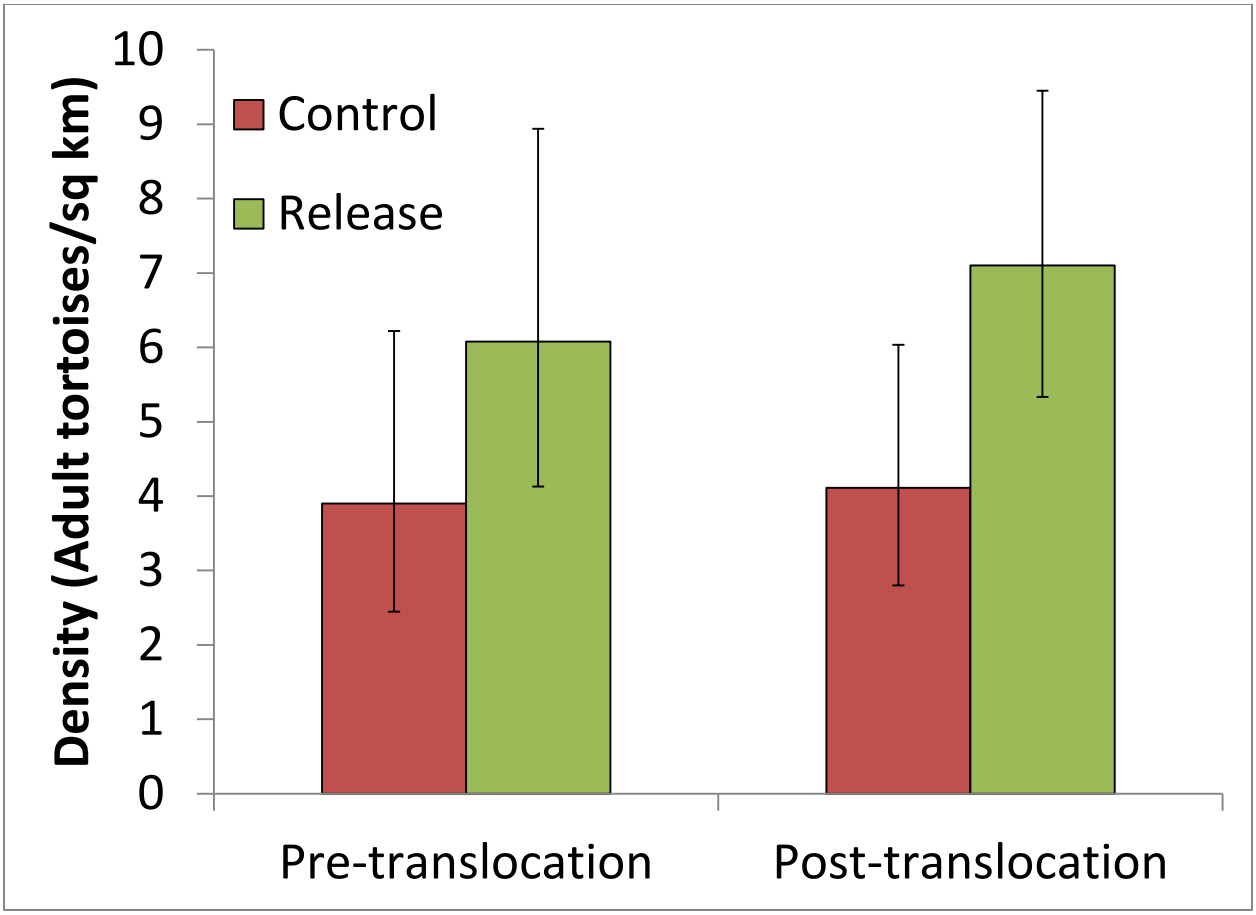

Figure. Average (±SE) adult (≤180 mm MCL) densities within the control and release areas.

Regarding health assessments of resident tortoises, 56 were conducted with 5 animals having a suspect titer of *Mycoplasma testudineum*. Seven had a positive *M. agassizii* titer, and 2 were suspect. Mild clinical signs of disease were observed in this group of tortoises, and 3 had body condition scores that were less than ideal (score = 3). Information about heavy metals exposure can be found in Cohen (2014).

Encounters with marked tortoises (residents and translocatees)

| | | Survey Year | | |
|---|---|---|---|---|
| Group | Original year marked | 2014 | 2015 | 2016 |
| Translocated | 2014 | | 11 | 14 |
| Residents | Previously unmarked | 75 | 53 | 74 |
| | 2014 | | 2 | 2 |
| | 2015 | | 2 | |
| | 2016 | | | 2 |
| | 2005 | | | 2 |
| Juveniles or in burrows and not marked | | 13 | 17 | 7 |

Two carcasses of translocated animals were reported in each of 2015 and 2016 (4 unique individuals). One other carcass reported in 2016 had been marked as a live resident animal on a different survey in 2005.

*Conclusions*

The local density in the release area (6.2 adults/sq km) is higher than the valley average (1.5 adults/sq km) based on the range-wide distance sampling program (USFWS 2015). Some local sites are expected to have higher than average densities and others lower. This site is above average and would not have been selected for a translocation based on the current operating guidance had more specific density information been available prior to the translocation. Local surveys at the scale of analysis are important! Nevertheless, no immediate signs of unusual mortality have been detected. It will be important to interpret results in the context of additional surveys some years from now.

*References*

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# Applying Dried Blood Spots for Toxic Heavy Metal Exposure to Mojave Desert Tortoises (*Gopherus agassizii)* in the Eldorado Valley Translocation Area

# DRAFT

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**OBJECTIVE.**

In this study we measure baseline concentrations of heavy metals in the blood of Mojave Desert tortoises (*Gopherus agassizii*; hereafter, "desert tortoise" or simply "tortoise"). We examined the use of a nonlethal and relatively non-invasive blood sampling technique known as Dried Blood Spot (DBS) analysis for monitoring of heavy metals and toxicants in desert tortoises.

Our objectives were to: (1) Develop and examine the utility of the dried blood spot (DBS) technique for measuring heavy metal exposure in wild free-raging desert tortoises (2) collect blood samples to perform heavy metal screening for these potential environmental toxins, (3) determine if a correlation exists between upper respiratory tract disease (URTD), cutaneous dyskeratosis (CD), and heavy metal exposure (4) test the diagnostic capability of the DBS technique to assist in identifying suitable habitat and populations for translocation (5) disseminate our findings publicly to create a foundation for a referenceable baseline data set.

**BACKGROUND.**

Recent approval by United States Fish and Wildlife Service (USFWS) gave authorization for the use of tortoise blood, scute, and bone samples for the purpose of environmental contamination studies (approval letter, June 3, 2014). A toxicological examination of desert tortoise blood using dried blood spots (DBS) on filter paper was employed to determine background levels (from both natural and anthropogenic sources) of potential environmental toxicants. The methods, results and interpretation of the findings from this study are presented here.

Nussear et al. (2012) described the increasing need for management tools to minimize and mitigate the effects of development on, and habitat encroachment of Mojave Desert scrub habitat, driving the extirpation of associated species such as the desert tortoise. Increasing urban development and alternative energy projects are ever encroaching on sustainable habitat and result in the removal of desert tortoises often to holding facilities, such as the former Desert Tortoise Conservation Center in Clark County, NV (Jacobson et al. 2014). Accumulation of these displaced animals has increased the need to reintroduce the animals to remaining habitats for either population restoration or augmentation purposes. In the case of military expansion (e.g. MCAGCC) or energy projects (e.g. Ivanpah Solar Electric Generating System) desert tortoises are moved a distance outside the designated project boundary on the basis of habitat quality, tortoise population structure and disease prevalence. Translocation of tortoises is increasingly proposed and used in these efforts. Despite the intention of this effort, various risks have not been fully evaluated, and long-term success has not been demonstrated. Data show that tortoises translocated into typical Mojave Desert scrub habitats can have variable success rates based on the potential for increased predation and hyperthermia incurred from longer time spent above ground and larger post translocation, first-year movements (Hinderle et al. 2014; USFWS, 2013; Nussear et al. 2012; Drake et al. 2012). Currently, there is a lack of baseline health parameter data on free-ranging tortoises. This lack of baseline information

is especially critical, as there continues to be emphasis in tortoise conservation through translocation, reintroduction, and other interventionist management.

Because these animals serve as sentinels for their respective ecosystems, information pertaining to emerging infectious diseases and toxins can be used to assess both the overall health of the Mojave Desert population of the desert tortoise and their ecosystem (Homer et al. 2000). This information can then be used to assist in management strategies to sustain these animals and their environment into the future.

## INTRODUCTION.

Desert tortoise morbidity and mortality have been studied for decades and attributed to several factors including upper respiratory tract disease (URTD) and cutaneous dyskeratosis. Tortoise mycoplasmosis is one of the most extensively characterized infectious diseases of chelonians. A microbial and pathological study (Jacobson et al. 1991) resulted in the identification of a new mycoplasma, *Mycoplasma agassizii* (Brown et al. 1995) and the confirmation of its causal relationship with URTD in desert tortoise (Brown et al. 1994). Cutaneous dyskeratosis is a disease state typified by shell lesions (Jacobson et al. 1995, 2014; Homer et al. 1998; Christopher et al. 2003). The causes of shell disease are not known, but nutritional deficiencies and environmental toxicosis affecting keratinized tissues are considered the major causes (Homer et al. 1996a, 1996b; Berry et al. 1997). Elevated levels of various elemental toxicants, arsenic (As) in particular, have been detected in the tissues of desert tortoises exhibiting clinical signs of CD (Jacobson et al. 1994; Homer et al. 1996, 1998; Berry et al. 2001; Seltzer et al. 2005; Foster et al. 2009). The increasing prevalence of URTD and CD and other signs of diseases in desert tortoises merits further investigation of the potential role of contaminants in their etiology.

### Toxicology - Desert Tortoises.

Chelonians possess several traits, including longevity, ectothermy, and various feeding preferences that make them good models for studying heavy metal bioaccumulation and biomagnification. However, the toxic effects of heavy metal exposure in tortoises have received significantly less attention compared to fish, birds, amphibians, and aquatic mammals.

With many tortoise populations declining, euthanizing adults for scientific study is neither sustainable nor conservation-minded, and efforts must be made to implement new non-lethal methods. Development of non-lethal methods will allow researchers to simultaneously collect larger sample sizes while minimizing effects on wild chelonian populations. Additionally, for bioaccumulative contaminants, non-destructive sampling techniques can enable investigators to repeatedly sample the same individual to monitor its exposure throughout its lifetime. Sampling multiple tissues will allow researchers to build mathematical correlations between less invasive samples (e.g., nail and blood) to concentrations that can be accumulated in tissues (e.g., muscle and eggs) that are often relevant to overall health, reproductive success, and transgenerational effects attributable to maternal transfer.

There is limited data regarding heavy metal and trace element levels in reptiles. Campbell (2002) provides a comprehensive review of such studies wherein only a handful links the contaminant effects with exposure concentrations and makes note of the development of several nondestructive sampling techniques for determining organic and inorganic contaminant concentrations in lizards and snakes. Diaz-Figueroa (2005) contains an extensive review of available literature with respect to Gopher Tortoises *(Gopherus polyphemus)* in the southeastern United States. Both of the above mentioned publications provide a good resource for understanding the current state of toxicological research on reptiles. However, the findings in this these reviews reveal very little relational data for comparison to toxins or heavy metals in the blood of desert tortoises.

Clinical signs potentially related to toxicity that can be observed when conducting physical examinations on desert tortoises in the field include; white or yellow discoloration, flaking and/or soft spots on the carapace (USFWS, 2013).
Jacobson (1994) suggested nutrient deficiency or heavy metal toxicosis as a cause of CD. The fact that larger, older tortoises in this study had more severe shell lesions supports a chronic, cumulative problem. Pathologists also report that the location and histologic appearance of cutaneous dyskeratosis lesions seen in tortoises are suggestive of a nutritional deficiency, a toxicosis, or both (Jacobson et al. 1994, Homer et al. 1998). Both deficiency diseases and environmental toxicants are known to affect keratin in other vertebrates. A wide variety of toxicants are responsible for lesions in the epidermal hard parts of domestic hoofstock, for example (Blood et al. 1989). Livers of several tortoises with CD were identified to have swollen and vacuolated hapatocytes, and three from the Chuckwalla Bench, Colorado Desert CA, where CD has been more commonly detected (Jacobson et al. 1994; Berry, 1997), had moderate hepatocellular anisokaryisis, suggested by the authors to be associated with toxic liver disease (Kelly, 1993). However, the sources of the toxins are not identified in these studies.

A report submitted by Berry (1997) summarized a subsequent study of the shell and limb lesions in tortoises at the Chuckwalla Bench, 32 ill or damaged tortoises (including some with similar shell lesions) were salvaged for necropsy from the Mojave and Colorado Deserts of California and the Sonoran Desert of Arizona (Homer et al. 1994, 1996a, 1996b). The salvaged tortoises with CD had elevated concentrations of toxicants in the liver, kidney, or plasma (e.g., barium, calcium, cadmium, chromium, magnesium, molybdenum, nickel, phthalates, and selenium in plasma), and/or nutritional deficiencies (e.g., low copper, zinc, selenium, plasma vitamin A). The authors suggest that the toxicants and/or nutritional deficiencies may be the cause of the shell disease in turn causing immunodeficiency that could render the tortoise vulnerable to bacterial and fungal infections, other diseases, and predation because of the thin shell (scutes) and loss of laminae.

In desert tortoises in the western Mojave Desert, liver mercury concentrations of tortoises with URTD (0.326 ppm) were significantly higher than those of healthy tortoises (0.0287 ppm) from the eastern Mojave Desert (Jacobson et al. 1991). Post-mortem examinations of several desert tortoises have revealed suspected elevated concentrations of heavy metals, including cadmium (Cd), mercury (Hg), lead (Pb), molybdenum (Mo), arsenic (As), selenium (Se), chromium (Cr), and nickel (Ni) in the livers and kidneys of ill tortoises (Homer et al. 1996a). Free-ranging tortoises that showed evidence of liver and kidney

degeneration have contained elevated Hg, Cd, or Pb concentrations (Homer et al. 1996b).

Seltzer and Berry (2005) developed a technique to measure trace element concentrations and their chronological uptake in the outer keratin layer or scute of desert tortoise shells. Patterns of trace elements and toxicants such as arsenic in transect profiles of scutes trace revealed patterns that, in all probability, reflect the actual chronology of elemental uptake. The results show an averaged range of 0.2 – 5.3 mg/kg of arsenic across the transected scutes for arsenic. Chaffe and Berry (2006) salvaged two ill tortoises from the Rand district. One of the two contained 15 mg/kg wet-weight of arsenic in scute, which they report as the highest concentration, recorded to date in necropsied tortoises.

Specimens analyzed from a mining-impacted area (Kelly-Rand Mining district, Kern County CA), and from sites on or adjacent to military bases (National Training Center, Ft Irwin, and Edwards AFB, CA) suggests that tortoise specimens collected from sites in the Kelly-Rand mining district and from sites near active-use military sites contain more bioaccumulated arsenic in scutes and more exogenous particles in scute and lung tissue than specimens collected from areas of minimal land disturbance (Foster et al. 2009).

Finally, Chaffe and Berry (2006), determined if naturally occurring elements likely to act as toxicants or contaminants to tortoises are readily available in the surface environment (soils and plants). Several elements, notably arsenic, were found to be unusual in several of the habitats studied. The authors suggest the need to build a database of chemical concentrations in selected organs of tortoises; and experimental research to test hypotheses about how specific toxicants or groups of toxicants affect general tortoise health, immune systems, susceptibility to diseases, and mortality. This area of research is currently being pursued by our research group as part of the ISEGS survivability study in the Ivanpah Valley, CA.

**Dried Blood Spot in Toxicology.**

Dried Blood Spots on filter paper have been used in human medicine since the 1960s, predominantly for screening in-borne metabolic disorders (Guthrie and Susi, 1963) and more recently, for toxicology (Stove et al. 2012). Dried Blood Spot analytical procedures are emerging as having practical diagnostic advantages for veterinary applications and for a wide range of biological and wildlife purposes.

Thus far, the ability to investigate the role of contaminants in desert tortoise health has been impeded by a lack of clinical data, by limited access to appropriate samples, and by the absence of a proven method for assessing the contaminant status of live animals that may or may not exhibit disease symptoms. It has been suggested that nondestructive sampling techniques, particularly analyses of blood, may be more easily applied in evaluations of contaminant exposure in the field to prevent excessive destructive sampling particularly in the case of threatened reptile species. Blood samples in the form of DBS can also be taken at more frequent intervals without having long-term effects on study animals and may be most applicable under conditions where repeated samples are desired (Hopkins 2001).

The Toxicology Section of the Diagnostic Center for Population and Animal Health

(DCPAH), Lansing, MI has been active in developing and improving technology to measure toxins including heavy metals in DBS on filter paper. Highlights of this development include, validation of measurement of six heavy metals (As, Cd, Pb, Hg, Se, thallium (Tl)) down to 10 ppb has been validated. In this study we expanded the screening panel to iron (Fe), a nutritional element and Thorium (Th), a radionuclide validated down to 10 ppb. Additionally, the lab is capable of examining pesticides and PCBs using the DBS method.

The measurement of heavy metals such as Pb have been well characterized in DBS sample preparations from wildlife and results correlate well with those observed in whole blood (Lehner et al. 2013). Accordingly, this diagnostic application has been extended to biomonitoring studies for heavy metals and other environmental contaminants, including organochlorine and organobromine persistent organic pollutants, which may affect avian and large game health (e.g., Dubay et al. 2006; Fisher et al. 2006; Trudeau et al. 2007; Yu et al. 2007). Shlosberg et al. (2011, 2012) successfully employed this method in a recent investigation on griffon vulture (*Gyps fulvus*) at two different sites in the Judea and Negev Deserts of Israel. Their study demonstrated the value of toxicological screening in the investigation of wildlife and the establishment of a baseline for contaminants in wildlife species, which is a primary goal of the DABSE (Database for Avian Blood Spot Examination) project (Shlosberg et al. 2011). An experiment using filter paper was conducted for ELISA detection of *Brucella* antibodies in caribou (Curry et al., 2001). The study presented how the detection of disease exposure in remote regions and under adverse conditions can expand wildlife disease surveillance across temporal and spatial scales. And more recently, Hansen et al. (2014) validated the use of whole blood on DBS filter paper for the detection of Hg in marine mammals, in this case bottlenose dolphin (*Tursiops truncatus*) or harbor seal (*Phoca vitulina*) blood was examined. The use of this technology was shown to have a valuable role in monitoring blood concentrations in wildlife populations and the advantage of being easy to use, store, and transport as compared with whole blood. A more complete review and compilation of these applications, including drugs of abuse, environmental contaminants, biotoxins, trace nutrients and heavy metals, have been presented elsewhere (Stove et al. 2012).

**SITE DESCRIPTION.**

According to the Eldorado Valley Translocation Plan (2013) the site encompasses approximately 46,000 acres (185 $km^2$) public lands managed by the BLM in Clark County. The study site consisted of four, 5 $km^2$ plots where health evaluations were conducted and DBS samples were collected.

**METHODS.**

Lehner et al., (2013) validated a novel DBS analytical procedure for the routine measurement of toxic heavy metals using whole blood on a single DBS by inductively coupled plasma mass spectrometry (ICP-MS). To take advantage of these validated methods, dried blood spots were used for collection of whole blood samples on filter paper. This offered the advantages that when the method of collection was applied to Desert tortoises it enabled simultaneous subsequent determination by both Enzyme-linked immunosorbent assay (ELISA) testing and heavy metal analysis during health evaluations. Health evaluations for Desert tortoises draw up to 0.25 -3 ml of blood from each individual

dependent on the weight of the animal at the time blood is drawn (USFWS 2013). No additional sampling was required using the DBS method, as only a small droplet of blood was necessary from the blood draw used for the ELISA sample and heavy metal analysis requires only 50 uL per DBS. DBS sample storage (Whatman 903® Filter Card) requirements are minimal, samples only need to be frozen for long-term storage and the cards are compact and do not use up valuable or unavailable freezer space.

**Field Collection.**

The DBS kit is shipped directly from Michigan State University Diagnostic Center for Population and Animal Health (DCPAH). The kit contains the filter paper card enclosed in a plastic zip-closed bag, each with a silica gel desiccant sachet and a Humonitor® strip which allows the user to discard the card if it shows exposure to the atmosphere and therefore too much moisture (Figure 1).

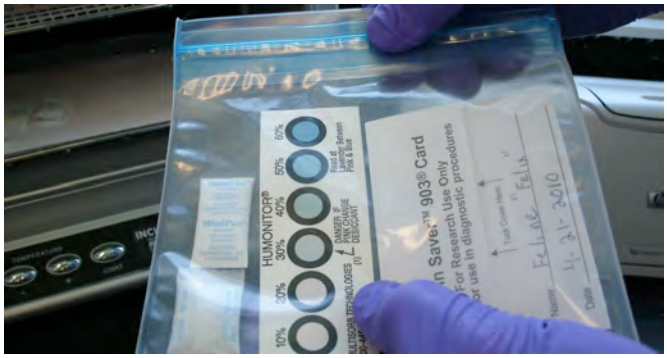


Figure1. DBS Kit containing Whatman 903® Filter Card, silica gel desiccant sachet and a Humonitor® strip

The DBS card can be prepared prior to field work to reduce the potential for contamination and minimize time spent handling and examining the tortoise. The field techniques developed for this study are novel yet straightforward and most likely applicable to many wildlife species where a DBS sample technique is employed. With the operator wearing gloves the DBS filter paper card with 5 spots per card is opened and mounted in a clean sealable plastic container (e.g. Tupperware®) applying a small piece of tape to the back-side of the card that will not come in contact with the blood samples. A duplicate card and container per 5 animals are prepared exactly the same and both are packed and brought to the field for the sampling effort. Duplicates were collected and analyzed on 10% of the samples as field methodology and analytical accuracy quality control measures.

Desert tortoises were accessible near entrances of their burrows or above ground during the Eldorado Valley health evaluations (late May – early June 2014). Health evaluations for the Eldorado Valley Translocation Study strictly followed the USFWS health assessment procedures for the desert tortoise (USFWS, 2013), along with published techniques describing the evaluation of health in chelonians (Jacobson et al. 1999; Berry and Christopher 2001). Blood was drawn from the subcarapacial vein as described in Hernandez-Divers et al. (2002) and USFWS (2013).

The subcarapacial area is first palpated by inserting a finger along the midline posterior to the skin-carapace junction to locate a depression anterior to the 8th cervical vertebra (USFWS 2013). This allows an estimation of the proper location of the injection site. A handler retrains and positions the tortoise at an angle that allows best access to the subcarapacial venipuncture site for the examiner. Upon obtaining the blood sample (0.25-3mL) the examiner aliquots 1-2 drops of blood onto the Whatman 903® Filter Card directly from the syringe used for the blood draw (Figure 2). DBS cards were sealed and allowed to dry in place so as to avoid any field contamination, while the remaining blood sample was placed into a heparinized microtainer for ELISA testing following the USFWS protocol.


Figure 2. (1) Blood aliquot to filter paper (2) Blood sample and labeling (3) Final sample covered and contained for drying in the field

**ANALYTICAL TECHNIQUES AND INSTRUMENTATION.**

Elements were determined in one 50 $\mu$L dried blood spot at the University of Michigan DCPAH. The spot was removed from the card using acetone-rinsed stainless steel scissors. Blood and blank paper spots were placed in separate 5 ml Teflon digestion vessels (Savillex, Minneapolis, MN), to which were added 250 $\mu$L concentrated nitric acid (Suprapur, Merck), followed by heating in an oven at 95$^{o}$C overnight. After cooling the vessels, increments of water were added until a 500 mg (±5 mg) mass was obtained, and the solution was centrifuged for 10 min at 3000 rpm. For quality control, Lyphochek-2 and -3 standards (Bio-Rad, Hercules, CA) were also run simultaneously. As, Cd, Pb, Hg, and Se were determined by using an inductively coupled plasma–mass spectrometry (ICP-MS) (7500ce ICP-mass spectrometer; Agilent, Santa Clara, CA). The limits of quantification (LOQs) were 10 ppb for As, Cd, and Se and 20 ppb for Hg and Pb.

Standard curves based on five points plus blank gave good refit values down to 10 ppb (Fe, As, Cd, Pb, Hg, Se, Tl, Th) or 20 ppb Hg. The limits of detection (LOD) and limits of quantitation (LOQ) were calculated analyzing repeated blanks for any background signal present in the untreated paper, determining the SD of the values (n = 10), and multiplying by 3.3 times or 10 times, respectively, according to established methods (Lehner et. al 2013). Blank spots from the paper gave values for elements lower than the LOQs.

Although acute poisoning concentrations remain unknown for desert tortoises this test panel method was shown to be suitable for the diagnosis of heavy metals in domesticated and other wildlife species. Normal blood concentrations of As, Cd, Hg, Pb and Tl are generally <50$\mu$g/L (Lehner et. al 2013).

**RESULTS.**

Concentrations of Hg, Tl, and Th were lower than the LOQ in all desert tortoises (Table. 1). Fe and Se were higher then the LOQ in all desert tortoises, but no statistical differences were found between the study plots or between males and females (Table. 1 & 2). One of the key questions for metal exposure is whether organisms with high levels of one metal also have high levels of other metals and which may co-occur together. There were no consistent patterns in relationships among metals for blood. There was no correlation between MCL (carapace length) and Fe or Se concentration. In fact, none of the heavy metal results correlated to MCL in this study. However, higher average and maximum concentrations of both Fe and Se were found in male desert tortoises (Table 2). Arithmetic means were 220 ppb (132-332) in males and 197 ppb (120-284) in females for Fe and 173 ppb (69-351) in males and 147 ppb (69-272) in females for Se. Male concentrations of Fe and Se were not significantly greater then females.

Arsenic was detected in one female and one male tortoise, FW8497 (23 ppb) and FW8430 (11 ppb) respectively. The female (FW8497) with the highest reported As concentration in this study also tested positive for *M. agassizii* and suspect for *M.testudineum*. The carapace and plastron were noted as having inactive peeling and flaking during the health evaluation, however, no additional abnormalities were identified (Appendix. 1). The male (FW8497) had serous discharge of the right eye at the time of the health evaluation but no other signs of CD or abnormalities. ELISA test results were negative for both *M. agassizii* and *M.testudineum* (Appendix. 1).

Cadmium levels were lower than the LOQ of 10 ppb in 30 of the 51 desert tortoises tested. The concentration range for the 21 tortoises (50% female and 30% male) that tested above the LOQ was 11 – 55 ppb, with the highest concentration of 55 ppb was found in FW8679, a female tortoise on plot 2. This individual was not reported to have any signs of CD during the health evaluation and tested negative for both *M. agassizii* and *M.testudineum.* Of the 21 tortoises where Cd concentrations were above the LOQ (41%), positive results were reported for *M. agassizii on* 63%, and 50% of the tortoises were identified as having signs of shell disease or CD, but no statistical differences were found across the study plots or between male or female tortoises (Table. 1 & 2). Interestingly, no Cd has been detected in the blood of study tortoises from the Ivanpah Valley (*n=118*) (Table. 3).

Lead was detected in the blood of 84% of all desert tortoises studied, and arithmetic means were 17 ppb (<10 – 37) in males and 19 ppb (<10 – 51) in females (Table. 2). No statistical difference was found between study plots or between males and females. However, the highest concentrations of Pb were found in three females, on three different study plots. A Pb concentration of 51 ppb was found in FW8454, a female on plot 2. This individual was not reported to have any signs of CD during the health evaluation and tested negative for both *M. agassizii* and *M.testudineum.* A female on plot 3 (FW8680) had a higher then average concentration of 45 ppb Pb in her blood. This tortoise tested positive for *M. agassizii* and negative for *M.testudineum*. A female on plot 4 (FW8539) had a concentration of 47 ppb, but ELISA test results were negative for both *M. agassizii* and *M.testudineum*. Neither tortoise was observed to have clinical signs or shell disease during the health evaluations.

**Table 1**. Concentrations of elements (ppb) in dried blood spots of desert tortoises from Eldorado Valley CA

| **Plot No.** | Fe | As | Se | Cd | Hg | Ti | Pb | Th |
|---|---|---|---|---|---|---|---|---|
| **1** ***(n=19)*** | | | | | | | | |
| **Detection %** | 100 | 0 | 100 | 32 | 0 | 0 | 37 | 0 |
| **Mean (median)** | 210 (205) | <10 | 151 (138) | 18 (18) | <20 | <10 | 17 (18) | <10 |
| **Range** | 123 - 332 | -- | 69 -351 | 12 - 25 | -- | -- | <10 - 24 | -- |
| **2** ***(n=10)*** | | | | | | | | |
| **Detection %** | 100 | 10 | 100 | 50 | 0 | 0 | 90 | 0 |
| **Mean (median)** | 193 (196) | 6 (5) | 157 (145) | 28 (21) | <20 | <10 | 25 (23) | <10 |
| **Range** | 120 - 307 | <10 - 11 | 69 - 335 | <10 - 55 | -- | -- | <10 - 51 | -- |
| **3** ***(n=17)*** | | | | | | | | |
| **Detection %** | 100 | 6 | 100 | 35 | 0 | 0 | 82 | 0 |
| **Mean (median)** | 210 (210) | 6 (5) | 171 (170) | 10 (5) | <20 | <10 | 18 (16) | <10 |
| **Range** | 145 - 278 | <10 - 23 | 107 - 272 | <10 - 27 | -- | -- | <10 - 45 | -- |
| **4** ***(n=5)*** | | | | | | | | |
| **Detection %** | 100 | 0 | 100 | 80 | 0 | 0 | 100 | 0 |
| **Mean (median)** | 217 (199) | <10 | 171 (144) | 10 (12) | <20 | <10 | 25 (21) | <10 |
| **Range** | 178 - 284 | -- | 122- 198 | <10 - 21 | -- | -- | 15 - 47 | -- |
| **ANOVA** | | | | | | | | |
| **Significance** | 0.09 | ID | 0.05 | 0.52 | ID | ID | 0.73 | ID |
| **Effect Size (partial $\eta^2$)** | 0.12 | ID | 0.15 | 0.05 | ID | ID | 0.03 | ID |
| **Bonferroni** | NA | ID | NA | NA | | ID | NA | ID |

**Table 2.** Concentration of elements (ppb) in dried blood spots by desert tortoise gender

| Gender | Fe | As | Se | Cd | Hg | Ti | Pb | Th |
|---|---|---|---|---|---|---|---|---|
| Male *(n=23*) | | | | | | | | |
| Detection % | 100 | 4 | 100 | 30 | 0 | 0 | 87 | 0 |
| Mean (median) | 220 (221) | ID | 173 (160) | 10 (21) | <20 | <10 | 17 (18) | <10 |
| Range | 132 - 332 | <10 - 11 | 69 - 351 | <10 - 27 | -- | -- | <10 - 37 | -- |
| Female *n=28)* | | | | | | | | |
| Detection % | 100 | 4 | 100 | 50 | 0 | 0 | 82 | 0 |
| Mean (median) | 197 | ID | 147 (143) | 12 (17) | <20 | <10 | 19 (21) | <10 |
| Range | 120-284 | <10 - 23 | 69 - 272 | <10 - 55 | -- | -- | <10 - 51 | -- |
| *t* -test | | | | | | | | |
| Significance | 0.07 | ID | 0.09 | 0.52 | ID | ID | 0.92 | ID |

**Table 3.** Concentration of elements (ppb) in dried blood spots of desert tortoise from Eldorado Valley, CA study plots and Ivanpah Valley, CA study site

| Study Site | Fe | As | Se | Cd | Hg | Ti | Pb | Th |
|---|---|---|---|---|---|---|---|---|
| Eldorado Valley *(n=51)* | | | | | | | | |
| Detection % | 100 | 4 | 100 | 41 | 0 | 0 | 84 | 0 |
| Median | 205 | 17 | 153 | 20 | <20 | <10 | 19 | <10 |
| Range | 120 - 332 | <10 - 23 | 69 - 351 | <10 - 55 | -- | -- | <10 - 51 | -- |
| Ivanpah Valley *(n=118)* | | | | | | | | |
| Detection % | 100 | 7 | 100 | 0 | 0 | 0 | 97 | 0 |
| Median | 223 | 14 | 115 | <10 | <20 | <10 | 29 | <10 |
| Range | 100 - 388 | <10 - 16 | 40 - 935 | -- | -- | -- | <10 - 172 | -- |

**DISCUSSUION.**

DBS sampling is a relatively new approach for measuring heavy metals. The authors and colleagues in the Ivanpah Valley as part of the ISEGS Effective Monitoring Program have conducted the only other samples known to have used this technique in desert tortoises. To the best of our knowledge, there does not appear to be any data specifically collected on heavy metals in the blood of desert tortoises for comparison to the results collected in this study. The data collected in the Eldorado Valley as part of health evaluations for possible translocation generally fall within the range of those collected in the Ivanpah Valley with several caveats (Table 3).

Cadmium is one of the most toxic heavy metals for living beings. Its environmental ubiquity and persistence allows accumulation in organisms of all types through continuous exposure at low doses (Martinez-Lopez et al. 2004). As with all of the heavy metals in this study we could not find any references in the literature about blood concentrations in *Gopherus agassizii.* There are a few studies that refer to Cd in the blood of amphibians and reptiles (See Birge et al. 2000; Burger et al. 2007; Burger 2008; Guirlet et al. 2008). Cadmium is particularly dangerous because plants growing in contaminated soils can absorb and accumulate Cd in large quantities, thereby introducing the metal into the potential food source (Monteiro et al. 2008, 2009).

Cadmium was a common element detected on the Eldorado Valley study plots (41% detection), but not detected in the Ivanpah Valley DBS samples. Cadmium concentrations from DBS ranged from <10 – 55 ppb (Table 2). This result was relatively similar to a study of blood in spur thighed tortoises(*Testudo graeca*) from southeastern Spain and northern Africa which had a range of 8 – 46 ppb (Martinez-Lopez et al. 2010). We could not find any references in the literature that relate tortoise Cd blood concentrations and its effect

on these species. Although low Cd levels in yolk has been shown to impact gonadal development in freshwater turtles, a positive correlation between egg and blood Cd in leatherback turtles (*Dermochelys coriacea*) were related to Cd concentrations ~80 ppb (Kitana and Callard 2008; Guirlet et al. 2008). These levels are higher than those found in the present study (Table 3). While no direct health effects were correlated to Cd concentrations this brings into question the threshold for tortoises and illustrates the need to further establish a range of reference intervals for heavy metals in this species.

Arsenic has been implicated in many desert tortoise studies for its potential to inhibit immunological response to mycoplasma and shell disease (Berry et. al 2001; Homer et al. 1996a, 199b; and others). FW8497, a female on plot 3 had the highest reported As concentration (23 ppb) in this study and tested positive for *M. agassizii* and suspect for *M.testudineum*. The highest concentration reported in the Ivanpah Valley to date is 16 ppb, and this tortoise does not exhibit clinical signs of disease and has remained negative for multiple ELISA tests. More information is needed to determine the health effects of As in tortoises particularly in relation to concentrations in blood samples as tissues and organs are known to be the major repository in most species (Matinez-Lopez et al. 2010, Day et al. 2005, Diaz-Figueroa 2005). While blood levels may be affected by recent changes in heavy metal (e.g. Hg) uptake, stability in the scute matrix has been shown to be preferable for approximating long-term exposure and bioaccumulation (Day et al. 2005).

Many anthropogenic sources of lead, most notably gasoline, lead-based paint, solder in food cans, pesticides, and shot and sinkers, have been eliminated or strictly regulated due to lead's persistence and toxicity. Because Pb does not degrade, these former uses leave their legacy as higher concentrations of Pb in the environment (Fisher et al. 2006). Martinez-Lopez et al. 2004 showed that lead in blood of tortoises is a good indicator of recent exposure, while chronic exposure can be estimated when concentrations in the accumulation tissue (bone) are available. Martinez- Lopez et al. (2010) results suggest that Pb blood concentrations higher than 150 pbb could be responsible for subletal effects in tortoises. However, the Pb concentration range (<10 – 51 ppb) in this study were below those found in turtles (*Trachemys scripta*) (90 ppb) from Oklahoma (USA) and spur thighed tortoises (*Testudo graeca*) (62 – 124) from southeastern Spain and northern Africa (Hays and McBee 2007; Martinez-Lopez et al. 2010). Our ongoing study of desert tortoises in the Ivanpah Valley have a range (<10 – 172 ppb) much higher then that of the Eldorado Valley plots. Currently, no clinical signs or ELISA test results suggest that these higher concentrations of Pb in the blood of desert tortoise in Ivanpah Valley are causing immunological degradation or signs of shell disease.

Sampling and sample handling in the desert environment can be a major challenge. Subcarapacial venipuncture requires skill and meticulousness to avoid contamination or dilution of blood with lymph, which can impact the DBS results, similar to ELISA testing. Our earlier trial runs with samples collected in the Ivanpah Valley have enabled us to more readily identify samples that may be of insufficient quality due to lymphatic dilution, alcohol at the puncture site or heparin in the needle hub. The blood droplet appears runny or light in color when placed on the filter paper even though no lymph is observed during the blood draw. In some instances the filter paper acts as a quality control test and can allow the examiner to reevaluate the quality of the blood draw. Additionally, the lab analyst

evaluates the bold spots prior to analysis and excludes spots they determine to be diluted or of insufficient size or quality. Finally, the robustness of Fe and Se concentrations on the filter paper also help to normalize and or flag samples.

Non-destructive sampling via blood, toenail clips, or biopsy is becoming an important aspect of bioindicator development (Hopkins et al. 2001; Burger et al. 2005). There is an increasing need for biomonitoring, particularly with industrialization and encroachment of wild lands, it is necessary to have non-destructive techniques that can be easily used with animals that are sentinel species, long lived and representative of a specific ecotype. Collection of DBS can help serve this purpose, allowing for examination of both spatial and temporal trends in contaminant distribution, uptake, turnover and threshold. While the levels of metals are most often higher in the tissues of chelonians and most if not all wildlife species, developing a sustainable non-invasive methodology through blood sampling provides an alternative for monitoring a threatened species. Opportunistically sampling tissues (e.g., organs, muscle, and bone) and blood of mortalities on projects (e.g. development or research based) overtime could lead to the development of a baseline relationship between the level of toxins in tissues and that of the less invasive and non-lethal sampling techniques.

One objective was to determine which metals could be detected in the blood of desert tortoise with an unknown history of natural or anthropogenic levels of toxic heavy metals. Herein we were able to establish a baseline for the relative presence of heavy metals in contrast to the health evaluations used to ascertain the site-specific potential for translocation efforts. It is very difficult to choose a “reference” site since metal contamination can be introduced into any environment from non-point atmospheric deposition as well as through point sources. Therefore it is extremely important to measure these potential toxicants at the local level and be able to compare them to different locations. Lastly, examining exposure at different geographical locations will allow for a comparison of uptake based upon the geochemistry of the sites.

## Conclusion.

Clearly the results presented in this study are preliminary and give precedence to further trial and examination. However, the utility of the DBS method for evaluating environmental quality and tortoise health is shown. The DBS method provides a non-invasive approach that can be incorporated to health evaluations with relative ease and low cost association. The need for this information is integral based on the lack of data available in relation to toxicological evaluations of tortoise health.

No previous study has presented values for a wide range of heavy metals in the blood of the desert tortoise or attempted to assess the possible effects on gender or location. Physiologically desert tortoises have a tremendous adaptive capacity and accordingly are able to tolerate extreme variations in habitat and seasonal climate. In order to develop reference intervals for heavy metal and toxicological data from wild free-ranging tortoises the data must be obtained across variations in habitat, season, and climate patterns such as drought or increases in temperatures from climate change.

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