Health Assessment Procedures for the Mojave Desert Tortoise (*Gopherus agassizii*): A Handbook Pertinent to Translocation

U.S. Fish and Wildlife Service
Desert Tortoise Recovery Office

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This document was developed to accompany a hands-on training course in conducting health assessments on Mojave desert tortoises for translocation screening and monitoring purposes. Wendland et al. (2009), Handbook on Gopher Tortoise (*Gopherus polyphemus*): Health Evaluation Procedures for Use by Land Managers and Researchers, served as a template and with permission of the authors, several sections were adapted or modified only slightly.

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Table of Contents

1. Introduction 4

Background 4

Objective 4

Scope 5

Approach 5

2. Preparing to Work with Desert Tortoises 6

Permits 6

Human-risk minimization protocols 6

3. Health and Disease Overview 7

Importance 7

How health is measured 7

4. Health and Diseases of Desert Tortoises 9

5. Conducting Health Assessments 12

Disinfection/sanitation protocols 12

Tortoise capture/handling 12

Physical examinations 12

Diagnostic tests 13

Biological sample collection 13

Biological sample storage 14

Hydration after voiding 14

6. Interpreting Results and Making Decisions 16

Interpreting results 16

Management decisions 17

References 18

Glossary 23

Appendix A: Disinfection and Sanitation 31

Appendix B: Completing the Health Assessments and Data Collection Form 33

Appendix C: Clinical Signs of Disease in Desert Tortoise 44

Appendix D: Body Condition Scores for Desert Tortoises 45

Appendix E: Biological Sampling Supplies 46

Appendix F: Protocols for Biological Sampling, and Sample Processing, Storage, and Shipment 48

- **Appendix F.1: Preparing for Biological Sampling (Before Handling the Tortoise)** 49
- **Appendix F.2: Ticks** 53
- **Appendix F.3: Oral Cavity Exam and Oral Swab** 54
- **Appendix F.4: Nasal Lavage** 56
- **Appendix F.5: Blood Sampling** 58
- **Appendix F.6: Rehydration** 62
- **Appendix F.7: Processing Biological Samples (After Handling the Tortoise)** 65
- **Appendix F.8: Shipping Samples** 68

Appendix G. Algorithm for Evaluating if Desert Tortoises are Suitable for Translocation 73

Appendix H: Disposition Plan Template 74

1. Introduction

Background

The Mojave desert tortoise (*Gopherus agassizii*) (i.e., all tortoises living north and west of the Colorado River in Arizona, Utah, Nevada, and California) is federally protected as Threatened under the Endangered Species Act (USFWS 1990). The Sonoran desert tortoise (*Gopherus morafkai*), south and east of the Colorado River, is listed as a Candidate under the Endangered Species Act, having been determined to be warranted for listing, but precluded by higher priority actions (USFWS 2010). State laws also protect the two desert tortoises. The protocols herein were developed for the Mojave desert tortoise, but could be appropriate for the Sonoran desert tortoise should that become needed.

Some projects that occur in desert tortoise habitat are not compatible with the continued existence of tortoises in and around the project sites. Recently, numerous energy-development projects (primarily solar and wind) have gained approval to construct such facilities in areas inhabited by desert tortoises. In an attempt to minimize impacts on desert tortoises, many projects propose to displace tortoises from the project sites and relocate them to other areas. Many steps must be taken to minimize the risks to the tortoises being moved, as well as to tortoises that they might encounter after translocation from the project site. Among the many precautionary steps is assessing the health of tortoises at both the project and recipient sites, as well as at any reference sites as may be required for specific projects.

While upper respiratory tract disease (URTD) is commonly assumed to be a threat to populations and was implicated in declines of the desert tortoise when it was federally listed, other diseases and conditions do exist. These conditions could have significant impacts on populations should pathogen spread be influenced by human-mediated movements of tortoises. Health assessments and subsequent decisions on whether or not translocation should be allowed must be done in concert with knowledge of the distance between proposed project and recipient sites, the prevalence of disease at the sites, the behaviors of tortoises after translocation, population density, food availability, and other factors that pertain to assessing the risk of the translocation. Lack of knowledge with respect to the precise impacts of potential infectious diseases present within desert tortoise populations, the impact of disease status relative to translocation of the species, and disease impacts to long-term population viability create a major dilemma for wildlife biologists, veterinarians, conservationists, and public policy makers. However, we do have enough information now to develop an approach to minimize known risks.

Objective

This handbook is designed to serve as a reference for biologists conducting translocation-related health assessments, as well as for individuals making decisions based on the results of the assessments. While health is only one of the many factors that need to be considered when planning the translocation of desert tortoises, it is a complex and important one. This handbook includes detailed protocols and photographic presentation of various conditions that may be encountered, and it is expected that formal, specialized hands-on training will be acquired by those individuals wishing to conduct health assessments.

Scope

While the information in this handbook was developed in response to projects affecting primarily the Mojave desert tortoise, generally it is applicable to all desert tortoises across their range. The health assessments are designed to minimize the risk of spreading disease within and among populations and to promote survival of individuals when relocating tortoises from project sites. Health assessments conducted with additional or different goals (e.g., health and disease-related research) may differ from the assessments described here. We do encourage, however, a standardized approach to health assessment data collection in order to monitor populations across projects and over temporal and spatial scales.

Approach

We present an overview of the importance of monitoring health and disease, followed by more specific information about the health assessments and decision points that are applicable specifically to relocating desert tortoises from project sites. Additional information is provided in the appendices. Some of the technical terms specific to tortoise biology and anatomy, as well as veterinary medical terminology, are defined in the Glossary.

2. Preparing to Work with Desert Tortoises

Permits

Field workers should be aware that several permits may be needed before they begin to handle and assess the health of desert tortoises. When working with the federally listed Mojave desert tortoise, a permit or incidental take statement from the U.S. Fish and Wildlife Service is required. In addition, each state wildlife agency requires permits authorizing handling activities. Permits may also be required by other federal or state agencies, so workers should thoroughly inquire about necessary permits prior to beginning work. Because conducting health assessments requires specialized skills, workers should also expect to provide documentation of training received and experience in conducting health assessments on desert tortoises to both state and federal agencies. Any research being conducted on vertebrate animals generally requires an approval from the Institutional Animal Care and Use Committee (IACUC) for academic institutions and industry or from other similar review panels in order to meet the Animal Welfare Act regulations.

Human-risk minimization protocols

Field workers must ensure that protocols are in place for disposal of medical waste, disposal of sharps, and response to accidental exposure to biological samples through needle sticks or other means. The protocols for biological sampling techniques in the appendices of this handbook include some details to address these issues.

3. Health and Disease Overview

Importance

Health is vital to the success and well-being of any population. Certain management activities may negatively impact the health of a population by imposing additional stressors to which the animals may have difficulty adapting. Implications of health and disease in wildlife management, especially in translocations, are a topic of increasing concern (Wolff and Seal 1993; Cunningham 1996; Kock et al. 2010). In the past, infectious diseases were not considered in wildlife management because it was generally believed that wild animal populations were large enough to adapt to, or deal with, any potential impacts from disease (Spalding and Forrester 1993). However, substantial increases in human-induced impacts to the natural environment have occurred over the past 50 years. The primary issues facing all species of wildlife, including desert tortoises, are habitat loss and habitat degradation due to human development activities (Mitchell and Klemens 2000).

In the case of desert tortoises, translocation is a management tool that has become increasingly popular. While translocation can be a useful tool to help restore viable tortoise populations, its misuse has the potential to cause great damage (IUCN 1987), and numerous safeguards must be taken to protect the translocated tortoises, as well as tortoises in the resident recipient population. Because desert tortoises are long-lived and do not reach reproductive maturity for 13-20 years, a disease outbreak that causes the death of a large number of tortoises may result in devastating population losses, making it difficult for the population to recover. A key step in minimizing disease risks is screening animals that will be translocated, as well as those in the existing population at the recipient site, for pathogens (Beck et al. 1993; Cunningham 1996). Because tortoise health can substantially influence the success of a translocation, baseline information should be collected before implementing the action and then again afterwards to measure the results.

How health is measured

Health is measured in all species using parameters such as food/water intake and fecal/urine output (physiological balance), body weight or condition in relation to a known standard, reproductive performance, blood biochemical parameters, social and environmental factors, amount of physical activity, availability of suitable habitat, absence of clinical signs of disease, and several other variables. Many of these variables are easy to measure in captive animals; however, most are extremely difficult to measure in wild species. The most common components of a comprehensive health assessment of animals include examinations and tests for:

- Physical condition, including evidence of trauma and body condition scoring
- Complete blood count and biochemical profile
- Vitamin and mineral assays
- Internal and external parasites
- Infectious diseases
- Reproductive capacity
- Exposure to environmental toxins

Unfortunately, normal reference ranges for some of these variables are simply not available for free-ranging wild desert tortoises (but see Christopher et al. 1999 and Dickinson et al. 2002). Increasing concern about tortoise health has led to several recent studies conducted at multiple institutions that ultimately will provide much health-related data in the near future.

4. Health and Diseases of Desert Tortoises

Disease is a natural phenomenon within wild animal populations, and epidemic outbreaks can have catastrophic effects on small or declining populations. To date, the available evidence indicates that upper respiratory tract disease is probably the most important infectious disease for desert tortoises (Hudson *et al*. 2009). Less is known about other diseases that have been identified in the desert tortoise (*e.g.*, herpesvirus, shell diseases, bacterial and fungal infections, and urolithiasis or bladder stones) (Jacobson *et al.* 1994, 1995; Homer *et al.* 1998; Berry *et al.* 2002; Origgi *et al.* 2002). Additional research is needed to clarify the role of disease in desert tortoise population dynamics relative to other threats and the level of effort we should expend in disease control as compared to other threats.

At least two pathogenic species of *Mycoplasma* known to cause upper respiratory tract disease in desert and gopher tortoises have been identified (*M. agassizii* and *M. testudineum*) (Brown *et al.* 1994, 1999, 2001; Brown *et al*. 2002). The pathogens are likely transmitted by contact with an infected individual or aerosols (airborne liquid droplets or solid particles). Once infected, tortoises may develop lesions in the nasal cavity, excessive nasal discharge, swollen eyelids, sunken eyes, lethargy, and possible death (Jacobson *et al.* 1991; Schumacher *et al.* 1997; Homer *et al.* 1998; Berry and Christopher 2001). These clinical signs, which may not always be evident in an infected individual, may also be symptomatic of other conditions, such as dehydration or infection with other pathogens (Brown *et al*. 2002). Various tests have been developed to detect the presence of antibodies to *M. agassizii* or to help determine active infection (Schumacher *et al.* 1993; Brown *et al.* 1995; Wendland *et al.* 2007; Hunter *et al*. 2008). Johnson *et al.* (2006) uncovered a positive link between tortoises with anti-*Mycoplasma* antibodies and the severity of clinical signs of upper respiratory tract disease, as well as with age class, with adults being more likely to test positive for presence of antibodies. However, in-depth study of the desert tortoise's immune system and epidemiological study of disease dynamics across space and time are necessary to more thoroughly understand the factors involved in the spread and virulence of the disease in the wild (Braun *et al*. 2011b; Boarman 2002; Sandmeier *et al*. 2009).

The most commonly described shell disease in desert tortoises is cutaneous dyskeratosis, which manifests itself as lesions along scute sutures of the plastron and sometimes on the carapace, which then spread to the scutes themselves (Jacobson *et al*. 1994; Homer *et al*. 1998). The shell can appear discolored, dry, rough and flakey, and there is also peeling, pitting and chipping through multiple cornified layers. In advanced cases, exposed areas become infected with bacteria, fungus, and exposed tissue and bone may become necrotic (Homer *et al*. 1998, 2001). Shell diseases have been seen in tortoise populations in the eastern Mojave and Colorado deserts of California but less so in the western Mojave Desert (Jacobson *et al*. 1994; Christopher *et al*. 2003). Shell diseases occur in all sizes and ages of desert tortoises but are usually more common in adults (Jacobson *et al*. 1994; Homer *et al*. 1998). It appears that shell diseases reflect metabolic and physiological changes that involve more than the shell itself (Homer *et al*. 1998, 2001). Little is known about the causes of shell disease; no evidence indicates a bacterial or viral origin, despite directed research efforts by pathologists to find one (Jacobson *et al*. 1994; Homer *et al*. 1998). 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). The extent to which shell diseases contribute to population declines in desert tortoises remains unclear (Jacobson *et al*. 1994).

In tortoises with herpesvirus infection, clinical signs include lesions and plaques on the tongue and hard palate (Johnson *et al*. 2006). The contribution of herpesvirus to population declines of the desert tortoise is unknown; however, herpesvirus infection has been reported in wild desert tortoises (Jacobson *et al.* accepted; Jacobson *et al.* 2011). In captive desert tortoises herpesvirus infections have been associated with illness and mortality (Johnson *et al*. 2006; Braun et al. 2011a). Clinical herpesvirus infections can be rapid and progressive, resulting in large die-offs in other species of vertebrate animals as well (Johnson *et al*. 2006). Therefore, at least in theory, herpesvirus could become a serious threat to desert tortoise populations.

There is evidence that harboring one disease may predispose desert tortoises to other diseases (Christopher *et al*. 2003); however, it is not known whether this is a cause or effect. That is, it is not known whether disease in an individual increases susceptibility to other diseases in that individual or whether an individual's baseline susceptibility to disease causes that individual to get more diseases. Nevertheless, positive nasal cultures for *Mycoplasma agassizii* had relatively high positive predictive values for tortoises with moderate to severe shell disease (Christopher *et al*. 2003).

In some cases, a disease may become apparent and get very severe soon after an animal has been exposed to the pathogen. Such diseases are generally called 'acute diseases' because the onset and progression of the disease is rapid and severe, sometimes resulting in death. The direct impacts of an acute disease to a population often become apparent quickly because these diseases usually spread through a population of animals very rapidly. In other cases, the disease may be more insidious, and exposure to the pathogen results in a chronic illness that progresses slowly over time. Animals with a chronic disease may have reduced reproductive capacity, may not grow normally, may be more susceptible to secondary infections or conditions such as predator attack or vehicular trauma, and in some cases, may have a reduced life span. Mycoplasmosis in tortoises is increasingly considered to be such a chronic disease (cf. Berish *et al*. 2010). Identifying the impacts of such diseases can be a difficult task. There is an abundance of literature describing the potentially severe, long-term consequences of chronic disease on animal populations (Spalding and Forrester 1993; Hess 1996; Heffernan *et al*. 2005). However, the full effect of chronic disease on a long-lived species such as the desert tortoise may take decades to be seen. Therefore, it is important that populations are monitored using standardized techniques so that any changes associated with health problems may be detected over time.

Table 1 provides a list of clinical signs that may be encountered when examining tortoises and some of the potential causes of these signs. Although it is not an exhaustive list of every potential problem that may be encountered when conducting physical examinations of tortoises, it covers the most common signs of illness.

Table 1. Clinical signs that may be observed when conducting physical examinations on desert tortoises in the field.

Clinical Signs	Possible Conditions/Causes
Behavioral	
Extending neck, increased respiratory effort, mouth gaped open	Respiratory disease (e.g., tracheal obstruction, reduced lung capacity secondary to coelomic cavity effusion, pneumonia - many potential causes such as bacterial and/or viral)
Weakness, non-responsive, outside burrow during cold weather	Severe debilitation (many potential causes)
Head	
Eye discharge (serous or mucous), red and/or swollen conjunctiva and periocular tissue	Eye infection, URTD*, abrasion/irritation, foreign bodies, nutritional imbalance
Nasal discharge (serous or mucous)	URTD*, clear nasal discharge sometimes seen after a tortoise has been drinking, do not mistake frothy oral discharge from stressed or overheated tortoise for nasal discharge
Erosion and/or depigmentation of skin around nares	Trauma, chronic nasal discharge associated with URTD*, skin infection
Asymmetrical or abnormally shaped nares	Normal, impacted with sand/soil, scarring or exudate secondary to chronic infection such as URTD*
Abnormal breath sounds (wet/crackling sounds)	URTD*, pneumonia (many potential causes)
Swollen tympanum (membrane over ear)	Abscess (bacterial infection), vitamin deficiency, trauma
Oral cavity	
Pale mucous membranes	Anemia, severe debilitation, shock
Ulcers, crusts, or plaques on tongue or inside mouth	Viral (herpesvirus, iridovirus), bacterial or fungal infection, embedded foreign body
Limbs/body	
Skin swelling	Abscess/infection, parasite, tumor, trauma, over hydration
Skin discoloration	Incomplete shed, infection, scar from prior injury
Emaciation/reduced muscle mass	Starvation, severe debilitation (many potential causes), chronic disease
Lameness, swollen joints	Trauma/injury, nutritional disease, metabolic disease (such as gout), neurological disease
Firm mass/object in coelomic cavity	Substrate ingestion, feces, eggs, urolith (bladder stone), tumor
Shell	
White or yellow discoloration, flaking	Healing traumatic injury, bacterial or fungal infection, dyskeratosis (nutritional deficiency, toxicity, autoimmune disease, infectious disease, metabolic disorder, or other)
Soft spots, especially at mid-carapace	Nutritional disorder, toxicity, trauma
Red blotches	Trauma, infection
Malformed shell	Trauma, nutritional disorder

* URTD: Upper respiratory tract disease caused by *Mycoplasma spp* or other pathogen.

5. Conducting Health Assessments

Disinfection/sanitation protocols

Caution must be taken whenever handling or sampling desert tortoises to ensure that field personnel do not aid in the spread of infectious microorganisms or contaminate samples. Cleaning refers to the physical removal of organic debris (dirt, feces, urine, blood, etc.) from objects or living tissue. Disinfection refers to the elimination or inhibition of the growth of microorganisms (except bacterial spores) on non-living objects, whereas antisepsis involves the same process for living tissue. In contrast, sterilization is the complete elimination or destruction of all forms of microorganisms (including bacterial spores) on non-living objects and is generally not possible in the field.

Field personnel must follow a standard protocol that includes the disinfection of all equipment. Field personnel should wear disposable latex or nitrile gloves whenever handling tortoises, and change gloves between individual tortoises. If gloves are torn when handling a tortoise, a new glove should be placed over the torn glove and an antiseptic should be used on the hands after handling of that individual is complete. A disinfection protocol is provided in Appendix A.

Tortoise capture/handling

Desert tortoises will be captured and transported according to existing protocols (USFWS 2009). Health assessments may occur at the capture site or within a project's quarantine pens.

Handling of tortoises should occur in a manner that minimizes stress. Effort should be made to keep the tortoise as close to ground level as possible and in its normal spatial orientation. Tortoises can be slightly rotated out of normal spatial orientation for necessary data and sample collection. Due to increased risks of voiding and stress, tortoises should not be turned upside down. Caution should also be taken to hold tortoises away from the handler's body (pants, shoes) to minimize risk of contamination from contact or voiding.

Handling a tortoise for health assessment and sample collection must be completed within 30 minutes or less. This time period does not include rehydrating tortoises that void.

Physical examinations

Health studies in wildlife typically include physical examinations and the collection of biological samples for a number of diagnostic tests (Christopher et al. 1999; Karesh *et al*. 1999; Berry and Christopher 2001; Hanni *et al*. 2003; Kilbourn et al. 2003; Deem *et al*. 2005; Deem *et al*. 2006; Uhart *et al*. 2006). Excellent guidelines have been published describing techniques for the evaluation of health in chelonians (Jacobson *et al*. 1999; Berry and Christopher 2001). Berry and Christopher (2001) provide sample data sheets and have helpful line drawings that show, in detail, how to examine the eyes and periocular area of desert tortoises. The manuscript also describes abnormal behaviors observed in desert tortoises that may be indicative of a health problem. Anyone planning to conduct a health study in wild tortoises should read that journal article (available online at http://www.jwildlifedis.org/cgi/reprint/37/3/427). The health assessments that are required in conjunction with translocations of desert tortoises include a physical exam, during which very

specific clinical signs of interest are evaluated, and collection of several biological samples. These health assessments are specifically designed to minimize risks when translocating tortoises and are not intended to be in-depth studies into the diseases that may affect an individual.

Performing a physical examination requires, at minimum, knowledge about normal tortoise behavior (attitude and activity) and physical appearance. For this reason, we consider conducting health assessments to be an advanced skill that novice tortoise biologists are not yet prepared to learn. Additional training is required if biological samples are going to be collected. The basic components of the physical exam include an overall assessment of the attitude and activity of the tortoise, an examination of the eyes, nares, beak, oral cavity, skin, muscle mass, and shell. Body condition scoring (BCS) is a system for evaluating the condition of domestic animals, and BCS has been modified for desert tortoises (Appendix D). The BCS is based on an evaluation of muscle mass and fat deposits in relation to skeletal features. A tortoise's body mass can vary greatly with hydration state or other physiological processes, so mass is of limited value when used alone. Other scoring systems exist such as condition index based on body mass and size (Nagy et al. 2002). The BCS is a rapid method of evaluating muscle mass and other characteristics and is currently being used by the U.S. Fish and Wildlife Service for these health assessments. Morphometric (i.e., body) measurements are used to determine the maturity of individual tortoises and to track growth over time. A brief explanation of the physical examination and procedures for completing the data collection forms are described in Appendix B. Appendix C provides photographic examples of numerous clinical signs that may be encountered when conducting these exams.

Diagnostic tests

Diagnostic assays (i.e., tests) greatly complement the physical examination because many diseases have an incubation or subclinical period when tortoises may not exhibit outward signs of illness. These diagnostic tests provide extremely valuable information regarding the immunological, nutritional, and physiological status of the animal and can indicate specific organ dysfunction or disease. Therefore, specific assays may detect a disease process before clinical signs develop. In some cases, the presence of a specific set of clinical signs observed in tortoises may prompt one to run diagnostic tests to try to determine the underlying cause of the signs.

Biological sample collection

Hands-on training is necessary to learn appropriate sample collection and handling techniques, and even more expertise is often required to interpret test results. Proper sample collection, processing, labeling, and handling is essential for meaningful, reliable, and repeatable test results. Samples that have not been collected, processed, labeled, or stored correctly may provide inaccurate results and can lead to inappropriate conclusions or decisions. For example, lymphatic vessels lie directly adjacent to the most commonly used blood vessels for sample collection in tortoises. Often blood samples are contaminated with clear lymphatic fluid, which may alter diagnostic test results dramatically, and therefore, when it occurs, it should always be noted on the sample tube and on data collection sheets, and results should be interpreted cautiously.

In this handbook and accompanying training, we instruct students on the particular techniques that should be used when collecting blood and oral cavity samples specifically for translocation-related heath assessments. Information regarding collecting nasal fluids is included in the handbook for

future reference. This technique may be necessary for specific research projects. The laboratories that will be analyzing these samples have specialized experience working with reptilian samples. This is important because reptilian blood cells and body temperatures differ from those of mammals, so standard laboratories may not have the appropriate laboratory setups or diagnostics. Sample handling and storage protocols, supply lists, and laboratory shipping information are included in Appendices E and F.

Biological sample storage

Even when diagnostic tests are not being performed at the onset of the project, blood samples, oral swabs, cloacal swabs, nasal lavages, and ectoparasites (e.g., ticks) may be collected, processed, and stored for future use in the event that a problem develops in the population. These stored samples can be used for retrospective studies in the event that a disease outbreak occurs, can be analyzed with new diagnostic tests that become available, or can simply be saved for analysis until sufficient resources become available in the future. The red blood cell (RBC) pellet that results from centrifugation of a blood sample to separate the serum should be saved because it may be used for DNA-based studies and, therefore, may be of value in the future. It is absolutely critical that blood products be stored appropriately to prevent degradation of the samples. Instructions for banking blood, nasal fluid, oral cavity, and cloacal samples are included in Appendix F.

Hydration after voiding

Voiding occasionally occurs while tortoises are handled for health assessment, data collection, and biological sampling. Tortoises store urine for long periods of time and use this for maintaining hydration and proper physiological balance. Loss of urine due to handling-induced voiding may have an adverse affect on long-term survivability based on a previous retrospective study (cf. Averill-Murray 2002).

Given the potential for adverse effects on health occurring from voiding urine, it is recommended that all tortoises that void urine be offered or administered fluids to replace voiding losses. Options for fluid replacement include injectable epicoelomic fluids, nasal-oral offering of fluids, and soaking in a shallow water bath. Each option has positive and negative aspects that will be discussed below. Injectable epicoelomic fluids and nasal-oral administrations are the preferred two of the three USFWS authorized methods of fluid replacement. For these two methods, a dose of 20ml/kg should be administered. A maximum volume of 60ml will be injected for all weights exceeding 3kg. Tortoises with an MCL < 100 mm should not be offered injectable epicoelomic fluids due to increased potential for harm if fluids are administered improperly. Instead, these small tortoises should be offered nasal-oral fluids or soaked as their primary route of fluid administration.

Epicoelomic fluid administration in desert tortoises has been commonly and safely used by veterinarians for treatment of dehydration as well as for other reasons. The technique of epicoelomic fluid administration in not technically difficult and with proper training can be performed by most biologists in the field. Epicoelomic describes a location between the coelomic cavity/membrane and the shell. This is a potential space similar to subcutaneous areas. The benefit of using this location versus other subcutaneous locations is that fluids in this area do not appear to affect mobility or the ability of the tortoise to withdraw its limbs into the shell prior to complete absorption of the fluids. This procedure has the benefit of providing of a known amount of fluid

replacement and does not require significant amounts of equipment. It does require advanced training and permitting by USFWS prior to use on federally protected desert tortoises.

Nasal-oral administration has been used successfully to administer fluids to tortoises that void. A dose of 20 ml/kg of drinking water should be offered to the tortoise. This is done by slowly emptying a syringe of water into the nares or mouth. Water should not be forced into the tortoise. If the tortoise does not accept water this way, epicoelomic fluids should be administered or the tortoise should be soaked. This process has the benefit of requiring less water to be carried by the biologist, and minimal equipment is needed; however, this method may not work for all tortoises.

Soaking is the last alternative for fluid replacement for tortoises that void. This requires a plastic container to hold water and it requires significantly more water than the other methods. The tortoise is placed in the plastic tote and water is added to the level between the skin-plastron junction and the chin. The tortoise remains in the tub soaking for 30 minutes. Tortoises may drink water through their nose or mouth or absorb it through their cloaca. Tortoises may void more urine while being soaked in an effort to eliminate urine with high solute levels and replace it with the fresh water being offered. If a tortoise voids in its soaking tub and additional fresh water is available, the water should be discarded and replaced with fresh water until the completion of the 30 minutes. Otherwise the tortoise can be soaked for 30 minutes in the water that contains the voided urine and urates. The tortoise should also be rinsed with water to remove urine odor on the limbs and shell, which might attract predators.

Complete protocols for all three of these methods are provided in Appendix F.6.

6. Interpreting Results and Making Decisions

Interpreting results

Data interpretation must be placed within the context of the overall goals, the questions asked, the limitations of the data collected, and the experience level of the individuals conducting the study. Although the collection of health related data from tortoises requires some level of basic training, interpretation and decision-making based on the results require a thorough understanding of normal tortoise biology and the health assessment tools used. For example, if clinical signs were observed in tortoises when conducting physical examinations, to interpret the findings one must be aware of the severity of the clinical signs (relative to normal), potential causes for the signs, and their biological significance. Alternatively, if clinical signs are not observed, managers must recognize that many diseases have subclinical periods (e.g., times when clinical signs are not exhibited) or have clinical signs that are intermittently expressed. Thus, although physical examinations are extremely useful, they do not provide conclusive evidence about the overall health of the individual tortoise.

There are a number of important considerations when interpreting results of diagnostic tests. For serological tests that detect antibodies, a positive result indicates that the tortoise has been exposed to a given pathogen, but it does not tell you if the tortoise is currently infected. The use of assays that have been validated properly and have had positive results correlated with pathological lesions is important to help make decisions regarding the significance of a positive test. Additionally, false positive and false negative results occur, which obviously complicates interpretation. Test performance varies with prevalence, stage of infection, concurrent diseases, immunological competence, experience of personnel, and cut-off values for positive tests. Tests should be applied and interpreted at the population level where they are effective at diagnosing disease within sick populations and confirming that healthy populations are uninfected. Conversely, tests are not as effective at identifying infected individuals among a mostly healthy population or uninfected individuals among a mostly sick population. We will be using the results of diagnostic tests to assist in selecting recipient sites (USFWS 2011), but not in determining an individual's eligibility for translocation. If hematology or biochemistry data are being used, one must be able to discern normal values from abnormal and additionally must understand the influence of season, reproductive status, and age on the test results. In particular, awareness of the limitations of the established sampling schemes, sample sizes, and methods selected for the project, as well as limits of the diagnostic tests mentioned above, are all very important considerations.

While field biologists will be preparing proposed disposition plans for the tortoises at their project sites after completing health assessments and receiving results of diagnostic tests, these plans MUST be submitted to the U.S. Fish and Wildlife Service's Desert Tortoise Recovery Office PRIOR TO relocating any tortoises. Final interpretation of results will be made by the U.S. Fish and Wildlife Service's Desert Tortoise Recovery Office in coordination with the state wildlife agencies, and partnering veterinarians and pathologists. Once interpretation is complete, USFWS will provide field biologists with an approved or modified disposition plan.

Management decisions

Most management decisions, including those based on the results of diagnostic assays and presence or absence of clinical signs, will have some degree of uncertainty associated with them. Management decisions must be made with a clear understanding of these limitations. The establishment of clear objectives greatly assists in weighing the importance of the limitations and making better management decisions based on findings of the study. It is important to note that definitive or clear-cut answers may not be available, and therefore judgments must be made based on the best available scientific information at the time. A strong commitment to follow-up monitoring, within a well-designed research framework, will be critical to document results of management decisions and to adapt conservation strategies as deemed necessary.

Our objectives with regards to translocation-related heath assessments are to minimize the risk of spreading disease within and among populations and to promote survival of individuals when relocating tortoises from project sites. Perturbation of populations will occur through translocation, and previous studies of desert tortoise translocations have given us information about likely movement patterns and behaviors. Using these data along with information about disease, we can devise a strategy that should meet our objective. Appendices G and H show the algorithm and disposition plan for making recommendations on whether or not individual tortoises should be translocated. The USFWS's guidance on translocation (USFWS 2011) describes how to choose appropriate recipient sites when considering the results of diagnostic tests and evaluation of clinical signs. As described above, the U.S. Fish and Wildlife Service's Desert Tortoise Recovery Office, in coordination with the state wildlife agencies and partnering veterinarians and pathologists, will make final decisions about translocating tortoises after reviewing data from the health assessments and from the proposed disposition plans that will be submitted along with those data.

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Glossary

Active (pertaining to lesions)
Status of a wound or lesion that is very recent or unhealed.

Aliquot
A portion of a total amount of solution or sample.

Anemia
A reduction in the number or volume of red blood cells in the blood.

Antisepsis
The elimination or inhibition of the growth of microorganisms (except bacterial spores) on living tissue (i.e., skin).

Atrophy
Decrease in size or wasting away of body part or tissue.

Axillary
The cavity or area around the forelimbs.

Beak
An external anatomical structure with a keratinized, horny covering, which serves as the mouth in some animals.

Bevel
The angled surface of the tip of the needle containing the opening.

Biochemistry panels
A number of tests relating to the chemistry of a living organism and vital processes that occur in living cells. Tests provide information regarding the function of major organ systems and metabolic processes.

Biological samples
Samples collected from living organisms or their products (i.e., blood, feces, urine, etc.).

Body condition score (BCS)
Body condition scoring is a system developed for domestic animals to estimate the average body condition of animals in a herd. This system provides managers a relative score based on an evaluation of muscle mass and fat deposits in relation to skeletal tissue. This scoring system has been adapted for the desert tortoise. A score of 1-3 is under condition, 4-6 is acceptable to good condition, and 7-9 is over-condition.

Catastrophic event
A sudden, short-lived, violent event that has a profound impact on a population (i.e., hurricane or other natural disaster, substantial population mortality associated with a disease, etc.).

Chelonians
Belonging to the order Chelonia, which includes the turtles and tortoises.

Choana
Opening of the upper respiratory tract in the upper oral cavity.

Coelomic Cavity
Internal cavity containing organs.

Complete Blood Count (CBC)
A series of tests that assess the quantity of each type of blood cell in a sample of blood, often including the amount of hemoglobin and the proportions of red blood cells and various white blood cells. Tests provide information regarding the immunological status of the animal and function of specific organ systems.

Chronic disease
A disease with slow progression and a long duration.

Cleaning
The physical removal of organic debris (dirt, feces, urine, blood, etc.) from an inanimate object or living tissue.

Clinical sign
An objective indication of some medical fact or characteristic that may be detected by a physician/veterinarian during a physical examination of a patient. A sign is not the same a symptom, which is subjective and observed by a patient.

Compressible
Capable of being flattened by pressure or pressed into a smaller space.

Conjunctiva
Mucous membranes that line the inner surface of the eyelids including the third eyelid.

Corneal opacity
A local or generalized loss of transparency of the surface of the eye.

Crust
An outer layer of solid material formed by drying of a bodily exudate or secretion

Debilitation
Being in a state of severe weakness; lack or loss of strength.

Disease
An abnormality in structure or function of a living organism. May be identifiable with clinical signs or may be subclinical (i.e., with no outward visible signs).

Disinfection
The elimination or inhibition of the growth of microorganisms (except bacterial spores) on inanimate objects.

Dyskeratosis
Abnormal development of keratin cells in the shell and skin of tortoises. Causes are many and may include toxins, auto-immune disease, infection, trauma, metabolic problems, or due to a nutritional deficiency.

ELISA (Enzyme-Linked Immunosorbent Assay)
A biochemical technique used to detect the presence of specific antibody or antigen in a plasma or serum sample.

Epicoelomic
A potential space between the coelomic membrane and the shell in reference to the location injectable fluids are administered.

Epidemiology
The frequency and distribution of disease in populations, and the detection of the source and cause of disease.

Eroded nares
Loss or wearing away of scales and/or skin around nares.

Etiologic agent
The cause or origin of a disease.

Evidence of foraging
Presence of food or coloration (green, pink) on beak.

False positive
A test result that is read as positive but actually is negative; a test that shows evidence of a disease or immune response when it is not present.

Feces
Bodily waste discharged through the digestive tract; excrement.

Generalized
Spread throughout most of an area

Hematology
The study of the nature, function, and diseases of the blood and of blood-forming organs.

Hydration
The process of providing an adequate amount of liquid to bodily tissues.

Hypersalivation

Increased saliva in or around the oral cavity.

Immunological
Pertaining to immunology, or the study of all aspects of the immune system, immunity from disease, the immune response, and immunologic techniques of analysis.

Impaction
Lodgment or instance of lodgment of something, such as food or debris, in a body passage or cavity.

Inactive
The condition of a wound or lesion that is no longer changing significantly or is healed.

Incubation
The maintenance of control over temperature, humidity, and oxygen concentration in order to provide optimal conditions for growth and development of an organism.

Incubation period
The time period between invasion of the body by a pathogen and development of the initial signs of disease. This period may range from days to years, depending on the type of disease.

Inflammation
A local response to cellular injury that is marked by redness, heat, pain, swelling, and often loss of function.

Lavage
The process of washing out a body cavity, such as the nasal sinuses, for diagnostic or therapeutic purposes.

LB
Luria-Bretani media used as a nutrient preservative for storing biological samples.

Lesion
An abnormal change in structure of an organ or part due to injury or disease

Lethargic
Sluggish or lacking energy.

Localized
Restricted to a particular place or area.

Lymph
A yellowish, transparent fluid that is composed of water, plasma proteins, chemical substances and lymphocytes (a type of white blood cell).

Lymphatic vessels

The vessels that collect lymph and transport it between tissues and the bloodstream.

Mental glands
Paired symmetrical glands on the chin.

Metabolic disease
A disease in which normal body metabolism is altered resulting in an excess, absence, or shortage of specific enzymes or substances needed for normalcy.

Microorganisms
An organism of microscopic or submicroscopic size, including bacteria, viruses, some fungi and protozoa.

Morphometric measurement
The measurement of forms and shapes (i.e., for these purposes, the measurement of tortoise body shapes, including plastron/carapace length, body thickness, body width, plastron concavity, and other body measurements).

Mucous
Thick viscous discharge that may be clear, cloudy, or discolored.

Multifocal
Having to do with two or more places or areas.

Naïve population
A population that has not been exposed to a pathogen of interest.

Naris (s), Nares (pl)
The external opening(s) of the nasal cavity in reptiles and birds; nostrils.

Necropsy
The examination of an animal's body after death; similar to an autopsy, which is an examination of a human's body after death.

Occluded naris
Narrowing or reduced opening of the naris due to debris, exudates, or scarring.

Oral mucosa
Membranes lining the oral cavity.

Packed cell volume (PCV)
The percentage of red blood cells relative to the total volume of blood in the sample.

Palpation
Physical examination by pressure of the hand or fingers to the surface of the body to determine the condition of an underlying organ or body part.

Pathogen
Any disease-causing agent; includes viruses, bacteria and other microorganisms.

Pathology
The study and diagnosis of disease through examination of organs, tissues, cells and bodily fluids.

PCR
The **polymerase chain reaction** (**PCR**) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Periocular
The tissues surrounding the eye including the upper and lower eyelids.

Physiological
Pertaining to physiology, or the study of the physical and chemical factors and processes associated with the normal functioning of a living organism.

Plasma
Non-cellular portion of the blood that includes clotting factors.

Plaque
A localized patch or flat area in the oral cavity. Plaques tend to have a white or yellow appearance.

Potential space
A space that can exist within the body because adjacent organs, membranes, etc. are not tightly adjoined. The space does not exist during normal functioning, but can be created during activities such as epicoelomic fluid administration.

Prefemoral
The cavity or area in front of the hind leg.

Prevalence
A ratio of the number of cases of a disease to the number of individuals at risk in the population for a given time period.

Recipient population
The population receiving individuals.

Sagittal crest
The ridge of bone running lengthwise along the midline of the top of the skull.

Scute
A bony external plate or scale, as on the shell of a turtle.

Serology
Dealing with the immunological properties and actions of serum (i.e. blood); evaluation of antigen-antibody reactions in a laboratory setting to determine if an animal has been exposed to a specific pathogen.

Serous
Clear watery discharge

Shock
A life-threatening medical condition where blood flow to the body tissues is inadequate, often resulting in reduced oxygen and nutrient delivery to the tissues, and sometimes cardiac arrest (the heart stops beating).

Sterilization
The complete elimination or destruction of all forms of microorganisms (including spores).

Subclinical
Not showing characteristic clinical signs or symptoms of a condition.

Temporal muscle
A large muscle that serves to raise the lower jaw and attaches to the sagittal crest.

Total Protein (TP)
A rough measure of all the proteins found in the plasma portion of the blood.

Ulcer
A localized defect or excavation of the surface of a tissue, usually produced by sloughing of necrotic inflammatory tissue.

Upper Respiratory Tract Disease (URTD)
An illness caused by an infection of the upper respiratory tract (nasal passages, nasopharynx, pharynx, larynx, and extrathoracic trachea). Can be caused by several bacteria, viruses, or fungi.

Urate
A salt of uric acid.

Urine
Waste material that is secreted by the kidney, is rich in end products of protein metabolism (such as urea, uric acid, and creatinine) together with salts and pigments, and forms a clear fluid.

Urolith
A calculus (i.e., stone or concretion) in the urine or the urinary tract.

Variable strain virulence
Differences in disease severity caused by different strains of a pathogenic microorganism.

Vent

External opening of the cloaca.

Void
To eliminate solid or liquid waste from the body.

Appendix A: Disinfection and Sanitation

Caution must be used whenever handling or sampling desert tortoises to ensure that pathogens (i.e., disease causing microorganisms) are not introduced to a field site through contaminated equipment. Equally important, field personnel must take necessary precautions to ensure that they do not aid in the unintended transmission of pathogens among the individual tortoises sampled or contaminate the samples that have been collected. Therefore, development and implementation of a step-by-step disinfection protocol is essential for field studies. An effective disinfection protocol must address the microorganisms being targeted, the characteristics of the disinfectant, and the impact a disinfectant may have on the environment. The health and safety of field personnel and desert tortoises are of vital importance.

A number of products are available for disinfection/sanitation purposes. The most common antimicrobial products fall within one of the following classes: alcohols (i.e., hand gels), chlorine (i.e., bleach), iodine/iodophors (i.e., povidone, iodine), chlorhexidine (i.e., Nolvasan), oxidizers (i.e., VirkonS), phenols (i.e., Lysol), quaternary ammonia (i.e., Roccal), and aldehydes (i.e., Wavicide). Each has varying effectiveness for different classes of microorganisms, and the reader is referred to the University of Nebraska - Lincoln Extension website entitled, 'Selection and Use of Disinfectants' for more information about disinfectants.[1] The effectiveness of any disinfectant or antiseptic is determined by the amount of time that the cleaning agent is allowed to contact the surface, concentration of the product used, the organic load (amount of dirt/debris), the level of microorganism contamination, the condition of the object being cleaned (cracks, crevices, wood vs. plastic surfaces), ambient temperature, and sometimes the environmental pH. In field conditions where high organic loads are almost always present, effective antisepsis and disinfection are not possible without first cleaning to remove excessive debris. Therefore, regardless of the agent used, an initial cleaning is required.

The protocol listed below describes methods to be used when working with desert tortoises. Certainly, other protocols may be developed to meet the needs of specific projects or management goals, upon approval from the U.S. Fish and Wildlife Service. Users should follow local and state regulations for transport and disposal of disinfectant solutions.

Disinfectant solutions

Solutions should be stored in dark bins or in opaque bottles and should be made fresh regularly (i.e., daily or weekly depending on storage conditions). Take care to keep all solutions off of skin and out of eyes. Gloves should be worn when using disinfectants. Appropriate choices as disinfectants include those listed below.

Trifectant®

This product is sold as a powder or tablet and is effective against viruses, bacteria (including mycoplasma), and fungi. It is also fairly resistant to inactivation by hard water and organic matter. Once mixed, the solution is stable for seven days. Contact time for disinfection is 5-10 minutes and metal instruments should not be soaked for more than ten minutes. A 1-2% solution should be mixed according to instructions on the packaging.

[1] Available through URL: http://www.ianrpubs.unl.edu/epublic/live/g1410/build/g1410.pdf

Chlorhexidine diacetate (e.g. Nolvasan®)
This disinfectant is sold as liquid and is effective against viruses, bacteria, and fungi. It should be prepared according to the instructions on the packaging.

Bleach
While no longer the preferred option, a standard bleach solution may be used (1:20 dilution of 5 percent household bleach in water). Bleach should be purchased in small bottles or dispensed into small bottles to minimize deterioration from opening/closing the lid. In the event of a high organic load, thoroughly remove or wash the organic material off before using bleach to disinfect to prevent the bleach from being inactivated. Bleach solutions are also readily inactivated by sunlight.

Protocol for cleaning and disinfecting equipment and sanitizing personnel

All equipment must be thoroughly cleaned and disinfected prior to arriving at a field site. Additionally, all equipment and work surfaces (if not on natural ground) must be cleaned before and after handling each tortoise. Efforts should be made to first remove organic debris (i.e., dirt, feces, etc.) by rinsing the area with water, brushing debris off with paper towels, or cleaning the equipment/work surface with the chosen disinfectant solution and wiping with paper towels. The equipment should then be thoroughly moistened with disinfectant at appropriate dilution and appropriate contact time and dried or allowed to air dry. Heat and direct UV light exposure will improve the disinfection process.

Gloves are to be worn and changed between handling individual tortoises to prevent the transfer of pathogens from one tortoise to another. Additionally, wearing gloves will protect field personnel from the effects of potentially irritating disinfectants and pathogens. Contaminated gloves should be removed after handling and cleanup and prior to the disinfection process. New gloves should be used while disinfecting equipment. All items touched with contaminated gloves should be considered contaminated (e.g. pens, clipboards, clothing). Skin must also be sanitized between tortoises, if contact with a tortoise and/or its excretions occurs. Acceptable sanitizing solutions include soap and water (if available) or a hospital grade ethyl alcohol hand wash (minimum alcohol concentration of 60%). Example product: Alcare Foamed Alcohol Scrub (Steris Co.). Follow manufacturers' recommended contact times for all sanitizing products. Removal of organic debris is essential for proper sanitation; therefore, a water rinse before using the product will be more effective when hands are extremely dirty. Alcohol hand washes should be allowed to air dry while rubbing hands vigorously in order to appropriately distribute the product.

Any individual that has broken skin as a result of a needle stick should notify their supervisor. Supervisors should have appropriate protocols in place and review them with all field personnel prior to the initiation of any fieldwork.

Appendix B: Completing the Health Assessments and Data Collection Form

This appendix provides an overview for conducting a physical examination of a desert tortoise and completing the data collection form, but it is not a substitute for appropriate training and hands-on experience. A thorough and accurate physical examination can only be performed if the individual performing the exam has knowledge of normal tortoise physical appearance and behavior. Prior training in assessing desert tortoise health is required. Physical examinations provide valuable information about the health of individual tortoises and insight into population level issues. Because the desert tortoise is a long-lived species, it is extremely important to use standardized techniques for data collection so that information can be compared over time.

This appendix provides a description of how to conduct a basic physical examination of a desert tortoise and follows the order of the provided data collection form. A minimum of two people will be needed in order to conduct a full health assessment that includes the collection of biological samples. One person will serve as the examiner and will also complete the data collection form. This person should be able to stay clean (i.e. not touch the tortoise or contaminated items) until he/she begins to collect the biological samples. The other person (handler) will handle the tortoise such that the examiner can complete the examination and collect samples. If a third person is available, this person (rather than the examiner) may record the data to further reduce contamination risks. It is important to complete certain observational aspects of the physical exam first, so that potential clinical signs are not affected by handling of the animal. For example, head restraint may cause eye bulging or serous discharge so it should be done after initial evaluation. Most of the health assessment can be conducted with minimal, if any, handling of the tortoise. We recommend using the order described below for a systematic health evaluation of every animal. Generally, the tortoise will be evaluated from a distance before direct contact (far to near), beginning at the head and working towards the tail (head to tail), and least invasive procedures will be conducted prior to more invasive procedures (least to most). This approach also helps field staff remember to collect all of the data consistently because they fill out the form as they proceed with the exam. Be sure to carefully describe all anomalies such that comparisons can be made with subsequent examinations. Handling a tortoise for health assessment and sample collection must be completed within 30 minutes or less. This time period does not include rehydrating tortoises that void.

Unless tortoises are being moved into a quarantine holding facility for their health assessment, they should not be moved far (<20 m, if possible) from the location at which they are found in the field. Before the handler touches the tortoise, find a flat shaded area with ample work space where you will not disturb a tortoise's shelter sites. If shade is not available, it can be created by placing a lightweight cloth over vegetation. Be sure to record the temperature where you plan to work to ensure that it falls within the requirements established by USFWS for handling tortoises. If the temperature is appropriate, lay out equipment in the order that it will be used and place it out of the reach of where you will place the tortoise. Depending on the substrate, the top few centimeters of soil can be cleared away, such that the tortoise is in contact with cooler soil while you work. Prepare all the biological sampling equipment and supplies and set them up in an orderly manner for easy access during the health assessment. An area on one side of the examiner should be designated for clean supplies and equipment and an area on the other side for contaminated items. After taking pertinent initial data (date, start time, project name, site description, tortoise ID, transmitter frequency if applicable, GPS location, temperature, and full names of the biologists), record the time at which you approach the tortoise and are within 2 meters of it. Take care not to

startle the tortoise as you approach it. While wearing gloves, the handler should gently lift the tortoise a short distance off the ground by grasping the sides of the shell and slowly carry it to the shaded area while maintaining it close to the ground in its normal spatial orientation.

While placing the tortoise on the ground in the work area, be sure that the tortoise does not touch any of the supplies or equipment. The examiner should perform as much of the visual observation portion of the health assessment as possible before the handler holds the tortoise again. Note that when completing the data collection form, right and left refer to the tortoise's right and left sides (not right and left from the examiner's perspective if looking at the tortoise head-on). Descriptions of how to complete each data field on the data collection form follow the presentation of the actual form below.

http://www.fws.gov/nevada/desert_tortoise/dtro/

Desert Tortoise Health Assessment (HA) Data Collection Form – Solar Projects **U.S. Fish and Wildlife Service April 2013**

Date (ddmmmyy):	Start time (24h):	Project name:	Site description / current pen #:	Tortoise ID #:	Transmitter frequency:

GPS datum:	UTM zone:	UTM easting:	UTM northing:	Temp °C:	Full name of biologist(s):

HA start time:	Attitude/activity:	Respiration:	Beak:
	Normal Lethargic/Weak	Normal Abnormal sounds Increased effort	Normal Abnormal Evidence of foraging

Left naris:	Left naris discharge and severity:	Right naris:	Right naris discharge and severity:
Normal Asymmetrical Eroded Occluded	None Serous: 1 2 3 Mucous: 1 2 3	Normal Asymmetrical Eroded Occluded	None Serous: 1 2 3 Mucous: 1 2 3

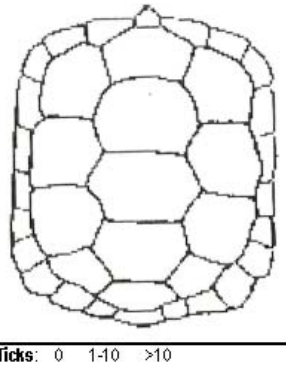
Left eye:	Right eye:
Normal Sunken Corneal opacity Partially closed Fully closed Serous discharge Mucous discharge Periocular swelling Periocular redness Conjunctival swelling Conjunctival redness	Normal Sunken Corneal opacity Partially closed Fully closed Serous discharge Mucous discharge Periocular swelling Periocular redness Conjunctival swelling Conjunctival redness

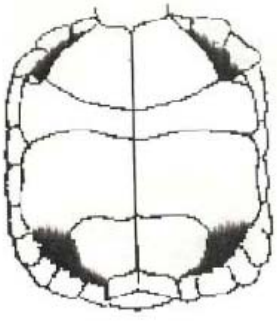
Skin lesion location:	Condition of skin lesion(s):	Coelomic cavity palpation:	Shell characteristics:
None Generalized Head Neck L / R forelimb L / R axillary region L / R hindlimb L / R prefemoral region Vent/tail	N/A Active Inactive	No mass L / R mass Not done	Compressible N/A Sunken +/ - Scutes Peeling/flaking keratin Bone exposed

Shell abnormality location:	Shell abnormalities (describe below):	Condition of shell abnormalities:	If present, circumstances of skin/shell trauma: N/A
Carapace Plastron N/A	None Localized Multifocal Generalized	N/A Active Inactive	Unknown Suspect canid bite Vehicle Other_____

Sex:	Initial weight:	Body condition score:	Photos (take all):
M F Unk	_____(g)	1 2 3 4 5 6 7 8 9	__Front face and body ___Left side face ___Right side face ___Carapace ___Plastron (take when measuring only if abnormal) ___Abnormalities

Label and describe trauma, anomalies, lesions, missing body parts, and identifying features.





Ticks:	Choana:	Tongue and oral mucosa:	# oral swabs collected:
0 1-10 >10 Location: Soft tissue Seams Scutes Eyes Nares Beaks Collected? N/A Yes No Removed? N/A Yes No	Not examined Normal Pale Reddened	Not examined Normal Pale Reddened Crust Ulcers Plaques Hypersalivation Impaction	_____

Time of blood draw (24h):	Total sample volume (blood and lymph) collected:	Total # hep tubes (number each):	Is the sample contaminated with lymph?	Void during processing:
	_____(ml)	0 1 2 3 4	Yes No Estimate the degree of lymph contamination: Small (1-10%) Moderate (11-29%) Severe (>30%)	None Urine/urates Feces

Post void weight:	MCL:	Width V3:	Height V3 :	Plastron:	Hydration method:	Post fluid weight:
N/A _____(g)	_____(mm)	_____(mm)	_____(mm)	_____(mm)	N/A Soak Nasal-oral Epicoelomic Fluid type _____ Vol _____(ml)	N/A _____(g)

End handling time (24h):	Disposition:	Blood processing time (24h):	Plasma color:	UFL plasma aliquots:	USFWS plasma aliquots:	Total tubes with RBCs saved:
	Wild capture location Same pen New pen _____ Other _____		Colorless Red Yellow Green	0 1	0 1 2 3	0 1 2 3 4

Total number of tubes/vials collected: _____ Revised April 4, 2013

Description of data fields on the data collection form

Date (ddmmmyy) – 2 digit day, 3 letter month, and 2 digit year (e.g. 04Mar11).

Start time (24h) – The time when the tortoise is located. This is not the time at which you begin assessing or touching the tortoise, but instead, it's the time at which you visually locate the tortoise. Use the 24 hour clock (e.g. 1:45pm is recorded as 13:45).

Project name – Name and phase of the project.

Site description/current pen # – If the tortoise is newly caught from the wild, provide a description of the capture location, or if the tortoise was already in captivity, provide the quarantine pen number.

Tortoise ID # - Each tortoise will be assigned a unique identification number from a series of numbers assigned by USFWS for that project. The tortoise may have been numbered prior to the health assessment or you may need to number it (refer to numbering protocols provided by the USFWS).

Transmitter frequency – Numbers of the radio transmitter frequency, if applicable. Usually includes 3 digits, a decimal, and 3 more digits (e.g. 164.020)

GPS datum – **Make sure your GPS is set to UTM, WGS 1984.** Datum is the model used to match the location of a feature on the ground to the coordinates of the feature on a map.

UTM zone – In the UTM system, the earth is divided into 60 zones of 6 degrees of longitude wide. Your GPS displays zone as 2 digit number (e.g. 11, 12 that may be followed by N denoting north of the equator).

UTM easting – 6 digit number displayed on your GPS. Easting is measured from the central meridian of the zone, which is given the value of 500,000 m, and increases as you travel east.

UTM northing - 7 digit number displayed on your GPS. Northing is measured relative to the equator, which is assigned a value of 0 in the northern hemisphere, and increases as you travel north.

Temp °C – Temperature in degrees Celsius measured 5 cm above the ground in the shade and protected from wind where you set up your equipment to process the tortoise, or as specified in the most recent USFWS Desert Tortoise Field Manual.

Full name of biologist(s) - First and last names of the biologists handling the tortoise and recording the data. List the Authorized Biologist primarily responsible for conducting the health assessment and biological sampling as the first name, followed by the name of the person assisting by handling the tortoise, then the name of person recording the data, if applicable.

HA start time – Health assessment start time. This is the time at which you approach a tortoise and get within 2 meters of it to begin assessing its condition, even without touching it or moving it.

Attitude and activity – Circle one.

Normal – Tortoise paddles its forelimbs when held, attempts to escape, and repeatedly retracts into its shell when handled; or is shy and tends to remain retracted into its shell when being handled, but has normal strength. During cold temperatures, activity may be minimal, but is considered normal for the temperature or time of year.

Lethargic/weak – Forelimbs may hang limp when tortoise is lifted, tortoise appears weak, is slow to respond to stimuli, and/or does not resist gentle tugging on the limbs.

Respiration – Circle all that apply.

Normal - No sound or a very faint, high-pitched whistle when expelling air out of their nares.

Abnormal sounds - Includes wet, crackling, or gurgling sounds associated with congestion.

Increased effort – Tortoise pumps forelimbs up and down symmetrically when breathing, may indicate lower respiratory disease or compromised lung volume. Open-mouthed breathing or neck extended while breathing may indicate increased respiratory effort, but this must be distinguished from occasional normal gaping.

Beak – Circle all that apply.

Normal – Usual shape, size, color, and texture. May have pieces of food-related debris or dirt stuck on it.

Abnormal – Unusual shape, size, color, or texture. Describe in area provided.

Evidence of foraging – Presence of food or coloration associated with food, usually green or black, but may be more colorful (e.g. pink) in spring depending on food sources.

Left/right naris – Circle all that apply.

Normal – Usual shape and/or size.

Asymmetrical - One naris is larger and/or wider than the other.

Eroded - Loss of scales and skin around naris opening.

Occluded – Plugged or reduced size of naris opening.

Left/right naris discharge – Circle one.

None – No discharge present.

Serous - Clear, watery discharge present.

Mucous – Cloudy, thick discharge present.

Severity of naris discharge – Circle one.

1. Mild - Moisture present around nares.
2. Moderate - Discharge coming out of the nares, but not running far from the nares themselves.
3. Severe - Discharge coming from nares that is running down the beak.

Left/right eye – Circle all that apply.

Normal – Usual shape, size, and color.

Sunken – Eye recessed within the orbit.

Corneal opacity- Eye is cloudy, hazy, or there is a loss of transparency of the cornea.

Partially closed – Eye is not fully open.

Fully closed – Eye is not open at all.

Serous discharge - Clear, watery discharge present.

Mucous discharge - Cloudy, thick discharge present.
Periocular swelling – Area around the eye is swollen.
Periocular redness – Area around the eye is abnormally pink or red.
Conjunctival swelling - Membranes around the eye are swollen.
Conjunctival redness – Membranes around the eye are abnormally pink or red.

Skin lesion location – Circle all that apply, describe in area provided, and draw on diagram.
None – No skin lesions.
Generalized – Widespread lesions in many locations all over the body.
Head
Neck
L/R forelimb – Circle left, right, or both.
L/R axillary region – Circle left, right, or both.
L/R hindlimb – Circle left, right, or both.
L/R prefemoral region – Circle left, right, or both.
Vent/tail

Condition of Skin lesions – Circle one, but for multiple lesions circle all that apply and describe in area provided.
N/A – Not applicable.
Active – Very recent or unhealed lesion.
Inactive - Old or healed lesion.

Coelomic cavity palpation – Circle one.
No mass – The tortoise was palpated but no masses were detected.
L/R mass – Circle left, right, or both.
Not done – The tortoise was not palpated.

Shell characteristics - Circle all that apply and describe in area provided. Note that these characteristics may not always be considered abnormalities, as in the case of sunken scutes on an older tortoise. Therefore, it is possible to circle a characteristic here, but record N/A in the section labeled "Shell abnormality location."

Compressible - Capable of being flattened by pressure or pressed into a smaller space.
N/A – There are no unusual shell characteristics.
Sunken – Scutes are sunken lower than the seams of the shell.
+/- Scutes – Circle (+) if there are more scutes than usual and circle (–) if there are fewer scutes than usual on the carapace and/or plastron.
Peeling keratin – Scutes are peeling on the carapace or plastron.

Shell abnormality location - Circle all that apply, describe in area provided, and draw on diagram. Note that it is possible to circle a "Shell characteristic" above, but record N/A in this section since not all characteristics that could be noted there are considered abnormal, as in the case of sunken scutes on an older tortoise. However, if you record something in the section "Shell abnormalities" below, then you must circle a location in this section.
Carapace
Plastron

N/A – There are no shell abnormalities.

Shell abnormalities (describe below) - Circle one, but for multiple abnormalities circle all that apply and describe in area provided.

None – No shell abnormalities.
Localized – Abnormalities are restricted to a particular place or area on the body.
Multifocal – Abnormalities found on two or more distinct places or areas on the body.
Generalized - Widespread lesions in many locations all over the body.

Condition of shell abnormalities – Circle one, but for multiple abnormalities circle all that apply and describe in area provided.

N/A – Not applicable.
Active – Very recent or unhealed abnormality.
Inactive - Old or healed abnormality, or genetic alteration, such as extra or too few scutes.

If present, circumstances of skin/shell trauma – Circle one.

N/A – Not applicable
Unknown – There is no way to determine the cause of the trauma.
Suspected canid bite - Suspected or known predation by coyote, dog, or other canid. Provide details, if known, in the area provided.
Vehicle – Trauma is suspected or known to be caused by a vehicle, including but not limited to a car, truck, military tank, ATV, dirt bike, dune buggy, etc. Provide details in the area provided.
Other _____ - Provide details if the other options do not provide an adequate description of the circumstances of the trauma.

Sex – Circle one.

M – Male
F – Female
Unk – Unknown sex

Initial weight (g) - Weigh the tortoise prior to extensive handling to avoid the risk of the tortoise voiding. Spring scales or electronic balances may be used. When using a spring scale, weigh the tortoise with the smallest scale appropriate for the individual to get an accurate weight.

Body condition score – Circle one. Scores range from 1 (emaciated) to 9 (morbidly obese). Descriptions of each body score are included in Appendix D.

Photos (take all*) – Photos should be taken with a good quality digital camera. Prior to taking photos of the tortoise, take a photograph of the data collection form clearly showing the Tortoise ID number, date, site, UTM location, biologists, etc. Whenever possible, take photos using a macro lens to show detail, such as those taken to show trauma and signs of disease.

Front face and body - Show nares, forelegs, gular, and anterior shell.
Left side face – Full frame close up.
Right side face – Full frame close up.
Carapace – Full frame close up.

Plastron (*take when measuring, but only if abnormal) - Take this photo when you are doing the plastron measurement (after sample collection) and only if the plastron is abnormal.
Abnormalities – Full frame close up.

Label and describe trauma, anomalies, lesions, missing body parts, and identifying features – On the line drawings of a tortoise carapace and plastron, draw in any trauma, anomalies, lesions, and identifying features of the tortoise, and circle or point an arrow to the location of missing body parts. Draw in the shape of the gular on the picture of the plastron. Provide a written description in the space provided for details about the tortoise that may require clarification.

Ticks (*Ornithodoros spp*) – Circle one. See Appendix F.2 for details regarding tick collection.

0 – No ticks were observed.
1-10 – 1 to 10 ticks were observed.
>10 – More than 10 ticks were observed.

Collected? - Circle one.

N/A – No ticks were observed so no ticks were collected.
Yes – Ticks were collected as per the tick collection protocols.
No – Ticks were present but not collected.

Removed? – Circle one.

N/A – No ticks were observed so there were no ticks to remove.
Yes – Ticks were removed as per the tick collection protocols.
No – Ticks were present but not removed.

Location – Circle one. Location where ticks were observed.

Soft tissue – Skin, including limbs, vent, and tail.
Seams – Areas between the scutes on the carapace and plastron.
Scutes – Keratinized plates of the carapace and plastron.
Eyes
Nares
Beak

Choana – Circle one. See Appendix F.3 for details regarding the oral cavity examination.

Not examined – An oral cavity examination was not conducted or it was conducted but the choana was not observed.
Normal – Pale pink or pink.
Pale – Lacking pink coloration, white, or slightly yellow
Reddened – Very dark pink to red.

Tongue and oral mucosa – Circle all that apply. See Appendix F.3 for details regarding the oral cavity examination.

Not examined – The oral cavity examination was not conducted or it was conducted but the tongue and oral mucosa were not observed.
Normal – Pale pink or pink.
Pale – Lacking pink coloration or white
Reddened – Very dark pink to red.
Crust - An outer layer of solid material formed by the drying of a bodily exudate or secretion.
Ulcers - Localized defects or excavations of the surface of a tissue, usually produced by sloughing of necrotic inflammatory tissue.

Plaques - Localized patches or flat areas in the oral cavity. Plaques tend to have a white or yellow appearance.
Hypersalivation - Increased saliva in or around the oral cavity.
Impaction – Lodgment of something, such as food or debris, in the oral cavity

 # oral swabs collected – Write the number of oral swabs collected. You can collect 0, 1, or 2 oral swabs. See Appendix F.3 for details regarding oral swab sample collection.

Time of blood draw (24h) – This is the time that you finished collecting the blood and removed the needle from the vein. Use the 24 hour clock (e.g. 1:45pm is recorded as 13:45).

Total sample volume (blood and lymph) collected – Total volume in milliliters of each blood collection attempt. Up to three attempts to collect total maximum volumes are allowed. See Appendix F.5 for details regarding blood sample collection**.**

Total # hep tubes (number each) – Circle one. You can have 0, 1, 2, 3, or 4 tubes containing whole blood and/or lymph samples.

Is the sample contaminated with lymph – Circle yes or no.

Estimate the degree of lymph contamination - Small (1-10%) Moderate (11-29%) Severe (>30%)

Void during processing – Circle all that apply.
None – The tortoise did not discharge bladder or digestive tract contents.
Urine/urates – Waste material that is secreted by the kidney as a clear fluid or as a semi-solid salt of uric acid that may be white, yellow, or brown
Feces – Bodily waste discharged through the digestive tract; firm excrement

Post void weight – If the tortoise voided during processing, weight the tortoise again at this point before rehydrating. Record the weight in grams. If the tortoise did not void, circle N/A.

Hydration Method – If the tortoise voided urine/urates, circle one, or if multiple rehydration methods were employed, circle all that apply and describe in the area provided. See Appendix F.6 for details regarding rehydration techniques.
N/A – The tortoise did not void urine/urates, so it was not provided with fluids.
Soak – The tortoise was placed in a plastic tote with shallow fresh water for 30 minutes.
Nasal-oral – The tortoise was offered fresh water ad lib with a syringe through the nares and mouth.
Epicoelomic – The tortoise was given an epicoelomic injection of sterile saline.

Fluid type – Fill in blank with fluid(s) used. If multiple rehydration methods were employed, describe in the area provided. See Appendix F.6 for details regarding rehydration techniques**.** If the tortoise did not void, skip this field.
Water – Fresh drinking water.
Saline – 0.9% sodium chloride.

Vol - Amount of fluids in milliliters injected into the tortoise or offered ad lib. Do not provide a volume if you used the soaking method. If the tortoise did not void, skip this field.

Post-fluid weight – If the tortoise voided, weight in grams after administration of fluids. If the tortoise did not void, circle N/A.

MCL – Straight midline carapace length measured in millimeters by holding the calipers directly over the center line of the tortoise and measuring from the center of the outer edge of the nuchal scute to the most caudal aspect of the carapace. Note that the most caudal end of the carapace may not be the edge of the supracaudal scute.

Width V3 - Width of the carapace measured in millimeters by holding calipers directly over the center line of the tortoise crosswise in the middle of the 3rd vertebral scute. Keep the calipers level with the ground.

Height V3 - Height of the carapace measured in millimeters by holding the tortoise so that one arm of the calipers is held across the center of the 3rd vertebral scute on the carapace and the other arm of the calipers is held in the same position across the plastron so the tortoise is between the arms of the calipers. The arms of the calipers should extend all the way from one side of the tortoise to the other while being held level against the tortoise's carapace and plastron.

Plastron – Length of the plastron measured in millimeters measured by having a handler tip the tortoise to one side (the tortoise's left or right side) so the examiner can see the plastron. The examiner then uses calipers to measure from the notch between the gular scutes to the notch between the anal scutes. If the calipers do not fit into the notch, then hold them outside the notch on the plastron where the notch comes to a V to get the most accurate measurement. Do not tip the tortoise too far to the left or the right and do not put it on its carapace to take this measurement.

End handling time (24h) – The time at which you complete the processing of the tortoise and return it to its original location (point of capture, pen, etc). Use the 24 hour clock (e.g. 1:45pm is recorded as 13:45).

Disposition - Circle one.

Wild capture location - For tortoises that were caught in the wild and are being placed back at their site of capture immediately following processing (i.e, the tortoise is not being brought into captivity after processing).

Same pen – For tortoises that were already in captivity at the time of processing and are being placed back in the same pen.

New pen – Provide the number of the new pen where the tortoise is being placed after processing, regardless if it was newly captured from the wild, or if it was already in captivity but moving to a new pen.

Other- Describe the new location.

Blood processing time (24h) – The time at which samples were removed from the cooler and placed in the centrifuge. Use the 24 hour clock (e.g. 1:45pm is recorded as 13:45).

Plasma Color – Circle one. This is the plasma color of the sample you will use for the "Hep plasma UFL" cryovial. This should be the best sample you collected for each tortoise, meaning the one with the least lymph contamination. Choose from colorless, red, yellow, and green.

UFL plasma aliquots – Circle one. You can have 0 or 1 aliquots of plasma for UFL. See Appendix F.7 for details regarding sample processing**.**

USFWS plasma aliquots - Circle one. You can have 0, 1, 2, or 3 aliquots of plasma for USFWS. See Appendix F.7 for details regarding sample processing**.**

Total tubes with RBCs saved – Circle one. You can have 0, 1, 2, 3, or 4 heparinized microtainers with RBCs in them to save. All of the plasma should have been pipetted out of these microtainers into the UFL or USFWS plasma and plasma/lymph tubes prior to storage, leaving only RBCs in the microtainers. See Appendix F.7 for details regarding sample processing**.**

Total number of tubes/vials collected – Count the total number of tubes/vials containing samples from the individual.

Appendix C: Clinical Signs of Disease in Desert Tortoises

Download at http://www.fws.gov/nevada/desert_tortoise/dtro/

Appendix D: Body Condition Scores for Desert Tortoises

Download at http://www.fws.gov/nevada/desert_tortoise/dtro/

Appendix E: Biological Sampling Supplies

Supplies for individual tortoise sampling
The following supplies will be needed to process a single tortoise. You can prepare zipper sealed plastic bags containing these supplies for individual tortoises to decrease preparation time in the field. It is also important to carry extras of all of these supplies in case of contamination or other unforeseen circumstances. We recommend laying out all the supplies from this bag before touching the tortoise to prevent potential contamination while processing the tortoise.

- (4) Alcohol or diluted povidone iodine wipes
- (2) Sterile cotton tipped plastic stick swabs for oral swabs
- (1) Sterile cotton tipped swab for cloacal swab
- (1) Small trash bag
- (1) Zipper sealed plastic bag to hold completed sample tubes
- (1) 20cc or 60cc luer lock syringe with (1) 22G 1.5", 22G 1", or 25G 1" needle for injectable epicoelomic fluid administration – syringe and needle sizes depend on size of tortoise
- (3) 1cc or 5 cc slip tip (luer) syringe with (3) 23G 1", 23G 1.5", or 25G 1" needle for blood sampling - syringe and needle sizes depend on weight of tortoise
- (1) 2.0ml snap top microtainer labeled "Ticks"
- (2) 2.0ml snap top microtainers labeled "Oral swab"
- (1) 2.0ml snap top Microtainer labeled "Cloacal swab"
- (4) 1.3ml heparinized snap top microtainers labeled "RBC"

If collecting nasal lavage samples

- (6) Pieces dry gauze
- (1) Plastic disposable pipet for nasal lavage sample processing
- (1) 50ml sterile conical tube for nasal lavage
- 1cc, 3cc, or 5cc slip tip (luer) syringe with 22G 1.5" needle for nasal lavage – syringe size depends on weight of tortoise

Supplies to keep in the cooler (must be with you while processing a tortoise)
The following supplies should be kept in a cooler for use with multiple tortoises.

- Frozen gel packs or dry ice
- (1) bottle liquid sodium heparin
- 1.8ml O-ring cyrovials with aliquots of LB in a zipper sealed plastic bag - 4 vials per tortoise
- (2) 250ml sterile saline bags encased to protect them from damage

General supplies to keep in the field kit
The following supplies should be kept in your field kit for use with all the tortoises that you process. Remember to keep your field kit free from contamination by laying out your supplies before touching a tortoise.

- Gloves
- Hand sanitizer
- Disinfectant in a spray bottle
- Sharps container
- Ultra fine permanent markers
- Tweezers, hemostats, and/or mutli-tool
- Bottle of water (to soak tortoise for rehydration or to clean tortoise face and nares)

- Paper towels
- Clipboard
- Data collection forms
- Extra packaged syringes – 1cc, 3cc, and 5cc slip tip (luer), 20cc and 60cc luer lock
- Extra packaged needles - 22G 1.5“, 22G 1”, 23G 1.5“, 23G 1”, 25G 1”
- Extra 1.3ml heparinized snap top microtainers labeled “RBC”
- Extra plastic disposable pipets
- Camera
- Calipers

Supplies to keep in your vehicle and/or lab
The following supplies and equipment should be kept in your vehicle and/or lab, in addition to extras of all the supplies listed above.

- Disinfectant
- Big cooler with dry ice
- Inverter
- Portable centrifuge
- Plastic disposable pipets
- 0.5ml O-ring cryovial labeled “Hep plasma UFL” – 1 vial per tortoise
- 1.8ml O-ring cryovials labeled “Hep plasma” – up to 2 vials per tortoise
- 1.8ml O-ring cryovials labeled “Hep plasma/lymph” – up to 2 vials per tortoise

The DTCC’s Research Coordinator, Angie Covert (702-526-3436), may be able to provide information on ordering supplies.

Appendix F: Protocols for Biological Sampling, and Sample Processing, Storage, and Shipment

These protocols describe the collection of biological samples from the tortoise; handling, aliquoting, and storage of the samples; and shipment of samples to the appropriate laboratories. Teams of two biologists (an examiner and a handler) will work together to collect samples, with at least one (the examiner) being certified and permitted to conduct all of the activities involved with sample collection. The handler must be trained in handling tortoises. Ideally, a third person will record data on the data form, but a third person is not required.

Conduct a physical examination of a tortoise prior to taking any samples. Follow the order of data collection as presented on the data form and as described in Appendix B. Prior to handling the tortoise, your sampling equipment should be removed from your field pack and/or cooler and placed in the order that you will need it. Avoid re-entering your field pack and supply bags while you work on an individual tortoise, but if you need to do so, remove your gloves to enter the pack and put on clean gloves before continuing with processing. If you forget to remove your gloves before entering your pack, disinfect everything you touched and put on clean gloves to continue. During the processing of a tortoise, you can avoid contaminating supplies and equipment by using the one-handed glove technique in which you wear gloves on both hands, but the glove on your dominant hand is one size too big so you can easily slide your hand into and out of it as needed to avoid contamination while handling samples, recording data, etc. In addition to preparing and laying out all of the necessary supplies and equipment prior to handling the tortoise, label all the sampling tubes you will be using for that animal with the tortoise ID and date. Appendix F.1 describes all of the preparations that should be made for sampling tortoises prior to handling the animal.

In general, samples collected with the intention of detecting an immune response (e.g., plasma for ELISA tests) require that the immune system be functioning and active. Because a desert tortoise's immune response is temperature dependant, thus depressed during periods of winter inactivity, it is important to collect such samples after the immune system resumes more typical active-season functioning. While individual activity does vary across the range of the desert tortoise, we consider blood samples to be used for ELISA tests valid when collected between 15 May and 31 October. Upon specific approval from USFWS, exceptions to the start date may be made. For example, a sample may be considered valid when collected four weeks after the date that the individual of interest left its hibernaculum or was first found active and above ground (not in association with a shelter site).

Appendix F.1: Preparing for Biological Sampling (Before Handling the Tortoise)

Label cryovials and microtainers

- Prepare cryolabels in advance (before going into the field) using a laserjet printer. Following is an example of the information to include on the label:

GoAg ID _____

Site _____ PI _____

Contents _____ Date _____

- Labels should always display GoAg to indicate that the sample came from a desert tortoise (*Gopherus agassizii*).

- Print your own site name and last name of your PI on the labels using a laserjet printer to avoid having to write these in the field.

- In place of the word "Contents", you will print ONE of the following contents on each label with your laserjet printer. Remember that you will be using multiple tubes for some of these samples so you will need to print more than one label for those samples. Example: You could need 4 RBC tubes for an individual tortoise so you will need to print 4 labels for RBCs and place one on each 1.3ml heparinized microtainer. Also be sure to print extra labels in case of unforeseen circumstances in the field

Contents	Number of tubes per tortoise	Type of tube
RBC	4	1.3ml heparinized microtainer
Hep plasma UFL	1	0.5ml O-ring cryovial
Hep plasma	3	1.8ml O-ring cryovial
Hep plasma/lymph	3	1.8ml O-ring cryovial
Nasal LB	3	1.8ml O-ring cryovial
Oral swabs	2	2.0ml snap top microtainer
Ticks	1	2.0ml snap top microtainer
Cloacal swab	I	2.0ml snap top microtainer

- While working in the field, you will record the tortoise ID next to GoAg ID using an ultra fine black permanent marker. Samples with unreadable or questionable ID numbers may be discarded when they arrive at the UFL or DTCC labs so be sure to record the ID clearly.

- Print the word "Date" using the laserjet printer but leave the space after it blank so you can record it while in the field using a black ultra fine permanent marker. The format of the date should read DDMMMYY.

- Complete the fields for “GoAg ID” and “Date” in the field with an ultra fine black permanent marker when you find a tortoise, but BEFORE approaching it. Complete these fields when you prepare and set out all of your supplies and equipment for processing.

- Note that if you purchase your biological sampling supplies from the DTCC, your sample tubes will be labeled for you, including the fields for your site and PI, but you will still need to complete the tortoise ID and date in the field.

Heparinize needles for blood sampling

In order to keep blood from coagulating when it is drawn, the needles and syringes need to be treated with the anticoagulant liquid sodium heparin. Prepare needles and syringes on the day that they will be used and if prepared before going into the field, store them in a zipper sealed plastic bag in a cooler at all times.

Supplies for heparinizing needles

- Bottle of liquid sodium heparin (to be kept in cooler)

- (3) 1cc or 3cc slip tip (luer) or luer lock syringe with (3) 23G 1”, 23G 1.5”, or 25G 1” - syringe and needle sizes depend on size of tortoise

Tortoise weight	Maximum blood volume to collect	Syringe size	Needle size
100-199g	0.25 ml	1cc	25G 0.5” or 1”
200-249g	0.5 ml	1cc	25G 0.5” or 1”
250-499g	0.75 ml	1cc	25G 1”
500-999g	1.5 ml	3cc	23G 1” or 1.5”
1000-1999g	2.0 ml	3cc	23G 1.5”
2000g+	3.0 ml	3cc	23G 1.5”

Procedure for heparinizing needles

- Take a bottle of liquid sodium heparin out of the cooler and use an alcohol swab to wipe the top of it.
- Attach the appropriate size needle and syringe for the tortoise you are sampling according to the table, and push and pull the plunger to loosen it.
- Remove the cap of the needle and insert the needle into the bottle of heparin.
- Tip the bottle upside down and draw in the heparin, ensuring that the bevel of the needle is in the liquid.
- When the syringe is full, keeping the bottle upside down, inject the heparin back into the bottle.
- Do not push the heparin out of the needle completely because you want a little heparin to remain in the hub.
- Remove the needle from the heparin bottle and recap it using the one-handed recapping technique (protocol below).

Safely discard heparinized needles and syringes that are not used on the day they were prepared. Note that heparinizing needles and syringes in advance instead of heparinizing them in the field as you need them can save you time in processing a tortoise. However, this could cause you to heparinize a large number of syringes that you may not need, and you will ultimately have to dispose of unused heparinized syringes and needles at the end of the day.

Draw saline for nasal lavage

Supplies for drawing saline for nasal lavage

- 250ml bag of sterile saline (0.9% sodium chloride - to be kept in cooler): Note – Use a marker to put a date on the saline bag. Discard fluids within 30 days after first usage, if they become discolored, or if the non-injection port parts of the bag are punctured with a needle.

- 1ml, 3ml or 5ml slip tip (luer) syringe with 22G 1 ½" needle for nasal lavage - syringe size depends on size of tortoise

Tortoise weight	Syringe size	Total volume of sterile saline	Volume of sterile saline per naris
100-249g	1cc	1.0ml	0.5ml
250-499g	3cc	2.0ml	1.0ml
500-999g	5cc	4.0ml	2.0ml
1000g +	5cc	5.0 ml	2.5 ml

Procedure for drawing saline for nasal lavage

- Select a syringe based on tortoise size (see table for proper size) and attach a 22G 1.5″needle to the syringe.

- Use an alcohol swab to wipe the port on a 250ml bag of sterile saline, and insert the needle into the port. Be sure to insert the needle directly into the port and be careful not to puncture any part of the bag. If the bag becomes punctured, dispose of it, as well as the needle and syringe, and start fresh with sterile supplies.

- Draw up the total volume of sterile saline required for the tortoise's weight (see table for volume).

- Carefully withdraw the needle from the port and either use a tool (*e.g*. multi-tool or hemostat; not your hand) to remove the needle from the syringe, or use the one-handed recapping technique to recap the needle and then remove the capped needle with your hand. Dispose of the needle in the sharps container.

- Place the bag of sterile saline back into the cooler and leave the syringe containing saline out such that the saline is not too cold when you are ready to inject into the nares.

One-handed needle recapping technique

Only use this technique to recap needles that have NOT been used on an animal (i.e., after heparinizing syringes and needles or after drawing up saline for nasal lavages).

- While holding the open needle on a syringe in your dominant hand, use your other hand to lay the cap of the needle on a solid surface with the open end of the cap facing you.

- Remove your hand and guide the needle into the cap without your hands touching it. You can use a solid object to hold the cap in place to make it easier to guide the needle in.

- Once the needle is inside the cap, continue moving your hand forward against the capped needle and syringe until the cap is pushed against a solid surface.

- Then angle the end of the syringe closest to you upwards with the needle inside the cap facing downward and push the needle to secure it inside the cap. Make sure it clicks closed.

- If you are heparinizing a syringe prior to going into the field, you may then place it in a zipper sealed plastic bag in a cooler for use that day.

- If you are drawing up saline for nasal lavage, remove the capped needle and place the saline-filled syringe with your clean supplies to be used while processing the tortoise.

Note that you must NEVER recap a needle that has been used on an animal, but you may use the one-handed recap technique to recap needles that are being ***prepared*** for use on an animal.

Appendix F.2: Ticks

Equipment and supplies

- 2.0ml snap top microtainer labeled “Ticks”
- Tweezers or hemostats

Ticks procedure

For all tortoises (translocatees, residents, and controls)

- Examine the entire body of the tortoise including the soft tissue and shell.
- If ticks are seen, record the number and locations on the data collection form.
- Collect up to 20 ticks in a snap top microtainer labeled “Ticks”.
- Recheck that the ID and date are correctly labeled on the tube.
- Put the microtainers in a zipper sealed plastic bag on ice in the cooler. They must remain cold until they are placed directly in the freezer.
- Record the number of ticks you collected on the data form.

For tortoises that are being translocated

- Carefully remove the remaining ticks with a solid object, such as a stick or rock and let them fall to the ground.
- Be sure that all visible ticks are removed to prevent translocating them to another field site along with the tortoise.
- If there are some ticks that are difficult to remove, use tweezers, hemostats, or even a toothbrush to scrub them off.
- Record the number of ticks you removed on the data form.

For tortoises that are NOT being translocated (residents and controls)

- Do not remove the remaining ticks.

Check your skin, clothes, and equipment for ticks that may have landed there or walked there after being removed from the tortoise, and remove any that you find. WEAR GLOVES. DO NOT HANDLE TICKS WITH YOUR BARE HANDS.

Appendix F.3: Oral Cavity Exam and Oral Swab

Equipment and supplies

- Sterile cotton tipped plastic stick swabs

- 2.0ml snap top microtainers labeled "Oral swab"

Oral swab procedure

- The handler is the only person that will touch the tortoise during this procedure. The examiner will wear gloves, but will not touch the tortoise.

- The handler holds the tortoise with one hand and places the other hand behind its head so that its neck is extended.

- The handler uses their thumb and middle finger to stabilize the head, leaving the index finger free.

- When stabilizing the tortoise's head, the handler holds the head behind the jaw at the neck, and avoids putting pressure on the jugular vein to minimize the chance of trauma.

- The handler uses the free index finger to gently push down on the mandible to make the mouth accessible.

- If you can't get the tortoise's head out or you can't open the mouth after a few attempts, and you believe the tortoise is experiencing stress, do not proceed. You can attempt to collect the sample during subsequent interactions with the tortoise. Another attempt may be made as soon as 24 hours from the failed attempt, if venipuncture was not conducted on the day of the failed attempt. If venipuncture was done on the day of the failed attempt, the next attempt should occur no sooner than 7 days later to allow for soreness associated with the venipuncture to resolve.

- Once the mouth is open, the examiner looks inside the oral cavity paying particular attention to the choana, tongue, and mucosa, and records the observations on the data form.

- The examiner gently inserts two cotton tipped swabs inside the oral cavity. Move them around the entire oral cavity to sample the choana, the tongue, the inside of the beak, and all mucosal surfaces; however, do not attempt to swab beneath the tongue. In addition to moving around the oral cavity with the two swabs, gently rotate the swabs as well to cover all surfaces of the swabs, and spend extra time swabbing abnormalities.

- The handler should not release the tortoise's head yet because the nasal lavage procedure will immediately follow.

- After collecting the samples, the examiner quickly places each swab in its own microtainer labeled "Oral swab". Put the cotton tipped end of the swab inside the microtainer so the plastic stick is pointed outward. Be sure not to contaminate the swabs by touching other surfaces or by touching them with your hands.

- Place these samples to the side, uncapped, in an area that you have designated for collected samples so you can quickly move on to the cloacal swab.

- Note: If the samples will not be safe if left uncapped (risk of blowing away, etc), you can follow the procedure for capping them in Appendix F.7.

Cloacal swab procedure
This should be the last sample collected from the tortoise. **Note:** Most projects are ***not*** permitted to collect cloacal swabs.

- Insert a cotton-tipped swab into the cloaca. Gently turn the swab and remove.

- Place the swab in the microtainer labeled "Cloacal swab".

- Put the cotton tipped end of the swab inside the microtainer so the plastic stick is pointed outward. Be sure not to contaminate the swab by touching other surfaces or by touching it with your hands.

- Place the sample to the side, uncapped, in an area that you have designated for collected samples so you can quickly move on to the nasal lavage.

- Note: If the sample will not be safe if left uncapped (risk of blowing away, etc), you can follow the procedure for capping it in Appendix F.7.

Appendix F.4: Nasal Lavage

Equipment and supplies

- 50ml sterile conical tube
- Appropriate size syringe and needle with saline drawn
- 0.75ml aliquots of LB working solution in 1.8ml O-ring cryovials labeled "Nasal LB" (to be kept in cooler)
- Plastic disposable pipet

Nasal lavage procedure

Note: Beginning in 2013, most projects will ***not*** be permitted to collect nasal lavage samples and these samples will not need to be routinely collected unless requested specifically by the researcher.

- The handler is still maintaining the tortoise's head extended outside the shell after the oral swab.
- If significant nasal discharge is present, the examiner immediately takes a sample of the discharge with a syringe (no needle) or pipet and places it directly into an LB cryovial and secures the cap. If no discharge is present, proceed to the next step. Note that the hand holding the LB cryovial must be wearing a clean glove that is free from contamination and the sample vial should be placed with other clean supplies for later processing.
- Clean head/nostrils. Use water soaked gauze or a cotton ball to wipe off nares, face, and chin, and allow to air dry.
- While still holding the head out, the handler positions the tortoise at a 45 degree angle (or more) so the cranial end (head) is pointed downward toward the person doing the flush.
- The tortoise should be no more than 0.5m off the ground and always held away from the handler so as to prevent contamination.
- The examiner holds an open 50ml sterile conical tube under the tortoise's chin. Avoid touching the tortoise with the tube so you can minimize sample contamination.
- With the hand that is stabilizing the head, the handler applies pressure to the ventral aspect of the head in the intermandibular space behind the chin to push the tongue dorsally into the choana. This will decrease the chance of the tortoise aspirating or swallowing the saline as the flush is performed.
- The examiner surrounds the left naris with the syringe tip (no needle!) and injects half of the saline directly into the naris so it comes out the right naris, reminding the handler to push up on the ventral aspect of the tortoise's intermandibular space.

- The examiner collects the fluid that comes out of the right naris and mouth into the conical tube.
- Repeat the process flushing through the right naris and collecting the fluid from the left naris and mouth into the *same* conical tube.

- After all fluid has been flushed through the nares, the handler should gently push under the chin with a pumping motion to encourage the last of the sample to be expelled into the conical tube.

- Cap the conical tube and set it to the side in an area you have designated for collected samples. It is assumed that there may be some contamination of the examiner at this point in the processing due to fluids so assume the conical is contaminated and treat it as such, and continue on with the processing. The examiner does not need to change gloves because they will be actively touching the tortoise during the blood sampling procedures that follow. But be aware not to touch your pen, clipboard, data form, or any other items that you want to remain free from contamination. This step is where laying out your supplies in advance is essential.

- If you collected discharge directly into an LB cryovial at the start of the nasal lavage process, recheck that the ID and date are correctly labeled on the tube and place it in a zipper sealed plastic bag in the cooler. Remember that this vial is not contaminated so do not touch it with the same gloves you used to touch the conical tube.

Appendix F.5: Blood Sampling

Equipment and supplies

- Alcohol or povidone iodine swabs, or 10% povidone iodine solution
- Gauze or cotton
- 1.3ml snap top heparinized microtainers with labeled "RBC"
- Appropriate size heparinized syringes and needles

Blood sampling volumes

The following table shows the maximum whole blood volume to collect based on tortoise weight and collection device. Note that blood is only drawn on tortoises over 100g and always with heparinized syringes and needles.

Tortoise weight	Maximum blood volume to collect	Syringe size	Needle size
100-199g	0.25 ml	1cc	25G 1"
200-249g	0.5 ml	1cc	25G 1"
250-499g	0.75 ml	1cc	25G 1"
500-999g	1.5 ml	3cc	23G 1" or 1.5"
1000-1999g	2.0 ml	3cc	23G 1.5"
2000g+	3.0 ml	3cc	23G 1.5"

Blood sampling procedure

- Blood will be drawn from the subcarapacial vein as described in Hernandez-Divers et al. (2002), and it is recommended that all individuals using this technique should read the publication.

- The subcarapacial venipuncture site is located underneath the carapace, posterior to the nuchal scute and beneath the anterior portion of the 1st vertebral scute, cranial or anterior to the 8th cervical vertebra.

- The site is accessed by inserting the needle a few millimeters posterior to the junction of the skin-carapace junction and then angling the needle dorsally toward the 8th cervical vertebra.

- While the handler restrains the tortoise's forelimbs, the examiner palpates the subcarapacial area by inserting a finger along the midline posterior to the skin-carapace junction and feels for the depression anterior to the 8th cervical vertebra. This allows you to estimate the proper location of the injection site, as well as determine the angle at which to bend the needle (see next step).

- The examiner removes the appropriate sized heparinized needle and syringe from the cooler. Prepare the needle by bending it upwards with the bevel facing up (using the cap, not your fingers!) to a position that will allow it to easily puncture the subcarapacial vein. It

should be bent at a slight angle to correspond with the angle of the dome between the nuchal and 1st vertebral scute.

- The examiner cleans the venipuncture site with alcohol or 10% povidone iodine solution using swabs, gauze, or cotton prior to insertion of the needle. Be careful not to get alcohol or povidone iodine in the tortoise's eyes.

- Before inserting the needle, the handler should position the tortoise at a level and angle that allows best access to the subcarapacial venipuncture site. The appropriate angle is likely to vary by size and doming of the carapace, but an upright position with the head approximately 75 degrees to the horizontal surface provides a good access point for the needle is often easier to see than when tortoises are held in other positions, especially on very large adults.

- Do not allow the needle to touch the head of the tortoise, which may be moving. With one finger on the beak, gently press the head in and downward toward the plastron and hold it in place, being careful not to touch or place pressure on the eyes.

- Insert the needle through the skin before pulling up on the plunger.

- Pull up on the plunger just slightly to locate the vein, pushing the needle in gently and slowly. You can move the needle very slowly and gently inward and outward with tiny motions along a single path of entry and exit. The needle should not be moved side to side or up and down.

- During the process of drawing blood, be careful not to push the plunger down in the syringe.

- When you see a flash of blood in the syringe, draw the maximum amount of blood indicated in the table for the size tortoise you are working with.

- If you cannot get the full amount of blood on the first or second try, you can try again, but you can only make 3 attempts to draw blood on a single tortoise in one day. If after 3 attempts, you cannot draw the minimum amount of blood required for ELISA testing (0.5ml), you can try again in seven days.

- Lymph
    - In order to be useful, blood should have as little lymph contamination (clear or yellow fluid) as possible and not be coagulated.

    - If the sample you collect is contaminated with lymph, the needle has likely been placed posterior to the subcarapacial venipuncture site.

    - Put the lymph-contaminated sample in a heparinized microtainer and be sure to note it on the label with an "L" so the sample can be transferred to the hep plasma/lymph cryovial after centrifuging.

    - If you have not used the maximum number of attempts, insert the needle anterior to the location of the previous stick and try to get a clean sample.

    - When placing a subsequent clean sample in a heparinized microtainer, do not combine the clean sample with the first lymph-contaminated sample.

- After removing the needle from the venipuncture site, if bleeding occurs, apply pressure with a cotton tipped swab and 10% povidone iodine solution for at least 30 seconds or until bleeding has stopped.

- Check the time so you can record it on your data form (Note: the person doing the venipuncture should wear a wristwatch, so that the time can be checked without touching anything).

- **DO NOT recap the needle**. Unglove one of your hands and remove the needle using a disinfected multi-tool or hemostats (not your hand). Immediately dispose of the needle directly into a sharps container.

- Use the clean hand to pick up a heparinized microtainer labeled "RBC". Use your gloved hand that is holding the syringe to slowly transfer the whole blood into the heparinized microtainer.

- Close the snap top lid with your clean hand and gently invert the microtainer two or three times to mix in the heparin to prevent coagulation and place the tube down temporarily in a location that has not been contaminated.

- If you collected too much blood to fit in a single heparinized microtainer, use as many heparinized microtainers as you need, always using your clean hand to hold the microtainer, cap it, and invert it.

- Dispose of the syringe in the trash bag and remove your other glove – both hands should now be ungloved and not contaminated.

- Recheck that the ID and date are correctly labeled on the tube.

- Record all the necessary information on the data collection form.

- Place the microtainers of blood in a zipper sealed plastic bag in a cooler for later processing. It is best if you can place a barrier, such as newspaper, between the zipper sealed plastic bag and the ice to prevent the cells in the sample from lysing.

<u>Multiple attempts to collect blood</u>

- An attempt is considered one full motion inward and outward with the needle, even if the needle does not leave the injection site. Any repositioning of the needle is considered another attempt and should not be done with the needle inside the skin.

- After each attempt to collect blood (with a maximum of 3 attempts in one session), if there is fluid in the syringe, follow the protocol above to transfer the fluid into a heparinized microtainer labeled “RBC”, and label the microtainers in the order in which their contents were drawn (1, 2, or 3). Also include on the label the letter “L” if the sample in that tube is contaminated with lymph.

- When completing the data collection form, record the total sample volume of all of the attempts combined, and circle the total number of tubes (microtainers) containing blood-related samples. There can be up to 4 tubes in total because in the case that you collect a clean sample of blood in your last attempt, you may have collected too much blood during that attempt to fit in a single microtainer so that sample will be divided into 2 tubes.

- Also record on the data collection form the estimated volume of lymph contained in each microtainer.

After blood sampling, collect all of the required morphological measurements. Record the end handling time when you have finished processing the tortoise and are returning it to its point of capture or placing it in a pen. If the tortoise voided during processing, continue to the rehydration protocol in Appendix F.6.

Appendix F.6: Rehydration

Options for fluid replacement include injectable epicoelomic fluids, nasal-oral offering of fluids, and soaking in a shallow water bath. Injectable epicoelomic fluids and nasal-oral administrations are the preferred two of the three methods of fluid replacement that the USFWS may authorize.

Fluids for rehydration are not prepared during pre-handling preparations for processing tortoises because only tortoises that void will be rehydrated. You can wait until you are ready to rehydrate the tortoise (i.e., after you have conducted all of the biological sampling) to prepare these fluids. However, before you rehydrate the tortoise, record a post-void weight on the data collection form.

<u>Injectable epicoelomic fluid administration</u>

Supplies for epicoelomic fluid administration

- 250ml bag of sterile saline (0.9% sodium chloride - to be kept in cooler)
- 20cc or 60cc luer lock syringe with 22G 1.5“, 22G 1”, or 25G 1” needle – syringe and needle sizes depends on size of tortoise

Tortoise MCL	Syringe size	Needle size	Total volume of sterile saline
150-180mm	20cc or 60cc	25G 1”	20ml/kg
180-200mm	60cc	22G 1.5” or 22G 1”	
200mm +	60cc	22G 1.5”	

Procedure for epicoelomic fluid administration

Epicoelomic describes a location between the coelomic cavity/membrane and the shell. This is a potential space similar to subcutaneous areas. The benefits of using this location versus other subcutaneous locations is that fluids in this area do not appear to affect mobility or the ability of the tortoise to withdraw its limbs into its shell prior to complete absorption of the fluids. This procedure has the benefit of providing a known amount of fluid replacement to the tortoise and does not require a lot of specialized equipment. It does, however, require advanced training and permitting by USFWS, if you would like this method to be among your options.

- Put on a fresh pair of gloves and select a syringe and needle based on tortoise size (see table for proper size). The needle size selected should go no further than the caudal aspect of the pectoral scute upon insertion.

- Use an alcohol swab to wipe the port on a 250ml bag of sterile saline, and insert the needle into the port. Be sure to insert the needle directly into the port and be careful not to puncture any part of the bag. If the bag becomes punctured, dispose of it, as well as the needle and syringe, and start fresh with sterile supplies.

- Draw up the total volume of sterile saline required for the tortoise’s size (see table for volume).

- Carefully withdraw the needle from the port and use a tool (not your hand) to remove the needle from the syringe. Dispose of the needle in the sharps container.

- The location of fluid administration is on the lateral aspect of the plastron anywhere from the caudal area of the pectoral scute to the cranial area of the abdominal scute, just inside the bridge.

- The handler should position the tortoise presenting the axillary fossa to the examiner. The handler can either restrain the forelimb into the fossa where the fluids are to be given or pull the forelimb outward from the fossa, forward and towards the head.

- Insert the needle bevel down in the lower outer aspect of the axillary fossa (above the ridge of bone at the skin junction) at approximately a 45˚ angle, directed caudally. When contact with the plastron bone is felt, move the needle caudally and parallel to the plastron and bridge.

- Pass the needle directly along the bones of the plastron to its appropriate depth (*i.e*. caudal aspect of pectoral scute).

- Pull the plunger out just slightly to ensure negative pressure occurs, and that no blood enters the hub. If there is no negative pressure or blood is seen in the hub, remove the needle from the tortoise, remove it from the syringe, and replace it with a new needle. Attempt to insert the needle into the tortoise again, aiming the needle more closely toward and in contact with the plastron.

- Once you have negative pressure in the syringe when pulling the plunger, steady the needle and inject the saline.

- Heavy pressure on the plunger may be needed to inject the fluids if a small needle size is used.

- If flow stops, slightly redirect the needle and continue administering the fluids.

<u>Nasal-oral fluid administration</u>
This procedure is easy to carry out with minimal equipment, but may not work for all tortoises. In such a case, use one of the other rehydration methods.

Supplies for nasal-oral fluid administration
- Syringe (any size, no needle)
- Fresh drinking water (20ml/kg)

Procedure for nasal-oral fluid administration
- The tortoise can be placed on the ground if it is not very active or the handler can hold the tortoise facing the examiner, near to and parallel with the ground, without restraining its legs.

- The examiner holds the water-filled syringe (no needle) facing downward just in front of the tortoise's face, very close to the nares and slowly pushes the plunger allowing the water to enter the nares and/or mouth.

- The tortoise will sometimes actively accept and drink the water, but if the tortoise refuses, do not force the tortoise to take in the water; use one of the alternate rehydration methods.

<u>Water bath</u>

Supplies for water bath rehydration

- Plastic tote slightly longer and wider than the tortoise
- Fresh drinking water

Procedure for water bath rehydration

- Place the tortoise in the tote and add water until it reaches the level between the skin-plastron junction and the chin.

- Allow the tortoise to soak for 30 minutes.

- Tortoises may drink water through their nose or mouth or absorb it through their cloaca.

- Tortoises may void more urine while being soaked in an effort to eliminate urine with high solute levels to replace it with the fresh water it is taking in. If a tortoise voids in its soaking tub and additional fresh water is available, the water should be discarded and replaced with fresh water until the completion of the 30 minutes. Otherwise the tortoise can be soaked for 30 minutes in the water that contains the voided urine and urates. At the end of the soak time, rinse the tortoise with fresh water to remove urine odor on the limbs and shell, which might attract predators.

<u>After rehydrating the tortoise</u>

- Complete the fields on the data collection form for hydration method, fluid type, and volume. Then weigh the tortoise again and record the post fluid weight.

- Record the end handling time now if you have finished processing the tortoise and are returning it to its point of capture or placing it in a pen.

Appendix F.7: Processing Biological Samples (After Handling the Tortoise)

Note: Change your gloves before processing the following samples to be sure they do not become contaminated.

Processing oral and cloacal swab samples

- Once you have completed all the biological sampling and rehydration (if necessary), cap the oral and cloacal swab samples.

- Hold the cotton tipped end of the swab inside the microtainer lifted just slightly off the bottom.

- Bend and break off the plastic stick so it will fit inside the vial when it is closed. You can also use a disinfected multi-tool to clip the stick.

- Close the snap tops tightly, make sure the vials are labeled properly, and place them in a zipper sealed plastic bag in the cooler.

- Recheck that the ID and date are correctly labeled on the tubes.

- Record on the data form the number of tubes containing oral and cloacal swab samples.

Processing nasal lavage samples

Note: The nasal lavage conical tube is considered contaminated so wear a glove on one hand while touching it and use your free hand to pick up the vials and pipet as needed to keep them free from contamination.

- Swirl the conical tube and take two LB cryovials from the cooler.

- Record on the data form the total amount of nasal lavage flushed and the estimated total collected after flushing.

- Make 2 aliquots by pipetting samples from the conical tube into the nasal LB cryovials at a 1:1 dilution
    - Pipet 0.75ml of the sample into the 1.8ml O-ring cryovial that contains LB (it has 0.75ml LB in it already so you can just add sample until the total amount of fluid in the vial is 1.5ml).

    - If you have extra sample fluids in the conical tube, pipet 0.75ml of the sample into the other 1.8ml cryovial that contains LB.

    - If you have a total of <0.75ml of sample in the conical tube after conducting the nasal lavage, pipet LB ***out of the cryovial*** into a trash bag until the amount of LB remaining in the cryovial equals the sample amount in the conical tube. Then pipet the sample into the cryovial.

- Secure the lids on the cryovials and recheck that the ID and date are correctly labeled on the tubes.

- Put the cryovials with the samples in a zipper sealed plastic bag on ice in the cooler. They must remain cold until they are placed directly in the freezer.

- Tighten the cap on the conical tube that contains the remaining flush and throw it away in a zipper sealed plastic bag. Put this in a trash bag, not the cooler, to make sure you don't confuse it with your samples later.

- Change gloves since the conical vial was considered contaminated.

- Record on the data form the total number of LB cryovials you collected with nasal lavage samples in them.

<u>Processing whole blood samples (at your vehicle or lab)</u>

Supplies for processing whole blood samples

- Centrifuge (with inverter, if at vehicle)
- 0.5ml O-ring cryovial with label for "Hep plasma UFL"
- 1.8ml O-ring cryovials with label for "Hep plasma"
- 1.8ml O-ring cryovials with label for "Hep plasma/lymph"
- Pipets

Procedure for processing whole blood samples

If you will be centrifuging blood samples in the field, test run the centrifuge from the vehicle's cigarette lighter using the inverter before your first day in the field. The centrifuge/inverter setup might not work on some older or smaller vehicles. Always try to keep the centrifuge cool and shaded.

- Centrifuge the blood as soon as possible after collecting the sample, and always within 4 hours, especially if the temperature is higher than 90F (32C). The longer the blood sits before centrifuging, the more likely it is to clot, which will render the sample useless.

- Before starting the centrifuge, ensure that the centrifuge is properly balanced by placing samples with the same amounts across from each other. If you do not have matching sample amounts, use tubes with water in the same amounts of the samples to balance the centrifuge.

- Record on the data form the time that you placed the samples in the centrifuge to spin (blood processing time).

- Spin the blood for the amount of time required by your centrifuge. This could be 3-10 minutes.

- While the blood is spinning, record the ID and date on the labels of the cryovials that you intend to use for each sample (one for "Hep plasma UFL", one to three for "Hep plasma" and/or "Hep plasma/lymph").

- When the centrifuge stops, record on the data form the plasma color of the sample you will use for the “Hep plasma UFL” cryovial. This should be the best sample you collected for each tortoise, meaning the one with the least lymph contamination.

- Use a pipet or syringe to draw out the plasma (clear layer on top of the red blood cells), being careful not to touch the red blood cells with the pipet. If you drop the sample with the lid closed or you touch the red blood cells with the pipet, re-centrifuge the sample before pipetting to ensure that no red blood cells are removed during the process. It is important to draw up only plasma with no red blood cells in it at all.

- Transfer 0.5ml of the plasma into a cryovial labeled “Hep plasma UFL”.

- Transfer the remaining plasma using the same pipet to cryovials labeled “Hep plasma” (or “Hep plasma/lymph” if applicable), and save the red blood cells in the original microtainer labeled “RBC”.

- Recheck that the ID and date are correctly labeled on all of the tubes (microtainers and cryovials).

- Record on the data form for each tortoise how may cryovials of plasma there are for UFL (UFL plasma aliquots), the total number of other cryovials containing plasma and plasma/lymph (USFWS plasma aliquots), and the number of heparinized microtainers you are saving that contain RBCs (all plasma should have been transferred out of these so only RBCs are left behind).

- Check the lids on the microtainers and cryovials several times to make sure they are closed tightly before placing them in a zipper sealed plastic bag.

- Freeze the samples immediately. If you do not have access to a freezer on site, place the samples in a cooler with dry ice and either transfer them to a freezer while they are still frozen or keep them on dry ice.
    - Note: If the freezer you will be using is a self-defrost freezer (like most residential freezers), be sure that it does not self-defrost at any time when there are samples inside it. Also remember not to leave samples in any location where they can become warm at any time and do not hold samples for more than a few seconds with warm fingers.

Appendix F.8: Shipping Samples

The samples that you collect will be shipped to two labs. If diagnostic tests will be performed as part of your study and they are not related to the required samples, please contact laboratories well in advance to determine if they have expertise performing the specific diagnostic tests you need, if they can accommodate your samples, and how they would like the samples to be collected, stored and shipped, as their protocols may differ from the ones presented here.

Shipping plasma samples to University of Florida for ELISA testing

- This protocol is only for the heparinized plasma samples in the 0.5ml cyrovials labeled "Hep plasma UFL".
- Samples should only be shipped to UFL Monday through Thursday.
- Call (352) 294-4086 or (352) 294-4071 before shipping to make sure someone will be in the lab to receive the samples the next day.
- Complete the UFL Mycoplasma Lab submission form.
    - All samples will be analyzed for antibodies to *Mycoplasma agassizii* and *Mycoplasma testudineum*. Mark the check boxes for ELISA *Mycoplasma agassizii and Mycoplasma testudineum*. Culture/PCR will not be done at this time.
    - If you complete the form by hand, you will need to scan it into a computer so you can email it to the lab after shipping the samples.
    - If you complete the form in Word or Excel, or on UFL's electronic form, you will also need to print it to include it in the shipment.
- If there is a time sensitive issue, such as relocation or temporary holding of animals, note it on the submission form so the lab can expedite your request whenever possible.
- Put the completed submission form inside a zipper sealed plastic bag.
- Put the samples in a separate zipper sealed plastic bag, making sure all caps are secure and cryovials are clearly labeled.
- Place the zipper sealed plastic bag with the samples in a small cooler with frozen gel packs. DO NOT use ice or any other substance that can melt into liquid. Note that you may ship the samples on dry ice.
- Place the zipper sealed plastic bag with the submission form in the cooler on top of the frozen gel packs and samples before sealing the package.
- Tape up the cooler or box. Note that hard sided coolers can usually be shipped without an additional cardboard box.

- Take the package to a FedEx office. Choose Priority Overnight for the delivery service. Sample shipments should always be shipped overnight to arrive at the lab no later than 11am unless otherwise instructed.

- Send to:

  Dr. Mary Brown
  University of Florida Mycoplasma Laboratory
  Room V2-234
  2015 SW Archer Rd
  Gainesville, FL 32608
  (352) 294-4086

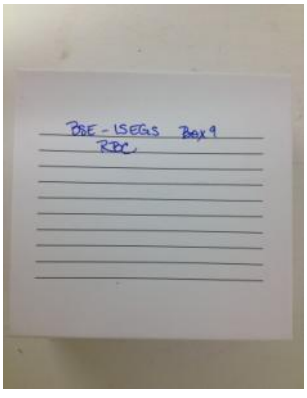
- After you ship the package, email the tracking number and a copy of the submission form as soon as possible to ALL of the following email address: mbbrown@ufl.edu, amburne87@gmail.com

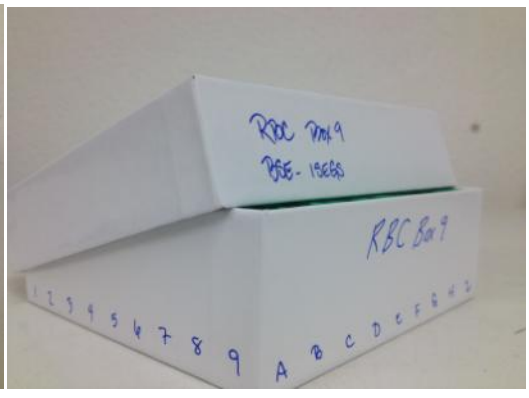
Shipping plasma, oral swab, nasal lavage, tick, cloacal swab, and RBC samples to the USFWS

- This protocol is for ALL samples EXCEPT the heparinized plasma samples in the 0.5ml cyrovials labeled "Hep plasma UFL".
- Samples should only be shipped to USFWS Monday through Thursday. Alternatively, arrangements can be made to drop off samples at the DTCC call 702-338-0104 to coordinate.
- Fill out the sample submission form (below).

Preparation for banking samples into freezer boxes

- Make sure all caps are secure and tubes are clearly labeled.
- As you are recording samples, place each sample in a labeled freezer box with 81 place dividers, organized by date, then by numerical order.
- The box should say the name of the project, the sample type it holds, and what number box it is if all the samples will not fit into one box.
- Box label should be on both the top of the lid and 1 side of the lid.
- The bottom of the box, when facing you will be lettered "A"-"I," and 1-9 when turned to the left, creating a grid "ID" for each slot used. See below





To bank all sample types:

- The sample log must be used to record all samples.
- Sample logs must have the following on each line for each sampled tortoise:
    - Bank location (bank location is the box number and box grid example: 1.1A-1.1I, 2.2A, etc).
    - ID #
    - Date sample was collected
    - Sample type
    - Project name on each line.

- Organize samples by date, then by numerical order.
- Record samples of the same type together, i.e. RBC with RBC, in a log for that sample type.
- Then, place the samples in the appropriate slots in the appropriate boxes.

- Place the banking boxes with the sample information in it on top of the gel packs and samples before sealing the package.

- Tape up the cooler or box. Note that hard sided coolers can usually be shipped without an additional cardboard box.

- Take the package to a FedEx office. Choose Priority Overnight for the delivery service. Sample shipments should always be shipped overnight to arrive at the office no later than 11am unless otherwise instructed.

- Send to:

  US Fish and Wildlife Office
  4701 N. Torrey Pines
  Las Vegas, NV 8913
  ATTENTION Desert Tortoise Conservation Center

- After you ship the package, call the DTCC Hotline at 702-488-8422 to inform staff that a package will be arriving at the USFWS office the following morning and provide the tracking number.

Sample Submission Form
USWFS Samples for Desert Tortoise Conservation Center

Use this form to submit up to 15 samples.

Project name			Site		BO #
PI name			Contact person name		
Contact phone			Contact email		
# plasma samples	# RBC samples	# nasal samples	# oral samples	# cloacal samples	# tick samples

Record each sample tube on a different line. For example, if Tortoise ID 001 has 3 sample tubes, then that tortoise ID should be recorded on 3 lines.

Desert Tortoise ID	Sample Date	Sample Type (plasma, RBC, oral, nasal, ticks)

Ship frozen samples overnight delivery (Mon thru Thur) in cooler to US Fish and Wildlife Service Field Office, 4701 North Torrey Pines, Las Vegas NV 89130, ATTENTION Desert Tortoise Conservation Center. Call the DTCC Hotline to inform them that you shipped the samples 702-488-9422.

Sample Submission Form for Tortoise Mycoplasma Testing
Mycoplasma Research Laboratory, University of Florida

Species of animal(s): _____

County and state of origin (free-ranging only): _____

Date of sample(s):_____

Identification #(s): _____

_____

Is this animal awaiting relocation? ☐ **Yes** ☐ **No** ☐

NOTE: Price discounts (20%) are available for larger numbers of samples (>20) that are submitted for testing or for large volume contracts. Large volume costs are reduced; discounted costs are $28/assay; $11 for dilution curve.

Test requested (please check appropriate box):

↑ ☐ **ELISA serology for *Mycoplasma agassizii*- $35 each.** This is the most commonly used test to detect exposure to *M. agassizii*, a cause of URTD in tortoises; submit serum or plasma.

↑↑☐ **ELISA serology for *Mycoplasma testudineum*- $35 each.** This test detects exposure to a second species of mycoplasma that can be associated with URTD and eye infections; submit serum or plasma. Please note that this test has not been extensively validated at this time.

↑↑☐ **ELISA serum dilution curves - $14 each**

↑ ☐ **Culture/PCR- $35 each.** To avoid false negative results, this test is recommended primarily for clinically ill animals with active nasal discharge; submit nasal lavage sample.

↑ ☐ **PCR sequence if amplicon obtained and RFLP suggests new species- $35.**

Person submitting sample(s): _____

Report results to:_____

Phone: _____ **Email** _____ **Fax:** _____

Preferred method of getting results (check one)↑ ☐ **Email** ↑ ☐**Fax** ↑ ☐**Mail**

Preferred method of billing (check one)↑ ☐ **Email** ↑ ☐**Fax** ↑ ☐**Mail**

Name & address of person to be billed:

_____

_____

_____

Email contacts: mbbrown@ufl.edu; amburne87@gmail.com

Please ship samples from Monday to Thursday only:

Dr. Mary Brown
University of Florida
Dept. of Infectious Disease and Pathology
2015 SW 16th Ave
Room V2-234/232
Gainesville, FL 32608
Telephone: (352) 294-4071 or 294-4086
Fax: (352) 392-9704

*****Please label tubes with investigator's name, tortoise species, tortoise identification name/number, and date sample collected.**

*****NOTE: Federal Express is the only carrier that delivers directly to our laboratory. Other carriers deliver to the VMTH, and samples may take 1-2 days to be delivered to our lab. In such cases, the quality of the samples may be compromised.**

For laboratory use only

Date Received:_____ Samples cold: ☐Yes ☐ No

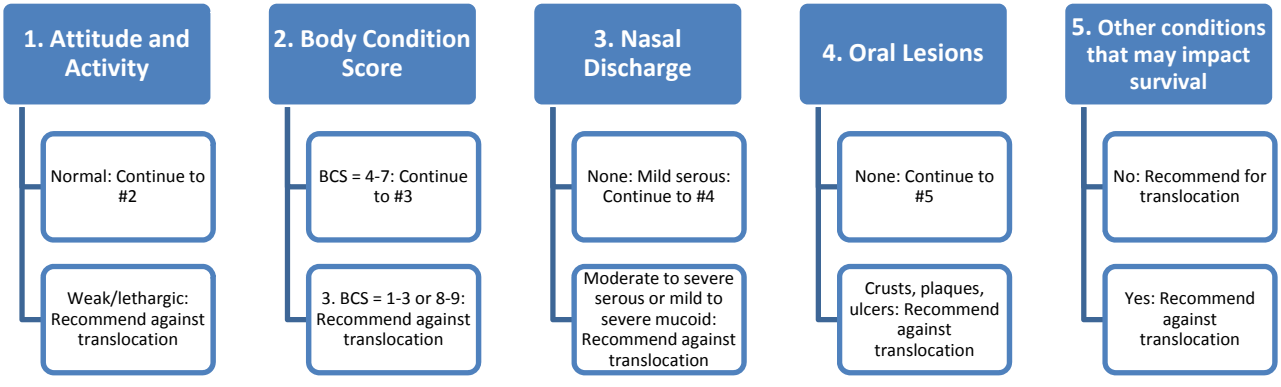
No. ELISA: Comments:

Appendix G. Algorithm for Evaluating if Desert Tortoises are Suitable for Translocation

Goals:

Relocate individuals that have high chances of survival.

Minimize the risk of spreading disease



All recommendations regarding translocation are to be recorded in the proposed disposition plan and submitted to the U.S. Fish and Wildlife Service's Desert Tortoise Recovery Office prior to relocating any tortoises.

Appendix H: Disposition Plan Template

ID	Sex	MCL	Mass	Attitude / behavior	BCS	Nasal discharge	Severity	Oral lesions	Other defect	MYAG ELISA Titer	MYTE ELISA Titer	Photos	Comments	Recommended disposition	Proposed release location (descriptive + UTMs)
##	M	##	##	Normal	1-9	None		None	No	<32	32	File #s		translocate	
	F			Lethargic /weak		Serous	1, 2, or 3	Crusts[1]	Yes[4]	64	128			on-site quarantine	
	U					Mucous	1, 2, or 3	Plaques[2]		32	64			requires further evaluation by FWS	
								Ulcers[3]							

[1]Crust = an outer layer of solid material formed by drying of a bodily exudate or secretion

[2]Plaque = any patch or flat area, in the oral cavity, plaques tend to have a white or yellow appearance with a dry surface compared to the pink moist tongue or mucous membrane

[3]Ulcer = a localized defect or excavation of the surface of a tissue, usually produced by sloughing of necrotic inflammatory tissue

[4]Describe in Comments