Presentation by Dr. Kristin H. Berry (U.S. Geological Survey, Biological Resources Division): Field Evaluations of Desert Tortoises for Health and Disease; Effects of Diseases on Populations

- **The Desert Tortoise and Upper Respiratory Tract Disease. (Jacobson, E. Prepared for the Desert Tortoise Preserve Committee, Inc., and the U.S. Bureau of Land Management. Revised November 1992)**

- **Mycoplasma agassizii Causes Upper Respiratory Tract Disease in the Desert Tortoise. (Brown, M.B., I.M. Schumacher, P.A. Klein, K. Harris, T. Correll, and E.R. Jacobson. Oct. 1994. Infection and Immunity, 62:4580-4586)**

- **Seroepidemiology of Upper Respiratory Tract Disease in the Desert Tortoise in the Western Mojave Desert of California. (Brown, M.B., K.H. Berry, I.M. Schumacher, K.A. Nagy, M.M. Christopher, and P.A. Klein. Oct. 1999. Journal of Wildlife Diseases. 35:716-727)**

- **Pathology of Diseases in Wild Desert Tortoises From California. (Homer, B.L., K.H. Berry, M.B. Brown, G. Ellis, and E.R. Jacobson. 1998. Journal of Wildlife Diseases. 35:508-523)**

- **Guidelines for the Field Evaluation of Desert Tortoise Health and Disease. (Berry, K.H. and Christopher, M.M. 2001. Journal of Wildlife Diseases. 37(3):427-450)**

The Desert Tortoise and Upper Respiratory Tract Disease

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BACKGROUND -- UPPER RESPIRATORY TRACT DISEASE IN CAPTIVE TORTOISES

A disease characterized by a mild to severe nasal discharge has been seen for many years in captive tortoises in Europe, England, and the United States. Although a complete list of the number of species of tortoises known to develop this disease is unavailable, it would be fair to say that until proven otherwise, all species of tortoises should be considered susceptible. In England, this disease is commonly seen in Greek (*Testudo graeca*) and Hermann's (*T. hermanni*) tortoises.[1] The disease has also been seen in free-ranging gopher tortoises (*Gopherus polyphemus*) in Florida (Jacobson, pers. comm.). At the Veterinary Medical Teaching Hospital, University of Florida, species of tortoises presented with nasal discharge include Greek tortoises, leopard tortoises *Geochelone pardalis*), radiated tortoises (*Geochelone radiata*), Indian star tortoises (*Geochelone elegans*) and gopher tortoises (*Gopherus polyphemus*). The disease has also been commonly seen in captive desert tortoises (*Gopherus [=Xerobates] agassizii*).[2]

Until 1990-1991, attempts at demonstrating or incriminating a casual agent were unsuccessful. Because of negative findings and the failure to incriminate a specific bacteria, a virus was considered as a possible cause.[3] In studies conducted on captive desert tortoises, a bacterial organism, *Pasteurella testudinis*, was isolated and incriminated as a possible cause.[4] However, *P. testudinis*, has also been isolated from healthy tortoises and the significance of this organism remains unknown.

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[1]Lawrence, K. and J.R. Needham. 1985. Rhinitis in long term Mediterranean tortoises (*Testudo gracea* and *T. hermanni*). Veterinary Record. 117:622-664.

[2]Jackson, O.F., and J.R. Needham. 1983. Rhinitis and virus antibody titers in chelonians. Journal of Small Animal Practice. 24:31-36.

[3]Snipes K.P., E.L. Biberstein, and M.E. Fowler. 1980. A *Pasteurella* sp. associated with respiratory disease in captive desert tortoises. Journal of the American Veterinary Medical Association. 177:804-807.

[4]Snipes, K.P., and E.L. Biberstein. 1982. *Pasteurella testudinis* sp. nov.: a parasite of desert tortoises. International Journal of Systematic Bacteriology. 32:201-210.

THE APPEARANCE OF UPPER RESPIRATORY TRACT DISEASE IN WILD TORTOISE POPULATIONS

In the 1970's desert tortoises with signs of the disease were observed on the Beaver Dam Slope of Utah, a site where many captive tortoises were being released. In 1988, desert tortoises at the Desert Tortoise Natural Area (DTNA), Kern County, California were seen with clinical signs of illness similar to that of captive desert tortoises. Signs included a mucopurulent discharge from the nares, puffy eyelids, eyes recessed into the orbits, and dullness to the skin and scutes. Based upon these clinical signs, Upper Respiratory Disease Syndrome (URDS) was used to characterize this syndrome.

Surveys of the DTNA in 1989 and 1990 revealed that many tortoises were ill with the disease, and shells of many tortoises indicated a major die-off was underway. Research on long-term study plots with marked tortoises showed that more than 70% of adult tortoises died between 1988 and 1992 (Kristin Berry, pers. comm.). Other surveys indicate that wild desert tortoises with URDS are also widespread in the western Mojave Desert of California, around Las Vegas Valley in Nevada, and on the Beaver Dam Slope of Utah and Arizona.

RESEARCH ON THE CAUSES OF UPPER RESPIRATORY TRACT DISEASE

In May 1989, with a contract from the U.S. Bureau of Land Management, we initiated studies on desert tortoises ill with URDS in an attempt to elucidate the responsible pathogens. During the course of these studies, the pathology of the disease was better understood and findings indicated that the upper respiratory tract was the major site of involvement.[5] Based on these findings the disease was determined to be a chronic upper respiratory tract disease and the acronym URTD was used. Today, URTD more appropriately designates this illness and should replace URDS.

Microbiologic investigations with URTD failed to incriminate a virus as a potential causal agent. *Pasteurella testudinis* was isolated from most of the ill tortoises examined and a previously unidentified *Mycoplasma* was also isolated from ill tortoises.[5] Electron microscopic studies confirmed the presence of *Mycoplasma* on the surface membranes of the upper respiratory tract of desert tortoises ill with URTD.

In 1992, research was conducted on transmission of the disease. The findings support the contention that *Mycoplasma* is the most likely cause of URTD. Koch's postulates have

[5]Jacobson, E.R., J.M. Gaskin, M.B. Brown, R.K. Harris, C.H. Gardiner, J.L. LaPointe, H.P. Adams, and C. Reggiardo. 1991. Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). Journal of Wildlife Diseases 27(2):296-316.

been fulfilled and a causal relationship between *Mycoplasma* and URTD has been established. Still, *Pasteurella* and other bacteria may affect the severity of the disease.

A serologic (blood) test has been developed at the University of Florida to determine exposure status of tortoises to *Mycoplasma*. Preliminary studies are very promising in that this test may ultimately be useful in assessing condition of tortoises.

Predisposing factors such as poor nutrition (resulting from habitat degradation), drought, and release of captive desert tortoises ill with URTD into the wild are also more than likely involved. The whole issue of release of ill pet desert tortoises needs to be publicized, because this practice should not continue. Transmission studies have clearly demonstrated the infectious nature of URTD. Thus, it is safe to assume that captive ill tortoises can transmit this disease to both captive and free-ranging clinically healthy tortoises.

TREATMENT OF UPPER RESPIRATORY TRACT DISEASE

Until recently, no antibiotics or combination of antibiotics have been efficacious for treating tortoises ill with URTD. With evidence that *Mycoplasma* is the etiologic agent of URTD and that *Pasteurella testudinis* and other gram negative bacteria may contribute to the severity of the disease, antibiotic therapy with enrofloxacin (Baytril, Mobay Corp., Shawnee, Kansas) at 5 mg/kg of body weight every other day for 10 treatments, is considered the therapy of choice. Additionally, injectable enrofloxacin should be diluted 1:10 in sterile saline and a small quantity (up to 0.5 cc) should be flushed up both nares of the affected tortoise utilizing a syringe and attached catheter of appropriate diameter. Flushing should be continued daily for 1 month (at least until the rhinitis has abated). Since enrofloxacin is very irritating to the mucous membranes surrounding the eyes, it is important to avoid contact of enrofloxacin with those tissues. It is important to maintain tortoises at an optimum environmental temperature during the course of treatment. While antibiotic therapy may result in clinical improvement and complete regression of clinical signs, this does not mean that this tortoise will be free of disease thereafter. Turtles may remain carriers of *Mycoplasma* for life with recurrence of the disease at some point in time in the future.

Results of clinical trials with these new drugs and drug combinations for treating tortoises ill with URTD are extremely promising for captive tortoises. Unfortunately the situation for ill free-ranging tortoises in not as promising. Since this disease more than likely is multifactorial, schemes for managing URTD in free-ranging populations are going to be difficult to develop and implement. Minimally tortoise hobbyists and veterinarians can make

a major contribution by getting the word out that captive tortoises should not be released to the wild. More than likely this practice has contributed to the spread of URTD in wild populations of desert tortoises.

SUMMARY

The following points should be remembered with regard to the desert tortoise and URTD:

1. URTD is a chronic infectious disease affecting not only the desert tortoise, but other tortoises as well.

2. Scientific evidence supports the belief that *Mycoplasma* is the infectious agent responsible for URTD.

3. Once infected with *Mycoplasma*, a tortoise may remain a carrier for life.

4. URTD is a transmissible disease. Because of this, tortoises showing clinical signs of illness should be isolated from healthy tortoises.

5. Different species of tortoises should not be kept together in captivity since foreign pathogens may be introduced into new hosts.

6. Although antibiotic treatment may result in complete remission of clinical signs, tortoises may still develop the disease at a future date.

7. Ill or formerly ill desert tortoises should never be released to the wild. Releases of captive tortoises may be responsible for disease outbreaks in the Mojave Desert.

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Mycoplasma agassizii Causes Upper Respiratory Tract Disease in the Desert Tortoise†

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The desert tortoise is listed by the United States government as a threatened species in part of its range. A major contributing factor in the decline of this animal has been the presence of an upper respiratory tract disease (URTD) which is characterized by a chronic disease which eventually leads to severe occlusion of the nares with viscous exudate and destruction of the respiratory epithelium. Electron microscopy of infected tissues demonstrated the presence of a mycoplasma-like organism attached to the respiratory surfaces. The mycoplasma was isolated and designated as a new species, with the proposed name *Mycoplasma agassizii*. The current study was designed to fulfill Koch's postulates and determine if *M. agassizii* was the etiologic agent of URTD. Clinically healthy animals with known antibody status were infused intranasally with pooled exudate ($n = 8$) from ill donor animals, with *M. agassizii* alone ($n = 9$) or in combination with *Pasteurella testudinis* ($n = 8$), with *P. testudinis* alone ($n = 9$), or with sterile broth ($n = 12$). The pooled exudate was culture positive for *M. agassizii*. Tortoises which received exudate or *M. agassizii* alone or in conjunction with *P. testudinis* were significantly more likely to develop clinical disease ($P < 0.0004$) than animals which received *P. testudinis* alone or the broth controls. Tortoises demonstrated a strong immune response to *M. agassizii*, and seroconversion was seen in all groups with clinical disease. *M. agassizii* was isolated from the upper respiratory tracts of clinically ill animals up to 6 months postinfection. On the basis of the results of these transmission studies, we conclude that *M. agassizii* is an etiologic agent of URTD in the desert tortoise.

Dramatic declines in the population of the desert tortoise, *Gopherus agassizii*, in the western Mojave Desert over the preceding 10 years resulted in designation of the animal by the Fish and Wildlife Service as environmentally threatened in 1990. Although a number of factors including habitat destruction, drought, and predation have contributed to the population decline, the appearance of an upper respiratory tract disease (URTD) was associated with major losses (8, 12). URTD has been seen in both captive (16) and wild (8) desert tortoises in the southwest United States and by us in captive and wild gopher tortoises (*Gopherus polyphemus*) in Florida. A similar disease has been seen in a variety of captive tortoises imported into England (9) and in tortoises submitted to the Veterinary Medical Teaching Hospital, University of Florida, of the following types: red-footed tortoise (*Geochelone carbonaria*), leopard tortoise (*Geochelone pardalis*), Indian starred tortoise (*Geochelone elegans*), and radiated tortoise (*Geochelone radiata*). In desert tortoises and other tortoises, the disease is seen as a rhinitis characterized by an intermittent serous discharge flowing or bubbling from the nares. On some days the nares will appear dry. As the disease progresses, the discharge becomes more tenacious and contains large numbers of inflammatory cells and bacteria.

Fowler examined desert tortoises with respiratory disease and concluded that no single microorganism was responsible (4). In his opinion, continued stress, especially resulting from malnutrition, was primarily responsible for the development of the disease. In another study, no major differences were observed in bacterial isolates from the respiratory tracts of captive and free-ranging desert tortoises (16). A bacterium belonging to the genus *Pasteurella* was isolated from the respiratory tract of both groups of tortoises, and eventually species status was proposed for these isolates under the name *Pasteurella testudinis* sp. nov. (15). Since this microorganism had been isolated from the respiratory tracts of ill and healthy desert tortoises, its significance was unknown.

Rhinitis also has been seen in long-term captive Mediterranean tortoises (*Testudo gareca* and *Testudo hermanni*), and a variety of microorganisms have been isolated from both ill and healthy tortoises of these species (9). As with desert tortoises, no major differences in bacterial isolations were noted. Because in many cases nasal swabs taken from tortoises with rhinitis do not yield bacteria, some investigators have suspected a viral agent as the cause of this disease (7). In another report, although not isolated from tortoises with rhinitis, mycoplasma was listed as a potential suspect microorganism (9).

In the 1970s, desert tortoises with signs of URTD were observed on the Beaver Dam Slope of Utah, a site where many captive desert tortoises were being released. In 1988, desert tortoises at the Desert Tortoise Natural Area, Kern County, Calif., were seen with clinical signs of illness similar to that of captive desert tortoises. Signs included a mucopurulent discharge from the nares, puffy eyelids, eyes recessed into the orbits, and dullness of the skin and scutes.

Recently, in examining free-ranging desert tortoises with URTD, we identified by electron microscopy a microorganism compatible with mycoplasma on the surface of the nasal

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mucosa of affected tortoises (8). A mycoplasma species (proposed *Mycoplasma agassizii* sp. nov.) was cultured from the nasal passageways of ill tortoises and was ultrastructurally similar to the pleomorphic microorganism seen in tissue section. The detailed description of this glucose-fermenting mycoplasma will be the subject of a later publication; however, the sequence of the 16S rRNA gene (GenBank accession number U09786) confirms that *M. agassizii* is a new species. Specific antibody to the mycoplasmal isolate correlated with the presence of lesions in the nasal mucosa (14), strongly suggesting an etiologic role for the mycoplasma.

Because of the previous high isolation rates of *P. testudinis* and the documented synergism of other infectious agents, including *Pasteurella* species, with mycoplasmas (1, 5, 13, 19), we designed a transmission study to determine if *M. agassizii* or *P. testudinis*, alone or in concert, could cause URTD in the desert tortoise.

TABLE 1. Summary of treatment groups used in transmission study

Group	No. of tortoises	Treatment[a]
I	9	0.5 ml of intranasal *M. agassizii* (10^9 CCU/ml)
II	9	0.5 ml of intranasal *P. testudinis* (10^9 CFU/ml)
III	8	0.25 ml of intranasal *M. agassizii* (10^9 CCU/ml) and 0.25 ml of intranasal *P. testudinis* (10^9 CFU/ml)
IV	9	0.5 ml of intranasal exudate (pooled nasal secretions)
V	12	Control[b]

[a] Tortoises were infused in the nares with infectious agent, exudate, or sterile broth control in the volumes noted.

[b] The control group contained tortoises which were infused with 0.5 ml of intranasal sterile TSB used to grow *P. testudinis* (n = 4), 0.5 ml of intranasal sterile SP4 broth used to grow *M. agassizii* (*n* = 3), or no infusion (no intranasal challenge) (*n* = 5).

MATERIALS AND METHODS

Tortoises. Sixty adult desert tortoises of both sexes and various weights (range, 1.5 to 4.6 kg) were acquired from the Desert Tortoise Conservation Center, Las Vegas, Nev., under special permit from the Fish and Wildlife Service. All tortoises were originally collected as wild tortoises in Las Vegas Valley and were considered healthy on the basis of physical examination and absence of clinical illness.

Individual outdoor pens (12 m^2) were constructed in groups of five around palo verde trees at a remote site at The Living Desert Museum, Palm Desert, Calif. All pens were placed in a circle around the tree and were separated from adjacent pens by a solid metal barrier. All pens around a single tree composed a treatment group. Tortoises were fed daily a mixture of commercially available vegetables and had access to native vegetation growing in their pens. Water was provided ad libitum.

Tortoises were acclimated for 10 months prior to initiation of the study and allowed to undergo normal hibernation prior to initiation of the transmission study. During the acclimation period, eight tortoises became clinically ill and were removed from the study, one tortoise died, and one tortoise required euthanasia as a result of severe debilitation.

Prior to challenge, each tortoise was restrained manually and 2 ml of blood was collected from the jugular vein. Blood was added to lithium heparin microtainer tubes (Becton Dickinson, Franklin Lakes, N.J.) and inverted gently several times to prevent coagulation. Packed cell volumes were determined on a portion of the sample; the remainder of the blood was centrifuged, and the plasma was removed and frozen in cryotubes in liquid nitrogen for future antibody determinations. The area around the external nares was cleaned with sterile saline. Nasal washes were collected by gently flushing the nares with 1 ml of sterile tryptose broth (Remel Laboratories, Lenexa, Kans.) via a catheter attached to a syringe. Following collection, the nasal washes were placed in cryotubes, frozen in liquid nitrogen, and transported on dry ice to the University of Florida for processing.

Because the antibody level of each animal was known prior to the start of the infection study and because of the limited number of animals available, we were unable to completely randomize treatment. Pen groups with the lowest preexisting levels of antibody to *M. agassizii* were chosen to receive the critical infections of *M. agassizii* or *P. testudinis*.

Infection groups. Treatment groups are summarized in Table 1. Group I animals (*n* = 9) received 0.5 ml (0.25 ml per nostril) of *M. agassizii* grown in SP4 broth (18) to a concentration of 10^9 color-changing units per ml. The *M. agassizii* strain used was originally isolated from the nares of a desert tortoise with URTD (8), was filter cloned, and was two passages from the primary isolation. The purity of the isolate was determined on the basis of immunostaining and 16S rRNA sequence analysis. Group II animals (*n* = 9) received 0.5 ml (0.25 ml per nostril) of *P. testudinis* grown in brain heart infusion broth to a concentration of 10^9 CFU/ml. The *P. testudinis* strain used was originally isolated from the nares of a desert tortoise with URTD (16). Group III animals (*n* = 8) received 0.5 ml (0.25 ml per nostril) of an equal mixture of *M. agassizii* and *P. testudinis*. Because of the possibility that an unidentified infectious agent was responsible for the disease, group IV animals (*n* = 9) received 0.5 ml (0.25 ml per nostril) of exudate (pooled nasal secretions) derived from desert tortoises with active clinical URTD. *M. agassizii* was cultured from these exudates. Group V animals were control tortoises which received either 0.5 ml (0.25 ml per nostril) of tryptose soy broth (*n* = 4), SP4 broth (*n* = 3), or no intranasal challenge of any type (*n* = 5). For all groups, nasal infusions were performed via a catheter connected to a 1-ml syringe.

One additional group of tortoises (*n* = 4) in which one animal had high levels of antibodies to *M. agassizii* but never had any clinical evidence of illness was identified. To determine if this animal was an active carrier, the solid metal barriers separating adjacent pens were removed and tortoises were allowed to have both visual and physical contact through the fencing material. These tortoises did not receive any treatments. Although not technically part of the transmission study, these tortoises were observed for clinical signs and antibody production.

Postinfection monitoring of tortoises. Following infection, all tortoises were observed daily for clinical signs of URTD. Clinical signs of URTD included lethargy, change in behavioral and feeding habits, and physical signs such as runny or wet noses. At 1, 3, and 6 months postinfection, blood was obtained and processed as described above. In tortoises with rhinitis, nasal washings were collected and processed as described above.

Microbial cultures. Nasal washes were received at the University of Florida frozen on dry ice. Samples were placed at −70°C until processing. Samples were thawed at room temperature. Half of the sample was submitted to the Clinical Microbiology Laboratory, College of Veterinary Medicine, for routine aerobic cultures. The remainder of the wash was cultured for mycoplasmas. The wash was serially diluted 10-fold in SP4 broth, and a running drop was placed on SP4

agar (18). Because of potential bacterial contamination due to field sampling, a portion of the first dilution in SP4 broth also was filtered through a 0.45-μm filter. Broth cultures were incubated at 30°C in ambient air. Agar was incubated at 30°C under 5% CO_2. Broth cultures were checked daily for acid production indicative of growth; agar plates were checked for growth at weekly intervals. All cultures were held for a minimum of 6 weeks. Isolations were most commonly seen at 3 weeks. Species confirmation was by growth inhibition with rabbit antiserum specific for *M. agassizii*. This antiserum did not cross-react with other known glucose-fermenting mycoplasmas, including *Mycoplasma testudinis*, *Mycoplasma pulmonis*, *Mycoplasma gallisepticum*, and *Mycoplasma pneumoniae*. In addition, selected isolates were confirmed on the basis of PCR amplification of the 16S rRNA gene followed by partial sequencing of the variable region of the gene.

ELISA procedure. The enzyme-linked immunosorbent assay (ELISA) procedure was performed as previously described (14). Briefly, *M. agassizii* PS6 was grown to mid-logarithmic phase in SP4 broth (18). Antigen was prepared by lysis of the cells in 0.05 M carbonate buffer, pH 10, for 30 min at 37°C followed by boric acid neutralization. The lysate was adjusted to 100 μg of protein per ml and stored at −70°C until use.

Each well of a microtiter plate (Maxisorp F96; Nunc, Kamstrup, Denmark) was coated with 50 μl of antigen at a concentration of 10 μg/ml in phosphate-buffered saline (PBS) containing 0.02% NaN_3 (PBS/A) and incubated at 4°C overnight. The wells were washed four times with PBS/A containing 0.05% Tween 20 (PBSA-T) by an automatic ELISA washer (EAW II; SLT-Labinstruments, 5082 Groedig/Salzburg, Austria) and then blocked with 250 μl of PBS/A containing 1% bovine serum albumin per well at room temperature for 60 min or at 4°C overnight. After four more washes, 50-μl samples of plasma diluted 1:2 and 1:10 with PBS/A were added to individual wells in duplicate and incubated at room temperature for 60 min. The wells were washed again as described. Portions (50 μl) of a biotinylated monoclonal antibody against desert tortoise immunoglobulin Y diluted 1:500 in PBS/A were added to the appropriate wells and incubated at room temperature for 60 min. After four washes, the wells were filled with 50 μl of alkaline phosphatase-labeled streptavidin (AP-Streptavidin) (Zymed Laboratories, Inc., San Francisco, Calif.) (1:1,000 dilution in PBS/A) and incubated at room temperature for 60 min. After the wells were washed four times with PBS-T, 50 μl of *p*-nitrophenyl phosphate disodium (Sigma, St. Louis, Mo.) (1 mg/ml prepared in 0.01 M sodium bicarbonate buffer, pH 9.6, containing 2 mM $MgCl_2$) was added to each well and incubated in the dark at room temperature for 60 min. The A_{405} of each well was determined in an ELISA plate reader (EAR 400 AT; SLT-Labinstruments). In each assay, the blank was the mean of two wells coated with antigen and incubated with the conjugate and the substrate only. Plasma of a desert tortoise, culture negative for *M. agassizii* and free of lesions indicative of URTD, was the negative control. Plasma from a desert tortoise infected with *M. agassizii* and having lesions indicative of URTD was the positive control. Positive and negative controls were included on each plate to determine interplate variation. All antibodies used in the ELISA were biotinylated. Antibody levels were expressed as the ratio of the A_{405} of the sample to the A_{405} of the negative control (8).

Pathological evaluations. Representative tortoises (n = 4) from each infection group were euthanatized with a concentrated barbiturate solution and necropsied. The head of each tortoise was bisected longitudinally. Swab specimens of the nasal mucosa were taken for mycoplasmal and microbial isolation. One side of each bisected head was fixed in neutral buffered 10% formalin, decalcified, embedded in paraffin, sectioned longitudinally at 7 μm, stained with hematoxylin and eosin, and examined by light microscopy. All samples were examined by a single pathologist, and the infection group to which a section belonged was not known. Lesions were classified as normal (no lesion), mild (no edema, limited numbers of inflammatory cells), moderate (moderate edema, moderate numbers of inflammatory cells with occasional incursion into the overlying mucosa, some disorganization of basal epithelium), and severe (marked edema, large numbers of inflammatory cells with incursion into the overlying mucosa, marked degeneration and necrosis of the basal epithelium).

TABLE 2. Outcome of transmission study to determine pathogenicity of *M. agassizii* and *P. testudinis* in the desert tortoise

Infection group and treatment[a]	Ratio with clinical signs (%)[b]	Ratio with seroconversion (%)[c]	Ratio with *M. agassizii* isolation (%)[d]
I MA alone	8/9 (89)	9/9 (100)	5/7 (71)
II PT alone	1/9 (11)	1/9 (11)	0/1 (0)
III MA + PT	7/8 (87)	8/8 (100)	3/5 (60)
IV Exudate[e] (n = 8)	8/8 (100)	6/8 (75)	5/7 (71)
V Control[f] (n = 12)	3/12 (25)	2/12 (17)	0/3 (0)
Broth (n = 7)	2/7 (29)	1/7 (14)	0/2 (0)
No treatment (n = 5)	1/5 (20)	1/5 (20)	0/1 (0)

[a] MA, *M. agassizii*; PT, *P. testudinis*.
[b] Groups receiving exudate or *M. agassizii* alone or in conjunction with *P. testudinis* were more likely to develop clinical disease, $P < 0.0004$; number with signs/number in group.
[c] Number that seroconverted/number in group. An animal was deemed to have seroconverted if the specific antibody as measured by ELISA either (i) increased from a negative to a positive value or (ii) the value increased by >0.1 ELISA unit during the course of the study.
[d] Isolation of *M. agassizii* was determined on the basis of direct culture of nasal flushes obtained from live animals which showed active clinical signs and is expressed as number with *M. agassizii*/number with active clinical signs. The isolates were confirmed as *M. agassizii* by a positive reaction with specific antiserum.
[e] One tortoise which received exudate died.
[f] Tortoises in the control group received either sterile broth or no infusions.

Statistical analysis. Binomial data were analyzed by the chi-square test. All other data were analyzed by analysis of variance, followed by Duncan's multiple range test used to determine differences among means. A P value of ≤0.05 was accepted as significant.

RESULTS

Clinical disease observations. Tortoises which received exudate or *M. agassizii*, alone or in conjunction with *P. testudinis*, were more likely to develop clinical disease ($P < 0.0004$ [Table 2]). Control groups and tortoises which received *P. testudinis* alone did not develop clinical disease.

All but one tortoise which received *M. agassizii* (group I) developed clinical disease, but only three tortoises were reported to be down (inactive or spending excessive time in their burrows) during the study. *M. agassizii* was recovered from five of seven tortoises with clinical disease in group I. Seroconversion to *M. agassizii* was seen in all group I tortoises (Table 2).

Infection with *P. testudinis* alone (group II) did not result in clinical disease (Table 2). One tortoise in this group had clinical signs of disease; however, this tortoise also demonstrated a rise in levels of antibody to *M. agassizii* a few weeks after clinical signs of illness were noted. However, attempts to isolate the mycoplasma were unsuccessful.

Although no tortoises which received both *M. agassizii* and *P. testudinis* (group III) were reported as down, seven of eight did develop clinical disease. It should be noted that these animals received half the amount of *M. agassizii* that animals in group I received, which could explain the less severe disease seen in this group. Seroconversion to *M. agassizii* was seen in all group III tortoises (Table 2).

Tortoises which received exudate (group IV) developed the most severe clinical signs. Six of eight tortoises were reported as down at some point during the study. One animal died but was not available for necropsy. Only two animals in group IV failed to demonstrate increased levels of antibody to *M. agassizii*. Both of these tortoises had initial levels of antibody to *M. agassizii* which remained stable and did not increase or decrease significantly.

No significant disease was seen in control animals (group V). Two broth control tortoises developed clinical signs of disease; signs were transitory in one animal but persistent in the other. Neither animal developed antibody to *M. agassizii* or had the mycoplasma isolated from nasal washes. One control animal which received no broth or other treatment developed clinical signs and demonstrated a rise in levels of antibody to *M. agassizii*, but attempts to culture *M. agassizii* were unsuccessful. This was the only control tortoise to be reported down and not active during the study.

Histopathology and lesion analysis. No significant lesions were observed in the control animals. Severe lesions were seen in all tortoises which received exudate ($n = 4$). In the group which received *M. agassizii* alone, severe lesions were seen in one, moderate lesions were seen in two animals, and mild lesions were seen in one animal. In the group which received both *M. agassizii* and *P. testudinis*, moderate lesions were observed in all tortoises.

Sections from the mucous and olfactory mucosae of normal control tortoises (Fig. 1) showed occasional small subepithelial lymphoid aggregates. Heterophils were rarely observed in the laminae propriae. No changes in the mucosal or glandular epithelia were noted, and no edema was observed. A lesion was characterized as mild inflammation (Fig. 2A) if multifocal, small subepithelial lymphoid aggregates were seen, if the lamina propria contained multifocal small numbers of heterophils, lymphocytes, and plasma cells, and if mild edema was seen in the lamina propria and minimal changes were noted in the mucosal epithelium. A lesion was classified as moderate (Fig. 2B) if multifocal to focally extensive lymphoid aggregates were observed, if diffuse, moderate numbers of heterophils, lymphocytes, and plasma cells which occasionally infiltrated the overlying mucosal epithelium were seen, and if moderate edema was noted in the lamina propria with proliferation and disorganization of the basal epithelium. A lesion was classed as severe (Fig. 2C) if focally extensive to diffuse bands of lymphocytes and plasma cells subjacent to and obscuring the overlying mucosal epithelium were seen, if large numbers of heterophils were found in the lamina propria and infiltrated the overlying mucosal epithelium, if the lamina propria was characterized by marked edema, if there was degeneration, necrosis, and loss of the mucosal epithelium with occasional erosion, and if proliferation of the basal cells of the epithelium with metaplasia of the mucous and olfactory epithelium to a basaloid epithelium was observed; occasional squamous metaplasia was also noted.

Antibody levels. A significant ($P < 0.004$) rise in antibody to *M. agassizii* was observed in tortoises receiving exudate as early as 1 month postchallenge (Fig. 3). Tortoises which received *M. agassizii* alone or in conjunction with *P. testudinis* also developed an antibody response, but the response was not observed as early as with exudate-challenged animals. By 3 months postinfection, tortoises which received *M. agassizii* (groups I and III, Table 1) had significantly higher antibody levels than did animals which received sterile broth (group V) or *P. testudinis* alone (group II). No difference in levels of antibody between animals receiving *M. agassizii* alone or in concert with *P. testudinis* was seen; however, tortoises which received the exudate did develop higher responses at 3 months postinfection than did those tortoises which received *M. agassizii* ($P < 0.01$).

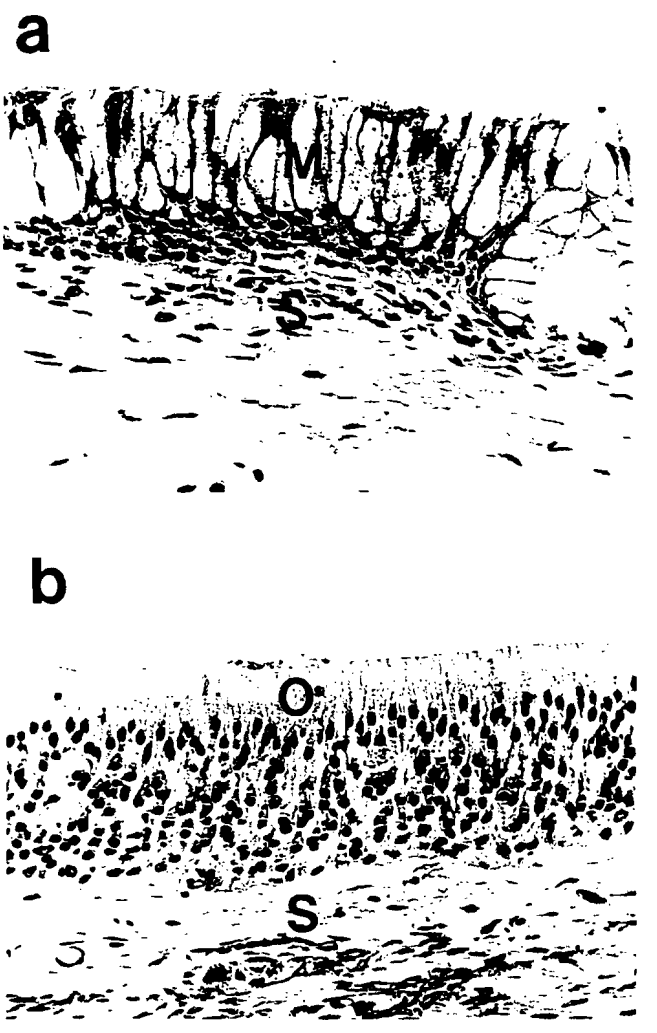

FIG. 1. Photomicrograph of the nasal cavity of a clinically healthy desert tortoise. (a) An area of mucous and ciliated epithelial cells (M) overlying a lamina propria submucosa (S) primarily consisting of connective tissue and small vessels. (b) An area of multilayered olfactory mucosa (O) overlying a lamina propria submucosa (S) consisting of connective tissue, vessels, and melanophores. Stain, hematoxylin and eosin; magnification, ×230.

DISCUSSION

Diseases caused by pathogenic microorganisms are an ever-present risk to animals in both captive and wild populations, especially during periods or conditions of captive breeding followed by release or translocation or ecosystem perturbation and habitat loss or fragmentation (6, 10, 11). Infectious diseases, their impact on population health, health status, and

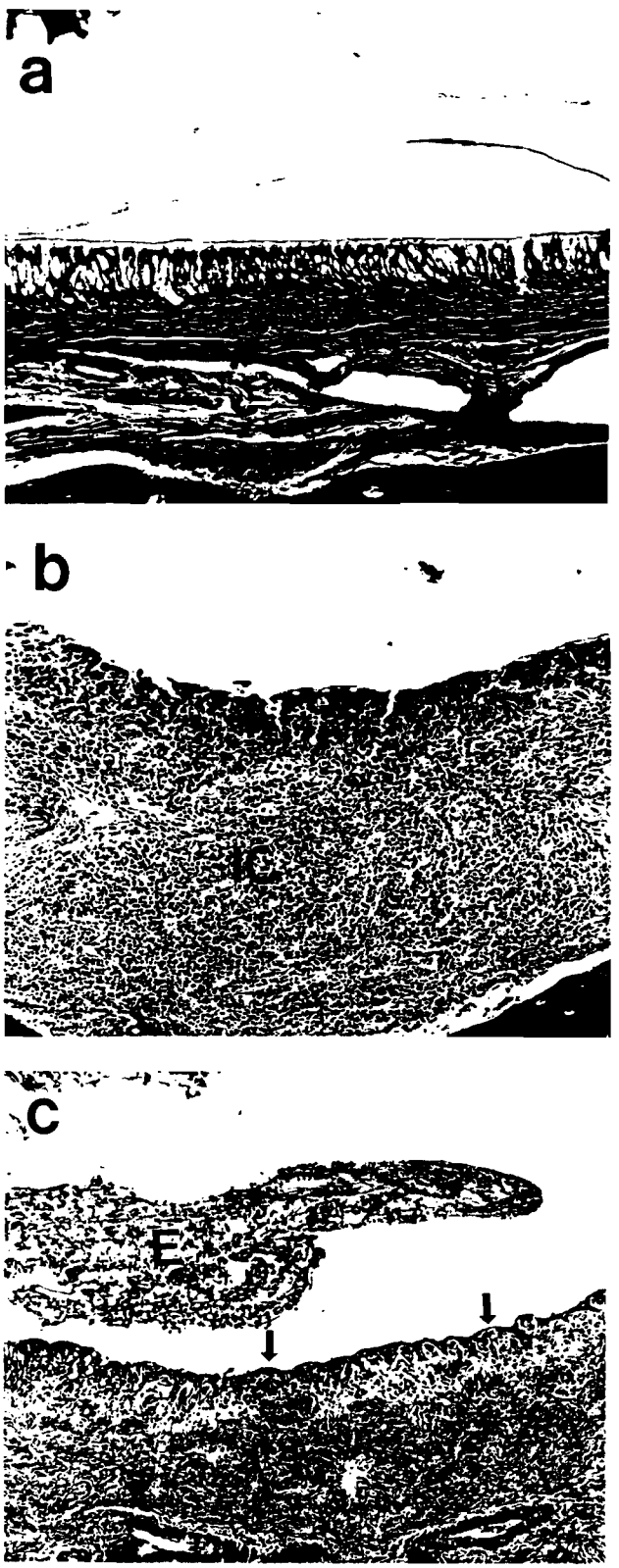

FIG. 2. Representative lesions observed in the upper respiratory tracts of experimentally infected desert tortoises. (a) Mild lesion in the choana characterized by infiltrates of lymphocytes and plasma cells into the lamina propria (arrows). Note that the overlying epithelium is intact. Stain, hematoxylin and eosin; magnification, ×113. (b) Moderate lesion in the nasal mucosa characterized by a dense mixed inflammatory cell infiltrate (IC) composed primarily of lymphocytes with few plasma cells, macrophages, and heterophils. Note that the overlying epithelium is disorganized and obscured by inflammatory cells. Stain, hematoxylin and eosin; magnification, ×113. (c) Severe lesion of the nasal sinus characterized by erosion of the normal epithelium (closed arrows) with the remaining epithelium composed of basaloid or squamous cells. Edema and mixed inflammatory cell infiltrates are seen (open arrows). Note the exudate (E) in lumen composed of heterophils and fibrin. Stain, hematoxylin and eosin; magnification, ×75.0.

population health monitoring, and the consequences of relocation or translocation on health are rarely considered in implementation and design of conservation projects. URTD in the desert tortoise is a prime example of the importance of infectious agents to population health in free-ranging wildlife. Because clinical disease may be silent, relocation of tortoises can pose health risks to other tortoise populations. Similarly, release of captive tortoises into wild populations could spread disease.

A major contributing factor in the decline of the desert tortoise and its federal listing as environmentally threatened has been the presence of URTD. Animals have a chronic disease which eventually leads to severe occlusion of the nares with viscous exudate and destruction of the respiratory epithelium (8). Electron microscopy of infected tissues (8) and association of antibody specific to *M. agassizii* (14) suggested, and the current study proves, a mycoplasmal etiology for URTD. Mycoplasmas cause respiratory disease in a number of animals including humans, rodents, pigs, and poultry (1). Although the species of mycoplasma which causes disease is different for each host, there are many common characteristics of the disease and URTD in desert tortoises shares many of these characteristics. Many apparently normal animals may carry the mycoplasmas without obvious ill effects (1). Disease caused by mycoplasmas is often clinically silent, slowly progressing, and chronic. The severity of the disease is exacerbated by environmental factors and stress, and other microbial agents may act synergistically to create even more severe disease (1, 5, 13, 19). URTD in the tortoise presents a very similar profile, with the presence of seemingly healthy animals which break with the disease after the stress of relocation or crowding. The types of lesions seen in other mycoplasmal respiratory infections also share many characteristics with URTD in the tortoise. In rats, *Mycoplasma pulmonis* causes focal loss of ciliary action, followed by extensive loss of epithelial cell layers with eventual complete destruction of the respiratory epithelium (1, 17). This is also seen in URTD (8). Most respiratory mycoplasmal infections are characterized by an increase in inflammatory cells, especially neutrophils. In tortoises, foci of inflammatory cells are seen (8). In electron micrographs, mycoplasmas frequently can be seen attached to the respiratory epithelium just above these areas of inflammation.

The most stringent requirements for establishment of the etiological role of an infectious agent in disease is the fulfillment of the Henle-Koch-Evans postulates (2, 3). Although it is particularly difficult to fulfill these postulates for a chronic disease in a free-ranging wild animal, the major postulates have been met. The initial *M. agassizii* isolate was obtained from the choana of a tortoise with clinical disease (8). Disease as evidenced by histological lesions was present significantly more often in exposed animals, as determined by both culture and serology (14). In the present study, the mycoplasmal isolate was cultured in vitro and produced clinical disease when

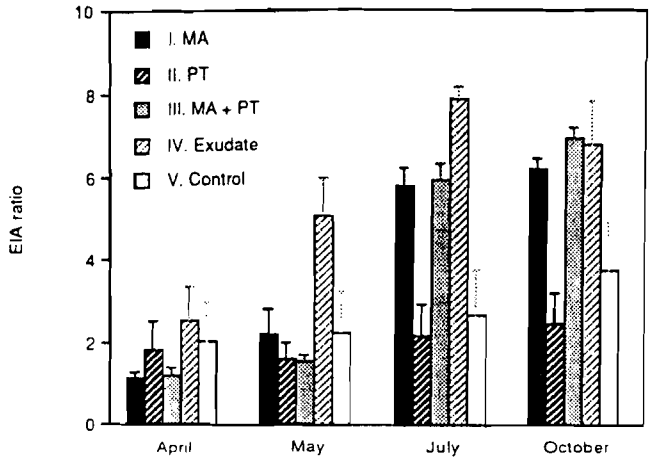

FIG. 3. Serum antibody response to *M. agassizii* in desert tortoises. Values are expressed as the enzyme immunoassay (EIA) ratios + standard errors. The EIA ratio is the ratio of the A_{405} of the sample to the A_{405} of the negative control. An EIA ratio of >2 was considered positive. Tortoises were inoculated in the nares with *M. agassizii* (MA; group I) *P. testudinis* (PT; group II), a combination of MA and PT (group III), exudate from ill tortoises (group IV), or sterile broth (control; group V). Serum values for April 1992 were obtained prior to challenge; all other samples were obtained postinfection. No significant differences were found among samples obtained in April. In May, group IV animals had significantly higher levels than the other groups ($P < 0.004$). In July and October, antibody levels in animals of groups I, III, and IV were significantly higher than those of animals in other groups ($P < 0.004$).

experimentally inoculated into the nares of healthy tortoises. Animals challenged with *M. agassizii* produced antibodies in response to the infection, and *M. agassizii* was isolated from the nares of animals which became clinically ill. The disease course as well as the specific immune response followed after a reasonable incubation period. Thus, we have fulfilled the majority of Henle-Koch-Evans postulates with regard to this disease. The lesions observed in the experimental infection studies were similar to those observed in the natural disease (8), and a range of lesions from mild to severe was seen.

A chronic infection was established, as evidenced by isolation of *M. agassizii* at 1 and 3 months postinfection. We have evidence (data not shown) that indicates some animals can remain infected after experimental challenge for up to 1 year. The transmission studies described in this report conclusively demonstrate that *M. agassizii* causes URTD in desert tortoises. Further, *P. testudinis* does not appear to have a role in the initiation of the disease, as indicated by the absence of clinical disease in challenged animals. A synergistic effect of *P. testudinis* and *M. agassizii* cannot be ruled out by this study but does not appear likely on the basis of severity of clinical signs observed in mixed infections.

The ability of exudate from donor tortoises to cause severe clinical disease may provide a clue as to transmission in the natural population. Direct contact and aerosol transmission of the mycoplasma is the most likely method of spread. However, one question that has yet to be addressed is the length of survival of *M. agassizii* in mucous droplets in the burrows of the tortoise and whether these could be a significant reservoir of infection. On the basis of other mycoplasmal infections, this is not a likely scenario but cannot be eliminated from consideration. The more severe disease seen in group IV animals may be a result of increased virulence after passage in the hosts. Alternatively, loss of virulence after culture in the laboratory could also explain the observed differences in disease severity between the groups. Other factors which could explain the differences in disease severity include a difference between the virulence of the strain used in the study and that of the strain in the donor tortoises, as well as the presence of other contributory factors in the exudate. The exudate undoubtedly contained host inflammatory cells and potentially inflammatory mediators which might enhance the pathogenicity of the mycoplasma.

Antibody profiles demonstrated that the serological test developed may be very helpful in screening for disease. Animals which were challenged responded with a specific antibody response. We do not know the exact temporal sequence of the response, but it appears that in most cases a strong response was developed within 2 months or less of exposure. The response is directed toward a number of mycoplasmal antigens as indicated by Western blot (immunoblot) analysis, and many proteins appear to be shared among different strains of *M. agassizii* (data not shown).

Most animals with preexisting antibody which developed clinical disease also showed an increase in the amount of specific antibody present (data not shown). However, there were some animals which had levels of preexisting antibody which remained stable even though clinical disease was present. Preexisting antibody did not appear to protect against development of clinical disease in animals challenged intranasally, suggesting that a humoral response alone may not be adequate for protection. There were some animals with stable antibody levels that did not show signs of clinical disease. One of these animals was housed in the communal pen. No other tortoises housed with the antibody-positive tortoise developed antibody or disease. This suggests several interesting possibilities. First, any antibody-positive animal should be considered suspect. Secondly, animals which show increases in antibody over time are likely to be carriers or express disease. Thirdly, some animals may be antibody positive but not spread disease, like the tortoise in the communal pen. This is the first suggestion that some animals can clear *M. agassizii* from the respiratory tract and recover from, or never get, clinical disease. This issue can be resolved with a more sensitive measurement of microbial presence. A PCR detection system is currently under development in an attempt to address the question of exposure versus infection.

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SEROEPIDEMIOLOGY OF UPPER RESPIRATORY TRACT DISEASE IN THE DESERT TORTOISE IN THE WESTERN MOJAVE DESERT OF CALIFORNIA

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SEROEPIDEMIOLOGY OF UPPER RESPIRATORY TRACT DISEASE IN THE DESERT TORTOISE IN THE WESTERN MOJAVE DESERT OF CALIFORNIA

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ABSTRACT: Several factors have combined with an upper respiratory tract disease (URTD) to produce declines on some population numbers of desert tortoises (*Gopherus agassizii*) in the western USA. This study was designed to determine the seroepidemiology of URTD in a population of wild adult tortoises at the Desert Tortoise Research Natural Area (DTNA) study site in Kern County (California, USA). Prior to initiation of the study, there was a dramatic decline in the number of individuals in this population. At each individual time point, samples were obtained from 12 to 20 tortoises with radiotransmitters during winter, spring, summer, and fall from 1992 through 1995. During the course of the study, 35 animals were sampled at one or more times. Only 10 animals were available for consistent monitoring throughout the 4 yr period. Specific antibody (Ab) levels to *Mycoplasma agassizii* were determined for individual tortoises by an enzyme-linked immunosorbent assay (ELISA) test. Specific Ab levels were not influenced by the gender of the tortoise. Levels of Ab and distribution of ELISA+, ELISA− and suspect animals were not consistently affected by season within a single year or for a season among the study years. Significantly more tortoises presented with clinical signs in 1992 and 1995. The profile of ELISA+ animals with clinical signs shifted from 5% (1992) to 42% (1995). In 1992, 52% of tortoises lacked clinical signs and were ELISA−. In 1995, this category accounted for only 19% of tortoises. Based on the results of this study, we conclude that URTD was present in this population as evidenced by the presence of ELISA+ individual animals, and that the infectious agent is still present as evidenced by seroconversion of previously ELISA− animals during the course of the study. There is evidence to suggest that animals may remain ELISA+ without showing overt disease, a clinical pattern consistent with the chronic nature of most mycoplasmal infections. Further, there are trends suggesting that the clinical expression of disease may be cyclical. Continued monitoring of this population could provide valuable information concerning the spread of URTD in wild tortoise populations.

Key words: Epidemiology, *Gopherus agassizii*, *Mycoplasma agassizii*, serology, upper respiratory tract disease.

INTRODUCTION

Dramatic declines in some populations of the desert tortoise (*Gopherus agassizii*) over the past 20 yr led the U.S. Fish and Wildlife Service (Portland, Oregon, USA) to list the species as threatened in 1990 under the Endangered Species Act of 1973, as amended (U.S. Fish and Wildlife Service, 1994). Several factors, primarily induced by human activities, have combined with an upper respiratory tract disease (URTD) to produce negative impacts on some desert tortoise populations in Arizona, Utah, and the western Mojave Desert of the western United States (U.S. Fish and Wildlife Service, 1994; Berry, 1997). Clinical signs of URTD have been observed in captive tortoises for many years (Fowler, 1977; Rosskopf et al., 1981) and in one wild population of tortoises at the Beaver Dam Slope (Utah; Jacobson et

al., 1991). In 1988, desert tortoises at the Desert Tortoise Research Natural Area (DTNA; Kern County, California, USA) were seen with clinical signs of illness similar to those observed in captive desert tortoises (Berry, 1997; Fowler, 1977; Jacobson et al., 1991; Knowles, 1989; Rosskopf et al., 1981). Clinical signs included a mucopurulent discharge from the nares, puffy eyelids, eyes recessed into the orbits, and dullness to the skin and scutes (Jacobson et al., 1991). The observations of clinical disease occurred at the time of precipitous population declines (Berry, 1997).

In an earlier study of free-ranging desert tortoises with URTD, a microorganism compatible with mycoplasma was identified on the surface of the nasal mucosa of affected tortoises (Jacobson et al., 1991). In a transmission study designed to fulfill Koch's postulates, *Mycoplasma agassizii* was identified as a cause of URTD in the desert tortoise (Brown et al., 1994). Like most respiratory mycoplasmal infections (Simecka et al., 1992), URTD is characterized by a chronic infection which may be subclinical and intermittent in disease expression (Jacobson et al., 1995; Schumacher et al., 1997). Although *M. agassizii* can be detected by culture and polymerase chain reaction tests (Jacobson et al., 1991; Jacobson et al., 1995; Brown et al., 1995), the most reliable method of diagnosis is serology (Schumacher et al., 1997).

A serological assay to detect specific antibody (Ab) to *M. agassizii* was developed (Schumacher et al., 1993, 1997), and this assay was applied to study the seroepidemiology of URTD in the DTNA population. Based on the clinical description of tortoises in the DTNA prior to and concurrent with the population declines (Berry, 1997) and the isolation of *M. agassizii* from adjacent areas in the Mojave Desert (Jacobson et al., 1991), it was hypothesized that URTD was a contributing factor to population losses in the DTNA. The purpose of this study was to evaluate the presence of specific Ab to *M. agassizii* in a population of desert tortoises which underwent catastrophic decline at the DTNA in the western Mojave Desert (Berry, 1997), to follow individual animals prospectively with respect to serology and expression of clinical signs of disease, and to address how Ab levels changed with season, year, and gender of the tortoise.

MATERIALS AND METHODS

The study area was located in the interior of the DTNA in the western Mojave Desert (Kern County; 35°10′N, 118°10′W, elevation 869–945 m) and was adjacent to a long-term desert tortoise study plot which was sampled for population attributes in the spring seasons of 1979, 1982, 1988, 1992, and 1996 (Berry, 1986a, b, 1997). This region of the DTNA was selected for the project because it was more than 1 km from areas with high levels of human activities and impact, such as off-road vehicle use, vandalism (for example, gunshots), and livestock grazing and protected within the interior of the preserve (Berry, 1986a, 1997). Virtually no people have visited the area since the early 1980's except for the research scientists. Thus, population changes, including mortality, due to anthropogenic influences were expected to be minimal.

When this study commenced in 1992, the desert tortoise population had declined substantially and altered in adult:juvenile ratio since earlier surveys in 1979 and 1982 (Berry, 1997). Between 1982 and 1992, the total population declined by about 86%, and the adult population declined by about 94% (statistically significant at the 95% confidence interval; Berry, 1997). The primary source of mortality in juvenile tortoises was raven predation (Berry, 1986b, 1997). The adult population increased between 1979 and 1982 because of tortoise protection from vandalism, off-road vehicle use, and livestock grazing within the fenced DTNA. At the next sampling time (1988), the adult population was declining and the first clinical signs of URTD were noted (Berry, 1997; Jacobson et al., 1991). From the time tortoises with signs of URTD were observed in 1988 (Jacobson et al., 1991; Berry, 1997) until the start of the study in 1992, the total population declined 76% and the adult population declined 90%. Since the mid-1980's, the primary source of adult mortality was presumed to be from URTD and probably predator attacks on debilitated animals (details to be reported elsewhere). When the study began, population densities of adults were estimated by

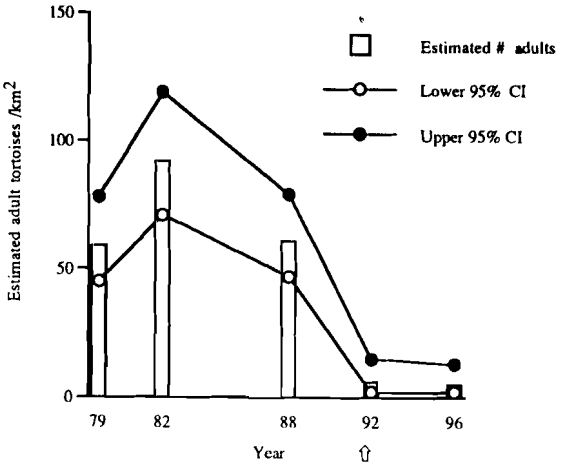

FIGURE 1. Estimates of desert tortoise population densities for the years 1979, 1982, 1988, 1992, and 1996 at the desert tortoise study plot in the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). Results are expressed as the estimated number of adult tortoises per km^2. The 95% confidence intervals for the upper and lower population intervals are shown.

the Stratified Lincoln Index (Overton, 1971) at 6 individuals/km^2 (Fig. 1).

Blood samples obtained for testing were part of a larger on-going study to assess overall tortoise health, to determine reference intervals for hematological and biochemical parameters, and to study water balance and energy flow (Peterson, 1996; Christopher et al., 1997). The animals selected for the research project were assumed to be healthy, and individuals with obvious signs of disease such as purulent nasal discharge were avoided. Blood samples were obtained by venipuncture from wild adult tortoises fitted with radio-transmitters and an identification tag on the posterior carapace. Exact numbers of tortoises sampled varied with the season and year (Table 1). From the winter of 1992 through the fall of 1995, four sample sets were obtained per year (n = 16 sample sets): in late winter (late February or early March), just prior to emergence from hibernation; in spring (May), during the time of peak activity; in summer (July/August), during the time of peak stress as a result of increased temperature and decreased rainfall; and in fall (October), during the time of decreased activity and initiation of hibernation. In 1992, samples were obtained from 12 to 14 tortoises; in 1993, from 14 to 16 tortoises; in 1994, from 13 to 15 tortoises; and in 1995, from 15 to 21 tortoises. Replacement tortoises were located and added to the study population as needed, when individuals disappeared or died, bringing the total number of individuals sampled during the study period to 35. The 35 tortoises were living in an area encompassing about 8 km^2 or 8% of the DTNA. The population density was so low during the 4 yr sampling period that virtually every adult located in the vicinity of a core study area of about 2 km^2 was eventually included in the study. Blood samples were centrifuged in the field. Samples of plasma were frozen above liquid nitrogen in the field, and were sent frozen on dry ice to the University of Florida (Gainesville, Florida, USA) for determination of Ab to *M. agassizii*. Samples were stored at −20 C in a manual defrost freezer until assayed usually within 2 wk of receipt.

At the time of capture, a field assessment of overall tortoise health (Christopher et al., 1997) was made. Assessment data for each tortoise included weight, carapace length at the midline (MCL), and packed cell volume (PCV). Signs of ocular disease (e.g., swollen eyelids, wet eyelids indicative of an ocular discharge or mucus in the eye), signs of nasal discharge, and condition of the chin glands were recorded. All tortoises were ≥180 mm MCL, allowing gender determinations to be made on the basis of presence or absence of gular horn, concavity of the posterior plastron, chin glands, and tail length. Photographic documentation was made of the shell, and, in 1994 and 1995, of the eyes and beak in tortoises with clinical signs (Jacobson et al., 1991). Throughout the study, all field assessments were made by the same two experienced senior investigators.

The ELISA procedure was performed as previously described (Schumacher et al., 1993). Antigen was prepared as previously described (Brown et al., 1996) using *M. agassizii* strain PS6 grown to midlogarithmic phase in SP4 broth (Tully et al., 1979). In each assay the blank was the mean of two wells coated with antigen and incubated with the conjugate and the substrate only. Plasma of a desert tortoise which was culture negative for *M. agassizii* and free of lesions indicative of URTD was used as the negative control (Schumacher et al., 1993). Plasma from a desert tortoise which was experimentally infected with *M. agassizii* and had lesions indicative of URTD was the positive control (Schumacher et al., 1993). Because of the limited volume of control sera available, new positive and negative controls were used beginning in 1994. Thus, all sera tested in 1992 and 1993 had one set of reference controls, and sera tested in 1994 and 1995 had a second set of reference controls. Positive and negative controls were included on each plate to determine interplate variation. Samples were categorized as positive if the ratio of sample absorbance to negative control absorbance was ≥3.0; samples were categorized as negative if the ra-

TABLE 1. Serological response of individual desert tortoises to *Mycoplasma agassizii* over a 4 yr period.

Individual tortoise	1992				1993				1994				1995			
	W	Sp	Su	F[a]	W	Sp	Su	F	W	Sp	Su	F	W	Sp	Su	F
Group I[c]																
D01M	N[b]	N	N	N	N	N	N	N		N	N	N	N[f]	N	N	N
D05M	P[b]	S[b]	P	P	S	P	S	P	P	P	P	P	P	P	P	P
D11F	N	N	N	N	N	N	S	N	N	N	N	N	N	N	N	N
D13F	N	P	S	S	N[f]	N	S	S	P	P	S	N	N	N	S	S
D15F	S		P	P	S	N	S	S	P	P	P	S	P	P	P	P
D25M	S	P	P	P	N	S	S	P	P	P	P	P	P	P	P	P
D26F	P	P	P	P	P[f]	P	P	P	P	P	P	P	P	P	P	P
D27M	N	N			N							N	N	N	N	N
D28M	N	N				N	N	N	N	N	N	N	N	N	N	N
D29F		N	N	N	N	N	N	N							N	N
Group II[d]																
D09M	P															
D10M	N				N											
D22M	N	N														
D30F[g]			N													
Group III[e]																
D31F		N	N	N	N	N	N	N								
D32M		P	P	P	N	N	N	P	P							
D33M		N	N	S	N	N		P	N							
D34F			N	N	N	N	N	P								
D35F			N	N	N	N	N	N	N							
Group IV[e]																
D36M							P		P	P	P	P	P	P		
D37M							P	P	P	P	P	P	P	P	P	P
D38M								N	N	N	N	N	N	N		
D39F										N	N	N	N	N		
D40M[g]										S	N	N	N			
D41M[g]												N	N	N		
Group V[d]																
D42F													P	P	P	P
D43M													N	N	N	P
D44F														P	P	P
D45M															N	N
D46F															N	N
D47F															N	N
D48M															N	
D49F																P
D50M																P
D51M																N

[a] W, Sp, Su, F = winter, spring, summer, fall

[b] Results are expressed as positive (P), negative (N) or suspect (S). If no result is noted, then the animal was not sampled at that time point.

[c] Group I is composed of tortoises which were present throughout the entire four year study period (92–95).

[d] Groups II and V are composed of tortoises which were present primarily during only 1 yr of the study period (1992 and 1994, respectively).

[e] Group III and IV are composed of tortoises which were present primarily during 2 yr of the study period (1992–93 and 1994–95, respectively).

[f] Tortoises with clinical signs of URTD at the time of sampling are denoted by a solid underline (ocular signs), a dotted underline (nasal signs or chin gland swelling), or solid double underline (both ocular and nasal signs).

[g] Tortoise found dead or transmitter found during course of study.

tio of sample absorbance to negative control absorbance was ≤2.0 (Schumacher et al., 1993). Samples with a ratio value between 2 and 3 were deemed suspect.

All statistical analyses were performed using a computer-assisted program (StatView, Abacus Concepts, Inc., Berkeley, California, USA). The effects of tortoise gender and season on Ab levels were analyzed by analysis of variance (ANOVA) (Armitage, 1977). Gender did not influence Ab levels, so all Ab comparisons were analyzed without consideration of gender. The distribution of positive, negative and suspect animals was analyzed by Chi square analysis (Armitage, 1977).

Changes in Ab levels of individual animals over time were evaluated by paired *t*-test (Armitage, 1977) using a computer-assisted program (StatView), with values compared only between the same season and only between study years 1992 and 1993 or between study years 1994 and 1995. Refinements in the ELISA during the course of the study included a change in reference standards necessitated by exhaustion of the original standards. This resulted in lower background values and decreased negative standard values. However, only the absolute ELISA values were affected; this change did not affect whether samples were deemed positive, suspect, or negative. The reference standard values used in 1992 and 1993 were comparable as were the reference standards used in 1994 and 1995. Therefore, absorbance values were compared between 1992 and 1993 data or between 1994 and 1995 data only. Because the determination of positive and negative status of samples was based on a ratio and was unaffected by the assay changes, these data could be compared among all four study years.

RESULTS

Estimates of desert tortoise population densities in the DTNA study plot are summarized in Figure 1. During the 17 yr of population monitoring, significant decreases occurred in the population densities from 1988 to 1992 ($P < 0.001$). The most dramatic decline in population occurred from 1988 to 1992 concurrent with, and subsequent to, the observation of clinical signs of URTD in the population in 1988 (Berry, 1997). This population decline has been described in detail elsewhere (Berry, 1997)

The serological response of individual tortoises during each sampling period (1992–96), as well as the individuals included within each sample, are shown in Table 1. Several patterns are apparent in the sample animals. Tortoises in Group I ($n = 10$) remained in the population consistently and were generally sampled throughout the entire study period. These animals provided a stable base and accounted for about 50% of the tortoises which were sampled. Three of the Group I animals (D27M, D28M, and D29F) were sporadically missing during sample periods, most notably in 1993 and 1994, but were still in the population in 1995. The remaining animals were consistent in their reactions in the ELISA with the exception of three tortoises (D13F, D15F, and D25M) which had fluctuations in their Ab status. Although animal D05M had three suspect values, this does not constitute a fluctuation in status since a suspect determination is considered to be a "gray zone" value which can be considered as an equivocal positive reaction. Groups II ($n = 4$) and V ($n = 10$) were composed of tortoises which were present primarily during only 1 yr of the study period (1992 and 1995, respectively). Group III and IV were composed of tortoises which were present primarily during 2 yr of the study (1992–93 and 1994–95, respectively).

In 1992, 19 tortoises were sampled; nine of 19 remained for at least four sample points and one was present twice in 1995 (Table 1). The remaining nine animals had disappeared from the population by spring of 1994. Of the missing animals, only three (D30F, D40M, and D41M) were confirmed as dead or had their radiotransmitters found. It was not possible to perform necropsies on any of these animals. As animals were lost to sampling, new animals were added. In both 1993 and 1994, three additional animals were added. Only those added in 1993 remained in the study by 1995. The largest influx of new animals occurred in 1995, with the addition of 10 new animals.

During the course of the study, 59% (13

of 22) tortoises with >3 samples retained their serological status or had only a single suspect sample. Nine tortoises (D01M, D11F, D27M, D28M, D29F, D31F, D35F, D38M, D39F, D40M) basically remained ELISA− throughout the study. Three animals (D26F, D36M, and D42F) remained ELISA+ throughout the study. Two animals (D13F and D33M) had results which were inconsistent. The ELISA+ values in these animals were near the cutoff value, and this may represent background noise or might represent a low level infection. There is insufficient data to adequately differentiate between these possibilities. Two animals (D34F and D43M) appeared to seroconvert but unfortunately were not available for follow-up sampling to determine if the seroconversion was real or spurious. The remaining 13 animals had too few samples to make any judgments as to their status.

The overall frequency of positive, negative, and suspect animals in the populations at each sample time is summarized in Figure 2. Distribution among the same season of different years was different only for winter of 1993 and 1994. The percentage of animals with positive reactions was significantly lower than expected in winter of 1993 and greater than expected in winter of 1994 ($P = 0.04$). This most likely is a reflection of fluctuations in levels in Group I animals (Table 1, D05M and D25M for example) and the changes in individual animals in the population (groups III and IV). In winter of 1993 the animals in Group III were all negative. By winter 1994, most of these animals had left the population and were replaced with ELISA+ tortoises. In addition, one of the original tortoises remaining had seroconverted.

There were no differences between the observed and expected frequencies for the seasonal distribution (Fig. 2) within a single year in 1992 ($P = 0.96$), 1994 ($P = 0.75$) and 1995 ($P = 0.87$). However, in 1993, significant increases ($P = 0.02$) were observed in the number of positive animals in fall and in the number of suspect animals in the summer. No other differences were significant. From Table 1, it is clear that the increased positive reactions in 1993 can be attributed to animals D34F and D33M, which became ELISA+, and possibly to D05M and D25M which had previously been suspect. The increase in suspect animals in the summer of 1993 can be attributed to negative animals which had increases in Ab (D11F, D13F, D15F) and a previously positive animal (D05M) which decreased in Ab levels.

Significant changes ($P = 0.002$) occurred during the four year study period with respect to the relationship between clinical signs and positive ELISA serology (Fig. 3). For the purposes of analysis, a decision was made to consider a tortoise as ELISA+ if any of the four sample times during the year were either suspect or positive. Similarly, a positive clinical sign at any point resulted in the animal being characterized as Sign+. The individual status at each sample point may be found in Table 1. When the results of testing from all time points for a given year were combined, there was no significant change in the percentage of tortoises in the population throughout the four year study period which were ELISA+ (Fig. 3, $\chi^2 = 1.75$, $P = 0.62$). However, the expression of clinical signs within the population changed significantly (Fig. 3, $\chi^2 = 22.3$, $P = 0.0001$). The ocular signs most commonly observed were swollen eyelids, wet eyelids indicative of an ocular discharge or mucus in the eyes, and wet or occluded nares. In 1992, only 16% of tortoises had clinical signs consistent with URTD at one or more sample times (Fig. 3). By 1995, 76% of tortoises had clinical signs consistent with URTD at one or more sample times (Fig. 3). Tortoises observed in winter of 1993, 1994, and 1995 as well as fall of 1995 had increased clinical evidence of URTD (Fig. 4). In 1992, 52% of tortoises lacked clinical signs and were ELISA−. In 1995, this category accounted for only 19% of the tortoises. Instead, the population pro-

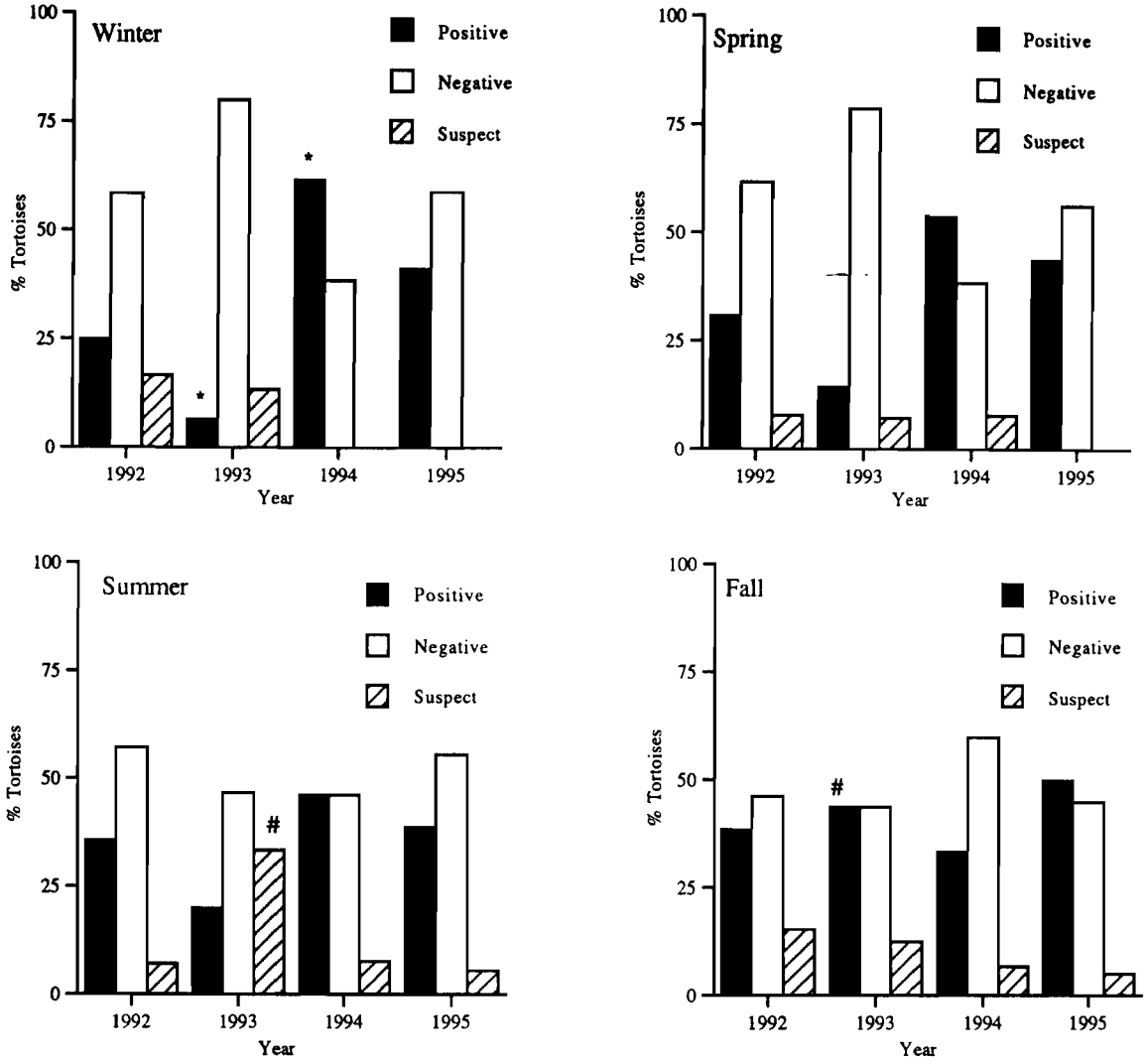

FIGURE 2. Distribution of tortoises with positive, negative or suspect ELISA values in desert tortoises from the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). In winter of 1993 and 1994, there were statistically significant differences in the distribution of tortoises with positive reactions as compared with the distribution in winters of 1992 and 1995, $P = 0.04$. In 1993, the distribution of positive tortoises increased in the fall, and the distribution of suspect tortoises increased in the summer ($P = 0.02$). No other differences were significant.

file had shifted to 42% of tortoises with both clinical signs and a positive ELISA result. The profile of ELISA+ animals with clinical signs also shifted from 5% (1993) to 42% (1995). The percentage of animals in the population which were ELISA+ yet free of clinical signs remained fairly constant (about 30%) until 1995, when it dropped to only 4%.

DISCUSSION

Seroepidemiology is a powerful tool for monitoring population health. Samples taken at a single point in time can provide a "snapshot" of the past exposure of a population to infectious agents. To understand the dynamics involved in the interaction between the host and infectious agent, it is necessary to follow populations prospectively over time. In any study of free ranging animals, there are limitations imposed by the ability to recapture animals at each time point as well as the inherent difficulties in sampling at relatively few times. The variable clinical expression of mycoplasmal infections (Schumacher et al.,

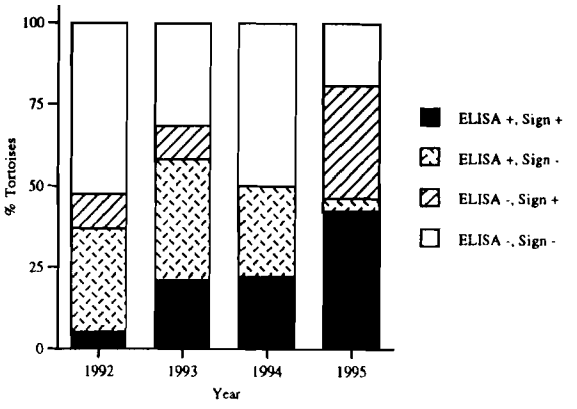

FIGURE 3. Comparison of ELISA results with presence of clinical signs in desert tortoises from the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). Results are expressed as the percentage of tortoises with positive or negative ELISA results in conjunction with the presence (Sign+) or absence (Sign−) of clinical signs. There was a significant difference among the 4 yrs, $P = 0.002$. Although the distribution of ELISA+ tortoises did not vary ($P = 0.62$), the distribution of Sign+ tortoises increased in 1995 ($P = 0.001$).

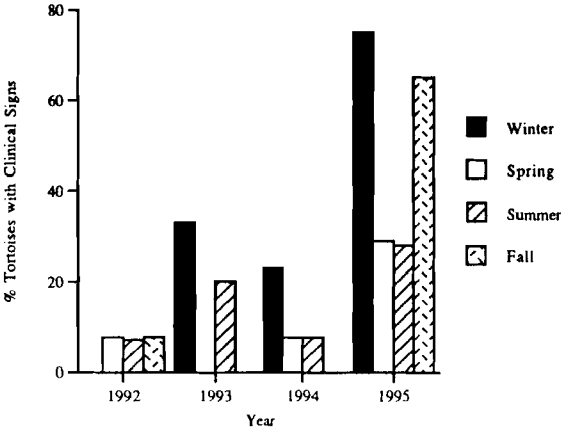

FIGURE 4. Distribution of tortoises with clinical signs in desert tortoises from the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). No significant differences were noted among seasons within a year in 1992 or 1994 ($P = 0.82$ and 0.21, respectively). Within a given year, there was an increase in tortoises with clinical signs only in winter and fall, 1995 ($P = 0.02$). For a given season compared among the 4 yr there were significant differences for winter and fall ($P = 0.001$), but not for spring ($P = 0.06$) or summer ($P = 0.29$).

1997; Simecka et al., 1992) can result in clinically ill animals which appear healthy. Thus, the reliability of clinical signs at any given sample time may be low. Similarly, individual antibody levels, particularly those that are close to the borderline cut-off values, may show variation. However, when coupled with repeated measurements over time, a more cohesive picture of the population will emerge. It also is important to remember that, while individual animals may be of interest, it is the overall picture of the group as a whole which provides the most accurate assessment of the population. The present survey is an excellent example of the value of continuously monitoring a population to obtain the status of a free-ranging wild population with respect to disease and overall health.

This study was initiated after the severe population declines occurred at DTNA. Based on the results of this study, we conclude that URTD was present in this population as evidenced by the presence of ELISA+ individual animals, and that the infectious agent is still present as evidenced by seroconversion of previously ELISA− animals during the course of the study. Although the animals in this study were not cultured or submitted for necropsy, tortoises within a 3.3 km area in the DTNA were used to document the pathology of URTD as well as cultural isolation of *M. agassizii* from the respiratory tract of ill animals (Jacobson et al., 1991).

There is evidence to suggest that animals may remain ELISA+ without showing overt disease, a clinical pattern which is consistent with the chronic nature of most mycoplasmal infections (Schumacher et al., 1997; Simecka et al., 1992). There are trends (Table 1, Fig. 4) which suggest that the clinical expression of disease may be cyclical. In captive animals, we know that known carriers of *M. agassizii* may appear clinically normal for long periods of time (up to 1 yr), then suddenly show classical signs of URTD (Schumacher et al., 1997). Therefore the presence or absence of clinical signs is an unreliable method of clinical diagnosis. In a recent study of 144 free-ranging tortoises in Nevada (Schumacher et al., 1997), a positive ELISA was

positively related to clinical expression of disease; 93% of animals with nasal discharge also had a positive ELISA. Approximately 34% of animals with no clinical signs tested positive by ELISA, indicative of a subclinical infection (Schumacher et al., 1997). The prevalence of subclinical infections (as defined by ELISA+, Signs−) in the DTNA was actually quite similar in 1992, 1993 and 1994 (32%, 37%, and 28%, respectively) to that observed in the Nevada (USA) population (Schumacher et al., 1997).

There are a number of questions which can be raised regarding the status of ELISA+, Signs− tortoises. Because of the management implications, it would be ideal to know if these animals continue to represent risks as carriers of infection. While the answer can never be definitive, we can speculate as to possible outcomes and the likelihood of each scenario. A tortoise which is ELISA+, Signs− could theoretically (1) recover from infection and clear the mycoplasma, (2) remain infected at low levels which preclude transmission or recrudescence of disease, or (3) remain infected, transmit the disease, and undergo a recrudescence of clinical disease expression. The first alternative is unlikely since chronic mycoplasmal infections rarely are cleared from a population (Simecka et al., 1992). This is in large part due to the nature of the association between the host and the mycoplasma. The mycoplasma may however be present in low numbers or sequestered. Secondly, some animals may remain at these low levels of infection and no longer transmit disease. Because these animals harbor the infectious agent, they are truly chronically infected but behave as convalescent animals in that they do not transmit disease. The increased prevalence of clinical signs in the DTNA population would argue against a population in which the organism is present in low numbers but no longer transmissible. The most likely explanation of the clinical pattern in the DTNA is that of a population in which has the disease has become established as a chronic disease. In a population of this type, one would expect a number of animals which were Ab positive as a result of prior exposure. The clinical manifestations would be cyclical, waxing and waning in severity, and shedding of the mycoplasma would be intermittent. This is consistent with other mycoplasmal respiratory infections (Simecka, et al., 1992). The observations in this study of seroconversion and increasing clinical signs in the sample tortoises are consistent with the establishment of a chronically infected population in the DTNA.

The observations that increased clinical signs were observed in 1995 in conjunction with increased ELISA+ symptomatic animals is intriguing. There might be several explanations for this observance. First, during the initial years of the study, ocular signs of URTD were not well established. Therefore it is possible that field workers assessing the tortoise health status became more proficient in identification of the clinical signs. If this is true, then one might expect similar increases independent of the season of the year. However, this was not the case as increased clinical signs were not reported in spring or summer of 1995. This suggests that the occurrence of clinical signs may not be wholly a function of improved recognition by observers in the field. A second explanation might be that the appearance of clinical signs is cyclical. ELISA− animals have seroconverted in 1994–95 study years, suggesting that the mycoplasma is still present in the population. Alternatively, the clinical signs might be the result of another unidentified infectious agent. Although this possibility cannot be ruled out, the increased percentage of ELISA+ Signs+ tortoises would tend to argue against this possibility.

ELISA− Signs+ tortoises also represent an interesting group of animals. In a previous study (Schumacher et al., 1997), this group represented only about 10% of tortoises tested. In the DTNA population, the percentage of ELISA− Signs+ tortoises

was ≤10% in all years except 1995, when that group accounted for 35% of the tortoises tested. It is especially interesting that during this same sample time the percentage of ELISA+ Signs+ tortoises in the population doubled. This would suggest that the most likely explanation of ELISA− Signs+ tortoises in 1995 might be a result of tortoises which have recently been infected and have not made a detectable antibody response. In experimental infections, the appearance of clinical signs can precede the production of detectable levels of antibody to *M. agassizii* (Schumacher et al., 1997). We cannot preclude the possibility that other viral or bacterial pathogens might produce similar clinical signs in the absence of *M. agassizii*; however, no additional pathogens have been confirmed to cause clinical signs compatible with URTD. The clinical signs associated with URTD, especially those of wet nares and eyes, may be associated with other stimuli, such as eating, drinking, dust irritation, or response to allergens. Because tortoises were observed under field conditions only once every four months, it is difficult to assess the possibility of these additional factors but it is unlikely that the increase seen in 1995 can be attributed entirely to these factors.

We have done preliminary studies in the gopher tortoise (*Gopherus polyphemus*) in Florida (USA) which suggest that preexisting Ab is not effective in preventing recurrence of disease, and in fact might result in more severe disease (McLaughlin, 1997). In an experimental transmission study of URTD (Brown et al., 1994), Ab responses could be measured within 1 to 2 mo of initial exposure to a relatively high number of *M. agassizii*. In a natural situation (i.e., exposure in the field to a subclinical carrier or ill animal), the initial number of *M. agassizii* encountered by a naive tortoise might be considerably less and a prolonged period could occur between exposure and development of measurable Ab.

The virulence of the individual field mycoplasmal strain would undoubtedly be important in the manifestation of clinical disease and immune response, but cannot be determined on the basis of a seroepidemiological study. Some animals in the DTNA do have increased levels of Ab, suggesting that at least some animals are still undergoing continued stimulation of the immune response, presumably via exposure to mycoplasmal antigens. As exposure to the pathogen increases, we predict that the number of animals which produce Ab, as well as the amount of Ab present, will increase.

We do not know how many of these animals will clear the infectious agent, develop disease, or become asymptomatic carriers. It is intriguing that the number of animals showing clinical signs is increasing in the population, which would tend to support the hypothesis that infection runs in cycles, with reexposure and newly exposed animals expressing signs in a cyclical manner. This could explain the observations of increased clinical disease in 1988, followed by a quiescent period in 1992–94, and a re-emergence of disease signs in 1995.

We do not know if tortoises which appear to have recovered from disease are protected upon subsequent challenge with the infectious agent. However, studies in our laboratory with respiratory mycoplasmosis in *G. polyphemous* demonstrated that animals with prior exposure (as indicated by presence of specific Ab and absence of clinical signs) are more severely affected when exposed to *M. agassizii* (McLaughlin, 1997). Long term monitoring is essential to fully determine the effects of the disease on the population.

Clinical signs compatible with URTD were recognized in this population in 1988 (Berry, 1997). The clinical signs were especially pronounced during the 1989–1990 seasons preceding the serological sampling times. Animals were observed with purulent nasal discharge. Clinically ill animals from this population were extensively evaluated in 1989 (Jacobson et al., 1991) and

had lesions consistent with URTD. It was from this study that the original isolations of *M. agassizii* were made. The factors which resulted in clinical expression of disease are not known. The periodic droughts and subsequent decreased forage availability typical of the Mojave Desert might have acted in concert with mycoplasmosis to adversely impact tortoise health. Clinical signs of mycoplasmal respiratory disease are known to be exacerbated by external stress and environmental factors (Simecka, et al., 1992).

Another more insidious aspect of the disease is the confounding factor it will undoubtedly pose to other scientific studies, especially those which investigate nutrition and reproduction of the desert tortoise. Any studies involving the tortoise should include the disease status of the animal to ensure that parameters and variables under study are not confounded by the disease. Both of these parameters have been severely influenced by respiratory mycoplasmosis in other species: most notably poultry, rodents, and swine (Simecka, et al., 1992). Conservation efforts which involve relocation, restocking, or translocation of tortoises in the wild as well as in captivity also may be impacted by the disease (Jacobson et al., 1995).

This study showed that a key factor which must be considered in the continued monitoring of free-ranging tortoise populations is that the introduction or removal of individuals from a sample population can influence interpretation of data, especially when the overall numbers of animals monitored is low. For example, half of the population studied in 1995 was different from the population members seen in 1992. Although Ab levels can give an idea of the magnitude of response by individual animals, the population profile as a qualitative assessment of ELISA+ animals may be more helpful. We have seen populations of *G. polyphemus* from different sites in Florida with ELISA+ animals ranging from 10 to >80% of the tested population (M. Brown, I. Schumacher, and P. Klein, unpubl. data). Clinical disease was rare except in populations with a high percentage of ELISA+ animals, paralleling what we have described in the DTNA population. Because of the long-term, chronic, and clinically silent aspects of URTD (Brown et al., 1994; Jacobson et al., 1991, 1995), it may well be that a minimum threshold of infected animals is required to see clinical disease.

Assessment of health status is particularly difficult in free-ranging animals (Jacobson et al., 1991; Schumacher et al., 1997). Seroepidemiology is a powerful tool for monitoring the spread of URTD in wild tortoise populations. Continued monitoring of populations is also essential for determining the predictive value of serological profiles in this disease. Changes in the percentage of ELISA+ animals within a population or changes in Ab levels could precede the appearance of clinical disease and provide an early warning of potential disease outbreaks in populations. Because URTD is clinically silent in the majority of animals, this early warning is especially important. Similarly, seroconversion of newly introduced animals in a population which has seemingly recovered from disease could indicate that the infectious agent is still present. Knowledge of the prevalence of infection in populations will allow better management decisions concerning possible geographical areas to be targeted for habitat preservation or populations which are at risk to acquire or to spread URTD.

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PATHOLOGY OF DISEASES IN WILD DESERT TORTOISES FROM CALIFORNIA

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ABSTRACT: Twenty-four ill or dead desert tortoises (*Gopherus agassizii*) were received between March 1992 and July 1995 for necropsies from the Mojave and Colorado deserts of California (USA). Diseases observed in these animals included cutaneous dyskeratosis ($n = 7$); shell necrosis ($n = 2$); respiratory diseases ($n = 7$); urolithiasis ($n = 3$); and trauma ($n = 5$). In tortoises with cutaneous dyskeratosis the horn layer of shell was disrupted by multiple crevices and fissures and, in the most severe lesions, dermal bone showed osteoclastic resorption, remodeling, and osteopenia. In tortoises with shell necrosis, multiple foci of necrotic cell debris and heterophilic inflammation within the epidermal horn layer were subtended by necrotic dermal bone colonized by bacteria and fungi. Of the seven tortoises with respiratory disease, five were diagnosed with mycoplasmosis. The diagnosis of mycoplasmosis was based on the presence of chronic proliferative rhinitis and positive serologic tests and/or isolation of *Mycoplasma* sp. Chronic fungal pneumonia was diagnosed in one tortoise with respiratory disease. In the three tortoises with urolithiasis, two were discovered dead, and the live tortoise had renal and articular gout. Traumatic injuries consisted of one tortoise entombed within its burrow, one tortoise burned in a brush fire, two tortoises struck by moving vehicles, and one tortoise attacked by a predator. While the primary cause of illness could be attributed to one or two major disease processes, lesions were often found in multiple organ systems, and a variety of etiologies were responsible for morbidity and mortality.

Key words: Desert tortoise, diseases, *Gopherus agassizii*, pathology, survey.

INTRODUCTION

Desert tortoises (*Gopherus agassizii*) inhabit the Mojave, Colorado, and Sonoran deserts of the southwestern United States. During the last 20 yr, it appears that some populations of desert tortoises have had significant declines (U.S. Fish and Wildlife Service, 1994). Depending on the location and region, the causes of population declines have been multifactorial and may be the result of an accumulation of factors over a long period of time. Since the free-ranging female desert tortoise requires 12 to 20 yr to reach reproductive age (Woodbury and Hardy, 1948) and produces small numbers of eggs (average = 4) in each clutch (Turner et al., 1986), recovery of severely affected populations could require centuries (U.S. Fish and Wildlife Service, 1994). On 2 April 1990, the U.S. Fish and Wildlife Service listed desert tortoise populations north and west of the Colorado River as threatened under the Endangered Species Act of 1973, as amended (Department of the Interior, 1990).

Disease has been one factor associated with recent declining numbers of desert tortoises (Jacobson, 1994). Cutaneous dyskeratosis, a shell disease, has been associated with high mortality in a protected population of desert tortoises at the Chuckwalla Bench Area of Critical Environmental Concern (Riverside County, California, USA) (Jacobson et al., 1994) and has been observed in tortoise populations elsewhere within their geographic range (Berry, 1997). The percentage of tortoises in the Chuckwalla Bench with the shell lesion increased from 1979 to 1990: from 68 to 97% in adults, and from 41 to 78% in immature tortoises (Jacobson et al., 1994). The lesion has been characterized as a loss of normal integrity of the cornified layer of the affected scutes. The cause of mortality was not determined in past studies as only shell biopsy specimens were collected from affected animals.

In 1988 desert tortoises with upper respiratory tact disease (URTD) were seen in the Desert Tortoise Natural Area (Kern County, California, USA) (Jacobson et al., 1991). Jacobson et al. (1991) found consistent chronic inflammatory lesions in the nasal cavity of free-ranging desert tortoises with URTD and, using electron microscopy, identified *Mycoplasma* sp. on the surface of nasal mucosal epithelium. Subsequently, *Mycoplasma agassizii* was demonstrated to be the causative agent (Brown et al., 1994) and an enzyme-linked immunosorbent assay (ELISA) test was developed for the detection of *M. agassizii*-specific antibodies (Schumacher et al., 1993). Free-ranging desert tortoises with signs of URTD or serological evidence of mycoplasmal infection have been found in other areas of the southwestern United States (Jacobson et al., 1995; Berry, 1997). Other potentially pathogenic bacteria isolated from nasal cavities and choanae of tortoises with URTD and healthy tortoises have included *Pasteurella testudinis, Aeromonas hydrophila, Klebsiella oxytoca,* and *Pseudomonas* sp. (Jacobson et al., 1991; Snipes et al., 1995).

Very little is known about other diseases of wild desert tortoises. Necropsy of ill, dying, and recently dead desert tortoises is a key approach in determining the causes of disease and mortality in different populations of these animals. In this study, we determined causes of illness or death in wild desert tortoises from all parts of their geographic range in California (USA) by gross and microscopic examination of tissues and by serologic, microbiologic, hematologic and toxicologic evaluations (Homer et al., 1994, 1996). In this paper, we describe the types of pathologic changes associated with a variety of diseases resulting in illness or death.

MATERIALS AND METHODS

Twenty-four tortoises from or near study sites throughout the Mojave desert and portions of the eastern and northern Colorado desert of California were examined from March 1992 through July 1995 (Table 1). Five tortoises were found moribund or dead and 19 ill tortoises were collected alive. Live tortoises were obtained when they showed signs of lethargy, weakness, inanition, weight loss, ocular and nasal discharges, swollen eyelids, or shell lesions. They were collected also following trauma by moving vehicles, fire, predation, or being entombed. Tortoises were shipped via air freight (live tortoises) or Federal Express (dead tortoises) to the University of Florida (Gainesville, Florida, USA). Dead tortoises were shipped on ice.

Blood was collected from a carotic artery, placed into tubes containing lithium heparin, centrifuged to collect plasma and stored at −20 C. A portion of plasma was submitted for an ELISA test to detect the presence of *M. agassizii*-specific antibodies, as described previously (Schumacher et al., 1993). After collection of blood, tortoises were euthanatized with intravenous pentobarbital and a necropsy was conducted. Organs were removed *in bloc*, and the head was sectioned longitudinally on the midline for examination of the nasal cavity. Tissue sections (approximately 0.5 cm wide) from all major organ systems were fixed in 10% neutral buffered formalin for 24 to 48 hr, embedded in paraffin, sectioned at 5 to 6 μm, and stained with hematoxylin and eosin and as necessary, with a variety of stains for bacteria, fungi and mucin (Luna, 1968).

Swab specimens of the choanae and colon of each tortoise were collected for aerobic bacterial isolation. Specimens were inoculated onto a Columbia agar with 5% sheep blood, Columbia CNA agar with 5% sheep blood, and MacConkey agar (all from Remel, Lenexa, Kansas, USA) and incubated for 48 hr at 37 C with 5% CO_2. To aid in the recovery of *Salmonella* sp., each colon swab also was inoculated into a Selenite broth (Remel) which was incubated at 37 C for 24 hr. The broth was then subcultured to a Hektoen enteric agar (Remel) and the plate was incubated at 37 C for 24 hr. Isolates were identified utilizing standard biochemical tests and the API 20E and NFT systems (BioMerieux Vitek, Inc., Hazelwood, Missouri). Swabs of nasal and choanal cavities were obtained for isolation of *Mycoplasma* sp. as described previously (Brown et al., 1994). Choanal swabs were obtained prior to opening the head. For nasal swabs, after the head was cut longitudinally the nasal septum was excised and the interior sinus cavity was swabbed.

Liver weights were divided by the whole body weight to determine the percent body weight of liver for each tortoise in the study except tortoises 24 and 30. The percentages were arcsine-transformed (Sokal and Rohlf, 1995), and the differences in the percentages

TABLE 1. Location, sex, midline carapace length (MCL), weight, condition when found, and major diseases and/or lesions of 24 desert tortoises collected or salvaged between March 1992 and July 1995 for necropsies from the Mojave and Colorado Deserts of California (USA).

ID	Location	Sex	MCL (mm)	Weight (kg)	Condition	Primary diseases/lesions
1	(34°51′N, 115°09′W) San Bernardino County	F	202	1.46	Moribund—attacked by a predator	Predation and acute bacterial pneumonia
3	(33°32′N, 115°28′W), Riverside County	M	208	1.77	Alive—shell lesions	Cutaneous dyskeratosis
4	(34°25′N, 114°40′W), San Bernardino County	F	188	1.52	Alive—shell lesions	Shell necrosis (mixed bacterial and fungal etiology)
5	(34°07′N, 116°16′W), San Bernardino County	F	255	2.23	Alive—nasal and ocular discharge	Fungal pneumonia; ulcerctine enteritis
6	(34°57′N, 117°26′W), San Bernardino County	F	191	1.15	Alive—entombed and lethargic	Cutaneous fungal infection; multicentric visceral inflammation
8	(33°32′N, 115°30′W), Riverside County	M	126	0.47	Alive—shell lesions	Cutaneous dyskeratosis
9	(33°32′N, 115°29′W), Riverside County	M	284	4.03	Alive—shell lesions	Cutaneous dyskeratosis
10	(33°32′N, 115°30′W), Riverside County	F	227	1.90	Alive—shell lesions	Cutaneous dyskeratosis Fungal dermatitis
11	(33°32′N, 115°29′W), Riverside County	M	193	1.30	Alive—shell lesions	Cutaneous dyskeratosis
12	(35°02′N, 115°12′W), San Bernardino County	M	286	4.25	Alive—burn injury, weak and lethargic	Burn injury; intestinal necrosis; multicentric visceral inflammation
13	(34°06′N, 116°09′W), San Bernardino County	F	230	1.45	Alive—weak, lethargic and weight loss	Mycoplasmosis
15	(34°59′N, 117°30′W), San Bernardino County	F	176	0.74	Moribund—multiple shell fractures	Blunt trauma, acute pneumonia
17	(34°36′N, 116°41′W), San Bernardino County	F	230	2.5	Alive—shell lesions	Shell necrosis (mixed bacterial and fungal etiology)
18	(34°07′N, 116°16′W), San Bernardino County	F	240	1.89	Alive—emaciated	Mycoplasmosis
19	(34°50′N, 117°04′W), San Bernardino County	F	252	2.3	Alive—nasal discharge	Mycoplasmosis
20	(34°59′N, 116°59′W), San Bernardino County	M	222	1.78	Alive—nasal discharge, weak, and emaciated	Mycoplasmosis
23	(35°06′N, 116°29′W), San Bernardino County	M	265	1.68	Alive—weak, lethargic and weight loss	Urolithiasis; renal and articular gout

TABLE 1 Continued.

ID	Location	Sex	MCL (mm)	Weight (kg)	Condition	Primary diseases/lesions
24	(35°22′N, 115°21′W), San Bernardino County	M	285	2.70	Dead	Urolithiasis
27	(34°50′N, 114°59′W), San Bernardino County	M	178	0.90	Moribund—multiple shell fractures	Blunt trauma
28	(34°40′N, 114°54′W), San Bernardino County	F	215	1.65	Alive—shell lesions	Cutaneous dyskeratosis; septicemia
29	(33°48′N, 115°46′W), Riverside County	F	245	3.17	Alive—nasal discharge and swollen eyelids	Mycoplasmosis
30	(35°13′N, 117°50′W), Kern County	M	260	?[a]	Dead	Urolithiasis
31	(35°21′N, 117°40′W), Kern County	M	271	3.73	Alive—nasal discharge, swollen eyelids and chin glands	Inflammation of chin glands, nasal cavity, eyelids, and salivary glands
32	(35°21′N, 117°40′W), Kern County	F	210	1.75	Alive—shell lesions and flaking of skin	Cutaneous dyskeratosis; multifocal necrotizing epidermitis with intralesional fungi

[a] Autolysis precluded accurate weight measurement.

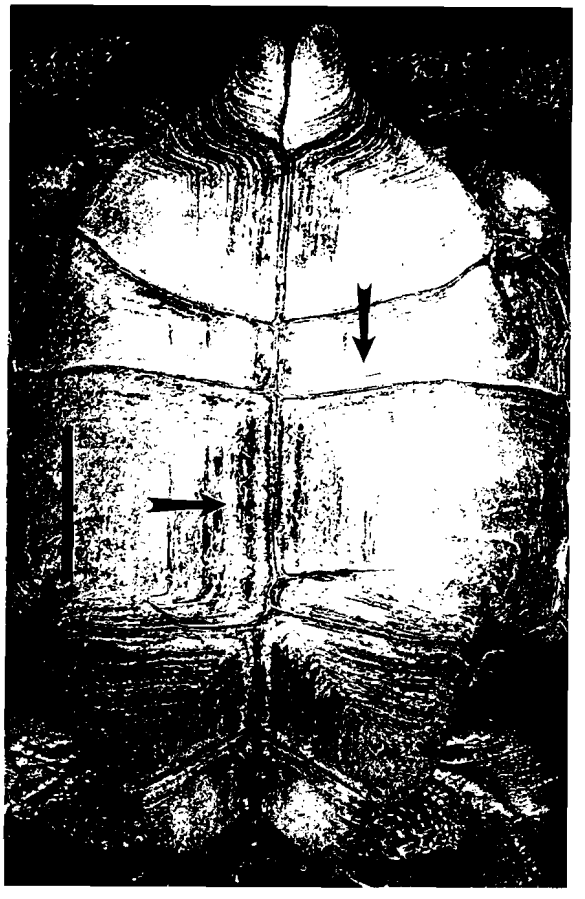

FIGURE 1. Cutaneous dyskeratosis of plastron from a desert tortoise in California (tortoise 10). Flaking and peeling extends from the seams into the scutes (arrows). Bar = 4.5 cm.

between tortoises with chronic respiratory diseases or urolithiasis and the remaining tortoises were compared using a *t*-test. Analyses were performed using SAS 6.12 (SAS Institute, Inc., 1988).

RESULTS

Desert tortoises ranged in size from 0.47 to 4.03 kg with a midline carapace length of 126 to 285 mm. Twenty-three tortoises were adults and one was a juvenile; 13 were females and 11 were males. Major diseases and/or lesions are listed in Table 1.

Seven tortoises (3, 8–11, 28 and 32) exhibited lesions consistent with cutaneous dyskeratosis. Gross shell lesions included white discoloration, flaking and peeling of the scutes, with irregular foci of pitting and chipping in the plastron and carapace.

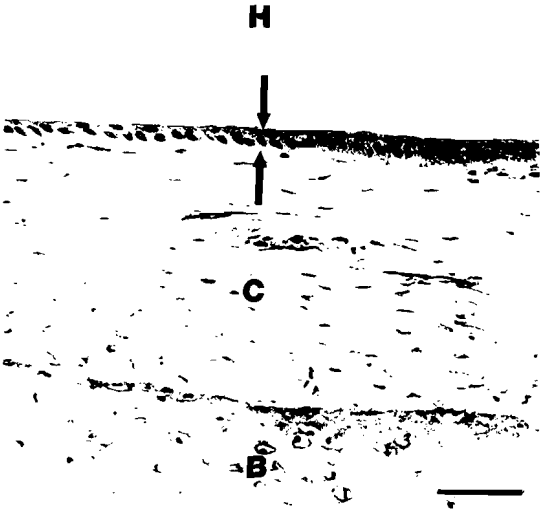

FIGURE 2. Photomicrograph of normal shell from a desert tortoise. The epidermis is comprised of uniform multilayered horn material (H), subtended by stratified cuboidal epithelium (arrows). The dermis is comprised of a collagenous layer (C) subtended by dermal bone (B). H&E. Bar = 55 μm.

Lesions were found most consistently in the plastron, usually on the midline, extending from the seams into the scutes (Fig. 1). Shell adjacent to these seams often had markedly accentuated ridges. In some areas, the white discolored scute could be peeled off in layers and was subtended by a thin layer of more normal appearing scute. Lesions in the carapace often encircled the scute just at the edges of the seams. The deepest scute defects exposed underlying bone, which was thin on cross-sectioning. Compared to normal shell (Fig. 2), areas with dyskeratosis were characterized microscopically by crevices, clefts and thinning of the epidermal horn layer (Fig. 3). In affected areas, the horn material changed abruptly from very compact pale staining substance to more eosinophilic fibrillar or fragmented substance (Fig. 3). Soil, plant debris, necrotic cellular debris and rare gram positive cocci were present in some lesions, and the epidermal epithelium was occasionally atrophic or hyperplastic. The external layer of the dermal bone was often irregular and com-

FIGURE 3. Photomicrograph of shell from tortoise with cutaneous dyskeratosis (tortoise 28). The horn material contains a cleft with fibrillar keratin and necrotic cellular debris (arrow). H&E. Bar = 115 μm.

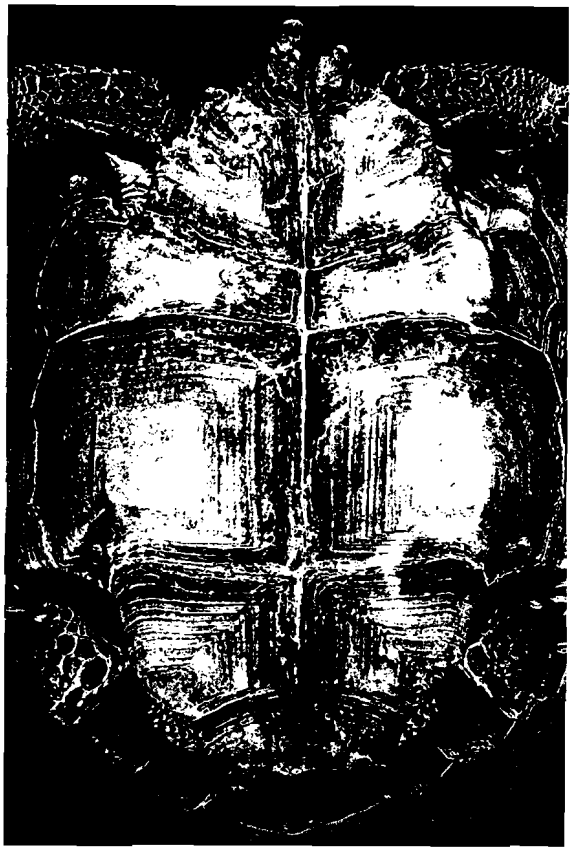

FIGURE 4. Shell necrosis of plastron (tortoise 17). Scute material has sloughed off the midline, exposing necrotic scute and dermal bone. The gular processes are irregular. Bar = 3 cm.

prised of woven bone with prominent cement lines. The most severely affected dermal bones exhibited osteoclastic resorption, osteopenia, widened osteoid seams, and an apparent increase in the number of osteoblasts lining the trabeculae. In one tortoise with osteopenia (28), mild to moderate dermal perivascular infiltrates of lymphocytes, macrophages and heterophils extended into the interosseous seams. In tortoise 10, the shell dermis was focally infiltrated by lymphocytes and the overlying horn layer was colonized by fungal hyphae. In tortoise 32, sections of skin contained intracorneal aggregates of necrotic heterophils with fungal hyphae and multifocal mild perivascular dermal infiltrates of lymphocytes.

In the two tortoises (4 and 17) with shell necrosis, several scutes had detached, revealing discolored dermal bone. In tortoise 4, the anterior scutes on the carapace and plastron had detached along the seams and the underlying bone was discolored white. On cut surface, the white dermal bone was subtended by more normal appearing light grey to cream/tan bone. In tortoise 17, scute material along the midline of the plastron was fragmented and peeling off (Fig. 4). The underlying scute material and exposed dermal bone were white to black, lusterless and had a powdery surface. Microscopically, the affected shell was characterized by discrete areas of epidermal, dermal and osseous necrosis (Fig. 5), often subtended by regenerative layers of epidermis and dermal bone that were continuous with epidermis and bone adjacent to the affected segment. Abundant necrotic cells were present within the horn material, on the surface of exposed necrotic bone and between foci of sloughing scute and underlying regenerative scute. Necrotic dermis and bone were colonized by a mixed population of gram positive and negative bacteria (Fig. 5) and aseptate, occasionally branching, 6 to 10 μm wide fungal hyphae. In areas of shell regeneration,

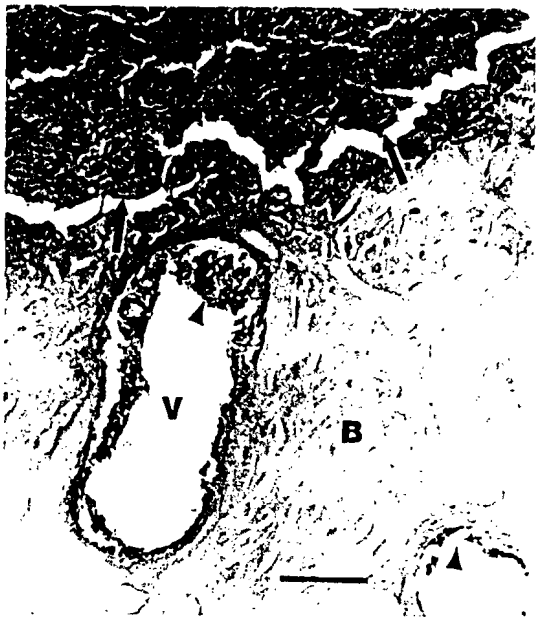

FIGURE 5. Photomicrograph of necrotic dermal bone (B) covered by necrotic cellular debris (arrows) in tortoise 4. Vascular channels (V) are colonized by bacteria (arrowhead). H&E. Bar = 28 μm.

FIGURE 6. Photomicrograph of nasal glands (G) of tortoise 18 with mycoplasmosis. Glands are disrupted by mixed leukocytic infiltrates (arrows). In two dilated glands, mucous epithelium is replaced by squamous cells (arrowheads). H&E. Bar = 55 μm.

the epidermal epithelium was multifocally hyperplastic, and the dermis was thickened by fibrous tissue and moderate mixed infiltrates of heterophils, lymphocytes, and macrophages.

Tortoises with mycoplasmosis (13, 18, 19, 20 and 29) had a clear watery, frothy or mucoid nasal discharge, the conjunctivae and eyelids were wet and swollen, and the globes were sunken in the orbit. Microscopically, the nasal cavity was characterized by proliferation, metaplasia and disorganization of respiratory and olfactory mucosal epithelium, associated with replacement of columnar mucous and ciliated epithelium by nonciliated polygonal cells, and compact cellular infiltrates of lymphocytes, plasma cells, macrophages and heterophils. Leukocytes extended into and encompassed mucous glands, resulting in disruption of the architecture (Fig. 6). The epithelium of some glands had undergone squamous metaplasia and hyperkeratosis, and debris accumulated in the glandular lumina. Low numbers of heterophils and focal accumulations of lymphocytes infiltrated edematous dermis and conjunctivae of the eyelids of some tortoises.

Two tortoises with clinical signs of respiratory disease as described above did not have mycoplasmosis. The lungs of tortoise 5 were slightly reddened and multifocally firmer than normal. Areas of pulmonary inflammation were characterized by proliferation and hypertrophy of epithelium (Fig. 7); interstitial infiltrates of heterophils, lymphocytes, and macrophages; septal smooth muscle hypertrophy; and scattered 3 to 6 μm wide, elongate, occasionally septate and branching fungal hyphae. A 1.0 cm irregular cavity in the mid left lung was filled with light tan caseous material composed of fibrin, cellular debris, necrotic leukocytes, numerous fungal hyphae and a population of 3 to 6 μm wide ovoid yeasts. Tortoise 31 had a green inspissated mucoid nasal discharge and swollen eyelids. The left chin gland

FIGURE 7. Photomicrograph of lung with proliferative pneumonia (tortoise 5). Epithelium lining air spaces is hyperplastic and hypertrophied (arrows). The hypercellular interstitium is infiltrated by a mixed population of leukocytes. H&E. Bar = 115 μm.

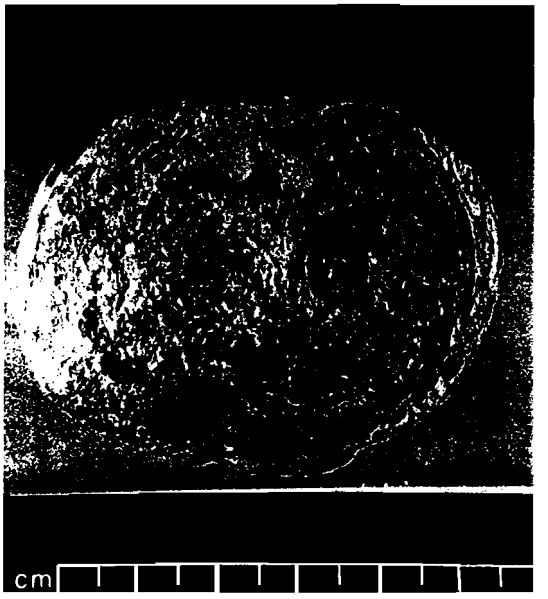

FIGURE 8. Urolith recovered from the coelomic cavity of tortoise 30.

was enlarged and exuded a small amount of grey fluid. The nasal cavity mucosa was infiltrated by heterophils. Both eyelids were edematous, with a mild infiltrate of heterophils. The chin gland contained perivascular infiltrates of lymphocytes and heterophils.

The urinary bladder of two tortoises (23 and 24) and the coelomic cavity of a severely autolyzed tortoise (30) for which only the shell remained, contained irregularly oval to round hard grey laminated uroliths (Fig. 8), measuring 3.3 × 3.3 × 3.0 to 5.6 × 4.4 × 3.8 cm and weighing 25 to 84 g. The bladder propria-submucosa was infiltrated focally by heterophils, and mucosal epithelium was hyperplastic and hypertrophied, with expansion of the apical cytoplasm by pale basophilic material that stained for mucin. In the live tortoise (23), the scapulohumeral joints and coxofemoral joints contained white thick chalky fluid (urate crystals). Multiple foci of renal tubular epithelial degeneration and necrosis were associated with tubular dilatation and intratubular and interstitial accumulation of urate crystals (Fig. 9). Nuclei of intact tubular epithelium were variably sized (anisokaryosis). Biochemical analysis of blood collected at four 3-mo intervals prior to death of tortoises 24 and 30 revealed a progressive elevation of blood urea nitrogen (25 to 306 mg/dl) in

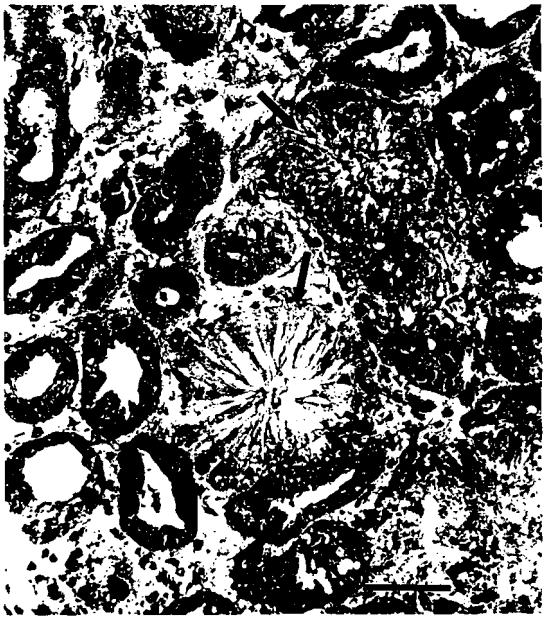

FIGURE 9. Photomicrograph of renal gout (tortoise 23). Several renal tubules are disrupted by radially arranged urate deposits (arrows). Epithelium of affected tubules is necrotic or sloughed. H&E, Bar = 56 μm.

FIGURE 10. Large intestine of tortoise with burn injury (tortoise 12). Discrete area of necrosis is well demarcated from adjacent unaffected intestine (arrows).

FIGURE 11. Photomicrograph of intestine from Figure 10. The mucosa is extensively ulcerated and there is a focal fibrinonecrotic exudate (arrowheads). The oxyurid-like nematode in the lumen facing the intact mucosa is considered an incidental finding. H&E. Bar = 115 μm.

both tortoises, and elevations of uric acid (5.6 to 12.9 mg/dl) in tortoise 24.

The interior of the burrow of tortoise 6 had collapsed, and the tortoise was tightly packed in dirt. The shell and skin were multifocally discolored brown/orange and were flaky. The stratum corneum of the skin contained aggregates of necrotic heterophils, the epidermis was focally necrotic and vacuolated, and the dermis was infiltrated by heterophils and scattered aggregates of lymphocytes. The horn layer of both skin and shell was colonized variably by 7 to 10 μm wide, occasionally branching fragmented fungal hyphae.

Tortoise 12 was collected following a brush fire. Black burn marks and foci of melted shell were present around the edges of the shell and the skin over the head, left foreleg and hind legs. Burned skin peeled easily, revealing thick creamy gray malodorous material covering blackened subcutis. The small and large intestines contained several 3 × 3 to 9 × 4 cm discrete transmural green/tan to dark purple, dull, friable foci encompassed by a red rim (Fig. 10). Microscopically, the small and large intestine contained multiple foci of acute mucosal to transmural necrotizing inflammation (Fig. 11).

Tortoises 15 and 27 were found moribund at the edge of a highway. The carapace and plastron were fractured through the scutes and bone along oblique lines or through the seams. The shell fractures extended into the coelomic cavity, and the intestine and liver were covered with blood and protruded through the fracture sites. The liver was multifocally lacerated and the spleen of tortoise 27 was ruptured, leaving only the stroma intact.

Tortoise 1 had chew marks associated with partial amputation of the cranium, maxilla and the dorsal neck region, ulceration of the tongue, and perforation of the trachea. A similar population of gram positive cocci, often present in pairs or small

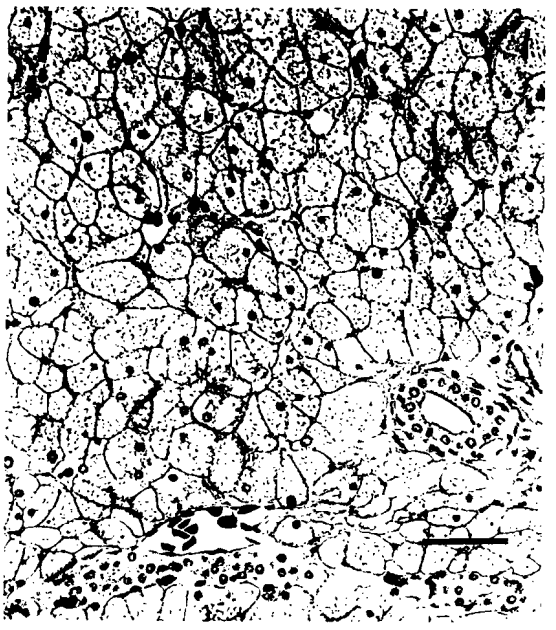

FIGURE 12. Photomicrograph of liver with vacuolar change (tortoise 8). Hepatocytes are swollen, compressing the sinusoids. Hepatocellular cytoplasm is compartmentalized by variably discrete vacuoles. H&E. Bar = 57 μm.

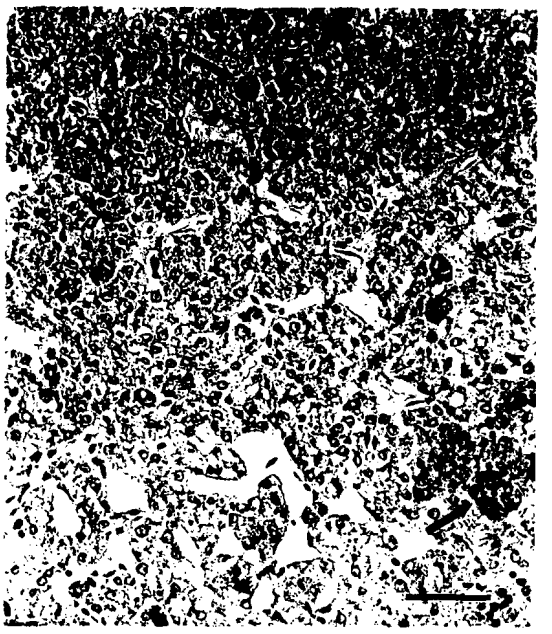

FIGURE 13. Photomicrograph of atrophic liver (tortoise 23). Variably sized (anisokaryotic) vesicular nuclei with prominent nucleoli are crowded due to loss of cytoplasm associated with atrophy. Cytoplasm is granular due to deposition of hemosiderin. There is increased deposition of melanin (arrows). H&E. Bar = 60 μm.

chains, colonized the lung, the ulcerated area of the tongue and the lumen of the esophagus.

Liver degeneration was found in 15 tortoises. Some livers varied from small and dark brown to swollen with rounded edges, pale tan coloration and friable consistency. A few pale livers floated in formalin. However, many livers with histopathologic changes were unremarkable on gross examination. Liver lesions included hepatocellular vacuolar change (Fig. 12), atrophy (Fig. 13), increased deposition and aggregation of melanin (Fig. 13), hemosiderosis, and anisokaryosis. Liver lesions were most severe in tortoises with chronic respiratory diseases and urolithiasis (5, 13, 18, 19, 20, 23, and 29). The ratio of liver mass to body weight for these tortoises was less than that of other tortoises in the study ($P < 0.001$), ranging from 0.95 to 2.2% of body weight compared to 2.1 to 6.0% for the other tortoises.

Degenerative skeletal muscle changes, found in 16 tortoises, were characterized by loss of striation, hyalinization, internalized nuclei and atrophy. The most severe changes of acute degeneration and necrosis occurred in traumatized tortoises. Tissue cysts of *Sarcocystis*-like protozoa were present in skeletal muscles of six tortoises (1, 3, 4, 17, 20, 29), sometimes associated with mild infiltrates of lymphocytes and heterophils, and fibrosis.

Pancreatic acinar cells in tortoises 5, 13, 19, 20, 23, and 29 were shrunken and devoid of zymogen granules, consistent with atrophy. Lymphoid depletion of the spleen and hypertrophy and intracytoplasmic mucus accumulation of urinary bladder epithelium were other changes seen in some tortoises with chronic respiratory disease and urolithiasis; the latter lesion was associated often with evidence of dehydration (sunken globes, dry tacky subcutaneous and coelomic tissues, and weight loss). One or more tortoises with cutaneous dyskeratosis (28 and 32), shell necrosis (4), respiratory disease (5, 18 and 19), and a history of trauma (1, 6 and 12) had multicentric inflammation of multiple coelom-

ic organs including heart, lung, intestine, ovaries, kidney, adrenal, and spleen. The most common change in the bone marrow of tortoises with multicentric inflammation was hyperplasia of heterophils. However, in two tortoises with severe visceral inflammation (1 and 12), the number of heterophils was markedly reduced. Three tortoises had cactus spines embedded in the intestine and stomach (24) or tongue (3 and 27) and pharynx (27), associated with granuloma formation or acute inflammation and bacterial colonization.

Serologic examinations were conducted only for tortoises that arrived alive. Tortoises 12 and 23 had a suspect ELISA result at the 1:10 dilution. Tortoises 13, 18, 19, and 20 had seropositive results at 1:5 and 1:10 dilutions.

Potential bacterial pathogens isolated from the choanae and colon included *Citrobacter* sp. *Klebsiella oxytoca, Pasteurella testudinis, Pseudomonas* sp., and *Xanthomonas maltophilia. Pasteurella testudinis* was the most frequently isolated bacterium (9 of 23 tortoises), and the only organism isolated from both the choanae and colon of individual animals (5 of 9 tortoises). *Mycoplasma* sp. was isolated from the choanae and nasal cavity of two tortoises (13 and 29).

DISCUSSION

Pathologic changes associated with cutaneous dyskeratosis appear to have two components; one is loss of integrity in the hard keratin of the scute and the other is resorption of the underlying dermal bone. The disease appears to be associated with a defect in keratinization based on the propensity of the lesion to center over the seams. The keratinization process occurs at the seams (Jacobson et al., 1994). The role of the epithelium in the pathogenesis of cutaneous dyskeratosis is unclear; the epithelial layer was sometimes atrophied or hyperplastic, but necrosis or ulceration of epithelium was not detected. Jacobson et al. (1994) suggested that cutaneous dyskeratosis could be caused by an infectious etiology, a toxic cause, or a nutritional deficiency. The absence of consistent shell inflammation and consistent bacterial or fungal colonization of the lesion tends to rule out an acute infectious etiology. The occasional bacteria and fungi that colonized the lesion were considered to be secondary invaders. Livers of several tortoises with cutaneous dyskeratosis had markedly swollen and vacuolated hepatocytes, and three from the Chuckwalla Bench (8, 9, and 10), where cutaneous dyskeratosis has been seen most commonly (Jacobson et al., 1994; Berry, 1997), had moderate hepatocellular anisokaryosis, suggestive of possible toxic liver disease (Kelly, 1993). There was gross and histologic evidence of dermal bone loss, although the relationship between the bone loss and the changes in the scute are not yet clear. It appears that bony changes occurred in the later stages of the disease when scute changes were most severe. The cause of decline in populations with high percentages of affected tortoises has not been determined. The anticipated end-stage of excessive scute disease and dermal bone loss would be perforation into the coelomic cavity. Although shell perforation was not detected in any of the tortoises of this study, areas of scute loss and osteopenia could have provided a portal of entry for the bacteria and fungi that colonized the scute. Most of the internal lesions were not immediately life threatening. However, the multicentric inflammation of one tortoise (28), pneumonia and fungal epidermitis of another tortoise (32), and fungal dermatitis of a third tortoise (10) would have likely resulted in the death of these three tortoises.

Features of shell necrosis not found in cutaneous dyskeratosis included sloughing of entire scutes, epidermal and bone necrosis, colonization of necrotic bone by bacterial and fungal organisms, extensive dermal inflammation, and shell repair. Bacterial and fungal cultures were not obtained from shell or skin, but *Klebsiella* sp. (tortoise 4), *Pseudomonas* sp. (tortoises 4

and 17), and *Xanthomonas maltophilia* (tortoise 17), all opportunistic pathogens associated with dermatitis (Rossi, 1996; Spencer, 1995), were isolated from the choanae. Shell repair occurred by infolding of hyperplastic epithelium from the margins of the lesion with undermining and extrusion of the necrotic portion similar to that described in other turtles with shell necrosis (Garner et al., 1997).

Most tortoises with mycoplasmosis were in moderately poor condition, as evidenced by dehydration and low body weight. All tortoises diagnosed with mycoplasmosis had nasal lesions similar to those reported by Jacobson et al. (1991, 1995). Tortoises also had inflammatory lesions in other tissues, and in some tortoises, visceral inflammation was multicentric, consistent with systemic disease. The livers of all tortoises and the pancreas of all but tortoise 18 were atrophied, consistent with chronic disease and/or malnutrition (Kelly, 1993). The species of *Mycoplasma* isolated from tortoises 13 and 29 has not yet been identified by serological or polymerase chain reaction analysis (Brown et al., 1995); it was not *M. agassizii* (Brown et al., 1994). However, the lesions in the affected tortoises were virtually identical to those described by Brown et al. (1994) in their transmission study using *M. agassizii*. Both tortoises were salvaged from Joshua Tree National Park, which is a highly frequented recreational area. Serologic testing helped to confirm the diagnosis for all affected tortoises except tortoise 29. The reason the ELISA test was positive for tortoise 13 but not 29 is not certain. Although antigens from the unnamed species of *Mycoplasma* were used to test the blood of tortoise 29, the ELISA test employed was developed for diagnosis of *M. agassizii* (Schumacher et al. 1993) and has not been standardized for detection of the other *Mycoplasma* sp. It also is possible that tortoise 29 was immunocompromised and did not have adequate antibody concentration. Suspect results in two tortoises (12 and 23) could represent early infection, immunosuppression, an ablated response or a nonspecific reaction.

Fungi were not cultured from the lung of tortoise 5 with histologic findings of fungal pneumonia, but based on the morphology of the fungal hyphae and ovoid yeasts, there appeared to be a dual infection with *Aspergillus* sp. and *Candida* sp. (Chandler et al., 1980). Both organisms have been isolated from pulmonary lesions in chelonians (Frye, 1991a). In a review of causes of mortality and diseases in tortoises, no reports on mycotic diseases in wild tortoises were found (Jacobson, 1994). Fungi often cause infection secondary to compromise of immune function (Jacobson, 1980). In tortoise 5, there was a marked reduction in numbers of splenic lymphocytes, possibly resulting in immunosuppression. *Citrobacter* sp., isolated from the colon, has been associated with septicemic cutaneous ulcerative disease of aquatic turtles (Rossi, 1996) and could have been responsible for ulcerative enteritis found in this tortoise. In turn, the intestinal lesion may have served as a portal of entry for the fungi.

Clinical signs and lesions in tortoise 31 resembled those of mycoplasmosis; however, serologic testing and culture for *Mycoplasma* were negative. This may not rule out mycoplasmosis, since rhinitis and blepharitis were acute. Lesions of mycoplasmosis in tortoises of this and other studies (Jacobson et al., 1991, 1995) were more chronic. Failure to isolate other pathogenic bacteria from the choanae tends to rule out a bacterial etiology. Another cause of upper respiratory and ocular disease in tortoises is vitamin A deficiency (Jacobson, 1994). However, squamous epithelial metaplasia, a hallmark of hypovitaminosis A, was not detected.

Three tortoises (23, 24 and 30) had urolithiasis. Factors that predispose animals to urolithiasis include excretion of calculogenic material in the urine, urinary pH, dehydration, vitamin A deficiency and supersaturation of urine by stone-forming salts (Maxie, 1993). While uroliths may be

tolerated to some extent, they may incite an inflammatory response in the urinary bladder and they occupy space in the urinary bladder that would normally hold fluid. In addition to heterophilic inflammation of the bladder wall, mucosal epithelial cells were hyperplastic and the apical cytoplasm was expanded with mucus. We believe the epithelial changes were either a reaction to the uroliths or an indication of dehydration. The latter is supported by the presence of similar changes in other dehydrated tortoises that did not have uroliths. The renal and articular lesions in tortoise 23 were consistent with gout (Frye, 1991b). Predisposing factors for gout include dehydration, pre-existing renal disease, exposure to a nephrotoxin, or excess animal protein in the diet (Maxie, 1993). However, the exact cause in desert tortoises is unknown. Based on the severity of the renal lesion and the lack of inflammation, a preexisting renal lesion was the likely cause of gout in this tortoise. The exact cause of death of tortoises 24 and 30 could not be determined. Dehydration and enteritis, associated with cactus spine penetration, were likely contributing factors in tortoise 24. Prior to death, both tortoises had biochemical evidence (elevated BUN and uric acid) of dehydration and/or renal insufficiency (Campbell, 1996); hyperuricemia also can be associated with gout. Renal damage could not be assessed due to advanced autolysis.

Two tortoises that sustained trauma (6 and 12) had multicentric visceral inflammation. Fungal dermatitis accounted for the flakiness of the skin of tortoise 6. Although fungi can be primary cutaneous pathogens, superficial cutaneous mycotic infections in reptiles usually occur secondary to some predisposing factor such as poor sanitation, high humidity, malnutrition or overcrowding (Jacobson, 1980). Heterophilic hyperplasia in the bone marrow was a response to the multicentric inflammation (Garner et al., 1996). Visceral inflammation in tortoise 12 was most severe in the intestine. The transmural intestinal necrosis was considered to be a post-burn complication, probably associated with impaired humoral and cell-mediated immunity (Griswold, 1993). Burn wound infection is usually caused by endogenous bacteria from the skin or gastrointestinal tract (Saxon and Kirby, 1992). *Citrobacter* sp. and *P. testudinis* were isolated from the colon, and the subcutis of the burned foreleg was colonized by bacteria. Unlike tortoise 6, bone marrow was depleted of heterophils, which was most likely the result of an overwhelming demand for heterophils in the intestine (Garner et al., 1996). Two tortoises (15 and 27) had evidence of acute blunt trauma as would occur subsequent to being struck by a moving vehicle. There was no other evidence of underlying disease in the tortoises. The cause of death of tortoise 1 was acute bacterial-induced bronchopneumonia, consistent with *Streptococcus* sp. etiology, likely occurring secondary to the head and neck lacerations.

Hepatic lesions seen in 15 tortoises were degenerative, but were otherwise nonspecific. Causes of lipidosis and hydropic degeneration overlap and include bacterial or environmental toxins, hepatocellular hypoxia, anorexia or dietary and metabolic imbalances (Kelly, 1993). Anisokaryosis represents a regenerative or toxic change, while hemosiderosis may have occurred due to inability to mobilize iron stores, as occurs in anemia of chronic disease (Smith, 1989). Iron-laden macrophages were reported to increase in livers of tortoises with URTD (Jacobson et al., 1991). The liver may undergo catabolism during starvation, resulting in atrophy (Kelly, 1993). Livers of chronically ill tortoises commonly contained increased amounts of melanin, suggesting that melanosis is a marker of chronic disease, as described in fish (Kennedy-Stoskopf, 1993). Melanin is not harmful. Conversely, melanin can neutralize free radicals and may have a bactericidal effect (Kennedy-Stoskopf, 1993).

The high frequency (16 tortoises) of

acute skeletal muscle lesions suggests that tortoises might be prone to exertional rhabdomyolysis (Bartsch et al., 1977). This lesion may have occurred when tortoises were restrained for blood collection. Muscle lesions seen in traumatized tortoises were also consistent with exertional rhabdomyolysis. Six tortoises had organisms resembling *Sarcocystis* sp. within muscle fibers, occasionally associated with mild chronic myositis. *Sarcocystis* spp. are generally not considered to be pathogens of reptiles (Barnard and Upton, 1994).

A number of chronically ill tortoises had a history of weight loss and were emaciated at the time of collection. In these tortoises, weight loss was an excellent indication of chronic disease and was often associated with muscle, pancreatic and/or hepatic atrophy. However, body weight versus carapace length cannot be used by itself to assess the health of tortoises because body weight is influenced by oviposition, time since hibernation, imbibition of water, defecation and micturition, and thickness of dermal bone (Jacobson et al., 1993). Liver as a percent of body weight may be a better indicator of chronic disease, since there was a significant difference between the percentage in tortoises with chronic respiratory diseases or systemic illness associated with urolithiasis and that of the other tortoises in this study.

This study was designed to address the types of diseases and associated pathologic changes occurring in wild desert tortoises, rather than the prevalence of disease. It was part of a larger investigation on density of desert tortoise populations. Only dead tortoises or those with obvious signs of illness were collected. Diseases may have a direct and obvious impact on mortality in populations of free-ranging desert tortoises. However, the effects of many diseases may be more subtle. Diseases may contribute to reduced growth, reduced reproductive vigor, and reduced survivorship of individuals and populations. Cutaneous dyskeratosis, for example, appears to contribute to or cause thinning of the scutes and dermal bone, thereby rendering the tortoise more vulnerable to other diseases such as fungal infections and multicentric visceral inflammation. Diseases also may inhibit or slow growth rates of individuals by reducing appetite, resulting in malnutrition. For females, reduction in growth rates limit carapace length and size, thereby limiting numbers of eggs produced annually. For juveniles, reduction in growth rates prolong the period of vulnerability to predators and environmental stresses.

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GUIDELINES FOR THE FIELD EVALUATION OF DESERT TORTOISE HEALTH AND DISEASE

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ABSTRACT: Field evaluation of free-ranging wildlife requires the systematic documentation of a variety of environmental conditions and individual parameters of health and disease, particularly in the case of rare or endangered species. In addition, defined criteria are needed for the humane salvage of ill or dying animals. The purpose of this paper is to describe, in detail, the preparation, procedures, and protocols we developed and tested for the field evaluation of wild desert tortoises (*Gopherus agassizii*). These guidelines describe: preparations for the field, including developing familiarity with tortoise behavior and ecology, and preparation of standardized data sheets; journal notes to document background data on weather conditions, temperature, rainfall, locality, and historic and recent human activities; procedures to prevent the spread of disease and parasites; data sheets for live tortoises to record tortoise identification, location, sex, body measurements and activity; health profile forms for documenting and grading physical abnormalities of tortoise posture and movements, general condition (e.g., lethargy, cachexia), external parasites, and clinical abnormalities associated with shell and upper respiratory diseases; permanent photographic records for the retrospective analysis of progression and regression of upper respiratory and eye diseases, analysis of shell lesions and evaluation of growth and age; and indications and methods for salvaging ill or dying tortoises for necropsy evaluation. These guidelines, tested on 5,000 to 20,000 tortoises over a 10 to 27 yr period, were designed to maximize acquisition of data for demographic, ecological, health and disease research projects; to reduce handling and stress of individual animals; to avoid spread of infectious disease; to promote high quality and consistent data sets; and to reduce the duration and number of field trips. The field methods are adapted for desert tortoise life cycle, behavior, anatomy, physiology, and pertinent disease; however the model is applicable to other species of reptiles. Comprehensive databases of clinical signs of disease and health are crucial to research endeavors and essential to decisions on captive release, epidemiology of disease, translocation of wild tortoises, breeding programs, and euthanasia.

Key words: Chelonian, desert tortoise, diagnosis, disease, field evaluations, *Gopherus agassizii*, health assessments.

INTRODUCTION

Most research on populations of wild animals is conducted by wildlife biologists, zoologists, and ecologists without collaboration with veterinary medical specialists. Many research projects, especially those involved with rare and endangered animals, could benefit from the contributions of veterinarians and other health specialists (Boyce et al., 1992) at every phase. Veterinarians and wildlife health specialists can assist in identifying diseases and their ecological significance to wild animal populations, determining the effects of anthropogenic impacts (e.g., stress), and developing management options for recovery and rehabilitation (Kirkwood, 1993, 1994).

Research on the desert tortoise (*Gopherus agassizii*), a species of the arid southwestern United States and Mexico, provides an excellent model for how interdisciplinary teams of research scientists developed techniques to evaluate health and diagnose disease. The tortoise was listed by the federal government as a threatened species under the Endangered Species Act (ESA) of 1973 (as amended) over approximately 30% of its geographic range in the arid southwestern USA and Mexico in 1990, because several populations were experiencing declines (Fish and Wildlife Service [FWS], 1994; Berry, 1997a). Two recently described diseases, upper respiratory tract disease (URTD) and cutaneous dyskeratosis, were associated with population declines in some areas (Brown

et al., 1994; Jacobson et al., 1995; Berry, 1997b). Upper respiratory tract disease is caused by *Mycoplasma agassizii* (Jacobson et al., 1991; Brown et al., 1994) and an as yet unnamed new mycoplasma organism (Brown et al., 1995). An enzyme-linked immunosorbent assay (ELISA) test was developed to measure antibodies to *Mycoplasma agassizii* in tortoises (Schumacher et al., 1993). URTD and exposure to mycoplasma, as evidenced by positive ELISA tests and presence of mycoplasma in nasal secretions by cultures or polymerase chain reaction tests, have been documented in tortoises at multiple sites in the Mojave Desert (Jacobson et al., 1995; Dickinson et al., 1995; Homer et al., 1998; Brown et al., 1999). Upper respiratory tract disease is a transmissible disease, often subclinical and generally chronic (Brown et al., 1994; Jacobson et al., 1995; Homer et al., 1998). Cutaneous dyskeratosis produces lesions on the shell and integument and is of unknown etiology (Jacobson et al., 1994), although environmental toxicants and nutritional deficiencies are suspected contributors (Homer et al., 1998).

We developed a model set of standardized field guidelines for collecting and analyzing qualitative and quantitative data on clinical and physical signs of health, disease, and trauma for wild desert tortoises. The guidelines and techniques were designed to maximize acquisition of data for demographic, ecological, health and disease research projects; to reduce handling and stress of individual animals; to avoid spread of infectious disease; to promote high quality and consistent data sets; and to reduce the duration and number of field trips. Techniques for recording journal notes and information about live tortoises were developed, tested, and revised between 1971 and 1998 at 27 study plots in the California deserts (e.g., Berry and Medica 1995; Berry 1997b) with >20,000 captures of wild tortoises. Most techniques for assessing health and disease were developed and tested between 1988 and 1998 at 36 sites in California with >5,000 captures of tortoises (e.g., Berry, 1997b; Henen et al., 1998; Homer et al., 1998; Brown et al. 1999; Christopher et al., 1999). These standardized field methods represent a productive collaboration between wildlife biologists, veterinarians and pathologists, and are applicable to other chelonians and reptiles.

PREPARATIONS FOR THE FIELD

Prior to initiating field work, project participants should familiarize themselves with the literature on wild desert tortoises to optimize time and expedite location of tortoises (e.g., FWS, 1994; Grover and DeFalco, 1995). The annual cycle of above-ground activity for tortoises varies according to location within the geographic range and depends on such environmental factors as number of freezing days per annum, timing and amounts of precipitation, day- and night-time temperatures, and the type of desert (FWS, 1994). The exact timing of above ground activity is also dependent on availability of forage, local weather patterns, and ambient daytime temperatures (Nagy and Medica, 1986; Ruby et al., 1994; Zimmerman et al., 1994; Henen, 1997), as well as the size and age of tortoises (Berry and Turner, 1986).

Wild tortoises are easily accessible (near entrances of their burrows or dens, or above ground) to the field worker about 1.7% of each year in the Mojave Desert (Nagy and Medica, 1986). They hibernate in late fall and winter, can be active above ground in late winter and spring, may estivate in summer, and may become active again in late summer and early fall. In the Sonoran Desert, the seasonal activity pattern is associated with monsoon rains, with tortoises active above ground primarily in summer and fall (Johnson et al., 1990). Immediately after emergence from hibernation in late winter and early spring, tortoises usually have a single activity period during the middle of the day, and shift to a bimodal pattern as ambient temperatures increase in late spring (Zimmerman

et al., 1994). During drought years, tortoises can be considerably more difficult to locate above ground. To ensure success in planning field work and locating tortoises, the field biologist should gather information on regional climatic patterns and local weather conditions, particularly precipitation during the previous year, from National Oceanic and Atmospheric Administration weather stations. The windows of activity when field workers can easily capture the tortoises are narrow, so each tortoise should be processed quickly to maximize encounters and sample sizes.

Field workers should familiarize themselves with the full repertoire of postures, behaviors, and display patterns of healthy desert tortoises (Ruby and Niblick, 1994) and the contexts in which they normally occur. Courtship in the Mojave Desert, for example, may occur in any month in which tortoises are above ground, with intense mating activity in both spring (April–May) and fall (August–November) (Rostal et al., 1994a: Ruby and Niblick, 1994). Nesting occurs between April and July (Turner et al., 1986; Rostal et al., 1994a). The timing of reproductive activities may be different in tortoise populations in the Sonoran and Chihuahuan deserts. Field workers also should be knowledgeable of abnormal behaviors and signs of ill health and disease by reviewing the literature on wildlife diseases.

Wild desert tortoises are similar to other members of the Testudinidae and exhibit a wide variety of responses when captured. They can be tame and curious, try to escape, or retreat tightly into their shells, posing difficulties for a thorough examination of the accessible soft parts (limbs, head, and tail). Since the species is threatened and protected under the ESA of 1973, as amended, efforts must be taken to reduce stress and handling time and to release the tortoise at the site of capture within 15 to 20 min. To ensure expeditious processing, new field workers should practice under an experienced supervisor on legally held captive desert tortoises or other chelonians.

Effective and efficient data collection can be accomplished by following written protocols and recording data on standardized forms printed on archival paper. These forms should document background environmental data, individual tortoise data, and data from physical examination of the tortoise. The forms can be modified to suit special projects and other species, and can be handwritten or directly entered into portable computerized databases in the field.

JOURNAL NOTES

Journal Notes should provide background data essential for interpreting whether the activities and behaviors of tortoises are typical of ill or healthy animals, as well as for identifying potential sources of trauma, illness, or disease. Journal notes should contain survey times, numbers of live and dead tortoises observed, starting and ending times of field work, time expended in searching for and processing tortoises, and observations of other animals (Fig. 1). Details of actual times spent in observing tortoise behavior from a distance as opposed to handling are recorded in more detail on other data sheets (Figs. 2, 3).

Daily weather conditions can substantially alter the interpretation of tortoise activity levels, behavior and physiology, so Journal Notes should contain a daily summary of weather conditions. For example, a rainfall event during late spring, summer or early fall can stimulate *en masse* emergence of tortoises to drink and rehydrate (Henen et al., 1998). In contrast, precipitation during cold weather in winter is unlikely to elicit emergence when tortoises are hibernating. Similarly, if air temperatures exceed 40, a panting tortoise may be interpreted as being overheated and unable to find shelter (an abnormal situation). Therefore the field biologist should begin each day by recording percentage and type of cloud cover, amount and tim-

JOURNAL

Desert Tortoise (*Gopherus agassizii*)
Paradise Mountains
San Bernardino County, California

Date _____

Start & Finish Times (PST) _____

Areas Searched (by section & grid no.; map also) _____

_____

Capture type 1: _____

Capture type 2: _____

Capture type 3: _____

Shells: _____

Other Capture types:

Names of field workers	Start & end times	Search times	Processing times	Total field time

	Temperatures (°C)				
Pacific Standard Time	1.5 m	1 cm	soil surface (shaded bulb)	Wind speed & direction	Cloud cover
0800					
1200					
1600					

min. temperature for the day: _____

max. temperature for the day: _____

HUMAN USES

People: _____

_____

_____

Vehicles (type & numbers): _____

_____

_____

Livestock: _____

_____

Shooting: _____

Other: _____

Other Notes: _____

FIGURE 1. Sample data sheet for Journal Notes.

ing of precipitation, temperatures, and wind speed. Air temperatures, recorded with a Schultheiss or Miller and Weber (Miller & Weber, Inc., Ridgewood Queens, New York, USA) quick-reading thermometer (0–50 C), are taken at 1.5 m, at 1 cm above the soil surface (shaded bulb) and on the soil surface (shaded bulb) at least three times daily (0800, 1200, and 1600 PST) and can be recorded also at the location of capture of each tortoise. Since many facets of tortoise behavior, physiol-

DO NOT ABBREVIATE

WRITE ON THIS SIDE ONLY

Data Sheet for Live Desert Tortoises

Field Worker _____ Tortoise ID Number _____

Study site name _____ Verification of ID _____

Study site number _____ Year first marked _____

Township _____ Range _____ Capture Type _____ Sex _____

Section _____ Grid no. _____ Date (day/month/yr) _____

COORDINATES (Reference SW corner) Time (PST): Start _____ End _____

_____ meters North, _____ meters East On Plot _____ Off Plot _____

County _____ ◄— Show location of tortoise in grid

State _____

TORTOISE LOCATION

Cover site type: burrow ☐ pallet ☐ shrub ☐ caliche cave ☐ rock shelter ☐

At cover site: entering ☐ exiting ☐ on mound ☐ inside ☐

Not at cover site: in open ☐ other ☐ _____ _____ _____

COVER SITE DATA

For tortoises ≤140 mm MCL

orientation _____

length _____ height _____

width _____ soil cover _____

location _____ _____

SURVEY TYPE

coverage 1 ☐ coverage 2 ☐ juv. search ☐ other ☐ _____ _____

TORTOISE ACTIVITY

resting ☐ walking ☐ basking ☐ feeding ☐

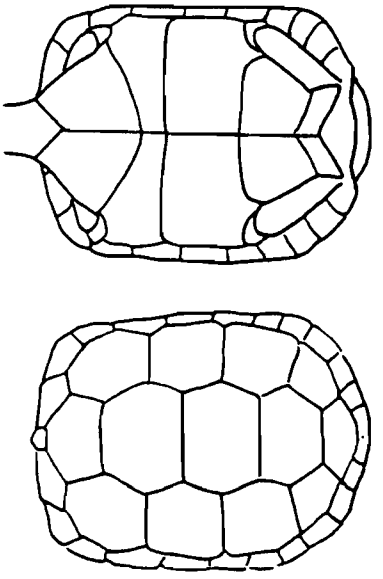
Interacting with other tortoise ☐ ID & sex of other tortoise _____

Interacting with other animals ☐ other species _____

Describe interaction: _____ _____

plants/items eaten (be specific): _____

SPACE FOR MORPHOMETRIC MEASUREMENTS, ECOLOGICAL DATA, Etc.



BODY MEASUREMENTS

carapace length at midline, MCL (mm) _____

plastron length (notch), PLN (mm) _____

weight (g) _____

void/feces (g) _____

total weight (g) _____

new growth: present ☐ absent ☐

epoxied #: present ☐ legible ☐

DRAW LOCATIONS OF NOTCHES (old and new), chips, and anomalies, etc.
Describe anomalies in numbering of marginals and any identification problems.

Other notes: _____ _____ _____ _____

Photo reference: roll _____ frames _____

Berry/livtort.3-1993(mac)

FIGURE 2. Sample data sheet for Live Desert Tortoises.

DO NOT ABBREVIATE **WRITE ON THIS SIDE ONLY**

Health Profile Form for Desert Tortoises

Field Worker _____ Tortoise ID Number _____

Study site name _____ No. ____ Year first marked _____

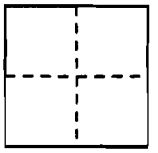
_____ MCL _____ Weight (g) _____

Township _____ Range _____ Capture Type _____ Sex _____

Section _____ Grid no. _____ Date (day/month/yr) _____

County _____ State _____ Time (PST): Start _____ End _____

On Plot _____ Off Plot _____ Shell Wear Class _____



← Location of tortoise in grid

BEAK & NARES	YES	NO	UNK
Beak/nares wet	☐	☐	☐
Beak/nose damp	☐	☐	☐
Nasal exudate present	☐	☐	☐
Exudate color: clear	☐	☐	☐
cloudy	☐	☐	☐
white	☐	☐	☐
yellow	☐	☐	☐
green	☐	☐	☐
Bubble(s) from nares	☐	☐	☐
One nare occluded	☐	☐	☐
Both nares occluded	☐	☐	☐
Dirt on nose/beak	☐	☐	☐
Dirt in nares	☐	☐	☐

FORELEGS (adjacent to face)			
Dried dirt on forelegs	☐	☐	☐
Moisture on forelegs	☐	☐	☐
Dried exud. on scales[1]	☐	☐	☐
Scales cracking[2]	☐	☐	☐

BREATHING:			
Smooth	☐	☐	☐
Wheezing	☐	☐	☐
Rasping, clicking	☐	☐	☐

EYES, CHIN GLANDS *Circle eyes or lids:*			
Eyes/lids whitened or discolored	☐	☐	☐
Eyelids swollen	☐	☐	☐
Eyes/lids wet	☐	☐	☐
Discharge from eyes	☐	☐	☐
Eyes sunken	☐	☐	☐
Eyes clear, bright	☐	☐	☐
Eyes dull, cloudy	☐	☐	☐
Chin glands draining	☐	☐	☐

INTEGUMENT[3]			
Integument dull	☐	☐	☐
Integument glossy	☐	☐	☐
Normal elasticity	☐	☐	☐
Abnormal skin peeling	☐	☐	☐

POSTURE/BEHAVIOR			
Alert, responsive	☐	☐	☐
Lethargic	☐	☐	☐
Can withdraw tightly into shell	☐	☐	☐
Limbs, head hanging limp or loose	☐	☐	☐

ORAL CAVITY[4]	YES	NO	UNK
Observed	☐	☐	☐
Discharge present	☐	☐	☐
Membranes pink	☐	☐	☐
Membranes pale, white	☐	☐	☐
Smells/mouth rot	☐	☐	☐

EVIDENCE OF SHELL/BONE DISEASE			
Lesions present	☐	☐	☐
Lesions active	☐	☐	☐
Lesions healed	☐	☐	☐
Scute laminae peeling	☐	☐	☐
Scutes missing/peeling	☐	☐	☐
Pitting	☐	☐	☐
Scutes depressed/concave	☐	☐	☐
Fungal areas	☐	☐	☐

EVIDENCE OF TRAUMA			
Head	☐	☐	☐
Gular	☐	☐	☐
Forelimbs	☐	☐	☐
Hindlimbs	☐	☐	☐
Shell	☐	☐	☐
Bone/scute replacement	☐	☐	☐

Describe: _____

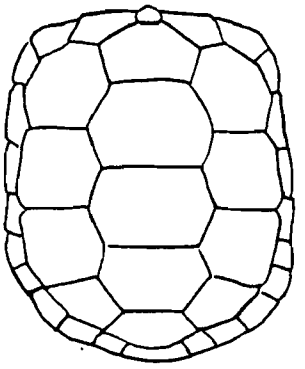
Soil dryness: wet ____ damp ____ dry ____

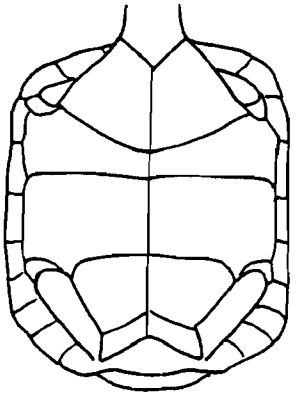
Last Precipitation (day/mo/yr) _____

Urine (vol) _____
Color _____
Viscosity _____
Particulates _____
color _____
Nasal wash sample collected _____
No. of needle sticks _____
Time of needle sticks _____
Location _____
PCV% _____
Other samples taken _____
Describe/draw parasites _____
_____
Other _____

LEGEND FOR DIAGRAM
- Pits in integument and/or shell
- Shell disease
- Trauma
- Ticks, mites

DRAW: shape of gulars, location of notches; chips, chews, shell damage, lesions; shell disease; shell abnormalities; scute concavities. Make new drawing at least once/year (spring).





OTHER NOTES: _____

Footnotes: 1 - Shiny integument, glossy with dried exudate. 2 - The integument can crack from effects of the exudate. 3 - Difficult, but try. For normal elasticity, gently pull skin on limb, note how quickly skin returns to position. 4 - Important. DO NOT try to open mouth. Make observations opportunistically, if tortoise opens mouth.

Berry/HealthProf.4-1995(mac)

FIGURE 3. Sample Health Profile Form.

ogy, and health are closely tied to nutrition and food intake, field workers should record the current and recent availability of fresh, green, succulent plants and recently dried plants used by tortoises for forage. The ability to observe and record such information presupposes that field workers have familiarized themselves with the diet and the locally preferred plant foods and are able to identify the plants in the field.

Journal Notes should contain detailed data on locality of study sites, e.g., latitude, longitude; township, range, and portion of section; universal transverse mercator (UTM) grid coordinates; county; and elevation. Some permanent sites (FWS, 1994; Berry and Medica, 1995) have survey poles at intervals of 100 to 165 m, so that locations of tortoises can then be estimated in meters by pacing to the nearest pole. At other sites, global positioning systems have been used to determine localities within 50 to 100 m. The precise locations of tortoises are critical for interpreting sources of trauma and toxicants and causes of some diseases.

All parameters related to human activities on and in the vicinity of the study site should be recorded both in Journal Notes and on a detailed map, because they may be critical factors in monitoring the long-term well-being of the population (Boyce et al., 1992; FWS, 1994). Examples include: distribution and densities of vehicle tracks, trails, paved and dirt roads; numbers and types of vehicles; numbers of visitors unrelated to research work and their purposes for visitation; sheep and cattle; observations of individual cats or dogs or packs of dogs; locations and types of refuse or hazardous waste; mining markers or stakes; mill sites; campsites; and evidence of shooting of firearms (shotgun shells, clay pigeons, targets). Historical information should also be recorded when deemed important: abandoned mines and mill sites, abandoned or active railroads, abandoned or active vehicle routes, previous military maneuver or bombing areas, ranching or farming operations, proximity to utility lines and incinerators, etc. Desert tortoises have been found with tar on scutes or caught in tar, with gunshot wounds (Berry, 1986), traumatic and fatal injuries due to military projectiles and tanks, and in the vicinity of hazardous waste materials. Desert tortoises may also become entangled in or consume foreign objects, e.g., string, rubber bands, surveyors tape, aluminum foil (K. Berry, unpubl. data), similar to reports of other chelonians (Balazs, 1985; Reidarson et al., 1994; Mader, 1996).

PROCEDURES TO PREVENT SPREAD OF DISEASES AND PARASITES

Special precautions must be taken to prevent transmission of pathogens causing diseases such as mycoplasmosis (Brown et al., 1994; Jacobson et al., 1995) within and between tortoise populations (Jacobson, 1993, 1994a; Berry, 1997b). The most likely sources of transmission of mycoplasmosis are direct contact, nasal exudate, and aerosols (Brown et al., 1994). The role of mucous droplets in burrows has not been studied and cannot be ruled out.

Each tortoise should be handled with a fresh pair of disposable gloves, which is placed in a plastic trash bag after use and discarded appropriately off-site. Each item of equipment (scales, calipers, ruler) touching the tortoise, including poles used to probe tortoise or other animal burrows and to tap tortoises from burrows (Medica et al., 1986), must be disinfected with a sodium hypochlorite solution (0.175%) or ethanol (70%) immediately after each use and before being replaced in the carrying case or pack. The sodium hypochlorite solution should be made fresh at least once per week, with both concentrated and diluted solutions protected from excessive heat and sunlight. Precautions must be taken to assure that the tortoise does not touch or rest on the field worker's limbs, clothing, or equipment without protective covering. Other options are to use disposable jump-suits and disposable plastic shoe covers. To prevent contamination, small

pieces of disposable paper or plastic sheeting can be placed under the tortoise or on the lap of the field workers. To prevent transmission of disease between study plots, field workers should not travel directly from one site to another without bathing and changing clothes and shoes. Clothes and shoes must be disinfected prior to use on other sites. Depending on the nature of the diseases present at the site, field vehicles may require thorough external and internal cleaning at a car wash.

Careful adherence to the above procedures can also help to reduce transfer of ticks, potential vectors of disease, to humans. The two species of ticks commonly observed on desert tortoises, *Ornithodoros parkeri* and *O. turicata* (Greene, 1983, 1986) are major vectors of the disease agents *Borrelia parkeri* and *B. turicatae* which cause American tickborne relapsing fever in people (Sonenshine, 1993). Humans are rarely involved in the cycle of transmission of these diseases unless they intrude into home sites or nests of the ticks, e.g., tortoise burrows. While no cases of borreliosis transmission from tortoise ticks to humans have been documented, field workers should take precautions when processing tortoises, because *O. parkeri* (and probably *O. turicata*) were found on 5 to 10% of wild desert tortoises in several tortoise surveys conducted between 1970 and 1980 (Greene, 1986). At one site, 43% of active tortoise burrows were infested with *O. parkeri*.

DATA SHEET FOR LIVE DESERT TORTOISES

The Data Sheet for Live Desert Tortoises (Fig. 2) is used for recording basic demographic and ecological data for each tortoise observed and/or captured and contains parameters useful for calculating condition indices and equations related to carapace length and mass. Desert tortoises are long-lived animals, requiring 12 to 20 or more years to reach sexual maturity, and may then live at least 70 or more years (Woodbury and Hardy, 1948: Hardy, 1976; FWS, 1994). Because of their longevity, careful records are essential for determining ecological and behavioral constraints; individual and population growth rates; recruitment of young into adult age classes; survivorship by cohort; causes of mortality; and frequency and types of trauma and disease. Critical parameters include: date, time and precise location of capture; unique tortoise identification number; type of capture (e.g., 1 = first capture, 2 = subsequent recaptures during the year [any year], 3 = first capture of the year for a previously marked tortoise, 5 = a marked tortoise found dead); sex, body measurements and weight; and activities and behaviors.

Each tortoise should be examined to determine whether it is a released captive or previously marked animal from a translocation project or an unauthorized translocation. Signs of previous captivity include: painted initials, numbers, or other writings on the shell; shell discoloration or stains from dyes, ink or paint; file marks or holes drilled in the marginal scutes of the carapace; caked dirt of a different color and type than the parent rock and soils of the study site; and fiberglass, epoxy, or other manufactured materials. Captive tortoises frequently have morphologic anomalies, such as pyramid-shaped scutes (Jackson et al., 1976). Tameness and curiosity are not valid criteria for assessing previous captivity of desert tortoises. Field workers should also ensure that the tortoise is a desert tortoise and not some other *Gopherus* spp. or exotic tortoise that was illegally released, by becoming familiar with dichotomous keys and descriptions of similar-appearing species.

Placing a unique identifying mark on a tortoise requires considerable care, because the identification number ideally should last the life of the tortoise. First, field workers must record physical anomalies (shape and number of scutes) on the carapace and plastron diagrams (Fig. 2). Second, based on scutellation, an identifying number is selected and notches are filed in the scutes with a triangular file.

Most tortoises ≥100 mm mid-carapace length (MCL) are notched on one or more of the marginal scutes using a standard numbering system. Tortoises <100 mm MCL are notched only on anterior or posterior marginal scutes either with a small triangular file or with nail clippers; the bridge (portion of the shell between the carapace and plastron) is avoided, because notches can penetrate to the bone in this area. Most notches are filed or cut into the keratin of scutes without penetrating to or notching the bone. When scutes are thin, the notch can expose a thin sliver of bone, which may stimulate replacement of both scute and bone and subsequent disappearance of the notch itself. Notches generally are evaluated each year a tortoise population is surveyed and remade or deepened when ambiguous or no longer clearly distinguishable. Notches have remained >20 yr on some desert tortoises, but may wear away as the tortoise ages, or may disappear if marginal scutes chip or are chewed by predators. Third, the identification number is placed on a scute as a supplemental identification. A dot or smear (about 5–8 mm in diameter) of cream-colored or pale yellow paint is placed on the areola or area formerly covered with the areola of the fourth right costal scute, a site with minimal abrasion, and allowed to dry. Then the number is written on the dried paint. The dot and number should be sufficiently small and obscure to preclude loss of the natural concealing colors of the tortoise shell. The number is covered with a small dot of Devcon (Devcon Consumer Products, Wood Dale, Illinois, USA) 5 min quick drying epoxy. The number may become obscured if the surface of the epoxy is scratched or covered with dirt, but it can often be read several years later when moistened and rubbed. The painted number reduces field time and handling, because field workers can rapidly identify the tortoise and determine if it was recently processed.

Additional forms of identification include passive integrated transponder (PIT) tags and radio transmitters. The PIT tags can be fastened with epoxy to the dorsal or ventral surface of marginal scutes (Boarman et al., 1998) or injected subcutaneously into the body (a practice which has not been perfected and which we do not advise). The first three forms of identification, coupled with the photographs described below, are essential.

On the first capture of the season and at subsequent capture intervals of two or more weeks, tortoises should be measured for MCL and plastron length from gular to anal notch. We prefer Starrett (L. S. Starrett Co., Athol, Massachusetts, USA) firm joint outside calipers and a 380-mm metal ruler (1 mm increments) for individuals >125 mm MCL, and dial calipers (130–150 mm, 0.05 to 0.1 mm increments) for individuals <125 mm MCL, although some researchers use tree calipers. Depending on the size of the tortoise, mass can be recorded using a 100 g Pesola (Geneva, Switzerland) scale (1 g increments) and varying sizes of Chatillon (John Chatillon and Sons, Kew Gardens, New York, USA) scales (1 kg, 20 g increments; 6 kg, 50 g increments; and 12.5 kg, 100 g increments). Tortoises can be suspended in clean plastic bags, or with disposable slings of surveyor's tape or string. Expensive and inexpensive electronic balances are also available but are not necessarily appropriate for carrying in a backpack for processing tortoises a few kilometers from the vehicle.

Several veterinarians have used the relationship of body weight to carapace length to evaluate clinical condition of tortoises, e.g., "Jackson's ratio" (Jackson, 1980; Spratt, 1990; Blakey and Kirkwood, 1995). For the desert tortoise, reliable predictions of health based on weight and carapace length data have not been fruitful, probably because so many different factors (sex, reproductive status, degree of hydration, morphology of the shell) contribute to weight (Jacobson et al., 1993). Another approach is the development of a condition index such as body mass (g) divided

by the cube of MCL (Wallis et al., 1999; see also Bonnet and Naulleau, 1994 for a different method).

The sex of each tortoise ≥180 mm MCL is assigned using several secondary sex characteristics: MCL, presence and condition of chin or mental glands (Alberts et al., 1994), size and curvature of the gular horn, the presence or absence of a concavity on the posterior plastron, and tail length. Reliable sexing of individuals <180 mm MCL requires laparoscopy (Rostal et al., 1994b) and is rarely done in the field. Smaller tortoises are assigned, unsexed, to juvenile (<100 mm MCL) or immature (100–179 mm MCL) size classes. Sexing a young or small adult (180–205 mm MCL) can be difficult, because the upturned gular horn and plastral concavity typical of males are unlikely to be well defined or fully developed until the tortoise is >210 mm MCL. Gular horns of males are often damaged by predators, and some males may not have an intact gular to evaluate. In contrast to males, the posterior plastron of a female is almost always flat or imperceptibly concave. The female gular is almost always flat, or only the lateral edges are slightly upturned. Tail length, a trait that changes with age, is longer in the male than the female. In young or small adults, the differences can be only a few mm. As the male ages and grows larger, tail length increases and differences between the sexes become more pronounced.

Two paired integumentary chin or mental glands are located below the mandibles (Alberts et al., 1994) and can be used to determine sex in adults. The volume of adult female chin glands is so small that secretion samples cannot be collected. In contrast the volume of adult male chin glands is greater, secretions can be collected, and the gland volume varies according to season. Male chin glands are relatively small in late spring and peak in size in late summer, a time when courtship, mating and aggressive behaviors frequently occur. Mean gland volume of males is also positively correlated with mean plasma testosterone concentration (Rostal et al., 1994a; Alberts et al., 1994) and is generally greater in dominant males than in subordinate males (Alberts et al., 1994). When the sex is in doubt or the field worker has limited experience, 35-mm slides should be taken of the head, chin glands, gular, posterior plastron and tail for retrospective evaluation by an expert.

The precise location of each tortoise is essential to record. Tortoises exhibit fidelity to burrows and dens, have established home ranges, and can spend a lifetime within limited, circumscribed home ranges or activity areas (FWS, 1994). As such, they can serve as sentinels of environmental conditions. When capture sites are accurately recorded, animals can be recaptured more easily for health evaluations, salvage, or demographic studies.

To determine whether the tortoise is or has been actively growing within the last few months, the seams between scutes should be inspected for the presence of a narrow (generally <2 mm) band of softer grey or lightly pigmented keratin. Within a few months the band will harden and form a new ring, gradually assuming the color of the portions of the scute adjacent to the seam. These lines or rings do not represent annular rings, because no rings or more than one ring may be formed in a single season (Zug, 1991).

THE HEALTH PROFILE FORM

The Health Profile Form (Fig. 3) was developed to assess health and well being of the tortoise and was revised several times between 1989 and 1998. It incorporates standard parameters used to evaluate captive chelonians (Jackson, 1987, 1991; Mautino and Page, 1993; Mader, 1996), as well as new parameters associated with recently described and commonly observed diseases. Field workers preferred the single page, circling or checking responses, and a limited protocol. We obtained the best results from the form

shown in Figure 3, coupled with photographs. There is some overlap in the Live Tortoise Form and the Health Profile Form, enabling the development and use of separate databases by interdisciplinary teams of research scientists.

The tortoise should first be observed from a distance, and if possible, before it responds with defensive or aggressive postures or movements. Critical factors include postures, particularly position of the head and limbs, and movement of the limbs and body; activities and behaviors; and general and specific locations in the environment. Shortly after emergence from hibernation in late winter or early spring, the normal suite of behaviors includes: basking at the mouth of the burrow or on the burrow mound with limbs fully extended and directed forward with the plastron on the soil, walking, foraging, resting in the shade of a shrub or tree, or (late in the day) facing into the burrow, partially down or at the end of the tunnel. Atypical and abnormal behaviors include: remaining overnight above ground in freezing temperatures or remaining in the same place outside the burrow for more than one day at any time of year. One abnormal posture signals chronic illness: the tortoise rests with head down and partially withdrawn, forelegs partially spread apart and with the dorsal surface rotated outward and forward. The limbs are limp and the tortoise appears lethargic and weak. Lethargy and weakness in a free-living tortoise are clinical signs of chronic disease. During the activity season (March–October), most tortoises should be alert and responsive under normal operating temperatures (Berry and Turner, 1986; Zimmerman et al., 1994), and able to withdraw head and limbs quickly and tightly into the shell when prodded. If environmental temperatures are at or near freezing, or skies are overcast and weather generally cold, the responses of a normal, healthy tortoise will be slower.

Observations of the limbs, head, beak, nares, eyes, chin glands, and oral cavity can be difficult or impossible to make if head and limbs are retracted tightly into the shell in a defensive posture. With field time at a premium, the field worker may have to abandon attempts to record most health data on such tortoises. If, however, the health profile evaluation is performed after the Data Sheet for Live Desert Tortoises is filled out, then the tortoise may relax and become curious. One technique to expose the limbs and head is to place the tortoise right side up on an inverted coffee can covered with a single-use clean paper towel. Some tortoises will extend head and limbs and flail, allowing an excellent view and an opportunity to photograph eyes, nares, and head.

The shell and integument should be evaluated when clean. Most shells have a little, easily removable dust and dirt. When wiped and rubbed free of dust and dirt, the integument should be glossy. After rain, some tortoises become so heavily caked in dirt or mud that the shell must be cleaned with a brush and the extremities rinsed with water prior to examination. For the shell and scales, important factors to consider are whether scales and scutes are clean and glossy (similar in appearance to the skin of a snake that has freshly shed) or are dull, dried-out in appearance, discolored, caked with dirt or mucus, or covered with fungi.

The general appearance of limbs and head are indicators of health status. An emaciated head, sunken eyes, and emaciated or cachectic limbs may be signs of dehydration, starvation or chronic URTD. Other factors to look for include swollen limbs, neck, and cloaca; and swellings in the inguinal or axillary area.

The beak, nares, eyes, and chin glands provide subtle signs indicative of health or disease. Since the desert tortoise lives in an arid environment and frequently experiences drought, dehydration, and accompanying weight loss (Henen et al., 1998), it may not always exhibit obvious clinical disease signs such as nasal and ocular discharges. Nasal and ocular discharges may

be intermittent. Therefore, the field worker must look for evidence of recent moisture associated with the eyes, nares, and beak. Tortoises with rhinitis or URTD may have wet or damp nares, and nasal exudate. The amount, color, consistency, and turbidity of any exudate (e.g., clear, cloudy, white, yellow, and green) should be recorded (Jacobson et al., 1991). Tortoises may blow bubbles from the nares or one or both nares may be occluded. On rare occasions, a healthy tortoise may exhibit what appears to be a clear nasal discharge, possibly associated with consumption of lush, succulent vegetation in spring. Dirt adhered to dried mucus on the beak or nares may be a sign of illness, but tortoises that have been drinking from depressions in the soil during a thunderstorm may also have dirt on the beak, nares and forelimbs. Tortoises with a tenacious exudate may have moisture or dried dirt on the medial surface of the forelegs from wiping the face, eyes, and beak with their forelegs. In severe cases, the integument between the scales of the forelegs may have cracked. Inflammation and congestion of the respiratory tract may alter breathing, so respiratory sounds should be evaluated for wheezing, rasping, and clicking noises. Severely affected individuals may extend their necks and open their mouths to breath. Consequently, breathing may look and sound labored.

The color, surface, and condition of the beak may reflect health status as well as recently consumed food items. When forage is plentiful, the beak should have green or other colored stains from recently consumed leaves, flowers, and fruits. Occasionally beaks will be caked with dried flesh of cactus fruits or dried sap from plants. In years when forage is plentiful, the observer should suspect illness in a thin, low weight, inactive tortoise that shows no evidence of recent food consumption or color on the beak. The chin or mental glands may be abnormally swollen and draining. If swollen, the dimensions of each gland should be measured to estimate volume (see Alberts et al., 1994 for measurements and formula).

The surface of the eye, appearance of palpebrae (eyelids), and periocular region should be examined closely for abnormal color; presence of dampness, mucus or drainage; and edema—all of which may be signs of URTD (Jacobson et al., 1991; Brown et al., 1994), rhinitis (Jackson, 1991) or other illnesses. The palpebrae are normally dry, unscaled, wrinkled, and delicate in appearance (Fig. 4A–C). The periocular area, separated dorsally and ventrally from the palpebrae by a furrow, is covered with small scales and is also normally dry and flat. The normal surface of the globe usually does not have visible strands or patches of mucus. To assess the eye and adnexal structures, we developed a grading scheme for the palpebrae and periocular areas. Palpebrae should be evaluated for swelling (edema) and dampness (Fig. 4D–L), and the periocular area surrounding the eye also may be swollen (Fig. 4E–K; also compare Fig. 4C with Fig. 4H). The degree of closure of lids on both eyes should be noted, as well as outward bulging, swelling or a sunken appearance within the orbit (compare Fig. 4C with Fig. 4H and 4L). Clinical signs (Figs. 3, 4) should be rated by degree of severity in each eye, with 1 = normal, 2 = mild, 3 = moderately severe, and 4 = severe or marked. Ratings may be accomplished with supplements (e.g., Appendix 1) to standard health forms (Fig. 3). Appendix 1 is for the well-trained or advanced field biologist working with diseases of the eye or upper respiratory tract.

The mouth of the tortoise is usually closed and separating the jaws is likely to induce additional stress. Unless the research program is focused on health and diseases, we recommend that data on the oral cavity be gathered opportunistically if the tortoise gapes or if the mouth is easily opened. The tongue is covered by a thick layer of cornified epithelium and the mouth has numerous mucous glands (Barboza, 1995). If the oral cavity is examined,

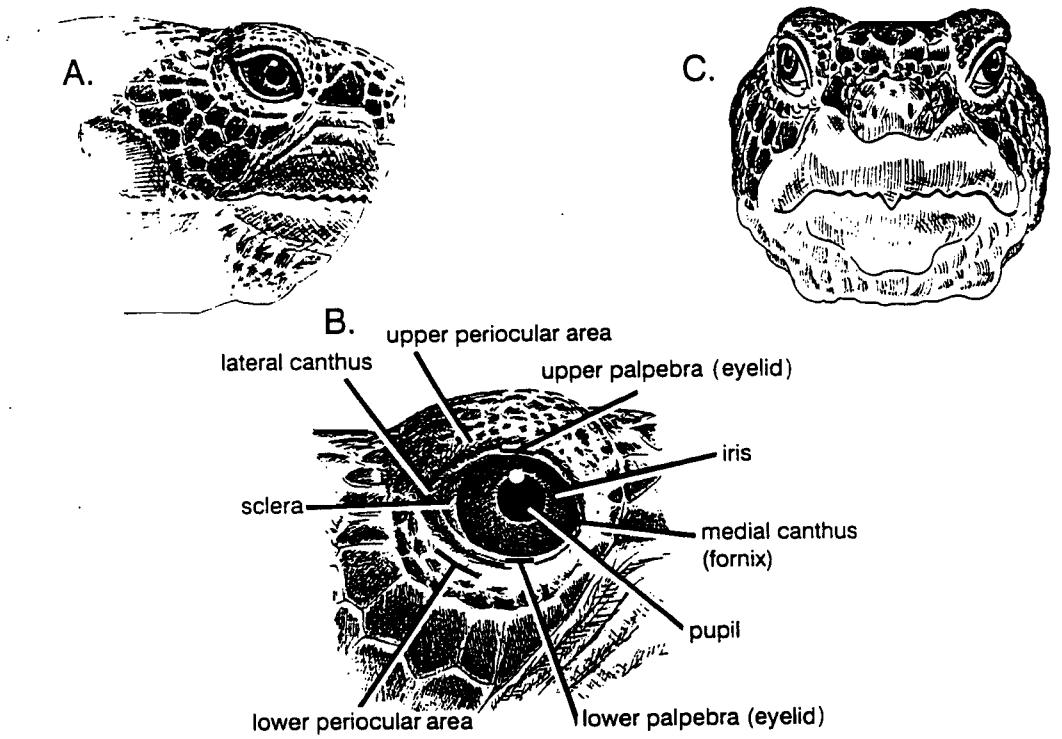


FIGURE 4. A–C. Line drawings of the desert tortoise head depicting anatomical landmarks. A. Lateral view of normal eye, palpebrae (eyelids) and periocular area in the context of the tortoise's head. B. Magnified lateral view of the normal eye, with upper and lower palpebra (lacking scales), periocular areas (scaled) and other anatomical structures denoted. C. Frontal view of the head and normal eye area.

the following data should be recorded: smell; general color and localized spots; and the presence of plaques, swellings, blisters, ulcers, stains, lesions, and foreign objects (e.g., embedded plant spines).

Wild desert tortoises >120 mm MCL are likely to have some lesions on their scutes, underlying dermal bone, and/or extremities. Occasionally to frequently, field biologists observe: chips of keratin and bone missing from marginal scutes; missing limbs, toenails, or scales on limbs; healed or healing tooth marks, chew marks or punctures (penetrating scute to bone) from predators; cutaneous dyskeratosis (Jacobson et al., 1994; Homer et al., 1998); depressions in scutes and underlying bone; and exposed, white or dark discolored bone, potentially indicative of necrosis (Homer et al., 1998). The location of all lesions should be drawn on the diagrams of scutes, described carefully, and photographed. Signs of predator attack should include notes on the potential predator (including feral dogs and cats), as indicated by size, location, and type of puncture, scratch or tear. The relative age of the wound or lesion should be recorded. Wounds or lesions may be fresh, in the process of healing, or evident as scars. Such data, when compiled over several years, can be used to: (1) compare survivorship of the different age classes of tortoises to predator attacks, and (2) measure predator pressures on populations. For example, the technique of recording scars of predator attacks has been successfully used with the scorpion mud turtle (*Kinosternon scorpioides*) to measure predation pressure by jaguars in different habitats (Acuña-Mesen, 1994).

Most desert tortoise populations contain individuals with cutaneous dyskeratosis, as manifested by discolored and flaky scutes.

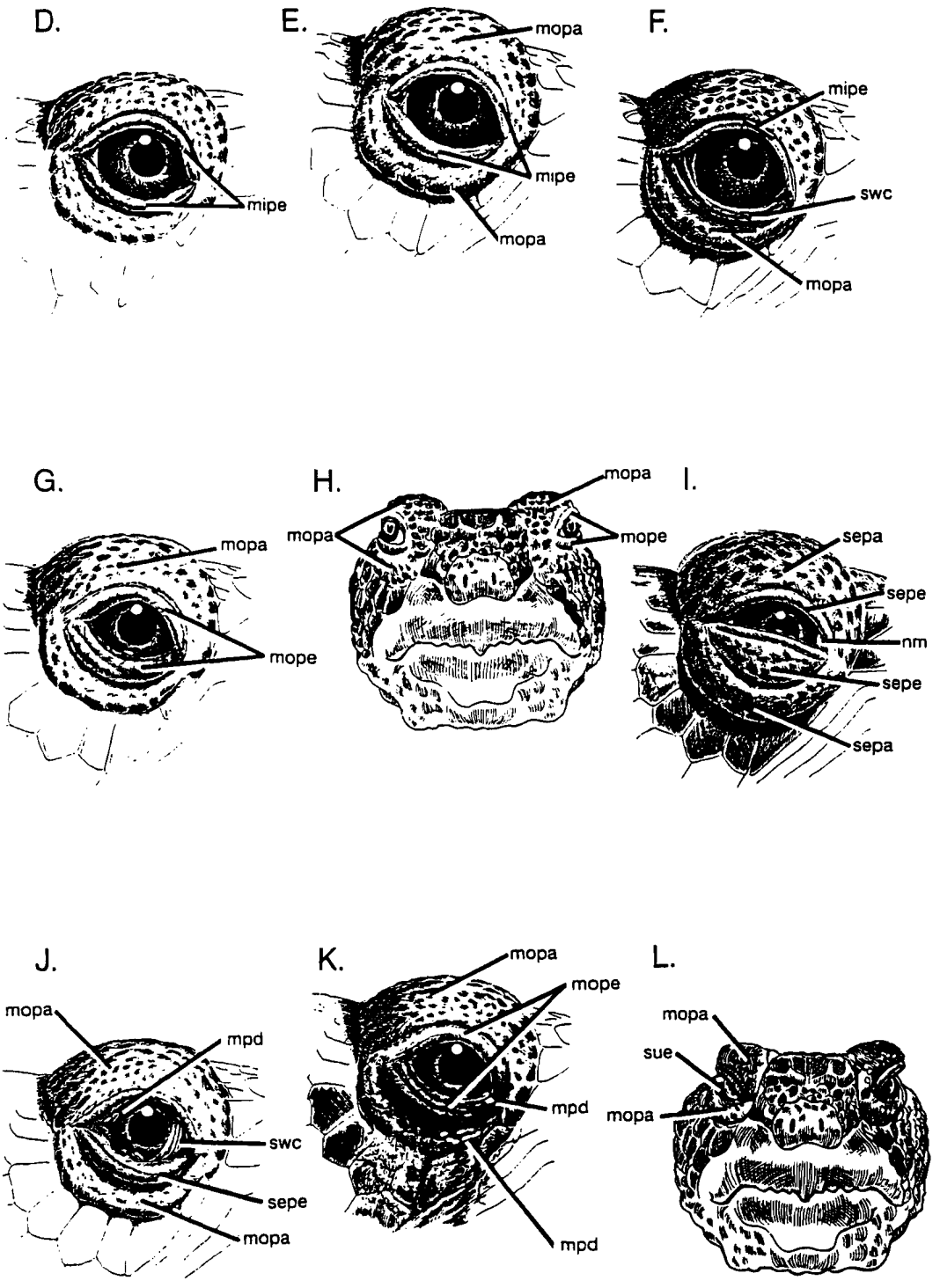


FIGURE 4. D–L. Same as 4 A–C showing ocular abnormalities commonly associated with upper respiratory disease infection and other ocular disorders. Abbreviations for D–L: mipe = mild palpebral edema; mopa = moderate edema of the periocular area; mope = moderate palpebral edema; mpd = mucopurulent discharge; nm = nictitating membrane; sepa = severe edema of the periocular area; sepe = severe palpebral edema; sue = sunken, recessed eyes; swc = swollen conjunctiva. D. Mild edema (chemosis) of the upper and lower palebrae. E. Moderate edema of the palpebrae, conjunctiva, and upper and lower periocular areas.

The lesions usually are associated with the seams of the plastron and spread outward from the seams in irregular patterns (Jacobson et al., 1994; Homer et al., 1998). The damaged portions of scutes are whitish grey, sometimes orange, slightly raised and flaking. In severe cases, tortoises with thin, peeling laminae and exposed bone may be more vulnerable to bacterial and fungal infections and predation (Homer et al., 1998). Cutaneous dyskeratosis and other shell diseases should be graded by distribution on the shell, severity, and approximate age of lesion or chronicity for each of three body regions, the carapace, plastron, and limbs (Table 1). A variation of the scale shown in Table 1 can also be used to record the presence of fungi, which may be present on tortoises that hibernated in damp or wet burrows.

Depressions in scutes should be recorded on the Health Profile Form and carefully photographed. Depressions in juvenile and immature tortoises (<180 mm MCL) may be due to malnutrition and metabolic bone disease, whereas in old adult tortoises the depressions may be a normal part of the aging process. Vermiculations between the scute and bone should be noted.

If the tortoise urinates (which frequently occurs when a hydrated tortoise is handled), the amount, color, viscosity and size of particles in the urine sediment should be evaluated. The color of normal urine is dependent on the level of hydration, with colorless, clear urine produced by a fully hydrated animal and very dark brown and concentrated urine typical of a tortoise dehydrated from prolonged drought (Nagy and Medica, 1986; Peterson, 1996a). The urine may be various shades of yellow, burgundy, or brown color and contain gelatinous material and precipitated urate crystals ranging from greyish white to pink, yellow, and brown in color. Since survivorship of tortoises may be affected by loss of bladder fluid (Averill-Murray, 1998), protocols for handling tortoises should minimize contact time. Fecal samples should be collected when available for analysis of internal parasites.

All ectoparasites on tortoises should be considered significant (Jacobson, 1994b). Ticks can injure a tortoise or transmit parasites, spirochetes, or viruses (Sonenshine, 1993). Records should be compiled by species of tick and should include (for each tick): numbers, attachment site or location in general (e.g., number and name of scute), specific attachment site or location (pit, chip, seam, new growth tissue, injury), size, developmental stage, sex, degree of engorgement, and activity (resting, feeding, moving) (Fig. 3). Recent attachment sites, such as small bloodied areas of seam between scutes, should also be recorded. Reference specimens should be collected and stored in appropriate museum collections, and the taxonomic identification should be confirmed (see Sonenshine, 1993 for methods). The ticks should be removed for accurate counts, identification, and determination of sex.

The most common ectoparasites recorded for desert tortoises are the nidicolous

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F. Mild edema of the upper and lower palpebra, moderate edema of the periorbital areas. G. Moderate edema of the palpebrae, with dorsal and lateral displacement of the eye from moderate edema within or adjacent to the orbit. Palpebra with this degree of swelling may appear translucent. H. Frontal view of 4G. I. Marked or severe edema of the upper and lower palpebrae and periorbital areas, with bulging of the eye laterally. The scaled periocular area is swollen into prominent folds or bags, resulting in partial closure of the eye. The nictitating membrane (3rd eyelid) is visible in the fornix (arrow). J. Similar to 4G, with mucus on the eyeball surface, spilling onto lower lid, and swollen conjunctiva. K. Moderate edema of the palpebrae and periorbital areas. Mucoid or mucopurulent discharge has accumulated in the medial canthus (fornix) area and spilled over onto the surrounding skin. Dirt may admix with the mucus, resulting in dried dirty crusts around the eye. L. The sunken eye, partially closed.

TABLE 1. System for grading shell lesions such as cutaneous dyskeratosis in desert tortoises. The carapace, plastron, and integument on limbs and head should be rated separately.

I. Shell lesions: source
   1 = From trauma
   2 = From disease (specify cutaneous dyskeratosis, necrosis, fungi, or other)
II. Distribution: specify by plastron, carapace, limbs, or head
   1 = Not present, no signs of lesions
   2 = Mild, lesions manifested primarily at seams, covers less than 10% of plastron (or carapace or limbs, etc.)
   3 = Moderate, covers 11%–40%
   4 = Severe, covers > 40%
III. Severity of lesions (from disease, e.g., cutaneous dyskeratosis)
   1 = No lesions
   2 = Mild, discoloration follows edges of lifting laminae, lightly discolored, flaking
   3 = Moderate, discoloration extends over several layers of laminae, edges of laminae flaking, scutes may be thin in small areas, and potential exists for small holes and openings exposing bone
   4 = Severe, some scutes or parts of scutes eroded away or missing and bone exposed, eroded, or damaged
IV. Chronicity of lesions (from disease, e.g., cutaneous dyskeratosis)
   1 = No lesions
   2 = Old lesions, no apparent recent activity, signs of regression or recovery; development of healthy, normal laminae is apparent at seams of scutes
   3 = Active, current lesions

Argasid ticks, *Ornithodoros parkeri* and *O. turicata* (Greene, 1986). Their life span is at least several years (20 years in the case of some argasid ticks), and they can survive long periods of starvation (Sonenshine, 1991). All stages of these ticks parasitize wild desert tortoises (Greene, 1986). They tend to be found on the posterior carapace, often attaching at the seams between scutes, or at the site of old injuries. Attachment at a site of injury is also typical of *Hyalomma aegyptium*, the tortoise tick that parasitizes *Testudo graeca* (Petney and Al-Yaman, 1985). Other ticks, e.g., *Amblyomma marmoreum* on *G. paradalis*, showed patterns of seasonal abundance, as well as gender preferences for site attachments (Rechav and Fielden, 1995).

PERMANENT PHOTOGRAPHIC RECORDS

Full-frame images of the head, carapace, plastron, and the fourth costal scute of each tortoise should be taken with 35-mm slide film at least once during each survey year for identification, to gather data on numbers of growth rings produced and how the growth rings change in appearance over time, to verify how contours of the shell age, and to confirm how damaged shell replaces itself over time. Additional photographs can be taken of recent or previously healed injuries to the head, limbs, or shell, or unusual abnormalities. The 35-mm slides are useful for confirming identification of tortoises which have not been observed for many years, which had very small notches when marked as juveniles and grew to large adults without being captured in the intervening period, or which have lost one or more notches from predator attacks. The relative sizes of scutes and seams form unique patterns which persist from the late juvenile sizes through life, much like a fingerprint. Slide transparencies and permanent notches on the shell were used to identify desert tortoises illegally taken from the desert in May 1993 and to support a court case (K. H. Berry, unpubl. data). Similarly, the British Chelonian Group has set up a registration program for captive tortoises using photographs for identification (Jackson, 1991).

Even when tortoises have died and only part of the shell persists, the identities can be determined by using a combination of 35-mm slides, numbers on the costal scutes, and notches in one or more scutes. Disarticulated scutes and bones can be reassembled and the pattern of scutes discerned on the external surfaces of the bones. Notches or the indentation from notches often can be seen in the marginal and bridge bones.

Permanent photographic records have

proved invaluable for retrospective analyses of progression and regression of signs of diseases in individual animals and populations, including cutaneous dyskeratosis and other shell lesions (Jacobson et al., 1994; Homer et al., 1998); URTD (Brown et al., 1999); traumatic injuries; and epidemiological research. Photographs also have proven to be a valid and reliable approach for grading trachoma in humans (West and Taylor, 1990). Close-up views of eyes and shells of the tortoises were especially critical for interpretation and grading of diseases and trauma (e.g., Jacobson et al., 1994; Brown et al., 1999; Christopher et al., 1999) and proved more reliable and consistent than the field evaluations.

Research veterinarians or health specialists can interpret slides and photographs and recommend whether to have a veterinarian visit the animal(s) in the field or to salvage the tortoise for necropsy. For consistent and effective interpretation, the film (manufacturer, brand, and speed) should remain the same for the entire project, because different types of films (with subtle color shading) render consistent interpretation difficult. For ease in storage, handling, and making comparisons, we recommend 35-mm slide transparencies and storage in archival slide sheets. New technologies, e.g., digital images archived on compact disks, are now available and offer numerous opportunities, such as automating assessments of health and disease and comparing different images of the same animal. For long-term projects with long-lived species, researchers should determine the level of detail available from film versus pixelated images, stability and longevity of the media, and ability to retrieve usable images after decades.

Cameras, including macro lenses, should be essential field equipment, and the ability to produce high quality, close-up photographs should be a job requirement. Lighting is critical for photographing animals, so skill with flash units should be another prerequisite for field workers.

SALVAGING ILL TORTOISES FOR NECROPSY

Necropsies of ill, dying, or recently dead wild tortoises provide a wealth of information about causes of death in populations and should be incorporated into field research protocols (Homer et al., 1998). Preparations for salvaging live or dead wild tortoises for necropsies must be made in advance by obtaining appropriate permits from the U.S. Fish and Wildlife Service and state fish and wildlife agencies, arranging for the services of a veterinary pathologist familiar with reptiles, identifying the types of tests to be made, and determining requirements of air freight lines (shipping boxes, shipping papers). If a forensic necropsy is required, a veterinary pathologist with formal training, board certification by the American College of Veterinary Pathologists, and experience with reptiles should be obtained (Wobeser, 1996).

More data can be obtained from a live tortoise than from a dead tortoise. Frozen remains are of limited value for most pathologic studies, other than gross visual examination and toxicant analyses. We ship live tortoises packed in loose newspaper in two sizes (13.5 cm high × 70 cm long × 70 cm wide; 25 cm high × 70 cm long × 70 cm wide) of specially made plywood boxes with screw-top lids cut with 27, 2-cm in diameter holes (nine holes on the top, six holes on each of three vertical sides). The boxes are designed to allow the tortoises to move about, but the limited vertical clearance inhibits climbing and overturning. Information about the live animal, shipping times and routes, name and phone numbers of the receiving veterinarian, the health and scientific research and salvage permits are placed in an envelope and taped to the top of the container. Recently dead (<48 hr) tortoises can be shipped chilled on ice in an ice chest via one of the 24 hr mail services. Frozen remains can be shipped on dry ice.

Decisions on criteria for salvage require advance planning and can be placed in

TABLE 2. General condition of 59 desert tortoises salvaged for necropsies between 1989 and 1996 on the senior author's scientific research permits.

Condition of tortoises at time of salvage					
			Ill		
	Dead	Dying	Alert[a]	Lethargic	Pathologist or reference
			12		Jacobson et al., 1991
					E. R. Jacobson and J. Gaskin (Bureau of Land Management [BLM] files, 1990)
	1		3	2	
	1		2	2	J. Klaassen (BLM files, 1991)
	2	3	11	8	Homer et al., 1998
	8	0	4	0	Homer et al., 1998
Totals	12	3	32	12	

[a] Ill but alert tortoises were generally salvaged on the basis of clinical evidence of upper respiratory tract disease or shell lesions.

three categories: (1) opportunistic salvage of recently dead tortoises, (2) opportunistic salvage of severely injured and dying tortoises, and (3) the deliberate and planned salvage of animals with specific behavioral abnormalities, signs of disease or syndromes for special research projects. We retrospectively evaluated records of 59 desert tortoises removed from the wild between 1989 and 1996 (Table 2), and developed salvage criteria using clinical signs of disease and abnormal behavior. The criteria for salvage are met when tortoises have one or more of the following attributes: (1) is severely injured and unlikely to survive as a result of vehicle-related or predator-caused trauma; (2) is lethargic, inactive, or non-responsive during the activity season; (3) is emaciated or severely dehydrated and of very low weight for the carapace length; (4) exhibits progressive weight loss over a 1- to 2-yr period, not associated with drought; (5) exhibits abnormally low growth rates over a several-year period; (6) exhibits weakness associated with limb atrophy; (7) exhibits cachexia with no apparent weight loss (may have uroliths); (8) is incapable of retracting limbs into the shell or is partially paralyzed; (9) has active shell lesions (from cutaneous dyskeratosis or necrosis, not trauma) covering ≥40% of the plastron or carapace; (10) has scutes sloughing or loose, if the loosening and sloughing are not part of a healing or scute replacement process from trauma; (11) has scales peeling or sloughing from the limbs or head in patches, not due to trauma; and (12) has moderate to severe edema of the palpebrae and periocular area, especially if accompanied by a mucopurulent nasal or ocular discharge and signs of chronic discharge on forelimbs, eyes, and beak. Salvage is inappropriate solely when a limb is lost from a predator attack, because some tortoises recover and function quite well in the wild. The monitoring of individual tortoises and environmental conditions will help to determine the cause and severity of some clinical signs of disease. For example, weight loss can be an early sign of disease (Jackson, 1980; Oettle et al., 1990), as well as a normal response to drought, hibernation, and estivation (Peterson, 1996a, b; Henen, 1997).

Subtle behaviors can provide evidence of illness and justification for salvage. Each of the lethargic and inactive tortoises and some of the alert and active tortoises (Table 2) provided one or more additional behavioral clues of their status for several weeks or months prior to death: they were active and above ground at inappropriate times of year, failed to emerge or were late (several weeks or months) in emerging from hibernation, failed to return to bur-

rows and typical sleeping places at night or during hot times of day (see also Oettle et al., 1990), remained in a resting position in one place day after day, and failed to eat when forage was readily available or failed to drink during a warm rain.

Decisions about salvage, whether for a specific research project or because the tortoise may have reached a "point of no return" can be difficult. An animal can only be evaluated in the field up to a point; without a necropsy there is no total certainty about physical status. Difficult cases may be resolved through a team effort between the wildlife health specialist, research veterinarian, and field biologist using a cell phone from the field (a requirement now for our field staff) or a visit to the field. No substitutes exist for experience, good judgment and common sense, however.

SUMMARY

Health assessments of wild animals are becoming more common, and often include blood sampling, complete blood counts and biochemical profiles, as well as analyses for vitamins, minerals, and organochemical compounds (e.g., Calle et al., 1994; Dunlap, 1995; Christopher et al., 1999). We recommend that the health assessments described herein become required and standard guidelines for prescreening any animal to be used in a research project, whether the research project is conducted by veterinarians, herpetologists, ecologists, or zoologists. Historically, most researchers have assumed that wild chelonians were healthy without evaluating clinical signs of disease or conducting lab tests. If research animals were ill and the information was not included in methods or results, the results and interpretations may be erroneous. Health assessments are also essential for any chelonian breeding program, as well as translocation, relocation, or repatriation programs (Jacobson, 1993, 1994a, 1994b; Cunningham, 1996).

The evaluation of clinical signs will be most reliable and effective when the clinician or field biologist has a broad knowledge of the wild animal's normal and abnormal appearance, postures, and behaviors by season and region, and a great deal of field experience. Field personnel are likely to be more reliable and consistent observers after viewing hundreds of animals with a wide range of conditions. When the species in question is rare, threatened, or endangered, field sample sizes are usually limited. In such cases the field team may gain experience using dozens of ill and healthy captive tortoises. Field personnel should also take precautions to prevent transmission of pathogens (e.g., Ahne, 1993; Cunningham 1996) from one individual animal to another and from one population to another.

Field personnel, wildlife health specialists, and veterinarians can use the data obtained through these methods to develop comprehensive databases on clinical and behavioral signs of health and disease for desert tortoises or other species. Clinical and behavioral signs should be quantified using consistent methodologies, and the relationships between clinical signs, behavioral data, and laboratory data compared. New statistical procedures are available to study links between behavioral characteristics and disease (e.g., Escós et al., 1995).

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APPENDIX 1. Supplemental system for grading the beak, nares, eyes, and chin glands of desert tortoises. Instructions: depending on subject, circle one or more options. Rating system: 1 = normal, 2 = mild, 3 = moderate, 4 = severe.

BEAK & NARES

Site and variables	Presence of moisture	Severity (rate 1–4)	Color and notes
Beak	dry/damp/wet	_____	no foraging evident vs. recent foraging evident (green beak, sap, etc.)
Right Nare	dry/damp/wet	_____	
Left Nare	dry/damp/wet	_____	
Exudate present	no/yes		
Right Nare	none/dried/wet	_____	N = none, C = clear, Co = cloudy W = white, Y = yellow, G = green
Left Nare	none/dried/wet	_____	
Bubble(s) from Nares			
Right Nare	no/yes	_____	
Left Nare	no/yes	_____	

Site	Degree of occlusion of nares Nares occluded
Right Nare	no/partial/complete
Left Nare	no/partial/complete

	Presence of dirt	Amount/Severity (rate 1–4)
Dirt on beak	no/yes	_____
Dirt on/in		
Right Nare	no/yes	_____
Left Nare	no/yes	_____

EYES: Palpebrae Area, Globe

Variable	Presence	Severity (rate 1–4)	Location
Condition of Globe			
Right Eye	yes/no	_____	clear/bright/mucus present/dull/cloudy
Left Eye	yes/no	_____	clear/bright/mucus present/dull/cloudy
Other obvious lesions			
Right Eye	yes/no	_____	corneal ulcers/cornel abrasions
Left Eye	yes/no	_____	corneal ulcers/corneal abrasions
Discoloration of globe			
Right Eye	no/yes	_____	Color and location: _____
Left Eye	no/yes	_____	Color and location: _____
Edema of palpebrae			
Right Eye	no/yes/unknown	_____	upper palpebra/lower palpebra
Left Eye	no/yes/unknown	_____	upper palpebra/lower palpebra
Edema of periocular area			
Right Eye	no/yes/unknown	_____	upper periocular area/lower periocular area
Left Eye	no/yes/unknown	_____	upper periocular area/lower periocular area
Discharge from Eye			
Right Eye	none/wet/dried	_____	
Left Eye	none/wet/dried	_____	
Crusts on palpebrae and periocular area			
Right Eye	no/yes	_____	upper palpebra/lower palpebra
Left Eye	no/yes	_____	upper palpebra/lower palpebra
Other lesions of the palpebrae and periocular area			
Right Eye	no/yes	_____	trauma, necrosis: palpebra/periocular area
Left Eye	no/yes	_____	trauma, necrosis: palpebra/periocular area

APPENDIX 1. Continued.

Degree of Closure of Palpebra			
Right Eye	normal (100% open)/partially closed (____ %)		
Left Eye	normal (100% open)/partially closed (____ %)		
Sunken/Recessed Eyes			
Right Eye	no/yes/unknown	_____	
Left Eye	no/yes/unknown	_____	
Eye Swollen or Bulging in Appearance			
Right Eye	no/yes/unknown	_____	dorsal/lateral
Left Eye	no/yes/unknown	_____	dorsal/lateral

CHIN GLANDS

Site	Size	Drainage	Severity	Color of Drainage
Right Gland	normal/swollen	present/absent	_____	none/clear/cloudy/white yellow/green
Left Gland	normal/swollen	present/absent	_____	

POSTURE/BEHAVIOR			
Behavior appropriate for time of day		yes/no	If no, describe _____
Behavior appropriate for season		yes/no	If no, describe _____
FORELIMBS			
Right	normal/abnormal	If abnormal, describe:	_____
Left	normal/abnormal	If abnormal, describe:	_____
HINDLIMBS			
Right	normal/abnormal	If abnormal, describe:	_____
Left	normal/abnormal	If abnormal, describe:	_____
OTHER			
Tail	normal/abnormal	If abnormal, describe:	_____
	normal/abnormal	If abnormal, describe:	_____

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APPENDIX 1. Supplemental system for grading the beak, nares, eyes, and chin glands of desert tortoises. Instructions: depending on subject, circle one or more options. Rating system: 1 = normal, 2 = mild, 3 = moderate, 4 = severe.

BEAK & NARES

Site and variables	Presence of moisture	Severity (rate 1–4)	Color and notes
Beak	dry/damp/wet	_____	no foraging evident vs. recent foraging evident (green beak, sap, etc.)
Right Nare	dry/damp/wet	_____	
Left Nare	dry/damp/wet	_____	
Exudate present	no/yes		
Right Nare	none/dried/wet	_____	N = none, C = clear, Co = cloudy W = white, Y = yellow, G = green
Left Nare	none/dried/wet	_____	
Bubble(s) from Nares			
Right Nare	no/yes	_____	
Left Nare	no/yes	_____	

	Degree of occlusion of nares
Site	Nares occluded
Right Nare	no/partial/complete
Left Nare	no/partial/complete

	Presence of dirt	Amount/Severity (rate 1–4)
Dirt on beak	no/yes	_____
Dirt on/in		
Right Nare	no/yes	_____
Left Nare	no/yes	_____

EYES: Palpebrae Area, Globe

Variable	Presence	Severity (rate 1–4)	Location
Condition of Globe			
Right Eye	yes/no	_____	clear/bright/mucus present/dull/cloudy
Left Eye	yes/no	_____	clear/bright/mucus present/dull/cloudy
Other obvious lesions			
Right Eye	yes/no	_____	corneal ulcers/corneal abrasions
Left Eye	yes/no	_____	corneal ulcers/corneal abrasions
Discoloration of globe			
Right Eye	no/yes	_____	Color and location: _____
Left Eye	no/yes	_____	Color and location: _____
Edema of palpebrae			
Right Eye	no/yes/unknown	_____	upper palpebra/lower palpebra
Left Eye	no/yes/unknown	_____	upper palpebra/lower palpebra
Edema of periocular area			
Right Eye	no/yes/unknown	_____	upper periocular area/lower periocular area
Left Eye	no/yes/unknown	_____	upper periocular area/lower periocular area
Discharge from Eye			
Right Eye	none/wet/dried	_____	
Left Eye	none/wet/dried	_____	
Crusts on palpebrae and periocular area			
Right Eye	no/yes	_____	upper palpebra/lower palpebra
Left Eye	no/yes	_____	upper palpebra/lower palpebra
Other lesions of the palpebrae and periocular area			
Right Eye	no/yes	_____	trauma, necrosis: palpebra/periocular area
Left Eye	no/yes	_____	trauma, necrosis: palpebra/periocular area

APPENDIX 1. Continued.

Degree of Closure of Palpebra			
Right Eye	normal (100% open)/partially closed (____ %)		
Left Eye	normal (100% open)/partially closed (____ %)		
Sunken/Recessed Eyes			
Right Eye	no/yes/unknown	_____	
Left Eye	no/yes/unknown	_____	
Eye Swollen or Bulging in Appearance			
Right Eye	no/yes/unknown	_____	dorsal/lateral
Left Eye	no/yes/unknown	_____	dorsal/lateral

CHIN GLANDS

Site	Size	Drainage	Severity	Color of Drainage
Right Gland	normal/swollen	present/absent	_____	none/clear/cloudy/white yellow/green
Left Gland	normal/swollen	present/absent	_____	

POSTURE/BEHAVIOR

Behavior appropriate for time of day		yes/no If no, describe	_____
Behavior appropriate for season		yes/no If no, describe	_____
FORELIMBS			
Right	normal/abnormal	If abnormal, describe:	_____
Left	normal/abnormal	If abnormal, describe:	_____
HINDLIMBS			
Right	normal/abnormal	If abnormal, describe:	_____
Left	normal/abnormal	If abnormal, describe:	_____
OTHER			
Tail	normal/abnormal	If abnormal, describe:	_____
	normal/abnormal	If abnormal, describe:	_____