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Mycoplasmosis and the Desert Tortoise (*Gopherus agassizii*) in Las Vegas Valley, Nevada

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ABSTRACT.–***Mycoplasma agassizii* is the cause of an upper respiratory tract disease (URTD) in certain populations of the desert tortoise, *Gopherus agassizii*, in the Mojave Desert of the southwestern United States. This disease and the resulting epizootic influenced the listing, by the federal government, of desert tortoise populations north and west of the Colorado River as threatened. As part of a lawsuit settlement, 875 desert tortoises were removed from specified properties in Las Vegas Valley, Nevada, between June 1990 and December 1991. Clinical signs of URTD were seen in 14.3% of the collected tortoises. Pathologic evaluations of tortoises submitted from the Desert Tortoise Conservation Center, Las Vegas Valley, Nevada, revealed that 8 of 12 tortoises submitted as clinically healthy had lesions consistent with URTD. The presence of lesions indicates that subclinical disease exists in this tortoise population and that determining health status of tortoises requires more sophisticated approaches than clinical appearance alone. The difficulty in accurately assessing health status of wildlife, including chelonians, is an example of one of the problems that conservation biologists will face when trying to evaluate and manage remaining populations of a wide range of threatened or endangered species. Protocols need to be developed as minimal guidelines that can be used in health assessment of those species to be either relocated or intensely managed.**

KEY WORDS. – **Reptilia; Testudines; Testudinidae; *Gopherus agassizii*; tortoise; upper respiratory tract disease; pathology; *Mycoplasma agassizii*; *Pasteurella testudinis*; Nevada; USA**

The implications of infectious diseases on the release of captive-bred animals and relocation of wild animals have surfaced as issues of major concern in the conservation biology of a wide range of vertebrates (Wolff and Seal, 1993). While infectious agents, such as viruses, bacteria, fungi, and protozoan and helminth parasites, represent the single largest component of biodiversity, it is only recently that population biologists have appreciated the extent to which various pathogens can influence the vitality and persistence of wildlife populations (Lyles and Dobson, 1993). As an example, the morbillivirus responsible for rinderpest entered Africa in 1888, and although a rinderpest pandemic ended in southern Africa in 1899, ecological and socioeconomic effects remain today (Meltzer, 1993). There is a growing realization that the potential for transport of infectious agents between captive and wild populations, between wild animals from different localities, and between domestic animals and wildlife is a real risk that must be assessed when initiating relocation or translocation programs. New wildlife pathogens are constantly being described, but the ability to screen animals for their presence is hampered by a lack of specific and sensitive diagnostic tests.

The desert tortoise, *Gopherus agassizii*, is the largest reptile native to the Sonoran and Mojave deserts of the southwestern United States. In 1988 desert tortoises with upper respiratory tract disease (URTD) (Fig.1) were seen in the Desert Tortoise Natural Area (DTNA), Kern County, California (Jacobson et al., 1991). In 1989 a detailed survey of the DTNA and nearby areas in the Rand Mountains and Freemont Valley indicated that 43% of 468 live desert tortoises showed clinical signs of this disease (Knowles, 1989). This disease rapidly spread through this population, resulting in a catastrophic decline. In a 1982 survey of a 2.6 sq km interior plot in the DTNA, 204 adults and subadults were counted. In the same plot in 1992, 13 were counted (K.H. Berry, *pers. comm.*). In part because of this disease, desert tortoises north and west of the Colorado River were listed as threatened by the federal government on 2 April 1990 (Fish and Wildlife Service, 1990).

Clinicopathologic studies on URTD in desert tortoises from the Mojave Desert indicated that there was diffuse, severe, subacute to chronic inflammation of the mucosa and submucosa of the entire upper respiratory tract (Jacobson et al., 1991). By electron microscopy, small (350 to 900 nm) pleomorphic organisms, resembling members of the genus *Mycoplasma*, were seen in close association with the surface epithelium of ill tortoises. An organism compatible with *Mycoplasma* was cultured from the nasal passageways of ill tortoises and has been tentatively named *Mycoplasma agassizii* sp. nov. (Brown et al., 1994). This represents the

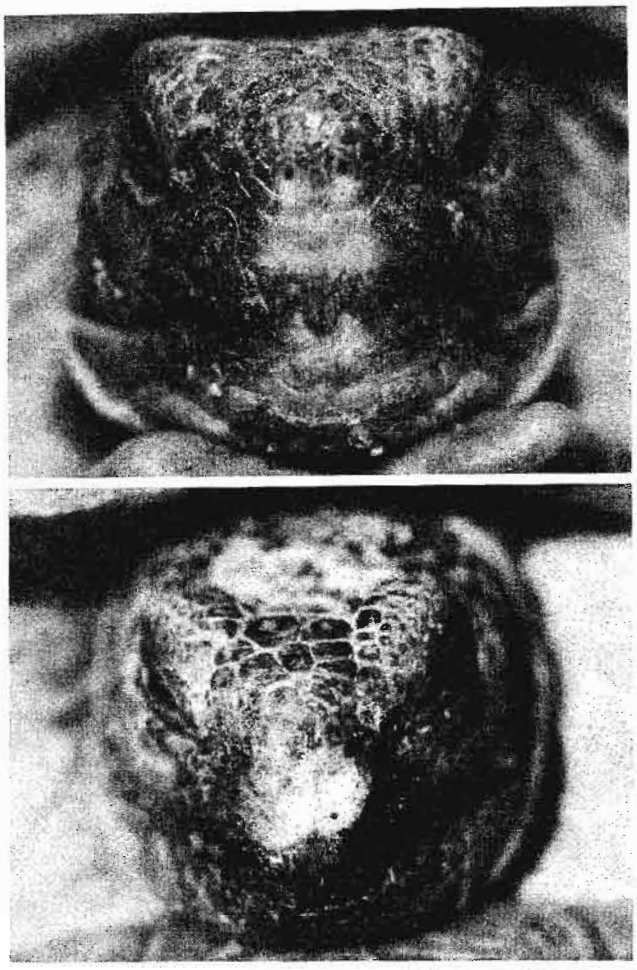

Figure 1. Top: Clinically healthy desert tortoise, *Gopherus agassizii*. **Bottom:** Desert tortoise with clinical signs of upper respiratory tract disease (URTD).

second *Mycoplasma* isolated from a reptile. Transmission studies confirmed *M. agassizii* as a cause of URTD (Brown et al., 1994).

As part of a lawsuit settlement between the U.S. Department of Interior, the City of Las Vegas, Nevada, Development Authority, and the State of Nevada, a permit (Federal Fish and Wildlife Permit PRT-747182) was granted for the removal of 875 desert tortoises from specified properties in the Las Vegas Valley between June 1990 and December 1991 (Hardenbrook, 1992). Clinical signs of URTD were observed in 125 (14.3%) ofthe 875 desert tortoises collected. The highest prevalence of URTD was from two tracts where previous releases of captive desert tortoises had occurred.

In an attempt to better understand URTD, clinicopathologic, microbiologic, and serologic studies were conducted on both clinically healthy desert tortoises and desert tortoises with signs of URTD. Here we report our findings documenting the occurrence of subclinical URTD in desert tortoises in Las Vegas Valley, Nevada.

MATERIALS AND METHODS

Tortoises Evaluated. — Four groups of three tortoises ill with upper respiratory tract disease (URTD) and four groups of three clinically healthy tortoises were received in April, July, and October 1991, and January 1992. All originated from Las Vegas Valley, Clark County, Nevada, and were collected from tracts of land as part of a lawsuit settlement agreement. Tortoises were determined to be ill with URTD if a nasal discharge was observed (Fig. 1). Tortoises were determined to be clinically healthy if found free of signs of URTD or signs of any other obvious health problems.

Serology. — Blood was collected from the jugular vein of each tortoise, placed in tubes coated with lithium heparin, centrifuged, and stored at -20°C. A portion of plasma from each tortoise was submitted for determining antibody titers for exposure to *Mycoplasma agassizii*, using an enzyme-linked immunosorbent assay (ELISA) specifically developed for the desert tortoise (Schumacher et al., 1993).

Pathology. — All tortoises were euthanized with a concentrated barbiturate solution. Heads of all tortoises were bisected longitudinally. For histopathologic studies one side of each bisected head was fixed in 10% buffered formalin, decalcified, embedded in parafffin, sectioned longitudinally at 7 μm, and stained with hematoxylin and eosin and the Brown-Hopps method for gram-positive and gram-negative bacteria. Sections of nasal cavities were examined by light microscopy and classified as either normal or exhibiting mild, moderate, or severe inflammation. Although classifying the severity of an inflammatory lesion is inherently subjective on the part of the pathologist, the following criteria were used in an attempt to classify the lesions as objectively as possible:

1) *Normal*: Occasional small subepithelial lymphoid aggregates; rare heterophils in the lamina propria; no changes in mucosal or glandular epithelium; no edema.

2) *Mild Inflammation*: Multifocal small subepithelial lymphoid aggregates; multifocally, small numbers of heterophils, lymphocytes, and plasma cells in the lamina propria; mild edema in lamina propria; minimal changes in mucosal epithelium.

3) *Moderate Inflammation*: Multifocal to focally extensive lymphoid aggregates; diffusely, moderate numbers of heterophils, lymphocytes, and plasma cells in the lamina propria, occasionally infiltrating the overlying mucosal epithelium; moderate edema in the lamina propria; proliferation and disorganization of the basal epithelium.

4) *Severe Inflammation*: Focally extensive to diffuse bands of lymphocytes and plasma cells subjacent to and obscuring the overlying mucosal epithelium; large numbers of heterophils in lamina propria and infiltrating overlying mucosal epithelium; marked edema of the lamina propria; degeneration, necrosis, and loss of the mucosal epithelium with occasional erosion; proliferation of the basal cells of the epithelium with metaplasia (transformation) of the mucous and olfactory epithelium to a basaloid epithelium; occasional squamous metaplasia.

Microbiology. — Swab specimens obtained from the nasal cavities of three ill and three healthy desert tortoises in April, July, and October 1991, and January 1992 were collected for aerobic bacteria isolation attempts. For isolation, samples were cultured on sheep blood agar and

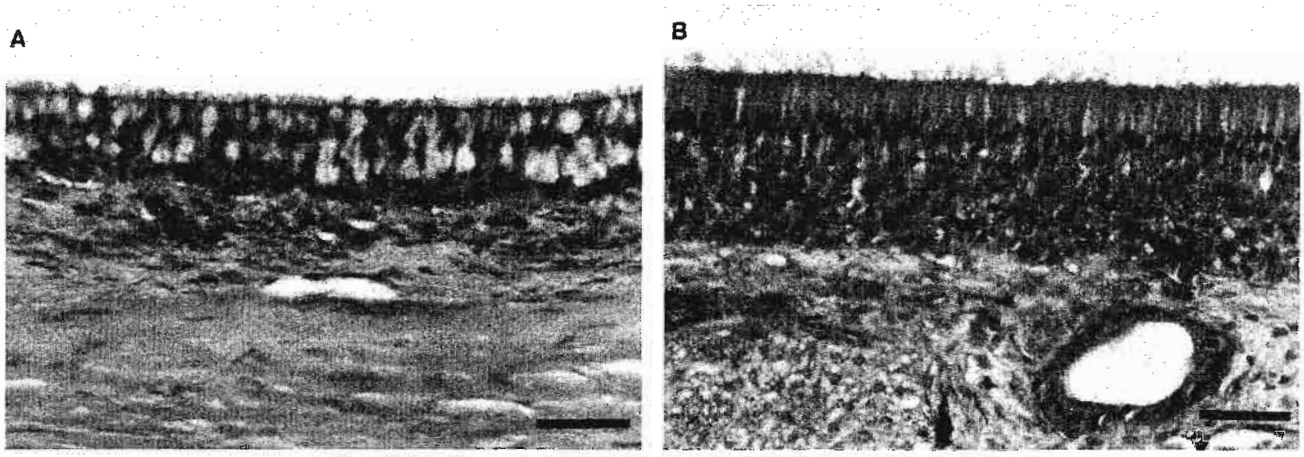

Figure 2. Photomicrographs of healthy desert tortoise nasal cavity, demonstrating both mucous epithelium (A) and olfactory epithelium (B). Hematoxylin and eosin stain. Bar = 50 μm.

MacConkey's agar and incubated at 37°C. All aerobes were identified using growth characteristics on various media and standard biochemical tests. All isolates of organisms consistent with *Pasteurella* were identified to species according to biochemical profiles listed for *P. testudinis* (Snipes and Biberstein, 1982).

For *Mycoplasma* isolation attempts, swabs were obtained from the left and right nasal passage, left and right choana, trachea, and lung of all tortoises necropsied. Uterine lavage and ovarian tissue were cultured for females. Testicular tissue and vas deferens were cultured for males. Isolation attempts were made in SP4 broth and on SP4 agar incubated to 30°C. At weekly intervals, plates were examined for growth with the aid of a dissection microscope. Broth cultures were examined for a color change biweekly. If a color change was observed, the broth was subcultured to agar and to fresh broth and the remaining culture frozen at -70°C. At one week a single blind passage was made to SP4

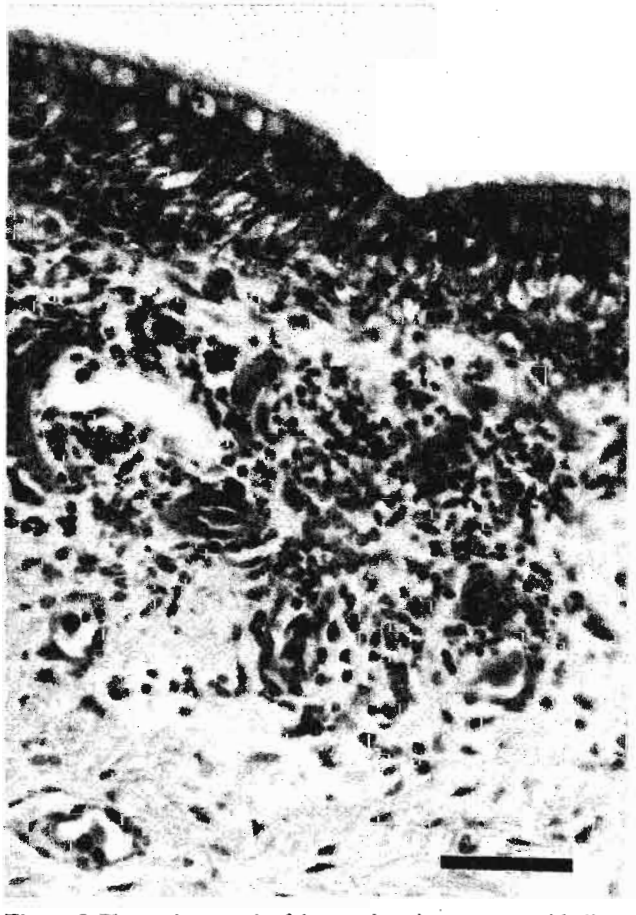

Figure 3. Photomicrograph of the nasal cavity mucous epithelium of a desert tortoise with moderate inflammation. Infiltrates of small mononuclear cells can be seen within the submucosa. Hematoxylin and eosin stain. Bar = 50 μm.

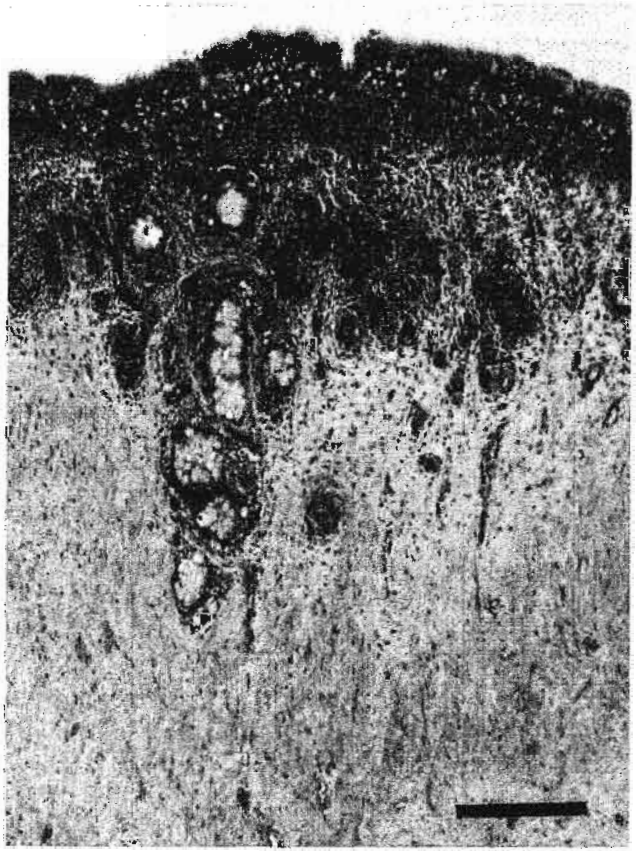

Figure 4. Photomicrograph of the nasal cavity olfactory epithelium of a desert tortoise with severe inflammation. The normal multilayered arrangement of epithelial cells has been replaced by proliferating basal epithelial cells. Numerous small mononuclear cells are seen within the submucosa, with aggregates around submucosal glands. Hematoxylin and eosin stain. Bar = 200 μm.

Table 1. Summary of nasal cavity histopathology, isolations of *Mycoplasma* and *Pasteurella*, and ELISA results for exposure to *Mycoplasma agassizii* in desert tortoises from Las Vegas Valley: April 1991 – January 1992.

	Clinically Healthy Appearing Tortoises					Tortoises with Clinical Signs of URTD				
Month	Tortoise No.	*Mycoplasma* Culture	*Mycoplasma* ELISA	*Pasteurella* (% of isolates)	Histopathology	Tortoise No.	*Mycoplasma* Culture	*Mycoplasma* ELISA	*Pasteurella* (% of isolates)	Histopathology
April	626	–	–	–	Not determined	708	–	+	20%	Severe
	743	+	+	10%	Mild	707	+	+	< 1%	Normal
	815	+	–	< 1%	Normal	963	+	+	30%	Moderate
July	376	+	–	< 1%	Normal	644	+	+	10%	Severe
	500	–	+	< 1%	Severe	728	+	+	–	Mild
	981	+	+	–	Moderate	687	–	+	–	Moderate
October	889	+	+	25%	Mild	663	+	+	35%	Severe
	875	–	–	< 1%	Mild	962	–	+	90%	Severe
	186	+	–	–	Normal	578	+	+	25%	Severe
January	066	–	+	–	Mild	716	–	+	–	Mild
	515	–	–	–	Mild	150	–	+	–	Mild
	065	–	–	–	Mild	609	–	+	< 1%	Severe

agar. When growth was observed on agar, agar plugs were removed aseptically, placed in SP4 broth, and treated as described for other broth cultures.

RESULTS

Pathology. — Histopathological findings on the nasal cavities of desert tortoises are summarized in Table 1. Of the 12 tortoises submitted as clinically healthy, only three had normal nasal mucosal epithelium (Fig. 2); eight had mild to severe lesions, and in one tortoise the nasal mucosa was lost during processing. Of 12 desert tortoises submitted with clinical signs of URTD, 11 had mild to severe lesions in the nasal cavity (Figs. 3 and 4), and one tortoise had a normal nasal mucosa.

Histologically the least severe lesions were characterized by small, focal, subepithelial, often perivascular aggregates of lymphocytes and plasma cells in the lamina propria. Accompanying the lymphoplasmacytic infiltrates were occasional heterophils and macrophages; there was mild edema and little or no change in the mucosal epithelium. The most severe lesions consisted of diffuse florid lymphoplasmacytic inflammation in the lamina propria. The mucous and olfactory epithelium was replaced by proliferating basaloid cells, with degeneration and necrosis of mucosal epithelium, erosions, and exudate in the lumen of the nasal passages.

Microbiology. — A variety of aerobic bacteria were isolated from those tortoises submitted as clinically healthy and those submitted with clinical signs of URTD (Table 2). While *Pasteurella testudinis* was isolated from tortoises with and without lesions in their nasal cavities, the organism was isolated as a greater percentage of the total isolates from tortoises with moderate to severe lesions. Of 18 desert tortoises necropsied in April, July, and October 1991, *Mycoplasma agassizii* was isolated from 6 of 13 (46%) that had histological lesions of URTD and also from 3 of 4 (75%) that had histologically normal nasal mucosa (Table 1). Few aerobic bacteria and no *Mycoplasma* were isolated from 6 ill or healthy tortoises necropsied in January 1992, when tortoises were hibernating.

In regard to *Mycoplasma* isolation, the nasal passage (n = 7) and choana (n = 9) were the best sites of isolation. Isolates were also obtained from trachea (n = 1), vas deferens (n = 1), and ovarian tissue (n = 1).

Serology. — Of the 11 tortoises submitted as ill with URTD and having lesions in their nasal cavities, all were seropositive for exposure to *M. agassizii* (Table 1); one tortoise submitted as ill, but with a histologically normal nasal cavity, was also seropositive. Twelve tortoises were submitted as clinically healthy. Three of these had histologically normal nasal cavities and were seronegative for exposure to *M. agassizii*; eight other tortoises were found to have histological lesions in their nasal cavities. Of these eight, five were seropositive, and the other three, with lesions categorized as mild, were seronegative.

Table 2. Microbial isolates from nasal cavities of clinically healthy desert tortoises and desert tortoises with upper respiratory tract disease (URTD). Only those isolates which were cultured as > 1% of the total microbial isolates per tortoise are listed. The numerator is the number of culture-positive tortoises, the denominator the number of tortoises cultured (n = 12).

	Healthy	URTD
Corynebacterium sp.	7/12	6/12
Staphylococcus sp.	7/12	3/12
α-hemolytic *Streptococcus*	5/12	1/12
Micrococcus	1/12	1/12
Pasteurella testudinis	2/12	6/12
Bacillus sp.	1/12	0/12
Achromobacter	2/12	5/12
Pseudomonas aeruginosa	1/12	1/12
Non-enteric gram-negative rod	7/12	8/12
Flavobacterium sp.	0/12	1/12
Mycoplasma agassizii	4/12	5/12

DISCUSSION

While nasal discharge has been seen in both captive and wild desert tortoises in Las Vegas Valley, Nevada, this study represents the first histologic, microbiologic, and serologic confirmation of URTD in this population. In this study the most disturbing finding was the presence of lesions of URTD in a majority of the tortoises submitted as clinically healthy. This finding reveals that subclinical disease exists in a substantial percentage of tortoises in Las Vegas Valley. Chronically infected animals serve as reservoirs, allowing trasnsmission of the causative agent to naive tortoises. Because of subclinical disease, determining the health status of a tortoise by physical clinical examination alone is impossible. While an ELISA test can be used to detect exposure to the causative agent by measuring *Mycoplasma*-specific antibody in plasma, it cannot determine the presence of active infection at the time of sampling. Additional diagnostic tests, such as a polymerase chain reaction (PCR) test for determining the presence of *Mycoplasma* gene nucleotide sequences in nasal secretions, will need to be developed to better ascertain the health status of tortoises. Such data are critically important when tortoises are being relocated, repatriated, translocated from one site to another (Dodd and Seigel, 1991), or used in research projects.

Pasteurella testudinis, a bacteria first described from captive desert tortoises with URTD (Snipes and Biberstein, 1982), was isolated from 11 of 19 tortoises with lesions in their nasal cavities. This bacteria was isolated from most tortoises in this study but represented a greater percentage of the total aerobic isolates in affected tortoises. This bacteria may contribute to the severity of the disease.

Mycoplasma agassizii was also cultured from three of four desert tortoises with histologically normal-appearing nasal cavities. There may be a couple of explanations for this. These tortoises may have been recently colonized with these organisms, and the tortoises were necropsied before lesions had a chance to develop. Second, pathogenic and nonpathogenic strains of *M. agassizii* may exist. Nonpathogenic strains of *M. agassizii* may exist as normal inhabitants of the nasal cavity of desert tortoises. Further work will be necessary to determine the pathogenic potential of the various isolates of *M. agassizii* in this study.

Antibody levels to *M. agassizii* were determined by an ELISA test (Schumacher et al., 1993). All tortoises submitted as clinically ill with signs of URTD were seropositive. Five of eight tortoises submitted as clinically healthy, but found to have mild to severe lesions in their nasal cavities, were also seropositive. Three tortoises with mild lesions in the nasal cavity were seronegative. Starting with the most likely explanation, seronegative responses may indicate that:

1) The tortoises had an early infection but were tested prior to the development of an antibody response.

2) The tortoises may have had normal nasal cavities, and the interpretation of the presence of mild lesions was incorrect.

3) If the tortoises did have mild inflammation, there may have been another causative agent.

4) The tortoises were incapable of eliciting an immunologic response to *M. agassizii*.

Because of land development and concomitant habitat loss, allowed as part of federally permitted Habitat Conservation Plans or other legal exceptions for this protected species, large numbers of desert tortoises are being displaced annually throughout their geographic range in the U.S. The disposition of these legally displaced tortoises, either collected from land scheduled for development or collected from federal land-disturbing projects, is a complex problem with no simple solution. In Las Vegas Valley, Nevada, pressures on desert tortoises and other animals are particularly great because of expanding retirement communities, theme parks, and casinos. Between June 1990 and September 1991, 875 desert tortoises were displaced and transferred to a conservation center where various research projects were conducted in an attempt to better understand their biology, health problems, and nutritional requirements. While tortoises were subjected to a quarantine period, with clinically ill and clinically healthy-appearing tortoises segregated into different pens, the findings of the current study indicated that most tortoises submitted as clinically healthy had lesions in their upper respiratory tract consistent with URTD. Tortoises with subclinical disease were more than likely intermixed in pens with clinically normal tortoises, potentially resulting in the spread of the causative pathogen.

The origin of *M. agassizii*, responsible for both the epizootic in the western Mojave Desert and clinical and subclinical disease in Las Vegas Valley, has not been determined. Since URTD is commonly seen in captive desert tortoises, and large numbers of these animals are kept as pets throughout their range, possibly a pathogenic strain of *M. agassizii* was introduced into these wild populations through captive releases. Because of the anxiety they generate in their owners, ill captives are commonly returned to the wild. Rather than releasing them into the wild, owners of desert tortoises with URTD should consider euthanasia. The ease with which desert tortoises can be captured and moved about makes mycoplasmosis a disease of major concern for the long-term survival of naive (unexposed) populations in the southwestern United States.

The desert tortoise has certain unique features that set it apart from other endangered and threatened species. Prior to federal listing, large numbers were, and still are, kept in captivity, often in backyards. Accurate figures are not available, but it is estimated that in excess of 40,000 tortoises are in captivity in Las Vegas Valley, Nevada (D.B. Hardenbrook, *pers. comm.*), and over 200,000 in California (K.H. Berry, *pers. comm.*).

In an attempt to reduce mortality and save large numbers of displaced tortoises in Las Vegas Valley, clinically healthy desert tortoises are adopted through a program approved by state and federal agencies responsible for enforcing the U.S. Endangered Species Act. There is no other species listed as endangered or threatened in the U.S. that is being handled in a similar manner. The historical perception of the desert tortoise as a popular pet has contrib-

uted to an apparently simple solution to a complex problem. This issue needs to be more thoroughly discussed so that scientifically and ethically sound disposition guidelines can be recommended.

Translocation is also being presented as a mitigation tool for the beneficial use of displaced tortoises. While translocation is appealing, significant health issues need to be considered and the risks of spreading infectious agents need to be minimized (Dodd and Seigel, 1991). In regard to mycoplasmosis and the desert tortoise, regardless of clinical appearance, all seropositive tortoises from known "hot spots," such as Las Vegas Valley, should be considered potential carriers of *Mycoplasma* and should not be released in areas where the disease does not exist. Clinically healthy, seropositive desert tortoises originating from populations where URTD has not been seen may be relocated to other sites where clinically healthy, seropositive tortoises have been identified. In such situations the relative numbers of seropositive and seronegative tortoises from donor and recipient populations should approximate one another. As with adoption, detailed guidelines for the translocation of desert tortoises need to be established. The Desert Tortoise (Mojave Population) Recovery Plan (Fish and Wildlife Service, 1994) states that "all potential translocatees should be medically evaluated in terms of general health and indications of disease, using the latest available technology, before they are moved," but it fails to recommend what to do with ill or subclinically affected tortoises. Because there is no known drug therapy for long-term improvement of tortoises with URTD, euthanasia rather than relocation should be considered for such tortoises. Healthy appearing sero-positive tortoises from populations in which URTD has been seen should be considered infectious and should not be released into areas where URTD has not been observed.

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