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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

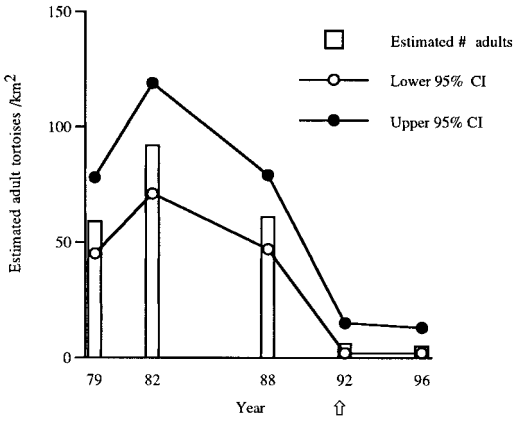

FIGURE 1. Estimates of desert tortoise population densities for the years 1979, 1982, 1988, 1992, and 1996 at the desert tortoise study plot in the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). Results are expressed as the estimated number of adult tortoises per km^2.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

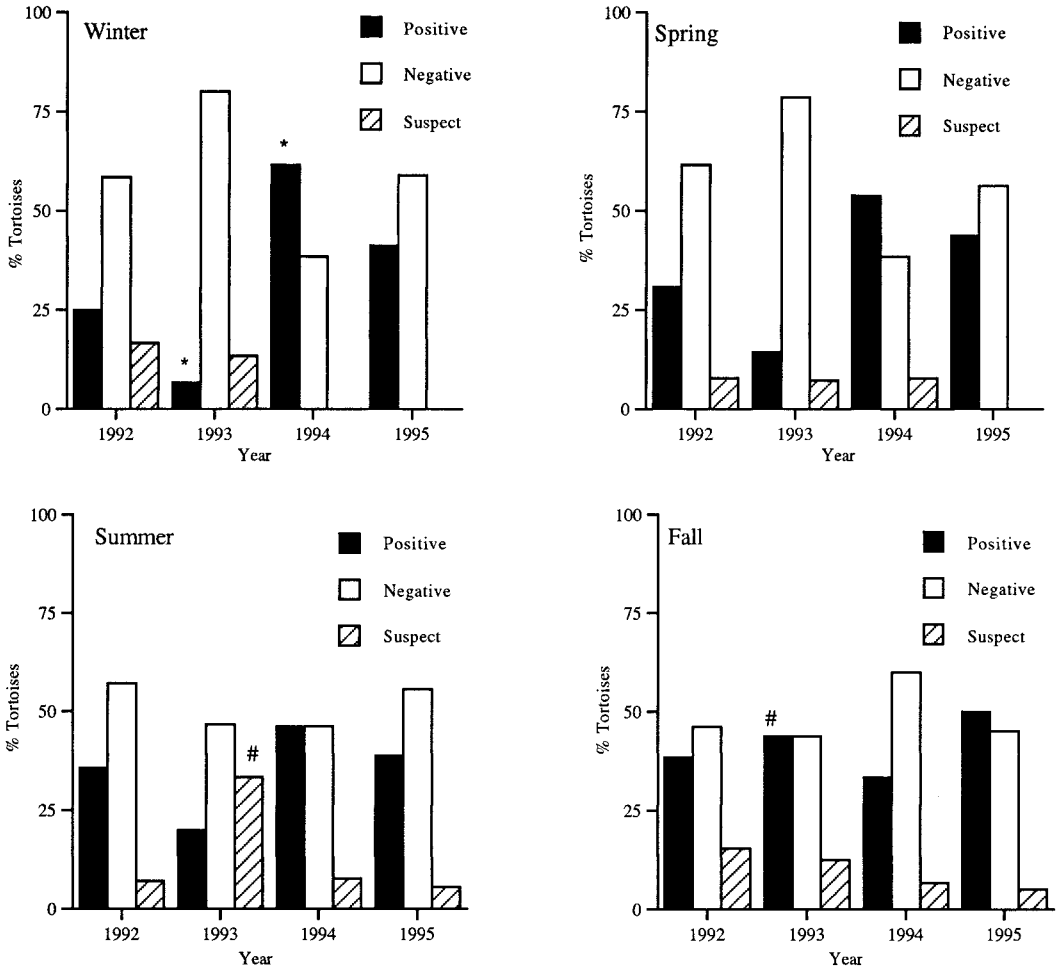

FIGURE 2. Distribution of tortoises with positive, negative or suspect ELISA values in desert tortoises from the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). In winter of 1993 and 1994, there were statistically significant differences in the distribution of tortoises 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.,

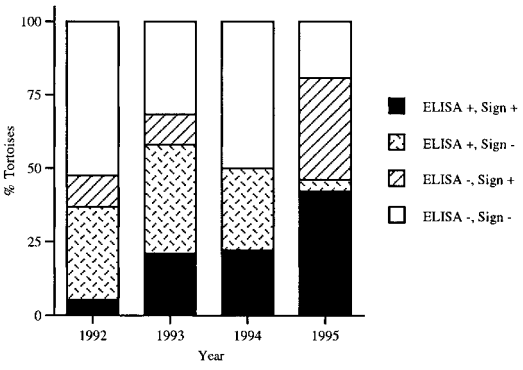

FIGURE 3. Comparison of ELISA results with presence of clinical signs in desert tortoises from the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). Results are expressed as the percentage of tortoises with positive or negative ELISA results in conjunction with the presence (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 ($P = 0.001$).

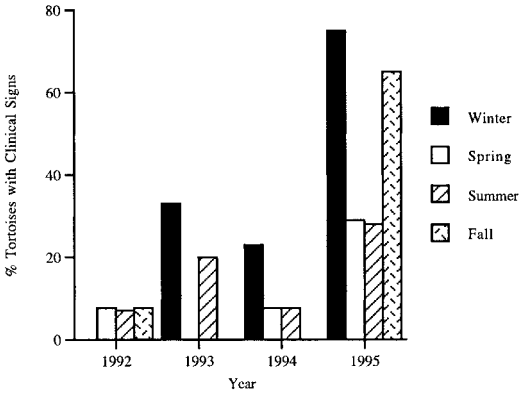

FIGURE 4. Distribution of tortoises with clinical signs in desert tortoises from the Desert Tortoise Research Natural Area (DTNA) in Kern County (California, USA). No significant differences were noted among seasons within a year in 1992 or 1994 ($P = 0.82$ and 0.21, respectively). Within a given year, 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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