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Centre de Recherche et de Conservation des Chéloniens

Health Assessment of Free-Ranging Hermann's Tortoises (*Testudo hermanni hermanni*) in Continental France

by

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ABSTRACT

The implementation of conservation measures for threatened species, such as animal translocation, inevitably involves the knowledge and control of diseases and pathogens likely to adversely affect the species. The western Hermann's tortoise (*Testudo hermanni hermanni*) is particularly affected by (1) a virus, Testudinid Herpesvirus (TeHV), responsible for lethal respiratory diseases and (2) a mycoplasm, *Mycoplasma agassizii*, which can lead to serious pathological issues. These agents are highly contagious and are frequently found in captivity. An assessment of the health status of wild populations began in 2012 on the entire continental distribution range. A total of 347 tortoises were sampled on 14 different sites. Physical examinations, blood samples, and nasal and oral swabs were performed on each individual. Herpesvirus was detected in 3.3% of tortoises *via* Polymerase Chain Reaction (PCR) test, which detects all forms of herpes. In contrast, one tortoise was found "suspicious" regarding the search for specific antibodies of genotypes TeHV-1 and -3 via the technique of serum-neutralization. *Mycoplasma* was detected in 8.1% of individuals by PCR. This study demonstrates for the first time the presence of highly dangerous pathogens (herpes) in wild populations of Hermann's tortoises in southern France and confirms the presence of another (*Mycoplasma agassizii*). It also highlights the threat posed by unauthorized releases into the wild of captive individuals, potentially carrying diseases.

Key words: *Mycoplasma agassizii*, Testudinid Herpesvirus, *Testudo hermanni hermanni*, tortoise, prevalence.

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Introduction

There is evidence that chelonians are undergoing a marked decline worldwide. Among more than 300 species, half are threatened with extinction[1]. Indeed, their life history, physiology and behavioral traits (i.e. long-lived species, low fecundity, late maturity, and limited dispersal abilities) make chelonians particularly vulnerable to natural and anthropogenic threats such as habitat loss and degradation, predation, pollution, unsustainable use, climate change, illegal capture in natural populations and commercial use (Guyot and Clobert 1997, Hailey 2000). In terrestrial tortoises, one of the most important threats comes from the release, deliberate or no, of ill captive-bred and exotic individuals into the wild which may introduce potentially destructive pathogens into naïve resident populations (Champagnon et al. 2012, Ewen et al. 2012, Griffith et al.1993, Kock et al. 2010, Snyder et al. 1996). A number of studies have identified pathogens causing terrestrial tortoise population declines (Holt et al. 1979). For example, the Upper Respiratory Tract Disease (URTD) has been weakening desert (*Gopherus agassizii*) and gopher (*Gopherus polyphemus)* tortoise populations in North America during the past three decades (Berish at al., 2010).

It has been shown that URTD is associated to a bacterial mycoplasm, *Mycoplasma agassizii*, which is spread when tortoises are in contact with one another (Mushinsky et al. 2006). It is thought that a pathogenic strain of *Mycoplasma* may have been introduced into wild populations in both California and Nevada by releases of captive desert tortoises (Jacobson et al. 1995, Berry 1997). Infected individuals are extremely lethargic and show clinical signs such as mucal discharge from the nares, wheezing

[1] As determined by the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group (TFTSG) and noted on the IUCN Red List of Threatened Species or in TFTSG draft assessments (Turtle Taxonomy Working Group 2010, www.iucnredlist.org).

breath, swollen and watery eyes (Smith et al. 1998; Berish et al. 2000). However, individuals may be carriers for *Mycoplasma* but may not become symptomatic until they are subjected to stress (e.g. new environment, vitamin deficiency), which can make infection difficult to detect (Berish 2000, but see McCoy et al. 2005). *Mycoplasma* have been associated with significant mortality in the eastern box turtles *Terrapene carolina carolina* (Feldman et al. 2006) and ornate box turtles *Terrapene ornata ornata* (Farkas and Gàl 2009), as well as in captive and pet *Testudo* species (Soares et al. 2004).

Another disease affecting terrestrial tortoises around the world comes from a virus known as Testudinid herpesvirus (TeHV) or "rhinitis-stomatitis syndrome". Four different genotypes of TeHV have been isolated (TeHV-1, -2, -3, and -4). Both TeHV-1 and TeHV-3 are present in Europe, with the latter being the most virulent in captive European tortoise. Herpesvirus (TeHV-1 and -3) has never been documented in wild tortoise populations excepted in *Testudo graeca* in Turkey (Marschang and Schneider 2007). Clinical signs include nasal and oral discharge, conjunctivitis and necrotic plaques on the oral mucosa and the tongue. Transmission mode is not fully understood but TeHV is extremely contagious and contaminated individuals, when then survive, may carry the illness for life (Origgi et al. 2000). Diagnosis of Herpesvirus and *Mycoplasma* infections is done using molecular-based tests like polymerase chain reaction (PCR) and serological assays from oral and nasal swabs, and plasma sample.

The western Hermann's tortoise (*Testudo hermanni* subsp. *hermanni*) is the last terrestrial species of tortoise remaining in France and is restricted to a very small area, in the Var department (southeastern France) and in Corsica. Classified as "near threatened" by the IUCN Red List of Threatened Species (Van Dijk et al. 2004) and as « vulnerable » by the French IUCN Red List of Threatened Species (UICN France, MNHN & SHF 2009), the western Hermann's tortoise faces a rapid and severe decline in its distribution range (Livoreil 2009). Because of reduced genetic variation in small

population, fragmented populations of *T. hermanni hermanni* in southern France are more susceptible to disease (Radwan et al. 2010). Additionally, the legal Hermann's tortoise trade in France is growing and more and more exotic and captive individuals, potentially carrying pathogens, are found into the wild.

Concerns about the potential impacts of infectious diseases on endangered *T. hermanni hermanni* populations have prompted health survey in wild and captive tortoises in southern France (Mathes 2001). However, the prevalence of these pathogens in southern France has not been extensively surveyed. This paper focuses on (i) analyzing the prevalence and distribution of TeHV (serotypes I and III) and *Mycoplasma agassizii* in free-ranging *T. hermanni hermanni* at 14 sites located within the French continental distribution area of the Hermann's tortoise (ii) correlating the infection status of individuals with physiological, physical and clinical parameters as well as geographical location and (iii) detecting the presence of exotic animals as well as tortoises from suspicious origin (i.e. captivity). We hypothesize that sites located nearby urbanized areas would be more prone to receive released or escaped diseased captive individuals. Gender may have an effect as females usually dispatch more in their environment, notably to lay their eggs, and thus have more chance to meet diseased tortoises.

Materials and Methods

Data collection

Monitoring of sampling sites. Fourteen study sites were sampled across the entire range of the western Hermann's tortoise (*T. hermanni hermanni*) in southeastern France (Var department, Figure 1). Sites were located near or within main population cores. Typically, vegetation at study sites consisted of mixed forests (mainly *Pinus pinea*, *P. halepensis*, *Quercus suber*, *Q. ilex* and *Q. pubescens*) and semi-open areas of scrubland vegetation (maquis mainly composed of *Erica sp.*, *Calluna vulgaris*, *Lavandula stoechas,*

Cistus sp. and *Filaria sp*.). Sites were surveyed from May to October 2012 and from April to July 2013, when this species is most active (Hailey et al. 1984). Each site was randomly surveyed in the morning and in the evening at days when climatic conditions were favorable to tortoise activity (no rain, no or low wind, mild temperature). Approximately 30 tortoises were sampled in each site.

Tortoise data. Tortoises were localized by sight, aurally or using wildlife detector dogs when available (see Nussear et al. 2008). Each specimen was individually marked with a unique notch code on the marginal scales (Plummer 1989). All tortoises were given a complete physical examination immediately upon capture. Body weights and morphologic measurements, including carapace and plastron length and width, were determined using a 5-kg, Kologn Electronic Scale and 25-cm caliper. Body weights were measured to the nearest 5 grams.

External sexually dimorphic characteristics were used to determine the sex of mature animals. Mature males have longer, thicker tails, hollow plastron, and grow to a much smaller body size than females. Given the high morphological resemblances between *Testudo* subspecies, individuals were thoroughly examined for the presence of traits specific to each subspecies (yellow colour of subocular scales, black plastral bands, supracaudal scute divided/undivided, ratio of pectoral and femoral seams) (for details see Soler et al. 2012).

The most common components of a comprehensive health assessment include examination for physical condition, biochemical profile, and infectious disease. Physical examination and biological sampling were conducted by four trained people. Caution was taken when handling and sampling tortoises to ensure that field personnel did not aid in the spread of infectious microorganisms or contaminated samples. All field personnel followed a protocol that included the disinfection of all equipment after use and hand cleaning using sanitizer. Physical examination was done in particular for eye

abnormalities (stomatitis, conjunctivitis), ocular and nasal discharge, and respiratory signs. Such clinical signs could suggest the possibility of an infection by a Herpesvirus or *Mycoplasma*. Overall activity and responsiveness, shell abnormalities (e.g. pyramidal growth, scales abnormalities, wounds such as burn marks, traces of dog fangs and bumps) and cloacae abnormalities were also noted.

Biological samples. Blood was collected from the subcarapacial venipuncture site (Hernandez-Divers et al. 2002) and only one sample was taken from each individual. The venipuncture site was cleaned with antiseptic and antifungal solution (Vétédine®, VETOQUINOL) using cotton bud prior to insert the needle. Blood extraction was done by means of plastic syringes and sterile needles (25G x 5/8", 0.5 x 16mm, TERUMO), used only once. In order to keep blood from coagulating when it was drawn, the needles and syringes were treated with the anticoagulant liquid lithium heparin. Between 0.5 and 1ml of blood was collected (depending on tortoise size) and samples were immediately placed in heparin containers. Samples were gently mixed and stored in cooler until centrifugation (1500 rpm for 5 min). The plasma was then removed and placed in two different tubes: a 50 µl aliquot for virology analysis and the rest for biochemical analysis. Because lymphatic systems are well developed in chelonian, obtaining blood samples from sites near the lymphatic system may result in hemodilution with lymph (Rohilla and Tiwari 2008). Thus, to avoid bias in the results, both lymph presence and quantity were noted and results were interpreted cautiously. None of the animals used in this study showed any ill effects related to blood sampling.

Because pathogens are present in body fluids during the acute phase of the infection, nasal and oral swabs were collected. Procedure for nasal lavage consisted in stabilizing tortoise's head while the handler surrounded a naris with the syringe tip and injected about 0.9 ml of sterile saline (0.9% sodium chloride, LAVOISIER) directly into the naris. As the flush was performed the handler immediately pulled up the plunger to collect the

fluid that came out. Process was repeated in the other naris. Fluid was then collected in a 50 ml sterile conical tube and kept in cooler.

For the oral swab, the handler stabilized tortoise head using thumb and middle finger. Using the free index, the mandible was gently pushed down to make the mouth accessible. Then a brush (Cervibrush + LBC, Endocervical sampler, CellPath) was gently inserted inside the oral cavity and moved around the entire oral cavity to sample the choana, the tongue, the inside of the beak, and all mucosal surfaces. Brush was cut from the plastic stick with pliers and stored in a conical tube containing 0.3 ml of sodium chloride to avoid desiccation of the mucous.

After that, tortoises were released at the place of capture, generally within 30 minutes. Oral and nasal swabs along with plasma aliquots were frozen at -15°C until they were shipped to Staaliches VetUAmt laboratory in Detmold, Germany, where they were tested for genetic material of TeHV and *Mycoplasma agassizii* and antibodies to TeHV respectively.

Oral and nasal swabs were analyzed by the Polymerase Chain Reaction (PCR) test which detects pathogen during the acute phase of the infection, when it is actively shed in the body fluids. To determine the presence of Herpesvirus antibodies in the bloodstream, a serum-neutralization (SN) assay was performed on plasma aliquots. Particularly, aliquots were tested for two serologically different strains of tortoise Herpesvirus (strains 1432 and 770) which correspond to TeHV type-1 and type-3. Thus, serological test only detects past exposure to pathogens as they are based on antibodies presence. The rest of plasma was left for biochemical analyses (Figure 2).

Serum biochemical parameters. Glucose concentration (mg/dl) was assessed directly on the field using the blood glucose meter: Accu-Chek® Performa. Serum biochemical parameters included sodium (mEq/l), potassium (mEq/l), calcium (mg/l), urea (g/l), uric acid (mg/l), triglyceride (g/l), cholesterol (g/l), serum glutamic

oxalacetic transaminase (SGOT) (U/l), and serum glutamic pyruvic transaminase (SGPT) (U/l). Analyses were carried out by LABAZUR laboratory for medical analyses (Le Luc, France) and realized under MODULAR de Roche automaton (ADVIA 2400 Siemens, Colorimetry, potentiometry).

Data analysis

A significance level or probability of a type-I error (α) of 0.05 was assumed for all analyses. Statistical analyses were performed using R 2.15.3 (R core team, 2012). Analyses did not concerned TeHV given the relatively small number of individuals infected by this disease.

Wilcoxon-test was used to evaluate difference in mean biochemical concentrations between PCR-positive for *Mycoplasma* tortoises and "negative" tortoises. The qui-square test of association was used to see whether there was any association between different clinical signs and *Mycoplasma* infection.

Mass-length relationships were used to calculate the body condition index (BCI) log (M/M'), where M is observed mass and M' is mass predicted from length, which is equal to residuals from the regression of log (M) on log (length). A body condition index (BCI) is a non-destructive and simple method to assess an individual's well-being indirectly. More well-hydrated and/or more well-fed individuals will display a higher BCI (Moore et al. 2006). We used Welch Two Samples t-test to see if mean BCI was significantly different between PCR-positive for *Mycoplasma* tortoises and other tortoises.

Non-parametric two-way ANOVA (Scheirer-Ray-Hare test) was used to evaluate whether sex, illness and the interaction of both have an effect on BCI.

Qui-square test was also used to see if there was an association between different types of sampling sites and *Mycoplasma* infection.

A logistic regression (GLM) was used to know if the prevalence of *Mycoplasma* was related to the study sites.

Results

Physical examination

Over the study period, 384 tortoises were sampled at 14 different study sites. Thirty seven of these were neither tested for *Mycoplasma* nor Herpesvirus (TeHV) and were thus not included in the subsequent analyses. Of the 347 remaining tortoises, 189 were identified as female, and 151 were identified as male. Sex of the remaining 7 tortoises could not be determined because tortoises were immature and did not yet display obvious dimorphic characteristics of either sex (Figure 3).

The most frequently observed physical abnormalities were: healed wounds consistent with trauma (52.4%) including regions of cracked and broken scutes, claws abnormalities (12.4%), shell abnormalities (10.5%), pyramidal growth (5.5%), missing limbs (2.8%), and missing portions of the tail (0.5%).

Exposure to pathogens

Of the 347 tortoises sampled, 335 were tested for *Mycoplasma* by PCR test, 209 were tested for TeHV by PCR test, and 216 were tested for TeHV by SN test. Twenty-seven (8.1%) of 335 tortoises were PCR-positive for *Mycoplasma*, indicating the presence of *Mycoplasma* or other mollicutes in their nasal passages (Figure 4). Positive (i.e. exposed) tortoises for *Mycoplasma* have been found in 10 of our study sites. From 209 oral swabs tested for TeHV, 7 were PCR-positive (3.3%). One “suspect” sample for TeHV-1 and -3 was identified out of 216 plasma samples (0.5%). This sample contained sequences from both TeHV types. Prevalence of TeHV-1 and -3 was the same (0.5%). None of the tortoises sampled were infected by both *Mycoplasma* and TeHV.

Biochemical parameters

Serum biochemical concentrations for PCR-positive for *Mycoplasma* tortoises and healthy tortoises are presented in table 1. Of the 10 values determined no significant differences in mean concentration of serum biochemical parameters were found between PCR-positive and PCR-negative for *Mycoplasma* tortoises (all $P \geq 0.106$, Table 2).

Clinical signs

During the study, all tortoises handled in the field (n = 347) were examined for clinical signs. Nasal discharge was rare (5/356; 1.4% of evaluated tortoises). Mild palpebral edema was also rare (4/353; 1.1% of tortoises); however minor swelling of the eyelids could be caused by environmental factors, unrelated to *Mycoplasma*. In all cases the symptoms were minimal. None tortoises showed ocular discharge. All tortoises with observed mild palpebral edema were negative to *Mycoplasma* and Herpesvirus. Only one tortoise with nasal discharge was PCR–positive for *Mycoplasma.*

None of these clinical signs were significantly associated with PCR-positive for *Mycoplasma* tortoises in this study (all $P \geq 0.0547$, Table 3). However, the chi-square test showed there was an association between *Mycoplasma* infection and three other clinical parameters. Ectoparasites ($\chi^2 = 14.65$, d.f. = 2, $P < 0.05$), pyramidal growth ($\chi^2 = 16.65$, d.f. = 2, $P < 0.05$) and mucous color ($\chi^2 = 6.19$, d.f. = 2, $P < 0.05$) were possibly associated with *Mycoplasma* infection (Table 3).

None of the 7 PCR-positive for TeHV tortoises showed clinical sign of disease.

Sex

Of the 27 PCR-positive for *Mycoplasma* tortoises, 19 were females and 8 were males. Chi-square test showed no association between sex and *Mycoplasma* infection ($\chi^2 = 3.5243$, d.f. = 3, $P = 0.3176$).

Body Condition Index (BCI)

Adult females weighed 807.1 ± 181.2 g (mean ± SE), with a range of 150 to 1597 g.

Adult males weighed 518.4 ± 96.2 g, with a range of 237 to 859 g. Positive and negative for *Mycoplasma* tortoises do not have significant difference in mean BCI (Welch Two Sample t-test, t = -1.76, d.f. = 23. 13, P = 0.09) (Figure 5). The effect of "sex" on BCI was highly significant (*Scheirer-Ray-Hare test; P = 0*), but *Mycoplasma* infection and the interaction of both were not significant (*P = 0.9* and *0.7,* respectively). The two lines are not strictly parallels so males and females are not responding to the infection in the same way. Males positive for *Mycoplasma* (1) show a lower mean BCI than negative males (0). Females show an even lower mean BCI when positive (1) compared to negative female tortoises (0) (Figure 6).

Geographical location

The logisitic regression between *Mycoplasma* prevalence and study sites showed that sites have no influence on the probability that a tortoise has the disease (GLM, P=0.92). Site categories were made in function of the type of habitat, and distance to the nearest urban area. The qui-square test showed there was an association between site category and *Mycoplasma* infection (χ^2, P = 0.0368; Table 4). More infected tortoises were found in the countryside category. Two sites out of 14 seemed to be free of pathogens.

Exotic tortoises

During the study period four *Testudo graeca* were found in the wild, all but one were PCR-positive for *Mycoplasma*. One hybrid *Testudo graeca* / *Testudo ibera*, 1 *Testudo boettgeri* and 9 hybrids were detected, all negative to both *Mycoplasma* and TeHV. Additionally, 11 hybrids tortoises found in the wild were brought at the Village des Tortues (Gonfaron, France) by walkers in 2012 and 2013.

Discussion

Prior work has documented cases of infection by *Mycoplasma* and Herpesvirus in wild *Testudo hermanni hermanni* in southern France. For example, Mathes (2001)

reported that 11 out of 96 wild tortoises were PCR-positive for *Mycoplasma* (11.5%) in southern France and all were negative to Testudinid Herpesvirus (TeHV). However, prevalence of these pathogens has not been extensively surveyed within the distribution area of the Hermann's tortoise in continental France. In this study we analyzed prevalence of *Mycoplasma* and TeHV among 14 sites located near or within main populations on 347 wild tortoises and correlated it with a number of environmental and physiological factors.

We found that 8.1% of wild Hermann's tortoises screened for *Mycoplasma* were PCR-positive. As for TeHV, 7 tortoises were positive to PCR test and negative to serum-neutralization test. This inconsistency may reflect a delay in the detectable immune response as it takes 6 – 8 weeks for an exposed tortoise to develop a detectable immune response (Brown et al. 2002, McLaughlin 1997). Finally, one tortoise was diagnosed as "suspect" for TeHV by serum-neutralization test.

Positive PCR test means tortoise is hosting the pathogen on its mucous and pathogen's DNA has been detected. A tortoise that is not harboring the pathogen (at the time of sampling) cannot be positive to PCR test. Positive serum-neutralization (SN) test reflects a contact between the pathogen and the tortoise immune system. In reaction to pathogen presence, tortoises produce neutralizing antibodies allowing them to control the infection and to get rid of the pathogen eventually. These antibodies persist in the body for a moment even when the pathogen is no longer present. Negative SN test means (1) tortoise has never been exposed to TeHV, (2) TeHV is in dormancy, or (3) TeHV has been eliminated by the tortoise (Fertard pers. comm.).

Although both *Mycoplasma* and TeHV have been isolated previously from captive *Testudo* in the Var department, this is the first description of prevalence of these pathogens in wild populations. Besides, TeHV prevalence is probably underestimated as not all populations have been sampled. Additionally, some situations can distort the

results and lead to “false negative” results. For example, if the elapsed time since infection is too short, serology may well be negative. Also, for a PCR test to be positive for TeHV, one need to detect the virus. But oral mucous of individuals that do not present clinical signs are less “rich” in pathogen DNA that individuals displaying clinical signs. This it is why it is important to complete PCR test with serology.

Due to the relatively high cost of serology and PCR analysis for TeHV, we sampled no more than 10 tortoises per site for these tests. Indeed, given the high contagion rate of this disease, 10 tortoises were thought to be enough to detect TeHV if present in a population. Nonetheless, every tortoise was screened for *Mycoplasma* by means of PCR when possible. However, tortoises were not tested serologically for *Mycoplasma*. The only laboratory able to perform this test being in the US and given the regulation concerning the shipping of biological samples with potential infection risk, this test was not carried out.

Interestingly we found shifts in *Mycoplasma* infection status in some tortoises sampled on more than one occasion. One tortoise was initially PCR-negative and subsequently converted to a PCR-positive status 3 weeks later. Another tortoise was PCR-positive and became positive 1 year later. Such changes might stem from a tortoise clearing the pathogen, laboratory errors or other unknown factors.

No significant differences in physiological parameters concentrations were found between PCR-positive and PCR-negative for *Mycoplasma* tortoises. *Mycoplasma* infection does not seem to impact tortoises physiology. In contrast to our findings, other studies (Jacobson et al. 1991) have found that ill tortoises had higher values for sodium, SGOT and cholesterol while values for phosphorus were significantly lower than those for controls. However it is well known that all clinical laboratory values (both hematology and plasma chemistry) change with season, age and sex in reptiles. As a result, changes in serial samples from a given tortoise over time would probably be

more significant than individual values. Besides, it is also possible that changes in physiological parameters concentrations had not occurred yet due to a recent infection.

Sex did not seem to be correlated with infection status in this study. This might be an artifact of the small sample size. However, more females were PCR-positive than males. This may be because females undertake important displacements to lay their eggs in spring and thus have greater chances to meet ill tortoises. This is consistent with the findings of Berish et al. (2010).

The majority of tortoises showed physical abnormalities such as carapace wounds. Carapace traumas as well as environmental stress or habitat degradation are predisposing factors that may be involved in epizootics of *Mycoplasma* in tortoises and other animals.

No correlations were found between clinical signs and infection status. Clinical signs such as nasal discharge found on some tortoises may be the result of pathogens other than *Mycoplasma*, or dust allergy for example. The reasons for the absence of clinical signs in infected tortoises are unknown. This is consistent with the results of Dickinson et al. (2005). Probably tortoises were recently infected and clinical signs had not appeared yet. Typically, clinical signs appear within 1 – 2 weeks post-exposure.

However a significant correlation was found between *Mycoplasma* infection and a number of factors which are ectoparasites, pyramidal growth and mucous color. Pyramidal growth is often the result of inappropriate living conditions in captivity, notably malnutrition. Thus, individuals with pyramidal growth may have spent some time in captivity where they may have been in contact with diseased tortoises. This result has to be cautiously interpreted given the small sample size. Further investigations are warranted to determine if there is a real link between infection status and these clinical signs.

Mycoplasma seemed to adversely impact tortoises regarding their Body Condition Index (BCI). Females seemed to be particularly impacted and showed a much lower mean BCI than healthy females.

Infected tortoises were more often found in the "countryside" category. This may be because owners who want to return their tortoises to the wild choose a place that seems to be favorable for its survival.

Mycoplasma and TeHV are known to be widespread in captive tortoises (Origgi 2012). Thus, captive tortoises represent a potential reservoir for diseases which may then affect natural populations. Because both pathogens have been implicated in the decline of wild tortoise populations, the control of these agents in captive tortoises must be considered. In addition to disease introduction, release of captive tortoises can lead to hybridism between subspecies of genus *Testudo* resulting in a loss of genetic specificity as well as increased infectious disease susceptibility (Goldberg et al. 2005; Edwards and Berry 2013). Besides, Hermann's tortoises are commonly collected by locals, tourists and may ultimately be released at other sites. The ease with which tortoises can be collected and transported has contributed to the spread of the disease. This problem should be managed through massive education, within public schools, on radio and television, and through literature given to travelers, at road sites rest areas. Because tortoises affected are reproductive adults, the consequences for populations may be disastrous. It is one of the longest lived terrestrial vertebrates - requiring 8 to 10 years for males and 12 to 18 years for females to reach reproductive age - and producing a small number of eggs in a clutch, thus we cannot expect to see recovery in our life time if populations become severely affected (Ballasina 1995; Hailey and Loumbourdis 1988).

However, no evidence of mortality due to *Mycoplasma agassizii* has been reported in wild *Testudo* ssp. populations so far. The effects these pathogens may have on the

studied populations are unknown. Pathogens may be present since generations in populations and tortoises may live with them well.

Nonetheless, tortoise managers should adopt the precautionary principle when handling wild terrestrial chelonians *in situ* (e.g. for genetic and/or sanitary studies, tracking, population census). Indeed, although it is short, the mean survival period outside the host amounts in days for *Mycoplasma* and in weeks for TeHV. Nasal and oral secretions, feces, and even urine are potentially contaminating products. TeHV is not very resistant in the environment but its survival is not negligible. For example, TeHV survival has been estimated to 7 days at 37°C and several weeks at 4°C (Fertard pers. comm.).

A monitoring of pathogens distribution and prevalence in wild populations of Hermann's tortoises is warranted on the long term. This would allow an assessment of the survival rate as well as the dynamic and the viability of infected populations. Further research should involve repeated sampling and higher numbers of screened sites in order to have an idea of the fate of infected tortoises and provide guidelines to managers.

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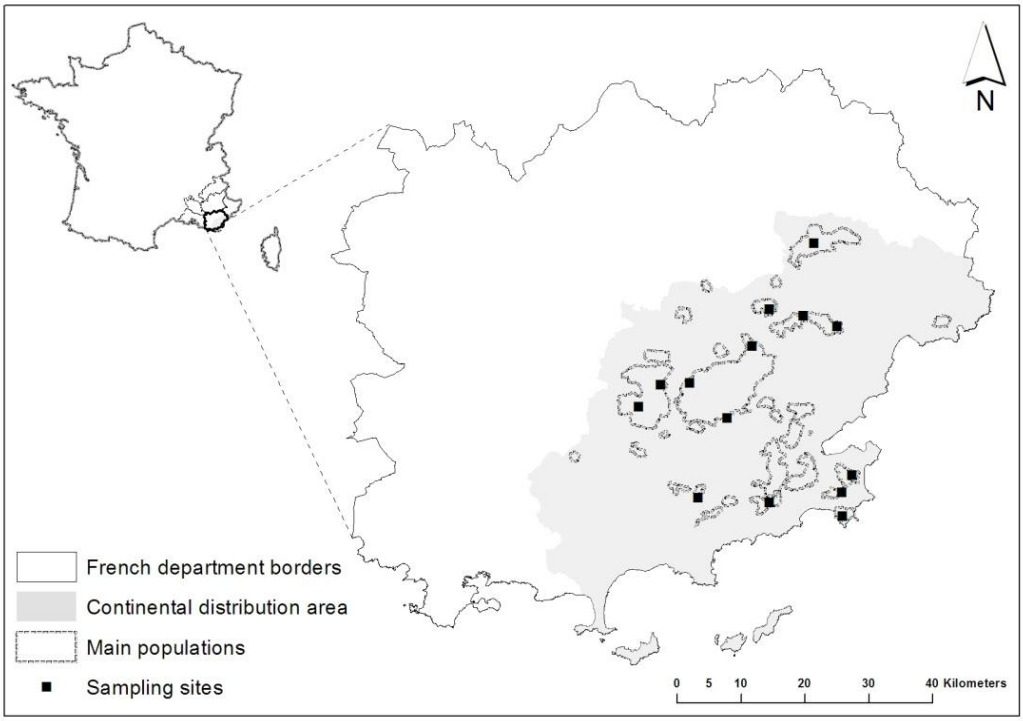

Figure 1: Map of sampling sites screened for *Mycoplasma* and TeHV in the Var department, France.

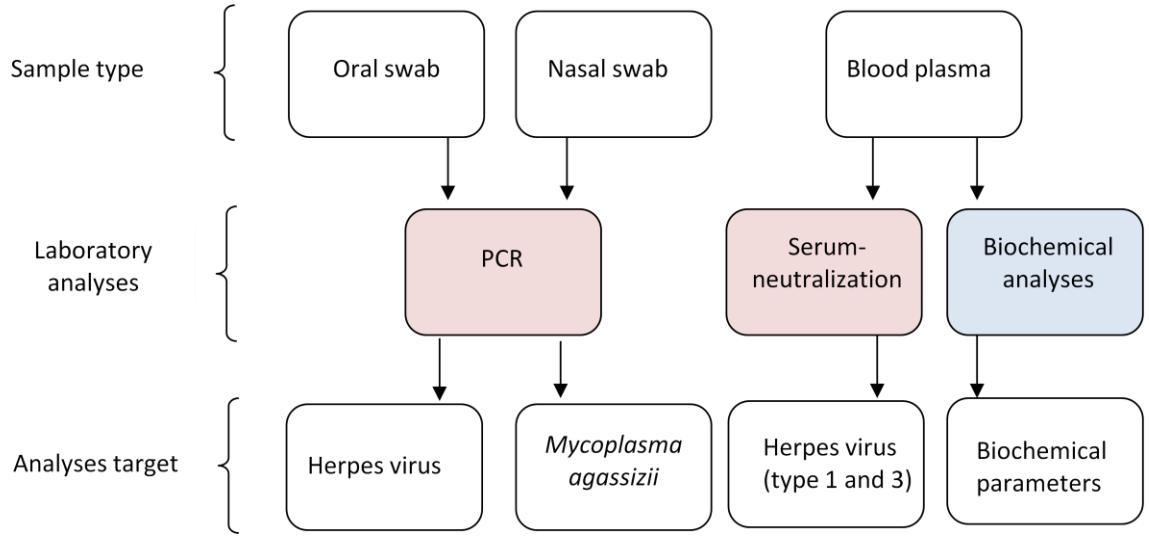

Figure 2: Summary of laboratory analyses performed on biological samples collected on tortoises. Pink refers to analyses carried out in Detmold lab, Germany; and blue indicates analyses carried out in France.

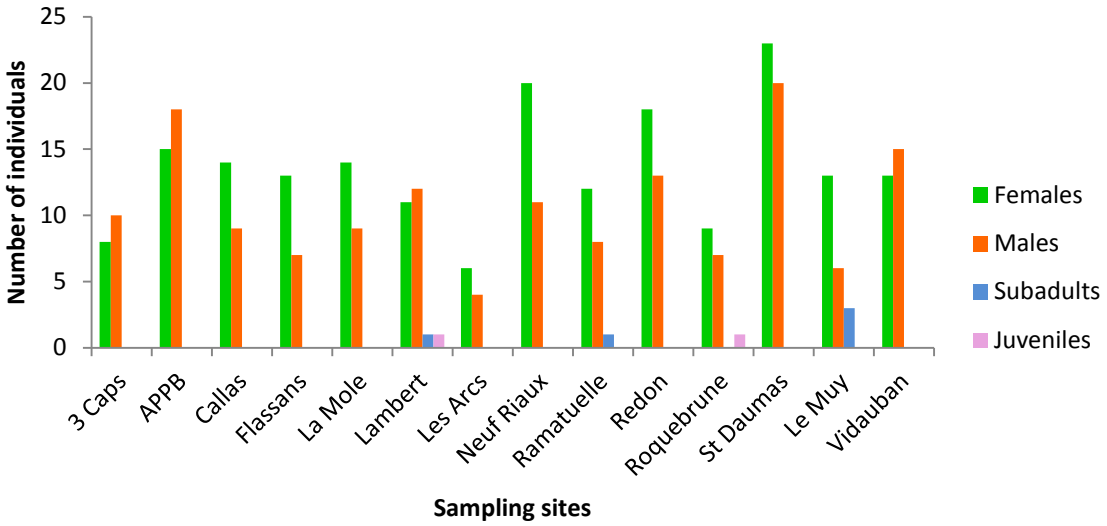

Figure 3: Number of males, females, sub-adults and juveniles found at each study sites between May and October 2012 and from April to July 2013. Juveniles and sub-adults (carapace length < 700 mm) are under-represented given the difficulty to detect them in the bushes.

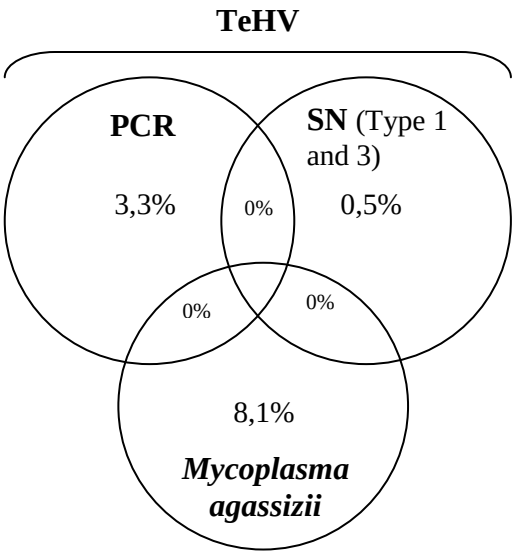

Figure 4: Prevalence of Testudinid Herpesvirus (TeHV) in southern France during sampling periods in 2012 and 2013 by means of Polymerase Chain Reaction test (n = 209) and serum-neutralization (SN) test (n = 216), and *Mycoplasma agassizii* by means of PCR (n = 335).

Table 1. Serum biochemical profiles (Sample size, N; mean, £; standard deviation, SD) for PCR - positive for *Mycoplasma* tortoises and "negative" tortoises. (PCR = Polymerase Chain Reaction test)

Determinant	PCR-negative for *Mycoplasma*			PCR-positive for *Mycoplasma*		
	N	£	SD	N	£	SD
Glucose (mg/dl)	231	77	30,35	18	77,35	29,82
Urea (g/L)	206	0,03	0,033	15	0,02	0,02
Uric acid (mg/L)	203	20,35	13,74	15	20,36	13,59
Sodium (mEq/L)	83	126,02	17,59	10	126,21	17,40
Potassium (mEq/L)	82	5,69	1,30	10	5,68	1,29
Calcium (mg/L)	205	121,10	76,82	15	122,52	78,21
Triglyceride (g/L)	82	3	2,29	8	3,04	2,32
Cholesterol (g/L)	81	1,59	0,77	10	1,61	0,77
SGOT (U/L)	175	142,73	154,63	12	146,14	158,13
SGPT (U/L)	50	9,75	5,89	7	9,67	5,87

Table 2: Results of Shapiro, Fisher and Wilcoxon tests conducted between PCR-positive for *Mycoplasma* and "negative" tortoises, for each biochemical parameter. (PCR = Polymerase Chain Reaction test)

		P-value		
Parameters	Infection status	Shapiro	Fisher	Wilcoxon
Glucose	Positive	0,008	0,01	0,20
	Negative	$9{,}47.10^{-15}$		
Potassium	Positive	0,2626	0,18	0,547
	Negative	0,069		
Calcium	Positive	0	0,734	0,855
	Negative	$1{,}13.10^{-12}$		
Triglycerides	Positive	0,61	0,909	0,189
	Negative	$7{,}1.10^{-6}$		
SGOT	Positive	0	$4{,}45.10^{-8}$	0,350
	Negative	$< 2{,}2.10^{-16}$		
Cholesterol	Positive	0.9912	0,684	0,198
	Negative	$2{,}9.10^{-5}$		
Sodium	Positive	0,0567	0,048	0,862
	Negative	$3.3.10^{-16}$		
Uremia	Positive	0	0,287	0,231
	Negative	$< 2.2.10^{-16}$		
Uricemia	Positive	0,6849	0	0,106
	Negative	$3.\ 23.10^{-11}$		
SGPT	Positive	0,063	0,343	0,791
	Negative	$2{,}08.10^{-8}$		

Table 3: Correlation of *Mycoplasma agassizii* PCR results with various health parameters in wild tortoises. (*) show significant correlations. (PCR = Polymerase Chain Reaction test)

Factor		*M. agassizii* +		*M. agassizii* -		X^2, df	*P*
Tonicity	Dynamic	12/136	(8.8%)	124/136	(91.2%)	0.903, 3	0.8247
	Calm	15/190	(7.9%)	175/190	(92.1%)		
	Lethargic	0/1	(0%)	1/1	(100%)		
	Unknown	0/8	(0%)	8/8	(100%)		
Ectoparasites *	No	25/324	(7.7%)	299/324	(95.3%)	14.6587, 2	0.0006
	Yes	2/3	(66.7%)	1/3	(33.3%)		
	Unknown	0/8	(0%)	8/8	(100%)		
Pyramidal growth *	Yes	6/18	(33.3%)	12/18	(66.7%)	16.7594, 2	0.0002
	No	21/311	(6.8%)	290/311	(93.2%)		
	Unknown	0/6	(0%)	6/6	(100%)		
Wounds	Yes	11/168	(6.5%)	157/168	(93.5)	2.3575, 2	0.3077
	No	16/157	(10.2%)	141/157	(89.8%)		
	Unknown	0/10	(0%)	10/10	(100%)		
Eyes	Closed	0/1	(0%)	1/1	(100%)	0.1912, 3	0.979
	Swollen	0/1	(0%)	1/1	(100%)		
	Normal	26/322	(8.1%)	296/322	(91.9%)		
	Unknown	1/11	(9.1%)	10/11	(90.9%)		
Nares	Normal	24/324	(7.4%)	300/324	(92.6%)	5.8702, 2	0.05312
	Obturated	1/3	(33.3%)	2/3	(66.7%)		
	Unknown	2/8	(25%)	6/8	(75%)		
Nasal discharge	Yes	1/5	(20%)	4/5	(80%)	4.2199, 2	0.1212
	No	24/322	(7.5%)	298/322	(92.5%)		
	Unknown	2/8	(25%)	6/8	(75%)		
Mucous color *	Normal	22/198	(11.1%)	176/198	(88.9%)	6.1965, 2	0.04513
	Abnormal	0/6	(0%)	6/6	(100%)		
	Unknown	5/131	(3.8%)	126/131	(96.2%)		

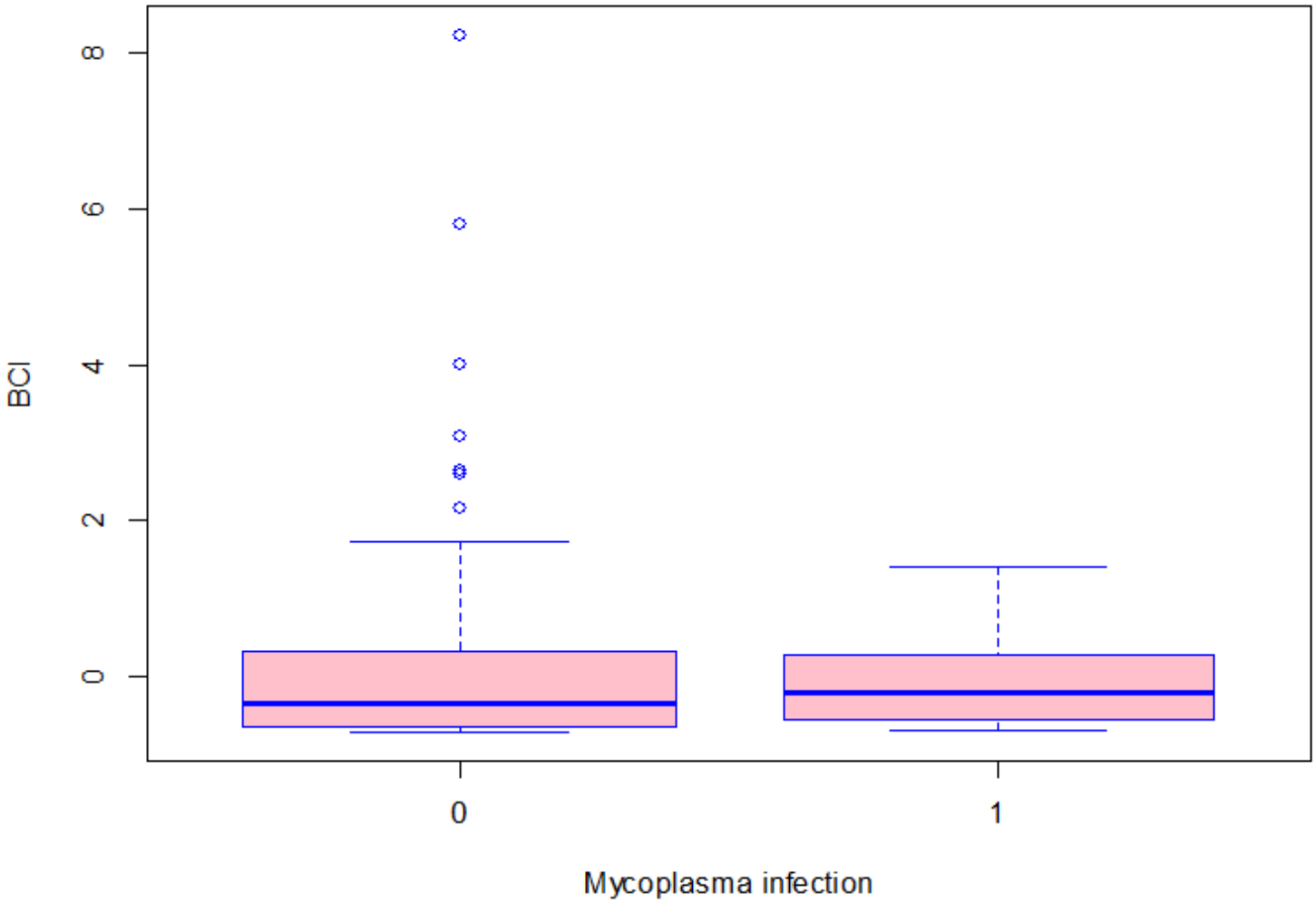

Figure 5: Boxplot of mean Body Condition Index (BCI) for negative (0) and positive (1) for *Mycoplasma* tortoises.

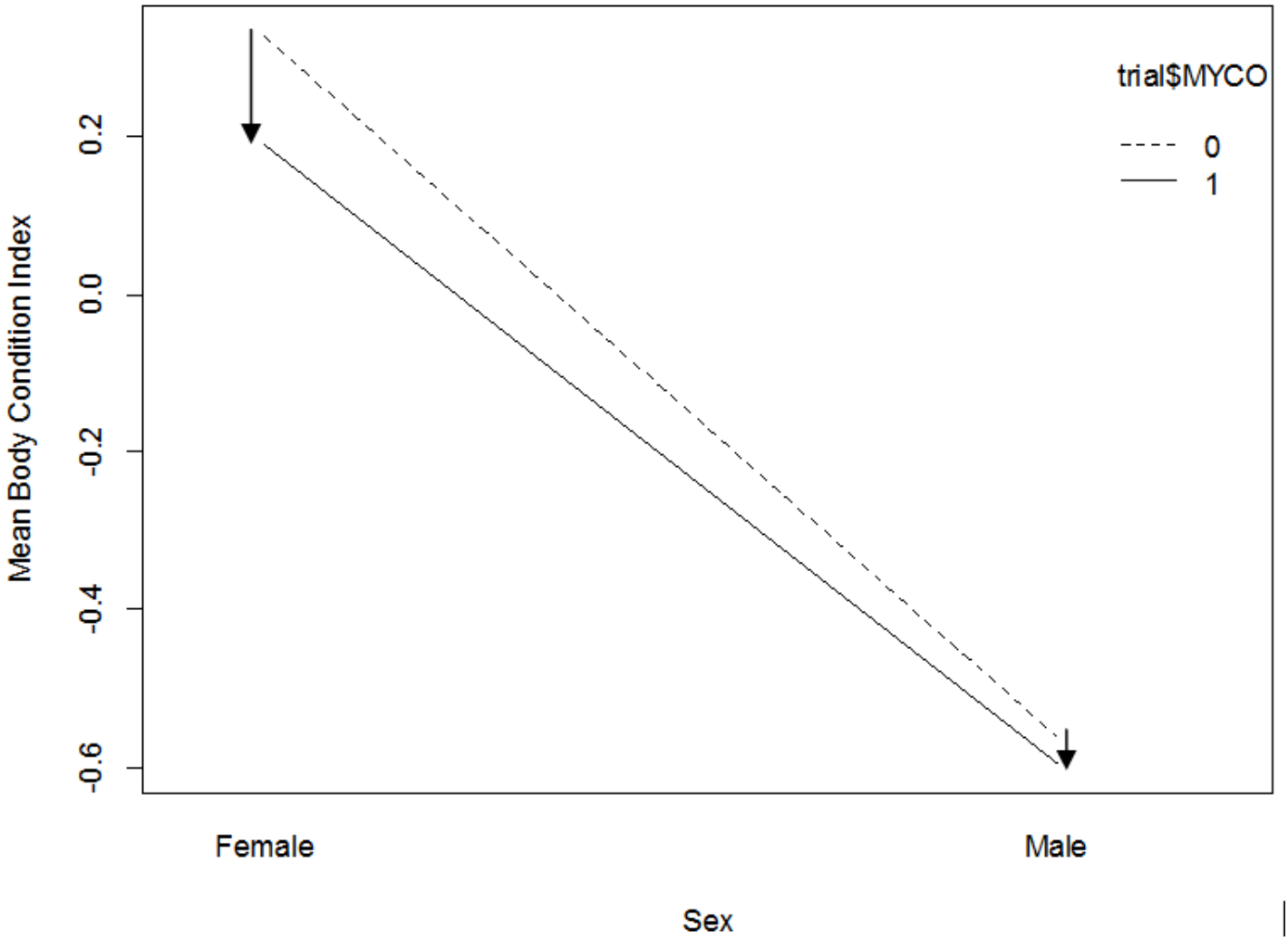

Figure 6: Interaction of sex and *Mycoplasma* infection (0: negative; 1: positive) on mean body condition index (BCI). Arrows show the difference in mean BCI for each sex between negative and positive for *Mycoplasma* tortoises.

Table 4: Correlation of *Mycoplasma agassizii* PCR results with site categories in wild tortoises. (PCR = Polymerase Chain Reaction test)

Factor		M. agassizii +		M. agassizii -		X^2, df	P
Site category	City	1/79	(1.3%)	78/79	(98.7%)	6.6018, 2	0.0368
	Village	9/97	(9.3%)	88/97	(90.7%)		
	Countryside	17/159	(10.7%)	142/159	(89.3%)		