Detection of Testudinid alphaherpesvirus, Chlamydia *spp.,* Mycoplasma *spp., and* Salmonella *spp. in free-ranging and rescued Italian* Testudo hermanni hermanni

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Summary
Testudo hermanni is included as near-threatened in the Red List of the International Union for Conservation of Nature, while *T. hermanni hermanni* is considered endangered in the Italian Red List. Appropriate management of smuggled or seized wild individuals is recommended before their reintroduction into the wild. Accordingly, a health monitoring study was carried out. During 2014-2016, 133 oral swabs and 121 cloacal swabs were collected from a total of approximately 180 free-ranging and rescued *T. hermanni hermanni* from eight different Italian regions to investigate the presence of DNA of *Testudinid alphaherpesvirus* (TeAHV), *Chlamydia* spp. and *Mycoplasma* spp. in the oral cavity, and *Salmonella* spp. isolates in the cloaca. *Mycoplasma* spp. was detected in 52 out of 87 (59.77%) of rescued and in 1 out of 46 free-ranging (2.17%) individuals; 33 out of 53 (62.26%) *Mycoplasma* spp. positive samples were typed as *M. agassizii* by PCR. *Salmonella* spp. was isolated from 45 out of 121 (37.19%) cloacal swabs, typed into 14 serovars, and characterized for complete antimicrobial susceptibility. A significantly different distribution of *Salmonella* spp. isolates was found in 2016 in comparison with 2014 and 2015, without any difference between free-ranging and rescued tortoises. All the tested tortoises were negative for TeAHV and *Chlamydia* spp. These results are considered a baseline information critical to monitor the dynamics of these microorganisms in free-ranging and rescued populations of *T. h. hermanni*, and to correctly approach the management of rescued animals and possible relocation programs.

Introduction

The Hermann's tortoise (*Testudo hermanni*), represented by two subspecies, is distributed in the northern Mediterranean area. The nominal form (*T. hermanni hermanni*) is found in the western Mediterranean, from Catalonia to Italy, while the eastern subspecies, *Testudo hermanni boettgeri*, inhabits the Balkans (Mazzotti 2016). The Hermann's tortoise is considered as near-threatened in the Red List of the International Union for Conservation of Nature (IUCN), while the Italian Red List reports *T. h. hermanni* as endangered (Rondinini *et al.* 2013). The Balkan subspecies has been widely traded and many tortoises have returned to the wild, either by escape or by release from captivity, sometimes even hybridizing with autochthonous individuals. Despite the Italian distribution of *T. h. hermanni* is relatively well known, some autochthonous wild population may include captive individuals of the Balkan form (Corti *et al.* 2013, Di Tizio *et al.* 2016). The Western subspecies (*T. h. hermanni*) occurs irregularly in the

Western Mediterranean area, in particular along the coasts of Spain and France. Even if it is protected by national and international laws, human activities in particular those which may cause habitat loss or illegal collection of wild animals, haevily contribute to the decline of this species. Rigorous monitoring, including close surveillance of infectious diseases, and appropriate management of smuggled or confiscated individuals are supported by the Italian Ministry of the Environment (Ministero dell'Ambiente e Tutela del Territorio e del Mare, MATTM), as part of the efforts needed to preserve natural population. However, specific information to plan possible relocation programs, without impacting biodiversity and respecting the ecosystem, are still limited. A multidisciplinary project on free-ranging and captive Italian *Testudo* spp., with special attention to *T. h. hermanni*, has been carried out to characterize the genetic background of the tortoises candidate to be relocated, their eco-ethology, morphology, and health conditions (MATTM 2019). More specifically, selected relevant infectious agents of tortoises were considered critical for the health assessment of tortoises part of the recollocation program. Within this scenario, surveillance of infectious diseases becomes very important when performing relocation programs (Hartley and Lysons 2001). Specific selection criteria for the choice of the microorganisms to detect were: 1) their ability to cause disease threatening individuals or populations either in captivity or in the wild, 2) the presence of asymptomatic carriers in their transmission cycle, and 3) the zoonotic risk for humans who handle the animals. Accordingly, Testudinid alphaherpesviruses (TeAHVs), *Chlamydia* spp., *Mycoplasma* spp., and *Salmonella* spp. were investigated.

Four genotypes of TeAHVs are known (TeAHV-1, TeAHV-2, TeAHV-3, TeAHV-4). The genotype TeAHV-3 is considered one of the most relevant pathogens of chelonians, especially in *T. hermanni*, because of its potential epizootic spread and mortality rates (Origgi 2012, Marenzoni *et al.* 2018). TeAHV-3 is rarely associated with fatalities in *T. graeca* or *T. marginata*, whereas it is often lethal in *T. hermanni*. Additionally, surviving infected animals, although apparently clinically healthy, can harbor the virus and shed it following reactivation, from its dormant condition (latency), typical of herpesviruses. Post-hibernation seems to be the period of major risk for reactivation (Origgi *et al.* 2015, Marenzoni *et al.* 2018). The consequences of the infection on the biodiversity could be considerable (Marschang *et al.* 2009, Martel *et al.* 2009).

The phylum *Chlamydiae* consists of intracellular bacteria, able to infect a wide range of animals and humans. The infection has been described both in free-ranging (Mitura *et al.* 2017) and captive reptilian hosts, including tortoises (Soldati *et al.* 2004, Hotzel *et al.* 2005, Kabeya *et al.* 2015, Taylor-Brown *et al.* 2015, Laroucau *et al.* 2020). The infection has been reported both in clinically healthy and ill captive tortoises in Italy (Di Ianni *et al.* 2015). However, no conclusive characterization of *Chlamydiae* responsible for reptile infections has been described, although *C. pneumoniae* and *C. psittaci* have been more frequently reported (Kabeya *et al.* 2015). Recently, new species and strains of *Chlamydia* spp. have been recognized in tortoises, suggesting also a risk of interspecies and zoonotic transmission, although their pathogenic role remains unclear (Mitura *et al.* 2017, Laroucau *et al.* 2020).

Mycoplasmas have been frequently detected in chelonians. Some species are considered commensal bacteria of the host (Farkas and Gal 2009, Di Ianni *et al.* 2015), whereas others can cause severe diseases of the upper respiratory tract, with chronic evolution and mortality. *Mycoplasma testudinis* has been reported as a non-pathogenic agent of the excretory tract of *T. graeca*, whereas *Mycoplasma agassizii* and *Mycoplasma testudineum* have been demonstrated to cause disease (upper respiratory tract disease) in both free-ranging and captive tortoises in the USA and Europe (Brown *et al.* 1995, Brown *et al.* 1999, Feldman *et al.* 2006, Lecis *et al.* 2011), and they are considered a threat for the management of wild tortoise populations (Jacobson *et al.* 2014). *M. agassizzi* has been reported in Italy in rescued tortoises, but its potential role as etiologic agent of disease has not been clarified in the infected tortoises (Lecis *et al.* 2011). Moreover, the isolation of specific species of *Mycoplasma* has been reported for only a few species of chelonians in captivity conditions, which makes it very difficult to understand whether the virulence of the microorganisms and the severity of the infection are linked to the microbial species or to the host (Kolesnik *et al.* 2017). Therefore, their pathological role still requires clarification.

Salmonella spp. are ubiquitous enteric Gram-negative bacteria, able to infect a wide range of host species, including mammals, reptiles, birds and insects. Reptiles are generally considered carriers and intermittent shedders of *Salmonella* spp.; some serovars are more frequently isolated in reptiles and are therefore called reptile-associated *Salmonella*, RAS (Bertrand *et al.* 2008, Harris *et al.* 2010, Pees *et al.* 2013, Bosch *et al.* 2016). Some of these have been implicated in a number of outbreaks involving humans, and especially young children (Bertrand *et al.* 2008, Pees *et al.* 2013), so that a European Directive defines infection by RAS as an emerging zoonosis (European Commission 2003, Pees *et al.* 2013). Although rarely, *Salmonella* spp. can also responsible for disease in reptiles, working as opportunistic pathogens generally associated with predisposing factors such as inappropriate environmental temperature (Pasmans *et al.* 2002,

Gonzalez-Candela *et al.* 2005, Sting *et al.* 2013). Isolates of *Salmonella* can be used also as indicators of antibiotic-resistance because of its role and the capacity to respond to antibiotic treatments (European Food Safety Authority and European Centre for Disease Prevention and Control 2018).

The aim of the present study was to investigate the presence of TeAHVs, in particular TeAHV-3, *Chlamydia* spp., *Mycoplasma* spp., and *Salmonella* spp. in both free-ranging and rescued Italian *T. h. hermanni*, monitored during a period of 3 years, to determine the infectious status of free-ranging tortoises and to elaborate appropriate health management guidelines for the rescued individuals to be relocated.

Materials and methods

Sample collection

From April to October of the years 2014, 2015, and 2016, oral swabs (OS) and cloacal swabs (CS) were individually collected from free-ranging and rescued *T. h. hermanni*. Collection of OS and CS was carried out only in individuals which could be sampled only with minimal restraint in compliance with animal welfare guidelines. Accordingly, not all the tortoises (approximately 180 individuals) were systematically sampled for both samples. Overall, 133 OS (from 46 wild and 87 rescued tortoises) and 121 CS (from 84 wild and 37 rescued tortoises) were sampled. Sampling type and year are described in Table I. A complete clinical examination was performed for each animal at the time of sampling, including a general health assessment, inspection of visible mucous membranes, and recording of visible gross lesions.

Free-ranging tortoises were captured in eight different regions of insular and peninsular Italy (Emilia Romagna, Abruzzi, Campania, Molise, Apulia, Calabria, Sardinia, Sicily) across their distribution range. Areas of sampling were identified during previous surveys carried out in the field, which are described in detail in MATTM (MATTM 2019). A non-probability sampling method (convenience sampling) was applied and tortoises were sampled when found in the wild.

Rescued *T. hermanni* included smuggled or confiscated animals housed in 10 different recovery centers, located in the eight different regions of Italy, selected at national level because considered representative of other centers present in the country. The average number of animals housed in each center was approximately n = 10, with two centers hosting much larger groups (> 100 animals); the management and the species of the hosted tortoises other than *T. hermanni* varied from center to center. All the animals were seized or confiscated in Italy (however, based on the genetic characterization, some animals were later found to be non-Italian, data not shown). Information concerning for how long the tortoises had been kept in the centers before testing was not available.

The OS and CS samples were collected by rotating sterile polyester swabs, dipped in 500 µL of phosphate buffered saline (PBS, pH 7.2), against the mucosa of the mouth or the cloaca. All the swabs were stored at 4 °C in PBS until analysis, which was performed within 24-48 hrs. No repeated sampling was performed.

Biomolecular investigation

Total DNA was extracted from 200 µL of each OS using a commercial kit (QIAamp DNA Mini kit, Qiagen, Italy), according to the manufacturer's instructions.

A consensus nested PCR protocol was performed to amplify a 225 bp-long highly conserved region of the DNA polymerase gene across the family *Herpesviridae* to identify any possible herpesviruses present in the investigated tortoises (VanDevanter *et al.* 1996). A nested PCR was used to identify TeAHV-3 in case of positivity (Marenzoni *et al.* 2018).

A PCR protocol targeting the 16S rRNA gene, discriminating between *C. pneumoniae* from *C. psittaci*, was used to detect *Chlamydia* spp. (Messmer *et al.* 1997).

A two-step protocol based on conventional PCRs targeting a fragment of 273 bp and 1,013 bp of the

Table I. *Description of the type and year of sampling of free-ranging and rescued* Testudo hermanni hermanni.

Year	Oral swabs			Cloacal swabs		
	Free-ranging tortoises	Rescued tortoises	Total	Free-ranging tortoises	Rescued tortoises	Total
2014	22	57	79	31	27	58
2015	18	30	48	22	10	32
2016	6	-	6	31	-	31
Total	46	87	133	84	37	121

16S rRNA gene was used to detect *Mycoplasma* spp. (Vojdani and Franco 1999, Lierz *et al.* 2007). In the case of positive samples, a PCR-based enzymatic restriction was used to confirm the presence of *M. agassizii* (Brown *et al.* 1995, Brown *et al.* 1999). Moreover, PCR products of the expected size were purified using a commercial kit (Qiaquick, Qiagen, Italy) and directly sequenced on both strands with the same primers as previously described (Biofab srl, Italy). The sequences were assembled and aligned using BioEdit (BioEdit 2009). The sequence similarity was checked against sequences available from GenBank using the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the species specificity of the PCR.

Screening of *Salmonella* serovars and antimicrobial susceptibility

The CS were processed according to the procedures indicated by the World Organization for Animal Health (OIE) guidelines to isolate and identify *Salmonella* spp. and its serovars (OIE 2015). Briefly, each CS was used to inoculate the enrichment medium, Rappaport-Vassiliadis broth (Oxoid, Italy), and incubated at 41.5 °C for 24-48 hrs. The samples were then subcultured at 37 °C on xylose lysine desoxycholate agar (XLD, Oxoid, Italy) and brilliant green agar (Oxoid, Italy) solid selective media. The results were read after 24 and 48 hrs of incubation. Identification of suspect colonies was performed using composite biochemical media and the commercial biochemical test API RAPID 20E (bioMérieux, Italy). *Salmonella* spp. isolates were serotyped by direct slide agglutination using specific antisera (Statens Serum Institut, Copenhagen, Denmark), according to the Kaufmann-White-Le Minor scheme.

Salmonella spp. isolates were tested for antibiotic sensitivity using the standard disk diffusion method of Kirby-Bauer on Mueller Hinton Agar (Oxoid, Italy). The following antimicrobial molecules (Oxoid, Italy) were tested: amoxicillin-clavulanic acid (30 µg), ampicillin (10 µg), nalidixic acid (30 µg), cephalothin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), sulfamethoxazole-trimethoprim (25 µg), sulfonamides (300 µg), and tetracycline (30 µg). The zone of inhibition around each disk was measured after 24 hrs incubation at 37 °C for each isolate. Results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI 2016) and the isolates were classified either as susceptible, intermediate, or resistant.

Statistical analysis

The apparent prevalence of each microorganism detected in infected tortoises was defined as the proportion of positive samples (based on the applied test) out of the total number of samples analyzed. Postulating the hypothetical target population as infinite, the number of animals sampled was used to estimate: 1) the maximum expected prevalence of infection with all the samples testing negative; and 2) the expected prevalence, with the corresponding 95% confidence interval (CI), if positive results were present. Specific tables for sample size and calculations were used (Cannon *et al.* 1982, Thrusfield 2005).

The chi-squared test, with Yates continuity correction, and Fisher's exact test were applied as appropriate to compare the proportions of positive samples based on wild or rescued origin and year of sampling for *Mycoplasma* spp. and *Salmonella* spp. A P value < 0.05 was considered statistically significant. Odds ratios (ORs) were calculated as a crude measure of the association between single risk factors and outcomes (isolation of *Salmonella* spp./PCR positivity for *Mycoplasma* spp.). OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 3.01., www.OpenEpi.com) was used for the analysis.

Results

None of the animal investigated showed detectable clinical signs.

All OS were negative for TeAHV and *Chlamydia* spp. DNA (0/133, 95% CI: 0.07-3.52%), consistently with an estimated prevalence lower than 2.23% for a hypothetical infinite population.

Mycoplasma spp. DNA was detected with an estimated prevalence of 39.85% (53/133, 95% CI: 31.57-48.72%). Results were reported in Table II. Prevalence was statistically different between rescued (52/87 OS, 59.77%, 95% CI: 48.69-69.98%) and free-ranging tortoises (1/46 OS, 2.17%, 95% CI: 0.11-12.97%, $P < 0.0001$), with rescued tortoises being 66.86 times (95% CI: 8.80-507.76) more likely to be positive. No differences in positivity were observed over years. Test positivity was not associated with detectable clinical signs. Thirty-three out of 53 (62.26%) *Mycoplasma* spp. positive samples were typed as *M. agassizii* by the specific PCR-based enzymatic restriction. The only positivity to *Mycoplasma* spp. detected in a free-ranging tortoise was determined not to be *M. agassizii*. The sequences of the PCR products of both 273 bp and 1,013 bp, obtained from three randomly selected tortoises, shared 98-99% identity with the homologous 16S rRNA gene sequences of *M. agassizii* (GenBank accession nos. KY212528-KY212536,

Table II. *Results of the PCR for* Mycoplasma *spp. and* Mycoplasma agassizii *in free-ranging and rescued* Testudo hermanni hermanni.

Year of sampling	Number of *Mycoplasma* spp. positive		Total (%)	Number of *Mycoplasma agassizii** positive		Total (%)
	Free-ranging tortoises (%)	Rescued tortoises (%)		Free-ranging tortoises (%)	Rescued tortoises (%)	
2014	1/22 (4.54)	31/57 (54,38)	32/79 (40.5)	0/1 (0)	20/31 (64.5)	20/32 (62.5)
2015	0/18 (0)	21/30 (70)	21/48 (43.75)	0/0 (0)	13/21 (61.9)	13/21 (61.9)
2016	0/0 (0)	ne	0/6 (0)	0/0 (0)	ne	0/0 (0)
Total (%)	1/40 (2.5)	52/87(59.77)	53/133(39.84)	0/1 (0)	33/52 (63.46)	33/53 (62.26)

ne = not executed; *PCR performed only on samples already positive for *Mycoplasma* spp.

NR025954, AF060821). Additionally, homologies were observed also with *M. agassizii* sequences detected previously in T. *marginata, T. graeca*, and *T. hermanni* in Italy, although with limited coverage (different amplified intervals) (HQ326165-HQ326177, Lecis *et al.* 2011). The three sequences were submitted to GenBank (accession numbers MF185252, MF185253, MF185254).

Salmonella spp. were isolated from 45 out of 121 CS (37.19%, 95% CI: 28.72-46.49%). The results of the isolation and typing of *Salmonella* spp. based on year and group of tortoises are reported in Table III. The geographical origin of the isolates is reported in Table IV. The isolates were collected in an official repository and have been made available to the scientific community for further studies at the Regional Reference Center for Pathogenic Enterobacteria, Istituto Zooprofilattico dell'Umbria e delle Marche 'Togo Rosati'. The distribution of *Salmonella* spp. isolates was significantly different according to the years of sampling, especially in 2016 (OR for 2016 vs. 2014 = 2.75, 95% CI: 1.06-7.11, P = 0.03, and OR for 2016 vs. 2015 = 3.12, 95% CI: 1.02-9.51, P = 0.03), whereas no statistical difference was found between free-ranging and rescued tortoises.

Discussion

Our results confirmed the presence of *Mycoplasma* spp. and *Salmonella* spp. in rescued

Table III. *Year of sampling, number of tortoises positive for* Salmonella enterica *subsp., apparent prevalence (Pr), and list of the corresponding serovars of* Salmonella enterica *isolated from free-ranging and rescued* Testudo hermanni hermanni.

Year of sampling	Number of positive free-ranging tortoises (Pr) and isolated serovars	Number of positive rescued tortoises (Pr) and isolated serovars	Total (Pr)
2014	8 (8/31, 25.8%)	7 (7/27, 25.9%)	15 (15/58, 25.9%)
	Abony (n = 1)	Abony (n = 5)	
	Hermannswerder (n = 1)	Miami (n = 1)	
	Langford (n = 5)	Newport (n = 1)	
	Wedding (n = 1)		
2015	5 (5/22, 22.7%)	3 (3/10, 30%)	8 (8/32, 25%)
	Abony (n = 1)	Hermannswerder (n = 2)	
	Hermannswerder (n = 1)	Lindern (n = 1)	
	Langford (n = 1)		
	Richmond (n = 1)		
	Salamae 17:b :enz15 (n = 1)		
2016	22 (22/31, 71%)		22 (22/31, 71%)
	Abony (n = 1)		
	Ferruch (n = 2)		
	Halle (n = 4),		
	Hermannswerder (n = 5)		
	Kottbus (n = 3)		
	Langford (n = 4)		
	Mikawasima (n = 1),		
	Newport (n = 1)		
	Zadar(n = 1)		
Total	35 (35/84, 41.7%)	10 (10/37, 27%)	45 (45/121, 37.2%)

Table IV. *List of the serovars of* Salmonella enterica *isolated in the present study, reporting the number of strains isolated and their geographical origin (Region).*

Serovar (n)	Geographical origin and number of isolates in free-ranging tortoises	Geographical origin and number of isolates in rescued tortoises
Langford (10)	Apulia (5)	-
	Calabria (2)	
	Calabria (1)	
	Calabria (1)	
	Molise (1)	
Hermannswerder (9)	Apulia (5)	Emilia Romagna (2)
	Abruzzo (1), Sicily (1)	
Abony (8)	Apulia (2)	Sardinia (4)
	Calabria (1)	Sicily (1)
Halle (4)	Campania (4)	-
Kottbus (3)	Campania (2)	-
	Molise (1)	
Ferruch (2)	Apulia (2)	-
Newport (2)	Apulia (1)	Sardinia (1)
Lindern (1)	-	Emilia Romagna (1)
Miami (1)	-	Basilicata (1)
Mikawasima (1)	Molise (1)	-
Richmond (1)	Calabria (1)	-
Salamae, 17 :b :enz15 (1)	Calabria (1)	-
Wedding (1)	Apulia (1)	-
Zadar (1)	Calabria (1)	-

Italian *T. hermanni*, as previously observed (Lecis *et al.* 2011, Pasmans *et al.* 2000, Corrente *et al.* 2004, Di Ianni *et al.* 2015, Bertelloni *et al.* 2016), and no molecular evidence of TeAHVs and *Chlamydia* spp. DNA. On the contrary, it is the first description of *Mycoplasma* spp. in free-ranging Italian *T. hermanni*, although with a very low prevalence. As reported previously (Lecis *et al.* 2011), *M. agassizii* was detected only in rescued Italian *T. hermanni*, but this is the first report documenting the lack of detection of *M. agassizii* in the free-ranging tortoises, raising biosecurity questions concerning the management of captive animals.

The lack of detection of TeAHV DNA by PCR confirmed that the tested free-ranging and rescued *T. hermanni* were not shedders of TeAHVs. Further serological investigations, not performed in this study, could be useful to complete the monitoring of the exposure to TeAHVs and eventually to exclude their circulation in Italian free-ranging *T. h. hermanni*. In the context of a possibly naïve population of free-ranging Italian tortoises, strategies to avoid virus circulation in captivity as well as the introduction of the virus into the wild must be implemented. *T. hermanni* is notoriously poorly resistant to herpesvirus and highly prone to get infected with lethal outcome, often secondary to the introduction of clinically healthy carriers, including *T. marginata* or *T. graeca* (Marschang *et al.* 1997, Origgi and Rigoni 2003, Martel *et al.* 2009, Origgi 2012, Marenzoni *et al.* 2018). Accordingly, avoiding the mixing of animals of different species and origins, as it can happen in a rescue center, is of critical importance; personnel of the rescue centers need to be educated in this direction. Repeated tests and frequent health checks should be performed, in particular on rescued tortoises, in order to increase the probability of detecting the virus, which can reactivate in unpredictable manner (Marenzoni *et al.* 2018).

Investigations for *Chlamydia* spp. suggest that *Chlamydiae* are not a common pathogen in Italian *T. hermanni*. However, considering that novel *Chlamydia* strains have been detected (Mitura *et al.* 2017, Laroucau *et al.* 2020), the protocol used in the current study, which was designed to detect in particular *C. pneumonia* and *C. psittaci*, might not be broad enough to identify these new microorganisms. Moreover, a limit of the study was that the investigations for *Chlamydia* spp. were restricted to oral swabs to detect respiratory disease, whereas these recent studies used successfully cloacal swabs, that maybe represent a better sample to improve the detection of *Chlamydia* spp. (Mitura *et al.* 2017, Laroucau *et al.* 2020). Detection of *M. agassizii* DNA in rescued tortoises only, raises concerns for relocation programs. Mycoplasmoses, especially those caused by *M. agassizii* and *M. testudineum*, are considered to have contributed to the decline of some wild populations of tortoises in the USA (Jacobson *et al.* 2014). In this perspective, the clear difference found in the prevalence of *Mycoplasma* spp. and *M. agassizii* among free-ranging and rescued animals suggests that *M. agassizii* is not a common component of the tortoise microbiome in the wild and its spread among free-ranging animals might have unpredictable consequences. Accordingly, realease of positive animals in the wild is not recommended. A previous study reported the presence of novel mycoplasmas similar to the pathogenic *M. agassizii* in a group of *T. graeca, T. marginata*, and *T. hermanni*, but it was limited to an Italian recovery center; the infection was weakly associated with respiratory signs (six animals with respiratory signs, of which three were *Mycoplasma* PCR positive vs three *Mycoplasma* PCR negative) (Lecis *et al.* 2011). Further investigations are needed to determine the actual pathogenic potential of *M. agassizii* in *T. hermanni* and the significance of this agent in *T. hermanni* disease ecology.

The estimated prevalence of *Salmonella* spp. in tortoises of the present study was 37.19% (95% CI: 28.72-46.49%). However, it may have been underestimated because of the intermittent shedding of *Salmonella* spp., considering that

only a single sampling was performed on each tortoise for practical reasons. A wide range of *Salmonella* prevalence has been previously reported in chelonians, varying from 0%-100%, probably depending on several factors including host species, feeding habits, environment, management, location and the *Salmonella* serovars involved (Pasmans *et al.* 2000, Hidalgo-Vila *et al.* 2007, Lecis *et al.* 2011, Percipalle *et al.* 2011, Pees *et al.* 2013, Marenzoni *et al.* 2015). The majority of the 14 serovars identified in the present study have been previously reported in Italian *Testudo* spp. (Pasmans *et al.* 2000, Corrente *et al.* 2004, Ebani *et al.* 2005, Percipalle *et al.* 2011, Dipineto *et al.* 2012, Bertelloni *et al.* 2016, Marenzoni *et al.* 2015), and the presence of the same serovars has been confirmed in the 1970s (Corsalini 1975) and in later studies (Pasmans *et al.* 2000, Corrente *et al.* 2004, Ebani *et al.* 2005, Percipalle *et al.* 2011, Dipineto *et al.* 2012, Bertelloni *et al.* 2016). Statistical analysis showed that no difference exists in the prevalence of *Salmonella* spp. infection between free-ranging and rescued tortoises, whereas differences are observed across time, with 2016 being associated with a prevalence of 71%. Many factors, that can stimulate differently a multiplication of bacteria, may have led to these results, such as temperature or environmental factors, or animal density, but they have not been considered herein as they were not relevant to the aim of the present work. Other studies reported differences in detecting also further microorganisms in chelonians over different years (Kabeya *et al.* 2015, Archer *et al.* 2017), but the factors influencing these trends are generally difficult to identify. Further studies are needed to better understand the predisposing conditions, possibly influencing these results.

All the *Salmonella* spp. isolated were completely susceptible to the antimicrobials tested, as commonly observed for *Salmonella* spp. isolated from wild animals (Corrente *et al.* 2004, Percipalle *et al.* 2011, Rubini *et al.* 2016). This supports that tortoises can be relocate into the wild with a very limited risk of introducing antimicrobial resistance into the environment through this route. The characterization of the antimicrobial susceptibility of the isolates could be a useful tool to monitor antibiotic-resistance in these animals and their environment.

The results obtained with this study, that analyzed and compared the different prevalences in free-ranging and captive tortoises, contribute to formulate and update guidelines for proper sanitary management and relocation of rescued tortoises, placing particular emphasis of the risk of introduction of new microorganisms into the wild (MATTM 2019). The different prevalence of relevant microorganisms (*Mycoplasma* spp. and *M. agassizii*) found between free-ranging and rescued animals and the absence of others (*Chlamydia* spp. and TeAHVs) suggest that monitoring of these infections is critical to prevent the spread of microorganisms in recovery centers and to preserve wild populations. In the future, the list of microorganisms to monitor should be extended to ranavirus, picornavirus, and ferlavirus, other known chelonian pathogens. The data regularly collected from this monitoring could be used to understand the relevance, the pathogenic potential, the host susceptibility to these infections, and draw a baseline to evaluate their possible evolution over time in these populations.

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