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Diversity of microorganisms in *Hyalomma aegyptium* collected from spur-thighed tortoise (*Testudo graeca*) in North Africa and Anatolia

Ana Cláudia Norte[1,2] | David James Harris[3] | Diogo Silveira[3] | Carolina Saramago Nunes[2] | Maria Sofia Núncio[2] | Eva Graciá Martínez[4,5] | Andrés Giménez[4,5] | Rita de Sousa[2] | Isabel Lopes de Carvalho[2] | Ana Perera[3]

[1] MARE - Marine and Environmental Sciences Centre, Department of Life Sciences, University of Coimbra, Coimbra, Portugal

[2] Centre for Vector and Infectious Diseases Research, National Institute of Health Doutor Ricardo Jorge, Águas de Moura, Portugal

[3] CIBIO/InBIO - Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Vairão, Portugal

[4] Departamento de Biología Aplicada, Universidad Miguel Hernández, Elche, Spain

[5] Centro de Investigación e Innovación Agroalimentaria y Agroambiental (CIAGRO-UMH), Miguel Hernández University, Elche, Spain

Correspondence
Ana Cláudia Norte, MARE - Marine and Environmental Sciences Centre, Department of Life Sciences, Calçada Martim de Freitas, Faculty of Sciences and Technology, University of Coimbra 3000-456 Coimbra, Portugal.
Email: acgnorte@ci.uc.pt

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Abstract

Ticks carry a diverse community of microorganisms including non-pathogenic symbionts, commensals, and pathogens, such as viruses, bacteria, protozoans, and fungi. The assessment of tick-borne microorganisms (TBM) in tortoises and their ticks is essential to understand their eco-epidemiology, and to map and monitor potential pathogens to humans and other animals. The aim of this study was to characterize the diversity of microorganisms found in ticks collected from the spur-thighed tortoise (*Testudo graeca*) in North Africa and Anatolia. Ticks feeding on wild *T. graeca* were collected, and pathogens were screened by polymerase chain reaction using group-specific primers. In total, 131 adult *Hyalomma aegyptium* ticks were collected from 92 *T. graeca* in Morocco (n = 48), Tunisia (n = 2), Algeria (n = 70), and Turkey (n = 11). Bacteria and protozoa detected included *Hemolivia mauritanica* (22.9%), *Midichloria mitochondrii* (11.4%), relapsing-fever borreliae (8.4%), *Ehrlichia* spp. (7.6%), *Rickettsia* spp. (3.4%), *Borrelia burgdorferi* s.l. (0.9%), *Francisella* spp. (0.9%), and *Wolbachia* spp. (0.8%). The characterization of *Rickettsia* included *R. sibirica mongolitimonae* (Algeria), *R. aeschlimannii* (Turkey), and *R.africae* (Morocco). *Hemolivia mauritanica* and *Ehrlichia* spp. prevalence varied significantly with the sampling region/country. We did not detect significant associations in microorganism presence within ticks, nor between microorganism presence and tick mitochondrial DNA haplogroups. This is the first report of *Francisella persica*-like, relapsing fever borreliae, *M. mitochondrii*, and *Wolbachia* spp. in *H. aegyptium* ticks collected from wild hosts from the South and Eastern Mediterranean region, and of *R. sibirica mongolitimonae* and *R. africae* in *H. aegyptium* from Algeria and Morocco, respectively. Given that *T. graeca* is a common species in commercial and non-commercial pet trade, the evaluation of the role of this species and its ticks as hosts for TBM is particularly relevant for public health.

KEYWORDS
Francisella, Rickettsia, tick-borne pathogens, ticks, tortoises, Wolbachia

1 | INTRODUCTION

Ticks are common parasites of vertebrates and carry a diverse community of microorganisms, some of which are pathogenic to humans and other animals. For this reason, ticks are considered one of the most important arthropod vectors of disease (Jongejan & Uilenberg, 2004). Furthermore, ticks themselves have an impact on their hosts given their blood-feeding habits, which can lead to decreased host body condition and anemia (Norte et al., 2013). Different tick species vary in their host specificity; while some ticks have a generalist behavior, feeding on different hosts species depending on their availability (e.g., *Ixodes ricinus*), others are more specific, feeding on a reduced spectrum of hosts (e.g., ornitophilic ticks such as *Ixodes frontalis*), or even on a single host species (e.g., *Ixodes lividus* feeds almost exclusively on sand martins *Riparia riparia* – Estrada-Peña et al., 2017; Hillyard, 1996). Host specificity may also vary with tick life stage; this is the case of *Hyalomma aegyptium*, whose larvae and nymphs feed on a variety of hosts, including mammals, birds, and reptiles, whereas adult stages feed almost exclusively on tortoises of the genus *Testudo* (Hoogstraal & Kaiser, 1960).

Feeding on a wide spectrum of hosts increases the opportunities for microorganism transmission among host groups, with consequences for the diversity of tick microbial communities that may include vertically transmitted symbionts, commensals, and pathogens, such as viruses, bacteria, protozoans, and fungi (Clay & Fuqua, 2010). These microorganisms may interact in different ways (Ginsberg, 2009), either directly maximizing each other's survival and transmission (Budachetri et al., 2018), or by affecting tick survival and activity (Herrmann & Gern, 2010). Alternatively, they may show antagonistic (e.g., by competition within the tick) (Moutailler et al., 2016) or neutral relationships. For example, the bacterial complex *Borrelia burgdorferi* s.l. and the protozoan *Babesia* sp. occur together in ticks more frequently than expected (Mather et al., 1987) while there is no apparent relationship between *Anaplasma* sp. and *B. burgdorferi* s.l. in *I. scapularis* (Schauber et al., 1998), and co-infections between these two pathogens in *I. ricinus* occurs at a relatively low frequency in Europe (May et al., 2015). Co-infection may also result in an increase in disease severity compared with disease caused by a single pathogen, and may hamper disease diagnosis (Diuk-Wasser et al., 2016).

Tick host specificity may affect the population structure of both tick and associated pathogens because, although ticks have low mobility, they can travel relatively long distances attached to their hosts (McCoy et al., 2003, 2013). Migratory birds and other animals with the potential for long-distance dispersal can transport ticks and their microorganisms to new areas, often creating new disease foci (Reed et al., 2003). On the other hand, ticks infesting small mammals or tortoises are likely to show more structured populations, and this also applies to their associated microorganisms (Norte et al., 2020; Vollmer et al., 2013). The interactions among microorganisms and their hosts is so intimate that they may not only mutually influence each other's fitness, but also genetic diversity and demographic dynamics (Barrett et al., 2008) which can be reflected in co-evolutionary patterns (Clayton et al., 2003; Criscione & Blouin, 2004).

Diversity and specific interactions of ticks with tortoises and infection by tick-borne microorganisms (TBM) remains poorly understood in some regions of the globe. In the Mediterranean region, the system formed by the tortoise *Testudo graeca*, the tick *Hyalomma aegyptium*, and their associated microorganisms is a valuable model to pinpoint co-evolutionary relationships and draw inferences regarding microbial interactions for several reasons. First, the distribution, diversity, and phylogeographic patterns of both *T. graeca* and *H. aegyptium* ticks are already known (Graciá et al., 2017; Silveira, 2016). Second, *T. graeca* has low mobility and reduced dispersal abilities (Graciá et al., 2013, 2020 ; Rouag et al., 2017). Third, *H. aegyptium* has high specificity towards *Testudo* tortoises during its adult stage (Siroký et al., 2006). Finally, *T. graeca* is one of the preferred tortoises in the commercial pet trade (Türkozan et al., 2008), and furthermore, they are frequently collected from the wild directly into households, increasing the risk of transmission of tick-borne diseases to humans and other domestic animals (Nijman & Bergin, 2017; Segura et al., 2020). Because *H. aegyptium* carries multiple bacteria and protozoan species pathogenic to humans, wildlife and domestic animals (Kar et al., 2020; Kumar et al., 2020; Paștiu et al., 2012; Široký et al., 2014), and adults and nymphs of this species have been reported to bite humans (Vatansever et al., 2008), TBM screening in this system will provide relevant information for public health.

Here, we investigate the presence of different bacteria and protozoa infecting *H. aegyptium* ticks attached to *T. graeca* across North Africa and Anatolia. Specifically, we screened for the presence of bacteria of the order Rickettsiales: *Ehrlichia* spp., *Wolbachia* spp., *Midichloria mitochondrii*, *Anaplasma* spp. and *Rickettsia* from spotted fever group (SFG) and typhus group (TG); *Francisella* spp. and relapsing fever (RF) and Lyme borreliosis spirochetes. In addition, we screened for the apicomplexan parasite *Hemolivia mauritanica*. We analysed putative associations between identified TBM and the mitochondrial DNA haplogroups of the ticks from which they were detected. Finally, we evaluated potential associations (co-variation) among these microorganisms.

2 | MATERIALS AND METHODS

2.1 | Tick collection

A collection of adult ticks from *T. graeca* hosts was performed in 27 localities across Morocco (15 sites), Algeria (7 sites), Tunisia (one site), and Turkey (4 sites; Figure 1). Further details on localities, sampling dates, and number of ticks collected per locality and host are available in Supporting Information 1. Tortoises were sampled, measured, and weighted during the day. Ticks were taken using pincers and transferred into microtubes with 96% ethanol. Not all ticks infesting the tortoises were collected or assessed, and, therefore, host infestation intensity was not calculated in this study. We collected a maximum of 5 ticks per single host. After data collection, all tortoises were released at the exact place where they were captured without marking because the same areas were not resampled. Ticks were identified in the laboratory morphologically using dichotomous keys (Földvari, 2005; Walker et al., 2014). The characterization of the mitochondrial DNA

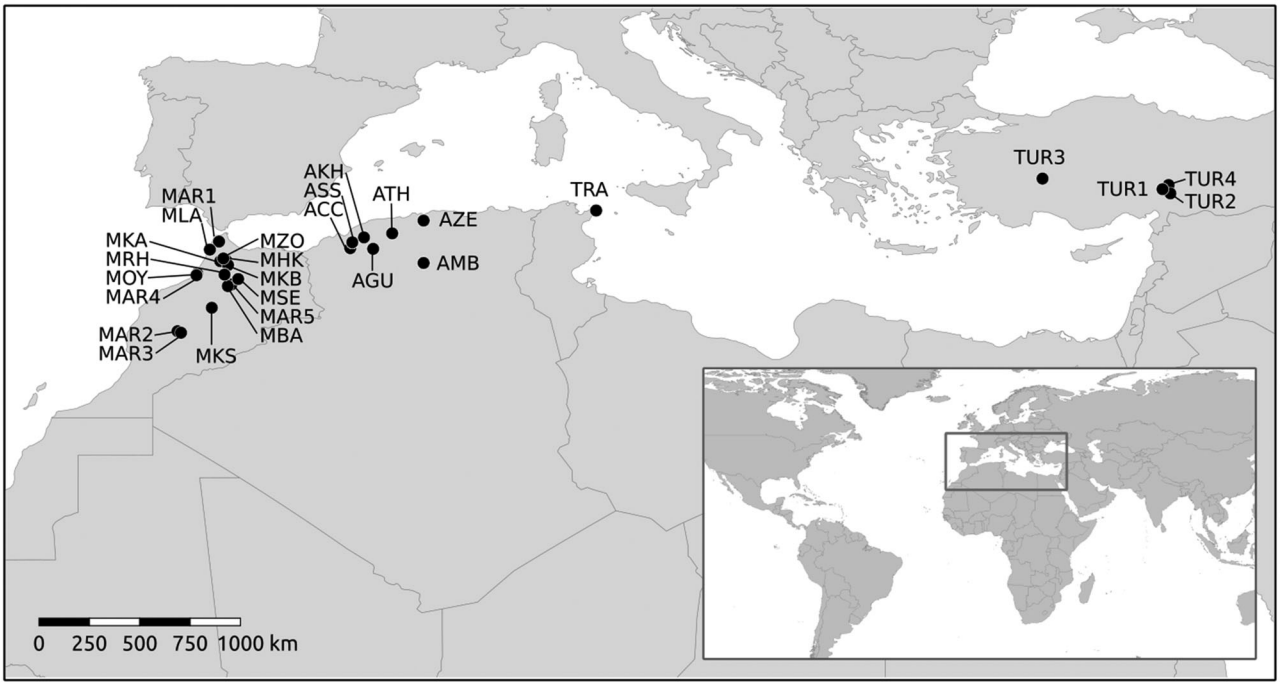

FIGURE 1 Map with the localities sampled in this study. The first letters of the locality codes indicate the country: M (Morocco), A (Algeria), T (Tunisia), TUR (Turkey). Detailed geographic location and number of samples collected in each locality is detailed in Supporting Information 1

haplotypes of the ticks included in this study was previously assessed in Silveira et al. (submitted; see below).

2.2 | DNA extraction and PCR detection of microorganisms

DNA was extracted from adult ticks using a saline method with ammonium acetate following Sambrook et al. (1989). Small incisions were performed in the abdomen cavity and under the scutum of the ticks to facilitate digestion. Molecular screening of each individual tick was performed by conventional and real-time Polymerase Chain Reaction (PCR) assays, according to previously described protocols (Table 1) for the detection of members of the family Anaplasmataceae (*Anaplasma*, *Ehrlichia* and *Neorickettsia*) and apicomplexans of the group of the hemogregarines (*Hemolivia* and *Hepatozoon*), relapsing fever group borreliae, *B. burgdorferi* sensu lato, *Francisella* and *Rickettsia*. In some specific cases, positive detections were confirmed by additional PCRs targeting other gene regions (Table 1).

2.3 | Sequence and phylogenetic analysis

All positive amplicons were purified with ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) and sequencing was performed for both strands of PCR products by the Sanger method, using the respective primers of different target genes. Gene sequences were manually adjusted, trimmed using the BioEdit Sequence Alignment Editor v 7.1.9 software, and further analysis was performed by comparison with the sequences available in the NCBI (GenBank) nucleotide database (https://ncbi.nlm.nih.gov/).

Pairwise distance (p-distances) between *M. mitochondrii* sequences were estimated in MegaX (Kumar et al., 2018).

2.4 | Statistical analyses

Difference in the number of ticks infected with pathogens among countries was analyzed using Fisher exact test. We tested whether the geographic location of the ticks (country, latitude, and longitude) affected the presence of microorganisms (those with a prevalence higher than 7% - *M. mitochondrii*, *H. mauritanica*, *Ehrlichia* spp., and relapsing fever borreliae) within the ticks. For this, we used Generalized Linear Models with a binomial distribution and logit link function. We started by running a full model including country, latitude, longitude, and the interaction between latitude and longitude and sequentially removed non-significant terms until we obtained a model with the lowest AIC. In these models, we excluded Tunisia from the analysis because only two ticks were analyzed from this location. After this, we assessed whether there was an association between tick mtDNA phylogeography identified in a previous study (Silveira et al., submitted), and the most prevalent TBMs. Based on two mitochondrial genes (cytochrome *c* oxidase subunit I (COI) and 12S ribosomal RNA (rRNA) Silveria et al. (submitted) identified a total of 31 different haplotypes grouped in two main haplogroups: A1 or *northern* haplogroup, which is abundant in the northern localities from Morocco and Algeria and also in Tunisia, and B1, which is present in Turkey, but is also widespread in inner Algeria,

TABLE 1 PCR method, primers, and probes used for microorganism screening in *Hyalomma aegyptium* ticks collected from *Testudo graeca* tortoises. (2) indicates reference used for PCR amplification conditions

Target microorganism(s)	PCR method	Target gene	Set primers and probe	Product size (bp)	Reference
Anaplasma spp., *Ehrlichia* spp., *Neorickettsia* spp., *M. mitochondrii* and *Wolbachia* spp.	Conventional PCR	16S rRNA	EHR16SD / EHR16SR	345	Brown et al., 2001; Hornok et al., 2008
Hepatozoon spp.; *Hemolivia mauritanica*	Conventional PCR	18S rRNA	HepF300 / HepR900	600	Ujvari et al., 2004; Harris et al., 2011
Relapsing fever group borreliae	Conventional PCR	16S rRNA	REC4 / REC9	523	Ras et al., 1996
	Real time PCR	flagellin	Flagellin F and R; flagellin probe	44	Cutler et al., 2010
B. burgdorferi sensu lato (s.l.)	Conventional PCR	flagellin	flaB outer F/R flaB inner F/R	390	Johnson et al., 1992
	Real-time PCR	flagellin	FlaF1A / FlaR1 (FlaProbe1)	132	Schwaiger et al., 2001
Francisella spp.	Real-time PCR	*tul4*	Tul4F / Tul4R TUL4 probe	91	Versage et al., 2003
	Real-time PCR	*Francisella tularensis* outer membrane protein *fopA*	fopA	204	EQADeBa, 2007
Rickettsia spp. (SFG and TG)	Conventional PCR	*ompB*	rompB_OF / rompB_OR	511	Choi et al., 2005
	Conventional PCR	*gltA*	RpCs877p / RpCs1258n	381	Regnery et al., 1991
	Conventional PCR	*ompA*	Rr190.70p / Rr190.602n	532	Regnery et al., 1991

and Morocco. These two haplogroups have been used in our analysis as a representation of tick phylogeography.

Finally, we also evaluated if there were any significant associations among the more common microorganisms (*M. mitochondrii*, *H. mauritanica*, and *Ehrlichia* spp.) within a tick, that is, if infection by different agents was independent or whether a tick infected with a given microorganism was more or less likely to be infected by others. For this, we used Schutler's variance ratio test (Schluter, 1984) to ascertain if there were significant associations between (a) *M. mitochondrii* and *Ehrlichia* spp., (b) *M. mitochondrii* and *H. mauritanica*, (c) *Ehrlichia* spp. and *H. mauritanica*, and (d) *M. mitochondrii*, *H. mauritanica*, and *Ehrlichia* spp. In this test, lack of a significant result indicates either that the parasites are independent or that positive and negative associations between pairs of parasites cancel each other (Forbes et al., 1994; Schluter, 1984). Since there were multiple cases of ticks collected from the same host, for this analysis we randomly selected a single tick from each individual host to control for the eventual influence of the vertebrate host on infection, that is if the tortoises act as a reservoir for a given microorganism.

3 | RESULTS

One-hundred and thirty-one adult *H. aegyptium* ticks were collected from 92 *T. graeca* hosts in Morocco (n = 48), Tunisia (n = 2), Algeria (n = 70), and Turkey (n = 11; Supporting Information 1). A higher prevalence of microorganisms was detected in ticks from Turkey (9 out of 11 ticks, 81.8%, were infected with at least one microorganism) than those from Algeria (35 out of 70, 50%) and Morocco (12 out of 48, 25%) (Fisher exact test, $p < 0.001$). However, TBM diversity was similar in these three countries (Fisher exact test $p = 0.854$; Table 2; Figure 2). In the two ticks collected in Tunisia, we did not detect any of the screened microorganisms.

Our screening detected the following microorganisms: *H. mauritanica* (22.9%), *M. mitochondrii* (11.4%), relapsing fever borreliae (8.4%), *Ehrlichia* spp. (8.4%), *Rickettsia* spp. (3.4%), *B. burgdorferi* s.l. (0.9%), *Francisella* sp. (0.9%), and *Wolbachia* sp. (0.8%). We did not detect *Anaplasma* spp. in the samples analyzed.

Hemolivia mauritanica was the most common microorganism found in the ticks with a prevalence of 22.9 % (30/131) and was found in Morocco, Algeria, and Turkey. Only one sample from Morocco (MSE) was positive for *H. mauritanica* in all the 15 localities sampled. In contrast, in Algeria, five out of the seven localities sampled were positive for *H. mauritanica*, while in Turkey, all sites (4 localities) revealed positive ticks. Country and longitude affected the prevalence of *H. mauritanica* with prevalence significantly decreasing from East to West (estimate ± SE = 1.07 + 0.31; reference category: not infected; Table 3). All the new sequences from this microorganism were identical (accession number MW295408) and were also identical to *H. mauritanica* previously retrieved from *H. aegyptium* from *T. graeca* (KC512766), as well as from blood of *T. marginata* from Greece (KF992710-KF992699) and *T. graeca* from Iraq (KF992700).

TABLE 2 Overall prevalence (%) of the microorganisms detected in *Hyalomma aegyptium* ticks attached to *Testudo graeca*. For each microorganism prevalence (number of positives/number of ticks tested) is given

	Ehrlichia spp.	*Midichloria mitochondrii*	*Wolbachia* spp.	*Hemolivia mauritanica*	Relapsing fever borreliae	*Borrelia burgdorferi* s.l.	*Francisella* spp.	*Rickettsia* spp.	Total number of ticks infected by at least one microorganism/ N ticks tested*
Algeria	14.3 (10/70)	10.0 (7/70)	0 (0/70)	32.8 (23/70)	8.6 (5/58)	0 (0/61)	0 (0/61)	2.3 (1/43)	35/70
Morocco	2.1 (1/48)	14.58 (7/48)	2.1 (1/48)	2.1 (1/48)	2.6 (1/38)	0 (0/38)	2.6 (1/38)	2.94 (1/34)	12/48
Tunisia	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0/2
Turkey	0 (0/11)	9.1 (1/11)	0 (0/11)	54.5 (6/11)	30 (3/10)	10 (1/10)	0 (0/10)	11.1 (1/9)	9/11
Total	8.39 (11/131)	11.45 (15/131)	0.8 (1/131)	22.9 (30/131)	8.4 (9/107)	0.9 (1/110)	0.9 (1/110)	3.4 (3/87)	

*Some ticks were tested only for some of the microorganisms due to limited DNA sample.

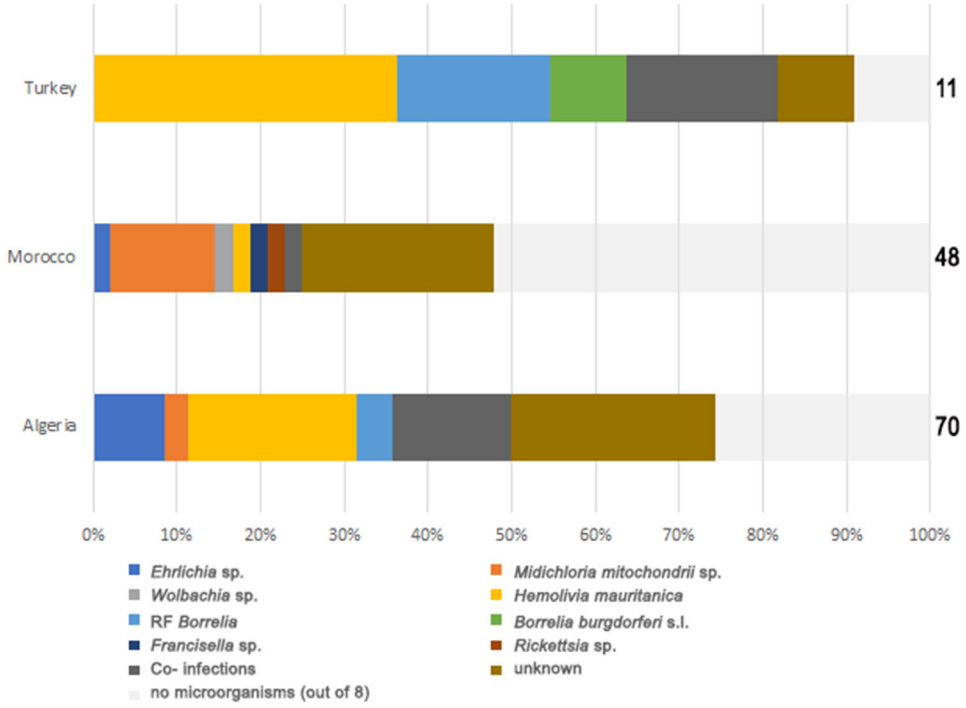

FIGURE 2 Prevalence of microorganisms in *Hyalomma aegyptium* collected from *Testudo graeca*. Co-infections include *Ehrlichia* spp., *H. mauritanica* and RF borreliae (n = 1), *H. mauritanica* and RF borreliae (n = 1), *Ehrlichia* spp. and *H. mauritanica* (n = 2), *Ehrlichia* sp. and *R. sibirica mongolitimonae* (n = 1), *M. mitochondrii* and *H. mauritanica* (n = 5) in Algeria; *M. mitochondrii* and RF borreliae (n = 1) in Morocco; *M. mitochondrii*, *H. mauritanica* and RF borreliae (n = 1), *H. mauritanica* and *R. aeschlimannii* in Turkey. Unknown refers to ticks tested negative for *Ehrlichia* spp., *M. mitochondrii*, *Wolbachia* spp., and *H. mauritanica* only and not tested for the remaining TBM. Numbers on the top of the bars represent the total number of ticks

Ehrlichia spp. overall prevalence was 8.39% (11/131), and was found only in Algeria and Morocco. Prevalence of *Ehrlichia* spp. decreased with latitude (estimate ± SE = 1.05 ± 0.51; reference category: not infected) and there was a significant effect of country on its prevalence (Table 3). All *Ehrlichia* sequences obtained in this study were identical (accession number MW293912). This sequence had 99.62% similarity with *Ehrlichia* sp. found, among others, in *Amblyoma* ticks in Brazil (MT514732) and Australia (KY425523), and *Haemaphysalis* ticks from Malaysia (KY046300) and China (KX987321). It also had 99.62% similarity with *Candidatus* Ehrlichia shimanensis (AB074459) and *E. ewingii* in *Haemaphysalis* ticks from China (MN148616).

Midichloria mitochondrii (overall prevalence = 11.45%, 15/131) and relapsing fever borreliae (overall prevalence = 8.4%, 9/107) were not associated with geographic location of tick collection. Although prevalence of relapsing fever borreliae was 30% in Turkey, this was not significantly higher than for the other countries (prevalence between 0 – 8.6%). We retrieved two different haplotype sequences of *M. mitochondrii* (p-distance = 0.022): one distributed across Morocco, Algeria, and

TABLE 3 Effects of geographic location of *Hyalomma aegyptium* collection on microorganism presence. Only fixed effects maintained in the best model are presented

	Country (χ^2, P)	Latitude (χ^2, P)	Longitude (χ^2, P)	Whole model χ^2	N	Whole Model P
Midichloria mitochondrii		1.47, 0.22	–	1.47	91	0.22
Hemolivia mauritanica	37.65, < 0.0001	–	19.48, < 0.0001	40.85	91	< 0.0001
Ehrlichia spp.	9.33, 0.0094	4.55, 0.033	–	10.01	91	0.02
Relapsing fever borreliae	–	2.07, 0.15	–	2.07	78	0.15

Turkey (accession number MW293914), and a second one, found in a single tick in Algeria (individual ATH4, accession number MW293913). The first sequence was 100% identical to *M. mitochondrii* sequences from several *H. dromedarium* infesting camels in Tunisia (MK416233 - 36) and from *H. detritum* samples in China (MG668797 - 98). The second one showed 100% identity with *M. mitochondrii* from the tick *Rhipicephalus sanguineus* (KY910125), and an uncultured bacterium from *Haemaphysalis wellingtoni* (AF497583) both collected in Thailand.

Overall prevalence of the other microorganisms tested was relatively low: ranging from 0.8% (*Wolbachia* sp.) to 3.4% (*Rickettsia* sp.). *Wolbachia* sp. (0.8%) and *Francisella* sp. (0.9%) were only detected in Morocco, *B. burgdorferi* s.l. (0.9%) was detected in Turkey, and *Rickettsia* sp. (3.4%) was detected in Algeria, Morocco, and Turkey (Table 2). The sequence of *Wolbachia* (accession number MW293915) matched with 100% identity with over 100 sequences of "uncultured bacteria," *Wolbachia* sp. and *W. pipientis* retrieved from mites (*Dermanyssus gallinae*, MT640298) and several insect groups, including flies (*Haematobia irritans*: CP042446, *Drosophila* sp.: MK940245), moths (*Carposina sasakii*, CP041215), fleas (*Pulex irritans*, MH521189), and hymenopters (*Ixodiphagus hookeri*, KU255240), among others. The *Francisella* sp. detected was most similar (93.7%) to *F. persica* (accession number MW207217).

Regarding the detection of *Rickettsia*, we identified three positive ticks: one in Turkey (1/9, 11.1%), one in Algeria (1/43, 2.3%), and one in Morocco (1/34, 2.9%). Sequence analysis identified *R. aeschlimannii* in the tick collected in Turkey. Our sequence was 100% identical to the sequences of *R. aeschlimanii* from Greece for *ompA* (491/491 bp; accession number KP675967) and France for *gltA* (341/341 bp; MK608659), and was not identical any other named species on the database. Similarly, for *gltA* it matched with 100% to *R. aeschlimannii* (e.g. MK608659), while for *ompB* it was 98% identical to the sequence MK028342 (455/464 bp). The sequence from the positive tick from Algeria showed 100% identity with *R. sibirica mongolitimonae* detected in ticks from Turkey for *ompA* (488/488 bp; MH500071), and Cyprus for *gltA*, (341/341 bp; accession number JF803902), while for the *ompB* fragment the most similar sequence on GenBank with 99% identity was *R. sibirica mongolitimonae* detected in a traveller patient in Algeria (DQ097083). The *Rickttesia* detected in *H. aegyptium* from Morocco was 100% identical to *R. africae* for both *gltA* (341/341 bp; MN025497) and *ompA* (482 bp; JQ691730), while for *ompB*, sequences on GenBank from various species including *R. africae*, *R. parkeri*, *R. peacockii*, and *R. slovaca* all showed matches over 98%. The only 100% match was to *Rickettsia* sp. from cattle in Zambia (LC565635).

Prevalence of *Ehrlichia* spp. was different between the two tick haplogroups (A1 and B1, Fisher's Exact test $P = 0.042$, n = 82), with probability of infection being higher in the widespread haplogroup B1 than in the northern haplogroup A1. However, because *H. aegyptium* mtDNA haplotypes are partially associated with geographic distribution, it seems likely that this is an indirect effect of geographic location of tick collection on *H. mauritanica* infection rates, rather than a direct association. Prevalence of other microorganisms (*H. mauritanica*, *M. mitochondrii*, and relapsing fever borreliae) was not associated with tick haplogroups (in all cases, Fisher Exact test, $P > 0.05$).

We detected 13 (13.8%, 12/87 considering only ticks with all TBM tested for) ticks with co-infections. The most common, detected in six (6.9%) ticks, was that of *M. mitochondrii* with *H. mauritanica*. We also detected co-infections between *Ehrlichia* spp. and *H. mauritanica* (n = 2), *Ehrlichia* spp. and *R. sibirica mongolitimonae* (n = 1), *H. mauritanica* and *R. aeschlimannii* (n = 1), *H. mauritanica* and relapsing fever borreliae (n = 1), and *M. mitochondrii* and relapsing fever borreliae (n = 1). Two ticks presented triple infections: one with *Ehrlichia* sp., *H. mauritanica* and relapsing-fever borreliae, and one with *M. mitochondrii*, *H. mauritanica* and relapsing-fever borreliae. However, we did not detect any significant associations between microorganism presence within ticks: (a) *M. mitochondrii* and *Ehrlichia* spp., (b) *M. mitochondrii* and *H. mauritanica*, and (c) *M. mitochondrii*, *H. mauritanica* and *Ehrlichia* spp. (Supporting Information 2).

4 | DISCUSSION

This study describes the diversity of microorganisms infecting *H. aegyptium* collected from *T. graeca* in North Africa and Anatolia. Although this tick species is relatively host-specific, the diversity of microorganisms it carries was high and included *M. mitochondrii*, *Wolbachia* spp., *Ehrlichia* spp., relapsing fever borreliae, *B. burgdorferi* s.l., *Francisella* spp., *Rickettsia* spp., and *H. mauritanica*. The highest microorganism diversity was found in ticks originating from Morocco, although this country also had the lowest prevalence, highlighting just how complex TBM distributions are.

To the best of our knowledge, this is the first report of *F. persica* - like, relapsing fever borreliae, and *Wolbachia* spp. in *H. aegyptium*. *M. mitochondrii* was found to be widespread in our study area, with positive specimens from Morocco, Algeria, and Turkey. This endosymbiont is common in the microbial communities of several tick species (Cafiso et al., 2016; Selmi et al., 2019), and was only recently reported for the

first time in *H. aegyptium* collected from two *T. graeca* from a marketplace in Qatar (Barradas et al., 2020a). Although the nature of this symbiotic relationship is still not fully understood (Cafiso et al., 2016), this symbiont is able to invade the mitochondrial intermembrane space, and it has been detected in different tick organs (Stavru et al., 2020). These peculiarities suggest far more complex interactions within the tick, namely enhancing tick's reproductive fitness, energy production, water balance, and cellular respiration, among others (Budachetri et al., 2018; Stavru et al., 2020). Indeed, a recent study also suggested that *M. mitochondrii* may assist tick cellular respiration during blood feeding, when ixodid ticks are thought to enter a hypoxic state (Stavru et al., 2020).

Other non-pathogenic tick endosymbiont such as *Francisella* -like endosymbiont (FLE) have previously been detected in *H. aegyptium* and *H. marginatum* from Israel, Hungary, Ethiopia, and Yemen (Azagi et al., 2017) and, *Francisella* spp. has been reported to be the most common bacteria in *H. aegyptium* ticks from Turkey (Keskin et al., 2017). Surprisingly, we identified only one putative *Francisella* sp. from one specimen from Morocco. Perhaps the identified *F. persica*-like is the bridge between *Francisella*-like endosymbiont found in ticks and the facultative intracellular pathogen *F. tularensis* (Larson et al., 2016). Nevertheless, these bacterial endosymbionts have been known to colonize tick salivary glands, and their presence in this organ does not ensure transmission to the vertebrate host (Klyachko et al., 2007).

Reports of *Wolbachia* spp. in ticks are scarce, and in our study area, *Wolbachia* has only been detected in one tick from Morocco (Sarih et al., 2005; Seng et al., 2009). Plantard et al. (2012) experimentally demonstrated that the detection of the bacteria *W. pipiens* in the tick *I. ricinus* is the indirect result of the infection of the tick by the endoparasitoid wasp *Ixodiphagus hookeri*, which carries the *Wolbachia* in the first place. The *Wolbachia* detected in our study was genetically identical to *Wolbachia* sequences retrieved from several insect species, including the one from the endoparasitic wasp described above. Although the mutual benefits of this relation are only vaguely known, this bacterium may induce an immune response in the ticks that could promote defense against pathogens (Haine, 2008; Tijsse-Klasen et al., 2011).

The most abundant microorganism detected was *H. mauritanica*. This apicomplexan species has a heteroxenous life cycle, which includes the tick *H. aegyptium* and *Testudo* tortoises (Široký et al., 2009). *Hemolivia mauritanica* is distributed from North Africa to the Balkans and Middle East regions (Široký et al., 2009). Prevalence is highly variable along its distribution range, although in general, values are higher in the eastern range decreasing toward the western and south (Široký et al., 2009). The highest prevalence has been reported in Romania (84%; Široký et al., 2009) and Iran (100%; Javanbakht et al., 2015). Regarding North Africa and Anatolia, prevalence reported include 51.9% in Turkey (Akveran et al., 2020) and between 6.25-30.4% in Algeria (Široký et al., 2009; Tiar et al., 2010), while there are no positive reports in Morocco or Tunisia (Harris et al., 2013; Laghzaoui et al., 2020; Široký et al., 2009). Our study also indicates a higher prevalence in Turkey than in Algeria. Interestingly, we detected for the first time a tick infected with *Hemolivia* in North Morocco (Sefrou). Studies show that *H. mauritanica* is highly specific towards its tick and tortoise host (Javanbakht et al., 2015). One possibility could be that this particular *Hyalomma* individual infected with *Hemolivia* could be phylogenetically closer to those from Algeria, which are commonly infected. However, this particular sample has the same mtDNA haplotype as ticks from nearby localities in Morocco (Silveira et al., submitted), and the *T. graeca* individual to which it was attached belonged to the subspecies *T. g. marokkensis*, which is distributed across the north of Morocco (Graciá et al., 2017). It seems likely, therefore, that *Hemolivia* is simply rarer in Morocco, so that it has not been detected in previous works. However, more studies are needed to understand the factors shaping the narrower distribution of *Hemolivia* regarding their *Hyalomma* and *T. graeca* hosts.

Ehrlichia bacteria are responsible for ehrlichiosis, and ticks infecting amphibians and reptiles are potential carriers of *Ehrlichia* strains pathogenic to humans (Andoh et al., 2015). The prevalence detected in our study (8.39%) was lower than those reported in *H. aegyptium* ticks infecting tortoises in Turkey (30.2%, Akveran et al., 2020). Although we were not able to identify the *Ehrlichia* species detected in our study, its 16S ribosomal RNA sequence was very similar (99.62%) to *E. ewingii*, one of the pathogenic species infecting humans (Harris et al., 2016).

Regarding the detection of rickettsiae in ticks collected from tortoises, the three species *R. aeschlimannii*, *R. sibirica mongolitimonae*, and *R. africae* are known to be pathogenic to humans (de Sousa et al., 2006; Nouchi et al., 2018; Raoult et al., 2002) and have been described in different regions of the globe, in Africa, Europe, and Eurasia (Parola et al., 2013). Moreover, *R. africae*, detected in one of the ticks from Morocco, is one of the most important causes of systemic febrile illnesses reported in travellers returning from sub-Saharan Africa (Jensenius et al., 2004). Although *R. africae* has been previously associated with *H. aegyptium*, it has been more frequently detected in ticks of the *Amblyomma* genus. The same occurs for *R. aeschlimannii* and *R. sibirica mongolitimonae* that have been also described to be associated with other species of *Hyalomma* or other tick species (Barradas et al., 2020b; de Sousa et al., 2006; Ereqat et al., 2016; Orkun et al., 2019). Our study corroborates previous descriptions of the presence of *R. aeschlimanii* in *H. aegyptium* collected from *T. graeca* in Turkey (Akveran et al., 2020; Orkun et al., 2014). We identify for the first time the presence of *R. sibirica mongolitimonae* in *H. aegyptium* in Algeria, although previous studies have detected *R. aeschlimannii*, and other pathogens, such as the Crimean-Congo virus in *H. aegyptium* ticks collected in this country (Bitam et al., 2009; Kautman et al., 2016). *Rickettsia sibirica mongolitimonae* infection has been reported in one traveler to southern Algeria (Fournier et al., 2005). Our finding of *R. africae* detected in Morocco adds new data regarding the several rickettsiae of the spotted fever group already identified in this country that can be implicated in human infections, including *R. aeschlimannii* in *H. marginatum*, *R. massiliae* in *R. sanguineus*, *R. slovaca* in *Dermacentor marginatus*, and *R. monacensis* in *I. ricinus* (Sarih et al., 2008).

Hyalomma aegyptium has been previously reported to harbour both *B. burgdorferi* s.l. (Kar et al., 2011) and *B. turcica* – a spirochete from the reptile-associated *Borrelia* group, a sister clade to RF *Borrelia* (Güner et al., 2004). *Borrelia turcica* is the most commonly reported *Borrelia* species from *H. aegyptium* and tortoises with detections often reported

from their ticks and tissues (Kalmar et al., 2015; Hepner et al., 2020). Unfortunately, we could not assess the species of *Borrelia* in our samples.

We assessed the presence of associations between infection by the tick endosymbiont *M. mitochondrii* and pathogenic agents (*H. mauritanica* and *Ehrlichia* spp.), hypothesizing this endosymbiont could exert positive effects on infection by other microorganisms, as has been shown for example with *R. parkeri* (Budachetri et al., 2018). However, this was not supported by our data: we did not detect any positive or negative associations between *M. mitochondrii* and *Erhlichia* sp., *or H. mauritanica* suggesting there are no synergistic or antagonistic interactions among them. Although we detected four ticks co-infected with *M. mitochondrii* and *H. mauritanica*, the sample size was relatively small for this analysis.

Different patterns of microorganism infection across disparate vector populations/ haplotypes could be expected if they had variable probabilities of infection either due to: (a) different contact rates of a given population/ haplotype with a microorganism or their vertebrate hosts, or (b) by a tick haplotype influencing the survival of a given microorganism within the tick (Gómez-Díaz et al., 2010; Hajdušek et al., 2013) and or (c) due to the lack of suitable environmental conditions in the niche exploited by a given tick lineage (e.g., presence of suitable hosts) precluding the microorganism to complete its life cycle. It has been shown that relatively isolated populations of vertebrate hosts are likely to increase population structure of their ticks and their microorganisms (McCoy et al., 2003; Norte et al., 2020; Vollmer et al., 2011, 2013). The mobility of *T. graeca* is relatively limited, with a range of movements varying between 0.29 and 5 ha (Rouag et al., 2017; Graciá et al., 2020). As such, the probabilities of long-distance dispersal are low, and these tortoises show notable phylogeographic structuring (Fritz et al., 2009). The *H. aegyptium* ticks it carries also present a geographic population structuring in the area covered by our study (Silveira et al., submitted). However, this does not seem to affect infection by the microorganisms studied: we did not detect significant differences in the microorganisms (*Erhlichia* sp., *M. mitochondrii*, and relapsing fever borreliae) carried by different tick haplotypes, although there was a tendency for the more widely spread haplotype (B1) to present higher prevalence of *H. mauritanica*. Although *H. aegyptium* is highly specific toward its tortoise host when adults, they will attach to other, more mobile, species such as hedgehogs and hares (Hoogstraal & Kaiser, 1960), and also lizards, birds, other mammals, and humans, thereby increasing the probability of contact with different microorganisms (Paștiu et al., 2012) that could partially explain the absence of clear infection patterns among tick haplotypes. With many factors involved, including aspects directly related to the microorganism and tick host (e.g., ability of the tick immune system to suppress the infection), it is difficult to distinguish between the effect of geographic location *per se* and tick haplotype on microorganism prevalence when these facets are correlated. Indeed, we found that geographic location affected both *Ehrlichia* spp. and *H. mauritanica* prevalence.

Our study contributed towards a better characterization of microorganisms circulating within the *T. graeca* – *H. aegyptium* host-parasite system, reporting for the first time the occurrence of relapsing fever borreliae of unknown pathogenicity and the endosymbiont *Wolbachia* spp. in this tick species.

Further studies regarding if the potential pathogens that *H. aegyptium* was shown to carry persist long-term in tortoises and if they can be transmitted to other feeding ticks and/or can be vectored by this tick species would be relevant. These results are pertinent for public health, and to identify infection risk related with translocation of pathogens and vectors into new areas, especially because this host species is widely collected for the pet trade. We also encourage public health authorities to reinforce surveillance and control within the commercial pet trade. Additionally, non-commercial trade is particularly relevant in this species (Segura et al., 2020), and educational campaigns are also needed to change the perception of locals toward tortoises, not as pets but as threatened wildlife species with zoonotic potential.

5 | SEQUENCE SUBMISSION TO A PUBLIC DATABASE

The GenBank accession numbers of partial sequences obtained in this study are: MT793826-28 (partial fragment *ompB* of *R. aeschlimannii*, *R. sibirica mongolitimonae*, *R. africae*); MT793829-31 (partial fragment *gltA* of *R. aeschlimannii*, *R. sibirica mongolitimonae*, *R. africae*); MT793832-33, MZ015009 (partial fragment *ompA* of *R. aeschlimannii*, *R. sibirica mongolitimonae*, *R. africae*); MW207217 (*F. persica* -like *fopA* gene fragment); MW295408 (*H. mauritanica* 18S rRNA gene fragment); MW293912 (*Ehrlichia* sp. 16S rRNA gene fragment); MW293913-14 (*M. mitochondrii* 16S rRNA gene fragment), and MW293915 (*Wolbachia* sp., 16S rRNA gene fragment).

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AUTHOR CONTRIBUTIONS

ACN, AP, DJH, and ILC designed the study and coordinated research; AG, AP, DS, DJH, and EGM collected samples; ACN, CSA, DS, ILC, MSN, and RS performed laboratory and data analysis; ACN wrote the original draft of the manuscript; ACN, AP, DJH, ILC, and RS wrote the main text. All authors reviewed and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article and in public databases.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All applicable international and national guidelines for animal care were followed. Samples were collected following the methodology approved by the ethics committee on animal experimentation of Miguel Hernández University and Generalitat Valenciana, under licence UMH.DBA.FBR.01.16

ORCID

Ana Cláudia Norte https://orcid.org/0000-0001-7833-4463
Ana Perera https://orcid.org/0000-0002-7466-4753

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