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Molecular detection of *Rickettsia* spp. in ticks and fleas collected from rescued hedgehogs (*Erinaceus europaeus*) in Portugal

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Abstract
Hedgehogs (e.g., *Erinaceus europaeus*, *E. roumanicus*) are wild mammals that frequently are observed near residential areas. The aim of this study was to investigate ticks and fleas collected from European hedgehogs in Portugal and to evaluate the prevalence of *Rickettsia* in those ectoparasites. Ticks and fleas were identified by morphological and molecular methods, and molecular detection by PCR and genotypic characterization of *Rickettsia* spp. was performed targeting *omp*B, *omp*A and *glt*A gene fragments. In total, 1892 ticks and 213 fleas were collected from 33 rescued European hedgehogs captured in seven districts of the north and centre of Portugal. Two tick species were identified – *Rhipicephalus sanguineus* accounted for 91 % (n = 1719) of the total ticks collected and 9 % (n = 173) were *Ixodes hexagonus*. All fleas were identified as *Archaeopsylla erinacei*. Regarding pathogen detection, *Rickettsia massiliae* DNA was found in 22 of the 212 tested *Rh. sanguineus*. None of the 48 *I. hexagonus* tested showed to be positive for rickettsiae. *Rickettsia asembonensis* DNA was identified in 55 *A. erinacei* fleas tested (n = 117). These results show that European hedgehogs are exposed to *R. massiliae* transmitted by ticks and to *R. asembonensis* via fleas suggesting that these mammals might be involved in the natural transmission cycle of these *Rickettsia* species. This study is the first report of *R. asembonensis* in fleas in Portugal.

Keywords *Rickettsia asembonensis* · *Archaeopsylla erinacei* · *Rhipicephalus sanguineus* · Hedgehog · Portugal

Introduction

The European hedgehog (e.g., *Erinaceus europaeus*, *E. roumanicus*) is a nocturnal and terrestrial insectivorous mammal that lives in many European countries. Although hedgehogs live in a wide variety of habitats, they are very well adapted to urban peridomestic environments such as gardens and parks (Speck et al. 2013). Furthermore, hedgehogs are often

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adopted and raised as pets at home. Hedgehogs can carry a large diversity of ectoparasites such as hard ticks (Acari: Ixodidae) and fleas that act as reservoirs and vectors in the transmission of tick and flea-borne pathogens to humans (de Sousa et al. 2006b; Marie et al. 2012; Orkun et al. 2019). The most common tick species collected from hedgehogs are *Ixodes hexagonus* (also known as the hedgehog tick), *I. ricinus*, and *Rhipicephalus sanguineus* (Jahfari et al. 2017; Khaldi et al. 2012; Marie et al. 2012; Szekeres et al. 2019). However, other tick species such *Hyalomma* spp have also been collected from various species of hedgehogs (Foldvári et al. 2011; Orkun et al. 2019).

Ixodes ricinus, *Rh. sanguineus* and *Hyalomma* spp. are well-known vectors for human and animal bacterial, viral and protozoan agents. *Rhipicephalus sanguineus* is the main vector of *Rickettsia conorii*, a spotted fever group (SFG) *Rickettsia* which causes Mediterranean spotted fever, one of the most severe rickettsioses in the Mediterranean basin (de Sousa et al. 2003). *Rickettsia massiliae*, a less pathogenic rickettsia also detected in *Rh. sanguineus* ticks removed from hedgehogs (Khaldi et al. 2012; Marie et al. 2012), has been sporadically implicated in human disease (Eremeeva et al. 2006; Fernandez-Soto et al. 2006; Vitale et al. 2006; Garcia-Garcia et al. 2010; Cascio et al. 2013; Zaharia et al. 2016).

Apart from ticks, hedgehogs are often parasitized by flea species, and the most common is *Archaeopsylla erinacei*. Fleas also have an important role in the transmission of several pathogens to humans, causing a range of mild to severe diseases (Bitam et al. 2010). *Rickettsia felis*-like organisms (RFLOs) such as *Rickettsia asembonensis* and *Candidatus* Rickettsia senegalensis have also been described in different fleas species collected mostly in non-European countries (Jiang et al. 2013; Hornok et al. 2018a). Although RFLOs are phylogenetically related to *R. felis*, they display considerable genetic heterogeneity and are therefore proposed as new species (Maina et al. 2019). *Rickettsia felis* causes disease in humans, but the pathogenicity of RFLOs still not very well defined (Maina et al. 2019).

Diagnostic tests of hedgehog carcasses revealed the presence of a wide range of tick-borne bacteria (Szekeres et al. 2019). However, to date, it is not clear whether hedgehogs develop disease from, nor whether they can function as a vertebrate reservoir of these pathogenic bacteria. As in Portugal rickettsiae are one of the most important tick-borne pathogens causing disease in humans and no systematic surveys on ectoparasites carried by hedgehogs have been performed, the aim of this study was to characterize which tick and flea species are parasitizing hedgehogs in some regions of the north and centre of Portugal, and to identify which *Rickettsia* spp. are circulating in those arthropods.

Materials and methods

Animals, ectoparasites and tissue sampling

From January 2017 to October 2018, a total of 51 European hedgehogs captured in North and Central Portugal were rescued and treated in a Rescue and Rehabilitation Centre (RRC) in Porto, Portugal. A standard procedure adopted by the RRC upon arrival of the animals is the removal of all ectoparasites. Ticks and fleas were collected from each animal using forceps and stored in 70 % ethanol at room temperature until further processing. Whenever available, a maximum of 10 ticks and 10 fleas were randomly selected from each animal and submitted to DNA extraction. The number of ectoparasites parasitizing hedgehogs varied a lot (ticks: 1-374; fleas: 1–63) and due to costs we were not able to test all of them. Because of that we have established a maximum of 10 ticks and 10 fleas per

hedgehog when available. During this period, all deceased hedgehogs (n = 11) infested by ectoparasites housed at the RRC were necropsied at the Veterinary Pathology Laboratory of ICBAS-UP. All liver and lung samples were collected and stored at − 20°C for further processing.

Morphological and molecular identification of ticks and fleas

Ticks and fleas were identified to species level based on morphological characters using taxonomic keys (Estrada-Peña et al. 2004; Zurita et al. 2018). To confirm tick and flea species identification by molecular methods, DNA of each individual tick/flea was extracted and a conventional PCR targeting mitochondrial genes was performed on a randomly selected sample of ticks (n = 10 *Rh. sanguineus* and n = 5 *I. hexagonus*) and fleas (n = 6). DNA was extracted using alkaline hydrolysis procedure according to previously described methods (Schouls et al. 1999). For tick identification a conventional PCR reaction targeting partial region of the 16S rDNA was performed, using the primer pair 16S + 1/16S-1 which amplifies 456 bp, as previously described by Black and Piesman (1994). Regarding the fleas, PCR was performed targeting a fragment of 780 bp of the cytochrome oxidase subunit II (Cox-2) gene using the primer set F-Leu/R-Lys (Zhu et al. 2015), as previously described (Hornok et al. 2018b).

Tissue DNA extraction

DNA extraction from hedgehog organs (lung and liver) was performed using the EXTRACTME® DNA tissue kit (Blirt, Trzy Lipy, Poland), according to manufacturer's instructions and DNA was stored at − 20°C for further analysis.

Molecular detection of Rickettsia spp.

DNA extracted from individual ticks, fleas, pulmonary and hepatic tissues were screened for *Rickettsia* spp. from the SFG and typhus group (TG) using a conventional PCR, targeting a 511-bp fragment of the rickettsial outer membrane protein B (*omp*B) gene, using the primer set rOmpB-OF/OR, as previously described (Choi et al. 2005). To confirm positive results, ticks and fleas were then tested for citrate synthase (*glt*A) rickettsial gene, using the primer pair RpCs877p/RpCs1258n which amplifies a fragment of 381 bp (Regnery et al. 1991).

For fleas, for a more detailed characterization we additionally tested for 17-kDa protein (*htr*A) gene that amplifies a fragment of 434 bp using the primer set 17K-1/17-K2 (Labruna et al. 2007), and outer membrane protein A (*omp*A) gene that amplifies a 532-bp fragment with the primer set Rr190.70p/Rr190.602n (Regnery et al. 1991).

All positive amplicons were purified with ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) and sequencing was performed for both strands of PCR products by Sanger method, using the respective primers of different target genes.

Mitochondrial 16S rDNA, Cox-2 and *Rickettsia* partial gene sequences were manually corrected, 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 (http://blast.ncbi.nlm.nih.gov/Blast).

Results

From January 2017 to October 2018, 33 (65%) *E. europaeus* hedgehogs rescued by RRC from seven districts of the north and centre of Portugal were infested with ticks and fleas (Fig. 1). In total, 1892 ticks and 213 fleas were collected from these mammals. Out of the total, 17 hedgehogs were parasitized only with ticks (52%), seven only with fleas (21%) and nine with mixed infestation (27%) (Table 1).

Of the total number of ticks collected, 1719 (90.9%) were identified as *Rh. sanguineus* and 173 (9.1%) were *I. hexagonus*. Of the *Rh. sanguineus*, 1693 (98.5%) were adults (n = 919 males, n = 774 females) and 26 larvae. Among the *I. hexagonus*, 144 (83%) were female, 11 nymphs and 18 larvae. The tick infestation per hedgehog ranged from 2 to 374 specimens. All fleas were identified as *A. erinacei* (n = 213). The number of fleas collected per hedgehog ranged from 1 to 63.

Molecular detection and sequence analysis of mitochondrial gene confirmed previous morphological identification of *Rh. sanguineus*, *I. hexagonus* and *A. erinacei* in all specimens tested. The analysis of *Rh. sanguineus* partial 16S rDNA mitochondrial tick gene sequences obtained showed 99.8% (402/403 bp) identity with *Rh. sanguineus* from Chile (KX632155) in 7 of the 10 tested *Rhipicephalus* ticks. The sequence analysis of the other three *Rhipicephalus* ticks were not able to identify species due to the less quality of the DNA that was extracted with alkaline hydrolysis. Previous tests in our laboratory showed that this extraction method did not affect the DNA detection for pathogens but for mitochondrial DNA it sometimes does not work and can be solved by better extraction methods using beads or columns.

Regarding *I. hexagonus*, all five sequences obtained in our study were identical and, when compared with GenBank database, showed 100% (410/410 bp) identity with *I. hexagonus* (AF549844), previously described in phylogenetics studies from the USA, and 99.8% with ticks analysed from Poland (AF001400). Sequence analysis of Cox-2 gene

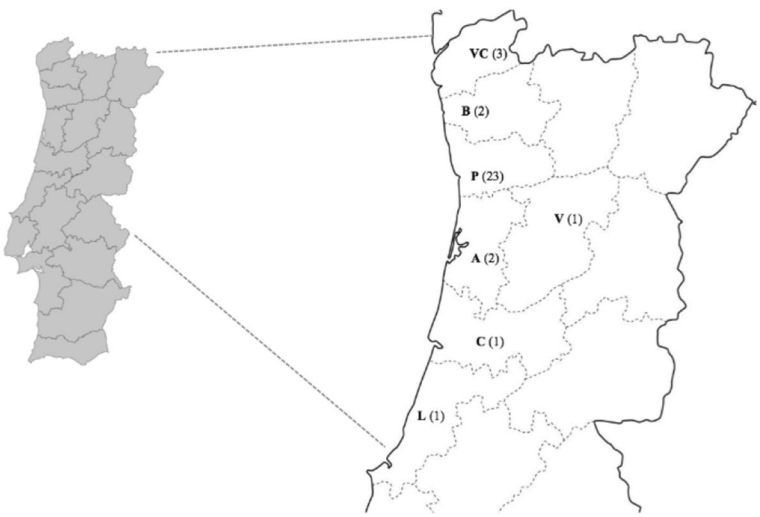

Fig. 1 Distribution in Portugal mainland of hedgehogs' collection sites. In parentheses (n) the number of hedgehogs rescued in that district. Viana do Castelo (VC); Braga (B); Porto (P); Aveiro (A); Viseu (V); Leiria (L); Coimbra (C)

Table 1 *Rickettsia* spp. detection in *Rhipicephalus sanguineus* s.l., *Ixodes hexagonus* and *Archaeopsylla erinacei* collected from 33 hedgehogs from Portugal, 2017–2018

District origin and hedgehog number		Season of hedgehog ectoparasite collection	Tick species and life stage*								*Rh. sanguineus* (no. positives/tested)	*I. hexagonus* (no. positives/tested)**	*A. erinacei* total fleas (no. positives/tested)	*Rickettsia* spp.
			Rh. sanguineus				*I. hexagonus*							
			F	M	N	L	F	M	N	L				
Viana do Castelo	1	Spring	4	30	–	–	–	–	–	–	5/10	–	–	*R. massiliae*
	2	Summer	–	–	–	–	1	–	–	–		0/1	14 (0/14)	
	3	Summer	22	50	–	–	–	–	–	–	5/10	–	–	*R. massiliae*
Braga	1	Spring	25	12	–	–	2	–	–	–	0/10	0/2	–	
	2	Spring	139	51	–	2	–	–	–	–	0/10	–	5 (0/5)	
Porto	1	Winter	10	22	–	–	–	–	–	–	3/10	–	–	*R. massiliae*
	2	Spring	8	2	–	–	–	–	–	–	0/10	–	–	
	3	Spring	44	31	–	1	–	–	–	–	4/10	–	–	*R. massiliae*
	4	Summer	–	–	–	–	2	–	–	–		0/2	1 (0/1)	
	5	Autumn	–	–	–	–	1	–	–	–		0/1	2 (2/2)	*R. asembonensis*
	6	Autumn	–	–	–	–	4	–	–	–		0/4	30 (12/30)	*R. asembonensis*
	7	Summer	–	–	–	–	12	–	6	5	0/10	0/10	7 (7/7)	*R. asembonensis*
	8	Summer	–	–	–	–	20	–	5	5	0/10	0/10	–	
	9	Spring	11	5	–	–	–	–	–	–	0/10	–	2 (0/2)	
	10	Spring	21	2	–	–	–	–	–	–	0/10	–	–	
	11	Spring	59	173	–	–	–	–	–	–	0/10	–	12 (0/12)	
	12	Spring	51	130	–	–	–	–	–	–	0/10	–	2 (2/2)	*R. asembonensis*
	13	Spring	172	48	–	–	5	–	–	–	0/10	0/5	–	
	14	Spring	1	2	–	16	–	–	–	–	0/10	–	–	
	15	Spring	–	2	–	7	–	–	–	–	0/9	–	–	
	16	Summer	3	22	–	–	–	–	–	–	0/10	–	–	
	17	Summer	5	17	–	–	–	–	–	–	5/10	–	–	*R. massiliae*
	18	Summer	44	65	–	–	–	–	–	–	0/10	–	–	
	19	Summer	13	21	–	–	–	–	–	–	0/10	–	–	

Table 1 (continued)

District origin and hedgehog number		Season of hedgehog ectoparasite collection	Tick species and life stage*								*Rh. sanguineus* (no. positives/tested)	*I. hexagonus* (no. positives/tested)**	*A. erinacei* total fleas (no. positives/tested)	*Rickettsia* spp.
			Rh. sanguineus				*I. hexagonus*							
			F	M	N	L	F	M	N	L				
	20	Summer	–	–	–	–	–	–	–	–		–	1 (0/1)	
	21	Summer	–	–	–	–	–	–	–	–		–	1 (0/1)	
	22	Autumn	–	–	–	–	–	–	–	–		–	15 (5/10)	*R. asembonensis*
	23	Autumn	–	–	–	–	–	–	–	–		–	63 (10/10)	*R. asembonensis*
Aveiro	1	Autumn	–	–	–	–	–	–	–	–		–	30 (7/10)	*R. asembonensis*
	2	Spring	140	234	–	–	94	–	–	8	0/10	0/10	–	
Viseu	1	Summer	–	–	–	–	3	–	–	–		0/3	–	
Leiria	1	Summer	2	–	–	–	–	–	–	–	0/2	–	–	
Coimbra	1	Summer	–	–	–	–	–	–	–	–		–	28 (10/10)	*R. asembonensis*
TOTAL			1719				173				22/212	0/48	213 (55/117)	

*F, female; M, male; N, nymph; L, larva. **All *I. hexagonus* tested were *Rickettsia* spp. negative

fragment revealed 99 % (529/535 bp) identity with *A. erinacei* (MG 637,370) described by Hornok et al. (2018b) in a phylogenetic study of comparative sequences of synanthropic flea species from Europe and Mediterranean area.

The results of *Rickettsia* detection and species characterization are regarding a sub-sample of the total ectoparasites collected. We identified *R. massiliae* in 22 of the 212 *Rh. sanguineus* tested and *R. asembonensis* in 55 of the 117 tested fleas (Table 1). All the positive ticks with *R. massiliae* were collected from five animals and the positive fleas for *R. asembonensis* were collected in eight hedgehogs (Table 1). None of the *I. hexagonus* tested was found infected with rickettsiae.

The comparison of our *R. massiliae* sequences (*omp*B sequence gene fragment) with other available sequences in GenBank database showed 99.6 % (462/464 bp) identity to *R. massiliae* MTU5 strain (CP000683) previously described in France.

Analysis of the *omp*B gene fragment sequences of the positive fleas showed 100 % (464/464 bp) identity with *R. asembonensis* detected in Peru (MK923741) and Asembo in Kenya (JN315972). As it was the first time that we detect this *Rickettsia* species in Portugal we have additionally confirmed our results with the characterization of *glt*A and *htr*A genes showing 100 % (341/341 bp) and 100 % (394/394 bp), respectively, with other sequences detected in Peru (MK923743, MK923744). No amplification was obtained for *omp*A gene. *Rickettsia* DNA was not detected in any of the tissue specimens collected from the 11 dead hedgehogs.

GenBank accession numbers of partial sequences obtained in this study are: MW114507 (Cox-2 gene fragment of *A. erinacei*); MW114503 (16S rDNA fragment of *I. hexagonus*); MW114506 (16S rDNA fragment of *Rh. sanguineus*); MK732016 to MK732024 (*omp*B gene fragment of *R. asembonensis*); MW114502 (*omp*B gene fragment of *R. massiliae*); MK732025 to MK732033 (*glt*A gene fragment of *R. asembonensis*); and MK862568 to MK862573 (*htr*A gene fragment of *R. asembonensis*).

Discussion

This research describes the results of a 22-month survey (Jan 2017–Oct 2018) regarding the characterization of ticks and fleas collected from rescued hedgehogs and reports the first identification of *R. asembonensis* in fleas in Portugal.

Ticks and fleas were collected from 33 hedgehogs rescued in seven districts in the north and centre of Portugal. Two ixodid ticks, *Rh. sanguineus* and *I. hexagonus*, were morphologically and genetically identified. *Rhipicephalus sanguineus* was the most prevalent tick species, accounting for 91 % of ticks collected. *Rhipicephalus sanguineus* has a worldwide distribution and in Portugal it is the most abundant species (Sanches et al. 2018). This tick species is well adapted to various ecological niches, and parasitizes a wide variety of wild and domestic animal hosts (Dantas-Torres et al. 2011).

Ixodes hexagonus is mainly found on carnivores and is geographically distributed across the Palearctic region (Estrada-Pena et al. 2017). The nidicolous behaviour of *I. hexagonus*, particularly of immature stages, supports the activity of all stages in the same host, place and throughout the entire year (Santos-Silva et al. 2011). In our study we corroborate this fact, as we found hedgehogs parasitized by all tick stages in various seasons of the year. In Europe *I. hexagonus* usually infests medium-size mammals that have a permanent dwelling such as carnivores (e.g., foxes, dogs, cats), mustelids and insectivores (hedgehogs) (Bernasconi et al. 1997). In Portugal, according to a nationwide network for surveillance

program, most of the *I. hexagonus* have been collected from dogs, but this fact is most probably related with accessibility to collect ticks from domestic animals compared to those collected from wild animals. The same observation was also reported for *I. hexagonus* collected from dogs and cats in other European countries (Krol et al. 2016).

Of the total number of *Rh. sanguineus* analysed, 10 % were positive for *Rickettsia* and all were identified as *R. massiliae*. Considered a mild pathogenic *Rickettsia*, it has been associated with only few human cases in Europe, such as in Italy (Cascio et al. 2013), France (Vitale et al. 2006), and Romania (Zaharia et al. 2016). *Rickettsia massiliae* was detected in Portugal for the first time in 1995 and various surveys performed in ticks collected from vegetation, humans and animals have estimated a prevalence of 4–18 % of *R. massiliae* in *Rh. sanguineus* (Bacellar et al. 1995; R. de Sousa, unpubl.). A similar finding reported that 11.2 % of *Rh. sanguineus* collected from Catalonia, Spain, were detected with *R. massiliae* (Beati et al. 1996).

All *I. hexagonus* tested in our study were negative for *Rickettsia* spp. However, other studies have detected *R. helvetica* in *I. hexagonus* collected from Belgian hedgehogs (Jahfari et al. 2017; Szekeres et al. 2019).

We report for the first time the presence of *R. asembonensis* in fleas collected in Portugal. *Rickettsia asembonensis*, a RFLO from spotted fever group of Rickettsia (SFGR) was identified for the first time in Kenya, and since then has been reported within Africa, Asia, Middle East, America and Europe (Maina et al. 2019). *Rickettsia asembonensis* has been isolated from the cat flea *Ctenocephalides felis*, but has also been detected in other flea species and in association with other arthropods such as ticks, e.g., *Amblyomma ovale* and *Rhipicephalus sanguineus* (Dall'Agnol et al. 2017; Troyo et al. 2016), and tropical rat mites (*Ornithonyssus bacoti*) from Egypt (Maina et al. 2019).

The percentage of positive-*Rickettsia* fleas (43 %) detected in our study was lower when compared with the prevalence (96 %) found in the same *A. erinacei* flea species collected in hedgehogs from Germany (Gilles et al. 2009). Although the flea species was the same as in the German study, our lower prevalence could be related with environmental conditions or to host abundance. *Rickettsia asembonensis* groups genetically related with other RFLOs that have been described worldwide such as *Rickettsia* sp. cf1 and 5 from South Carolina, USA (Reeves et al. 2005), *Rickettsia* sp. SE313 from Egypt (Loftis et al. 2006), *Rickettsia* sp. ARV5606 from Peru (Forshey et al. 2010), and *Rickettsia* sp. Hf56-2 from Germany (Gilles et al. 2009). Although the full pathogenic spectrum of RFLOs remains unknown, there is a report of DNA detection of RFLOs (*Rickettsia* sp. RF2125) in the blood sample of one Malaysian patient with clinical symptoms (Kho et al. 2016). That fact is not surprising considering that closely related *R. felis* has been associated with human disease.

We were unable to identify *Rickettsia* DNA in hedgehog tissue specimens. However, in previous studies the molecular detection of infectious agents such as *Anaplasma phagocytophilum*, *Borrelia afzelii*, *B. spielmanii*, *B. miyamotoi*, *R. helvetica*, and *Bartonella* species in hedgehog tissue samples suggested that these mammals may play a role in the transmission cycle and can develop transient rickettsiemia infecting non-infected ticks that parasitize this hosts (Skuballa et al. 2010; Szekeres et al. 2019). A larger study would be required to more accurately determine the prevalence and distribution of vector-borne agents carried by these mammals in Portugal. Nevertheless, the results of this study seem to indicate that rickettsiae are important pathogens carried by hedgehogs ectoparasites in Portugal.

The detection of *R. asembonensis* brings the number of SFGR species found in ticks (*R. conorii*, *R. mongolitimonae*, *R. slovaca*, *R. aeschlimannii*, *R. helvetica*, *R. massiliae*, *R. monacensis*, *R. raoulti*, *R. lusitaniae*) (Bacellar et al. 1995; de Sousa et al. 2006a; Milhano

et al. 2010, 2014; Sousa et al. 2008) and fleas (*R. felis*) (De Sousa et al. 2006b) in Portugal to 11. Surveillance and understanding of the potential tick and flea-borne pathogen transmission/infection routes for humans are fundamental to mitigate public health impact of vector-borne diseases. In Portugal, the nationwide network for surveillance of mosquitoes and ticks has been extremely important not only to identify the vector species but also to identify pathogens that are circulating in those vectors and can be transmitted to man. Being aware of which pathogens are circulating in each country can help to improve our perception and identification of clinical manifestations that can be related to these pathogens. The fact that until today we have detected two species of *Rickettsia* (*R. felis* and *R. asemboensis*) in fleas collected from hedgehogs can be a warning that these hosts can be important in the spread of fleas and their pathogens for humans. Bearing in mind that hedgehogs are very well adapted to urban peridomestic environments such as gardens and parks (Speck et al. 2013) and are often adopted and raised as pets at home.

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Authors' contributions Designed the study: PFB and RdS; sample collection: PFB and TLM; processed samples and extracted DNA: PFB; performed PCR and sequencing: PFB; Phylogenetic analysis: PFB, JRM and RdS; analysed data and wrote the manuscript: PFB, IA and RdS; reviewed the manuscript: IA, PF and FG. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval and consent to participate This study was approved by the Institute of Biomedical Sciences Abel Salazar animal ethics commission (191 /2017), from University of Porto, Portugal, as complying with the Portuguese legislation for the protection of animals (Law No. 92/1995 and Decree-Law no. 113/2013).

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