Molecular survey of *Hepatozoon* infection of *Teira dugesii* in the Azores

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Abstract

Molecular survey of Hepatozoon *infection of* Teira dugesii *in the Azores*. Hemogregarine parasites are found in many vertebrates, being most prevalent in reptiles, with lizards being the second most common hosts after snakes. *Hepatozoon* is the most widespread of the four genera that parasitize reptiles by infecting red blood cells. The *Hepatozoon* lifecycle requires blood–sucking invertebrates as vectors, and vector abundance can determine the parasite prevalence. To compare parasite prevalence between a large island and an islet without standing water, we analysed blood samples of the Madeiran wall lizard, *Teira dugesii,* at Praia Islet and Graciosa Island in the Azores, Portugal. We found a comparatively low prevalence of *Hepatozoon*, belonging to a new genetic line. The prevalence of this new parasite on the larger Graciosa Island was eight times higher than that for Praia Islet, which has no standing water sources. Our results are in line with a generally higher prevalence of blood parasites in sites with higher vector abundance.

Key words: *Teira dugesii*, *Hepatozoon*, Azores, Molecular analysis, Island size

Resumen

Estudio molecular de la infección de Teira dugesii *por* Hepatozoon *en las Azores*. Las hemogregarinas son parásitos que se encuentran en numerosos vertebrados, principalmente en reptiles, de los cuales el grupo más común son las serpientes seguidas de las lagartijas. *Hepatozoon* es el género más abundante de los cuatro que parasitan reptiles infectando los eritrocitos. El ciclo vital de *Hepatozoon* necesita invertebrados hematófagos como vectores, cuya abundancia puede determinar la prevalencia de los parásitos. Para comparar la prevalencia de los parásitos entre una isla grande y un islote sin agua estancada, analizamos muestras de sangre de lagartija de Maderia, *Teira dugesii* en el islote de Praia y en la isla Graciosa en las Azores, en Portugal. Encontramos una prevalencia comparativamente baja de *Hepatozoon*, perteneciente a una nueva línea genética. La prevalencia de este nuevo parásito en la isla Graciosa, de mayor tamaño, fue ocho veces superior a la del islote de Praia, que carece de fuentes de agua estancada. Nuestros resultados están en consonancia con la prevalencia generalmente más elevada de parásitos hemáticos en sitios con una mayor abundancia de vectores.

Palabras clave: *Teira dugesii*, *Hepatozoon*, Azores, Análisis molecular, Tamaño de isla

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Introduction

Parasites are an important, yet often poorly known, component of biodiversity (Morrison, 2009) and parasite studies have been used to answer complex questions of host–specificity and coevolution. Parasites play an important role as a crucial factor in evolution. As they constantly co–evolve with their host, they have a direct impact on natural communities and play an important role in ecosystems (Dobson and Hudson, 1986). A meta–analysis of 38 experimental studies on the cost of parasites in wild populations showed a moderately negative impact (Watson, 2013). Parasitic infections constantly challenge the immune system, and the influence of parasites was shown to be at least as strong as the influence of predation (Watson, 2013). Other stressors can be poor nutritional conditions, extreme climatic conditions and reproductive efforts (Quillfeldt et al., 2004), which can lead to a higher susceptibility to infections and even to death (Shutler et al., 1999). At the same time, parasitic infections can have a negative impact on the nutritional status, and thus lead to decreased reproductive success due to diminished manifestation of sexual ornaments and weakened physical condition (Hamilton and Zuk, 1982; Read, 1991; Zuk, 1992).

From an evolutionary perspective, parasites can act as a significant selective agent and contribute to the development and maintenance of rare host genotypes and speciation (Haldane, 1992). At the genetic level, parasites are a major driving factor for the development of the complex immune system of vertebrates (Hedrick, 1994) and may structure host genotypic polymorphism (Clarke, 1979) and thus determine genetic structure (Shykoff et al., 1991).

Hemogregarines are a group of the phylum Apicomplexan, Adeleorina, which are intracellular parasites. The Apicomplexa are a poorly studied group, with only about 0.1 % of species described (Morrison, 2009). Hemogregarines are the most prevalent parasites in reptiles, with lizards being the second most commonly affected hosts after snakes (Smith, 1996). Currently, four genera of hemogregarines are known to parasite reptiles: *Hepatozoon* (Miller, 1908), *Haemogregarina* (Danilewsky, 1885), *Karyolysus* (Labbé, 1994) and *Hemolivia* (Smith , 1996; Petit et al., 1990; Smith and Desser, 1997; Telford, 2009). The genus *Hepatozoon* is the most common genus among aquatic and terrestrial reptiles and is widely distributed among all other groups of tetrapods (Telford, 2009). In 1908, Miller described the genus *Hepatozoon* infesting leucocytes in rats. The first record of *Hepatozoon* in reptiles was made by Hoare (Hoare, 1932), who described sporogony of *Hepatozoon pettiti* (Thiroux, 1910, 1913) in tsetse flies (*Glossina palpalis*) that feed on infected Nile crocodiles (*Crocodylus niloticus*). This group can be very common in reptiles. *Hepatozoon* spp., for example, was found in 100 water pythons (*Liasis fuscus*) and seven other species of snakes with 100 % prevalence (Ujvari et al., 2004). In general, our knowledge of hepatozoonosis in wild animals like reptiles is far less than our understanding in pets and livestock, and further studies are required. In short–lived species, such as lizards,

hemogregarine infections are normally non–detrimental to host condition (Amo at al., 2005b).

Over 300 species are described within the genus *Hepatozoon*, and the wide variety of morphological characteristics, life–histories and hosts is extensive (Smith, 1996). *Hepatozoon* has been found to be paraphyletic (Barta et al., 2012; Maia et al., 2014). Thus, it was suggested to divide the genus into two genera, as all species of *Hepatozoon* except those that infect carnivores form a monophyletic group (Smith and Desser, 1997).

The present study was carried out in the Azores archipelago on an introduced species of lizard, the Madeiran wall lizard *Teira dugesii* (Milne–Edwards, 1829). The introduction of new species can introduce new pathogens, such as parasites, which could facilitate host switching to new naïve hosts (Gurevitch and Padilla, 2004). In Iberian water frogs *Pelophylax perezi*, which have been introduced in the Azores, one individual from São Miguel Island was found to be infected with *Hepatozoon* (Harris et al., 2013). *Hepatozoon* is commonly found in lizards and other reptiles in Portugal, Spain and the Maghreb region of North Africa (Maia et al., 2011, 2012, 2014), while Hepatozoonosis was not found in *Podarcis* sp. lizards In North America (Burke et al., 2007).

Studies in birds indicate that the prevalence of parasites is usually higher in freshwater inland habitats than in marine coastal habitats (Mendes et al., 2005) and higher on larger islands than on smaller ones (Lindström et al., 2004) due to the presence of more vectors. Vector distribution has been proposed as a factor affecting habitat choice in shorebirds, allowing them to make lower investments in immunofunction when living in saline areas due to lower risk of contact with vector–borne transmitted pathogens (Piersma, 1997).

Given that *Hepatozoon* sp. Is, on one hand, prevalent in reptiles worldwide and was already present in frogs in the Azores, and, on the other hand, that more studies are needed to answer questions regarding systematics, lifecycle and distribution, our aim was to determine the prevalence of *Hepatozoon* sp. in *T. dugesii* in the Azores.

Specifically, we tested the following hypotheses: *Hepatozoon* sp. is present in *Teira dugesii* in the Azores considering that it is widespread and very common among reptiles in similar habitats.

The prevalence of *Hepatozoon* infections is higher on the main island Graciosa than on the small islet of Praia because the likelihood of an infection on the former is higher due to the presence of freshwater bodies and thus more areas allowing vector reproduction and a larger Madeiran wall lizard population.

Material and methods

Study site

The study took place in the archipelago of the Azores, Portugal, in the subtropical northern Atlantic on Graciosa (fig. 1, 39º 3' 5" N 28º 0' 51" W, surface area
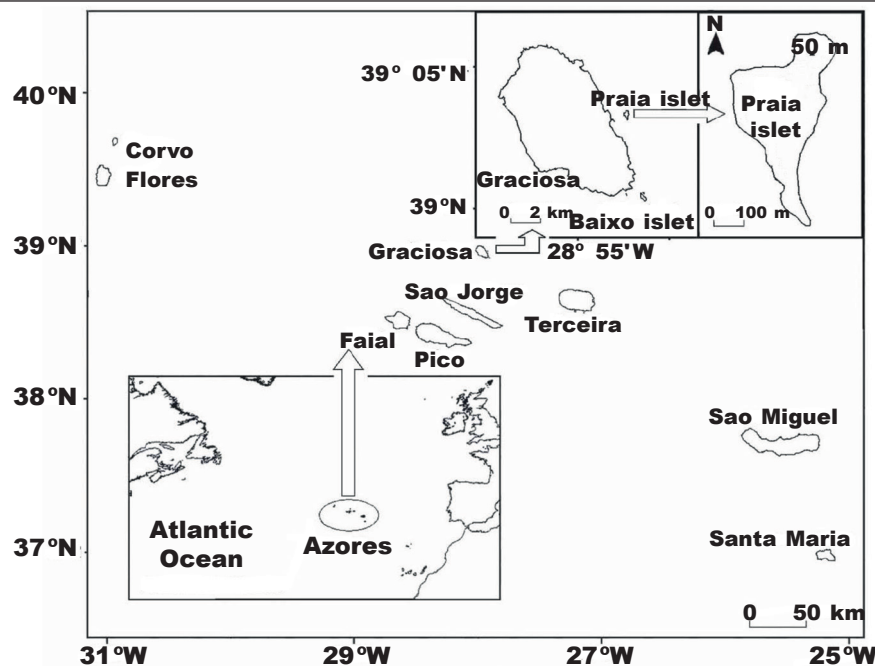
Fig. 1. Location of Graciosa and Praia Islet within the Azores archipelago (adapted from Bried and Neves, 2014).

Fig. 1. Ubicación de la Graciosa y del islote de Praia en el archipiélago de las Azores (adaptado de Bried y Neves, 2014).

60.66 km²) and the nearby small Praia Islet (fig. 1, 'Ilhéu da Praia', surface area 0.12 km²; stands about 1 km offshore Graciosa). Praia islet is uninhabited and has no water sources. Graciosa Island, in contrast, has about 5,000 inhabitants, several farms and open water tanks for livestock, as well as natural water sources. Graciosa is classified by UNESCO as a Biosphere Reserve, and Praia Islet is a breeding site for several seabird species. Between 1995 and 1997, rabbits (the only mammals on Praia Islet, introduced about 50 years earlier; Bried and Neves, 2014) were completely eradicated to restore seabird habitat, to stop soil erosion through overgrazing, and to protect seabird nests from being destroyed. After their eradication, native plants were reintroduced and successfully spread, and invasive exogenous plants were removed. Seabird breeding numbers have since increased (Bried et al., 2009). Today Praia Islet is a natural reserve and protected area, and entry is restricted.

Madeiran wall lizard *Teira dugesii*

The Madeiran wall lizard is a relatively new species in the archipelago of the Azores, Portugal (Malkmus, 1995). Here the subspecies *Teira dugesii dugesii* can be found, the sturdiest of all the subspecies. Other subspecies are *Teira dugesii jogeri,* a native inhabitant of the archipelago of Madeira (Madeira, Deserta Grande, Bugio, Porto Santo and some smaller islets, Brehm et al., 2003; Arnold et al., 2007) and *Teira*

dugesii selvagensis (Bischoff et al., 1989) from the Selvagens Islands. *Teira dugesii* was introduced to the Azores around 1860 via vessels and, lacking competitors, it rapidly multiplied. It is the only established terrestrial reptile in the archipelago of the Azores (Malkmus, 1995).

Teira dugesii can colonize different habitats from sea level to 1850 m above sea level but it becomes rarer at higher altitudes. In the Azores, they are almost exclusively found 4 km within the coastline and not at elevations higher than 220 m (Malkmus, 1995). They can inhabit open and rocky areas but also more heavily vegetated habitats. In the Azores, *T. dugesii* usually lives on lava fields, agricultural areas and gardens with stonewalls and villages in close proximity to the coast. In some places, this species can reach very high densities. Due to the temperate to sub–tropical climate in the region, this species does not hibernate in the Azores. They are territorial and males can show quite aggressive behavior, especially during the mating season (Glandt, 2010). Common threats for *T. dugesii* are cats, dogs, rats, birds of prey, and gulls. According to the IUCN, *T. dugesii* is listed as of Least Concern.

Hepatozoon species and their life cycle

Hemoparasites have a complex lifecycle with different life stages, and they require more than one host to complete it. All *Hepatozoon* species in general follow

the same lifecycle, consisting of sexual gametogony and asexual sporogony inside an hematophagous invertebrate host and merogony followed by gametogony inside a vertebrate intermediate host. Most of the life stages are haploid; only the zygote is diploid. Environmental factors determine the later sex of parasite clones (Lucius and Loos–Frank, 2008).

The first invasive stadium of Apicomplexans is the wormlike sporozoite, which penetrates host cells of different tissues, e.g. blood cells, to develop into a trophozoite. After maturing, asexual schizogony occurs and the daughter cells differentiate into merozoites. Normally, several schizogony cycles occur and then the merozoites develop into gametocytes, which later differentiate into gametes. The fusion of a microgamete and a macrogamete will result in a diploid zygote. During sporogony, the sporont accrues and further differentiates into sporoblasts. The sporoblasts then divide and build sporozoits (Lucius and Loos–Frank, 2008). The meront stage of *Hepatozoon* can be found in different tissues and organs, depending on the species. In reptiles infected by *Hepatozoon* species, the gamont stage can usually be found in the erythrocytes, in birds and mammals most often in leucocytes (Lucius and Loos–Frank, 2008; but see Merino et al., 2014).

In contrast to most protozoic or bacterial pathogens that are passed on by vectors, *Hepatozoon* is not transmitted during blood sucking through the salivary glands of a hematophagous arthropod or annelid but by swallowing and ingesting of the oocyst–carrying arthropod by the intermediate (vertebrate) host. Definitive hosts are blood–sucking invertebrates (Smith, 1996). Most studies of life cycles of *Hepatozoon* species infecting reptiles have been conducted on mosquitoes of the genera *Culex* sp., *Aedes* sp. and *Anopheles* sp., but ticks, mites, heteroptera and leeches can also act as vectors (Smith, 1996; Telford, 2009). Another pathway of transmission would be predation of an infected vertebrate host by another vertebrate. Here, the cystic stadium from the prey tissue is consumed (Smith, 1996). One example would be the transmission of *Hepatozoon domerguei*: A lacertid lizard that was infected with oocysts throughconsumption of an infected mosquito developed cystic forms of the parasite and was predated by a snake in which *H. domerguei* developed meronts and gamonts (Landau et al., 1972).

In the vertebrate host, *Hepatozoon* infections can lead to clinical inflammation and can have a negative effect on hemoglobin concentration. The immune response is dependent on the adaptation to a specific host, the localization in the tissue, and the host´s immune status (Jacobson, 2007). In general, there is still little information about the clinical effects on reptiles (Maia et al., 2011).

Field work

Field work took place from May to July 2016. Blood samples of the Madeiran wall lizards on Graciosa were collected on 18 June 2016. Lizards were caught by hand or in traps made from empty 5 liter plastic water containers with tomato juice and pieces of fresh or dried fruit used as bait. The traps were stabilized by stones and roof tiles. Individuals were measured, weighed, sexed according to coloration and the presence of femoral pores, and examined for ticks and mites. We detected only one mite on a single male lizard. The base of the tail was disinfected using ethanol. A blood sample was then drawn from the caudal (tail) vein (see Divers and Mader, 2005), with a sterile insulin syringe (0.33 x 12.7 mm, 29G), and a drop was transferred onto a Whatman FTA classic card. We took 33 blood samples on Praia Islet and 32 on Graciosa. We also made 48 blood smears from individuals that provided sufficient blood and that were also sampled on FTA cards for genetic analysis (24 at Praia and 24 at Graciosa). We used blood as the tissue for parasite detection because, as stated above, in reptiles, the gamont stage can usually be found in the erythrocytes (Lucius and Loos–Frank, 2008). After the procedure, the lizards were released at the capture site.

Laboratory analyses

In the laboratory, a 2x2 mm piece of the dried blood sample was cut out of the FTA classic card. The DNA was then isolated using an Ammonium–acetate protocol (adapted from Martínez et al., 2009). The final DNA–concentration of the sample was determined with a NanoDrop2000c UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA). In total, we successfully isolated DNA from 60 blood samples, 30 from Praia Islet and 30 from Graciosa. The analyses with the NanoDrop2000c UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA) confirmed the presence of nucleic acid (DNA) in all 60 of our isolated samples (from below 10 ng/µl up to 32.5 ng/µl).

These 60 DNA samples were screened for parasite presence using the polymerase–chain–reaction (PCR). We used the primers HepF300 (5'–GTTTCTGACCTATCAGCTTTCGACG–3') and Hep900 (5'–CAAATCTAAGAATTTCACCTCTGAC–3') that target a part of the 18S rDNA gene in *Hepatozoon* spp. (Ujvari et al., 2004). The primers were designed to amplify *Hepatozoon* parasites and thus adequate for this study, but they are also found to amplify other parasite species such as *Eimeria* and *Sarcocystis* (Harris et al., 2012).

The PCR reactions were run in a 16 µl mixture containing 2.5 µl of template DNA, 8 µl Hot Star TaqQiagen Plus Master Mix Kit (stock conc. 2x), and 1 µl of each primer. The reactions were cycled at the following parameters using a peqSTAR 96Q thermal cycler (Peqlab): 95 °C for 5 min (polymerase activation), 35 cycles at 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min, and a final extension at 72 °C for 10 min. We also run positive and negative PCR controls.

The PCR–amplicons were visualized using QIAxel (Qiagen) high–resolution capillary gel electrophoresis. Five samples showing the strongest peaks during gel electrophoresis were Sanger sequenced by Seqlab–Microsynth (Göttingen, Germany).

Table 1. Blood parasite species, GenBank accession number (G Num), host species, location, authors, and sequences obtained in this study and reference sequences for the phylogenetic relationships in figure 2 obtained from Genbank. Author's abbreviations: 1, this study; 2, Maia et al. (2011); 3, Maia et al. (2012); 4, Maia et al. (2014); 5, Tomé et al. (2014); 6, Tomé et al. (2016); 7, Tateno et al. (2013).

Tabla 1. Especie de parásito hemático, el número de muestra, la especie hospedante, la localización, los autores y las secuencias obtenidas en este estudio para las relaciones filogenéticas en la figura 2 obtenidas de GenBank. (Para las abreviaturas de los autores véase arriba).

Blood parasite	G Num	Host species	Location	Author
Lizards				
Sample L_35	MH201396	*Teira dugesii*	Azores: Graciosa	1
Sample L_56	MH201397	*Teira dugesii*	Azores: Graciosa	1
Sample L_58	MH201399	*Teira dugesii*	Azores: Graciosa	1
Sample L_61	MH201398	*Teira dugesii*	Azores: Graciosa	1
Hepatozoon sp.	HQ734792	*Podarcis vaucheri*	Morocco: Lake Tislit	2
Hepatozoon sp.	HQ734794	*Podarcis vaucheri*	Morocco: Lake Tislit	2
Hepatozoon sp.	HQ734799	*Timon pater tangitanus*	Morocco: 15 km N of Azrou (Balcon d'Ito)	2
Hepatozoon sp.	HQ734801	*Timon pater tangitanus*	Morocco: Agoudal	2
Hepatozoon sp.	JX531955	*Podarcis bocagei*	Portugal: Viana do Castelo	3
Hepatozoon sp.	JX531916	*Podarcis hispanica*	Spain: Alba de Tormes	3
Hepatozoon sp.	JX531920	*Podarcis lilfordi*	Spain: Balearics Islands, Cabrera	3
Hepatozoon sp.	JX531925	*Podarcis bocagei*	Portugal: Viana do Castelo	3
Hepatozoon sp.	JX531955	*Podarcis bocagei*	Portugal: Viana do Castelo	3
Hepatozoon sp.	JX531957	*Podarcis hispanica*	Spain: Alba de Tormes	3
Hepatozoon sp.	JX531958	*Podarcis bocagei*	Portugal: Viana do Castelo	3
Hepatozoon sp.	JX531973	*Podarcis lilfordi*	Spain: Balearic Islands, Cabrera	3
Hepatozoon sp.	KJ189415	*Podarcis bocagei*	Portugal: Gêres	4
Hepatozoon sp.	KJ189416	*Podarcis bocagei*	Portugal: Gêres	4
Hepatozoon sp.	KJ189417	*Podarcis hispanica*	Portugal: Gêres	4
Hepatozoon sp.	KJ189418	*Podarcis hispanica*	Portugal: Gêres	4
Hepatozoon sp.	KJ189421	*Podarcis bocagei*	Portugal: Gêres	4
Hepatozoon sp.	KJ189422	*Podarcis hispanica*	Portugal: Gêres	4
Skinks				
Hepatozoon sp.	HQ734796	*Eumeces algeriensis*	Morocco: close to Ouazzane	3
Geckos				
Hepatozoon sp.	KJ408510	*Cerastes cerastes*	Morocco: Figuig	5
Hepatozoon sp.	KJ408527	*Psammophis schokari*	Morocco: Kaar Es Souk	5
Hepatozoon sp.	KU680423	*Tarentola angustimentalis*	Spain	6
Cats				
Hepatozoon felis	AB771546	*Prionailurus iriomotensis*	Japan: Okinawa	7

Data analyses

Forward and reverse sequences were aligned in CLC Main Workbench 7.6.4. and checked for quality. Variable bases and conflicts in nucleotides were resolved when possible by visually inspecting the traces of the aligned forward and reverse sequences. One of five sequences was discarded due to low quality. The final four sequences (length 471 bp) were used in a Blast search to find matching sequences. Sequences with the highest similarity (97–98 %) were downloaded. These sequences (N = 22) and the results from our own samples (see table 1) and *Hepatozoon felis* (AB771546.1, Tateno et al., 2013) as outgroup were aligned in BioEdit using CrustalW multiple alignment and very long overhangs were removed. The resulting
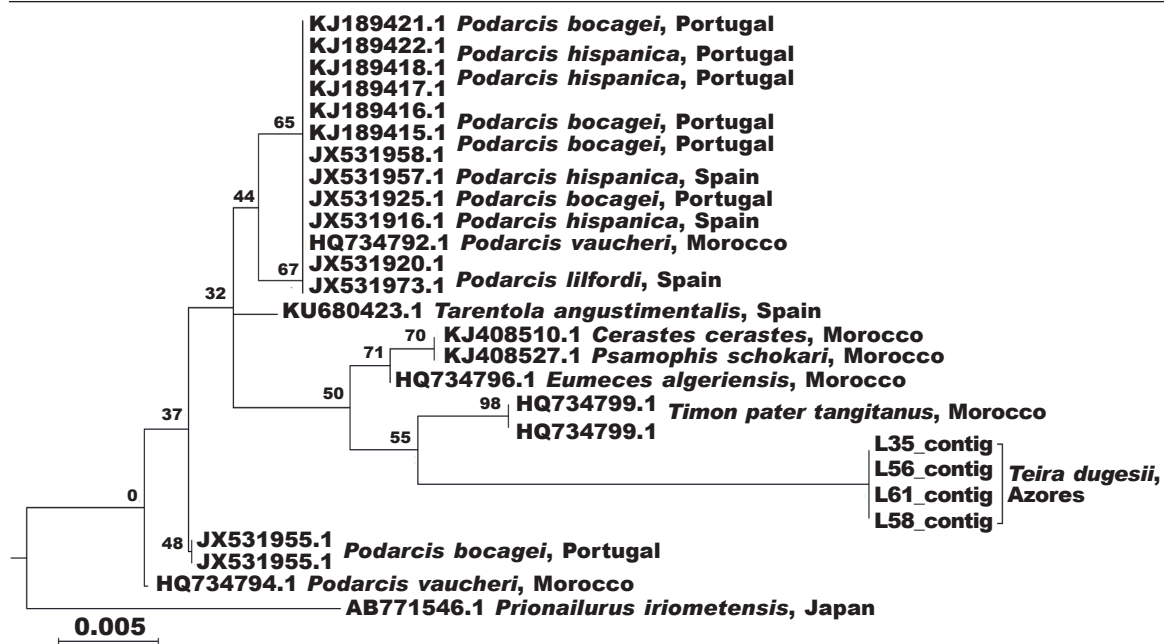
Fig. 2. Phylogenetic relationships for blood parasites found in *Teira dugesii* on Graciosa and Praia islet inferred with maximum–likelihood. Branch support is shown above the nodes. Table 1 shows details for the samples used (22 reference samples, four study samples, one outgroup).

Fig. 2. Relaciones filogenéticas de los parásitos hemáticos encontrados en Teira dugesii en la Graciosa y el islote de Praia, inferidas con la máxima probabilidad. El soporte de las ramas se indica encima de los nodos. En la tabla 1 se muestra información detallada sobre las muestras utilizadas (22 muestras de referencia, cuatro muestras del estudio y un grupo externo).

number of variable positions was 23 out of 471 and the number of parsimony–informative positions was 18 out of 471. The overall mean p–distance was d = 0.012. A matrix of pairwise distances is provided as supplementary material (table 1S). A maximum–likelihood phylogenetic tree was constructed based on 1000 bootstrap replications by fitting the best model (T92, Tamura 3–parameter, with gamma distribution) in Mega 6.0 (fig. 2).

To obtain images of the parasites and infected cells (erythrocytes), blood smears were stained with Giemsa stain and a monolayer of blood cells was scanned with a light microscope (125x, oil immersion, Primo Star ZEISS).

To test if the prevalence of blood parasites increased with the size of the lizards, we carried out a GLM with length as dependent parameter and island, sex and PCR result as independent factors, in R 3.4.2. (R Core Team, 2017).

Results

One of 30 samples from Praia Islet (3.3%) and eight of 30 samples from Graciosa (26.7%) were PCR positive, with a significantly higher prevalence at the larger Graciosa Island (Fisher's Exact Test, P = 0.035).

All infected lizards were female (Fisher's exact test, P > 0.001). The lizards sampled on Graciosa were about 5 mm smaller (females: 6.3 ± 0.4 cm, males: 7.2 ± 0.3 cm SD) than the lizards sampled on Praia (females: 7.1 ± 0.2 cm, males: 7.7 ± 0.4 cm SD). The length differences between the lizards on the two islands and between sexes were statistically significant (GLM with length as dependent parameter and island, sex and PCR result as independent factors; effect of island: $F_{1, 56}$ = 23.6, P < 0.001, effect of sex: $F_{1, 56}$ = 32.8, P < 0.001). However, the length was not different for individuals with positive or negative PCR results (GLM as above, $F_{1, 56}$ = 1.0, P = 0.312).

The PCR–products from five samples with the highest signals were selected for sequencing, one from Praia (L_35) and four from Graciosa (L_55, L_56, L_58, L_61). The Blast search revealed a 97–98 % similarity with already published *Hepatozoon* sequences from other lacertid lizards and reptiles from Portugal, Spain and Morocco (table 1). These 22 reference sequences and one of *Hepatozoon felis* as outgroup were used to generate a maximum likelihood phylogenetic tree (fig. 2). The *Hepatozoon* sequences from the present study formed one cluster and were closest to the sequence of a parasite from a lacertid lizard from Morocco, *Timon pater tangitanus* (Maia et al., 2011). Microscopic examination of the slides of the sequenced

samples confirmed an infection with a blood parasite (i.e. presence of gamonts in erythrocytes) for three samples (L_35, L_56 and L_58, fig. 3), but no infection was observed for sample L_55, probably due to a very low intensity of infection.

Discussion

The aim of the present study was to examine whether *Hepatozoon* sp. infect *Teira dugesii* in the Azores and to compare the prevalence of *Hepatozoon* infections on the main island Graciosa with the small islet of Praia. Using genetic and microscopic methods, we detected the presence of *Hepatozoon* sp. in *Teira dugesii* red blood cells at Graciosa and Praia Islet in the Azores. On the main island Graciosa we found a prevalence of *Hepatozoon* sp. of 26.7 % and of only 3.3 % on Praia Islet, supporting the hypothesis for a higher prevalence on the larger island. Microscopic examinations confirmed the infection of erythrocytes as found in other reptiles (Lucius and Loos–Frank, 2008).

Few studies have characterized the apicomplexan parasites in reptiles at the molecular level, and the relationships of many of these protozoan species are unresolved (Morrison, 2009; Jirku et al., 2009). Phylogenetic analyses based on microscopy methods are intricate due to the scarcity of differential phenotypic traits, which qualifies molecular phylogenetics based on genetic data as the best method to shed more light on the subject (Morrison, 2009).

All of the infected lizards exhibited female–colouration but a larger sample size and genetic sex determination would be required to confirm sex as it is possible that some sub–adult males had not yet completely developed the typical male color pattern. An equal rate of infection with haemogregarines was observed among males and females of the ocellated lizard (*Timon lepidus*) in Spain (Amo et al., 2005a), while other studies of blood parasites of lizards suggested that males had a higher prevalence (Olsson et al., 2000; Klukowski and Nelson, 2001), probably due to the immune suppressive effects of testosterone, at least during the reproductive period (Roberts et al., 2004). Pregnant females, on the other hand, need to use a great amount of energy and metabolites for the development of eggs, energy that cannot be used for defense against parasites (Amo et al., 2005a). During our fieldwork, we found burrows of lizard eggs and observed frequent territorial fights among the males, indicating that sample collection overlapped with the breeding season.

The occurrence of *Hepatozoon* species varies significantly among lizard families, with the highest prevalence detected in lacertids, to which *T. dugesii* belongs (Maia et al., 2012). However, our study on Graciosa Island and Praia islet revealed a relatively low prevalence of *Hepatozoon* compared to the 90 to 100 % prevalence reported in *Podarcis hispanica* in the Iberian peninsula (Harris et al., 2012; Maia et al., 2012), and 70 % for *Podarcis lilfordi* in the Balearic Islands (Harris et al., 2012). The lower prevalence
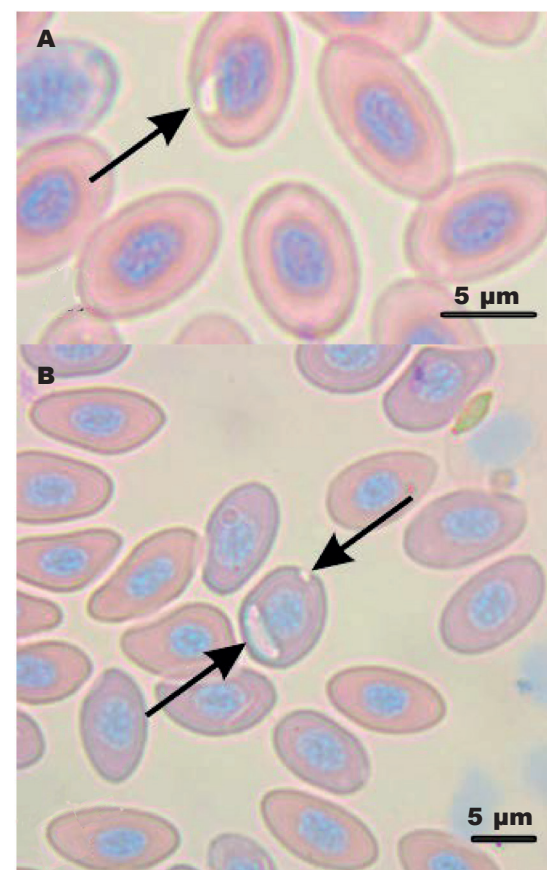

Fig. 3. Erythrocytes of the lizard *T. dugesii* from the Azores infected with a *Hepatozoon* blood parasite in samples L_35 (A) and L_56 (B), indicated with an arrow.

Fig. 3. Eritrocitos de la lagartija T. dugessi *de las Azores infectados con un parásito hemático del género* Hepatozoon *en las muestras L_35 (A) y L_56 (B), indicadas con una flecha.*

in the Azores and the Balearic Islands when compared to the Iberian peninsula might be partly due to a lower diversity of vectors in the Atlantic islands. The diversity of *Culicoides* species, for example, is much lower in the Azores than in mainland Portugal (Ramilo et al., 2012).

In *Timon lepiduss*, 72 % of all adults but no juveniles were positive for haemogregarines (Amo et al., 2005a), and prevalence or intensity of infection in adults did not differ between seasons or in relation to body condition. A positive correlation has been found between the intensity of *Hepatozoon* infection and body size and thus age in different short–lived lizard species (Maia et al., 2014), suggesting that the intensity of infection increases with longevity due to more encounters with parasites and reduced immunocompetence in older animals (Amo at al., 2005b; Palacios et al., 2011). In contrast to most protozoan

or bacterial pathogens transmitted by vectors, *Hepatozoon* is not transmitted during blood–sucking but by the vertebrate host swallowing the oocyst–carrying arthropod (Smith, 1996). Several vector species have been identified as infested with *Hepatozoon* oocysts or sporocysts of reptilian origin: the tsetse fly *Glossina palpalis* (Chatton and Roubaud, 2013; Macfie, 2016), ticks *Amblyomma dissimile* (Ball et al., 1969) and *Hyalomma* cf. *aegyptium* (Paperna et al., 2002), mites *Ophionyssus* sp. (Shanavas and Ramachandran, 1990) and *Hirstiella* sp. (Lewis and Wagner, 1964), predatory bugs *Triatoma arthurneiva* (da Rocha, 1975) and *Triatoma rubrovaria* (Osimani, 1942) and the sandfly *Lutzomyia vexator occidentis* (Ayala, 1970). Although to our knowledge there are no studies on arthropods parasites in the Azores, several Culicoide species recently reported to the archipelago (Ramilo et al., 2012) represent plausible vectors.

Lizards may acquire mites or ticks from their conspecifics when they share favorable places such as basking spots and refuges. They have been identified as vertebrate hosts of ixodid ticks (Dantas–Torres et al., 2008). *T. dugesii* has been found to host immature stages of *Ixodes ricinus* (De Sousa et al., 2012). The lizards that we examined during our field work showed virtually no ectoparasites, except for a mite on one male. Mosquitoes could also be vectors of *Hepatozoon* if ingested (e.g. shown for *Schellackia*: Lainson et al., 1976). On Praia Islet, where there are no freshwater reservoirs, virtually no mosquitoes were present, in contrast with Graciosa, which could be an explanation for the higher prevalence of hepatozoonosis. Superficial examinations of seabirds studied on Praia Islet revealed almost no ticks or mites, and only bird lice were frequently detected. On Madeira Island, *T. dugesii* was infested with ticks in one of the two studied areas (De Sousa et al., 2012).

Recently, four distinct main lineages of *Hepatozoon spp.* were found in wall geckos of the genus *Tarentola* from European and African countries adjacent to the Mediterranean Sea (Tomé et al., 2016). Two of these lineages clustered closely together not only with those previously known from individuals of the genus *Tarentola* and other species of geckos, but also with those from other reptiles and from rodents. A higher abundance of rodents could thus be another explanation for the eight times higher prevalence of *Hepatozoon* infections on Graciosa than on Praia Islet. The only mammals known to be present on Praia were rabbits, but they were eradicated by 1997 (Bried and Neves, 2014), while rats, ferrets, lifestock, cats and dogs are present in Graciosa, and all these mammals can also be hosts for *Hepatozoon* (Baneth et al., 2003).

Some studies have suggested that *Hepatozoon* sp. infections are not host–specific and the parasite has the ability to switch easily between different host species (Maia et al., 2011). Other studies, however, have found a narrow host–specificity for some *Hepatozoon* sp. regarding vertebrate hosts (Telford et al., 2001), and the definitive invertebrate host (Carreno et al., 1997). Therefore, further studies are needed regarding relationships between lizard hosts, the arthropod *Hepatozoon* sp. vectors and other potential vertebrate hosts.

In conclusion, the findings from this research supported both our hypotheses. We found a previously undescribed genetic lineage of *Hepatozoon* sp. infecting *Teira dugesii* in the Azores and a higher prevalence of *Hepatozoon* infections on the main island Graciosa than on the small islet of Praia. Further studies in the Azores are necessary to determine the potential role of other vertebrate species in the circulation of *Hepatozoon*, such as rats, cats, dogs, birds of prey and seabirds.

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Table 1s. Matrix of pairwise p–distances between each of the 22 reference sequences, the four sequences from this study and the outgroup (AB771546). For identity of the reference samples please see table 1 in the main text: A, JX531955.1; A, HQ734792.1; B, HQ734794.1; C, HQ734796.1; D, HQ734799.1; E, HQ734801.1; F, JX531916.1; G, JX531920.1; H, JX531925.1; I, JX531955.1; J, JX531957.1; K, JX531958.1; L, JX531973.1; M, KJ189415.1; N, KJ189416.1; O, KJ189417.1; P, KJ189418.1; Q, KJ189421.1; R, KJ189422.1; S, KJ408510.1; T, KJ408527.1; U, KU680423.1; V, L35_contig; W, L56_contig; X, L61_contig; Y, L58_contig; Z, AB771546.1.

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	0,004												
B	0,002	0,006											
C	0,008	0,008	0,011										
D	0,015	0,015	0,017	0,008									
E	0,015	0,015	0,017	0,008	0,000								
F	0,004	0,000	0,006	0,008	0,015	0,015							
G	0,004	0,004	0,006	0,008	0,015	0,015	0,004						
H	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004					
I	0,000	0,004	0,002	0,008	0,015	0,015	0,004	0,004	0,004				
J	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004			
K	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000		
L	0,004	0,004	0,006	0,008	0,015	0,015	0,004	0,000	0,004	0,004	0,004	0,004	
M	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000	0,000	0,004
N	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000	0,000	0,004
O	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000	0,000	0,004
P	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000	0,000	0,004
Q	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000	0,000	0,004
R	0,004	0,000	0,006	0,008	0,015	0,015	0,000	0,004	0,000	0,004	0,000	0,000	0,004
S	0,011	0,011	0,013	0,002	0,011	0,011	0,011	0,011	0,011	0,011	0,011	0,011	0,011
T	0,011	0,011	0,013	0,002	0,011	0,011	0,011	0,011	0,011	0,011	0,011	0,011	0,011
U	0,004	0,004	0,006	0,008	0,015	0,015	0,004	0,004	0,004	0,004	0,004	0,004	0,004
V	**0,023**	**0,025**	**0,025**	**0,023**	**0,023**	**0,023**	**0,025**	**0,025**	**0,025**	**0,023**	**0,025**	**0,025**	**0,025**
W	**0,023**	**0,025**	**0,025**	**0,023**	**0,023**	**0,023**	**0,025**	**0,025**	**0,025**	**0,023**	**0,025**	**0,025**	**0,025**
X	**0,023**	**0,025**	**0,025**	**0,023**	**0,023**	**0,023**	**0,025**	**0,025**	**0,025**	**0,023**	**0,025**	**0,025**	**0,025**
Y	**0,023**	**0,025**	**0,025**	**0,023**	**0,023**	**0,023**	**0,025**	**0,025**	**0,025**	**0,023**	**0,025**	**0,025**	**0,025**
Z	0,021	0,025	0,019	0,025	0,028	0,028	0,025	0,025	0,025	0,021	0,025	0,025	0,025

Tabla 1s. Matriz de las distancias observadas (distancias p) entre pares de las 22 secuencias de referencia, las cuatro referencias de este estudio y el grupo externo (AB771546). Para consultar la identidad de las muestras de referencia, véase la tabla 1 en el texto principal: A, JX531955.1; A, HQ734792.1; B, HQ734794.1; C, HQ734796.1; D, HQ734799.1; E, HQ734801.1; F, JX531916.1; G, JX531920.1; H, JX531925.1; I, JX531955.1; J, JX531957.1; K, JX531958.1; L, JX531973.1; M, KJ189415.1; N, KJ189416.1; O, KJ189417.1; P, KJ189418.1; Q, KJ189421.1; R, KJ189422.1; S, KJ408510.1; T, KJ408527.1; U, KU680423.1; V, L35_contig; W, L56_contig; X, L61_contig; Y, L58_contig; Z, AB771546.1.

N	O	P	Q	R	S	T	U	V	W	X	Y	Z
0,000												
0,000	0,000											
0,000	0,000	0,000										
0,000	0,000	0,000	0,000									
0,000	0,000	0,000	0,000	0,000								
0,011	0,011	0,011	0,011	0,011	0,011							
0,011	0,011	0,011	0,011	0,011	0,011	0,000						
0,004	0,004	0,004	0,004	0,004	0,004	0,006	0,006					
0,025	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**				
0,025	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,000**			
0,025	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,000**	**0,000**		
0,025	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,025**	**0,000**	**0,000**	**0,000**	
0,025	0,025	0,025	0,025	0,025	0,025	0,023	0,023	0,021	0,036	0,036	0,036	0,036