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Absence of haemoparasite infection in the fossorial amphisbaenian *Trogonophis wiegmanni*

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$\rm SUMMARY$

Blood parasites such as haemogregarines and haemosporidians have been identified in almost all groups of vertebrates. However, very little is known about biodiversity of these parasites and their effects on some major groups of reptiles such as amphisbaenians, a distinctive group with many morphological and ecological adaptations to fossorial life. Conditions of the fossorial environment might also affect host-parasite relationships. We investigated the presence and the potential prevalence of three genera of haemoparasitic aplicomplexan blood parasites (*Hepatozoon, Plasmodium* and *Haemoproteus*) in the amphisbaenian *Trogonophis wiegmanni*, a fossorial worm lizard species from North West Africa. Blood parasite infection was not detected in *T. wiegmanni*, both in visual surveys of blood smears and using molecular methods to detect DNA of such parasites in the blood of the potential amphisbaenian hosts. We discuss how conditions of the fossorial environment might affect blood parasitaemias in amphisbaenians as well as in other fossorial reptiles.

Key words: amphisbaenians, fossorial environment, haemoparasites, reptiles.

INTRODUCTION

Birds, mammals and reptiles harbour intracellular haemoparasites (i.e. plasmodiids, haemogregarines), which can cause serious damages to their hosts (Davies and Johnston, 2000; Goater *et al.* 2014). However, in comparison with other vertebrates, the amount of studies on blood parasites of reptiles is comparatively small (Telford, 2008). In many reptiles, it is common to find infection by haemoparasitic apicomplexan, a group of blood-borne intracellular sporozoans that includes, among others, the genera *Hepatozoon, Plasmodium* and *Haemoproteus* (Jacobson, 2007; Telford, 2008).

Although several studies have shown that many reptiles may tolerate haemoparasite infection and suffer little or no pathogenic effect (e.g. Wozniak *et al.* 1996; Amo *et al.* 2005; Vardo-Zalik and Schall, 2008), heavily blood infected lizards and snakes may show anaemia and immunosuppression, resulting in impaired growth, lower body condition, and decreased reproductive output and juvenile survival (e.g. Schall, 1990; Amo *et al.* 2004). However, in comparison with lizards or snakes, other major groups of reptiles, such as amphisbaenians, have

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received very little attention in the study of their blood parasites (Telford, 1984, 2008).

Amphisbaenians are one distinctive major group of fossorial reptiles with important morphological and functional adaptations to the underground life (e.g. reduced vision, elongated body and loss of limbs in most species) (Gans, 1978, 2005) and a suite of original responses to ecological demands (e.g. Papenfuss, 1982; Martín *et al.* 2013*a, b*). In addition, conditions of the fossorial environment might also affect host-parasite relationships. However, prevalence and genetic diversity of haemoparasites remain almost unknown for amphisbaenians (Smith, 1996; Gans, 2005; Telford, 2008). Here, we present the results of a survey for blood parasites in a North African population of the amphisbaenian *Trogonophis wiegmanii*.

MATERIALS AND METHODS

We conducted field work during two weeks in March 2014 (spring) and two weeks in September 2014 (autumn) at the Chafarinas Islands (Spain), a small archipelago located in the southwestern area of the Mediterranean Sea (35°11'N, 02°25'W), 2.5 nautical miles off the northern Moroccan coast (Ras el Ma, Morocco). This archipelago consists of three islands: Congreso (25.6 ha), Isabel II (15.1 ha) and Rey Francisco (13.9 ha). Vegetation is conditioned by

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the aridity of the warm, Mediterranean climate (i.e. average annual precipitation is 300 mm), the high soil salinity, and the guano accumulation from numerous seabird colonies (García *et al.* 2002). Thus, current vegetation is dominated by bushes adapted to salinity and drought, such as species of *Salsola*, *Lycium* and *Suaeda* genera (García *et al.* 2002). In general, the soils are shallow and immature, characterized by a thin A horizon over the original volcanic rock, and their characteristics have a deep effect on amphisbaenians condition (Martín *et al.* 2013*a*, 2015). In these islands, amphisbaenians are abundant and are always found buried in the substrate or under rocks (Martín *et al.* 2011, 2013*a*, *b*).

We searched for amphisbaenians in the three islands by lifting all stones found. We captured adult amphisbaenians (n = 75) by hand, gathered morphological measurements and took blood samples in situ, and released animals at their exact point of capture in less than 5 min. To avoid sampling the same individual twice, we avoided sampling the same area twice. We collected blood samples from the caudal sinus at the base of the tail by gently piercing the skin with a 23 G, 1", $0.60 \times$ 25 mm needle for each amphisbaenian and taking a droplet of blood with a heparinized capillary. We made blood smears by blowing a drop of blood onto the microscope slide. Smears were air-dried until coagulation. In the laboratory, the smears were fixed in absolute methanol for 10 min, and then stained in Giemsa diluted 1:9 with phosphate buffer (pH 7.2) for 40 min before their examination for parasites. On mounted slides, we scanned smears entirely at $200 \times$ along the length of the slide, looking for haemoparasites. Further, we looked for potential intraerythrocytic parasites at 400× by looking for the number of parasites in several random fields until totally about 2000 erythrocytes (Amo et al. 2004, 2005). Blood smears were checked under the microscope twice by two different researchers (J. M. and M. G.), who had previous experience with haemoparasites of reptiles (e.g. Amo et al. 2004, 2005; Garrido and Pérez-Mellado, 2013).

Additionally, we stored a few drops of blood in 500 µL SET buffer (0.015 м NaCl, 0.05 м Tris, 0.001 M EDTA, pH 8.0) at room temperature for molecular analyses. Genomic DNA was extracted from blood samples in the laboratory using a standard chloroform/isoamylalcohol method (Sambrook et al. 2002). To detect the presence of Hepatozoon spp. haemoparasites, we used a polymerase chain eaction (PCR) to amplify the 18S RNA gene of Hepatozoon spp. as described previously (Harris et al. 2011). Briefly, a nested PCR reaction with primers HEMO1 and HEMO2 (Perkins and Keller, 2001), and then primers HEPF300 and HEPR900 (Ujvari et al. 2004), were used for the Hepatozoon identification of infection. Additionally, detection of haemosporidian parasites

was made using primers and PCR protocol designed to amplify a portion of the cytochrome b gene of avian Plasmodium and Haemoproteus (Hellgren et al. 2004). This latter protocol has been successfully used for testing *Plasmodium* infection in reptiles (Davis et al. 2013). Briefly, diluted genomic DNA $(25 \text{ ng } \mu \text{L}^{-1})$ was used as a template in every PCR assay for detection of the parasites using nested-PCR protocols. The amplifications were evaluated by running $2.5 \,\mu\text{L}$ of the final PCR on a 2% agarose gel. All PCR experiments contained one negative control for every eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all potential positives were true.

RESULTS

We extracted blood from 75 adult amphisbaenian individuals. Visual careful examination at the microscope of the blood smears in two different scans made by two different observers did not result in the finding of any haemoparasite. To confirm these negative results, molecular analyses of 30 blood samples did not either yield any positive result for genomic DNA of either *Hepatozoon*, *Plasmodium* or *Haemoproteus*.

DISCUSSION

This is one of the very few studies that focused on the detection of haemoparasite infection in amphisbaenians (Pessoa, 1968; Telford, 1984; Lainson, 2003). Up to our knowledge, within fossorial reptiles, just a single amphisbaenian species, Amphisbaena alba, was found infected by haemogregarines (Haemogregarina amphisbaenae) in Brazil (Pessoa, 1968) and Venezuela (Telford, 1984). A review of the species of the genus Hepatozoon that infect reptiles (Smith, 1996) cited the mentioned H. amphisbaenae infecting A. alba as the only case of an intracellular protozoan infecting a fossorial reptile. However, Lainson (2003) was unable to encounter H. amphisbaenae in 43 blood samples of A. alba collected in various localities from Brazil. Lainson (2003) concluded that most probably the haemogregarine described by Pessoa (1968) did not belong to Haemogregarina genus. Likewise, Lainson (2003) suggested that the intracellular parasite that Telford (1984) found in just a single specimen of A. alba from Venezuela was probably the same species as Pessoa (1968) did. Our results, based on a wide survey of Trogonophis wiegmanni, indicate that there is a lack of haemoparasite infection in this amphisbaenian, at least in these island populations.

We propose several alternative hypotheses, not mutually exclusive, to explain the negative records of blood parasites in T. wiegmanni. First, the

absence of blood parasites has been commonly attributed to the lack of appropriate vectors in the environment (Bennett et al. 1992; Martínez-Abraín et al. 2004). Haemoparasites recognized of reptiles can be transmitted either by the ingestion of an invertebrate vector (mainly mites, ticks or leeches) that previously fed on infected reptiles (Smith, 1996; Telford, 2008) or either by the bite of the arthropod vector (Telford, 1984, 2008; Klein et al. 1987). While the former is considered the main transmission mechanism for haemogregarines (sensu lato), including all Hepatozoon spp., Plasmodium and Haemoproteus are most commonly infected through the bite of arthropod vectors, mainly flies and mosquitoes. Hence, either one or the other mechanism predominates, the inclusion of an arthropod vector is needed to complete the life cycle of the mentioned haemoparasites in reptiles. In this sense, fossorial habits of amphisbaenians might reduce the probability of a vector relationship evolving. This has been previously pointed out to explain why haemogregarines are found in all families of snakes except in those families containing species with strictly fossorial habitats, such as Typhlopidae, Leptotyphlopidae and Uropeltidae (Telford, 1984, 2008; Smith, 1996). The lack of infection by haemoparasites in our study population of amphisbaenians might also be due to the scarcity of suitable vectors (mites, ticks, mosquitoes, etc.) in the fossorial environment and the absence of these prey in the diet of amphisbaenians (Martín et al. 2013b). In fact, we have never observed mites or ticks as ectoparasites of T. wiegmanni in this population (Martín, unpublished results). Moreover, seabirds, which are very abundant in the study islands, are widely considered as potential dispersals of pathogens to remote islands, as they can act as dispersers of their arthropod vectors (Dietrich et al. 2011) and are infected by haemoparasites (Quillfeldt et al. 2011). However, the absence of haemoparasites in T. wiegmanni suggests that the role of seagulls is negligible in this case.

Second, it has also been proposed that marine and arid habitat seem to represent an unsuitable environment for potential vectors of haematozoan parasites (Little and Earlé, 1994; Piersma, 1997; Mendes *et al.* 2005). Therefore, island isolation or extreme arid and salinity conditions in the Chafarinas archipelago (Martín *et al.* 2015) might affect the presence of invertebrate hosts and therefore the prevalence of haemoparasites in amphisbaenians. However, we have found in the same study area blood parasites (*Hepatozoon*) in epigeal skinks (Fam. Scinicidae) and lizard (Fam Lacertidae) species that are sympatric with this amphisbaenian and share similar habitats but that are not fossorial (Martín, unpublished results).

Third, it might be possible that amphisbaenians could be free of blood parasites due to good

immunological capabilities (Ricklefs, 1992). However, other types of internal parasites, such as coccidians and nematodes, have been often found in amphisbaenians, also in our study species and population with high prevalences (e.g. Lainson, 2003; Megía-Palma *et al.* 2015).

Finally, the absence of blood parasites may also be attributed to lack of suitable host-parasite assemblage (Medeiros *et al.* 2013), such as problems faced by parasites to complete their life cycles in reptile species that spend their lives underground, or absence of physiological compatibility between haemoparasites and amphisbaenians.

We conclude that haemoparasites are absent in this population of the amphisbaenian *T. wiegmanni*. Further studies should consider whether this is a particular situation restricted to this species or to the particular island conditions of this population, or whether this is a general characteristic, related to the fossorial environment, of amphisbaenians and other fossorial reptiles.

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