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

JOSÉ MARTÍN[1]*, MARIO GARRIDO[2], JESÚS ORTEGA[1], ROBERTO GARCÍA-ROA[1], ALEJANDRO IBÁÑEZ[1,3] *and* ALFONSO MARZAL[4]

[1] *Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain*
[2] *Mitrani Department of Desert Ecology, Swiss Institute for Dryland Environmental and Energy Research, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben-Gurion, Israel*
[3] *Department of Evolutionary Biology, Zoological Institute, Technische Universität Braunschweig, Mendelssohnstr. 4, 38106 Braunschweig, Germany*
[4] *Departamento de Anatomía, Biología Celular y Zoología, Universidad de Extremadura, 06071 Badajoz, Spain*

(*Received 12 March 2016; revised 24 April 2016; accepted 29 April 2016; first published online 25 May 2016*)

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 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

* Corresponding author: Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain. E-mail: Jose.Martin@mncn.csic.es

doi:10.1017/S0031182016000950

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 × 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× 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 M NaCl, 0·05 M 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 identification of *Hepatozoon* 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 ng μL^{-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 μL 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

https://doi.org/10.1017/S0031182016000950 Published online by Cambridge University Press

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*. 2013*b*). 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. Scincidae) 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.

ACKNOWLEDGEMENTS

We thank two reviewers for helpful comments. The field station of the 'Refugio Nacional de Caza de las Islas Chafarinas' provided logistical support. We thank Juan Ignacio Montoya, Javier Díaz, Gonzalo Martínez, Angel Sanz, Francisco López, Alfredo Ruiz and Javier Zapata for friendship and help in the Islands. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

FINANCIAL SUPPORT

This work was supported by the 'Organismo Autónomo de Parques Nacionales' (J. M.), with additional financial support from Ministerio de Economía y Competitividad projects CGL2011-24150/BOS (J. M.), CGL2014-53523-P (J. M.) and CGL2015-64650 (A. M.).

REFERENCES

Amo, L., López, P. and Martín, J. (2004). Prevalence and intensity of haemogregarinid blood parasites in a population of the Iberian rock lizard, *Lacerta monticola*. *Parasitology Research* **94**, 290–293.

Amo, L., Fargallo, J. A., Martínez-Padilla, J., Millán, J., López, P. and Martín, J. (2005). Prevalence and intensity of blood and intestinal parasites in a field population of a Mediterranean lizard, *Lacerta lepida*. *Parasitology Research* **96**, 413–417.

Bennett, G. F., Montgomerie, R. and Seutin, G. (1992). Scarcity of haematozoa in birds breeding on the Arctic tundra of North America. *The Condor* **94**, 289–292.

Davies, A. J. and Johnston, M. R. (2000). The biology of some intraerythrocytic parasites of fishes, amphibia and reptiles. *Advances in Parasitology* **45**, 1–107.

Davis, A. K., Benz, A. C., Ruyle, L. E., Kistler, W. M., Shock, B. C. and Yabsley, M. J. (2013). Searching before it is too late: a survey of blood parasites in *Ctenosaura melanosterna*, a critically endangered reptile of Honduras. *ISRN Parasitology* **2013**, Article ID 495304, 1–6.

Dietrich, M., Gómez-Díaz, E. and McCoy, K. D. (2011). Worldwide distribution and diversity of seabird ticks: implications for the ecology and epidemiology of tick-borne pathogens. *Vector-Borne and Zoonotic Diseases* **11**, 453–470.

Gans, C. (1978). The characteristics and affinities of the Amphisbaenia. *Transactions of the Zoological Society of London* **34**, 347–416.

Gans, C. (2005). Checklist and bibliography of the amphisbaenia of the World. *Bulletin of the American Museum of Natural History*. **280**, 1–130.

García, L. V., Marañón, T., Ojeda, F., Clemente, L. and Redondo, R. (2002). Seagull influence on soil properties, chenopod shrub distribution, and leaf nutrient status in semi-arid Mediterranean islands. *Oikos* **98**, 75–86.

Garrido, M. and Pérez-Mellado, V. (2013). Prevalence and intensity of blood parasites in insular lizards. *Zoologischer Anzeiger – A Journal of Comparative Zoology* **252**, 588–592.

Goater, T. M., Goater, C. P. and Esch, G. W. (2014). *Parasitism. The Diversity and Ecology of Animal Parasites*, 2nd edn. Cambridge University Press, Cambridge.

Harris, D. J., Maia, J. P. M. C. and Perera, A. (2011). Molecular characterization of *Hepatozoon* species in reptiles from the Seychelles. *Journal of Parasitology* **97**, 106–110.

Hellgren, O., Waldenström, J. and Bensch, S. (2004). A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium*, and *Haemoproteus* from avian blood. *Journal of Parasitology* **90**, 797–802.

Jacobson, E. (2007). *Infectious Diseases and Pathology of Reptiles*. CRC Press, Florida.

Klein, T. A., Young, D. G., Telford, S. R. and Kimsey, R. (1987). Experimental transmission of *Plasmodium mexicanum* by bites of infected *Lutzomyia vexator* (Diptera: Psychodidae). *Journal of the American Mosquito Control Association* **3**, 154–164.

Lainson, R. (2003). Some coccidial parasites of the lizard *Amphisbaena alba* (Reptilia: Amphisbaenia: Amphisbaenidae). *Memórias do Instituto Oswaldo Cruz* **98**, 927–936.

Little, R. M. and Earlé, R. A. (1994). Lack of avian haematozoa in the Phasianinae of Robben Island. *Ostrich* **65**, 343–344.

Martín, J., Polo-Cavia, N., Gonzalo, A., López, P. and Civantos, E. (2011). Structure of a population of the amphisbaenian *Trogonophis wiegmanni* in North Africa. *Herpetologica* **67**, 250–257.

Martín, J., López, P. and García, L. V. (2013*a*). Soil characteristics determine microhabitat selection of the fossorial amphisbaenian *Trogonophis wiegmanni*. *Journal of Zoology* **290**, 265–272.

Martín, J., Ortega, J., López, P., Pérez-Cembranos, A. and Pérez-Mellado, V. (2013*b*). Fossorial life does not constrain diet selection in the amphisbaenian *Trogonophis wiegmanni*. *Journal of Zoology* **291**, 226–233.

Martín, J., López, P., Gutiérrez, E. and García, L. V. (2015). Natural and anthropogenic alterations of the soil affect body condition of the fossorial amphisbaenian *Trogonophis wiegamnni* in North Africa. *Journal of Arid Environments* **122**, 30–36.

Martínez-Abraín, A., Esparza, B. and Oro, D. (2004). Lack of blood parasites in bird species: does absence of blood parasite vectors explain it all. *Ardeola* **51**, 225–232.

Medeiros, M. C. I., Hamer, G. L. and Ricklefs, R. E. (2013). Host compatibility rather than vector-host-encounter rate determines the host range of avian *Plasmodium* parasites. *Proceedings of the Royal Society of London, Series B* **280**, 2947–2954.

Megía-Palma, R., Martínez, J., Acevedo, I., Martín, J., García-Roa, R., Ortega, J., Peso-Fernández, M., Albaladejo, G., Cooper, R. D., Paranjpe, D. A., Sinervo, B. R. and Merino, S. (2015). Phylogeny of the reptilian *Eimeria*: are *Choleoeimeria* and *Acroeimeria* valid generic names? *Zoologica Scripta* **44**, 684–692.

Mendes, L., Piersma, T., Lecoq, M., Spaans, B. and Ricklefs, R. E. (2005). Disease-limited distributions? Contrasts in the prevalence of avian malaria in shorebird species using marine and freshwater habitats. *Oikos* **109**, 396–404.

Papenfuss, T. J. (1982). The ecology and systematics of the amphisbaenian genus *Bipes*. *Occasional Papers of the Californian Academy of Sciences* **136**, 1–42.

Perkins, S. L. and Keller, A. K. (2001). Phylogeny of nuclear small subunit rRNA genes of hemogregarines amplified with specific oligonucleotídeos. *Journal of Parasitology* **87**, 870–876.

Pessoa, S. B. (1968). Sobre uma hemogregarina de *Amphisbaena alba*. *Gazeta Médica da Bahia* **68**, 75–78.

Piersma, T. (1997). Do global patterns of habitat use and migration strategies co-evolve with relative investments in immunocompetence due to spatial variation in parasite pressure? *Oikos* **80**, 623–631.

Quillfeldt, P., Arriero, E., Martínez, J., Masello, J. F. and Merino, S. (2011). Prevalence of blood parasites in seabirds – a review. *Frontiers in Zoology* **8**, 26.

Ricklefs, R. E. (1992). Embryonic development period and the prevalence of avian blood parasites. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 4722–4725.

Sambrook, J., Fritch, F. J. and Maniatis, T. (2002). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Schall, J. J. (1990). Virulence of lizard malaria: the evolutionary ecology of an ancient parasite-host association. *Parasitology* **100**, S35–S52.

Smith, T. G. (1996). The genus *Hepatozoon* (Apicomplexa; Adeleina). *Journal of Parasitology* **82**, 565–585.

Telford, S. R. (1984). Haemoparasites of reptiles. In *Diseases of Amphibians and Reptiles* (ed. Hoff, G. L., Frye, F. L. and Jacobson, E. R.), pp. 385–517. Plenum Press, New York.

Telford, S. R. (2008). *Hemoparasites of the Reptilia: Color atlas and text*. CRC Press, Boca Raton, Florida.

Ujvari, B., Madsen, T. and Olsson, M. (2004). High prevalence of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) infection in wáter pythons (*Liasis fuscus*) from tropical Australia. *Journal of Parasitology* **90**, 670–672.

Vardo-Zalik, A. M. and Schall, J. J. (2008). Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* **135**, 1363–1372.

Wozniak, E. J., Kazacos, K. R., Telford, S. R. and Mclaughlin, G. (1996). Characterization of the clinical and anatomical pathological changes associated with *Hepatozoon mocassini* infections in unnatural reptilian hosts. *International Journal of Parasitology* **26**, 141–146.