Fitness effects of *Hepatozoon* blood parasites in selected lizard species

Maria Isabel V. B. Damas Moreira

MSc in Biodiversity, Genetics and Evolution
Mestrado em Biodiversidade, Genética e Evolução

Faculty of Sciences of University of Porto
Faculdade de Ciências da Universidade do Porto

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Supervisor
Dr. David James Harris, Researcher, CIBIO

Co-supervisor
Dr. Ana Perera, Researcher, CIBIO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Cover pictures taken by Isabel Damas Moreira

[…]

[Nothing at the first can appear more difficult to believe than that the more complex organs and instincts should have been perfected, not by means superior to, though analogous with, human reason, but by the accumulation of innumerable slight variations, each good for the individual possessor]

Charles Darwin

The Origin of Species

[The nature of science is such that the earth is constantly changing under our feet as old ideas are revisited and changed or thrown out altogether while their replacements are presented, challenged, and modified in turn]

John Alcock

Animal Behavior

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[Obrigada]

ABSTRACT

Recent findings show that parasites play a major role in host populations' ecology and dynamics. The phylum Apicomplexa is a very interesting and diverse group of obligate unicellular blood parasites, with a vast distribution. Their study has become progressively more important as documented works reveal that emergent diseases caused by Apicomplexa pose serious health risks both for animals and humans. In many Apicomplexans, pathogenicity is still poorly understood, such as in the haemogregarine genus *Hepatozoon*, the most widespread Apicomplexan parasite detected in reptiles, especially in lacertids. Although in a broad sense haemogregarines seem to have a low impact on their natural reptilian hosts, the fitness impact of harbouring parasites of the genus *Hepatozoon* is still controversial.

In order to understand the impact of *Hepatozoon* in three species of lacertids, we conducted three studies in which we assessed the influence of *Hepatozoon* infections on different host traits and fitness indicators. In two studies, we applied the Phytohaemagglutinin skin-testing (PHA) technique for estimating host immune response, and scored the number of circulating blood cells. In addition, one of these experiments incorporated two performance tests, sprinting speed and bite force, considered to be key traits for host survival and increased fitness. The aim of the last study was to investigate possible changes in host behavior induced by these parasites, by assessing lizards' flight-initiation distance from a simulated predator.

Differences in prevalence between localities and species were remarkable across the three studies. However, parasite intensity revealed no consistent pattern with host size, with very different results between studies. For all experiments no sex effects were detected, while the impact on body condition was found to be different among studies. *Hepatozoon* presence appeared to have little or no impact on the immune response of the lizards, and no significant correlation was found between immune reaction or parasitemia load and haematological pathologies. Increased parasitism was correlated with maximum bite force but only due to an overall relationship with size, while no effects of *Hepatozoon* parasites were detected on locomotor performance or escape behaviour.

Overall our results showed that all parasites belong to the same genetic lineage, exhibiting a very widespread nature. Broadly, results from these experiments suggest that *Hepatozoon* presence has little or no impact on their hosts, which has seen by the lack of significant effects on important host traits including immune response, bite force and sprinting speed. These studies contribute to increase the knowledge on the impact of *Hepatozoon* parasites in lizards, and highlight the importance of further investigations to determine the real impact of these widespread hemoparasites in other aspects of host life history, which is essential information prior to making conservation efforts.

KEYWORDS

Podarcis bocagei; *Podarcis vaucheri*; *Scelarcis perspicillata*; *Hepatozoon*; haemogregarines; immune system; performance; flight-initiation distance.

RESUMO

Descobertas recentes mostram que os parasitas têm um papel importante na ecologia e na dinâmica dos hospedeiros. O filo Apicomplexa é um grupo diverso e interessante de parasitas sanguíneos obrigatórios, com uma vasta distribuição. O seu estudo tem-se tornado cada vez mais importante à medida que trabalhos documentados revelam que doenças emergentes causadas pelo Apicomplexa representam sérios riscos de saúde tanto em animais como em humanos. A patogenicidade de muitos apicomplexas é ainda mal compreendida, como o género *Hepatozoon* (haemogregarine), o qual é o apicomplexa mais encontrado em répteis, especialmente em lacértidos. Embora num sentido lacto as haemogregarines pareçam ter baixo impacto em répteis que são hospedeiros naturais, o impacto de parasitas do género *Hepatozoon* no *fitness* do hospedeiro é ainda controverso.

De modo a compreender o impacto do *Hepatozoon* em três espécies de lacértidos, realizámos três estudos para avaliar qual o impacto das infecções causadas por *Hepatozoon* em diferentes características e indicadores de fitness do hospedeiro. Em dois estudos, aplicámos o teste cutâneo da Fitohemaglutinina (PHA) para estimar a resposta imune do hospedeiro, e em ambos o número de células sanguíneas circulantes foi também registado. Uma destas experiências incorporou ainda dois testes de performance, a velocidade máxima de corrida e a força da mordida, consideradas características chave para a sobrevivência dos lagartos e ligadas a um maior fitness. O objectivo do último estudo foi investigar eventuais alterações no comportamento do hospedeiro, induzidas por estes parasitas, avaliando a distância de início do escape do lagarto utilizando um predador simulado.

As diferenças na prevalência entre os locais e entre espécies foram notáveis ao longo dos três estudos. Contudo, a intensidade de parasitas não revelou nenhum padrão relacionado com o tamanho do hospedeiro, apresentando resultados muito diferentes entre os testes. Em todas as experiências não foram detectados efeitos no sexo, enquanto o impacto na condição física mostrou diferenças entre os estudos. A presença do *Hepatozoon* aparentou ter nenhum ou baixo impacto na resposta imune dos lagartos, e não foi encontrada uma relação significativa entre a reacção imunitária, ou a carga parasitária, e patologias hematológicas. O aumento do parasitismo apresentou-se correlacionado com a força máxima de mordida, mas devido a uma relação geral com o tamanho corporal, enquanto nenhum efeito dos parasitas *Hepatozoon* foi detectado nas aptidões locomotoras ou no comportamento de fuga.

No geral, os nossos resultados mostraram que todos os parasitas pertencem à mesma linhagem, exibindo uma natureza muito dispersa. Os resultados destas experiências sugerem que a presença de *Hepatozoon* tem pouco, ou nenhum, impacto nos seus hospedeiros, como se vê pela falta de efeitos significativos em características importantes para o hospedeiro, como a resposta imune, força da mordida e velocidade de corrida. Estes estudos contribuem para aumentar o conhecimento do impacto dos parasitas *Hepatozoon* nos lagartos e salientar a importância de haver mais investigações para determinar o impacto real destes hemoparasitas, amplamente disseminados, informação essencial para se basearem esforços de conservação.

PALAVRAS-CHAVE

Podarcis bocagei; *Podarcis vaucheri*; *Scelarcis perspicillata*; *Hepatozoon*; hemogregarinas; sistema imunitário; performance; distância de início do escape.

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ABBREVIATIONS

ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
BC	Body condition
DNA	Deoxyribonucleic acid
GLM	Generalized Linear Model
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
rRNA	Ribosomal ribonucleic acid
SVL	Snout-vent length
SD	Standard Deviation

CHAPTER I

INTRODUCTION

[The majority of living organisms are parasitic in one form or another, be they a virus invading a sea coral, a tapeworm within the guts of your dog, a cuckoo chick in a reed warbler's nest or a lion stealing a hyæna's kill on the African plain]

Peter J. Hudson

Ecology of wildlife diseases, 2002

1.1. PARASITES

Every living organism interacts with many other organisms, in interactions that are the product of natural selection acting over evolutionary time to produce organisms well suited to their environment (Poulin 2007). Parasitism is one of the most successful lifestyles and a major component of biodiversity (Poulin & Morand 2000; Combes 2001).

Increasing evidence suggests that parasites play a crucial role in ecosystems, by interacting in population processes, shaping entire community structures and significantly reducing host fitness in the wild (Hudson *et al.* 2002, 2006; Pedersen & Fenton 2007). This can become more complex considering that parasites can form communities themselves within their hosts (see Poulin 1999). It has even been suggested that the health of an ecosystem can be estimated using inhabiting parasites (Hudson *et al.* 2006).

However, and despite the understanding that parasites can add an unexplained source of variation to data (Schall 1983), to most ecologists, parasites "have been not only invisible but unimportant" until 30 years ago (Poulin 1999), both individually but also considering interaction with their hosts (Deviche *et al.* 2001). The pioneering work of Hamilton and Zuk (1982) greatly changed this viewpoint. These authors advanced a theory that places parasites in a central position in sexual interactions: using North American passerines they suggested that certain blood parasites were involved in the elaboration of sexual signs, in which more resistant individuals could invest more energy into sexual signals and therefore exhibit a higher fitness.

However, even with the gradual improvement knowledge, many parasite species remain undescribed (Poulin 1996) and this is particularly true for the important Apicomplexan parasites.

1.2. APICOMPLEXA

The protist phylum Apicomplexa Levine 1970 is a large unicellular clade, composed of a diverse array of obligate intracellular parasitic organisms (Morrissette & Sibley 2002; Morrison 2009) that probably evolved from free-living, photosynthetic organisms (Gubbels & Duraisingh 2012). Its name derives from the apical end, common to every member of the clade, which plays a key role during the host cell invasion process.

In a review paper concerning current knowledge of Apicomplexa, Morrison (2009) stated that this "is the only large taxonomic group whose members are entirely parasitic and is, therefore, presumably of major interest to parasitologists". Regarding the concept of what is a parasite, it is clear that all Apicomplexan are, by definition, detrimental to the fitness of their host. Despite of classic debates, parasites can be commonly referred to as "organisms living in or on another living organism, obtaining from it part or all of its organic nutriment, commonly exhibiting some degree of adaptive structural modification, and causing some degree of real damage to its host" (Price, 1980). This said, it is easy to realize how remarkable it was when, one year after Morrison's review, Saffo *et al.* (2010) discovered an Apicomplexan mutualistic marine endosymbiont, accentuating the interest of this phylum, but simultaneously highlighting the lack of knowledge we possess on the biology of the group.

It is accepted that Apicomplexa (Fig. 1) is the most poorly-known group regarding its biodiversity, with estimates of only 0.1% of the comprised species described (Adl *et al.* 2007; Morrison 2009). Yet it is suggested that every vertebrate and invertebrate animal in the world hosts at least one Apicomplexan species (Morrison, 2009; Šlapeta & Morin-Adeline 2011). This is a crucial point, as Apicomplexa encompasses several important pathogens (Levine, 1988). Their study has become progressively more important since studies reveal that emergent diseases caused by Apicomplexa pose serious risks for both animals and humans.

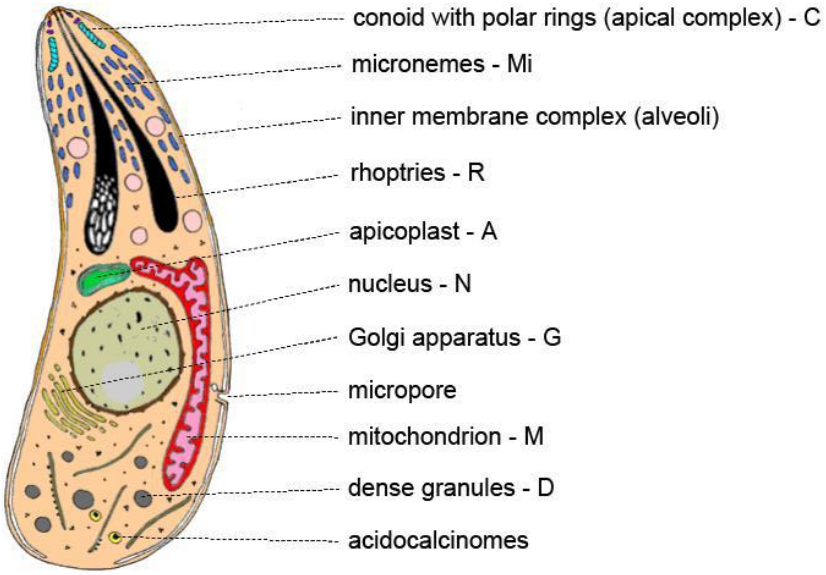

Fig. 1 – Schematic representation of an Apicomplexan parasite and its constituents (in Šlapeta & Morin-Adeline 2011).

1.2.1. IMPORTANT PATHOGENS

The medical importance of some Apicomplexan organisms fuelled much research and there is today a bias towards the study of those genera. The most studied lineages include the piroplasms *Babesia* and *Theileria*, the malaria parasite *Plasmodium* (the three belonging to Hematozoa clade), the Coccidians *Eimeria* and *Toxoplasma* and also the *Cryptosporidium*, following the Apicomplexan taxonomic structure proposed by Šlapeta and Morin-Adeline in 2011. It is easy to realize why these are the most studied genera when considering their general impact. For instance, the genus *Babesia* can cause babesiosis in a large variety of hosts, imposing high medical and veterinary implications. The cattle industry has seen its economy gain decreased by this disease, reaching a global scale of concerning (Späth *et al.* 1990; Bock *et al.* 2004), and in humans the disease can even occasionally result in deaths (Homer *et al.* 2000).

The resemblance between the symptoms of babesiosis and malaria, caused by parasites from the *Plasmodium* genus is noteworthy and not restricted to the human host. A study comparing naïve mice and humans suggested that diseases caused by these two Apicomplexan parasites are likely to be conceptually identical (Clark & Jacobson 1998). These authors reported that humans become ill with a small parasitic load, while naïve mice tolerate high infections, and this was the same in both *Babesia* and *Plasmodium* genera.

Plasmodium is the most studied genus within the Apicomplexan group. It is the most eminent parasite for humans since it is the causative agent of malaria disease, and as such is responsible for more than 1 million human deaths annually (Mackintosh *et al.* 2004; Manguin *et al.* 2010). Likewise this parasite severely affects domestic and wild animals, and can truly affect an ecological community. Around 1920, introduced avian malaria decimated Hawaiian endemic bird populations naïve to this disease (van Riper III *et al.* 1986). In brief, this sole parasite was able to define the distribution and abundance of these species through an entire island. Similar prominence is given to *Plasmodium azurophilum* in shaping the distribution of *Anolis wattsi* and *Anolis gingivinus*, in Caribbean Islands (Schall 1992). This parasite is responsible for the geographic patterns among the two species, by mediating the competition between them (Schall 1992). Moreover in other work developed with lizards (*Sceloporus occidentalis*), it was possible to conclude that malarial infection with *Plasmodium mexicanum* blocks the ability of males to compete for females, since non-infected lizards were dominant in social interactions, both towards other males and females (Schall & Dearing 1987).

All these iconic representative parasites present in so many taxa, with great medical importance and public health consequences, have been subjected to extensive genome sequencing for biomedical advances (Tarleton & Kissinger 2001; Carlton 2003), overshadowing other Apicomplexan genera in number of studies, such as the less well known haemogregarine *Hepatozoon,* the most common hemoparasite found in reptiles (Telford 2009).

1.2.2. HAEMOGREGARINES

Revised by Telford (2009), haemogregarines (Adeleorina) are the most common and widely distributed hemoparasites in reptiles, with a confirmed presence in every order of living reptiles and also in all main terrestrial tetrapod order (Levine 1988).

The life cycle of many haemogregarine parasites remains to be elucidated, but generally it is heteroxenous, requiring multiple hosts (both invertebrate and vertebrates) to complete their life cycle (Manwell 1977; Telford 2009). The range of hosts these parasites use is highly variable. Ticks, mites, leeches, and mosquitoes have been shown to transmit these parasites to a diverse array of vertebrates, including many species of reptiles, amphibians, fishes, birds and mammals (Manwell 1977; Telford 2009). Research has shown that haemogregarines present a great taxonomic diversity, variation in life cycles and hosts, and cosmopolitan distribution, suggesting they are an ancient and successful group (Perkins & Keller 2001).

1.2.2.1 PATHOGENICITY IN REPTILES

Episodes of haemogregarine induced mortality or severe pathogenicity are rarely reported in natural hosts (Manwell 1977; Bouma *et al.* 2007), but it remains to be established what are the costs associated with harbouring these parasites, as the identified effect in reptiles (and other types of hosts) is varied and very ambiguous. This is important, since eventual variations in host susceptibility to disease can influence host community ecology.

There are some studies containing a few reports of anomalies such as erythrocyte plasma membrane alterations or slight anemia (Nadler & Miller 1984; Telford 1984; Wozniak *et al.* 1994). Most studies essentially state that haemogregarines seem to have a low impact on their natural hosts and that infection is not always reflected in the

immune system condition, with little evidence showing that it can noticeably affect reptile health (Wozniak *et al.* 1996; Sperry *et al.* 2009).

In lizards, some evidence points that haemogregarines can destroy erythrocytes, and this can lead to reduced hemoglobin concentrations and reduced capacity for oxygen transportation (Oppliger *et al.* 1996; Veiga *et al.* 1998; Jacobson 2007). Moreover there are records of immature red blood cells production associated with parasite infection (Oppliger *et al.* 1996; Jacobson 2007). These physiological effects, together with the activation of the immune system to fight the infection, may affect the ability to allocate energy to other processes and, as a downstream effect, reduce host fitness (Oppliger & Clobert 1997; Lochmiller & Deerenberg 2000).

Lizard's body condition is an easy and simple trait to assess, and is thus normally included in studies of haemogregarine prevalence and intensity. Revising the general outcome from those works, it is apparent that there are no consensual conclusions regarding haemogregarines impact based on only this trait. There are studies showing that haemogregarine load can be associated with poorer lizard condition in lizards (Garrido & Pérez-Mellado 2013), or contrariwise with better condition (Amo, López, *et al.* 2005; Molnár *et al.* 2013). In one of these studies, on the host *Podarcis muralis*, the authors suggest that this better condition might reflect the mortality of infected lizards with reduced condition (Amo, López, *et al.* 2005). Furthermore, other studies detected no relationship between haemogregarine load and body condition (Amo, Fargallo, *et al.* 2005).

In the lacertid *Zootoca vivipara* it has been demonstrated that lizards with higher parasite intensities were those exhibiting low endurance at birth with reduced growth and activity rate, but were also those with lower predation risk (Clobert *et al.* 2000). More commonly, the same lizard species show also fitness-related effects, such as slower running speed (Oppliger *et al.* 1996) and reduction of tail regeneration rate (Oppliger & Clobert 1997). Nevertheless, other works such as those performed by Schall (1986) in the lizard *Cnemidophorus arubensis* reported avirulent haemogregarines with no sign of impact besides an augment in the resting metabolic rate. However, this is an insular species which may have evolved with low pressure of parasites.

Studies in mate selection appear to represent a good model to assess the impact of haemogregarines infections. It has been shown that *Psammodromus algirus* females react differently to femoral gland secretions of males according to health and parasite load (Martín *et al.* 2007), confirming that haemogregarines can be involved in female

mate choice. Additionally, other works show that male coloration seems to be directly linked with parasite infection. In fact, this appears to be the only lizard host trait that constitutes a coherent signal of haemogregarines impact. For example males of *Lacerta viridis* with fewer blood parasites develop more intense coloration, indicating their resistance to infection (Molnár *et al.* 2013). Likewise, regarding the colour of the polymorphic lizard *Podarcis melisellensis*, males with yellow or orange ventral colour (higher levels of testosterone) were always less infected than white individuals (Huyghe *et al.* 2010) and in *P. muralis* males with brighter, more yellowish ventral colorations, and more attractive femoral secretions, revealed less parasites (Martín *et al.* 2008). Yet a lab based work with testosterone application in the latter species revealed an immunosuppression of the immune system but no linkage with endoparasites (Oppliger *et al.* 2004). Contradicting the pattern found in every study concerning male coloration, the results of an earlier work revealed that brightly coloured males of *Cnemidophorus arubensis* were more likely to be infected than blandly coloured males of the same body size (Schall 1986). Yet the author discusses this as a consequence of brightly coloration being associated with increased age, more aggressiveness and propensity to participate in chases, which let the animals more exposed and for a longer time to the arthropod vector (the transmitter of haemogregarines).

In a more ambitious approach, a very elegant study proposed that the haemogregarine *Hemolivia mariae* can shape lizards activity (Bouma *et al.* 2007), as already described for other Apicomplexan (Schall 1992; van Riper III et al. 1989). The authors observed that infected *Tiliqua rugosa* individuals had smaller home ranges than those not infected but, contradictory, lizards with larger home ranges were those more susceptible to infection under experimental conditions. The most important outcome of this study was the prospect of lizards sacrificing defense against pathogens with increased activity, possibly to maintain home ranges and increase mating chances. This illustrates that, at least in the Australian *T. rugosa*, haemogregarines can be very important in host life history.

Because the different genera within this group are so difficult to distinguish when observed in the peripheral blood (Smith 1996), the general term “haemogregarine” is used to report their occurrence, using the group as a whole (Keymer 1981), without distinguishing between each of the parasite genus of haemogregarines in microscope inspections (Smith 1996). Nevertheless, molecular tools and current knowledge are starting to tackle some of the major questions. It is now acknowledged that the most commonly reported haemogregarines are members of the genus *Hepatozoon*, from the Hepatozoidae family (Zhu *et al.* 2009; Telford 2009).

1.2.3. GENUS *HEPATOZOON*

Hepatozoon (Apicomplexa, Adeleorina, Hepatozoidae) is a genus of unicellular intraerythrocitic parasites that can infect a wide range of hosts (Smith 1996). Although *Hepatozoon* was first described over 100 years ago (Miller 1908), the comprehension of its transmission and the impact on the host are still scarce. The advent of molecular diagnostic techniques revealed that lack of knowledge can negative consequences, since new studies have identified *Hepatozoon* spp. with increasing frequency in both wild and domestic animals (Mathew, Bussche, *et al.* 2000; Criado-Fornelio *et al.* 2009; Gimenez *et al.* 2009; Maia *et al.* 2011; de Bortoli *et al.* 2011; Pawar *et al.* 2012; Harris *et al.* 2012). Manwell (1977) stated that "there is a likely possibility that numerous species of *Hepatozoon* remain to be discovered" but was still far from appreciating the overwhelming actual number (Smith *et al.* 1999). In effect, diversity might surpass prior expectations, especially since new species keep being revealed during regular herpetofauna surveys (for example, Harris *et al.* in press; Desser 1997; Telford *et al.* 2001; O'Dwyer *et al.* 2013).

Hepatozoon parasites can be found worldwide in an extensive range of invertebrates (Wozniak & Telford 1991; Watkins *et al.* 2006; Baneth *et al.* 2007) and terrestrial vertebrates (Levine 1988; Smith 1996), including birds (Merino *et al.* 2006), mammals (Pawar *et al.* 2012), reptiles (Maia *et al.* 2011), amphibians (Barta *et al.* 2012), and even fishes (in Davies & Johnston 2000).

1.2.3.1. LIFE CYCLE

As with all haemogregarine parasites, *Hepatozoon* life cycle is complex and variable, comprising three major processes: gametogony, sporogony and merogony (Leander 2003). Miller described in 1908, with surprising precision, the general processes underlying *Hepatozoon* life cycle, and since then, numerous studies have aimed to further elucidate *Hepatozoon* life cycle and modes of transmission (Ball *et al.* 1967; Pessôa *et al.* 1974; Nadler & Miller 1984; Wozniak & Telford 1991; Desser *et al.* 1995; Smith *et al.* 1999). Smith (1996) in a review article, proposed a view of *Hepatozoon* life cycle that is still largely accepted.

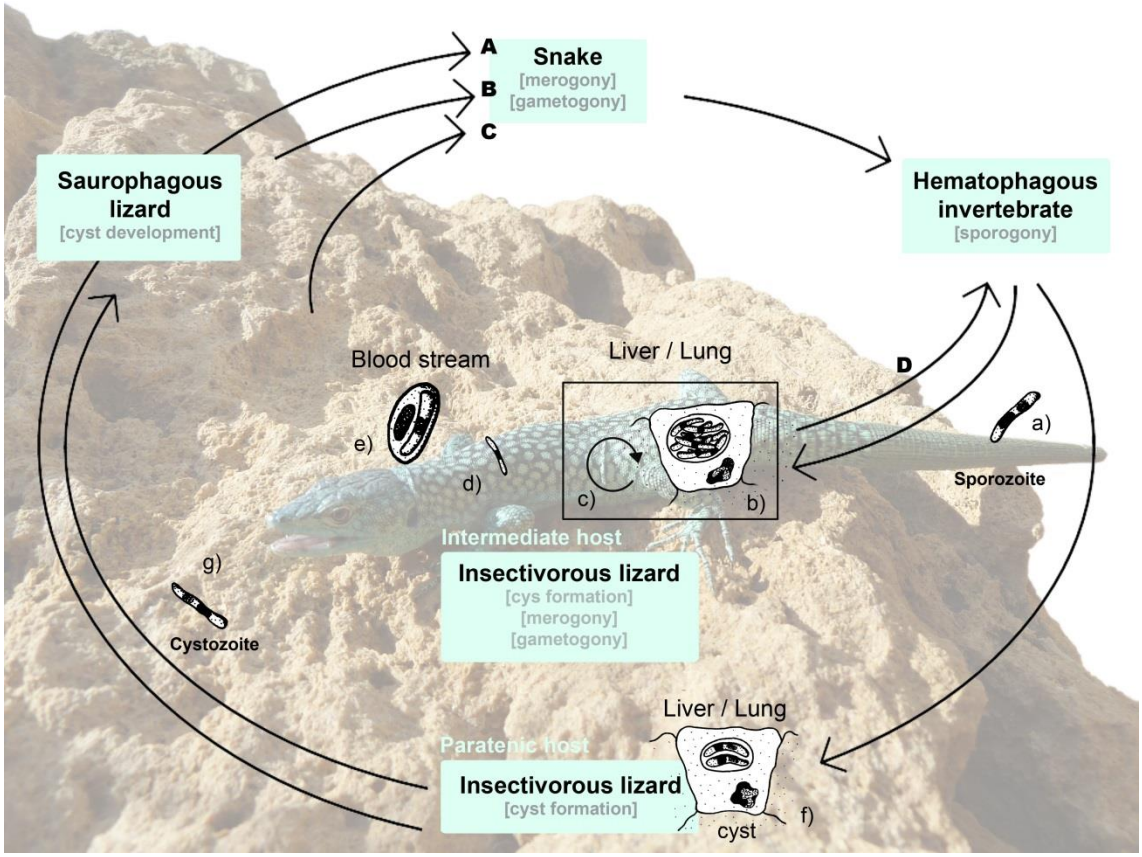

Fig. 2 - Diagram regarding several possible *Hepatozoon* life cycles. Scheme and picture by Isabel Damas Moreira.

Hepatozoon parasites have a heteroxenous life cycle alternating between hematophagous invertebrate hosts, where its sexual reproduction takes place (definitive hosts), and vertebrate hosts, where asexual reproduction occurs (intermediate hosts) (Smith 1996). The invertebrate hosts can be as varied as ticks, mites, fleas, mosquitoes, but in reptiles the most common reported *Hepatozoon* vectors are *Culex* mosquitoes (Wozniak & Telford 1991; Smith *et al.* 1994, 1996; Smith & Desser 1998).

Hepatozoon spp. present a wide variation to this life cycle, distinct in their complexity and number of used hosts. For reptiles, the most commonly described life cycle involves, besides the invertebrate vector, two different vertebrates: a first and second vertebrate hosts. The second (or final) vertebrate host has the role of intermediate host and can be a mammal or a bird but it is usually a snake (Smith *et al.* 1994, 1996; Smith & Desser 1998; Sloboda *et al.* 2008), that becomes infected by predation of the first vertebrate host (Smith 1996). These first vertebrate hosts are snake prey items, as rodents (Wiger 1977; Sloboda *et al.* 2008), amphibians (Smith *et al.* 1994, 1996; Smith & Desser 1998) and lizards, such as geckos and lacertids (Landau *et al.* 1972; Tomé *et al.* 2012). I will briefly describe the processes that occur in the proposed common life

cycle for *Hepatozoon* spp. infecting reptiles based mainly on Smith *et al.* 1994; Smith 1996; Baneth *et al.* 2007; Jacobson 2007; Johnson *et al.* 2009; Telford 2009). For a schematic view, see Fig. 2 with an illustration of the general strategies a parasite might adopt when parasiting insectivorous lacertid lizards.

Within a hematophagous invertebrate, the parasite goes under sporogony, which results in the production of sporozoites. These infective stages are transmitted to the first vertebrate host (Fig. 2 a)) when the invertebrate feeds on it (Sloboda *et al.* 2007; Viana *et al.* 2012), or when the vertebrate ingested the infected invertebrate (Smith 1996; Johnson *et al.* 2009; Allen *et al.* 2011). In this new host, sporozoites enter in the cells of the liver or lungs, and cystic formation occurs (Fig 2 c) and f)). Here, two strategies can be adopted: either merogony and gametogony take place in this host (therefore it assumes the role of intermediate host), or the parasite does not reproduce and forms cysts instead (acting the first vertebrate as paratenic host). The paratenic host serves as a bridge between the definitive (hematophagous invertebrate) and the final intermediate host (e.g. snake) (Fig. 2 A). When the first vertebrate is a paratenic host, another host can even occur before the parasite reaches the final vertebrate host, such as a saurophagous lizard that predates the insectivorous lizard (Fig. 2 g)) and is posteriorly eaten by a snake (Fig. 2 B).

Alternatively, when the first lizard host functions as intermediate host, the cystic development occurs alongside with asexual reproduction. In this case, sporozoites undergo multiple fission to produce meronts (Fig. 2 c)), in a phenomenon called merogony. Meronts mature into merozoites (Fig. 2 d)), which enter the blood stream and infect the red blood cells of the vertebrate host. Merozoites use their apical complex to enter erythrocytes and, after invasion, they transform into gamonts. (Fig. 2 e)). Gamonts represent the familiar stage of the parasite life cycle when screening blood smears under the microscope. A gamont is observable within an erythrocyte, which has its nucleus flattened and placed laterally as a result of parasite invasion (Fig. 3) (Manwell 1977; Telford 2009). Despite the great diversity of *Hepatozoon* spp., all species display morphologically similar gamonts occurring in vertebrate blood cells at this point of its life cycle (Smith 1996; Herbert *et al.* 2010).

Then, gamonts have two possible pathways to reach the invertebrate host: the invertebrate feeds upon the lizard and gets infected (Fig. 2 D); or a saurophagous vertebrate eats the lizard, becomes infected and, only after, the parasite is transmitted to the invertebrate when it takes a blood meal (Fig. 2 C). In the first case, the parasite

used only two hosts, but in the second pathway, the parasite exploited one invertebrate and two vertebrate hosts.

Summaryzing, *Hepatozoon* species can present three possible life cycle strategies: two-host, obligate three-host or optional three-host types of lifecycle. Common to every *Hepatozoon* species is the requirement of one invertebrate hosts and a minimum of one vertebrate. However, knowing which and how many hosts the parasite needs to inhabit, represents a major challenge as it can be different for each *Hepatozoon* species (reviewed in Smith 1996).

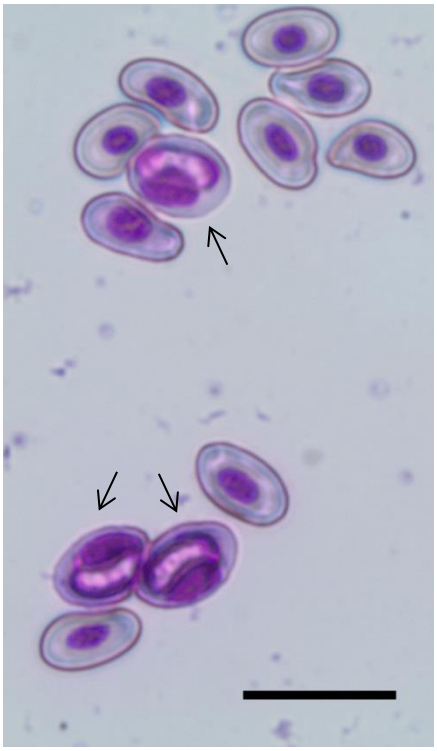

Fig. 3. - *Hepatozoon* parasites (arrows) from *Podarcis vaucheri*. Black bar = 20µm Picture taken by Isabel Damas Moreira.

The process of infection of the vertebrate host during the cycle is still not clear due to all dietary habits and varying ecology of the different hosts (Telford 2009) but stronger evidences point to transmission of the parasite though predation or ingestion of the hematophagous invertebrate (Desser 1990; Wozniak & Telford 1991; Smith *et al.* 1994; Smith & Desser 1998). However some typical haemogregarine vectors (like mosquitoes) are normally not consumed by snakes and thus the addition of an intermediate host could have been an evolutionary strategy to overcome this problem. This was first proposed more that a century ago in 1899 by Langmann (*in* Smith 1996). Plausably, the passage from the first to the second vertebrate host is believed to occur mainly through predation (Telford 2009; Allen *et al.* 2011; Viana *et al.* 2012; Tomé *et al.* 2012).

1.2.3.2. IMPACT ON THE HOST

1.2.3.2.1. NON-REPTILE HOSTS

It is now acknowledged that some *Hepatozoon* spp. can be significantly harmful in species including cats and dogs, and thus the interest of this genus has been rising in veterinary research (Baneth *et al.* 1998; Panciera *et al.* 2000). Once deleterious effects were noticed in domestic animals, more studies started to emerge regarding

Hepatozoon spp. presence (Criado-Fornelio *et al.* 2009; Gimenez *et al.* 2009; de Bortoli *et al.* 2011) and pathogenesis, particularly in cat and dog hosts.

Impact can differ for different *Hepatozoon* species, as it is the case of *Hepatozoon canis* and *Hepatozoon americanum*, the two most common species of this parasite in canids. While the latter is highly virulent and frequently provokes fatal skeletal and cardiac myositis (Panciera *et al.* 2000), *H. canis* is better adapted to the host producing less severe effects (Baneth *et al.* 2003). However *Hepatozoon* can be an opportunistic parasite when the immune system of an animal is already compromised (Dwyer *et al.* 2006; Pawar *et al.* 2012) and "less severe effects" can still mean skeletal pain and recumbency (Baneth *et al.* 2007). For example in Brazilian dogs infested with *H. canis,* the subjects exhibited anemia, weight loss, diarrhoeas, fever, and anorexia, among other symptoms, but all dogs exhibited concomitant diseases other than hepatozoonosis (Dwyer *et al.* 2006). Similar scenario can be described for cats, with most of significant clinical abnormalities being reported in animals with *Hepatozoon* but believed to be attributed to other concurrent diseases (Baneth *et al.* 1998).

It is especially due to this literature, allied with the urgent need for realizing which animals were parasitized by *Hepatozoon* spp. and for understanding the mechanisms that were underlying its infection, that most of the information available on vertebrates was gathered. During this process, alongside the development of molecular tools, some studies of *Hepatozoon* in wild animals also started to be performed. This was probably not only a consequence of the awareness of *Hepatozoon* as a very widespread genus with a significant impact on domestic animals, but also from the consolidation of wild animals as "reservoirs of diseases" (Simpson 2002).

The identification of clinicopathological characteristics in domestic and wild canids and felids also led to questioning what could be the effects on other wild hosts, as for instance in reptiles. Adding some interest to this is the fact that reptiles, as amphibians and birds, display nucleated erythrocytes, contrary to mammals. Besides that, researchers soon discovered that reptiles should warrant further research as it was found that at least for snakes, *Hepatozoon* is the most common intracellular protozoa (Jacobson 2007; Zhu *et al.* 2009; Telford 2009).

1.2.3.2.2. REPTILE HOSTS

Only approximately 25 years after the first description of this genus (Miller 1908), a reptilian *Hepatozoon* was described, in Nile crocodiles, during an investigation with

tsetse flies from Uganda (Hoare 1932). However, since then these has been considerable confusion and contradiction in the literature.

It is clear from a revision of the literature that pathological studies performed in reptiles are biased towards snakes, a common final vertebrate host for *Hepatozoon* (Telford 2009). However, it is believed that the occurrence of the first vertebrate host is the more important step during the transmission of *Hepatozoon* to snakes (Sloboda *et al.* 2007), deserving at least a spotlight for that reason.

General processes regarding erythrocyte invasion are broadly accepted, but the real consequences of this cell invasion are still debatable. In microscope inspections it is clearly visible that an erythrocyte invaded by a gamont often display a different morphology in size and shape than a non-infected one (Ball *et al.* 1969; Jacobson 2007). This can have implications at the cell function level also due to the nucleus being typically displaced from its original position (Smith 1996). In reptiles, several functions have been also attributed to erythrocytes: it can be involved in sugar transport, calcium homeostasis, haemoglobin, redox homeostasis, and it is suggested that they have a direct role in the immune response (reviewed in (Morera & Mackenzie 2011). Nevertheless, the major function associated with these cells is respiratory gas exchange (oxygen and carbon dioxide transport). It is proposed that this alteration in mature erythrocytes can lead to a decrease of its capacity for carrying oxygen, which in turn forces the immune system to react by discharging immature erythrocytes into the blood stream. This process was not only observed with haemogregarine infections (e.g. Oppliger *et al.* 1996) but also with *Plasmodium* infections (e.g. Schall *et al.* 1982). Consequently, the reduction of haemoglobin concentrations and oxygen transport can result in individuals with anemia and weaker fitness (Jacobson 2007). Sometimes erythrocytes can exhibit encapsulated gamonts, which may be associated with improvement of survival against the vertebrate host immune defence (Telford 2009) but this is still not well understood.

Even with the occurrence of invasion and altered erythrocytes, it is rarely reported that an infected cell is lysed by a haemogregarine (Telford 2009), and this might be an indication of low general damage to the host. In fact, severe infections are seldom translated into significant illness or lesions in tissues (Jacobson 2007). There are only punctual documented cases in snakes that link haemogregarines with dangerous lesions, and most of the times it is associated with observations of parasite forms in the liver (Keymer 1976), but also associated with *Hepatozoon* species (Wozniak *et al.* 1994, 1998).

The general suggestion that reptilian *Hepatozoon* might be well-adapted with a low pathogenic effect on the host is what is expectable in a long-term coevolution scenario (Nadler & Miller 1984; Telford 1984) and so led to the appearance of different experimental designs, focused on understanding *Hepatozoon* impact on unnatural naïve hosts, by experimentally infecting them.

Before that, an earlier study developed in this sense showed that a *Hepatozoon* species, *H. tupinambis*, can cause clinically significant inflammatory disease in an unnatural snake host, when transferred from the natural lizard host (Pessôa *et al.* 1974). Curiously, those authors could detect that the usual *Hepatozoon* ability to infect blood cells was verified in the artificially infected host, but there was a tendency to lose this characteristic, as infection progress. Once again, the dangerous effect in neonatal hosts was shown, as both of the young snakes in the study died a few weeks after the experimental infection (Pessôa *et al*. 1974). Wozniak and Telford (1991) reported high mortality in mosquitoes and dangerous pathologies in lizards with artificial infection by a *Hepatozoon* sp. Later, Wozniak and his team (1996) discovered harmful effects in three lizard species, never before detected in natural hosts, when infected with *Hepatozoon mocassini*. Infection eventually led to severe lethargy and anorexia due to induced necrotizing inflammatory lesions.

Furthermore, in the same way as with other blood parasites, the invasion of erythrocytes might lead to a decrease in haemoglobin levels leaving the host more susceptible to infections (Smith *et al.* 1999), as already observed in other taxa (Atkinson & van Riper III 1991), which in turn possibly reflects reduced metabolic rate. The invasion of white cells can also take place but it is a rare event, and might imply different implications (Godfrey *et al.* 2011). The mentioned decreasing metabolic rate is considered the main cause for some pathogenic effects studied in natural infected reptile populations. It is proven to negatively affect, for example, tail regeneration rate in *Zootoca vivipara* when infected by haemogregarines (Oppliger & Clobert 1997), or the growth rate of water pythons, *Liasis fuscus*, when infected with *Hepatozoon* (Ujvari *et al.* 2004; Madsen *et al.* 2005). In the latter work the authors could even ascertain that *Hepatozoon* parasites were having a negative impact on female reproductive output as well as on juvenile survival (Madsen *et al.* 2005). Here the parasite has a clear role in the population structure and dynamic, because since only those snakes with fewer parasites can reach older ages, *Hepatozoon* is basically regulating their demography. A study in the same python species found an association between higher *Hepatozoon* loads and a decrease in host humoral immune response (Ujvari & Madsen 2005).

Perhaps one of the most interesting studies assessing *Hepatozoon* impact is that performed in keelback snakes, *Tropidonophis mairii*, where natural hosts were shown to be in an apparent neutral association with the parasite, after 10 fitness variables were tested (Brown *et al.* 2006). Curiously, a sympatric snake exhibited opposite results for the same evaluated fitness parameters in a previous study (Madsen *et al.* 2005). Also interesting in the work with keelbacks, was the fact that snakes did not generate more immature erythrocytes in response to heavier parasitic infection, contrary to previous findings described above (e.g. Oppliger 1996). More recently Xuereb *et al.* (2012) found a similar pattern in the Eastern Foxsnake, *Pantherophis gloydi*, detecting no effect of *Hepatozoon* spp. in two host fitness correlates. Some of these works suggest that whatever effect might be verified in host adult life, it is expected that newborn hosts are more susceptible to the effect of *Hepatozoon*, due to its size and due to a non-fully developed immunity (Madsen *et al.* 2005; Ujvari & Madsen 2005; Brown *et al.* 2006).

In order to understand evolutionary interactions occurring between *Hepatozoon* parasites and their hosts, it is necessary to investigate the extent to which parasites may cause detrimental effects on host life history traits, such as growth, condition, performance and survival.

1.2.4. BLOOD PARASITES DETECTION

Apicomplexan organisms are among the most demanding organisms to work with (Morrison, 2009), particularly certain groups. Simply by screening under the microscope, species can be identified through detection of parasite stage in invertebrate hosts (Herbert *et al.* 2010), but in vertebrates most haemogregarines cannot be reliably identified to the genus level mainly due to two reasons: first, the gamont stage observed in the cells of intermediate hosts can have general similar appearance for every member of the haemogregarine group (Smith 1996; Telford 2009); and second, the same parasite lineage can exhibit different morphology (Fig. 4, a), which may be considered a different species, when in fact it is simply at different phases of development (Jakes *et al.* 2003; Roca & Galdón 2010) or because the gamont cytoplasm itself can possess vacuoles (Telford 2009) (Fig.4, b). Some experienced researchers in this area, such as Telford (1984, 2009) or Desser (1997), advise against base identification solely on morphology. Therefore, a complete but still

economically viable study might rely on microscopy, for intensity and morphological inferences, and genetic analysis, for parasite identification (e.g. Sloboda *et al.* 2007).

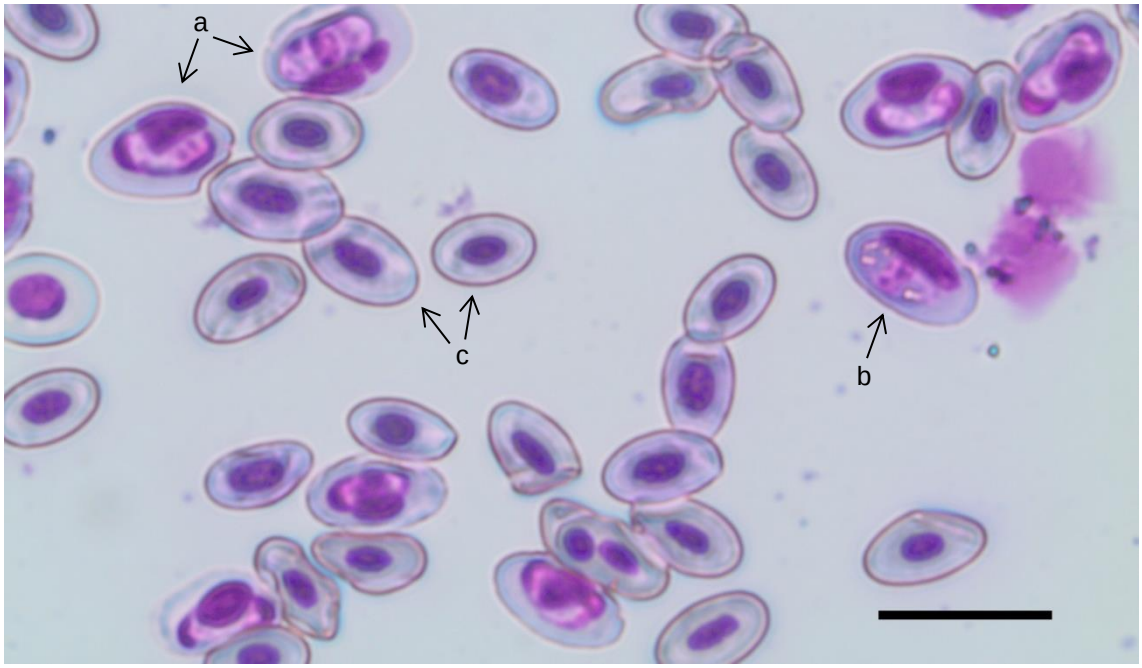

Fig. 4 - Different morphology of *Hepatozoon* parasites (a) with one gamont cytoplasm exhibiting vacuoles (b), surrounded by healthy erythrocytes, such as in c. Black bar = 20µm. Picture by Isabel Damas Moreira.

1.2.4.1. COLLECTION OF SAMPLES FOR STUDIES WITH BLOOD PARASITES

Both tissue and blood can be used to obtain *Hepatozoon* parasites from a lizard host (e.g. Harris *et al.* 2011; Maia *et al.* 2012). However, the use of blood can enhance the process because blood smears from intermediate hosts can be readily used to easily detect gamont stages of these parasites, and molecular techniques may be more effective using blood samples (Maia et al. submitted). Nevertheless, tissue samples are typically taken for other molecular studies of the host, and thus assessments of these may allow a wider sampling of host individuals.

Tissue samples are generally acquired by cutting a tail tip from the lizard, which is preserved in 96% ethanol at room temperature, and blood can be obtained from the caudal vein as a result of this procedure (Duguy 1970). Although Duguy (1970) advise that this is a difficult technique to apply in lacertids, good quality samples can be obtained when lizards are warm, easing the blood fluidity (Damas-Moreira, personal observation). The blood obtained through this process is used for two purposes: microscopic examination (blood smears) and genetic analyses (blood dots in whatman filter paper).

For microscopic examination, the blood is smeared across a glass slide (accordingly with Fig. 5), fixed with absolute Methanol on the day of collection, stained with the standard Giemsa coloration in the following days (1:9 of distilled water for 55 minutes) and air-dried (Moody 2002; Valkiunas 2005). For genetic analysis, a blood drop can also be stored in Whatman filter paper (Fig. 6) and kept at -20ºC (Harris *et al.* in press). Attention should be paid during the preparation of blood smears to aspects that can adulterate the samples, namely dust, excessive heat, humidity or sun exposure, and mainly delay in fixation and staining, as this can damage the quality of the sample (Valkiunas 2005; Valkiunas *et al.* 2008).

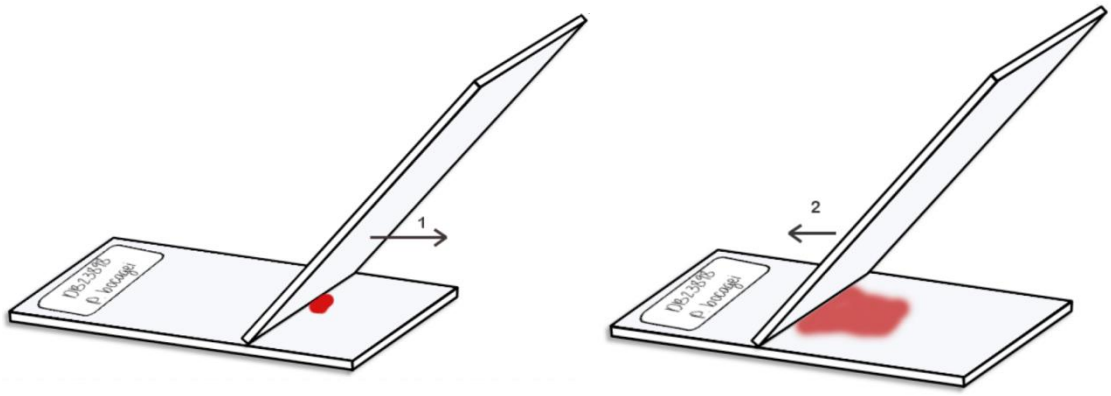

Fig. 5 - Procedure to obtain blood smears: a drop of blood is placed in the horizontal slide; the second slide is then pull against the drop (1), making the blood spread along the edge by capillarity, and finally pushed across the horizontal glass slide (2). Illustration by Isabel Damas Moreira.

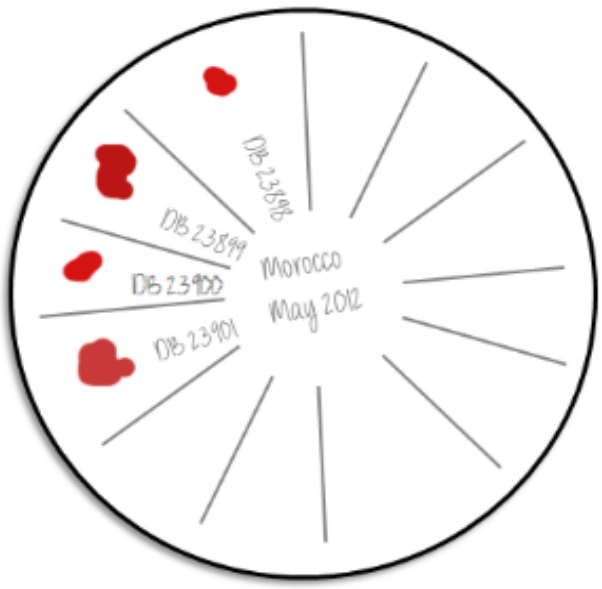

Fig. 6 - Illustration of blood stored in Whatman filter paper for posterior use for DNA amplification. Based on Telford 2009. Illustration by Isabel Damas Moreira.

1.2.4.2. MICROSCOPY

Since Antonie van Leeuwenhoek developed microscopy techniques in the 15th century, little has changed regarding the detection method of many parasitic diseases (in Ndao 2009). Microscopy has been for a long time the standard tool to detect, identify and quantify blood parasites (Moody 2002; Valkiunas 2005) and is widely used in the study of Apicomplexan parasites, including haemogregarines (e.g.Amo *et al.* 2004; Amo, Fargallo, *et al.* 2005; Brown *et al.* 2006; Roca & Galdón 2010). In general, this is the main source for the current knowledge on parasites.

Currently, microscopy comprises several advantages that still only few modern and expensive methods have. It allows quantifying the parasitic intensity, and simultaneously observing the parasite morphology, the developmental stages of infection and which type of blood cell is being parasitized by the gamont (Moody 2002). It is also the only available technique to study the morphology of gamonts in the blood cells and to easily detect multiple infections in the same host cell (Jovani *et al.* 2004). The main advantage comparatively to other methods is clearly the fact of being inexpensive and easily available in laboratories (Moody 2002; Valkiunas *et al.* 2008). Comparatively to other methods, microscopy can be time consuming but nowadays available software incorporated in the microscope interface, such as the *Cell^B Olympus*® software allied with built-in cameras, and software, such as the *ImageJ*® with a cell counter plug-in, can ease the work and reduce the time at the microscope.

Using the microscope, parasite intensity can be assessed by counting the number of infected cells within a stipulated time, but this is not considered the best way to proceed, as different researchers have different skills and thus will have great variances in the final number of analysed cells (Moody 2002; Valkiunas *et al.* 2008). Hence, the most commonly used technique for measuring parasite intensity is counting the number of infected cells within a restricted number of cells, defined by the researcher (Margolis *et al.* 1982; Bush *et al.* 1997). In most studies within Apicomplexa, this number can vary between 2000 (Amo *et al.* 2004; Amo, Fargallo, *et al.* 2005; Amo, López, *et al.* 2005), 5000 (Schall 1996; Austin & Perkins 2006), and 10000 cells (Godfrey *et al.* 1987; Valkiunas *et al.* 2008). Similarly, to specifically measure *Hepatozoon* infection levels, the number of counted cells can range from 1000 (Wozniak *et al.* 1998; Brown *et al.* 2006) to 2000 (Madsen *et al.* 2005; Salkeld & Schwarzkopf 2005; Ujvari & Madsen 2005), or even until 10000 erythrocytes (Sloboda *et al.* 2007; Godfrey *et al.* 2011). However, as pointed out, this is a number defined by the researchers, as it can depend on the quality of samples and the requirements of

the individual study. Nevertheless, for hematozoa quantification, counts of 2000 cells can be considered enough (Godfrey *et al.* 1987).

1.2.4.3. MOLECULAR DETECTION

Allying microscopy techniques with molecular detection of the positive samples can be a good way to study intensity and prevalence of Apicomplexan parasites and simultaneously correctly identify the parasite visible in microscope inspections. The molecular detection is commonly performed using the classic Polymerase Chain Reaction (PCR) (Ndao 2009; de Waal 2012), but inherent to this technique is the impossibility of measure the intensity of infection (Perandin *et al.* 2004). Recently, real-time PCR technique allow to accurately quantify parasitemia levels but this is still not a routine methodology due to its high costs (Bell & Ranford-Cartwright 2002; Perandin *et al.* 2004; Mangold *et al.* 2005). Through this, it is clear the complementarity of both microscopy and classic molecular techniques in order to make inferences regarding parasite prevalence and intensity.

DNA is normally extracted using a commercial kit, following the manufacturer's instructions (e.g. DNeasy Blood & Tissue kit (Qiagen, Washington D.C.), or using traditional high salt methods (Sambrook *et al.* 1989). *Hepatozoon* identification is normally performed using PCR reactions with parasite-specific primers (e.g Perkins & Keller 2001; Ujvari *et al.* 2004), targeting part of the 18S rRNA gene region. This gene has been universally used for phylogenetic reconstructions of protists (Adl *et al.* 2007) and, particularly, of Apicomplexans (Morrison & Ellis 1997; Mathew, Van Den Bussche, *et al.* 2000; Šlapeta *et al.* 2003; Adl *et al.* 2007; Morrison 2008; Perkins *et al.* 2011). In fact, practically all published *Hepatozoon* and related parasite sequences belong to the 18S rRNA gene region, providing valuable information on the taxonomy of the group.

The advantages of sequencing the 18S rRNA gene region are worth noting. Among other reasons for its particular interest (see Allsopp & Allsopp 2006), the use of this region enables identification of some Apicomplexan parasites up to the species level, and its extensive use led to a great abundance of available sequences, allowing easy comparisons with freshly obtained sequences (Šlapeta *et al.* 2003; Barta *et al.* 2012). However, although in the past the interpretation of amplification results was considered simple, as the success or absence of amplification usually indicates if the sample is parasitized or not, recent studies have shown that many unrelated organisms may be amplified, including other Apicomplexan parasites (Harris *et al.* 2012, 2013) or even

fungi (Tomé *et al.* 2012), and therefore sequencing is recommended to confirm the expected parasite is amplified.

Certainly the 18S rRNA gene region is the most commonly used for *Hepatozoon* identification in reptiles, so that a considerable amount of data is available for comparison with new sequences (Ujvari *et al.* 2004; Sloboda *et al.* 2007; Vilcins *et al.* 2009; Herbert *et al.* 2010; Harris *et al.* 2011; Maia *et al.* 2011, 2012; Tomé *et al.* 2012), particularly in lacertids (Maia *et al.* 2011, 2012).

1.3. THE HOSTS

1.3.1. LIZARDS: HOST MODELS

As stated before, most works concerning *Hepatozoon* biology have been performed in snakes (Jacobson 2007; Zhu *et al.* 2009; Telford 2009) and although it is not clear the exact role of the lizard in the life cycle (i.e. if it acts as a paratenic or intermediary host), its occurrence can be considered the more important step in the transmission of *Hepatozoon* to snake hosts (Sloboda *et al.* 2007).

Reptiles have numerous attributes that make them suitable and popular systems for physiological and ecological studies (Pough *et al.* 1998). Lizards are perfect for studies regarding natural host-parasite interactions as they are easy to observe, capture and manipulate (Arnold 1981) and, particularly in Europe, lacertids can occur in populations of high density allowing researchers to acquire suitable sample sizes (Schall 1990). Moreover, lizard performance at key tasks, such as running endurance or sprint speed, can be assessed easily and quickly, which gives an immediate insight into the individual performance, body condition and reproductive success. This makes lacertids ideal models for this type of studies, particularly as haemogregarines are common blood parasites in this group (Jacobson 2007; Telford 2009).

1.3.2. HOST SPECIES

Since several years ago, our research group has been conducting field work on reptiles and amphibians through the coastal region of the North of Portugal and Morocco (especially central Morocco). During this course of time, analysed lizards were frequently parasitized by haemogregarines, which was later demonstrated to be

Hepatozoon (Maia *et al.* 2011, 2012; Harris *et al.* 2012). This agrees with the recent awareness that *Hepatozoon* is the most common blood Apicomplexan parasites in lacertids (Jacobson 2007, Telford 2009).

Consequently for this work we focused on these areas, as they appear to be relevant sites for making inferences concerning parasite-host interactions between *Hepatozoon* and lacertids, as there are known to be high prevalence and intensities. The genus *Podarcis* can be a key model since it is abundant and has a wide distribution, is able to display high infection levels of haemogregarines (Oppliger *et al.* 2004; Amo, López, *et al.* 2005; Roca & Galdón 2010; Harris *et al.* 2012), and was already used in studies concerning pathogenicity of haemogregarines (Oppliger *et al.* 2004; Martín *et al.* 2008; Huyghe *et al.* 2010).

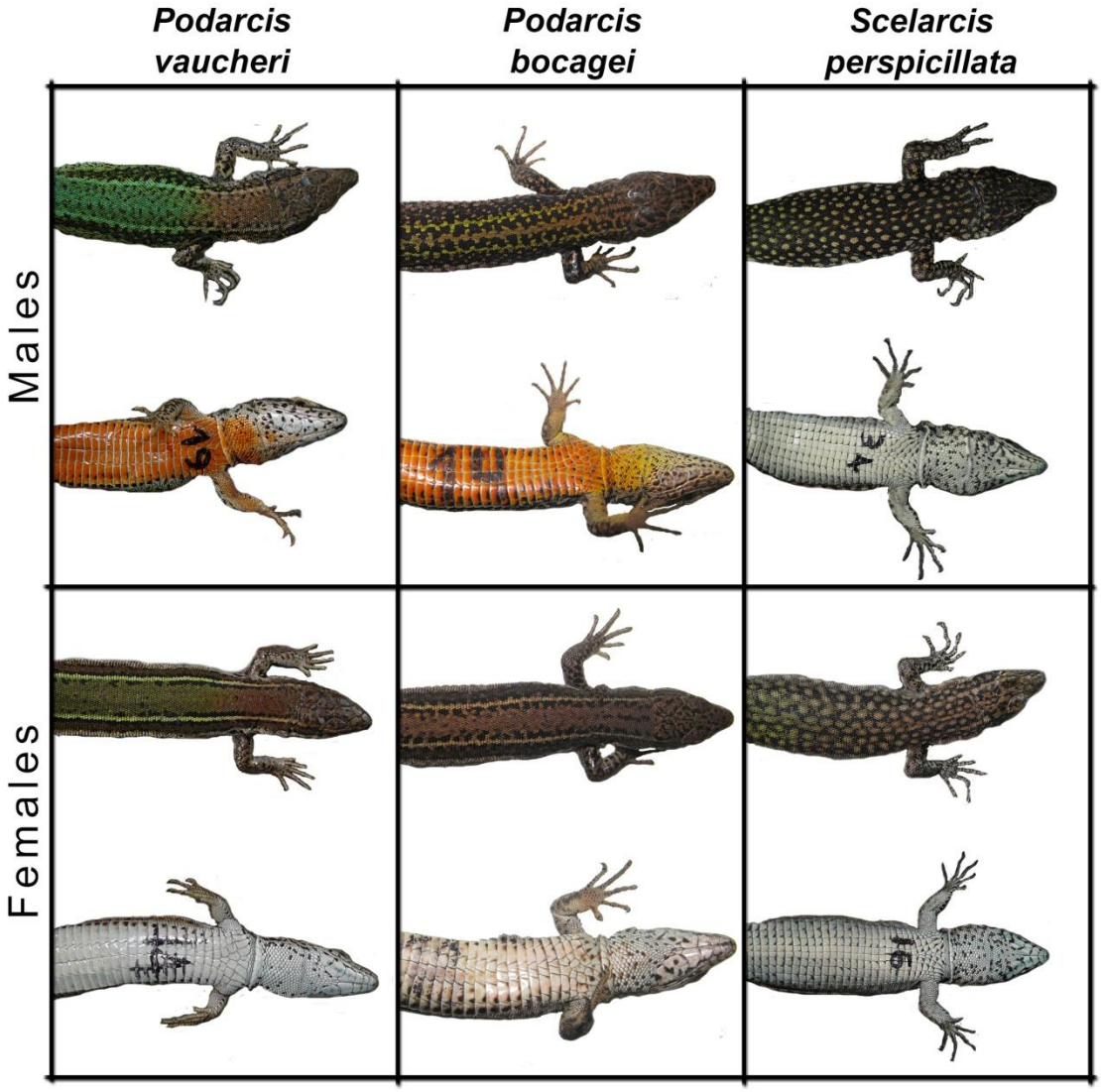

Fig. 7 - Dorsal and ventral pictures from both genders of the three studied species: *Podarcis vaucheri*, *Podarcis bocagei* and *Scelarcis perspicillata*. Pictures taken by Isabel Damas Moreira and Daniele Salvi.

Podarcis populations normally are ecological generalists, living in high densities and spread through the Mediterranean basin, occupying a wide range of habitat types (Arnold 1987). For the present works, the model species were *Podarcis bocagei*, *Podarcis vaucheri* and another lacertid that is sympatric with *P. vaucheri* in Morocco, *Scelarcis perspicillata* (Fig. 7).

Podarcis bocagei (Seoane 1907) is the predominant member of the genus in north-western Portugal and present a clear phylogeographic pattern, now evidently separated from the other Iberian *Podarcis* belonging to the *P. hispanica* complex (Pinho *et al.* 2008; Kaliontzopoulou *et al.* 2012). According to the current taxonomy, *Podarcis vaucheri* is the only species representing the genus in Morocco (Busack *et al.* 2005), with ample distribution though through central Morocco (Bons & Geniez 1996), in high densities in favourable places (Schleich *et al.* 1996).

A related lacertid species, *Scelarcis perspicillata*, occurs in sympatry with *P. vaucheri* in numerous Moroccan sites (Bons & Geniez 1996; Kaliontzopoulou *et al.* 2008). This association can represent an interesting model to understand the parasite-host interactions between two different host species, under the same environmental conditions and with similar ecological requirements and higher water-dependence (Schleich *et al.* 1996).

1.4. OBJECTIVES

Without knowledge of the effects of a specific parasite on a host species, it is impossible to evaluate the importance of the parasite as an evolutionary force (Price 1980).

For the first time, the impact of *Hepatozoon* blood parasites is investigated on *Podarcis* and *Scelarcis* lizards. The studies integrate ecological and genetic data, in order to evaluate pathogenicity of *Hepatozoon* parasites in selected lizards.

Conducting three surveys in several sites from both Portugal and Morocco, the impact on host fitness will be assessed through the immune system, performance and escape behaviour, both in the field and laboratory conditions.

Through the analysis of these host-parasite systems, prevalence and intensity pattern for *Hepatozoon* parasites will also be further assessed, for each species and sample site.

Through all of this, the aim is to gain further knowledge regarding *Hepatozoon* ecology, and to develop new insights into the overall effect of these parasites on the hosts.

CHAPTER II

MANUSCRIPTS

[Because one of the fundamental characteristics of life is that it is present on our planet in discrete forms]

Claude Combes

The Art of Being a Parasite

MANUSCRIPT I

VARIATION IN *HEPATOZOON* PARASITEMIA LEVELS IN SYMPATRIC LIZARD SPECIES: DO PARASITES AFFECT THE IMMUNE SYSTEM?

Isabel Damas-Moreira[1,2], João P. M. C. Maia[1,2,3], Beatriz Tomé[1]

Daniele Salvi[1], D. James Harris[1], and Ana Perera[1]

[1] *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal*

[2] *Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre FC4 4169-007 Porto, Portugal*

[3] *Institut de Biologia Evolutiva (CSIC-UPF). Passeig Maritim de la Barceloneta, 37-49. 08003 Barcelona, Spain*

ABSTRACT

Assessment of parasites is essential for studying populations' ecology and in understanding their dynamics. The phylum Apicomplexa is diverse group of obligate unicellular blood parasites, with a vast distribution. In many, the pathogenicity is still poorly understood, such as in genus *Hepatozoon*, the most widespread apicomplexan parasite detected in reptiles. Molecular diagnostic techniques have identified these parasites with increasing frequency in both wild and domestic animals, revealing in most cases severe deleterious effects in dogs and cats. However, results are not clear and ambiguous for most other hosts, including reptiles.

Our study focuses on two sympatric lizard species, *Podarcis vaucheri* and *Scelarcis perspicillata*, from three Moroccan sites, to which an immune test (PHA skin-testing technique) was applied to assess the levels of individual immune condition. In particular, this study will analyse how the infection load and prevalence vary between the sympatric species, and how the immune system is affected. 18S rRNA sequences were used to confirm parasite identity and results showed that parasites belong to the same *Hepatozoon* lineage. Our results showed that parasites were widespread, with differences in prevalence between localities and species. However, results suggest that *Hepatozoon* presence does not modify the immune response of these hosts, with no identified significant impact. The immune response was not correlated with circulating blood cell values and was significantly higher in smaller individuals.

KEYWORDS

Podarcis vaucheri; *Scelarcis perspicillata*; *Hepatozoon*; immune system; sympatry

INTRODUCTION

Parasites represent a major component of biodiversity, playing an important role in ecosystems (Pedersen & Fenton 2007). In fact, there is evidence showing that parasites are such a key part in ecosystem dynamics, that they can even shape an entire host community structure (Hudson *et al.* 2006; Pedersen & Greives, 2008). For example, apicomplexan parasites are responsible for well-known cases of severe impact on structure of ecosystems and host communities, such as the introduced avian malaria in Hawaii that devastated endemic bird populations naive to this disease (Van Riper III *et al.* 1986), or the case of parasite-mediated competition of two *Anolis* lizards in the Caribbean island of St. Maarten by a single Apicomplexan parasite species (Schall, 1992). Knowing the costs for the host to harbour such parasites is thus a central question when studying parasites' impact on ecological systems.

Apicomplexa Levine 1970 is a variable and widespread phylum of obligate intracellular parasites (Morrissette & Sibley 2002; Morrison 2009). They generally exhibit a heteroxeneous life cycle with one hematophagous invertebrate host and at least one vertebrate host, although it normally comprises two (Smith 1996; Criado-Fornelio *et al.* 2009; Telford 2009). It is a diverse phylum of parasites with high impact on animal communities and public health such as species within the genera *Plasmodium, Eimeria* or *Babesia*, but also parasites for which pathogenicity is still poorly understood, such as the haemogregarine genus *Hepatozoon* (Miller 1908). Despite being poorly known, the advent of molecular diagnostic techniques, particularly targeting the 18S ribosomal RNA gene (Mathew *et al.* 2000), has led to new studies not only identifying *Hepatozoon* with increasing frequency in both wild and domestic animals (e.g. (Mathew *et al.* 2000; Criado-Fornelio *et al.* 2009; Gimenez *et al.* 2009; de Bortoli *et al.* 2011; Pawar *et al.* 2012), but also shedding new light onto their phylogenetic relationships (e.g. Maia *et al.* 2011; Barta *et al.* 2012).

In recent decades it has been determined that *Hepatozoon* spp. cause hepatozoonosis in domestic animals, particularly cats and dogs, leading to severe impact on their health (Baneth *et al.* 1998; Panciera *et al.* 2000), and occasionally resulting in death (Karagenc *et al.* 2006). However, this is not always the case, and some *Hepatozoon* species may be better adapted to the host producing less severe effects (Baneth *et al.* 2003). This show how there is still much to be known and how the full assessment of *Hepatozoon* pathogenicity for other hosts might be of great importance.

Studies in reptiles reveal very ambiguous results (Telford 2009). Certain studies in pythons associated long-term parasitic infection with negative impacts on host life-history and stated that only those reptiles harbouring low *Hepatozoon* intensity were able to achieve older ages (Madsen *et al.* 2005; Ujvari & Madsen 2005), larger body size (Madsen & Ujvari, 2006) and exhibited a stronger humoral immune response (Ujvari & Madsen, 2005). However, this is not always reported. In the snake *Tropidonophis mairii*, little evidence was found between *Hepatozoon* intensity and several measures of fitness, indicating an apparently benign association between parasite and host (Brown *et al.* 2006). This study becomes even more interesting as a sympatric water python studied previously (Madsen *et al.* 2005) appeared to have strong correlations between the levels of *Hepatozoon* load and the same evaluated host fitness traits. These differences in sympatric snake species are highly important, as they may reflect to some extent the coevolution pattern of these parasites, and the enormous variation of effects *Hepatozoon* species can cause.

Since *Hepatozoon* infect mostly the red blood cells of a lizard (Jacobson 2007; Telford 2009), the study of the impact of this parasite on the immune system response can be considered of major importance, as erythrocytes are involved in vital functions (reviewed in Morera & Mackenzie 2011). The immune response can be assessed through several methods, such as counting the circulating blood cells and comparing differences in their ratio among infected individuals (Shutler *et al.* 2009). Another standard technique for measuring immunocompetence in vertebrates is the Phytohaemagglutinin (PHA) skin-testing technique (Kennedy & Nager, 2006). This method is frequently used in lizards (e.g. López & Martín 2005; Calsbeek *et al.* 2008) and has already been demonstrated in the genus *Podarcis* (Oppliger *et al.* 2004; Sacchi *et al.* 2007; Huyghe *et al.* 2010), for example. The technique consists of a PHA intradermal application *in vivo* in one of the hind-foot pads, which triggers a highly complex proinflammatory response, that induces a swelling of the foot pad when the lizard exhibits a good inflammatory response (Vinkler *et al.* 2010). Hence, individuals with greater response, should have better immune condition (Vinkler *et al.* 2010), and eventually higher survival probability (Gonzalez *et al.* 1999).

The sympatric lacertid lizards *Podarcis vaucheri* (Boulenger 1905) and *Scelarcis perspicillata* (Duméril & Bibron 1839) from Morocco are good models to investigate this potential impact of *Hepatozoon* on the first vertebrate host. Not only do they occur in sympatry in a wide distribution through Morocco (Bons & Geniez 1996; Schleich *et al.* 1996), but also because the most common blood apicomplexan parasites in lacertids are haemogregarines (Jacobson 2007; Telford 2009), and both species typically

display high infection levels (Oppliger *et al.* 2004; Roca & Galdón 2010; Maia *et al.* 2011; Harris *et al.* 2012). Moreover the genus *Podarcis* was already used for inferring the impacts of haemogregarines on life traits (Oppliger *et al.* 2004; Martín *et al.* 2008; Huyghe *et al.* 2010).

Therefore the main goal of this study is to assess the prevalence and intensity of *Hepatozoon sp.* on two sympatric lizard species from different localities in Morocco. The PHA skin test was used to assess if presence of parasites is related to any variation in the immune system response, to allow inferences to be drawn regarding the impact of *Hepatozoon* parasites on their sympatric lizard hosts. It is expected that if parasites are negatively affecting the lizards, a reduction of the immune response would be detected.

MATERIAL AND METHODS

This study was carried out in May 2012 at three sites in Morocco: Debdou (33.8725ºN, 3.0388ºW), Oukaimeden (31.2010ºN, 7.8554ºE) and Mischleiffen (33.4054ºN, 5.1033ºE). In each locality, two lizard species living in sympatry were studied, *Podarcis vaucheri* and *Scelarcis perspicillata*. Adult individuals were captured by hand or using nooses and kept in individual bags during 24 hours for Phytohaemagglutinin (PHA) testing.

The immune response was measured for each individual using the PHA test. A small dot in the right hind-foot pad was drawn with a permanent marker to be used as a reference point for injection and thickness measurements. The initial thickness of the foot-pad was measured 3 times in that same point using a digital calliper (±0.01 mm). Afterwards the foot pad was injected with 0.02 ml of PHA solution (Sigma-Aldrich) (López & Martín 2005; Amo *et al.* 2007), using a different syringe for each animal. To analyse how the lizard immune system reacted to the injected PHA, the right foot pad was measured 3 times, 24 hours later. The difference in the swelling between the measurements after and before injection was taken as a metrical index of the intensity of the immune response. To minimize inaccuracies as much as possible, all measurements were taken by the same person (I.D.M.).

After the experiments, several variables including species, sex, snout-vent length (SVL) and weight were recorded. Finally, before releasing at the sample sites, lizards were photographed, a small piece of tail tip stored in 96% ethanol for genetic analyses and a

drop of blood smeared across a glass slide and air-dried for posterior examination under a microscope. The slides were fixed in the field with methanol for 2 minutes, and once in the laboratory, they were stained with Giemsa for 45 minutes and analyzed under an Olympus CX41 microscope. Individual parasitemia levels were estimated as the percentage of infected red blood cells per 2500 cells; prevalence was considered as the percentage of infected host individuals within a population. For the cell counting, pictures were randomly taken in sequences through the slide at x400 using the Cell^B 3.4 Olympus® software and the following cell types were counted using the ImageJ 1.46® program: parasitized erythrocytes, un-parasitized erythrocytes, immature erythrocytes, thrombocytes and leukocytes (lymphocytes, monocytes, heterophils, eosinophils, azurophils, and basophils).

After the microscope screening, positive samples were randomly selected from each population, to confirm parasite identity using molecular methods. Parasite DNA was extracted from selected *Hepatozoon* positive samples using High Salt methods (Sambrook *et al.* 1989). A fragment of the 18S rRNA gene was amplified using the primers HepF300 and HepR900 (Ujvari *et al.* 2004) and the conditions from (Harris *et al.* 2011). PCR products were purified and sequenced by a commercial sequencing facility (Macrogen, The Netherlands). Sequences were blasted in GenBank to confirm their identity.

Prior to statistical analysis all continuous variables were log transformed to meet normality and homoscedasticity assumptions. A proxy for each animals' body condition was calculated (SVL / weight) and in order to determine if this body condition estimate (BC) or snout-vent length should be included as a covariate in the following analysis, non-parametric Spearman correlations were performed between SVL or BC and the variables *prevalence*, *intensity* and *immune response* using the function *cor* of the R package (R Development Core Team 2012). Differences in SVL and BC among *species*, *locality* and *sex* were analysed using Analysis of Variance (*ANOVA*) with the function *lm* in R (R Development Core Team 2012).

Differences in parasite prevalence among localities, species and sexes were analysed with a Generalized Linear Model (*GLM*) with a logistic regression function where the full model included *presence* of the parasite as response variable, and host characteristics (*SVL* or *BC, species, locality* and *sex*) and their interactions, as factors (Venables & Ripley 2002; R package MASS; R Core Team 2012). Post-hoc tests were performed to assess which group was responsible for the differences detected.

Regarding intensity of parasites, we performed an Analysis of the Covariance (*ANCOVA*) using a linear model (function *lm* implemented in R, R Core Team, 2012) with parasite *intensity* as response variable, SVL or BC as covariate, and host characteristics (*species, locality* and *sex*) and their interactions as factors. The interaction factors with covariate was used to test the assumption of slope homogeneity (Engqvist 2005). Post-hoc tests were performed to assess which group was responsible for the differences detected.

When assumptions for *ANCOVA* were not met, permutational analyses of covariance *ANCOVA* were used. This procedure is a good alternative to sum-of-squares-based *ANCOVAs*, in cases where data do not meet normality and homoscedasticity assumptions (Anderson 2001). Permutational *ANCOVAs* with the same models tested using parametric procedures, based on 1000 permutations were calculated using the function a*donis* implemented in the package *Vegan* (Oksanen *et al.* 2012) of the R software (R Development Core Team 2012).

In order to have a first estimation of any putative relation between immune response and parasites was analysed in two ways. First, we analysed differences in the swelling response between infected and non-infected individuals, using an *ANCOVA,* with *swelling response* as dependent variable and the host characteristics (*SVL, prevalence, species, locality* and *sex*) as factors, the latter being used to test slope homogeneity (Engqvist 2005). Furthermore, in order to understand how the swelling response was related to the intensity of parasite infection, a similar analysis was run including only infected lizards, using the factor *intensity* instead of *prevalence*. Also, correlations between swelling response and intensity within each group (species and localities) were assessed using Spearman correlation methods.

The possible association between swelling response and the levels of certain blood cell types was tested with *ANOVAs* using a linear model with the immune response as dependent variable, and the number of thrombocytes, immature erythrocytes and leukocytes as factors. The different leukocyte types were not analysed separately due to the uncertainty associated with its differentiation in some cases.

RESULTS

From the genetic analysis results, parasites from 13 hosts were all confirmed as *Hepatozoon* spp. belonging to the same lineage (lineage 2 as described in Maia *et al.*

(2012). Body length (SVL) showed differences among species (F=40.77, df=1, p<0.001) with *Scelarcis* being larger than *Podarcis*, localities (F=15.60, df=2, p<0.001), with individuals from Oukaimeden being the longest and Debdou the shortest, and sexes (F=6.43, df=1, p=0.012) with males being longer than females. Interactions between these factors were not significant (in all cases, p>0.05). Regarding body condition (BC) *P. vaucheri* had higher SVL / Weight ratio than *S. perspicillata* (F=14.62, df=1, p<0.001), individuals from Mischleiffen showed worse BC and Debdou the better one (F=8.89, df=2, p<0.001), and females had better BC than males (F=37.76, df=1, p<0.001). Therefore SVL and body condition were considered in further analysis.

Parasite prevalence did not differ between species (χ^2=220.73, p=0.502), but it did differ among localities (χ^2=193.06, p<0.001), with almost 80% individuals infected in Oukaimeden, against 50% infected in Mischleiffen and 33% in Debdou, and sexes (χ^2=188.84 p=0.040) with prevalences of 59% in males versus 46% in females (Table 1). We also found differences in the interaction species*locality (χ^2=170.56 p<0.001) and locality*sex (χ^2=163.20 p=0.029). Parasite intensity differed among localities (F=0.0637, df=2, p=0.030; Table 1), and also among localities interacting with body size (F=0.2607, df=2, p=0.001). However, when splitting the populations in species, localities, and genders, we observed no correlation between host SVL or BC and the intensity of infection (in all cases, p>0.05).

Table 1. Descriptive statistics of *Hepatozoon* intensity and prevalence among sites, species and genders.

	Debdou				**Oukaimeden**				**Mischleiffen**			
	Podarcis vaucheri		*Scelarcis perspicillata*		*Podarcis vaucheri*		*Scelarcis perspicillata*		*Podarcis vaucheri*		*Scelarcis perspicillata*	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
N	30	14	8	13	26	7	7	17	16	5	11	6
Mean intensity (%)	0.236	0.113	0.383	0.665	1.233	0.623	0.097	0.270	0.212	0.123	1.327	2.902
±SD	0.310	0.065	0.642	0.594	2.905	0.447	0.048	0.448	0.205	-	1.378	2.516
Prevalence (%)	33.33	21.43	62.50	23.08	92.31	100.00	42.86	64.71	31.25	20.00	90.91	50.00
	30.23		40.00		93.94		58.33		28.57		76.47	
	33.33				78.95				50.00			

Regarding the immune response, body size (SVL) was negatively correlated with swelling response (Spearman correlation, R=-0.271, p<0.001), meaning that larger lizards tend to have a weaker immune response. Given this correlation, swelling response was corrected by SVL in further analysis. However, BC was positively correlated with swelling response (R=0.209, p=0.008), meaning that immune response was higher in animals with better body condition. Interestingly, we did not find differences in the immune response between infected and non-infected lizards. However, differences in the immune response were detected among localities (F=8.56,

df=2, p<0.001). When accounting only with infected individuals, the swelling response of the parasitized animals differed among sexes (F=8.04, df=1, p=0.007) and localities (F=3.99, df=2, p=0.026), but it was not related to parasite intensity. The analysis concerning the effect of circulating blood cellular values, no significant association was found among cell types and the immune response nor parasite intensity (for all, p>0.05).

DISCUSSION

Studies made in lizards are typically focused on the analysis of parasite prevalence and intensity in populations from a single species. To our knowledge this study is the first to assess the parasitemia of *Hepatozoon sp.* in two sympatric genus of lizards (*Podarcis* and *Scelarcis)* incorporating phylogenetic, ecological (different localities), and host (species, sex, size, and the different cells) characteristics simultaneously. The preliminary assessment of parasite prevalence between the two host species suggest that *Hepatozoon* tend to be generalistic, being constrained mainly by the host distribution and accessibility, rather than host specificity (Mccoy *et al.* 2001; Sloboda *et al.* 2007).

However, our results reveal some local differences in parasite prevalence between the two host species. In Debdou prevalence was similar between species, but there were divergences among species for the other two localities. Oukaimeden was the local with higher number of infected individuals, with a higher prevalence in *P. vaucheri* (94% prevalence in *Podarcis* versus 58% in *Scelarcis*), while *S. perspicillata* exhibited more parasitized specimens in Mischleiffen (28% prevalence in *Podarcis* versus 76% in *Scelarcis*). Regarding differences between sexes, we observed higher prevalence in males than in females. These results might be related with the immunosuppressive effect of hormones such as testosterone, which would make males more susceptible to parasites than females (Klein 2004). Moreover, we did also find differences in parasite intensity between localities or species, but not between species or sexes, which might be indicative of a similar level of virulence in both sympatric hosts. On the other hand, the differences in prevalence between species within each site might point to different levels of host resistance (Oppliger *et al.* 1999).

Our results indicate that parasite load is not correlated to body size or body condition for both species at all the sampled sites. Previous works showed that haemogregarines can be positive or negatively correlated to body size and condition, with incongruent

results even within the same host genus. In *Timon lepidus* (formerly *Lacerta lepida*), the prevalence but not the intensity of infection by haemogregarines was positively correlated to the adult size, but not to body condition (Amo, Fargallo, *et al.* 2005) whereas *Lacerta viridis* was shown to have both condition and body size positively correlated with blood parasite intensity (Molnár *et al.* 2013). In *Podarcis muralis* those harbouring higher haemogregarine load had better body condition (Amo, López, *et al.* 2005), while the opposite trend was observed in the insular *Podarcis lilfordi*, where higher prevalence and parasite load in individuals with lower condition was observed (Garrido & Pérez-Mellado 2013).

The immune response was negatively correlated to SVL but positively linked to body condition, which means that lizards with greater immune response were smaller but with higher body condition. However, swelling response was not related to parasitemia, i.e., was not related to the presence of the infection, nor to parasite intensity in infected individuals. The fact that we did not find any significant correlation between parasite intensity with body condition and swelling response, in any of the three localities and for both species, may suggest that *Hepatozoon* sp. does not noticeable prejudice the immune inflammatory response of both selected lizard hosts from Morocco in wild conditions.

So far, few studies attempted to use the PHA skin-testing technique to look for haemogregarine effects in reptiles and most of them did not conduct direct comparison between these two factors, such as in assessments with the species *Iberolacerta cyreni* (Amo *et al.* 2007), *Psammodromus algirus* (Martín *et al.* 2007) or *Podarcis* (Oppliger *et al.* 2004; Martín *et al.* 2008). To our knowledge, the only studies that compare directly these two variables were performed in *Podarcis muralis* (Amo *et al.* 2006) and *Podarcis melisellensis* (Huyghe *et al.* 2010). The former study found a negative correlation between immune response and haemogregarine load. In the second work, as in our study, the authors did not observe any relationship between the immune response and haemogregarine infection levels but, since their primary objective was to test the morph specific variation in parasite load and cellular response across seasons, they did not discuss this particular result.

Some reptile species are capable of tolerating haemogregarine infections, experiencing no or slight negative impact (Wozniak *et al.* 1996). This may be the typical case of *Hepatozoon*, which is generally assumed to have low pathogenicity in snake species (Nadler & Miller 1984; Telford 1984). In fact, Brown and colleagues (2006) go further, and claim that the infection on keelback snakes (*Tropidonophis mairii*) may be benign

for the host, with insignificant consequences on the host fitness. Furthermore, other studies with haemogregarine parasites displaying the same trivial consequences were observed in other snakes, including *Boiga irregularis, Stegonotus cucullatus* and *Elaphe obsolete*, when assessing general haemogregarine impact on reptile health (Caudell *et al.* 2002; Sperry *et al.* 2009). Nevertheless, it has been hypothesized that these parasites can provoke clinically significant inflammatory disease in unnatural hosts, but little or no pathogenic change in their natural hosts (Telford, 1984; Wozniak *et al.* 1996).

Since *Hepatozoon* thus seems to have limited impact on the hosts from the present study, it is possible that the interaction between host and parasite may be relatively stable in these populations (Combes 2001). This can also be supported by the absence of a correlation with the number of leukocytes, which also provides a measure of the immune function (Norris & Evans 2000), or with the amount of immature erythrocytes, which are indicators of anemia and could possibly indicate a weaker immune system (Oppliger *et al.* 1996; Telford, 2009). As postulated in other host-parasite systems (Toft & Karter, 1990; Thompson, 1994), this stability can be the outcome of the gradual coevolution between this *Hepatozoon* lineage and the two lizard genera living in sympatry in Morocco. In the face of constant exposures over considerable time, the host defences could have evolved towards a mild effect for the lizard immune system; and such mild effect could present an advantage for the parasites since it would allow them to maintain the host alive as long as is needed to ensure propagation (Combes 2001). Besides this, *Hepatozoon* might display more complex actions and be able, for example, to escape from immune system detection or manipulate leukocyte investment, as suggested by Shutler *et al.* (2009). Wherever origin it might have, this consequential mild effect could enhance effective transmission to other vertebrates, thus continuing the life cycle of the parasite (Thompson 1994; Combes 2001, 2005).
In sum, our conclusions point to a near-negative effect of parasitism by *Hepatozoon sp.* on the immune system of wild *P. vaucheri* and *S. perspicillata* from Morocco, in accordance with some previous works developed in reptiles and amphibians (Brown *et al.* 2006; Shutler *et al.* 2009). This study highlights the limitations of the assumption that *Hepatozoon sp.* prevalence and intensity in reptiles is related to a lower fitness compared to exhibiting no or low parasitemia levels. These results also highlight the need of improving our understanding regarding the *Hepatozoon* impact on different hosts.

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MANUSCRIPT II

TESTING THE IMPACT OF *HEPATOZOON* PARASITES ON THE PERFORMANCE AND IMMUNE SYSTEM OF *PODARCIS BOCAGEI*

Isabel Damas-Moreira[1,2], D. James Harris[1], João P. M. C. Maia[1,2,3], Beatriz Tomé[1], Veronica Gomes[1,4], Antigoni Kaliontzopoulou[1,5], Miguel A. Carretero[1], and Ana Perera[1]

[1] *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal*

[2] *Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre FC4 4169-007 Porto, Portugal*

[3] *Institut de Biologia Evolutiva (CSIC-UPF). Passeig Maritim de la Barceloneta, 37-49. 08003 Barcelona, Spain*

4 Cátedra "Rui Nabeiro", Biodiversidade, Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Universidade de Évora, Évora, 7004-516, Portugal

5 Department of Ecology, Evolution, and Organismal Biology, (515) 294-0133, Iowa State University, Ames, IA, USA

ABSTRACT

Parasites can shape population dynamics by acting at several levels on the host, including its ecology and behaviour, but how this interference takes place still warrants further research. Haemogregarines (Apicomplexa) are the most common hemoparasites in reptiles, and among them, the genus *Hepatozoon*, is now gaining more interest as molecular tools reveal its wide occurrence in a large array of hosts. *Hepatozoon* gamonts invade host erythrocytes, thus diminishing the hosts' ability to transport oxygen as a consequence of the reduction in the red blood cells. Although in a broad sense haemogregarines seem to have a low impact on their natural reptilian hosts, the impact of harbouring parasites of the genus *Hepatozoon* is controversial.

In the present study, the consequences of *Hepatozoon* parasitism on host immune system and performance was assessed in four populations of the lacertid *Podarcis bocagei* using three host fitness indicators: swelling response (PHA), maximum sprinting speed and maximum bite force. Prevalence and intensity of parasitism were high in the four populations analysed. We detected that larger individuals hosted more parasites, although there were no differences between sexes. Humoral immune response was lower in larger and more parasitized individuals. However, haematological pathologies that can arise from haemogregarine infection were not observed in our study. Results showed that increased parasitism was correlated with maximum bite force but no effects of *Hepatozoon* parasites were detected on the sprinting speed, which varied locally, mostly due to slight differences in morphologies of the different host populations.

KEYWORDS

Podarcis bocagei; *Hepatozoon*; immune system; sprinting speed; bite force

INTRODUCTION

At a time when emerging diseases pose actual threats to wildlife and human health, there is a real need to find out more about parasite ecology and evolution (Poulin 2007). Parasites can interfere with host population dynamics by acting at several levels of its ecology and behaviour, but how this interference takes place still requires much research (Hudson *et al.* 2002, 2006). The study of blood parasites belonging to the haemogregarine genus *Hepatozoon* (Apicomplexa: Adeleorina: Hepatozoidae) is recently gathering interest as molecular tools reveal its wide distribution in a large array of hosts (Harris *et al*. in press; Smith *et al.* 1999; de Bortoli *et al.* 2011; O'Dwyer *et al.* 2013; Pinto *et al.* 2013).

Haemogregarines are blood parasites transmitted by hematophagous invertebrates and are considered the most common hemoparasites in reptiles (Telford 2009). Therefore the relationship between *Hepatozoon* and their reptile hosts has been investigated (e.g. Brown *et al.* 2006; Ujvari & Madsen 2005), but nevertheless its pathology and effect on the host still need to be properly addressed.

Although in a broad sense haemogregarines seem to have a low impact on their natural reptilian hosts (Manwell 1977; Bouma *et al.* 2007), the impact of harbouring *Hepatozoon* is still unclear. For example, while some authors found that older lizards harbour more parasites (Gupta *et al.* 2013), others found the exactly opposite trend, with younger subjects exhibiting more parasites (Godfrey *et al.* 2011). The same ambiguity can be found in other animal traits. High *Hepatozoon* load can be directly linked with slower growth rates and worse body condition in pythons (Ujvari *et al.* 2004; Madsen *et al.* 2005) while further research found a surprising lack of correlation between parasitemia levels and fitness consequences in other snakes (Brown *et al.* 2006; Xuereb *et al.* 2012).

Some experimental evidences show, however, that *Hepatozoon* can severely affect the health of infected reptiles (Pessôa *et al.* 1974; Wozniak & Telford 1991; Wozniak *et al.* 1996). On the other hand, much less information is available regarding the immune system reaction of naturally infected reptiles, although it is suggested that mounting an immune response against this pathogen is associated with physiological costs (Ujvari & Madsen 2005).

The majority of studies indirectly infer about the consequences for the immune condition of an infected individual, by assessing fitness traits, which can reflect the

performance of the immune system. For example, higher haemogregarine burdens were found to be associated with smaller home ranges (Bouma *et al.* 2007), or less colourful males, but exhibiting better body condition (Martín *et al.* 2008; Molnár *et al.* 2013). Likewise, also negative associations between haemogregarine load and locomotor speed (Oppliger *et al.* 1996) or tail regeneration rate (Oppliger & Clobert 1997) can point to an indirect negative effect in the immune system function. Locomotor performance is essential for lizards' survival, and the aptitude to reach a certain maximum speed can be directly reflected in tasks such as foraging, mating or escaping from predators (e.g. Clobert *et al.* 2000), being a good trait to infer the fitness of an animal (Husak 2006).

In the present study, we investigate the impact of *Hepatozoon* sp. in the immune system and in performance behaviours of *Podarcis bocagei* (Seoane, 1885). This small lacertid, endemic to the north-west Iberian Peninsula, has a well-known phylogeographic structure (Pinho *et al.* 2008) while morphological variation has also been assessed (Kaliontzopoulou *et al.* 2012), and it is known to harbour *Hepatozoon* parasites (Maia *et al.* 2012). In addition, the intensity and prevalence patterns in four localities in the North of Portugal are also reported and compared.

MATERIAL AND METHODS

Adult males (n=20) and females (n=20) of *Podarcis bocagei* were collected during October 2012 at four Portuguese periurban localities: Madalena (41.100229º, -8.650075º), Mindelo (41.307928º, -8.720400º), Gião (41.299560º, -8.674506º) and São Mamede do Coronado (41.274195º, -8.571028º). Individuals were noosed and transported to the laboratory, in Vairão, Porto. Each animal was placed in individual terraria, under natural light conditions and with food and water supplied *ad libitum*. The lizards had an adaptation period of two days before starting the performance experiments. For each individual, two performance tests (bite force and sprinting speed) were measured. Lizards' sex, weight and snout-vent length (SVL) were recorded, and individuals with recent tail autotomy or amputations were excluded. After trials we tested for individual immune response.

Both performance tests were carried out under a controlled temperature of about 31°C (similar to the selected body temperature of this species; Ferrand de Almeida *et al.* 2001) in order to ensure their best performance. Bite force was measured 5 times with a SAUTER FH20 external (Sauter GmbH, Albstadt, Germany) mounted on a vertical

holder and connected to a Kistler charge amplifier (type 5058A, Kistler Inc., Winterthur, Switzerland). The highest value from the total of measurements for each individual was recorded.

Locomotor performance was carried out in order to obtain the maximum sprint speed for each individual. This test took place in a racetrack 1m long divided into 10 segments, and made of cork, which provides good traction (Bauwens *et al.* 1995; Kaliontzopoulou *et al.* 2013). All runs were filmed with a CANON digital camera at 50 frames per second. To ensure that maximal locomotor capacity was recorded, each individual was tested three times. Afterwards, the videos were visualized frame by frame, and the number of frames per second selected for each 10 cm interval. Among them, the maximum speed recorded over any 10cm interval of each individual was selected as maximum sprint speed for statistical analyses, following Kaliontzopoulou *et al.* (2013).

Two days after the tests finished, we tested the lizards' immune response using the PHA skin-testing technique. A dot was marked on the lizard right hind-foot pad and its thickness measured 3 times with a digital caliper (±0.01 mm). This mark ensures that injection and measurements are performed at the same point. The foot pad was then injected with 0.02 mL of Phytohaemagglutinin (PHA) solution (Amo *et al.* 2007; Martín *et al.* 2008), using a different syringe for each animal.

To evaluate the reaction of the lizard immune system to the injected PHA, the right foot pad was measured again 3 times 24 hours after (Smits *et al.* 1999). The difference in the swelling between average measurements after and before injection was used as a metrical index of the intensity of the immune response. This reaction takes place because the PHA intradermal application in vivo activates a complex proinflammatory reaction that induces a swelling of the foot pad when the lizard exhibits an inflammatory response (Vinkler *et al.* 2010), exhibiting a good immune condition. All the measurements were taken by the same person (I.D.M.) while another (D.J.H.) performed all the injections.

Before releasing the lizards at their sample sites, a piece of tail tip was taken and stored in 96% ethanol for genetic analyses (Sevinç *et al.* 2000). Resulting blood was smeared across a glass slide and air-dried. The slides were fixed with absolute Methanol for 2 minutes, stained with Giemsa (1:9 distilled water) for 45 minutes and air dried. Examination took place using an Olympus CX41 microscope. Individual parasitemia load was considered as the percentage of infected red blood cells per 2500 cells and prevalence was estimated as the percentage of infected individuals

within a population. For the cell counting, pictures were randomly taken across the slide at x400 using the Cell^B 3.4 Olympus® software and several cell types were counted using the ImageJ 1.46® program: erythrocytes, immature erythrocytes, parasitized erythrocytes, thrombocytes and leukocytes (lymphocytes, monocytes, heterophils, eosinophils, azurophils, and basophils).

Parasite DNA was extracted from selected *Hepatozoon* positive samples using High Salt methods (Sambrook *et al.* 1989) and an 18S rRNA gene fragment (Ujvari *et al.* 2004) amplified using standard protocols (Harris *et al.* 2011). PCR products were sequenced using an external facility (Macrogen, The Netherlands), and sequences blasted in GenBank in order to confirm the identity of the parasite.

DATA ANALYSIS

Three distinct analyses were performed, holding as dependent variables the immune response for the first model and bite force and maximum sprint for the second and third models, respectively. Prior to statistical analysis all continuous variables were log transformed to meet normality assumptions and a proxy for each animals' body condition (BC) was estimated as SVL / weight. In order to first understand the population dynamics and variation of parasite infection, some analysis regarding the host traits and infection values were carried out. Post-hoc tests were performed to assess which group was responsible for the differences detected. Differences in SVL and BC among *locality* and *sex* were analysed using Analysis of Variance (*ANOVA*) with the function *lm* in R.

Differences in hosts' body size and body condition were first investigated. In order to describe levels of prevalence and intensity of parasitism in the populations analysed, differences in parasite prevalence for all models were analysed with a Generalized Linear Model (*GLM*) with a logistic regression function where the full model included *presence* of the parasite as response variable, SVL or BC as covariables, host characteristics (*locality* and *sex*) as factors and the interactions of the factors with the covariate (R package *MASS*, Venables & Ripley 2002; R Core Team 2012). The interaction factor was used to test the assumption of slope homogeneity (Engqvist 2005).

In order to determine if SVL or BC are correlated with parasite intensity of infected individuals, non-parametric Spearman correlations were performed between *SVL* and *intensity* for the global dataset and for each sample site, using the function *cor* of the R

package (R Development Core Team 2012). To analyse the parasite load among localities and sexes, an Analysis of Covariance (*ANCOVA*) with *lm* function was carried out, with SVL or BC as covariables.

In order to analyse the factors affecting the immune response, non-parametric Spearman correlations were first tested to analyse association between SVL or BC and the swelling response. Afterwards, *ANCOVAs* were performed to check if the immune response differed between infected and non-infected lizards, accounting for an effect of the *locality* and *sex,* and correcting for SVL. Likewise, the relationship between swelling response and parasite intensity in infected individuals was carried out with a similar *ANCOVA* model, but using the variables *intensity* instead of *prevalence*. To make further inferences regarding this, non-parametric Spearman correlations between immune response and parasite intensity were performed within each sample site. Finally, interactions between swelling response, parasite intensity and the quantity of each different cell type were analysed through *ANCOVAs*, using SVL or BC as covariables.

In the second model, the maximum bite force was investigated. Initially, a non-parametric Spearman correlation was performed in order to assess SVL interference in this lizard trait. Therefore, SVL was used as covariable in the final model. Then, an *ANCOVA* was carried out to understand eventual variations in bite force of infected and non-infected lizards, adding *localities* and *sex* to the model and SVL as covariable. A similar *ANCOVA* model was used on infected individuals but including parasite intensity instead of prevalence in the model. Non-parametric Spearman correlations were also conducted on only parasitized lizards to assess the association between intensity and bite force, in total and for each locality individually.

Finally, maximum sprinting speed was tested against parasite infection. We performed an *ANCOVA* (with SVL as covariable) to assess variations in maximum sprinting speed for infected and non-infected animals, with localities and sex incorporated, and then, a similar *ANCOVA* model was used but using parasite intensity instead of *prevalence*. Finally, non-parametric Spearman correlations was used to infer about the relation of intensity with sprinting speed in total, and for each locality.

Given that in some cases *ANCOVAs* assumptions were not met, permutational analyses of covariance *ANCOVA* were used to confirm the results. This procedure is a good alternative to sum-of-squares-based *ANCOVAs*, in cases where data do not meet normality and homoscedasticity assumptions (Anderson 2001). Permutational *ANCOVAs* based on 1000 permutations were calculated using the function a*donis*

implemented in the package *Vegan* (Oksanen *et al.* 2012) of the R software (R Development Core Team 2012).

RESULTS

From the genetic analysis results, all parasites were confirmed as *Hepatozoon* spp. belonging to the same lineage (Maia *et al.* 2012). Differences in body condition and size were detected among localities (BC: F=4.478, df=3, p=0.005; SVL: F=14.437, df=2, p<0.001), and BC also varied between sexes (F=22.021, df=1, p=<0.001), with females exhibiting better body condition than males. Madalena and Mindelo had the individuals with the worse body condition, but the smaller average size was from Gião, while the largest average lizards were found in Madalena and intermediate ones in Mindelo and São Mamede do Coronado.

We found apparent differences in prevalence among localities but differences disappear after including in the model SVL as covariate (p>0.05), with SVL being the only significant variable (Table 2.). These differences in prevalence are thus, the result of different body sizes in the populations. In effect, infected individuals are on average larger than non-infected individuals (F=41.905, df=1, p<0.001).

Table 2. Descriptive statistics of *Hepatozoon* intensity and prevalence among sites and genders.

	Gião		**Madalena**		**Mindelo**		**São Mamede do Coronado**	
	Males	Females	Males	Females	Males	Females	Males	Females
N	20	20	20	18	20	18	20	20
Mean intensity (%)	0.557	0.194	0.216	1.717	2.085	0.305	0.418	0.698
±SD	0.738	0.138	0.145	3.835	5.04	0.463	0.384	0.96
Prevalence (%)	35.00	55.00	75.00	72.22	65.00	77.78	60.00	55.00
	45.00		73.68		71.05		57.50	

Analysing only the infected lizards, intensity did not differ between sexes (F=0.126, df=1, p=0.718) or localities (F=0.882, df=3, p=0.448), but a general positive correlation between body size and parasite intensity indicated that larger lizards are more heavily infected (R=0.412, p<0.001). This correlation was detected within all localities (for Mindelo, São Mamede and Gião, p<0.05), with the exception of Madalena (R=0.140, p=0.402). Not significant effects were found in the interaction between factors (F= 0.070, df=3, p=0.059).

The analysis of the immune response showed a negative correlation between swelling response and SVL, with larger animals having a reduced swelling response (R=-0.210,

p=0.009). Interesting, larger animals are also more commonly infected and have more parasites. Regarding the relation between swelling response and body condition, animals with worse BC had a reduced immune response (R=0.260, p=0.001). After correcting by SVL, immune response was not significantly different between sexes (F=1.606, df=1, p=0.207), localities (F=1.283, df=3, p=0.283) or prevalences (F=0.127, df=1, p=0.722). Using the same approach, the same non-significant results were verified for infected individuals, when studying the relation between immune response and intensity for each locality and sex (p>0.05 in all cases). In fact, the results showed no correlation between the swelling response and the intensity of infection, in any of the studies localities (for all four sample sites, p>0.05). Finally, there was no difference in the swelling response or intensity depending on the cell types, and considering SVL as covariate (p>0.05 in all interactions).

The analysis of maximum bite force showed, as expected, that bigger animals bite harder (R=0.806, p<0.001). There are also differences in the bite response between sexes (F=532.070, df=1, p>0.05) and the interaction prevalence*sex (F=11.150, df=1, p=0.001) after SVL correction. Similarly, the *ANCOVA* performed on infected animals also showed differences in bite force due to SVL (F=1152, df=1, p<0.001), intensity (F=3.963, df=1, p=0.048) and sex (F=501,df=1, p<0.001). These results indicate that even after correcting by SVL, males bite harder than females. A general positive correlation was also found between intensity and bite force in infected individuals (R=0.340, p<0.001). However, when investigating each locality separately, this significant correlation was only detected in São Mamede do Coronado (R=0.336, p=0.034, other localities p>0.05).

Finally, maximum sprinting speed was tested and it was verified that it is related to both SVL and locality (p<0.05 for both), and that, these differences among localities maintain after correcting by SVL (F=5.654, df=3, p=0.001). Considering only infected lizards, results are similar, with differences in maximum speed among localities (F=6.301, df=3, p=0.001), but no effect of intensity or sex was detected on sprint speed (p>0.05). This lack of differences was confirmed when analysing correlation was detected among speed and intensity within each locality (for all, p>0.05).

DISCUSSION

It is worth noting that every locality displayed *Hepatozoon* infections in both sexes, which can show some stability in the presence of this parasite in this area (Combes 2001). Prevalence and intensity had high values (similar to Roca & Galdón 2010, in

Podarcis) and has been demonstrated here that prevalence and intensity is high and do not differ between the four sample sites. This might be associated with a similar host resistance for *Podarcis bocagei* between localities (Oppliger *et al.* 1999), also taking into account that all analysed parasites are from the same lineage.

Our results indicate that infected individuals are larger than non-infected, and larger hosts exhibit lower swelling response. However, when removing the effect of the animal size, no direct association was found between the amount of parasites and the immune response. Therefore, it is clear the influence of body size in both parasitism and immune response. Since larger lizards are presumably older (Duellman & Trueb 1985), they may be more exposed to diseases and vectors for a longer time, thus showing higher parasites intensity (Schall 1986; Ujvari & Madsen 2005). In the same way, larger animals had a lower immune response, which some authors suggest to be the result of an ageing process (Cichon *et al.* 2003; Amo *et al.* 2005; Ujvari & Madsen 2005). A decline in immune function as individuals grow older can also be related to gradual alterations of the immune system and increased vulnerability to infections (Miller 1996). However, more studies to assess the effect of parasitism and age are needed, using more accurate methodologies such as skeletochronology or capture-recapture methods.

In this sense, our results resemble those from a previous study conducted in water pythons (*Liasis fuscus*) parasitized by *Hepatozoon*. As in our experiment, the authors detected humoral immune response to be lower in larger and more parasitized individuals, but also in animals with worse body condition (Ujvari & Madsen 2005). The reduced immune response allied with poor body condition may suggest that setting up an inflammatory reaction is likely to entail energetic costs (Demas *et al.* 1997; Bonneaud *et al.* 2003; Madsen *et al.* 2005; Ujvari & Madsen 2005). Larger body size and lower body condition, associated with more parasites, was already detected in insular *Podarcis lilfordi* (Garrido & Pérez-Mellado 2013).

The sex effect on the bite force is well studied, as male lizards bite harder due to the larger head sizes (Herrel *et al.* 1996; Verwaijen *et al.* 2002). In our study larger lizards exhibited better bite performance, and parasitism was correlated with bite force, which is ultimately related to animal size. Therefore, we show that the higher parasite load in lizards with greater bite force is mostly associated with their size, suggesting that the effect of parasites in bite force is not significant. As mentioned, this may be a direct outcome from longer exposure time to vectors and disease (Schall 1986; Garrido & Pérez-Mellado 2013). Also, bigger animals with stronger bite force are related with

dominance, and therefore their higher aggressiveness can place them in situations with more risk of getting bitten by the hematophagous vector (Schall 1986). Similarly, more dominant males can take over the best territories for foraging and basking, which will probably have greater vector presence, as suggested by Garrido & Pérez-Mellado (2013), or have more agonistic encounters with other lizards, who might pass to them their mites or ticks (Amo *et al.* 2005), both vectors of *Hepatozoon* (Smith 1996). As such, we argue that parasites are not negatively affecting bite force capacity of *P. bocagei*, since more parasitized animals were those exhibiting higher maximum bite force, which is directly associated with advantages in combats and copulations (Herrel *et al.* 1996, 2001; Husak *et al.* 2006).

Interestingly, we detected no effects of *Hepatozoon* parasites on the sprinting speed, which indicate that they are not affecting the locomotor performance at this level in this species. Our findings are consistent with previous studies detecting no impact of parasites on fitness determinants (Weatherhead 1990; Eisen 2001; Caudell *et al.* 2002; Xuereb *et al.* 2012). Some studies report that haemogregarines can negatively affect lizards' locomotor performance (Oppliger *et al.* 1996; Clobert *et al.* 2000), but that has not been verified in our study. Instead, our results agree with the findings recently published where no linkage was found between ectoparasite parasite load and running speed (Ekner-grzyb *et al.* 2013). More interestingly, a work detecting *Hepatozoon* in keelback snakes (*Tropidonophis mairii*) also reported avirulent effects of this parasite in their crawling speed (Brown *et al.* 2006).

Moreover, haematological pathologies that can arise from haemogregarine infection were not observed in our study. Leukocytes are the most important cells involved in the immune response (Jacobson 2007) and changes in their levels due to haemogregarine infection have already been documented (Bonadiman *et al.* 2010). Similarly, an increment of immature erythrocytes' production is also described as a reaction of the immune system against parasitism (Schall *et al.* 1982; Oppliger *et al.* 1996; Clobert *et al.* 2000). However, we did not detect either changes in leukocyte ratio (Shutler *et al.* 2009) or increased immature erythrocytes circulation (Schall 1986; Brown *et al.* 2006). Despite the limitations of the study, our results suggest that intraerythrocytic gamonts do not elicit anemia (as described in Keymer 1981; Jacobson 2007) or a significant decrease in oxygen transport capacity (as in Schall 1983), which is indicative of parasites causing no measurable host erythrocyte destruction (Schall 1986). Ultimately, this absence of cellular reaction might indicate that *Hepatozoon* parasites can be benign, or at least not harmful, prompting no host investment in cell ratio alterations, as there was no need to counteract deleterious effects from these parasites. The apparent

little or no evident effect of *Hepatozoon* infection on host fitness may also imply an evolved stable interaction between hemoparasites and their hosts at these localities.

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MANUSCRIPT III

CONSEQUENCES OF *HEPATOZOON* INFECTION ON THE ESCAPE DISTANCE IN THE LACERTID LIZARD, *PODARCIS VAUCHERI*

Isabel Damas-Moreira[1,2], D. James Harris[1], Daniela Rosado[1,2]

Isabel Tavares[1,2], João P.M.C. Maia[1,2,3], Daniele Salvi[1], and Ana Perera[1]

[1] *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal*

[2] *Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre FC4 4169-007 Porto, Portugal*

[3] *Institut de Biologia Evolutiva (CSIC-UPF). Passeig Maritim de la Barceloneta, 37-49. 08003 Barcelona, Spain*

ABSTRACT

Nowadays it is widely accepted that parasites can play a significant role in community structures in which they occur, and ultimately in the ecosystems. Furthermore, infection by parasites might be associated with considerable deterioration of host fitness. While the apicomplexan parasites belonging to the genus *Hepatozoon* can provoke severe deleterious effects in some mammals, impact on other hosts, such as reptiles, is still ambiguous.

We here assessed the effect of *Hepatozoon* on *Podarcis vaucheri* flight-initiation distance from a simulated predator, a behaviour that is determinant for a successful escape and is likely to thus have major implications on a lizard's survival. We found that flight-initiation distance was not dependent on the time of the experiment or tail condition. Subadults exhibited worse body condition and, within the infected group, also higher parasitemia levels. Escape distance was not associated with any of the studied features however, which is indicative of a low or no impact of *Hepatozoon* in this behaviour. This may be related with the remarkably high prevalence (more than 96%) among this *P. vaucheri* population.

KEYWORDS

Podarcis vaucheri; *Hepatozoon*; flight-initiation distance

INTRODUCTION

Parasitism is a major selective force driving organisms' life history traits, inducing changes in host population dynamics, and ultimately affecting the structure of the communities (van Riper III *et al.* 1986; Schall 1992; Hudson *et al.* 2002, 2006; Pedersen & Fenton 2007). Infection by parasites causes a relocation of host energetic resources, usually associated with a deterioration of host body condition (Stien *et al.* 2002; Garrido & Pérez-Mellado 2013), and with negative effects on reproductive success (Dyrcz *et al.* 2005), and survival (Martínez-de-la-Puente *et al.* 2010).

The apicomplexan haemogregarines (Apicomplexa: Adeleorina) are the most common blood parasites in reptiles, but despite this, knowledge regarding the impact on the host is still scarce and contradictory (Bouma *et al.* 2007; Telford 2009). For instance, in reptiles, higher frequency of haemogregarines has been related to poor body condition in some studies (Garrido & Pérez-Mellado 2013), but to better body condition in others (Amo, López, *et al.* 2005; Molnár *et al.* 2013). Similarly, studies show that haemogregarines can infect more younger reptiles (Madsen *et al.* 2005; Brown *et al.* 2006a; Godfrey *et al.* 2011) or, on the other hand, older animals (Schall 1986; Amo *et al.* 2004; Salkeld & Schwarzkopf 2005; Molnár *et al.* 2013), or that its intensity can differ between genders (Schall 1986; Salkeld & Schwarzkopf 2005). Nevertheless, no substantial lethal consequences were found in naturally infected hosts (Manwell 1977; Brown *et al.* 2006; Jacobson 2007; Bouma *et al.* 2007; Telford 2009; Xuereb *et al.* 2012). In addition, other works report no relation between haemogregarine load and host body condition (Amo, López, *et al.* 2005), age (Garrido & Pérez-Mellado 2013) or gender (Oppliger *et al.* 1996; Smallridge & Bull 2000; Amo, López, *et al.* 2005).

Within the haemogregarine group, the genus *Hepatozoon* (Miller 1908) belonging to the Hepatozoidae family, is one of the most common intracellular protozoa in reptiles (Jacobson 2007; Zhu *et al.* 2009; Telford 2009). Although the deleterious effects of some *Hepatozoon* species in domestic animals like cats and dogs (Baneth *et al.* 1998, 2003, 2007; Panciera *et al.* 2000; Dwyer *et al.* 2006) are well-known, the impact for reptile hosts is still uncertain and vague. It is associated to slower growth rates and low body condition levels in pythons (Ujvari *et al.* 2004; Madsen *et al.* 2005), while other studies in snakes reveal no correlation between parasitemia levels and fitness (Brown *et al.* 2006; Xuereb *et al.* 2012). Moreover, *Hepatozoon* gamonts infect erythrocytes, causing oxygen deficit as result of the destruction of red blood cells (Jacobson 2007; Telford 2009). Therefore, consequences of parasitism might be especially evident in

situations in which high transportation and consumption of oxygen is required, such as escaping from predators. In previous studies, parasite load was found to reduce antipredatory performance by decreasing both running speed (Oppliger *et al.* 1996) and tail regeneration rate (Oppliger & Clobert 1997).

Several traits can be used to assess lizard's condition, and predators' escape mechanisms can be of major importance. For example, the distance a lizard allows potential predators to approach before running away (the flight-initiation distance), is determinant for a successful escape (Cooper 2006). The optimal escape theory, originally developed by Ydenberg and Dill (1986) is based on the principal that a prey will only escape from its predator when the risk of predation equals the cost of escaping. These costs are not only energetic but also include abdicated foraging (Cooper & Perez-Mellado 2004), missed social opportunities (Ydenberg & Dill 1986) and potential reduction of body temperature, due to less basking opportunities (Martín & López 1999).

In this study we analyse the influence of the haemogregarine *Hepatozoon* on the flight-initiation distance in the lizard *Podarcis vaucheri* (Boulenger 1905). Following the optimal escape theory and previous studies, if parasites negatively affect host fitness or locomotor performance, we propose that this can be reflected in its flight-initiation distance. Thus we hypothesize that lizards with higher parasite load will have a faster response and run to the shelter sooner than non-parasitized lizards, as *Hepatozoon* may decrease their locomotor speed which can be compensated for running away before the predator gets as close.

MATERIAL AND METHODS

The tests were performed in May 2013 in Oukaimeden (Morocco, 31.2010ºN, 7.8554ºE) on a total of 55 individuals of *Podarcis vaucheri*. Of these, 24 were males (including 3 subadults), and 31 were females (including 7 subadults). According to previous studies, individuals above 45 mm snout-vent length were considered adults, and below, subadults (Kaliontzopoulou, unpublished data).

The study area consists mainly of rock outcrops with fissures or little rocks among grass. At this location, *Podarcis vaucheri* presents high densities and *Hepatozoon* parasites have been previously reported with high prevalence (Maia *et al.* 2011) and variable parasitic intensity (known from previous expeditions; MANUSCRIPT I).

Escape distance trials consisted of standardized approaches to lizards simulating a predator attack. This protocol has been extensively used in lizards (Rand 1964; Martín & Lopez 1995; Cooper & Perez-Mellado 2004). The approaching technique is crucial to the success of the study, as it can interfere with the lizard behaviour in escaping. Speed and direction of the approach, and the distance to the refuge might influence a lizard response (Cooper 1997, 2003, 2006; Martín & López 1999; Cooper *et al.* 2003). Therefore, in order to minimize the effects of all these variables, approaches were always performed in the same way by the same person wearing the same clothes. The researcher (D.R.) simulating the predator slowly walked towards the lizard, with arms next to the body and making no abrupt movements until the lizard fled. The distance between the researcher and the place where the lizard was at the moment they escaped was recorded (Cooper 2006) using a laser distance measurer. The distance between lizards and nearest refuge at the time of fledging was not recorded, as all lizards were no more than 30 centimetres away from a shelter (I.D.M., personal observation). Only one trial was performed per individual lizard that were basking at the initiation of the trial. Those in movement, foraging, performing social interactions, or that showed signs that detected the presence of the researcher before the beginning of the approach test were not considered.

After each approach trial, lizards were captured. Tail condition (intact, regenerated or recently lost) and time were recorded for each test. Individuals were measured, weighed and a piece of tail tip was taken and stored at 96% ethanol for genetic analyses (Sevinç *et al.* 2000). Blood resultant from the tail was smeared across a glass slide and air-dried for posterior treatment in the laboratory. All animals were released at their sample site.

Blood smears were fixed with absolute methanol for 2 minutes, stained with Giemsa (1:9 distilled water) for 45 minutes and air dried. Prevalence and parasite intensity were examined under an Olympus CX41 microscope. Prevalence was estimated as the percentage of infected individuals within a sampled population and the individual parasitemia load was considered as the percentage of infected red blood cells up to 2500 erythrocytes. For these counts, pictures were randomly taken from the blood slide at x400 using the Cell^B 3.4 Olympus® software, and infected and non-infected erythrocytes were counted using the ImageJ 1.46® program.

Molecular methods were used to confirm the identity of the detected parasites. DNA was extracted from 5 randomly selected *Hepatozoon* positive samples using the high salt method (Sambrook *et al.* 1989). A fragment of the 18S rRNA gene was amplified

using the primers HepF300 and HepR900 (Ujvari *et al.* 2004), as described in Harris *et al.* (2011). Positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen, The Netherlands). Sequences were then blasted in GenBank to confirm their identity.

Prior to statistical analysis all continuous variables were log transformed to meet normality assumptions and a proxy for each animals' body condition (BC) was estimated as SVL / weight. In order to infer if the hour of capture or tail condition were interfering with the flight-initiation distance, an Analysis of Variance (*ANOVA*) with the function *lm* implemented in R (R Core Team 2012) was performed for each variable independently. In order to assess the need to include SVL and BC as covariates in the analysis, Spearman correlations with all the remaining continuous variables were performed using the function *cor* of the R package (R Development Core Team 2012). Since body size was not associated with any other variable, it was removed from further analysis.

Differences between sexes and ages differed in body size and body condition were assessed using an ANOVA (*lm* function, R, 2012). Differences in parasite prevalence between sexes and ages were tested using a Generalized Linear Model (*GLM*) with a logistic regression (R package *MASS*, Venables & Ripley 2002; R Core Team 2012), while differences in parasite intensity (considering only positive samples) were tested with an Analysis of the Covariance, *ANCOVA* (with *lm* function) including BC as covariate.

To evaluate if BC, SVL or intensity of parasitism were related to flight-initiation distance, non-parametric Spearman correlations were performed using the function *cor* of the R package (R Development Core Team 2012). Differences in flight escape distance were assessed using an *ANCOVA*, where we tested the effect of host *sex*, *age* and *presence* of the parasite, including BC as covariate. Finally, an *ANCOVA* was performed only on infected individuals, to assess for any effect of sex and age, after correcting by BC and intensity as covariates.

RESULTS

All parasite sequences analysed blasted to *Hepatozoon*, and were part of the same lineage of *Hepatozoon* infecting lizard hosts described in Maia *et al.* (2012). From the 55 individuals analysed, only 2 were not parasitized, giving a total prevalence of 96.3%. Intensity of infection varied, from 0 to 11.7% of erythrocytes infected, with a total mean

intensity (± SD) of 1.86 % (± 2.44). Six individuals were exhibited more than 5% of infected erythrocytes and two of these lizards, both females, displayed more than 10% or erythrocyte infected, with one reaching almost 12% of infected cells.

Table 1. Description of the number of individuals from each population (N), mean of the flight-initiation distance (m) and of intensity of infection (%) and prevalence among genders and ages.

		Males		Females	
		Adult	Subadults	Adult	Subadults
	N	21	3	24	7
Distance	**Mean**	1.870	1.424	1.959	1.536
	± SD	0.856	0.222	0.774	0.434
	Mean± SD	1.814 ± 0.815		1.863 ± 0.727	
Parasites	**Mean intensity**	1.72	1.52	1.51	3.11
	± SD	1.90	1.61	2.46	3.76
	Prevalence (%)	100.00	33.33	95.83	100.00
	Mean intensity ± SD	1.77 ± 1.85		1.93 ± 2.84	
	Prevalence (%)	95.65		96.67	

Flight-initiation distance was not dependent on the time of the day ($F=0.971$, $df=1$, $p=0.328$), nor tail condition ($F=0.241$, $df=2$, $p=0.787$). Therefore, both variables, hour and tail status, were not considered in further analysis. Males and females included in the study had similar body size ($F=2.232$, $df=1$, $p=0.141$). Body condition, however, differed between sexes ($F=38.510$, $df=1$, $p<0.001$) and ages ($F=37.918$, $df=1$, $p<0.001$). Thus, females had lower BC than males, while adults had higher BC than subadults. Body condition was similar among infected individuals ($F=0.308$, $df=1$, $p=0.581$) and parasite prevalence did not differ between sexes or ages (in all cases, $p>0.05$). However, when considering only infected individuals, intensity of parasitism was not associated with sex ($p>0.05$), but it differed between age classes, with younger lizards having a higher parasite load ($F=4.136$, $df=1$, $p=0.048$).

Flight-initiation distance was not correlated either to body condition ($R=-0.102$, $p=0.458$) or to parasite intensity ($R=0.024$, $p=0.861$). Moreover, escape distance did not differ between infected and uninfected lizards ($F=0.007$, $df=1$, $p=0.933$), sexes ($F=0.714$, $df=1$, $p=0.402$) or ages ($F=1.175$, $df=1$, $p=0.284$) after correcting for BC. Finally, after considering only infected individuals, we did not find differences in escape distance between sexes and ages (*ANCOVA*, using BC as covariate, in all cases, $p>0.05$)

DISCUSSION

Our results show that the escape behaviour measurements appear to bee independent of parasite intensity. Nevertheless, this is not related to a higher mortality of heavily infected individuals, since several of the individuals exhibited high parasitemia levels, when compared to other studies with *Podarcis* (low parasitemia levels (0.1%): Amo, López, *et al.* 2005); high levels and close to ours (0.9%): Garrido & Pérez-Mellado 2013). *Hepatozoon* intensity and prevalence for this locality exhibited thus a very interesting pattern. Prevalence was extremely high, with only one male and one female not infected with *Hepatozoon*, from the 55 collected lizards. Clearly, this high difference in the sample size of infected and non-infected lizards, meant that we could not adequately compare the two classes (infected vs. uninfected), but only parasitemia levels.

Hepatozoon prevalences in lizards are highly variable. Some studies reported 20% of infected individuals in a population (Herbert *et al.* 2010; Godfrey *et al.* 2011; Gupta *et al.* 2013) and few works described populations exhibiting more lizards parasitized; the highest level were reported in populations of *Podarcis bocagei* (74.7%) and *P. carbonelli* (69.7%) from the North-western Portugal (Roca & Galdón 2010), or in *Eulamprus quoyii* lizards from Australia (66%) (Salkeld & Schwarzkopf 2005). In general studies with haemogregarines, *Podarcis muralis* from Central Spain (Amo, López, *et al.* 2005) exhibited 58.1% of individuals infected and in the same province but a few years later, displayed 78.6% (Martín *et al.* 2008). Our prevalence values are the highest scored for *Podarcis* in a continental population, as a study conducted in insular *Podarcis lilfordi* verified that 95% of the population was infected with haemogregarines, although with an average of only 1% of individual parasitemia levels (Garrido & Pérez-Mellado 2013). This may be the result of the different availability of competent vectors, although this still need to be verified.

Our study revealed that younger individuals were more heavily infected, which is the opposite finding of some studies with general reptilian haemogregarines (Schall 1986; Amo *et al.* 2004; Molnár *et al.* 2013) and even with *Hepatozoon* (Salkeld & Schwarzkopf 2005). However, this result is also in agreement with some other works conducted in *Hepatozoon* spp. in reptiles, in which parasite intensity declined with increased host size or age (Madsen *et al.* 2005; Brown *et al.* 2006; Godfrey *et al.* 2011). This may be explained by natural selection removing susceptible reptiles and only those displaying lower parasite load are able to survive until older ages (Madsen *et al.* 2005; Brown *et al.* 2006), or simply occur because younger individuals have had

less opportunity to acquire immunity, which might lead to higher infection intensity (Hudson & Dobson 1997) and plausibly lower body condition, as we verified.

Finding no signs of parasites inducing changes in this anti-predator tactic can indicate that *Hepatozoon* presence and load have no impact in the flight-initiation distance of *P. vaucheri* from Oukaimeden. The apparent trivial impact of *Hepatozoon* on the flight-initiation distance may indicate a solid and stable evolutionary interaction between host and parasite (Combes 2001; Amo, López, *et al.* 2005). Perhaps, it could be indicative that lizards from this population opt to tolerate the infection rather than to fight it (Sheldon & Verhulst 1996), creating a balance between the strong presence and a limit in parasite intensity.

Nevertheless, we detected more than 96% of infected individuals in overall sample, with a mean intensity of almost 1.9% in the total of infected lizards, regardless of sex or age. The displayed pattern of parasitemia is clearly closer to those found in snakes (ranging from 90 to 100%, Ujvari *et al.* 2004; Madsen *et al.* 2005; Ujvari & Madsen 2005; Brown *et al.* 2006), even accounting for possible fluctuations in different seasons. Both intensity and prevalence levels can indicate how well established this parasite is in this population, while the mean intensity (~2%) might be the result of immune system counterbalancing the high prevalence values in the population (Keymer & Read 1990). Thus, our study system presents several features that can be promising for further studies of *Hepatozoon* infections and their impacts on hosts.

Moreover, flight-initiation distance might be influenced by other variables, allied or not with the occurrence of parasites. For example, it would be appropriate to consider other endo or ectoparasites, as studies in lizards revealed that their presence can also interfere in host fitness, including nematodes (Amo, Fargallo, *et al.* 2005), ticks (Main & Bull 2000) or mites (Sorci *et al.* 1995). Despite the negative result obtained in this study, this intricate system clearly warrants further investigation.

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CHAPTER III

GENERAL DISCUSSION

[Natural selection favours host genes to kill the parasite whereas it simultaneously favours parasite genes to survive in the host]

Claude Combes

Fitness of Parasites: Pathology and Selection, 1997

I started this thesis by saying that interactions among organisms are shaped by natural selection, in order to produce organisms well-suited to their environment (Poulin & Morand 2000; Combes 2001; Poulin 2007). *Hepatozoon* parasites appear to have responded very well to this force, as they seem to be extremely well adapted to the hosts studied. Although previous works revealed their ability to inflict some degree of virulence in hosts (Baneth *et al.* 2003), including reptiles (Madsen *et al.* 2005), in general, we found that *Hepatozoon* have little or no observable effect in our study species, as also suggested in some other studies with *Hepatozoon* spp. (Nadler & Miller 1984; Brown *et al.* 2006; Shutler *et al.* 2009; Xuereb *et al.* 2012) and general reptilian haemogregarines (Telford 1984; Jacobson 2007).

The invasion of the erythrocytes by a hemoparasite is suggested to lead to some damage to host cell functions, such as limiting the erythrocyte oxygen-carrying function and gas exchange, decreasing haemoglobin values, eliciting the release of immature red blood cells and provoking anemia (Wozniak *et al.* 1994; Oppliger *et al.* 1996; Jacobson 2007; Telford 2009, but see CHAPTER 1). Thus, if erythrocyte invasion lead to these severe consequences, a heavily parasitized lizard cannot possibly function as well as a conspecific that lacks such parasites. However, no indications for a negative impact on the immune system, or such deleterious effects were perceived in our studies, even when a lizard displayed more than 10% of parasitized blood cells. Some explanations for this will be discussed below.

3.1. CAUSES FOR LOW IMPACT

Currently, morphology of immune circulating cells are well documented in reptiles (Campbell 2004; Martínez-Silvestre *et al.* 2005; Jacobson 2007; Troiano *et al.* 2008; Telford 2009; Bonadiman *et al.* 2010; Sacchi *et al.* 2011). We analysed levels of erythrocytes, immature erythrocytes, thrombocytes and leukocytes (azurophils, basophils, eosinophils, heterophils, lymphocytes, and monocytes). The amount of leukocytes circulating in the blood can provide a good measure of the immune function (Norris & Evans 2000). The fact that we found no significant differences in the amount of circulating leukocytes might indicate that *Hepatozoon* spp. are able to evade the immune system detection. This idea is suggested to be associated with the fact that, instead of parasitizing the most important cells involved in the immune response, leukocytes (Jacobson 2007), *Hepatozoon* typically infect erythrocytes (Shutler *et al.* 2009), as also verified for our study.

Nevertheless, the invasion of erythrocytes occurs, and that has consequences in its functions, as already explained above. Yet, perhaps the invasion of an erythrocyte may not lead to a noticeable decrease in the exchange rate of oxygen and carbon dioxide. As this function is related with erythrocyte surface area to size ratio (Hartman & Lessler 1964), an invaded cell might compensate the invasion by altering the surface area, minimizing the impact on its function.

Anemia is also considered a possible consequence of an erythrocyte's invasion. As shown for some Apicomplexan genera, such as *Babesia* or *Plasmodium*, the infection results in the haemolysis of read host blood cells. Thus, anemia is observed with severe infections (Homer *et al.* 2000; Stoute *et al.* 2003), as the result of the incapacity of the body to produce enough new red blood cells to replace the ones being destroyed (Campbell 2004). It is widely accepted that *Plasmodium* provoke anemia in reptiles (Ayala 1970), and documented works report moderate to severe anemia for some haemogregarines (Jacobson 2007; Telford 2009). However, haemolysis was not detected during microscope surveys either for this thesis or by other colleagues from CIBIO (JPMCM and BT, pers. comm.) and thus we suggest that anemia caused by *Hepatozoon* infections was not taking place in any of the studied lizard species. This is also consistent with the lack of association we found between the percentage of immature erythrocytes and parasite load. These immature cells are another usual immune reaction to combat haemoparasitic infections, in an attempt to compensate the eventual decreased number of erythrocytes (Schall 1983; Oppliger *et al.* 1996; Telford 2009). In general therefore, we did not found haematological pathologies, in agreement with some previous authors (Schall 1986; Brown *et al.* 2006; Shutler *et al.* 2009).

3.2. CONSEQUENCES OF LOW IMPACT

There are many situations where it may be advantageous for a parasite to cause detrimental effects on host fitness. However, there are multiple advantages for not causing it as well (Fellous & Salvaudon 2009). An interesting idea is that parasites often evolve towards a reduction or elimination of their fitness costs because, ultimately, they rely on host availability (Brown *et al.* 2006; Bouma *et al.* 2007). The parasite may also take direct advantage from the lack of impact on the host. By not severely affecting the lacertid, *Hepatozoon* parasites can eventually pass on to a predator that eats the lacertid, thus exploiting two vertebrate hosts successively (Schmid-Hempel 2011), and this may be a common pathway within the *Hepatozoon* life

cycle (Smith 1996). Possibly, *Hepatozoon* do not usually provoke lethal effects in lizards, but instead its presence can be reflected by alterations in physical and chemical cues, without directly harming the host. In this sense, the host may suffer indirect consequences to its fitness, but this is a bearable risk compared to the efforts and energy invested in the defence against these parasites from immediate lethal consequences. This trade-off might be reflected in the high prevalence but relatively low intensity we detected in the three studies (Keymer & Read 1990). By altering these traits, the parasite plausibly maximizes its transmission to other hosts, not only to vertebrate hosts, but also to the hematophagous invertebrates.

As an example of a similar situation, a very recent study showed an apparent preference of mosquitoes for infected rather than non-infected frog hosts with *Hepatozoon clamatae* (Ferguson *et al.* 2013) and, similarly, children infected with gametocytes of *Plasmodium falciparum* have been shown to be more attractive to mosquitoes (LaCroix *et al.* 2005). In both cases, it is suggested that this preference is related to modified olfactory cues in the infected hosts. Besides changes in olfactory cues, *Hepatozoon* can also induce chemical and visual alterations. For example, it has been proven that lower haemogregarine infection levels were associated with males displaying femoral secretions more attractive to females (Martín *et al.* 2008) and also more colourful patterns (Martín *et al.* 2008; Molnár *et al.* 2013), which is of major importance for their interactions during courtship, and ultimately, their reproductive success. On the other hand, in other species male lizards which exhibited brighter colours were shown to have increased probability of infection (Schall 1986).

3.3. PREVALENCE, INTENSITY AND SPECIFICITY

Regardless of its nature, such lack of pathological impact, in so many host traits, implies an evolved equilibrium between the parasites and their hosts (Amo, López, *et al.* 2005). Evidence shows that, although parasites have shorter generation times, hosts can also promptly respond to parasitism in order to minimize its impact on their fitness (Schmid-Hempel 2009). For instance, the same study that detected the devastation of endemic bird populations naive to malaria disease, also reported the development of immunogenetic and behavioural responses against parasite impact, in a short period of time (since 1920 to date) (van Riper III *et al.* 1986).

The way the host react towards the infection impact is of considerable importance in the parasite-host system. High prevalences with low intensities (<10%, as considered in Caudell *et al.* 2002), as we found in our studies, can be an evidence of a cost of parasitism. This might be the outcome of the reaction of the host, as tolerating high prevalence may involve the maintenance of the immunity system to keep the parasite load at a relative low level (Keymer & Read 1990).

Among localities (Fig. 8), different prevalences and intensities were found, which can be due to habitat characteristics, and to a different availability and infectivity of both vector and vertebrate hosts (Gupta *et al.* 2013). However, *Hepatozoon* appears to have low specificity, as we found the same lineage to be parasitizing all the host species with relatively high prevalence in all sample sites. Previous studies suggest that *Hepatozoon* can have high flexibility and low specificity, especially concerning the first intermediate host (Sloboda *et al.* 2007), as it easily adapted when transferred to new reptile hosts in laboratory tests (Booden *et al.* 1970; Pessôa *et al.* 1974). Plausibly, a given parasite may be able to infect several host types within the same community (Kaltz & Shykoff 1998) and eventually become specialized on different host species (Mccoy *et al.* 2001).

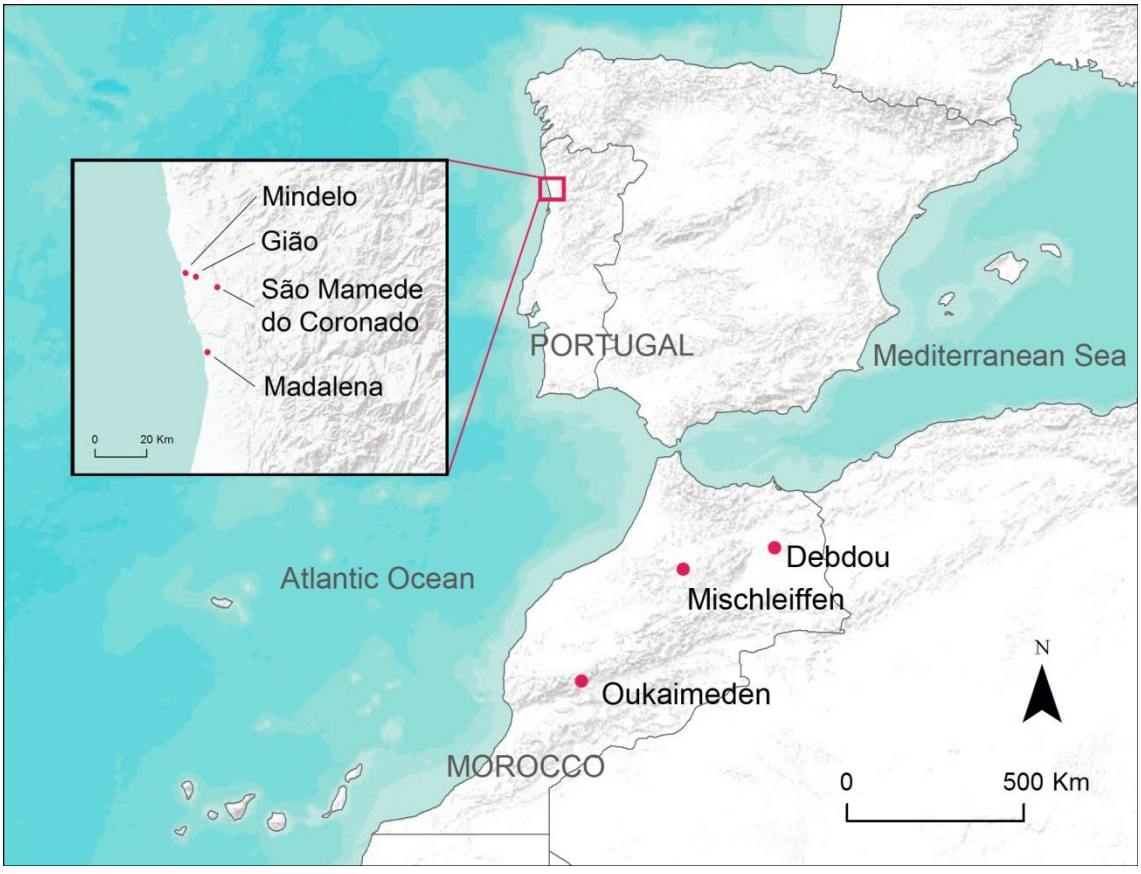

Fig. 8 – Map with the sample sites from the three experiments through Morocco and Portugal. Designed by Isabel Damas Moreira and Beatriz Tomé.

In the first two studies, only adult lizards were considered, and while in the first assessment no correlation was found between *Hepatozoon* parasites and size, in the second one larger adults were more parasitized. The only study comprising subadults (MANUSCRIPT III) showed that these had worse body condition and higher *Hepatozoon* levels and therefore, suggesting an effect related to age, as seen in previous studies (Ujvari & Madsen 2005). Clearly, there is huge ambiguity in our general results from the three manuscripts regarding the age, size and body condition of parasitized lizards. Here, perhaps a seasonal effect can be occurring between the works performed in May, and the one performed in October, but that does not explain the different results verified in Oukaimeden between the first (May 2012) and third manuscript (May 2013), as it was performed in the same season. Moreover, such seasonal effect is rarely reported (Amo, López, *et al.* 2005; Godfrey *et al.* 2011).

3.4. LIFE CYCLE

Small insectivorous lizards, as the three species in study, are sometimes considered paratenic hosts in the life cycle of *Hepatozoon* (Smith 1996). This means that these hosts can function as a bridge between the vector and final vertebrate host, in which parasite reproduction would not take place. However, the fact that we did find gamonts invading blood cells indicate that asexual reproduction is occurring, and that all hosts analysed were intermediate hosts, likely displaying cyst formations in organs as well, such as in the liver (Roca & Galdón 2010). Clearly, the genus *Podarcis* had already being confirmed to play the role of intermediate host in the transmission of haemogregarines (Amo, Fargallo, *et al.* 2005; Martín *et al.* 2008; Roca & Galdón 2010) and now *Scelarcis* is confirmed to play the same role. Therefore, lacertids might perform a more prominent part in the transmission of these parasites, rather than simply being transportation until the final vertebrate host.

Studies performed in sympatric species might be a good opportunity to study coevolution patterns. Understanding why the effect of parasitism is so divergent among sympatric species in some studies (Madsen *et al.* 2005, and Brown *et al.* 2006) but not in others (MANUSCRIPT I) might help to disentangle the role of the parasites on the populations, and the factors shaping it (host-specificity or ecological requirements). Brown *et al.* (2006) further suggest that the biogeographic histories of each host sympatric species might also be considered relevant factors, since it can reveal different periods of exposure of the host species to the parasites. In the studies

conducted in Morocco, *Hepatozoon* is infecting different hosts (*P. vaucheri* and *S. perspicillata* at least) and the different intensities and prevalence levels observed in some localities might be associated to this, or to different virulence levels (Toft & Karter 1990). However, the detected lack of impact in both species, under the same ecological variables at three different sites, can strongly suggest that these host-parasite systems are coevolving over a long evolutionary time.

3.5. CONSERVATION

The importance of parasites with relative avirulent effect for the hosts has been highlighted recently, and evidence suggest that, "in fact it is precisely these parasites that are most likely to be a powerful force in the maintenance of species diversity and the structuring of ecological communities" (Fenton & Brockhurst 2008).

It is now understandable that the simplistic assumption of parasites as *Hepatozoon,* to cause deleterious effects on host fitness is being reviewed (Brown *et al.* 2006). Through experiments performed during this thesis, along with already extensively described works, evidence is gathering that may indicate a non-observable impact of *Hepatozoon sp*. on its lizard host, and this may constitute important information from a conservation perspective. How parasite burden and pathology affect captive animals is of much interest, especially when large resources are being placed into captive breeding programs of endangered species.

For example, work carried out in India revealed the presence of *Hepatozoon canis* and *Hepatozoon felis* in several wild and endangered mammals, including the emblematic Bengal tiger (Pawar *et al.* 2012). Similarly, endangered lizards might harbour *Hepatozoon* spp., such as the tuatara *Sphenodon punctatus* (Herbert *et al.* 2010; Godfrey *et al.* 2011). Evidence may indicate that if an endangered lizard is already infected with *Hepatozoon* spp, no severe consequence from its presence should be imposed (Reardon & Norbury 2004). However, if the species to protect does not exhibit *Hepatozoon* infection, conservationists should be concerned about the existing vectors or other infected vertebrates. For example, as showed for other studies and in the presented ones in this thesis (Roca & Galdón 2010; Harris *et al.* 2012), the genus *Podarcis* is a very widespread lacertid displaying high prevalence of *Hepatozoon* and therefore can present a powerful transmitter of *Hepatozoon* to naïve species. As we also showed, *Hepatozoon* may present low host specificity, and thus the continuous

contact between a non-infected lizard and infected vertebrates may elicit transmission between species. Since naïve species have different susceptibility and tolerance to parasites, its introduced infection may result in dramatic pathologies and eventual devastation of an endangered population (Jones & Shellam 1999; Hudson *et al.* 2006).

Furthermore, parasitism does not act alone on the host fitness, but instead results from several interactions with other organism and abiotic factors (Poulin 2007). For example, it is now acknowledged that perhaps up to 100 frog species have gone extinct within one decade probably as a result of the interaction between climate change, anthropogenic factors and a chytrid fungal pathogen (Pounds *et al.* 1999). Therefore, all these interactions and the possible presence of other parasites, must be taken into account when inferring about the impact of these parasites in conservation efforts.

3.6. FUTURE REMARKS

Hepatozoon may play an intricate role in the evolution of their reptile hosts and what is most intriguing is the fact that there are almost no definitive conclusions regarding the effects of these parasites on their hosts. For future research, I advise few guiding lines to be taking into account in order to enhance research efforts in this area.

Through assessments in host individuals, *Hepatozoon* does not seem to have a strong and clear effect, but sometimes differences are reported when assessing its effect at a population level (Ferguson *et al.* 2013), and therefore investing in population analysis might be a good chance to find new evidences. We have an ongoing research project regarding the effect at population level, comprising more than 200 male individuals from 9 different Portuguese sample sites, that we expect will provide some new clues regarding the effect of *Hepatozoon* at population level. Moreover, parasite impact on lizards' thermoregulation and its bond with habitat altitude will also be assessed, two features that are intrinsically associated with oxygen transportation and can thus represent a closer approach to the alteration of erythrocyte's function by blood parasites.

We now know, as was previously demonstrated with *Podarcis muralis* from Central Spain, that prevalence can vary a lot within a few years (Amo, López, *et al.* 2005; Martín *et al.* 2008), and probably intensity for each individual as well (see intensities and prevalence in Oukaimeden for MANUSCRIPTS I and II). So in order to assess the possible temporal variation in parasite load, capture-recapture techniques could be a

viable option to infer about this temporal dynamics, as performed in studies with different purposes (Salkeld & Schwarzkopf 2005; Godfrey *et al.* 2008, 2011; Huyghe *et al.* 2010).

Furthermore, our understanding of *Hepatozoon* ecology and transmission remains limited, particularly in wildlife populations. There is an urgent need for understanding the actual vectors and hosts of each *Hepatozoon* species since without that knowledge, no precise conclusions can be drawn about its impact on the hosts (Price 1980). Although this is very difficult to achieve, the system should be studied as a closed circuit in which every element is considered, understanding which *Hepatozoon* species is present, and analysing its life cycle and impact on every host participating in its life cycle (as in Ferguson *et al.* 2013). Reaching a close circuit, other tests can be carried out, as for example, manipulation of hosts' infection (Wozniak & Telford 1991). The immune reaction could be assessed through more aggressive methods, as it is to artificially infect hosts naïve to *Hepatozoon* in order to understand how the infection can harm a lizard that was never before exposed to the disease. This was done previously in several taxa, and results showed that the novel infection led to severe pathological effects on the unnatural host (Pessôa *et al.* 1974; Wozniak & Telford 1991). Nowadays, these processes could be clarified with the help of molecular tools, especially if all the hosts are identified.

Particularly, the nature of the final invertebrate hosts has to be acquired as this is essential to fully understand the *Hepatozoon* life cycle. For instance, it was discovered in works with babesiosis (a disease caused by the Apicomplexan *Babesia*) that the disease would appear in animals with more contact with ticks, as this was the origin of the infection in the population (Healy 1989). This can much help not only in clarifying *Hepatozoon* life cycle, but also its general transmission processes, which can ultimately be essential in understanding its impact for reptile hosts. This major gap in *Hepatozoon* studies was already overcome in some cases and few studies can already link a *Hepatozoon* species with its vector, being able to identify both to the species levels (Reardon & Norbury 2004; Godfrey *et al.* 2011). Moreover, knowledge of the parasite's development in both the vector and the reptile is necessary for accurate classification at generic level if molecular tools are not used (Jacobson 2007).

Free-ranging reptiles are infected and infested with a great diversity of endo- and ectoparasites (Jacobson 2007). Thus, we agree with Xuereb *et al.* (2012) when they suggest that it would be better to consider other nonhemoparasites in future studies of host behavioural impact. However, some studies that tried this found no significant

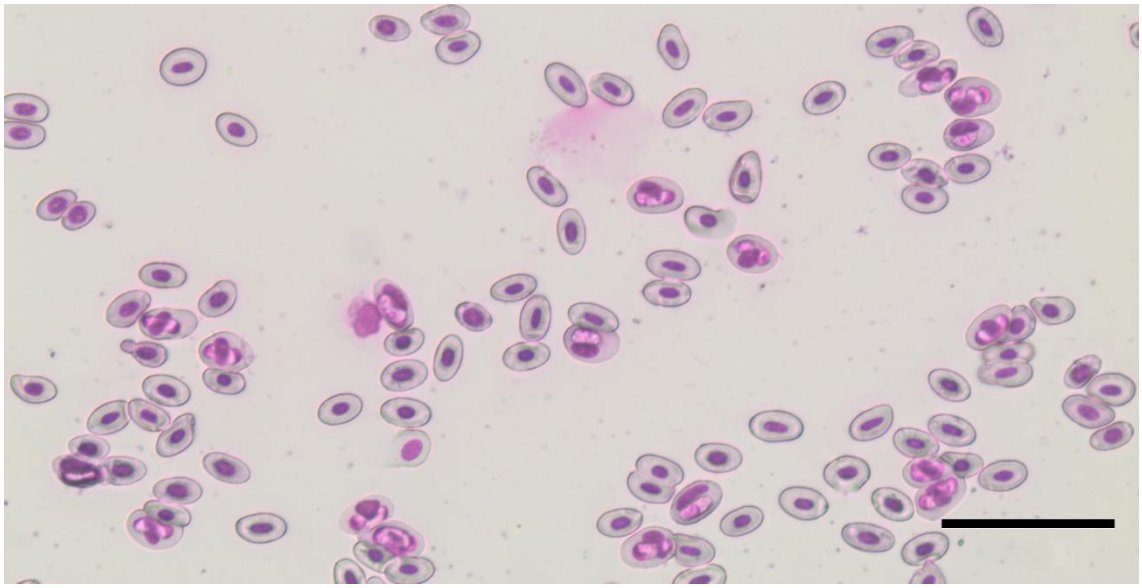

Fig. 9 - Picture of the blood sample of the most infected lizard for the three studies, exhibiting around 14% of infected cells. *Podarcis vaucheri* from Oukaimeden. Black bar = 50 µm. Picture taken by Isabel Damas Moreira.

results between harbouring, for example, ectoparasites and decrease in host fitness (e.g. Bull & Burzacott 1993; Ekner-grzyb *et al.* 2013). We have also ongoing works regarding both hemo and intestinal parasites from lizards to understand if in fact there is some correlation between these two types of parasites. Similarly, the possibility of multiple hemoparasite occurrence should not be discarded from influencing our results, as PCR alone can miss multiple infections when one lineage is preferentially amplified, as may sometimes happen with *Hepatozoon* (Tome *et al.* 2012).

There is clearly still much to do in this area, not only for *Hepatozoon* parasites, but for all haemogregarines infecting reptiles. An interesting observation during the experiments was the existence of outliers regarding parasitic intensity, i.e., the occurrence of individuals much more heavily infected comparatively to the rest of the population (Fig. 9). Instinctively, one might think that those lizards would exhibit a poorer condition, but such suppositions were not verified and higher parasite loads were not noticeably reflected in the fitness of the animals. Brown and colleagues also observed the same in keelback snakes, with some extremely infected snakes (up to 64% of intensity) demonstrating no evidence of fitness impact (Brown *et al.* 2006). This type of parasitized individuals should therefore be subjected to further research, in populations with high prevalence and infection rate as in Oukaimeden, as they might indicate the extent of infection up to which the host can tolerate parasite infection. This sample site might comprise a promising host-parasite system for further studies on *Hepatozoon* infections and their impacts to hosts. Also interesting in this locality, is the similar prevalence in the two consecutive years (MANUSCRITPS I and III), showing that

the chance of becoming infected may be stable over time for *Podarcis vaucheri* (a similar conclusion was attained in the work of Schall 1986).

As described before, most studies of haemogregarine impact in reptiles consider it as a whole group, not even distinguishing the parasite family surveying through microscopy (but see CHAPTER 1). Therefore our studies represent already a progress in this direction, as we could genetically identify the parasites up to the genus, and even to a specific lineage within the genus. However, still much needs to be done in developing more variable genetic markers. Scientific studies require a stable taxonomy based on a robust phylogeny (Morrison 2009). The 18S rRNA region is becoming widely used, but may not allow discrimination deeper than the genera due to its generally slow evolution pattern, given its structural and functional constraints. This can represent a major problem in analysing parasite impacts, as different identified species may produce different effects on the host. This was already verified in dogs, for example, where two distinct *Hepatozoon* species had highly contradictory disease syndromes: while *Hepatozoon americanum* provoked severe damages, *Hepatozoon canis* revealed no significant impact (Baneth *et al.* 2003). However, for this large funds and efforts are required (Mathew *et al.* 2000), which are normally invested only in Apicomplexan species of higher medical or veterinary importance (Walker *et al.* 2011).

Theory suggests that host-parasite interactions are a powerful evolutionary force. However, although most arguments are based on the idea that parasites reduce host fitness, the effects for the hosts to harbour parasites are and will remain controversial (Bouma *et al.* 2007). Without the understanding of the nature and consequences of harbouring *Hepatozoon* spp., but mostly understanding the extent of the impact on the host species, it is impossible to fully assess the importance of the parasite and the impact it has on its various hosts (Price 1980).

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