Assessment of how various factors impact H*epatozoon* prevalence and intensity of infection in several Iberian *Podarcis* species.

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O Presidente do Júri,

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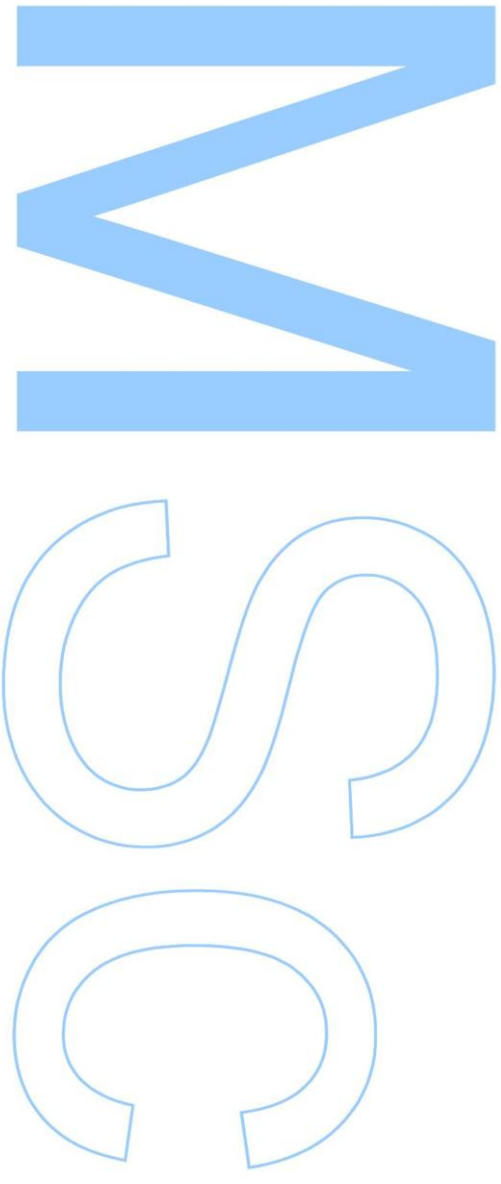

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ABSTRACT

As research regarding parasites increases, so does our understanding of these organisms' role in the ecosystem. Studies have shown that parasites can have a significant impact on host behavior and fitness, thus shaping host populations and communities. Knowing and predicting the distribution of parasite populations, allow us to develop preventive measures. However, due to the complex host-parasite interactions, determining which factors shape the distribution of parasites is rarely straightforward, as many climatic variables, as well as host population traits, can play a role in it.

The *phylum* Apicomplexa, a diverse group of obligate blood parasites, includes some of the most threatening parasites to public health, but is still poorly studied. The genus *Hepatozoon* is the most widespread group of Apixomplexan parasites in reptiles.

To understand the distribution of *Hepatozoon* in the Iberian Peninsula, parasite prevalence and intensity were assessed in several *Podarcis* species, and variation of both traits was tested for different factors: host sex and age, altitude, several climatic factors. Additionally, we performed a second study, in which we tested for significant differences of erythrocyte size of *P. bocagei* and *P. vaucheri* in varying altitudes. Changes in erythrocyte morphology correlated with high-altitude environments, as possible evolutionary adaptations to low levels of available oxygen, have been described in the literature. Since blood parasites, such as *Hepatozoon*, can inflict changes in the host erythrocytes morphology, this may translate in loss of fitness.

In the first study, prevalence and intensity of infection varied greatly between and among populations. Regarding host sex and age, no significant differences were found for either prevalence or intensity. *Hepatozoon* prevalence varied significantly in some *Podarcis* species for different climatic variables, whereas intensity only showed significant differences for maximum temperature of the warmest month. These results could indicate that climatic variables may be suitable to understand prevalence variation, but insufficient for intensity of infection. Finally, we created a preliminary model of *Hepatozoon* distribution in the Iberian Peninsula. It is a positive strong approach, but now sampling effort is needed in order to test the model's robustness.

As for the second study, erythrocyte size varied significantly between different altitudes for the Moroccan populations of *P. vaucheri*, but not for *P. bocagei*. To clarify if such considerable differences are the result of differences between host species, instead of different altitudinal ranges, variation in erythrocyte size should be assessed in a higher number of populations and for other *Podarcis* species.

Keywords *Hepatozoon, Podarcis bocagei, Podarcis vaucheri, Podarcis carbonelli, Podarcis hispanica*, prevalence, intensity, climatic variables, erythrocyte size, altitude.

RESUMO

Com o aumento de estudos sobre parasitas, também a nossa capacidade de compreender o papel destes organismos no ecossistema aumenta. Estudos revelam o impacto significativo que parasitas podem ter no comportamento e *fitness* dos hospedeiros e, consequentemente, a sua influência na estrutura das comunidades. Conhecer e prever a distribuição de populações de parasitas, permite-nos desenvolver medidas preventivas. Contudo, devido às complexas interações entre hospedeiro e parasita, determinar quais os fatores mais determinantes na distribuição dos parasitas não é simples, uma vez que esta pode ser influenciada pelo clima ou características das populações dos hospedeiros.

O filo Apixomplexa, um grupo diverso de parasitas sanguíneos obrigatórios, incluí alguns dos parasitas mais importantes para a saúde pública, sendo, contudo, ainda pouco estudado. O género *Hepatozoon* é o grupo de parasitas Apicomplexa mais comum em répteis.

Para compreender a distribuição de *Hepatozoon* na Península Ibérica, prevalência e intensidade destes parasitas foram avaliadas em várias espécies de *Podarcis*, e a variação foi testada para diferentes factos: sexo e idade do hospedeiro, altitude e vários factores climatéricos. Adicionalmente, realizámos um segundo estudo no qual diferenças no tamanho de eritrócitos em populações de *P. bocagei* e *P. vaucheri* a diferentes altitudes foram analisadas. Diferenças na morfologia de eritrócitos como possível adaptação evolutiva a habitats de elevada altitude, foram descritas na literatura. Considerando que parasitas sanguíneos, como *Hepatozoon*, podem provocar alterações na morfologia dos eritrócitos do hospedeiro, tal pode provocar perda de fitness.

No primeiro estudo, prevalência e intensidade de infeção variaram consideravelmente entre as várias populações. Considerando sexo e idade do hospedeiro, diferenças significativas não foram observadas. Variações de prevalência foram observadas entre diferentes variáveis climáticas, enquanto intensidade apenas mostrou diferenças significativas para temperatura máxima do mês mais quente. Resultados podem indicar que variáveis climatéricas são adequadas para compreender variação de prevalência, mas insuficientes para intensidade de infeção. Por fim, desenvolvemos um modelo preliminar de distribuição de *Hepatozoon* na Península Ibérica. Embora seja uma forte abordagem, mais amostragens são necessárias para testar a robustez do modelo.

No segundo estudo, tamanho de eritrócitos variou significativamente entre diferentes altitudes para as populações *P. vaucheri* de Marrocos, mas não para *P. bocagei*. Para esclarecer se esta diferença considerável de resultados se deve a diferenças entre as espécies de hospedeiros ou diferenças nas altitudes testadas para cada espécie, as mesmas análises deviam ser realizadas em mais populações e diferentes espécies de *Podarcis*.

Palavras-Chave *Hepatozoon, Podarcis bocagei, Podarcis vaucheri, Podarcis carbonelli, Podarcis hispanica*, prevalência, intensidade, variáveis climatéricas, eritrócitos, altitude.

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Abbreviations

ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
GLM	Genelarized Linear Model
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
rRNA	Ribosomal ribonucleic acid
SD	Standard Deviation

CHAPTER I

INTRODUCTION

Parasites

The definition of a parasite, even though it may seem straight forward at first, generates debate and disagreement on some aspects regarding this group of organisms. It has been suggested that parasites are, in general, “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). The diversity of parasitic organisms is immense, and they vary greatly in their physiological characteristics, size and shape and numerous other morphological characteristics including effective population size, mechanisms of infection, occupation of different micro-habitats within their hosts and different life cycles (Poulin, 2011). The differences are remarkable and there are different evolutionary paths followed by the parasite species, resulting in their organization along distinct taxonomic groups. Unfortunately parasites are often overlooked, even though research highlights their importance in ecosystems.

A considerable percentage of biodiversity in most given ecosystems consists of parasites (Toft, 1986, Poulin and Morand, 2004). Hudson et al. (2002) showed evidence of how parasites can significantly reduce host-fitness, thus interacting with population processes and deeply affecting community structure. On the other hand, some parasites even consume environmental toxins ingested by their hosts (Sures, 2004), which can have beneficial effects to their hosts and the ecosystem. It seems undeniable that parasites play an important role in the equilibrium of the ecosystem. Recent research suggests that parasites are of crucial importance regarding the evaluation of the health of an ecosystem (Hudson et al., 2006). It has also been argued that parasites could be ideal biological models to study the mechanisms of speciation and processes of diversification (de Meeûs et al., 1998), due to their varying evolutionary histories from independent transitions to parasitism, to sympatric speciation (Poulin and Morand, 2000). Especially from a contemporary perspective when so much attention is being given to global conservation and loss of biodiversity, increasing our knowledge about parasite groups is of clear importance - how can we preserve that which we don't know? Due to the dependence on their host, specialist parasites may be extremely susceptible to coextinction. Actually it is commonly expected that conservation measures targeting host species will also yield positive results to the host's parasites. This however has been shown to be inaccurate and most, if not all, endangered parasite species are not listed in the IUCN list (Gomper &

Williams, 1998; Whiteman & Parker, 2005), which only highlights the lack of scientific concern for this group of organisms.

Another, more ingenious, perspective on parasites is the ability to use information regarding parasitic populations to make inferences about their hosts. Assuming that a particular parasite is transmitted vertically along host generations, one can take advantage of the genetic characteristics of parasites to try to answer questions regarding the host's history (Clay, 1949; Page, 2003). This is particularly true in cases where vertebrate hosts have low genetic variability within and between populations (Kieser, 1991; Hoelzel et al., 2002). In most host-parasite complexes, parasites have generally higher mutation rates and lower generation time than their hosts. This allows for higher genetic variance in parasites DNA and the ability to infer the host's evolutionary history, which would not perhaps be possible using the hosts' genetic information (Whiteman & Parker, 2005; Funk et al., 2000; Rannala & Michalakis, 2003). For example, Falush et al. (2003) used *Helicobacter pylori*, a chronic gastric pathogen of humans, and managed to detect different parasitic populations, with distinct geographical distributions, that were consistent with the human populations and also found a potential relationship between the genetic variation of the different parasite populations and possible past migrations of the human populations. Incredible results such as this one serve as clear evidence to the potential usages of parasites' genetic data.

Horizontal transmission of parasites, from one population to another, can cause obstacles to data analysis similar to the ones caused by horizontal transmission of genes (Page, 2003). Even so, horizontal transmission can provide useful information on past host dispersal (Criscione & Blouin, 2004) and the patterns of parasite transmission between different hosts (Rannala & Michalakis, 2003). Parasites generally have no free-living form or a low dispersal capable one in the cases when it exists, during its life-cycle, leaving their dispersal highly dependent on the mobility of the host.

Despite the importance of studying parasite populations and their impact on the ecosystem, research of this group of organisms lags behind research of free-living organisms.

Apicomplexa

The phylum Apicomplexa Levine 1970 consists of a large and diverse group of poorly studied unicellular protists and its classification is a conservative one, with little consideration to modern molecular data (Morrison, 2009). The organisms of this phylum are, in the majority of cases, obligate parasites. They share a characteristic apical structure from which the name of the group derives, constituted by several components that are crucial for the process of host cell invasion (Adl et al., 2005). The Apicomplexa is generally divided into several taxonomic groups: the gregarines, the cryptosporidians, formerly considered to be coccidians (Adl et al., 2005), and, forming a separate clade, the piroplasms, haemosporinids and coccidians.

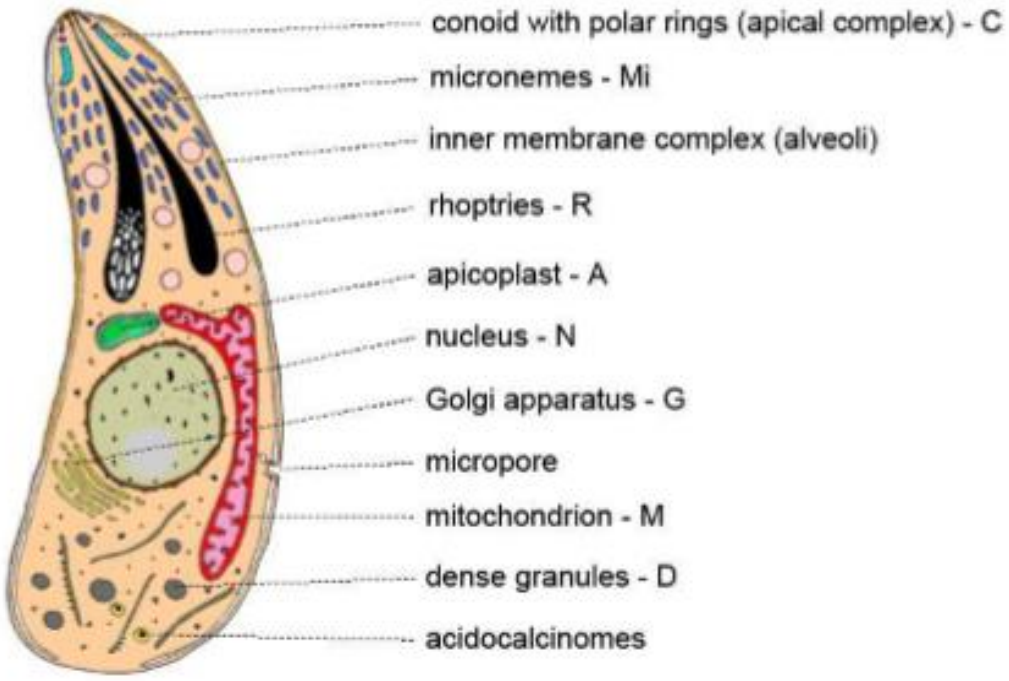

Fig.1 – Schematic representation of an Apicomplexan parasite and its constituents. (in Slapeta & Morin-Adeline, 2011)

Apicomplexa includes some parasitic species highly important to human health and economy, some of which are responsible for an estimate of 1 million human deaths per year and economic damage of US $1 billion per year. Some of the most anthropogenically relevant parasites and, as such, some of the most focused species

in research studies are *Eimeria* spp., *Cryptosporidium* spp., *Toxoplasma gondii*, *Neospora caninum*, *Theileria* spp. and *Plasmodium* spp. (Hans-Peter, 2009).

Eimeria spp. represents one of the most important pathogen of poultry, causing drastic loss of weight and sometimes death of the host, and has a considerable economic impact. *Cryptosporidium* spp. is a widespread enteric pathogen of animals, including humans, and is the cause of the disease cryptosporidiosis, which can be life-threatening to certain demographic groups such as children and immunodeficient individuals, and even though recent, substantial advances have been made in genetic and epidemiological analysis of these parasitic organisms, molecular diagnostics for clinical purposes are still inadequate. *Toxoplasma gondii* is a globally distributed, successful parasite, capable of infecting any warm-blooded animal across various ecosystems, while *Neospora caninum* is a cyst-forming parasite, initially found in dogs and associated with neuromuscular disorders, but later acknowledged to be one of the main causes of abortion in cattle. Species of the genus *Theileria* are also important pathogens for the cattle industry, being responsible for livestock death in the Mediterranean, Africa, Middle East, China and India. Finally, *Plasmodium* is the most studied apicomplexan genus, responsible for the disease malaria and significant mortality estimates both in humans and various other vertebrate groups (Hans-Peter, 2009). The negative impacts of all these parasites to human welfare have stimulated genome sequencing studies in order to find biomedical advances and solutions (Tarleton & Kissinger 2001; Carlton 2003).

How is it, therefore, that a phylum encompassing some of the most important parasites to mankind is also one of the most poorly studied groups, in terms of its biodiversity? It is estimated that 1.2 to 10 million species of the Apixomplexa phylum exist, but only around 6000 are currently described (Adl et al., 2007).

Hemogregarines

Hemogregarines (Apicomplexa: Adeleorina) are protozoan parasites capable of infecting a variety of hosts, including many reptile species. In fact, they are the most common blood parasite in reptiles and can be found present in each order of living reptiles (Telford, 2009). There are three families of hemogregarines (Telford, 2009): the *Hepatozoidae*, *Haemogregarinidae* and *Karyolysidae*. The erythrocytic stages in the

vertebrate's circulatory system are very similar across the hemogregarines. Thus, the three families of hemogregarines are distinguished by very different developmental patterns in their invertebrate hosts (Herbet et al., 2010; Morsy et al., 2013). In fact most hemogregarine species were initially described as *Haemogregarina*, until the importance of sporogonic patterns in the invertebrate host for taxonomic identification was acknowledged (Smith, 1996).

Due to the veterinary importance of these parasites, being pathogenic to hosts such as domestic dogs (Baneth et al., 2003), the research focus is extremely biased towards some groups of parasites, while hemogregarines of wild hosts such as reptiles are often overlooked, as for example the *Hepatozoon* genus, the most common and widely distributed in reptiles (Telford, 2009).

Hepatozoon

The genus *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina: Hepatozoidae) consists of a diverse group of apicomplexan blood parasites and was first described in rodents. It is considered as a single genus, through assessment of morphological and ecological characteristics, but more recent molecular research consistently leads to finding new *Hepatozoon* species, and it has even been suggested by Smith and Desser (1997) that the genus should be split in two different genera. Over 300 species have been described to this genus, but some species description include only characteristics of the gamont stage on the vertebrate host, which is easier to detect and observe but is often insufficient for taxonomic purposes, and many others have been described only because they were in a new host or locality (Smith, 1996).

Species of *Hepatozoon* have been found in various invertebrates, which are the definitive host. These include triatomid bugs (Osimani, 1942), some mosquitoes species (Robin, 1936; Mackerras, 1962, Ball et al., 1967; Nadler and Miller, 1984), lice and fleas (Wenyon, 1926; Clark et al., 1973), flies (Smith, 1996), ticks (Levine, 1988) and mites (Miller, 1908). *Hepatozoon* hemogregarines also infect a variety of different vertebrates. As for vertebrate hosts, Hepatozoon can infect many different species of mammals (Gimenez et al., 2009; Criado-Fornelio, 2006), are common parasites of frogs and toads worldwide (Harris et al., 2013; Davies and Johnston, 2000), have been found to infect birds (Biedrzycka et al., 2013), although less common when comparing

to other groups of hosts, and have been described the most in lizards and snakes (Smith, 1996). Both for invertebrate and vertebrate hosts, *Hepatozoon* species generally show low levels of host specificity (reviewed in Smith, 1996; Barta et al., 2012). This would provide them significant flexibility, especially coupled with diverse life cycles. On the other hand, both Smith et al. (1994) and Telford (2001), reported that *Hepatozoon* parasites showed considerable host specificity to snake species, highlighting the need of further investigation to elucidate questions on host-parasite relationship regarding *Hepatozoon* species.

Due primarily to its low socio-economic relevance, this group of organisms has not been the focus of as much research as many other parasite groups, hence information about its diversity, and other specific characteristics such as its relationship with their hosts, is limited. Only after the description of negative effects on domestic dogs and cats infected by *Hepatozoon* spp. (Panciera et al., 2000; Baneth et al. 2003), did this group start to receive more attention.

Life-Cycle

Due to, partly, the wide range of invertebrates and vertebrates as potential hosts, the life cycle of *Hepatozoon* hemogregarines can be quite complex. An additional layer of complexity is added when you consider the possibility of the formation of dizoic cysts in the internal organs, liver or lungs, of the infected vertebrate host (Smith, 1996). However, there are still some developmental features that are common among *Hepatozoon* species: (1) all *Hepatozoon* parasites require at least one invertebrate host and one vertebrate host (heteroxenous lifecycle); (2) sporogonic development and oocyst formation occurs in a hematophagous invertebrate host, while merogonic and gamontogonic development occurs in a vertebrate host that ingests the, or is parasitized by, the infected invertebrates; (3) the formation of latent cysts in the vertebrate hosts is a unique characteristic of the *Hepatozoon* genus, as suggested by Desser (1990). There is also variation between different types of vertebrate hosts, but I will focus on reptiles.

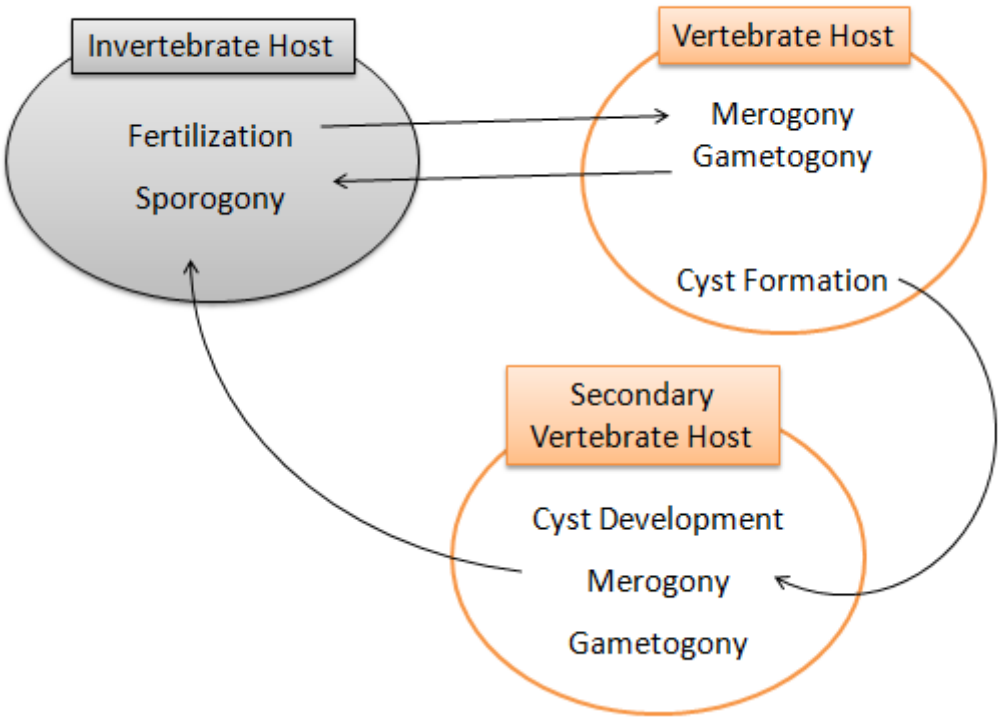

Fig. 2 – Diagram of a possible and simplified *Hepatozoon* life cycle, by Henrique Estrela.

The simplest possible life cycle of a *Hepatozoon* species requires one invertebrate host and only one vertebrate host (Desser et al., 1995; Smith, 1996). Gamonts are ingested by an invertebrate host feeding on the blood of the infected vertebrate. Inside the now infected invertebrate, micro- and macro-gamonts associate in syzygy, thus allowing fertilization. Then, sporogony occurs resulting in the production of sporozoites. It is at this stage that the parasite can be transmitted to the vertebrate host, for example, through ingestion of the vector or while the infected invertebrate blood feeds. In the visceral tissues (liver, for example) of the vertebrate host, the sporozoites produce meronts, through merogony. Meronts will mature into merozoites and enter the blood stream. Using their apical complex, merozoites will enter inside erythrocytes and change into gamonts, through a process called gametogony, completing their life cycle. This stage can be observed under the microscope. So in the life cycle of Hepatozoon spp., the invertebrate host has the role of definitive host, while the vertebrate host is called the intermediate host (Telford, 2009).

The life cycle described above can become more complex when cysts form in the infected vertebrate and more than one vertebrate host is used by the parasite. Inside the vertebrate host sporozoites can accumulate, usually in the liver or lungs, and cystic formation can occur. The vertebrate host can now act as a paratenic host – a host not necessary for the completion of the parasite's lifecycle. In lizards, the paratenic host serves as a bridge between the invertebrate-definitive host and the intermediate host, or final vertebrate host, such as a snake (Landau, 1972). A second vertebrate host can predate on the initial paratenic host, acting as an additional host, before the parasite reaches the final vertebrate host. Moreover, the sporozoites can simultaneously undergo merogony, producing meronts that will later mature and enter the blood stream, and form latent cysts in the definitive host, adding to the complexity of *Hepatozoon* species life-cycle.

Morphological Features

With varying life history stages, diverse invertebrate and vertebrate hosts and different possibilities of life cycles, *Hepatozoon* parasites could potentially have morphological differences which could serve as a tool to differentiate between them. However such potential differences are not always clear or rigorously described.

Available data regarding sporogonic stages of 4 North American *Hepatozoon* species, infecting snakes, reveal variation in features such as oocyst and sporocyst dimensions, the number of sporocysts per oocyst and the number of sporozoites per sporocyst (Ball et al., 1967; Ball et al., 1969; Nadler and Miller, 1984; Smith et al., 1994). However there have not been enough studies with information about variation of sporogonic features across different *Hepatozoon* species. Most morphological identifications are based on the parasite stages in the vertebrate host, such as gamonts (Telford, 1984).

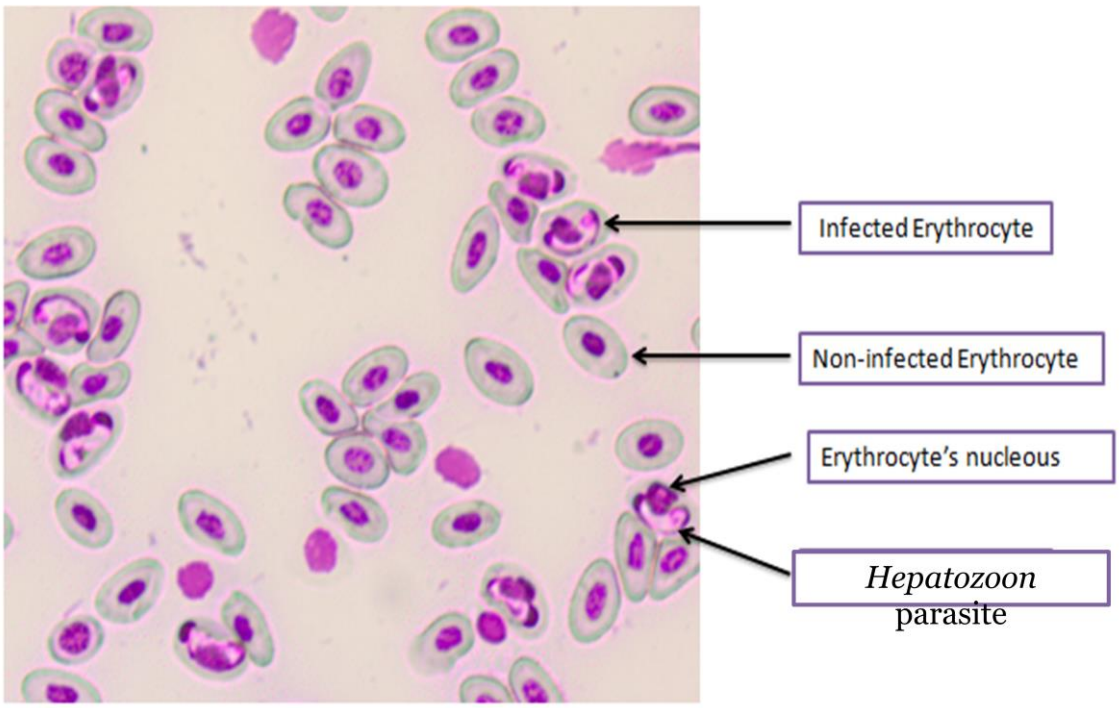

Fig. 3 – Picture of microscopic observation of a blood slide. Picture by Henrique Estrela.

Attempts to assess variation in gamont morphology often fail to identify characters that can be used in species diagnosis. There are several obstacles to describing species of *Hepatozoon* based on gamontogonic features: similar morphology of gamonts between different species, misinterpretation of immature and mature gamonts as different species; possibility of gamonts of two or more parasite species infecting the same host, especially considering the apparent low host specificity of some *Hepatozoon* species; mistaking microgramonts and macrogamonts for two different species; potential of the same parasite species presenting different characteristics depending on the host (Smith, 1996).

Using morphological characteristics of the different parasitic stages to describe species, although useful and a source of important information, is therefore quite inaccurate, leading to a limitation in describing *Hepatozoon* species. Combining this information with molecular markers is therefore a more reliable approach (Harris et al, 2014; Abdel-Baki et al., 2014; Maia et al., 2014).

Pathogenicity

The increase of interest on *Hepatozoon* organisms arose from the negative, and sometimes severe, effects of these parasites that were described in domestic host species, such as dogs and cats (Criado-Fornelio et al. 2009; Gimenez et al. 2009; de Bortoli et al. 2011). In the case of canine hosts, the effects of *Hepatozoon* infection can vary (Baneth et al., 2003). *H. americanum* can cause fatal skeletal and cardiac myositis, while *H. canis* is less severe on the host. It has been suggested that *Hepatozoon* parasites may be opportunistic pathogens taking advantage of an organism with an immune system already compromised, and, as such, much of the symptoms observed in the host may be caused or intensified by other pathogens (Dwyer et al. 2006; Pawar et al. 2012; Baneth et al. 2007). The same hypothesis was also proposed in the case of cats infected with both *Hepatozoon* and other pathogens (Baneth et al., 1998).

With the increase of studies regarding the pathogenicity of *Hepatozoon* in domestic species, wild species started to receive more attention and there was an interest to understand the impact of these parasites in wild populations, especially since reptiles, amphibians and birds have nucleated erythrocytes, which is not the case in mammals. In reptiles most studies were performed using snakes. In this host, *Hepatozoon* parasites impact is rarely severe (Wozniak et al. 1994, 1998), with very mild effects on individual fitness (Xuereb et al., 2012). It has been described that *Hepatozoon* can have significant impact on female reproduction and juvenile survival (Madsen et al. 2005; Pessôa et al. 1974), but otherwise low pathogenic effects have typically been reported. This led to the suggestion that *Hepatozoon* is very well adapted to reptile hosts (Nadler & Miller 1984; Telford 1984). This hypothesis seemed to be supported with further experiments in which certain non-natural hosts for *Hepatozoon* were deliberately infected. The negative impact on these hosts was much more severe, causing significant inflammatory disease in some cases and general neonatal mortality (Pessôa et al. 1974; Wozniak and Telford, 1991).

There have been studies showing a clear correlation between *Hepatozoon* presence and pathogenic symptoms in the host. However, it is still sometimes unclear if *Hepatozoon* is the main cause for such negative effects, or if the parasites are acting as opportunistic pathogens. Futhermore, research on this topic is extremely biased towards a more veterinary perspective, concerned with pathogenicity of *Hepatozoon* on domestic species, such as dogs and cats. When such research widens its scope to wild-life, it is found that negative effects of *Hepatozoon* infection are more severe in

non-natural hosts than natural ones (Pessôa et al. 1974; Wozniak and Telford, 1991, Wozniak et al., 1996). More specifically in reptiles, the investigation is focused on certain groups of species like snakes, but even in these hosts the results are sometimes unclear. In lizards the results are similar and the question about the impact of *Hepatozoon* on these hosts remains to be fully answered. Damas-Moreira et al. (2014) reported no significant differences in fitness between *Hepatozoon* infected and non-infected *Podarcis*.

Effects of environmental factors on Host-Parasite Interactions

Some of the most important questions about parasites regard their interactions with their hosts. These interactions depend deeply on the inherent features of both the parasite and host, such as parasite prevalence and intensity of infection, rate and mode of parasite transmission, host mortality and defense mechanisms against parasites (Combes, 2001). These features can be, either negative or positively, affected by environmental conditions, such as climatic variables (Merino and Moller, 2010). In light of current global climate changes, it is crucial to understand how climatic variables shape parasite population structure and behavior, not only in a conservational point of view, for wildlife, but also in a social-economic perspective, as many parasites are pathogens of livestock, domestic animals and humans.

Even though temperature is the most relevant variable associated with climate changes, others factors such as precipitation and wind can also play an important role (Moller et al., 2013). For example there is evidence that some parasites have been altering their date of emergence (Merino and Moller, 2010; Poulin, 2006; Poulin and Mouritsen, 2006) and prolonging their active periods (Ogden et al., 2006; Cadenas et al., 2007). This could offer an advantage to parasites, but, at the same time, a longer breeding season could benefit the host. For example, in the case of bird populations a more extensive breeding season would reduce the constraints of a strict migration timing (Dunn and Winkler, 2010).

Results of studies on the effects of climatic variables on parasites have been conflicting. Moller (2010) showed a drop in virulence of a directly transmitted mite, while its host suffered from decreased cell-mediated immunity, related to climate change. Blood parasites and avian malaria incidence was shown to have increased

over the last years and researchers think this is related to an increase in adequate habitat for the vectors of malaria (Garamszegi, 2011; Pascual et al., 2009). Not just for malaria, but also for other diseases, climatic variability can have unexpected influence on the distribution of vectors of various parasites (reviewed in Githeko et al., 2000). Moller et al. (2013) tested the effect of climatic changes in the interaction between 24 european birds and 89 parasite populations, revealing a very balanced proportion of positive and negative effects for both the parasites and the hosts. Interestingly, the authors suggested that changes in parasite abundance occurred not by direct influence of the climatic variables, but through the indirect effects of temperature changes on the hosts, showing just how complex is the interface of climatic variability and parasite-host interaction.

Most models predict that population size and geographical range of parasite populations will not change with alterations in climate, whereas their distributions will move northwards (Merino and Moller, 2010). However, these models are based on scarce empirical information and, as such, actual changes in parasite populations are difficult to predict. Predicting future changes in the host populations is increasingly complicate, for not only the response to the climatic changes but also the response to the changes in parasites need to be considered.

Even though there is evidence of a connection between climatic changes and host-parasite interaction changes, the results have been contrasting and conflicting among different parasite and host species. This can be a consequence of a lack of development in this scientific area. Furthermore, it can be a result of a limited selection of hosts and parasites. Most studies focus on either birds or mammals as the vertebrate hosts, whereas no attempt has been made to study the effect of climate on the relationship of parasites and their reptile hosts. The understanding of host-parasite dynamic and how this is influenced by environmental variability should not be restricted to hosts or parasites of particular importance to humans. Such fundamental knowledge can be used and extrapolated across different host-parasite systems.

Hosts: Why *Podarcis* spp.?

To perform studies about parasite populations and parasite-host interactions, especially concerning a group of parasites less studied as the *Hepatozoon* species, the host species chosen ideally should have some characteristics: 1) easily observed, captured and manipulated in the wild; 2) occur in high densities, thus being able to provide good sample sizes; 3) have, to some extent, a wide geographic distribution; 4) show high levels of parasite prevalence and intensity; 5) be well described in the literature. *Podarcis* spp. presents all of these favorable features. Lizards in general, *Podarcis* species included, are easily manipulated on the field (Arnold, 1981), can occur in populations of high density (Schall, 1990) and have been shown to have consistently high levels of *haemogregarine* infection, including *Hepatozoon* parasites (Maia et al., 2012; Harris et al., 2012). Some *Podarcis* species also occur in sympatry, which allows the study of *Hepatozoon* impact in different species under similar environmental conditions. Another important aspect to consider is that *Podarcis* populations, as with several lizards, probably play an important role, as either a paratenic or intermediate host, in the life cycle of *Hepatozoon* parasites and the infection of predators, such as snakes (Tomé et al., 2014).

The *Podarcis* (Lacertidae) genus is composed of lacertid lizards distributed along the Mediterranean basin and represent some of most common reptile groups of Southern Europe (Arnold, 1987; Harris et al., 2005). The phylogeny of this genus is still partially unresolved (Harris and Arnold, 1999; Oliverio et al., 2000; Poulakakis et al., 2003). Several *Podarcis* species occur in the Iberian Peninsula, such as *P. bocagei, P. vaucheri, P. carbonelli* and the *P. hispanica* complex which probably corresponds to different species. For this thesis prevalence and intensity levels, as well as inferences of host-parasite interactions, were evaluated in these different *Podarcis* species.

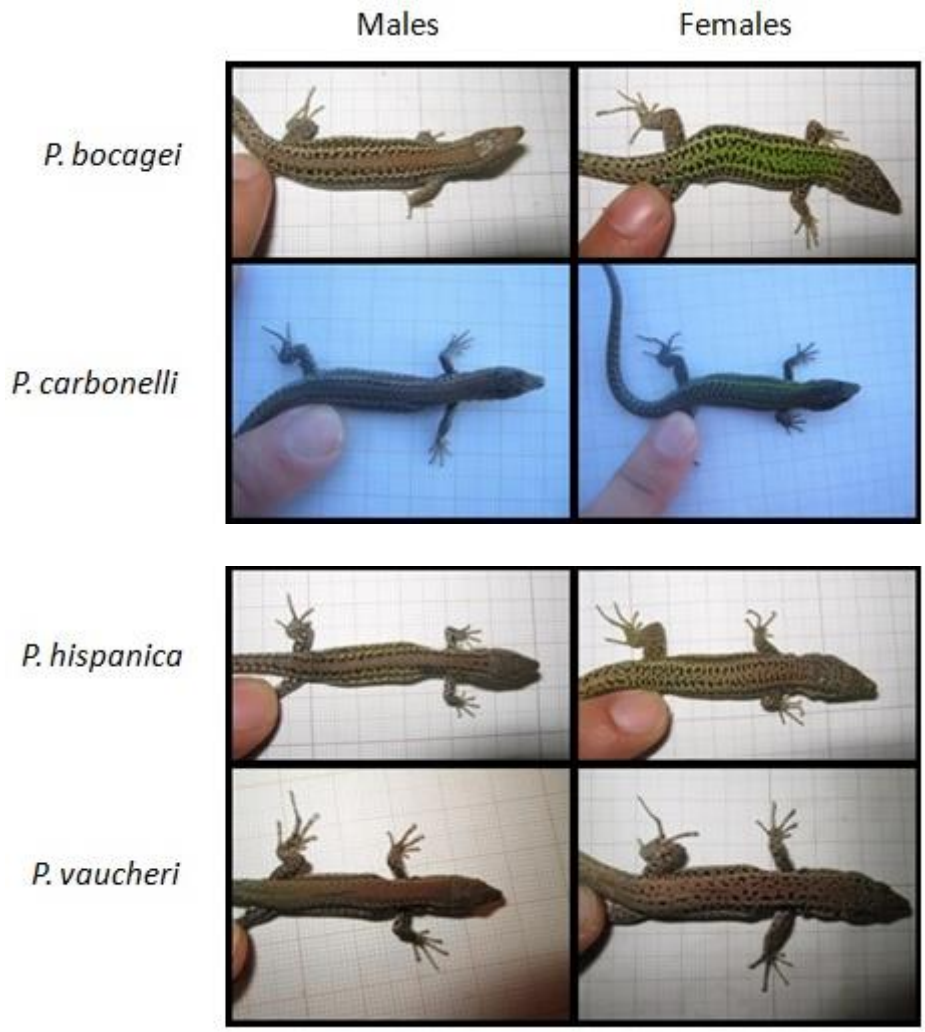

Fig. 4 – Dorsal pictures of both males and females for *P. bocagei, P. carbonelli, P. hispanica II and P. vaucheri.* Pictures taken by Guilherme Dias, scheme by Henrique Estrela.

Podarcis bocagei (Seoane, 1884) is an abundant species within its distribution and is able to occupy different types of habitats. It is generally distributed north of the river Douro, with some regions of Espinho and Serra da Freita e Gralheira as exceptions, requiring relatively humid climates (Sá-Sousa, 2001).

Previously considered a subspecies of *P. bocagei*., *Podarcis carbonelli* (Pérez-Mellado, 1981) was later described as a separate species in light of morphological and genetic studies (Harris & Sá-Sousa, 2002). It is distributed south of the river Douro and along the coastal line of Portugal. *Podarcis carbonelli* can also be found in some regions of Spain, such as Cáceres and the National Park of Doñana. IUCN classified

this species as "Vulnerable", with loss of habitat due to primarily anthropogenic factors being one of the major threats.

Before being recognized as a full species by Klemmer (1959), *Podarcis hispanica* was initially described as *Lacerta oxycephala hispanica* by Steindachner (1870) and later considered to be a subspecies of *Lacerta (Podarcis) muralis* (Alonso-Zarazaga, 1998). Then, Sá Sousa (2000) described two distinct, morphologic and genetically, types of *P. hispanica* in Portugal: type I and type II. Genetic data later indicated that *P. hispanica* was a species complex, with many distinct genetic lineages within the Iberian Peninsula (Kaliontzopoulou et al. 2011), several of which have since been recognized as full species. This *P. hispanica* complex is distributed across the Iberian Peninsula, South of France, and in certain Atlantic and Mediterranean islands.

P. vaucheri taxonomy suffered continuous changes in the last years. It is relatively recent the suggestion of elevating the subspecies *P. hispanica vaucheri* to its own state of species, *P. vaucheri* (Oliverio et al. 2000), with various genetic studies supporting this (Busack et al., 2005; Pinho et al., 2008), although the taxonomic status of some populations in southern Morocco, and the distinction between forms in Algeria and Tunisia remains complex (Lima et al., 2009; Kaliontzopoulou et al. 2011). This species *sensu strictu* is distributed on all North-West Africa and South of Spain.

Detecting *Hepatozoon*

The molecular detection of *Hepatozoon* parasites involves several steps, from the capture of the host, which, in the case of this thesis, is *Podarcis* spp., to the analysis of sequences obtained through the amplification of DNA. Each step is of equal importance for the final goal of obtaining our genetic data. Some methodologies complement each other. Thus, it is pertinent that the several techniques and methods used should be described in detail.

Sampling

Capturing lizards can prove to be a challenge, due to their high level of activity during the day. Once captured, a small piece of tissue from the end of their tail is sampled. The tissue is preserved in 96% ethanol at room temperature to be used for molecular studies of both the host and its parasites. After removing the tip of the tail of the lizard, bleeding can occur and blood samples are both sampled for blood smears and blood dots in Whatman filter paper. The sampling of blood samples may not always be possible due to coagulation and lack of fluidity.

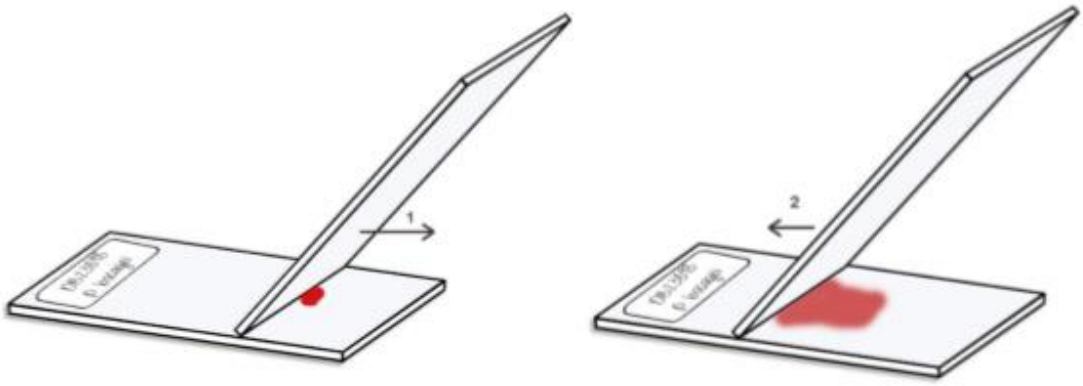

Fig. 5 – Technique to obtain blood smears: drop of blood is place in the bottom slide; second slide is place on top of the first, making contact with the blood drop (1); by sliding the slide on top, the blood, by capillarity, will smear across the bottom slide (2). Illustration by Isabel Damas Moreira.

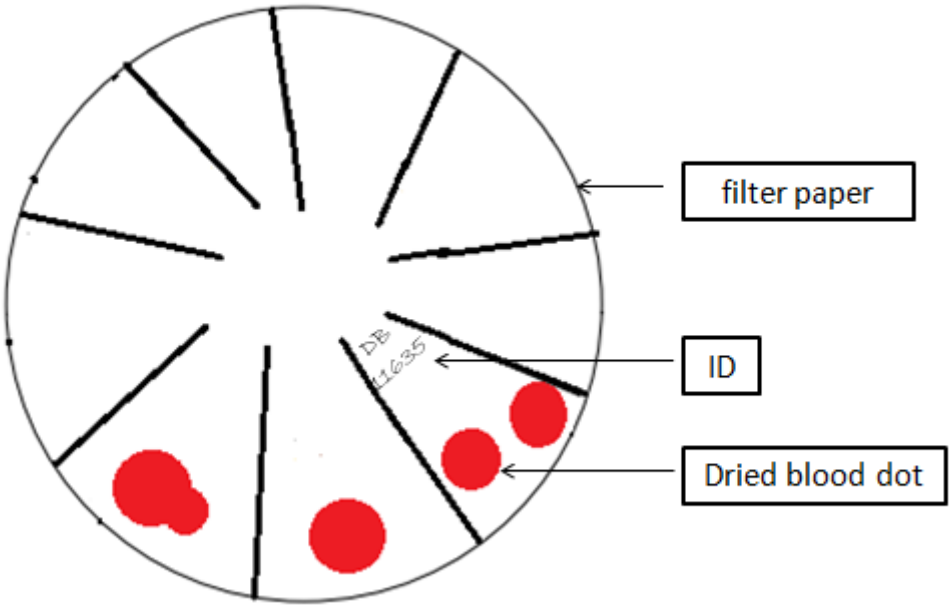

Fig. 6 – Example of Whatman filter paper. Based on Telford, 2009. Image by Henrique Estrela

Both the tissue and blood samples can be used to detect *Hepatozoon* parasites (Maia et al., 2014). Blood smears are obtained by smearing blood across a glass slide, air dried, fixed with absolute methanol as soon as possible, later stained with Giemsa coloration (1:9 distilled water) and finally left to dry at ambient temperature. Stained slides can be viewed through microscopy for detection of parasite gamont stages and morphologic identification. They also provide an important tool for estimating prevalence and intensity of infection. On the other hand, the tail tissue and blood dots are used for molecular analysis, such as DNA amplification.

Microscopy

The immense time that microscopy has been used for scientific purposes is evidence of its great usefulness. Microscopy can be used in the detection of parasites in their different developmental stages, description of morphologic features of parasites, assessment of prevalence (percentage of infected individuals in a given population) and intensity (percentage of parasites in a given individual) levels and identification of which host cells are being targeted. It serves several purposes and is fairly inexpensive, comparing to other modern, laboratory methods. It is easy to understand why microscopy is widely used for studying several groups of parasites, including *Hepatozoon*, even in the face of technological advances and innovation. Even some inherent disadvantages of microscopy, such as its time consuming usage, have been mitigated by modern software like Cell^B Olympus® that allow to easily capture pictures or make precise measurements for later observation and analysis, with built-in cameras.

As mentioned, microscopy is also a tool that enables the assessment of intensity levels. This can also be performed with molecular studies, such as real-time PCR technique, but its high cost can be prohibitive. The most used technique to evaluate parasitic intensity, and the one used for this thesis, is to count the number of infected cells within a total number of cells previously defined (Margolis et al. 1982; Bush et al. 1997). The number of total erythrocytes counted regarding *Hepatozoon* studies vary greatly between different studies and researchers preferences, from 1000 (Wozniak et al. 1998; Brown et al. 2006) to 2000 (Madsen et al. 2005; Salkeld &

Schwarzkopf 2005; Ujvari & Madsen 2005), and sometimes going up to a total of 10000 (Sloboda et al. 2007; Godfrey et al. 2011). In this thesis, this value varied due to, exclusively, the quality of the slides, but never dropped below 2000 total erythrocyte count, which is generally considered to be enough to give accurate results (Godfrey et al. 1987).

Molecular Analysis

Identifying species from the Apicomplexa group by description of their morphologic features is possible only when observing the developmental stages in the invertebrate host (Herbert et al. 2010), whereas in the vertebrate host the gamont stage creates many obstacles for such identification. Gamonts between different parasite species can be remarkably similar, leading to the incorrect identification of two species as being the same. On the other hand, gamonts of the same species during their development phases may have distinct morphologic characteristics in different hosts or even within the same host. It is indeed suggested that identification of parasite species base on morphology, although may be useful as a first approach, should always be complemented with additional information.

Analysis of molecular data can be useful to confirm the observations made with microscopy. Nucleotide sequences data can complement effectively the limitations of morphologic description of parasites in the vertebrate host.

DNA can be extracted from tissue samples using a commercial kit, following the manufacturer's instructions (e.g. DNeasy Blood & Tissue kit (Qiagen, Washington D.C.)) or using a high salt method (Sambrook et al., 1989). In this thesis, the high salt method was used for the extraction. *Hepatozoon* identification was done by performing PCR reactions with parasite-specific primers targeting a region of the 18S rRNA gene. This gene is considered to be a good genetic marker for reconstructing the phylogenetic relationships among protists, including Apicomplexa organisms (Morrison and Ellis, 1997; Šlapeta et al., 2003; Adl et al., 2007; Perkins et al., 2011). The rRNA genes in general have some characteristics that facilitate their amplification, such as abundance of their transcripts in the cell, and have both highly conserved regions that

enable the design of primers and more variable regions that provide phylogenetic information (Perkins et al., 2011). Furthermore, the 18 rRNA is commonly used for *Hepatozoon* studies, thus it provides a useful data base for comparing sequences. However, it should be taken into account that phylogenetic results based on this gene alone, reflect its evolutionary history, which may not be congruent with the species evolutionary history.

Objectives

Information regarding the interaction between *Hepatozoon* parasites and reptile hosts is not only lacking, but also research is biased towards snakes. However other reptiles such as lizards are as important in the life cycle of *Hepatozon*. Many lizard species are common intermediate hosts, which represent the most important phase in the life cycle of these parasites (Sloboda et al., 2007).

Prevalence and intensity of infection can be described, respectively, as the percentage of infected individuals in a population and the mean number of parasites found in each individual. Both these estimates are useful when trying to comprehend the impact of parasites on their hosts. By sampling several *Podarcis* spp. populations from the Iberian Peninsula, estimating *Hepatozoon* prevalence and intensity of infection in each population, and complementing with it with genetic data of these parasites, several questions will be addressed regarding this host-parasite complex. For example, are there significant differences of prevalence and intensity between males and females of *Podarcis* populations? Are there changes in the degree of infection between different individual growth stages of the lizards? Are there differences of both prevalence and intensity levels within and among *Podarcis* populations?

Additionally, the effects of several climatic variables on the prevalence and intensity levels of *Hepatozoon* parasites are tested. This information can hopefully shed light on our understanding of how climate influences these organisms and their distribution. This is useful not only for the study of *Hepatozoon*, but also for a wider array of other parasitic groups. Geographic and climatic research on parasites is of increasing relevance, even though it is scarce. Thus, studies regarding these

questions, even if directed at a specific host-parasite complex, can serve as example for future investigation.

As an additional, side project, the correlation of erythrocyte size in two different *Podarcis* species - *P. bocagei* from the Iberian Peninsula and *P. vaucheri* from Morocco - and altitude was also assessed. In other groups of animals it has been described that erythrocyte size changes with altitude variation. Such changes in red blood cells' morphology may act as adaptations to high altitude environments, in which there is lower concentration of oxygen in the atmosphere. In mammals red blood cells size seem to increase with an increase of altitude, while in amphibians the correlation between the two factors appears to be negative. Unfortunately, such correlation in reptiles has been poorly investigated and results have not been consistent across different studies. If, in fact, erythrocyte morphology in reptiles changes as a response to prevent anemia, then *Hepatozoon* parasites, by infecting the erythrocytes and changing their morphology, could cause anemia to the host.

CHAPTER II

MANUSCRIPTS

Manuscript I

Assessment of *Hepatozoon* Prevalence and Intensity in Iberian *Podarcis* species: what environmental factors can explain the distribution of *Hepatozoon* parasites?

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Abstract

Various studies have shown the importance of parasites in the ecosystem. They can influence host behavior and fitness, for example, and thus shape population and community structure. There are also several species of parasites with direct impact to public health and human welfare. Understanding the host-parasite relationship is therefore crucial for both conservation measures and understanding the spread of diseases. For example, knowing how future global climatic changes will affect parasite populations may help to predict future distributions. We assessed variation of *Hepatozoon* prevalence and intensity across several *Podarcis* species in the Iberian Peninsula. We also performed a preliminary model of *Hepatozoon* distribution patterns. There were no significant differences in prevalence and intensity across host sexes and ages. Significant differences in prevalence across different climatic variables were detected for *P. carbonelli, P. hispanica II* and *P. vaucheri*. Intensity also showed a weaker, but still significant association to climatic variables

Keywords

Hepatozoon, *Podarcis*, prevalence, intensity, climatic variables

Introduction

Parasites play an important role in the ecosystem. Not only do they represent a considerable percentage of the ecosystem's biodiversity, they also shape the population structure of the hosts through their significant effect on host fitness (Hudson et al., 2006; Johnson and Hoverman, 2012; Mouritsen and Poulin, 2003). Furthermore, parasites can have an effect on host behavior (Ferguson et al., 2013) and competition between hosts (Hudson et al., 2006), ultimately shaping community structure. For these reasons, it has been proposed that parasites can act as a reasonable indicator of an ecosystem's health (Hudson et al., 2006).

There is also a huge socioeconomic impact of certain parasite species to take into account. Some pathogens are responsible for over 1 million human deaths per year and US $1 billion per year economic cost. Some of the most well-known, and studied, parasites belong to a diverse group of apicomplexan blood parasites, including *Plasmodium* species, the agent responsible for the malaria disease that has been described to infect cattle populations and humans, or *Eimeria* spp., which is one of the most important pathogens of poultry. Species of the genus *Hepatozoon*, also apicomplexan blood parasites, although overlooked in the past, has recently received more scientific focus, mainly due to being the most common and abundant pathogen in reptiles and having been described as important pathogens in domestic dogs and cats.

Host-parasite relationships are dynamic and complex, and studying such relationships is essential to understand the impact of parasites in their hosts and, consequently, in the ecosystem. Parasite populations are not influenced solely by inherent characteristics of the host, such as population size and densities, or resistance to mechanisms of infection (Combes, 2001), or those of the parasite such as life cycle or host specificity, but also by geographical and environmental factors (Pérez-Rodríguez et al., 2013).

The relation between parasite and the environmental variables is not easy to disentangle and may vary greatly depending on several aspects intrinsic to the parasite, such as its life cycle, which determine its dependence to the external environment. For example, in direct life cycle helminth parasites with fecal-oral transmission, adequate environmental conditions, such as temperature or humidity, are determinant in ensuring egg viability and development during the free-living stages (Poulin and Dick, 2007; Poulin and Mouritsen, 2006). Also, in the case of vector-borne parasites, the presence of suitable environmental conditions is determinant for vector

survival and thus effectiveness of parasite transmission (Garamszegi, 2011; Pascual et al., 2009).

Several studies report an increase of incidence of malaria over recent years, and some authors suggest this may be the result of an increase of suitable habitat for malaria's vectors due to temperature changes (Garamszegi, 2011; Pascual et al., 2009). On the other hand, Moller et al. (2013) found a very balanced impact of climatic variance in parasite populations of European birds.

In this study, we investigate the distribution and levels of parasitism of *Hepatozoon* parasites in Iberian wall lizards, *Podarcis* species. For this we use microscopy to assess prevalence and parasitaemia levels, and molecular methods to characterize genetically the parasites found. Futhermore, we perform a preliminary analysis to try to understand which factors may explain the distribution and levels of parasitaemia observed

Material and Methods

Sampling

A total of 317 Individuals of 5 different *Podarcis* species were collected at 30 locations across the Iberia Peninsula (Table 1).

Species	Locality	n	Coordinates	Altitude	Prevalence (%)
P. hispanica II	Castillo	7	39.06117; -3.00633	738	0
	NP de Cornalvo	11	39.01983; -6.17617	302	0
	Valencia del Ventoso	4	38.22767; -6.483	431	0
	Beja	5	38.01783; -7.86433	258	0
	Saelices	7	39.91967; -2.80467	923	0
	Villanueva de Córdoba	5	38.31533; -4.62533	707	0
	Riopar el Viejo	17	38.49983; -2.419	970	5,9
	Casar de Cáceres	15	39.575; -6.42933	394	0
	Évora	16	38.5714; -786433	280	100
	Arroyo	2	39.3605; -4.35833	771	0
	Albacete	2	38.97817; -1.85767	694	0
	Santa Maria da Feira	7	40.92072; -8.54293	175	85,7

P. vaucheri	Doñana National Park	12	37.15217; -6.3367	3	58,3
	Almonte	9	37.2536; -6.5611	61	100
	Matalascañas	31	37.0030; -6.5626	11	67,7
	Torcal de Antequera	4	36.95467; -4.5445	1209	0
	Playa de la Vibora	2	36.49; -4.77567	18.1	0
	Alcala la Real	10	37.46133; -3.92867	984	0
	Jaén	8	37.7863, -3.775	432	0
	Jaén2	8	37.7863; - 3.775	432	0
P. carbonelli	Vale de Rossim	5	40.40298; -7.58664		100
	Santa Maria da Feira	10	40.92072; -8.54293	175	60
	Silvalde	11	40.98758; -8.65281	3	72,7
	Cabo Raso	15	38.70933; -9.48533	4	13,3
	Vale de Rossim	9	40.40298; -7.58664	1444	22,2
	Hinojos	11	37.2865; -6.339418	47	0
	Esmoriz	10	40.95945; -8.65281	5	40
	Doñana National Park 2	31	36.99083; -6.44507	6	0
	Matalascañas	9	37.0030; -6.5626	11	0
P. bocagei	Aguda	4	41.0569; -8.65569	4	50
	Botanico Porto	4	41.15334; -8.64341	60	50
P. hispanica I	Vale de Rossim	17	40.40298; -7.58664	1445	58,8

Table 1 – All populations from the different *Podarcis* species in our dataset. N represents number of individuals sampled, while altitude is given as meters above sea level. Prevalence is give by the percentage of infected individuals.

During sampling, all host individuals were identified and tissue from the tip of the tail was collected and preserved in 96% ethanol for genetic analysis. Whenever natural bleeding from the end of the tail occurred, blood samples were also collected in Whatman filter paper. In addition, when possible, blood smears were also done for microscopic examination. Smears were air-dried and fixed in methanol for 2 minutes prior to staining and observation under the microscope. After sampling, all *Podarcis* individuals were released at the capture site, after basic information such as sex, size and locality were recorded.

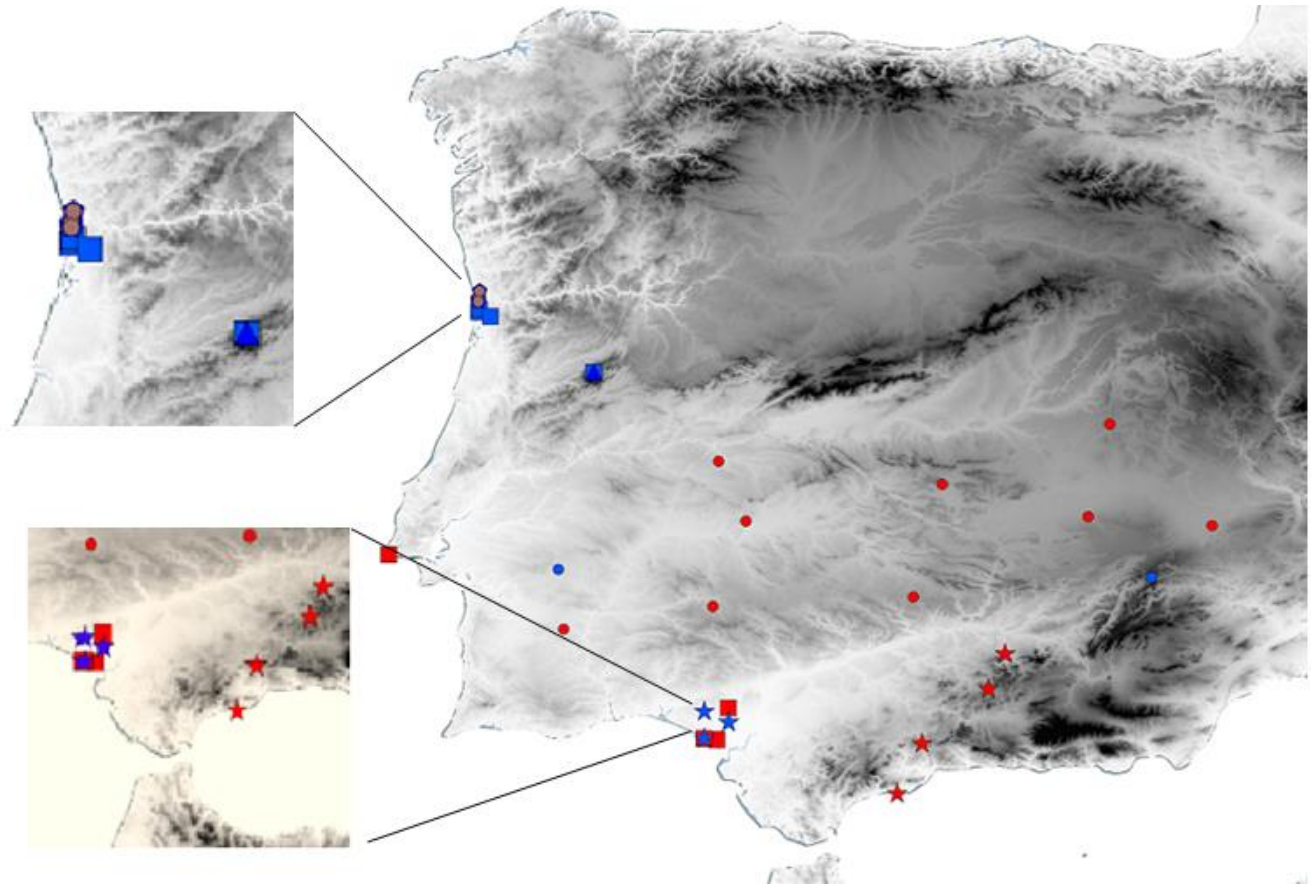

Fig. 7 – Distribution of the sampled population in the Iberian Peninsula. Circles represent *P. hispanica II* populations, stars represent *P. vaucheri* populations, squares represent *P. carbonelli* populations, the single triangle represents *P. hispanica I* population. Populations with no infected individual sampled are illustrated in red, and populations with at least 1 infected individual sampled are illustrated in blue. Dark pentagons represent infected populations of *P. bocagei*.

Microscopy

Blood smears obtained during sampling were later used for microscopy. We utilized a microscope (brandt) with a built-in camera to observe the slides containing the smears and detect the presence of *Hepatozoon* parasites. In vertebrate hosts these parasites form gamonts inside the host's erythrocytes, allowing for an easy detection through microscopy.

Intensity of parasitism was estimated as the number of infected cells in counted blood cells. Pictures of the blood smears were taken with the built-in camera and the Cell^B Olympus® software. Twelve pictures were taken per slide. Cell count varied with the quality of the slide. However, number of erythrocytes counted never dropped below 2000.

Molecular Analysis

Tissue sampled from the tail of the individuals and blood samples stored in Whatman blood dots were used for molecular analysis. DNA of 24 infected individuals identified in the microscope was extracted using the high salt method (Sambrook et al., 1989). In order to indentify *Hepatozoon* spp. we performed PCR reaction using parasite-specific primers (HEPF300) targeting a region of the 18s rRNA gene. Conditions for PCR consisted of 3 initial minutes at 94 ºC, followed by 35 cycles at 94 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 1 minute, and a final extension at 72 ºC for 10 minutes.

Phylogenetic Analysis

Sequences obtained were blasted in GenBank in order to discard contaminations and to confirm the identity of the amplicons. This is necessary as these primers are known to sometimes amplify fungus and other apicomplexan parasites (Maia et al., 2012, Tomé et al., 2013). Resulting sequences were analyzed with Geneious 5.6.2 (Drummond et al., 2012). Electropherograms were checked manually and consensus sequences for every individual were created. Some heterozygous positions in the sequences were detected and given the IUPAC code. What these heterozygous positions represent is not clear – they are either the result of existence of different *Hepatozoon* isolates in the same individual, or variation within the multiple copies of the 18S rRNA gene of the same parasite (Maia et al., 2012).

Other *Hepatozoon* sequences available in GenBank were also included in our alignment. The final alignment consisted of 101 *Hepatozoon* sequences, 565 base pairs long. Through the Modeltest 3.06 software, we used the AIC criterion (Posada and Crandall, 1998) to choose the most adequate model of evolution. Bayesian analysis was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist, 2001). The analysis ran for 10^7 generations, saving one tree every 100 generations. Trees generated were combined, after applying a burnin of 25%, in a consensus tree.

Statistical Analysis

Since presence of the parasite has a binomial distribution (individuals are either infected, value=0, or not, value=1), we used a logistic regression modeled using a binomial distribution to assess if there were differences in the prevalence of parasites across the different *Podarcis* species. For this we used the command "glm" with the family option set to binomial (R development Core team, 2012). There were significant differences across the different species, so consequent analyses were performed for each host species separately. For each *Podarcis* species, a model including locality, sex and age (adult, sub-adult and juvenile) was tested. We also analyzed differences of prevalence with a second model including altitude and various climatic variables such as mean annual temperature, minimum temperature of the coldest month, maximum temperature of the warmest month, annual precipitation, precipitation of the driest month and precipitation of the wettest month.

Intensity levels were also analyzed, using a dataset containing only the infected individuals. We used Shapiro's Test to assess if our dataset followed a normal distribution. Since the dataset was not normally distributed, we performed permutational (m)ANOVAs, which generates distribution curves based on permutational re-sampling of the data. As with the analysis regarding prevalence, we first tested for variation of intensity levels between species. Since no significant differences were detected, we used for the rest of the analysis a dataset consisting of all *Podarcis* populations. Differences of parasitic intensity between sex and developmental stage of the host were tested.

Preliminary analysis of the environmental factors modeling the presence parasite

To model the presence of *Hepatozoon* in the Iberian Peninsula we used our dataset of 33 populations of *Podarcis*. Environmental data was obtained from WorldClim databases, choosing a scale of 5km. Due to this resolution, we excluded sampling localities that were too close together, ending up with a total of 26 populations from our initial dataset.

We used Quantum-GIS, specifically its sampling points' plugin, to obtain the environmental data of our sampling points. Then, in R, we ran a Generalized Linear

Model including 14 climatic variables. Our initial environmental data included a total of 19 different climatic variables. Of these, 5 variables (Mean Diurnal Range, Isothermality, Temperature Annual Range, Mean Temperature of the Wettest Quarter, Mean Temperature of the Driest Quarter) had to be removed from the model.

In QGIS we created a 5x5 km grid and extracted our environmental information for the center of each pixel. Then, in R, we used the Predict function to evaluate, based on our model, the probability of *Hepatozoon* presence, or not, for each pixel of our grid. Results of the prediction were viewed in QGIS.

Results

A total of 317 individuals were analyzed, of which 92 were found to be parasitized through microscopy, from 27 different populations. In 12 populations none of the individuals sampled were found to have parasites, whereas the other 15 populations had at least 1 individual parasitized. Two populations tied regarding the highest ratio of infected individuals per total samples: individuals both from *P. hispanica II* population sampled in Évora and *P. carbonelli* sampled in Vale de Rossim were all parasitized (16/16 and 5/5, respectively). The highest intensity percentage was 18.8%, an individual of *P. hispanica II* from Évora.

In our dataset we also had sympatric populations in 3 locations: one, in Santa Maria da Feira, between a population of *P. carbonelli* and a population of *P. hispanica II*; another in Vale de Rossim, between *Podarcis hispanica I* and *P. carbonelli*; and finally, in Matalascañas, between *P. carbonelli* and *P. vaucheri*. One thing to note is that *P. carbonelli* is common in all the contact zones of our data. In the sympatric populations of Vale de Rossim and Santa Maria da Feira, both species presented individuals infected with parasites. However, in Matalascanãs, even though we observed a high percentage of infected individuals of *P. vaucheri* (21 out of 31), in *P. carbonelli* none of the individuals showed presence of parasites.

1. Prevalence

There were significant differences of prevalence detected between species ($p=5.048^{-5}$), so consequent analyses were performed per species. This meant we had

to remove the only population we had of *P. hispanica I* from our prevalence analysis. When we analyzed each host species separately we did not find significant variation in parasites prevalence between host sex and age in any of the *Podarcis* species.

1.1 *P. bocagei*

For *P. bocagei* there was no significant variation of prevalence between different altitudes, and there were also no significant differences regarding any of the climatic variables included in the model.

1.2. *P. carbonelli*

When performing analysis for *P. carbonelli* populations we found significant differences in prevalence across different altitudes only ($p=3.65^{-12}$). We repeated the analysis, but only considering the climatic variables (altitude was removed from the model), and found a significant relationship between annual precipitation and precipitation of the driest month. Removing the last variable, we ran once again the analysis. Significant differences in prevalence were found for maximum temperature of the warmest month ($p=6.98^{-8}$), minimum temperature of the coldest month ($p=6.834^{-9}$) and annual precipitation ($p=0.005651$).

1.3. *P. vaucheri*

Significant differences in prevalence were detected for altitude ($p=1.848^{-11}$). However, when we removed altitude from the analysis, the results showed significant differences of prevalence for mean annual temperature ($p=5.295^{-10}$), maximum temperature of the warmest month ($p=0.01319$) and minimum temperature of the coldest month ($p=4.256^{-6}$).

1.4. *P. hispanica II*

The results of the analysis for *P. hispanica II* populations were similar to those of *P. vaucheri*. Significant variation of prevalence was again found between different altitudes only ($p=3.505^{-16}$). However, when removing altitude variation from the model,

significant differences of prevalence were found for mean annual temperature (p=0.04319), maximum temperature of warmest month ($p=8.15^{-17}$) and minimum temperature of coldest month ($p=7.323^{-8}$).

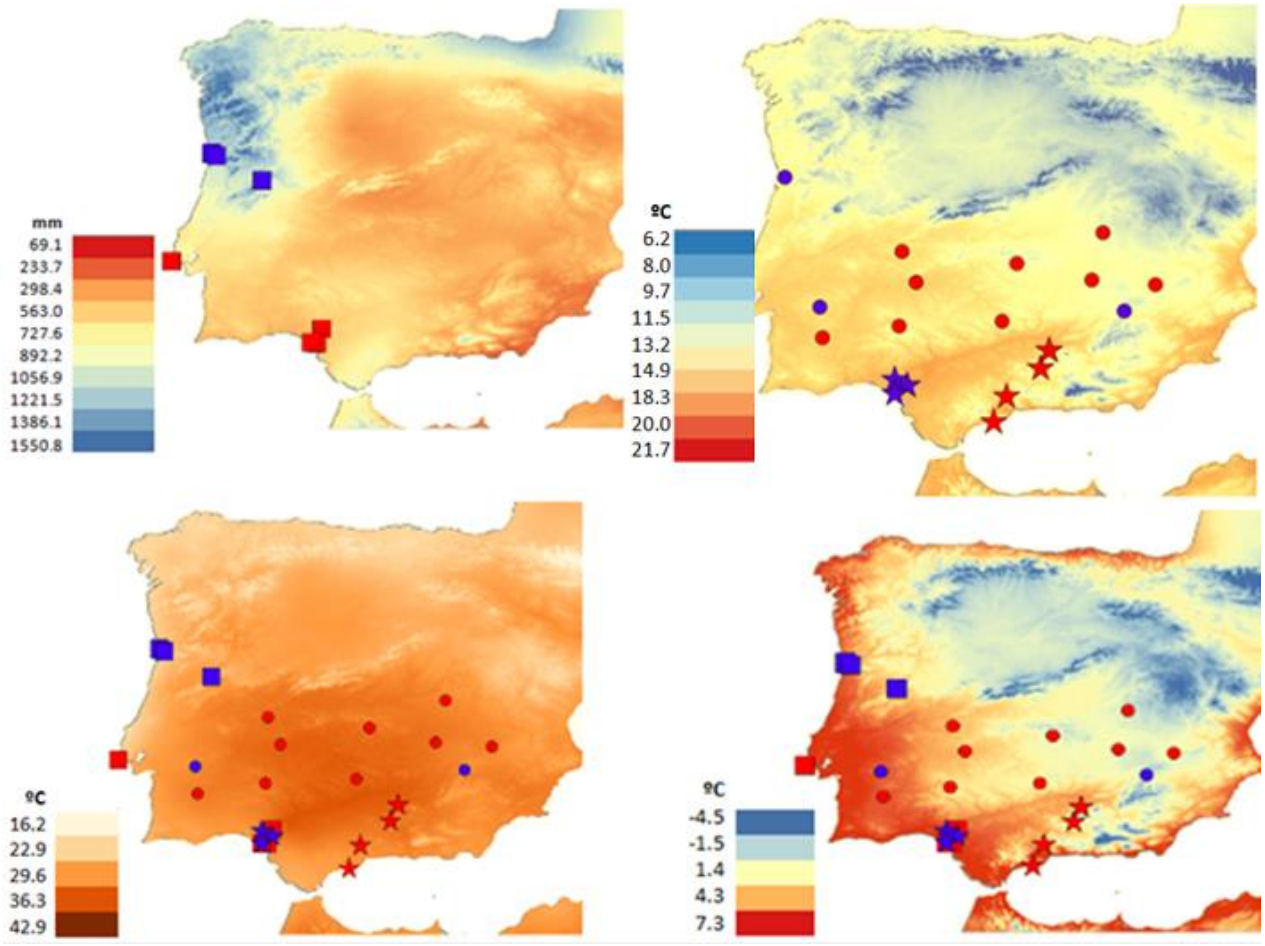

Fig. 8 – Variation in Annual Precipitation (top right), Annual Mean Temperature (top left), Maximum Temperature of the Warmest Month (bottom right) and Minimum Temperature of the coldest Month. Squares represent P. carbonelli populations, circles represent P. hispanica II populations and stars represent P. vaucheri. Red populations have no infected individuals in our samples, while blue ones have at least one individual in which we detected *Hepatozoon* parasites. ºC = Celsius degrees, mm = millimeters.

2. *Intensity*

There was no significant variation of intensity between different host species. Thus, we performed the additional analysis including all the individuals in the same dataset, regardless of their species. There were no significant variation in intensity between sexes and age of the host. The results showed significant differences in intensity for maximum temperature of the warmest month (p=0.048) and minimum temperature of the coldest month (p=0.018).

3. Phylogenetic Analysis

Consensus tree obtained with Bayesian analysis indicate that sequences obtained in this study, from individuals of the Iberian Peninsula, are part of the same lineage. This lineage includes various other *Hepatozoon* isolated from reptiles – both lizards and snakes. As found in other estimates of relationships (eg Tomé et al. 2014), it is the sister taxa to a lineage found in carnivores, such as domestic dogs.

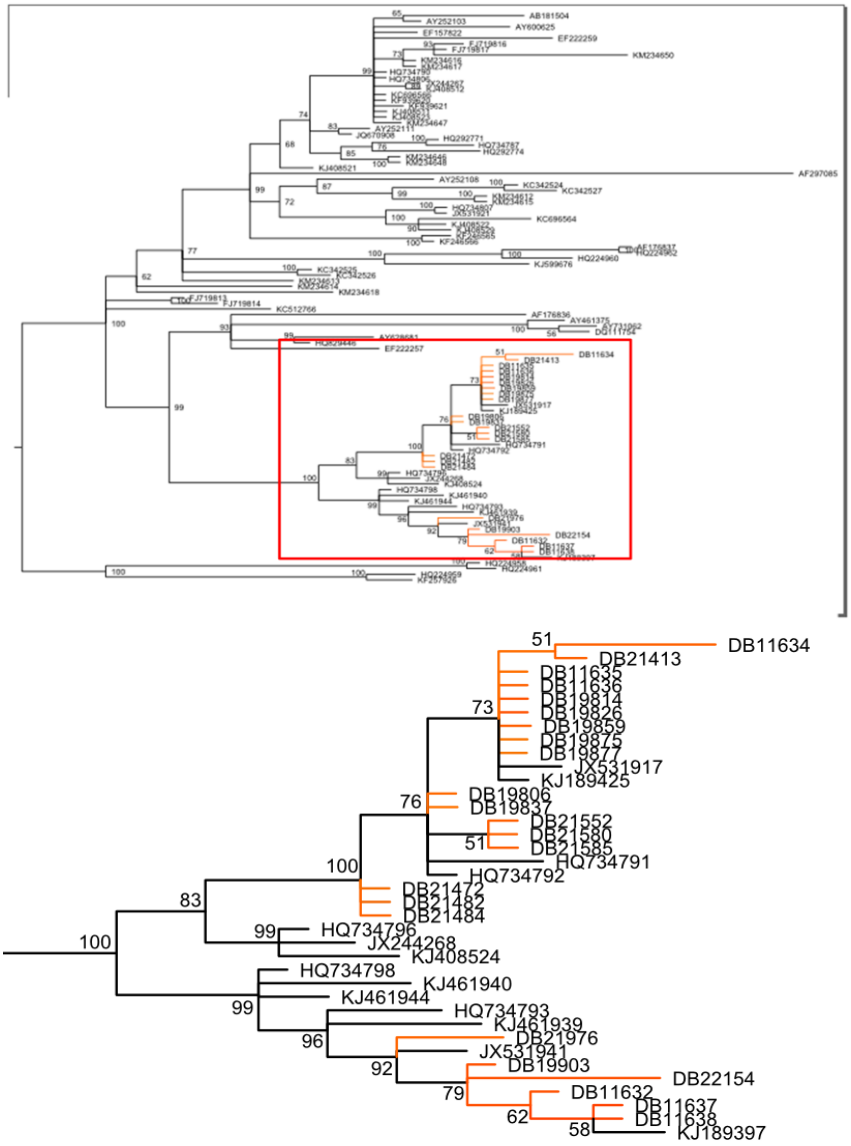

Fig. 9 – Bayesian estimate of relationships of *Hepatozoon* species based on 18S rRNA gene sequences. Bayesian posterior probabilities are given near the nodes. Bottom image consists of a zoom in of the selected portion of the first image. Sequences obtained in this study are highlighted.

4. Model of Hepatozoon prevalence

Our model for *Hepatozoon* prevalence across the Iberian Peninsula was viewed with QGIS. Our populations are in accordance with the model's prediction. All positive populations are located in areas where the model predicts the existence of *Hepatozoon* and all negative populations are located in areas where the model does not predict the occurrence of the parasites.

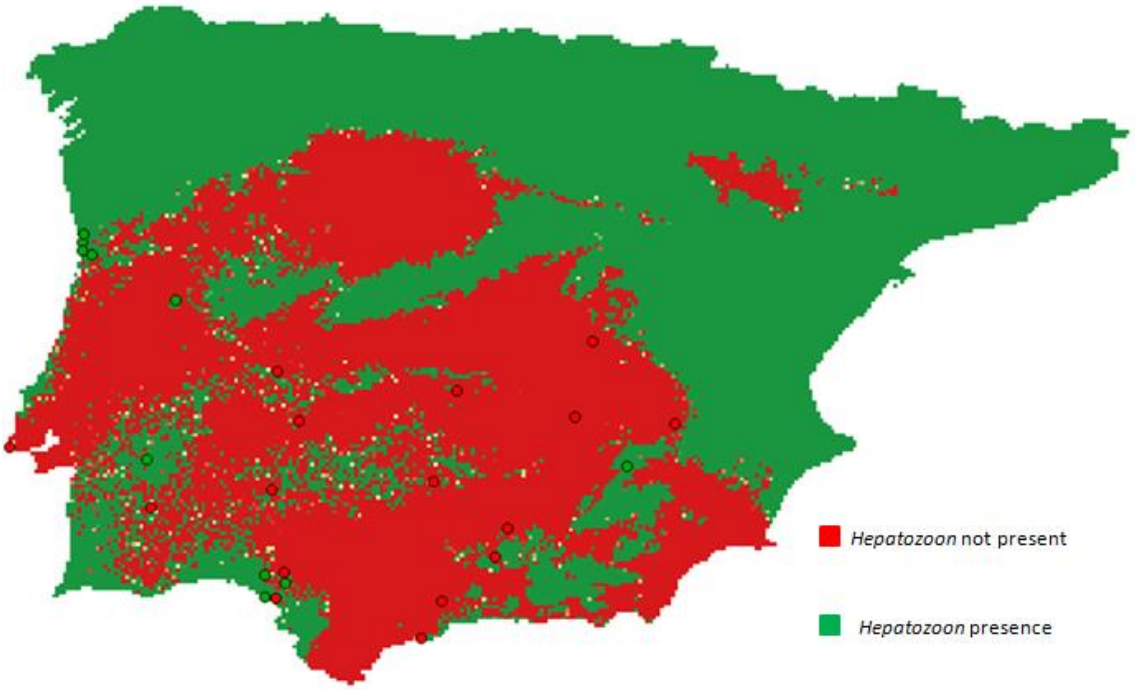

Fig. 10 – Prediction of presence, or not, of *Hepatozoon* parasites in the Iberian Peninsula.

Discussion

Although the main focus of this study was not to obtain genetic data of *Hepatozoon* parasites and assess its phylogeny, it was important to sequence some individuals and confirm that *Hepatozoon* in Iberian Peninsula belong to the same lineage, thus minimizing any confounding effects when assessing the variation of prevalence and intensity of infection across the different locations and host species. Our results confirmed that all the *Hepatozoon* that were sequenced belonged to the

same clade, and so could be treated as single unit for the purposes of the modeling approach.

Our results showed that prevalence varied among the *Podarcis* species sampled and, in some cases, between populations of the same species. For instance, prevalence in *Podarcis carbonelli* varied from populations where all individuals sampled were found to be infected (Vale de Rossim), to populations where no individual was infected (El Acebuche). This variation across the different species could be an artifact of sampling bias in some cases, such as the *P. bocagei* population in Aguda where the number of individuals sampled was 4. However, in some other cases, such as *P. hispanica II*, our results include 16 out of 16 infected individuals from a population of Évora and, contrastingly, 15 out of 15 non-infected individuals from the same species, but in a different locality. We had populations where we registered 100% prevalence for *P. carbonelli* (Vale de Rossim, n=5), *P. hispanica II* (Évora, n=16) and *P. vaucheri* (Matalascañas, n=9), but not for *P. hispanica* I and *P. bocagei*. However, sampling for *P. bocagei* included only 8 individuals from 2 different populations and for *P. hispanica I* we only had 1 population of 17 individuals, thus the results can be partially biased by non-representative sampling.

Intensity values also varied within and between species, and even within the same population. The highest value of intensity observed (18%) was from a *P. hispanica II* individual from Évora, but low levels of intensity (0.3% or 0.6%) were also found in that same population. Contrasting results in infection rates, of both Prevalence and Intensity, between populations of the same host species and between different species is not unexpected (Badge et al., 2003), and similar varying results obtained in this study have been described in previously published studies (Maia et al., 2014).

We found no significant differences both in prevalence and intensity between sexes and different developmental stage, in any species considered in this study. Studies have shown that parasite prevalence can significantly differ between male and female hosts, or not (references). Sex differences in behavior and parasite susceptibility could be correlated with differences in parasite prevalence between sexes (Zuk and McKean, 1996; Klein, 2004). Regarding the age of the hosts, our results could be biased by a significantly lower number of immature hosts sampled (only 13), compared with adults captured.

There were significant variations in prevalence across different species. This result can be directly attributed to actual differences between species or it can indicate differences in localities associated with each species. Nevertheless, further analyses

were performed per species to avoid confounding effect. Prevalence was significantly different only across different altitudes for *P. carbonelli, P. hispanica II* and *P. vaucheri*. The additional analysis of variance performed without considering altitude as a factor, showed significant differences of prevalence across different climatic variables. Altitude can be strongly correlated with climatic variables such as temperature and precipitation. That could explain why significant differences are only found between different altitudes, when this factor is included in the model. Significant differences in prevalence were explained more often by differences in temperature and precipitation. Studies have shown that, although changes in precipitation should not be overlooked, variation in temperature usually has a greater impact in parasite infections (Moller et al., 2013). However, significant differences in prevalence between populations of *P. bocagei* were partly explained by variation of annual precipitation. Populations with no infected individuals sampled were located in southern Spain, where annual precipitation is significantly low, while populations with infected individuals were located in north of Portugal, where precipitation levels are undeniably higher (Fig. 8).

Variation in intensity was not so obviously related with climatic variables. There were no significant differences between species, as with prevalence. Intensity varied significantly with minimum temperature of the coldest month. Even though there was also significant variation for maximum temperature of the warmest month, the p-value for this variable was extremely close to the 0.05 threshold.

Overall therefore, these results show that prevalence seems to be more affected by the climatic variables considered compared to intensity. This could indicate that perhaps variation in climatic variables could have a significant impact on *Hepatozoon* vectors. Once individuals are infected, intensity's levels may depend on either different climatic variables not considered in this study, or other factors. For example, studies have shown that population sizes and population densities of the hosts can be related to higher intensity levels (Badge et al., 2003; reviewed in Hudson et al., 2002).

Further studies are required to elucidate on how *Hepatozoon* parasitaemia levels vary. Although we showed obvious relationships between several climatic factors and prevalence levels, more variables should be tested, such as landscape factors. Furthermore, host's demographic characteristics, such as population density, should be taken into account.

Manuscript II

Does Altitude shape red blood cells in *Podarcis*? Testing variation in erythrocyte size of *P. carbonelli* and *P. vaucheri* at varying altitudes

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Abstract

Across different taxonomic groups there is vast literature describing different adaptations from species to high-altitude environments. Differences can be detected in the respiratory system, with development of thinner pulmonary membranes, or in the circulatory system. In the blood, adaptations vary immensely from hemoglobin concentration to red cell count or even erythrocyte size and morphology. We analyzed the erythrocyte size (Length, Width and Area) of two different *Podarcis* populations at varying altitudes: *P. bocagei* in the Iberian Peninsula and *P. vaucheri* in Morocco. No significant differences were found in erythrocyte size of *P. bocagei* across different altitudes. However, for *P. vaucheri*, which had more contrasting populations in terms of altitude differences, showed significant differences in erythrocyte width and area.

Keywords

Erythrocyte, width, length, high altitude, *P. vaucheri, P. bocagei*.

Introduction

Hematological studies are most often conducted either on humans or economically relevant species. Specifically on humans, there is a vast body of research concerning hematopoiesis and erythropoiesis processes, blood composition, detailed description of blood cell types and even different evolutionary adaptations to high altitude of populations in Asia, Africa and America (Sheinfeldt and Tishkoff, 2010). Adaptations to high altitude pressures can be observed both in the respiratory and circulatory system, and include changes in erythrocyte size (reviewed in Monge and Leon-Velarde, 1991).

Comparatively, hematological research on reptiles is scarcer. Among the different studies there is reasonable diversity on the focused characteristics of the circulatory system of reptiles. Morphological studies vary from erythrocyte descriptions of turtles and tortoises populations to the observation of leukocytes and thrombocytes of blue tongue lizards (Ugurta et al., 2002; Canfield and Shea, 1988). Some researchers have tried to evaluate the effect of several factors, such as seasonality and sex, on blood formation of reptiles (Hutton, 1960; Haggag et al., 1966; Altrland and Thompson, 1958). Cline and Waldmann (1962) showed a significant depression of erythropoiesis at low environmental temperature in immature American alligators.

Erythrocyte size has been found to vary substantially across the different species of the *Reptilia* class. Some of the largest erythrocytes were found in *Sphenodon punctatus*, turtles and crocodiles, while the smallest were found in lizards (reviewed in Arikan and Çiçek, 2013; Hartman & Lessler, 1964; Saint Girons & Saint Girons, 1969; Saint Girons, 1970) Aquatic species of turtles were found to possess larger erythrocytes than the terrestrial species (Ugurtas et al. 2002). Also, erythrocyte sizes of lizards vary greatly across different families and sometimes even within the same family (reviewed in Arikan and Çiçek, 2013). The effect of altitude on erythrocyte size of reptiles and possible adaptations of these organisms to such environments is not clearly correlated (Ruiz et al., 1993). In general, studies across different groups of animals do not have consistent results when it comes to the relationship of altitude and red blood cells' dimensions. In a recent study, erythrocyte size among populations of *Hypsiboas cordobae* decreased with altitude (Baraquet, 2013). On the other hand, the effect of altitude on the morphometry of red blood cells in bovines was tested, and the authors found that the populations from higher altitudes had significantly larger erythrocytes (Adili et al., 2013).

Due to the reduced amount of data regarding erythrocyte variation with altitude in reptiles, the contrasting results within this group have to be considered with caution. It is required further studies with similar and consistent results to fully capture the adaptations of reptiles to high altitude environments. Therefore, in this study we attempt to increase data regarding this subject and perhaps better understand it.

Methods

Acquiring the Data

Four Moroccan populations of *Podarcis vaucheri* and eight Iberian populations of *Podarcis bocagei* were selected (Table 2). From each population, 10 adult individuals were captured.

Species	Location	Altitude	Coordinates
P. bocagei	Montesinho	1378	41.98; -6.80
	Castro Laboreiro	1005	42.03; -8.16
	Vila Pouca de Aguiar	676	41.45; -7.67
	Gerês	306	41.72; -8.17
	São Mamede do Coronado	123	41.29; -8.57
	Subportela	50	41.69; -8.72
	Mindelo	23	41.30; -8.74
	Madalena	0	41.10; -8.66
P. vaucheri	Bab Taza	1155	35.02; -5.20
	Debdou	1518	33.87; -3.04
	Oukaimeden	2751	31.21; -7.86
	Tislit Lake	2261	32.20; -5.64

Table 2 – Altitude and coordinates of the populations sampled in this study. n=10 for each population, altitude is given in meters above sea level.

During sampling, all host individuals were identified and tissue from the tip of the tail was collected and preserved in 96% ethanol for genetic analysis. Blood obtained from natural bleeding of the tail was smeared across a glass slide. Every slide was fixed with absolute Methanol, stained afterwards with the standard Giemsa coloration (1:9 of distilled water for 55 minutes) and air-dried (Moody 2002).

The slides were observed through a microscope with a built-in camera (brandt). Using Cell^B Olympus® software, one picture was taken from each slide of each individual. Using the same software, the length and width of 100 randomly selected erythrocytes were measured. The area of each erythrocyte was subsequently calculated, assuming an ellipsoid shape of the cells.

Analyzing the Data

Bartlett test was used to evaluate the homocedasticity of the variables (erythrocyte length, width and area), and was concluded that the variance of the groups we were comparing was similar. Then, to assess the distribution of the variables, we performed a Shapiro-Wilk test ($P > 0.05$), which confirmed the variables followed a normal distribution.

Differences between males and females were tested for, with analysis of variance (ANOVA). Only one population of *P. bocagei* and *P. vaucheri* had equal number of males and females. In each of these 2 selected populations, we had 5 adult males and 5 adult females. No significant sex differences were found (t tests, $P > 0.05$) in length and width for both populations, so we conducted the following analysis ignoring the sex of the individuals in our dataset.

Differences between populations were compared by analysis of variance (ANOVA). Whenever significant differences were detected between different populations, we performed pairwise Tukey HSD tests to obtain more detailed information and determine which populations were significantly different from each other. Finally, correlation between the variables (Length, Width and Area) and altitude was assessed, using Pearson's correlation test.

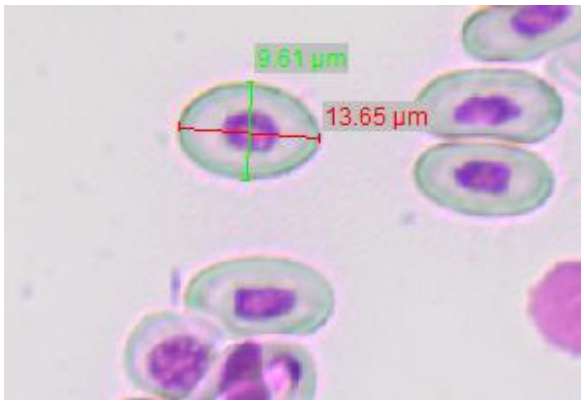

Fig. 11 – Image with measures of width and length of an erythrocyte. Picture by Henrique Estrela.

Results

1. *P. bocagei*

The biggest, on average, erythrocytes observed (in length, width and area: 15.67 µm, 8.50 µm and 133.31 μm^2, respectively) belonged to the population of Vila Pouca de Aguiar. Madalena had the shortest erythrocytes, with an average length of 15.04 µm (Table 4). This value was very similar to the average length of Mindelo (15.05 µm). The lowest values of erythrocyte width (7.69 µm) and area (115.75 μm^2) were observed in the population of Gerês.

Concerning the 8 Iberian populations of *P. bocagei*, the ANOVA analysis showed no significant differences in erythrocyte length, width and area between populations (Table 3). Pairwise Tukey HSD test showed that the population of Gerês differed from the populations of Vila Pouca de Aguiar and Castro Laboreiro ($P < 0.05$), both concerning the erythrocyte width and area.

	Species	
Variable	*P. bocagei*	*P. vaucheri*
Lenght	p = 0.0907	p = 0.6301
Width	p = 0.6074	$p = 3.37^{-06}$
Area	p = 0.2317	p = 0.0259

Table 3 – Descriptive results for p-values obtained with ANOVA. Differences in length, with and area across different altitudes for *P. bocagei* and *P.vaucheri* were tested.

Population	Length (±SD)	Width (±SD)	Area (±SD)
Madalena	15.04 (±0.47)	8.18 (±0.45)	122.97 (±31.07)
Mindelo	15.05 (±0.46)	8.10 (±0.36)	121.81 (±30.94)
SubPortela	15.25 (±0.52)	8.23 (±0.58)	125.54 (±31.40)
São Mamede Coronado	15.06 (±0.79)	8.01 (±0.67)	120.85 (±30.81)
Geres	15.07 (±0.51)	7.69 (±0.38)	115.75 (±30.16)
Vila Pouca de Aguiar	15.67 (±0.45)	8.50 (±0.38)	133.31 (±32.36)
Cabo Laboreiro	15.45 (±0.66)	8.38 (±0.47)	129.43 (±31.88)
Montesinho	15.21 (±0.70)	7.96 (±0.32)	120.98 (±30.84)

Table 4 - Average values (in µm) of erythrocyte length, width and area of eight populations of *P. bocagei.* Each population had 10 individuals. SD = Standard Deviation.

Pearson's correlation test did not indicate any significant correlation between altitude and any of the erythrocytes measures (length, width or area) for populations of *P. bocagei*.

2. *P. vaucheri*

For *P. vaucheri* populations, the longest, on average, erythrocytes observed, belonged to the population of Debdou, with an average length of 14.85 µm (Table 5). It was also this population that showed the highest values of erythrocyte area, with an average of 94.79 μm^2. However, the erythrocytes with the highest width values, on average, were observed in the Oukaimeden population.

Population	Length (±SD)	Width (±SD)	Area (±SD)
Bab Taza	13.50 (±0.85)	7.39 (±0.51)	78.48 (±9.11)
Debdou	14.85 (±0.91)	8.13 (±0.48)	94.79 (±8.61)
Tislit Lake	13.68 (±0.76)	8.45 (±0.49)	90.92 (±9.34)
Oukaimeden	13.86 (±0.61)	8.45 (±0.41)	92.01 (±6.02)

Table 5 - Average values (in µm) of erythrocyte length, width and area of four populations of *P. vaucheri.*

The ANOVA results for the 4 Moroccan populations revealed significant differences between populations for erythrocyte width and area (Table 3).Through a pairwise Tukey HSD we found that when considering erythrocyte width and area, it was Bab Taza population that differed significantly from all the others ($P < 0.05$).

Pearson's correlation tests showed that although there was a significant correlation between width and area with altitude (Fig. 12, 13), there was no significant correlation between length and altitude. Correlations for both width and area were positive (0.606 and 0.352, respectively).

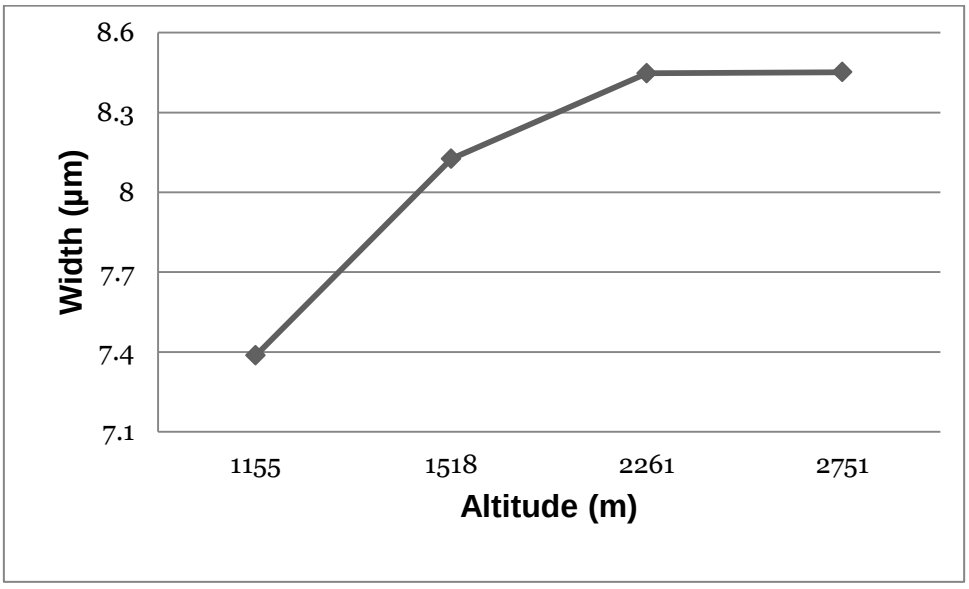

Fig. 12 – Graph of variation of mean erythrocyte width of *P. vaucheri* populations across different altitudes.

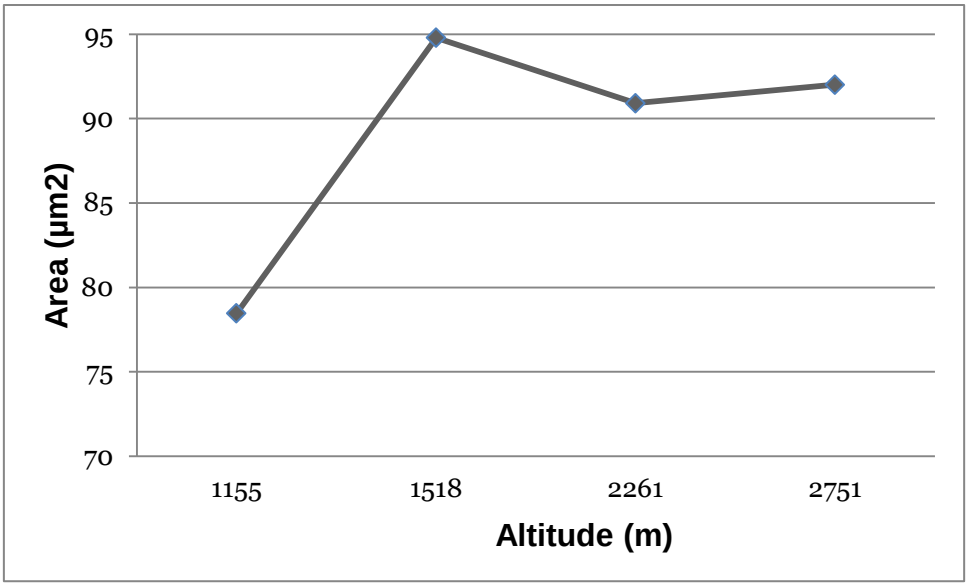

Fig. 13 – Graph of variation of mean erythrocyte area of *P. vaucheri* populations across different altitudes.

Discussion

Our results are drastically different between the two *Podarcis* species. The populations of *Podarcis bocagei* were not significantly different from each other concerning erythrocyte length, width and area. Lower altitude populations (Madalena, Mindelo, Subportela, São Mamede Coronado) did not reveal any significant differences from the other populations. On the same note, the highest altitude population, Montesinho, also did not differ from any other *P. bocagei* population. This indicates that either for these altitudes or for *P. bocagei*, erythrocyte sizes don't appear to change with altitude. The fact that there was no significant correlation between length, width and area with altitude, seems to support that hypothesis.

When using a wider array of altitudes between populations, even though with a different species (*P. vaucheri*), there looked to be a clearer relationship between erythrocyte size and altitude. This could indicate that significant differences of erythrocyte size between different altitudes may only be detected when such variation in altitude is considerably high. The population from Debdou was significantly different from the other three populations when accounting for erythrocyte length and had considerably longer erythrocytes, on average. However, we did not find a significant correlation between length and altitude across the different populations. Concerning

erythrocyte width and area, it was Bab Taza population which differed significantly from the others, presenting lower averages of red cells width and area. Even though there was no correlation found between erythrocyte length and altitude in *P. vaucheri* populations, width and area showed significant positive relationship with altitudes.

Although differences detected in our study between *P. bocagei* and *P. vaucheri* are possibly explained by the altitude variation in populations of both species, it should be considered that inherent characteristics of both *Podarcis* may also play a role. Even so, erythrocyte size changes were detected across different altitudes in *P. vaucheri*. Are these differences the result of adaptations to high-altitude environments? Further studies could test for a correlation between morphology of erythrocytes and host fitness. Furthermore, it would be interesting to assess variation in erythrocyte size across different altitudes for other *Podarcis* species.

CHAPTER III

GENERAL DISCUSSION

General Discussion

1. Prevalence and Intensity

High variation in parasite prevalence and intensity across populations of the same species or between populations of different species is not an unexpected observation (Badge et al. 2003). In fact such variation has been recorded not only across different groups of hosts, but also for entirely different parasites, such as malaria parasites in birds (Knowles et al. 2011, Wood et al. 2007, Bensch and Akesson 2003), helminth parasites in fish populations (Poulin and Dick 2007) and *Hepatozoon* parasites in reptiles (Maia et al. 2012). Parasite infection and parasitaemia levels depend on a combination of both abiotic and biotic factors. Climatic effects affecting vector or parasite development (Rogers and Randolph 2006) or effects of host age on immunity (Palacios et al. 2007, Damas-Moreira 2014, Ujvari and Madsen 2005) are some of the factors that can play a role in both prevalence and intensity levels.

In one of our studies, we observed within species variation of both prevalence and intensity. For example, *Podarcis carbonelli* populations sampled in the North of Portugal were found to be infected by *Hepatozoon*, while populations in the South of Spain did not have a single infected individual. These differences could be caused by the contrasting climatic conditions between these two areas: North of Portugal is usually colder with higher precipitation rate, while the South of Spain has considerably higher temperature means along the years and is drier. Indeed significant differences of prevalence were attributed to variation in temperature of warmest and coldest months and annual precipitation. However, also in the South of Spain, several *P. vaucheri* populations were all infected. In this case, general climatic conditions between infected *P. vaucheri* populations and non-infected *P. carbonelli* are similar. Prevalence variation between these populations most likely cannot be explained on a macro-habitat perspective. Specific characteristics of micro-habitats occupied by these two species could be the answer for the different prevalence levels. Although very few studies indicate it, some have shown a relationship between the host's microhabitat use and its parasites (Brito et al. 2014, Dwyer et al. 2014). The differences in

prevalence between closely distributed populations can also be the result of intrinsic host characteristics, such as population size and density (Badge et al. 2003, Morand & Poulin, 1998, Earn et al. 2000). Our data also include two populations in sympatry, one with no apparent infection (*P. carbonelli*) and another with high percentage of infected individuals (*P. vaucheri*). Studying such populations can provide insightful information regarding host-parasite interactions.

Much like prevalence, intensity varied across different species and different populations within the same species, but also varied between individuals of the same population. Significant differences in intensity were only explained by one of the climatic variables used (maximum temperature of the warmest month), even though prevalence showed significant variation for several climatic factors. Prevalence offers information on whether an organism is infected or not, and such infection status can be correlated with genetic resistance of the host (Westerdahl 2007) or degree of exposure to infective vectors (Sol et al. 2000). Specifically, the contact with infective vectors can be influenced by climatic factors. Several studies have shown that changes in temperature can increase infection rates by increasing the distribution of suitable habitat for infective vectors (Garamszegi 2011, Pascual et al. 2009, Pérez-Rodríguez et al. 2014). On the other hand, intensity offers more insight about host-parasite relationship after the infection occurs. As reviewed in Reece et al. (2009), variation in parasite intensity can reflect the hosts' ability to control established infections or the parasites ability to regulate its own replication rate when faced with different hosts.

Our phylogenetic analysis revealed that all *Hepatozoon* sequences are part of the same lineage. Considering that these parasites were found in different *Podarcis* species, and that the same lineage occurs in various other snakes and lizards, this can indicate low host specificity. Even though there was significant variation of prevalence across the various hosts, some populations of each *Podarcis* species was found to have relatively high prevalence. Previous studies have suggested low host specificity of *Hepatozoon* both in snakes (Telford et al. 2004) and lizards (Landau et al. 1970, Paperna and Lainson, 2004; Damas-Moreira, 2014). Low host specificity means the parasite has the ability to infect several host types within the same community, which could lead to a self-regulation of the parasites' replication rates and variation in prevalence and intensity across different hosts. In all these cases we are discussing low vertebrate intermediate host specificity – of course the final invertebrate host specificity is a separate issue. Indeed, since it is not clear in many cases which species are acting as the final invertebrate host, this is one of the key aspects that should be examined in the future.

2. Modelling and Predicting Parasite incidence

Being able to model the incidence of any parasite and predicting its distribution, not only spatially but also temporally, represents a powerful tool to anticipate emergence of diseases and to better understand how the host-parasite complex is influenced by different factors (Peterson 2008, Fuller et al., 2012). Most research trying to model parasite distribution is based on climatic variables, such as temperature, humidity or precipitation (Merino and Moller, 2010; Poulin and Mouritsen, 2006; Halimi et al., 2014; Olwoch et al., 2009), but few others have also taken into account other factors such as landscape features (Pérez-Rodríguez et al., 2013).

However, interpreting the effect of biotic and abiotic factors on parasite distribution can be complicated due to the relationship between the parasites and their hosts. It can be challenging to determine whether a particular environmental factor is correlated directly with the occurrence of a parasite or indirectly by affecting the hosts (Hance et al., 2007; Cardon et al., 2011). Moller et al. (2013) suggested an indirect effect of climatic variables on parasites abundance through their hosts. Futhermore, changes in parasite incidence may also be the result of the effect of environmental variables on the vectors of the parasites (Gage et al., 2008).

Modeling the distribution of parasites and predicting their spatial occurrence becomes increasingly complex when adding temporal variation. For example, trying to predict the distribution of parasite populations in the future can be biased by inaccurate prediction of future changes in the environmental factors considered for the model.

In our study we performed a preliminary modeling test of the distribution of *Hepatozoon* in the Iberian Peninsula. Our model was based in data obtained from our 27 *Podarcis* spp. populations and 14 climatic variables. Although it is, as mentioned, a preliminary approach in attempting to model the parasites' distribution across the Iberian Peninsula, we obtained 100% concordance between our model's predictions and our sampled populations. All infected populations were located in areas where our model predicts the occurrence of *Hepatozoon*. The same could be observed for our non-infected populations in areas where parasite incidence was not predicted. These

positive results demonstrate that modeling the distribution of *Hepatozoon* may be possible. Still, improvement of the model is necessary, for example by increasing sampling across the Iberian Peninsula. Also, more climatic variables and, perhaps more important, other factors such as landscape features and host population attributes should be added to the model. Locations with low prevalence, for example, can be mistaken for areas where there is no occurrence of parasites, and a robust and well supported model should not be influenced by sampling bias.

3. Erythrocyte size vs Altitude

Variation in altitude can sometimes be related to differences in hematological characteristics such as hemoglobin concentration, red cell count or erythrocyte size and morphology.

The size of red blood cells (RBC) has been shown to vary across different species. In a recent study, erythrocytes of *Hypsiboas cordobae* seemed to be significantly smaller in lower altitude populations (Baraquet, 2013). In mammals, namely bovine populations, erythrocyte has been shown to increase in size in higher altitude populations (Adili et al., 2013). On the other hand, in 1986, Yamaguchi et al. found not only that high-altitude camelids had smaller erythrocytes than low-altitude camelids, but also that the small RBC had higher oxygen transfer rates. In reptiles there have been no studies where the correlation between altitude and erythrocyte size has been properly tested. Ruiz et al. (1993) found no significant differences in erythrocyte volume between 27 different lizard species at altitudes ranging from 4600m to sea-level. However the authors in this study were more interested in testing other hematological values, than erythrocyte length or width.

In our study, erythrocytes from *P. bocagei* populations did not present significant differences in size across different altitudes. The observed results for these populations can be biased by low variation of altitudes (from sea-level to 1378m). This possibility becomes more plausible, when we considered the *P. vaucheri* populations from Morocco. In these populations, erythrocyte width and area significantly increased in higher altitudes.

Adaptations to high altitudes are not limited to erythrocyte size. A high oxygen-hemoglobin affinity, a thin-walled pulmonary vascular tree, absence of chronic mountain sickness and lower erythrocyte volume are some of the described adaptations to high altitude habitats (reviewed in Monge and Leon-Velarde, 1991). Erythrocyte size has been shown to be negatively correlated with its total number of cells (Hawkey et al., 1991; Ruiz et al., 1993), and animals of higher altitude populations have higher amount of RBC, but lower mean volume per RBC. However, our studies appear to indicate a different adaptation to high altitude environments, which involves larger erythrocytes and, consequently, higher erythrocyte volume. Further research would be required to fully comprehend adaptations to high altitude pressures in *Podarcis* populations.

4. Future Projects

Even though the studies included in this thesis have had interesting results and provide information to further understand questions regarding host-parasite interaction of *Hepatozoon* and *Podarcis*, and the adaptations of lizards to high altitude environments, there is still many issues that remain to be addressed. Although several recent studies have assessment of the relationships of *Hepatozoon* parasites in reptiles (Harris et al. 2011, Tomé et al. 2012; Damas-Moreira, 2014), many of their relationship with the vertebrate intermediate hosts remains to be clarified. Particularly in reptiles much of the data is from Europe and North Africa, and only by including samples from other regions will biogeographic patterns become discernible. Even more important there is very little information concerning the final invertebrate hosts, and this is clearly a major field to be explored in the future.

Being able to model *Hepatozoon* distribution provides tremendous information about intrinsic characteristics of the parasites and, potentially, about its relationship with different hosts. Some authors have attempted to model *Hepatozoon* populations' distribution, for instance in snakes (Davis et al., 2012) and birds (Pérez-Rodríguez, 2013). In our model we try to predict *Hepatozoon* distribution in the Iberian Peninsula. However, with only 27 populations sampled and 14 climatic variables accounted for, the model is still in a preliminary stage. Additional sampling should be conducted,

serving both to add robustness to the model and test its predictions. The distribution of our sampled populations shows a clear lack of data in the North of the Iberian Peninsula, the northeast region and the coastal line of Portugal. Obtained information about *Hepatozoon* prevalence in these areas is crucial for the development of a more robust model. If possible, including variables other than climatic could provide a better representation of the parasites distribution. For example, microhabitat differences can be playing a role in the variation of infection in certain populations.

Also interesting would be to investigate, in more detail, *Hepatozoon* parasites in sympatric populations, since such populations can give unique information. Since these populations share the same macro-environment characteristics, significant differences in infection rate and parasitaemia can only be explained by particularities in micro-habitats or different vectors, for example. These complex systems between parasites and sympatric hosts can also unravel evolutionary questions. Studies have shown that parasites may play a role in sympatric host diversification (Buckling and Rainey, 2002). In one of the studies of this thesis, we had three sympatric populations of *Podarcis*, and although in two of them both populations had parasites, in the third one only one population was infected. Our data was not sufficient to understand these differences, but an effort should be made increase our information about these populations. Could parasites provide crucial information about host sympatry? Why is it that in two, apparently sympatric populations, we did not detect a single infected individual in one of them? Could this indicate that both populations are not in considerable contact with each other? Or could this be the result of different vectors? If such, why do we have different vectors for different *Podarcis* species?

Further investigation is needed not only to clarify the results from both studies of this thesis, but also to pursue new questions that we can pose to our data and our results.

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