Universidade de Aveiro Departamento de Biologia
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Ana Patrícia Dos Reis Marques

Herpetofauna em cavidades do Maciço Calcário Estremenho

Herpetofauna in caves of the Estremenho Karst Massif

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Ana Sofia Pereira Serrenho Reboleira, Professora Associada da Universidade de Copenhaga e coorientação científica do Doutor Sérgio Miguel Reis Luís Marques, Investigador de Pós-Doutoramento do CESAM da Universidade de Aveiro e do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave anfíbios, répteis, grutas, biomarcadores, metais, pesticidas.

resumo

Grutas e escassez de água à superfície são algumas das caraterísticas e peculiaridades da paisagem cársica. A água rara à superfície pode ser encontrada numa intrincada rede subterrânea. Em Portugal, os maciços calcários estão maioritariamente distribuídos em duas grandes regiões, as Bacias Lusitânica e Algarvia. O Maciço Calcário Estremenho é o maior do país (cerca de 900 Km^2), estando praticamente toda a área incluída no Parque Natural das Serras de Aire e Candeeiros (PNSAC). Nesta área são conhecidas mais de 2000 grutas e uma grande variedade de habitats. A herpetofauna presente nesta região compreende 15 anfíbios e 19 répteis, mas pouco se sabe acerca do uso que estes animais fazem das grutas. Para tentar colmatar esta lacuna, o presente estudo pretendeu fazer o mapeamento da presença e distribuição de espécies em grutas. Como principais resultados registou-se a presença de três espécies (*Tarentola mauritanica; Salamandra salamandra* e *Pleorodeles waltl*) e a observação pela primeira vez em Portugal de reprodução em *P. waltl*. Este estudo indicou a necessidade de mais trabalhos futuros com a perspetiva de conservação dos locais e das espécies neles existentes.

Para além do escasso conhecimento acerca da presença de herpetofauna em grutas, também não existe informação relevante acerca do impacto das massas de água no desenvolvimento de algumas espécies, nomeadamente, anfíbios. A importância destas massas de água deve-se ao facto de o sistema cársico ser vulnerável a atividades humanas que libertam contaminantes para o ambiente e que facilmente se infiltram nos lençóis freáticos e em nascentes, dispersando-se por longas distâncias. Deste modo, o segundo objetivo, foi perceber como as massas de água, selecionadas da zona de amostragem, afetam o desenvolvimento de girinos de *Hyla arborea*. Os resultados não mostraram alteração no crescimento dos girinos nem se verificou um número de anomalias e de mortes elevado. As respostas a nível de stress oxidativo apresentaram diferenças significativas para a peroxidação lipídica entre o controlo e um dos locais, muito provavelmente relacionada com o nível de salinidade. Ao nível da atividade enzimática das enzimas antioxidantes não existiram quaisquer diferenças nos animais expostos às diferentes massas de água. Os resultados das análises aos pesticidas selecionados, apesar de não apresentarem valores elevados, mostram a presença de pesticidas, num dos locais.

Este estudo indica que a água dos locais amostrados pode não apresentar ameaça imediata aos anfíbios, sendo, no entanto, necessários trabalhos futuros a fim de concluir o estado das massas de água do maciço Estremenho ao longo do ano, e seus impactos na biodiversidade.

keywords amphibians, reptiles, caves, metals, pesticides, biomarkers.

abstract

The karst landscape has among other features and peculiarities, caves and scarce water at surface. The water rare at surface can be found underground in a complex hydrological network. In Portugal, karst massifs are distributed by two major zones, Lusitanian and Algarve basins. The Estremenho karst massif is the biggest massif of the country (around 900 Km^2) and most of it is covered by the Serras de Aire and Candeeiros Natural Park (PNSAC). It has more than 2,000 caves and a great variety of habitats. In this territory are present 15 amphibians and 19 reptiles species, but the knowledge of the use of caves by these animals is scarce. To fill this gap, this study aimed to map the presence and distribuition of herptofauna in caves. The major results were the presence of three species (*Tarentola mauritanica; Salamandra salamandra,* and *Pleorodeles waltl*) with report of *P. waltl* reproduction for the first time in Portugal. This study highlighted the need of more future works with conservation of the sites and species that they harbour in view.

In addition to the scarce knowledge on the presence of herpetofauna in caves, there is no significant information about the impact of water bodies on development of some species, namely amphibians. The importance of this water bodies is due to the vulnerability of the karst system to human activities that release contaminants to the environment and that can easily reach the groundwater table and springs, spreading over large distances. Thus, the second aim of this study was to see how the selected water bodies in the sampling area affect the developmento of early stages of *Hyla arborea*. The results showed no difference in larvae growth nor was reported high number of anomalies or mortality. The oxidative stress responses showed statistical significant difference on lipidic peroxidation between control and one of the sites, most likely related with the level of salinity. In terms of enzimatic activity of the antioxidant enzymes there were no differences on the animals exposed to the different water bodies. The results of the analyses of the selected pesticides, although with low values, show the presence of pesticides in one of the sites.

This work show that water from the sampled sites is not a threat to the amphibians, but future works are needed to conclude the water bodies state of the Estremenho massif throught the year and its impacts on biodiversity.

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Chapter I

General Introduction

Herpetology is the study of amphibians and reptiles (Loureiro, *et al.,* 2008; Vitt & Caldwell, 2014). These two groups aren't closely related evolutionarily, however, they are usually studied together because of their occurrence in the same places and many similarities in physiological, behavioral and ecological features.

Global interest in this field of study has increased and the innovative technologies allow its rapid evolution, leading to new discoveries and advances in many areas. For example, due to their small climatic niche (Bonetti & Wiens, 2014), many studies used amphibians and reptiles to infer paleoclimate conditions (Blain *et al.*, 2010, 2013; Bailon *et al*., 2015; Lobo, *et al*., 2016; Suárez-Bilbao *et al*., 2016).

In Portugal, the zoological studies started during the Pombaline Reform of the University (1772), in the eighteen century (Loureiro *et al*., 2008; Martins, 2014). The first Portuguese scientific work using binominal nomenclature entitled "*Florae et Faunae Lusitanicae specimen*" was done by Domenico Agostino Vandelli, a professor at the University of Coimbra (Loureiro *et al*., 2008; Reis, 2017). This study, published in 1797, refers seventeen species of Portuguese herpetofauna (Guimarães, 2017). Another notable person, in the history of Portuguese Herptofaunistic studies, was Barboza du Bocage, which contributed to the progress of science being even called the "father of zoology" (Sacarrão, 1968 in Madruga, 2013). He had a particular interest in amphibians and reptiles, having written in 1863 "Lista dos mamíferos e répteis observados em Portugal", the second list that included the Portuguese herpetofauna and described the new genus and species: *Chioglossa lusitanica*.

After Bocage, other naturalists contributed to the knowledge of this subject, being considered the golden age of the Portuguese herpetology, namely Adolpho Möller, Eduardo Sequeira, Lopes-Vieira, Bettencourt Ferreira, Augusto Nobre, Paulino de Oliveira, Oskar Boettger, Eduardo Boscá, López Seoane and Jacques Vladimir von Bedriaga (Loureiro *et al*., 2008). Some events, like the regicide, the fall of the monarchy, the turbulent beginning of the republic, the two World Wars and the Spanish Civil War in Spain in the first part of twentieth-century, lead to a decrease of herpetological studies. From the seventies to nowadays, the interest in herpetofauna has grown, mainly in the last twenty years. For example, José Miguel Cei (Cei, 1962, 1972, 1973; Crespo & Cei, 1975), an italo-argentinian herpetologist, a pioneer in the use of new technics to systematic and evolution of amphibians (e.g., electrophoreses), reorganized the herpetological collections of Bocage Museum, resulting in publications of catalogs of amphibians (Crespo, 1971), reptiles (Crespo, 1972) and an addendum (Crespo, 1975). These documents present, for the first time, maps with the location, although without precision, where the specimens were found.

Already in the eighties, Rudolf Malkmus (Malkmus 1979, 1981, 1982, 1984, 1985, 1987) added an extraordinary contribute on distribution and ecology of Portuguese herpetofauna. His first book, “Die Amphibien und Reptilien Portugals, Madeiras und der Azoren” (Malkmus, 1995), was published in 1995 and compiled data about the ecology and distribution of these groups. An updated English version was available in 2004. But Malkmus wasn’t the only one who contributed to the field, other investigators worked and researched on morphology, ecology, conservation and geographic conservation (Ferrand de Almeida *et al*., 1983; Perez-Mellado,1984; Ferrand de Almeida, 1985; Thireau *et al*., 1985; Barbadillo, 1987; Paulo & Vicente, 1989).

Another important event was the publications of “Livro Vermelho dos Vertebrados de Portugal” (SNPRCN, 1990), about the conservation status of the vertebrates of Portugal, and later, in 2005 the second version of the red book (Cabral *et al*., 2005). Other important works, dealing with the species distribution, were published over the years, including the Field Guide “Anfíbios e Répteis de Portugal”, edited by FAPAS in 2001, and “Atlas dos Anfíbios e Répteis de Portugal” (Loureiro *et al*., 2008)., with a second edition in 2010. The data about the distribution of amphibians in Portugal is still incomplete and the present dissertation intents to be a contribution to improving the knowledge in this field.

Amphibians

The class Amphibia encompasses four subclasses, of which three are extinct. Being that all modern amphibians belong to the subclass Lissamphibia, which is divided into three orders: Anura (frogs and toads), Caudata (salamanders), and Gymnophiona (caecilians) (Cannatella *et al.,* 2009; Gardner & Rage, 2016). The total species known is 7,782, where 6,866 species of anurans belong to 55 families, 709 species of caudates belong to 10 families, and 207 species are distributed by 10 families of Gymnophiona (AmphibiaWeb, 2017). Thus, the amphibians are one of the terrestrial vertebrates with the most diverse radiations (Pyron & Wiens, 2011).

Figure 1 Representation of extant amphibian taxonomy

Biology and Ecology

Amphibians can live both in water as in land, and their name literally means “living on both sides”. However, a huge variety of species exhibit different life cycles, from water-born newt or tadpole - that grow into an adult salamander or frog - to caecilians, living in the soil (Schoch, 2014).

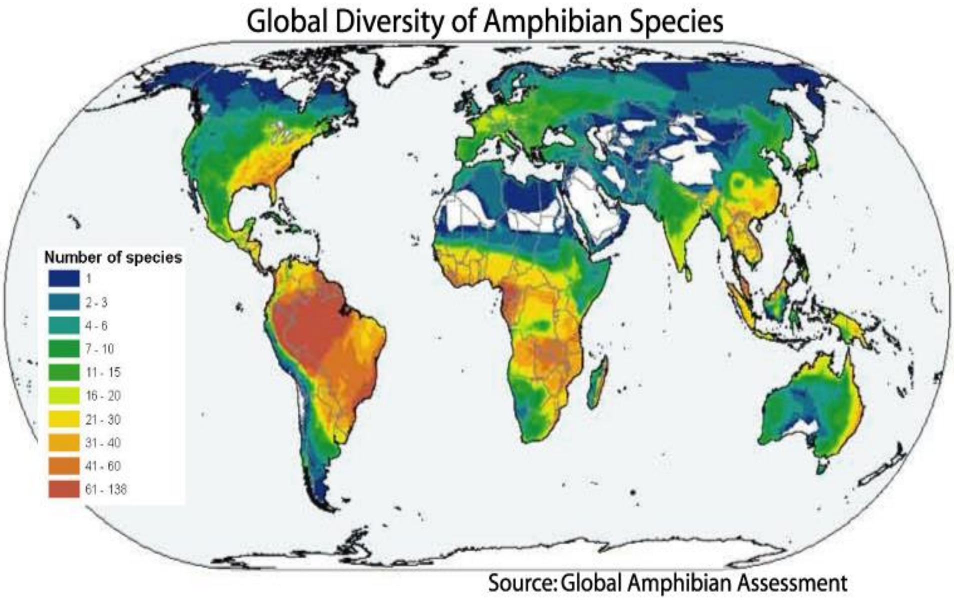

Figure 2 Distribution of amphibian diversity worldwide. Map retrieved from AmphibiaWeb (2017)

It is possible to find these animals worldwide (Fig. 2), from tropical rainforest to some of the driest deserts, with exception of the coldest regions of the globe (Wells, 2007; Loureiro *et al*., 2008). Amphibians have a very permeable skin, allowing rapid passage of both water and respiratory gases (Vitt & Caldwell, 2014). For this reason and although unable to live in saltwater, they are dependent on water.

Another characteristic of amphibians is their lack of ability to maintain constant body temperature, therefore, as ectothermic creatures, they need external heat sources to maintain body temperature for their activity (Wells, 2007). These features are determinative to amphibian’s distributions and species richness since the environmental variables and surrounding landscape restrain the areas in which they find the ideal conditions to live (Buckley & Jetz, 2007; Manenti *et al.*, 2013b). Some of these variables are temperature, precipitation, evapotranspiration, primary productivity, and physiography. Both temperature and precipitation are important to amphibian species richness, but the latter has a stronger role (Qian *et al.*, 2007; Qian, 2010). However, the relevance of these variables is species dependent, as there is a great variability of species environmental requirements (Menin *et al*., 2007).

Not all the species of lissamphibians are completely aquatic, in fact, all three orders (Anura, Caudata, and Gymnophiona) include entirely aquatic, entirely terrestrial, and semiaquatic species (Wells, 2007). This variety of life strategies is possible due to adaptations to water scarcity (Vitt & Caldwell, 2014). For example, *Litoria caerulea*, a frog from Australia, remains active during the dry season, as it collects water from condensation. Basically, it emerges from its refuge on cool, dry nights and stays outside until its temperature drops to as low as 12.5ºC, and when it returns to the warmer refuge, condensation occurs on its dorsal surface (Tracy *et al.,* 2011).

Amphibians have a great variety of reproductive strategies, which include chemosignals (Woodley, 2015), different mating strategies, from external to internal fertilization, viviparity or oviparity (Wells, 2007; Buckley, 2012), parental care behaviour (Brown *et al.*, 2011; Lantyer-Silva *et al.*, 2014; Tumulty *et al.*, 2014; Kupfer *et al*., 2016) and modes of reproduction (Crump, 2015). The most diverse order is Anura, showing around 42 reproductive modes, and although rare (less than 1%), they have internal fertilization (Sandberger-Loua *et al.*, 2016; Sandberger-Loua *et al.,* 2017). Only 17 species are known or assumed as being viviparous (Sandberger-Loua *et al.*, 2017).

Internal fertilization is shared by all caecilians and majority of caudates (Wake, 2015; Sandberger-Loua *et al*., 2017). Salamanders have three types of reproductive strategies. They can be oviparous, water dependent, or viviparous and ovoviviparous, water independent. In some salamanders' species, the metamorphosis is never completed, maintaining a larval phenotype (Buckley, 2012).

Within caecilians, there are oviparous and viviparous species. The former has an aquatic larval stage with later metamorphose or a direct development into terrestrial juveniles. The latter has two modes of viviparity differentiated by the independence of the new-born (Wells, 2007; Sandberger-Loua *et al*., 2017) According to the type of reproduction and parental investment, the offspring number and quality vary (Kupfer *et al.*, 2016).

Considering that eggs do not have a hard shell they are more susceptible to dryness. So usually the eggs are deposited in water, however, in the cases of organisms laying eggs on land (Menin *et al*., 2007) (still dependent on humid environments), the use of leafs or soil as nests is common (Vitt & Caldwell, 2014). Some frogs involve their eggs in foam that is deposited in water surface or in superficial depressions on land (Crump, 2015), eventually the foam disappear and depending on the nest's location, the tadpoles can fall into the water, be washed by rain into close streams or ponds or even be able to develop completely in the foam, emerging as froglets. But many species change the timing of hatching when the conditions are unfavorable to eggs or larvae (Warkentin, 2011). After hatching, the larvae live in water or out of it until the time they undergo metamorphosis transforming into adults, adapted to live on land (Vitt & Caldwell, 2014).

In the last years, many species and life strategies were discovered, but much more is being found (Iskandar *et al.*, 2014) and waiting to be found (Crump, 2015).

Conservation

Due to its complex life cycle and physiology, amphibians are vulnerable to environmental changes. Indeed, climate changes, which can promote alterations in temperature, precipitation or ultraviolet radiation exposure, have been pointed out as one of the major causes of their decline (Stuart *et al*., 2004). Generally, the causes may vary from region to region and within different populations of the same species (Blaustein & Bancroft, 2007). A variety of factors influences the reaction to the change or potential harmful agents, such as synergetic interactions between two or more agents, differences between species and different life stages (Blaustein & Bancroft, 2007; Sasaki *et al.*, 2015; Davis *et al*., 2017).

Amphibians are the class of vertebrates most threatened (Catenazzi, 2015) as they are declining at an alarming speed on account of six main causes: infectious disease, climate chance, contaminants, land use change, introduced/exotic species and commercial use (Collins, 2010; Meredith *et al*., 2016). Starting by diseases, there are the *Ranavirus* (Teacher *et al.*, 2010) and the chytrid fungus, *Batrachochytrium dendrobatidis* (Hyatt *et al*., 2007) and the recently-discovered *B. salamandrivorans* (Martel *et al*., 2013). The *Ranavirus* can cause mass mortality events (Kik *et al*., 2011), mostly with individuals in metamorphosis and is characterized by systemic hemorrhage and tissue necrosis, leading to organ failure (Blaustein *et al*., 2012). There is no sign of extinctions caused by ranaviruses (Collins, 2010) because, as referred before, the virus affects the larval or juvenile stages. However, they are a strong selective force as they provoke fluctuations on the population and local extinctions (Brunner *et al*., 2015). Hoverman *et al.* (2011) found that species with reproduction in semi-permanent ponds that have a fast development as larvae and have limited territory sizes are more susceptible to infection. Another alarming situation is the co-infection of ranaviruses and chytrid fungus (Warne *et al*., 2016; Rosa *et al*., 2017). Chytridiomycosis has a very negative effect on amphibians, having been responsible for the extinction of more than 200 species (Kilpatrick *et al.*, 2010; Vredenburg *et al.*, 2010; Martel *et al*., 2013), and so it is considered the disease that poses highest threat to biodiversity.

The two known fungus have different niches and hosts, while *B. dendrobatidis* affects anurans, *B. salamandrivorans* affects caudates and prefers lower temperatures than the former (Martel *et al*., 2013). The danger of these fungus resides in the fact that they are highly pathogenic (Martel *et al*., 2014), having a large global distribution (Olson *et al*., 2013; Spitzen-van der Sluijs *et al*., 2016). Infection may then occur as shown by Scheele *et al*. (2015), where perennial water sources may act as source habitat for the amphibian chytrid fungus and then is taken to transitory ponds by the animal. Therefore, it´s possible that these ponds in open landscapes offer a refuge from the fungus. There is already a treatment for the chytrid fungus, whether with only temperature (Blooi *et al*., 2015a) or with the aid of medication (Blooi *et al*., 2015b). Amphibians have natural defenses against the fungus, but environmental and anthropogenic stressors prejudice their capacity to prevent infection (Blaustein *et al*., 2012; Catenazzi, 2015). Such stressors can be pollutants that are going to interfere with hormone systems and impacting on their reproductive development and function (Orton & Tyler, 2015). Another example is the effects of contaminants like pesticides, more precisely atrazine, that induces feminization in males, affecting reproduction of the species (Rohr & McCoy, 2009; Hayes *et al*., 2010). Altogether, pesticides and fertilizers affect the amphibians differently, depending on their chemical classes (inorganic fertilizers, organophosphates, chloropyridinyl, phosphonoglycines, carbamates, and triazines) reducing survival and growth of individuals (Baker *et al*., 2013). An isolated contaminant may not be lethal, but the result of multiple contaminants at the same time can cause lethal and sublethal effects (Sparling *et al*., 2015). This together with the already undermined population by the transference of contaminants by female progenitor and bioaccumulation (Bergeron *et al.*, 2010; Todd *et al*., 2011; Metts *et al*., 2013) makes it even more susceptible to mortality (Willson *et al*., 2012). The effects can even vary with the presence of predators, inducing antipredator behaviour and morphology (e.g. growth) (Blaustein *et al*., 2011; Relyea, 2012). Although it’s given importance to the effects of contaminants on amphibians, some authors argue (Kerby *et al*., 2010) that they are not so sensitive to chemical pollution as was thought. Another study (Sasaki *et al*., 2015) reports that alterations in the resource availability and microclimate in simultaneous with structural changes of terrestrial habitat can have stronger consequences than metal pollution by itself.

The speed at which climate changes are occurring is much greater than what living beings experienced in recent past (Beaumont *et al*., 2011) 1000 times superior (Pimm *et al*., 2014). Human actions responsible for causing environmental changes, habitat loss, fragmentation (Tittensor *et al*., 2014; Catenazzi, 2015) with emphasis on habitat conversion or land use are resulting in increased decline of biodiversity (Vié *et al*., 2009; Newbold *et* *al*., 2015) affecting as well as ecosystem services (Lawler *et al*., 2014). These declines lead to reductions of the size of populations at local and regional scales, decreasing genetic diversity. Populations with fewer individuals are vulnerable to stochastic environmental and demographic events, diminishing the population even more (Catenazzi, 2015). The majority of habitat conversion of forest is for agricultural fields, either for grasslands, that occupy around 26% of the planet (excluding areas with ice), or for farming fields that occupy around 12%. Expansion continues, destroying more and more habitats (Foley *et al*., 2011). Still, some species are found in these modified habitats, where vegetation structure plays an important role offering refuge to species and working as biological corridors between habitats (Robinson *et al*., 2013). But habitat matrix of these patches is also fundamental on abundance and occupancy of species (Watling *et al*., 2011). Even though agriculture is prejudicial to amphibians, that occurs mainly in zones with a long agricultural background (Piha *et al.*, 2007). And not all have a negative impact, for example, amphibian biodiversity is not affected by traditional pastoral activities in comparison with natural landscapes (Manenti *et al*., 2013a) as these two habitats create environmental heterogeneity important for the coexistence of different species (Keller *et al.*, 2009).

Composition and change in habitat cover combined with climate change alter species distributions as the area of thermally suitable habitat for amphibians is reduced. Thus, species distribution will be defined by their thermal tolerances. (Nowakowski *et al*., 2017), and will have two responses, contract their ranges while moving to different directions and altitudes or expanding their ranges (Duan *et al*., 2016).

Climate change on its own (Loyola *et al*., 2014; Cruz *et al*., 2016) and in synergy with other factors (Weir *et al*., 2016; Rollins-Smith, 2017) have a great impact on biodiversity. In response to it, amphibians, in addition to changes in their distribution, they can alter the time of activity (Catenazzi, 2015) and reproductive phenology (Green, 2017). A recent study portrays the possibility of morphological adaptation (change in the number of thoracic vertebrae) of salamanders in response to climate change (Ficetola *et al*., 2016).

Another stressor to amphibiofauna are invasive species (Bellard *et al*., 2016b). Ficetola *et al*. (2011) studied the impact of *Procambarus clarkii* on amphibians and verified that it may have two implications, avoidance of invaded locals, or lower reproductive success if breeding occurred in these sites due to predation. *Gambusia holbrooki* is an invasive fish and it predates amphibians too, being 30% of his diet constituted by tadpoles (Remon *et al*., 2016). Additionally, competition, debilitation of the native amphibian's immune system, hybridization (Bucciarelli *et al*., 2014) and disease transmission (Hatcher *et al*., 2012; Young *et al*., 2017) are caused by alien species. This problematic is considered the second most common threat to the species extinction (Bellard *et al*., 2016a).

Commercial trade is also the source of invasive species and their associated diseases (Herrel & van der Meijden, 2014). It's likely that *Batrachochytrium salamandrivorans* came to Europe from Asia through amphibian pet trade (Martel *et al*., 2014) and this and other diseases are being introduced by the same way (Kolby *et al*., 2014; Kolby & Daszak, 2016; Wombwell *et al*., 2016). Beside pet trade, this taxon is used for medical and educational research, and harvested for consumption (Auliya *et al*., 2016b). The market of frog legs is responsible for overexploitation as tons of frogs are collected at several countries demand, more precisely 90-230 million individuals per year (Warkentin *et al*., 2009). Consume also occur in large number at local and regional scales (Mohneke *et al*., 2010). This overexploitation puts many species at risk and some of them are already threatened (Auliya *et al*., 2016b).

People's attitudes toward amphibians has a strong influence on wildlife, whether by human values or folklore, which result in persecution, capture and killing. They are one of the most despised of vertebrates, because of misconceptions from interpretations of folklore and ancient myths and negative values to them associated. The attitudes change depending on gender, age or education. Phobias, cultural issues and emotional reactions are among the irrational fears for these animals (Ceríaco, 2012). Amphibians are also used to medicinal, and cultural purposes objects (Mohneke *et al*., 2009, 2011; Alves *et al.*, 2013).

Aesthetics greatly influences the preservation of species, once support given to them by the public is inferior (Ceríaco, 2012; Prokop & Fančovičová, 2013). However, a greater knowledge on the species change people attitudes toward species they are unfamiliar with. So, providing more information for example in educational institutions is important to change the idea that people have about these vertebrates (Tomažič, 2011; Prokop & Fančovičová, 2013 Perry-Hill *et al*., 2014; Sousa *et al*., 2016).

In sum, disturbance in species and ecosystems are caused by factors at different scales, both temporal and spatial (Piha *et al.*, 2007). As well by differences in life histories and species' interactions (Davis *et al*., 2017). Take that into consideration is essential when aiming for an effective management and conservation of wild species (Piha *et al*., 2007; Davis *et al*., 2017). Since, although important, the implementation of reserves or protected habitats it´s not completely effective when the losses are caused by diseases (Clulow *et al*., 2014).

Some plans are already in action, such Amphibian Conservation Action Plan (ACAP), that pretend to take actions to prevent amphibian declines. One short-term solution is Amphibian Ark which consists in having animals in captivity as a way to avoid extinctions and reintroduce populations in the wild (Catenazzi, 2015). Another measure of conservation passes by the use of reproductive technologies for captivity breeding (Clulow *et al*., 2014).

Reptiles

Reptiles belong to Class Reptilia and are divided in Archosauria - Order Crocodylia (crocodiles) and Order Aves (birds); Order Testudines (turtles) and Lepidosauria – Order Squamata - Amphisbaenia (amphisbaenians), Sauria (lizards), Serpentes (snakes); and Order Rhynchocephalia (tuataras) (Vitt & Caldwell, 2014; Uetz *et al.*, 2017). They totalize 10,639 species as of 15 October 2017, from which around 10,150 species are squamates, and tuataras are represented by a single species (Uetz *et al*., 2017). These numbers refer only to non-avian reptiles. Birds are considered reptiles because they had an origin in Archosauria (Vitt & Caldwell, 2014). However, in this work, when referring reptiles, birds are excluded.

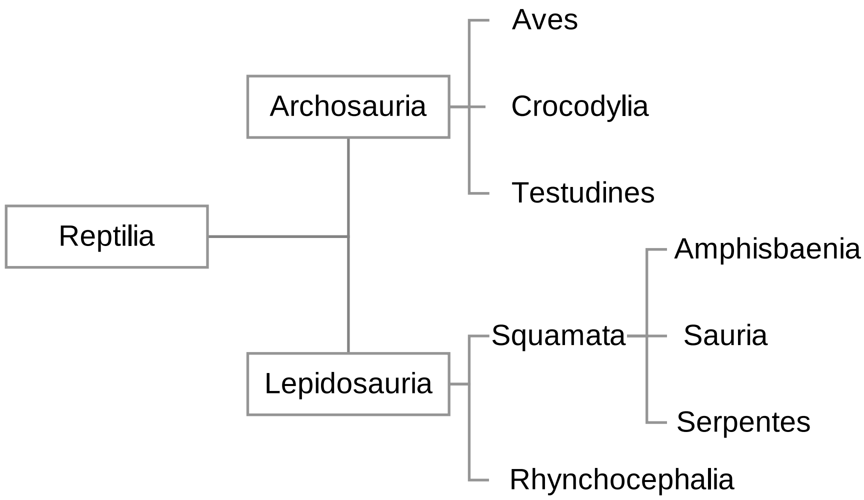

Figure 3 Diagram of reptile taxonomy

Biology and Ecology

Reptiles are also between the groups of terrestrial vertebrates more diverse (Bland & Böhm, 2016; Tingley *et al*., 2016). The word reptile comes from Latin and means “to creep” (Harding, 2006).

Like amphibians, reptiles are widely spread around the planet, except in the continent Antarctica (Loureiro *et al*., 2008; Pough, 2013). But unlike amphibians, it is possible to find reptiles in a marine environment like turtles, iguanas, and snakes (Wallace *et al*., 2016).

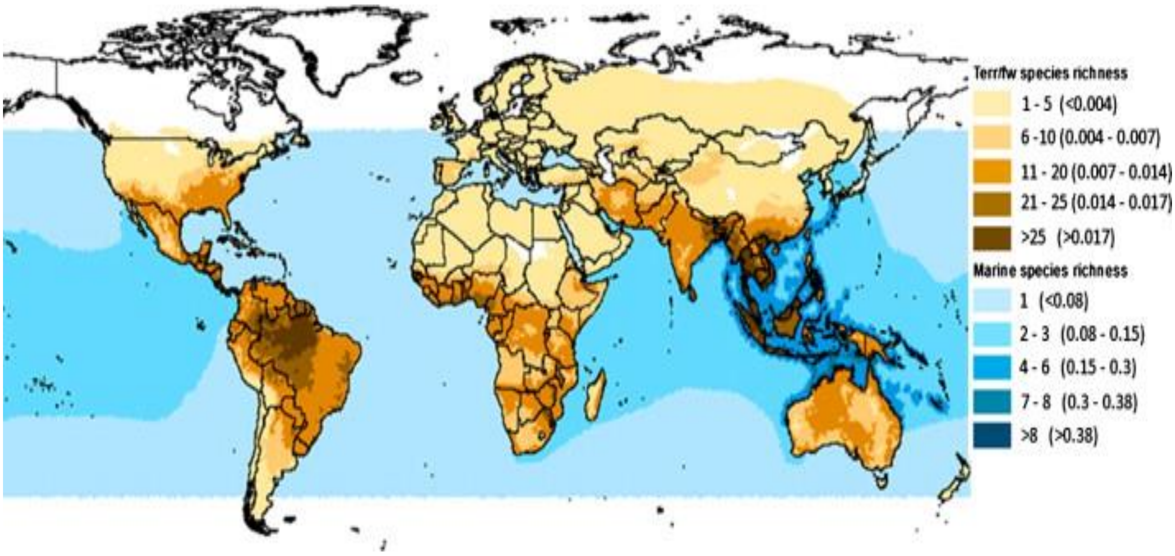

Figure 4 Map of the world distribution of reptiles. In yellow/brown colour are terrestrial species, and in blue are marine species. Retrieved from Böhm *et al*. (2013)

As ectotherms, they are influenced by external temperature (Jarvie *et al*., 2014; Pough, 2017), body temperature mark several aspects of reptiles' life such as growth, embryonic development, fecundity, survival and in some species even sex determination (Vitt & Caldwell, 2014). With latitude and its geographic variation in climate their life history, behavior, physiology and ecology change (Weatherhead *et al*., 2012; Hileman *et al*., 2017).

Within Reptilia, there is a vast morphology between orders. Turtles do not have teeth, they have shells formed by a dorsal carapace and a ventral plastron, their form varies greatly from flat to irregular and from soft to hard. Crocodiles are characterized by their body size, elongate snout, the lack of lips and interlocked exposed teeth. Snakes and five of the six families of amphisbaenians are distinguished by not having limbs. But between them there are differences, snakes have a long forked tongue, spectacle over the eyes and don't have an external ear opening. Amphisbaenians more characteristic trait is the presence of scales in a ring-like pattern around their bodies. The Tuatara do not have a male copulatory organ, a cloacal slit parallel to the body. Finally, lizards have a body form similar to crocodilians, although with differences such cloacal slit perpendicular to the body, and some share traits with snakes (forked tongues and no eyelids but spectacles) (Pough, 2013; Vitt, 2016).

Reptiles are amniotes, they have an amniotic egg that allows reproduction on land and they can be viviparous or oviparous. In oviparous species (crocodilians, turtles, and tuatara) (Pough, 2017), construction of nests for egg deposition depends on the species.

Some bury their eggs in sand or ground, others lay eggs in damp soil or rotting logs, or even in ant or termite nests or in rocks fissures (Vitt & Caldwell, 2014). A reason why reptiles are so affected by changes in temperature is because of their temperature-dependent sex determination, which means that the sex of hatchlings is determined by the incubation temperature during a certain period of development called thermosensitive period (Pezaro *et al*., 2016).

Among reptiles, the group of lizards, snakes, and amphisbaenians have a spectrum of complex reproductive modes, presenting viviparity and oviparity in the same family, genus and its seen as well in populations of the same species (Pyron & Burbrink, 2014; Griffith *et al*., 2016). It is estimated that in squamates the viviparity has a repeated origin of at least 115 times (Blackburn, 2015; Griffith *et al*., 2015). But Pyron & Burbrink (2014) found that instead of independent origins, there is an intricate pattern of various shifts between egg laying and live birth. Many are the hypotheses proposed to explain viviparity evolution, one of them is the cold-climate hypothesis. This hypothesis defends that viviparity develops as an adaptation to cold temperatures, with the uterus and behavioral thermoregulation the mortality and slow development caused by low temperatures is reduced (Lambert & Wiens, 2013; Shine, 2014). An alternative hypothesis is maternal manipulation hypothesis in which prolonged time in uterus allows better incubation conditions (Shine, 2014) and manipulation of thermal conditions during embryogenesis by females can improve fitness-related phenotypic traits in offspring (Sites *et al*., 2011).

Although rare, changes from viviparity to oviparity its possible, breaking Dollo's law that an organism cannot reverse to a condition expressed by an ancestor (Lynch & Wagner, 2010; Fenwick *et al*., 2012; Wright *et al*., 2015).

Like in many others hypothesis, there are those who defend the former (Watson *et al.*, 2014; Feldman *et al*., 2015) and whom refute it and accept the lattest (Li *et al*., 2009). Lambert & Wiens (2013) show results that defend the cold-climate hypothesis, viviparity evolution is favored by cold climates, but mostly in the tropics. They also support that viviparous species live in higher elevations and have much smaller latitudinal and temperature niche ranges.

Another interesting characteristic of reptiles, in particular, squamates, is the fact that they are the only vertebrates having true parthenogenesis (Sites *et al*., 2011). In this, females produce offspring without males, in other words, they clone themselves as the offspring is genetically identical to their mothers. Parthenogenesis is found in more than 50 squamates (Vitt, 2016). Reptiles with this reproductive mode have their origin in interspecific hybridization (Manríquez-Morán *et al*., 2014), sometimes secondary and tertiary hybridization events occur given origin to triploid and tetraploid species, reproducing as well by parthenogenesis (Lutes *et al*., 2011; Cole *et al*., 2014; Abdala *et al*., 2016).

These vertebrates have a diversity of life histories, but data about life histories of the majority of reptile species is scarce, so management programs for species are affected and thus their conservation (Vitt, 2016; Hileman *et al*., 2017).

Conservation

Humans with direct and indirect actions are causing great loss in biodiversity. In the specific case of reptiles, around 21% of the species are endangered or vulnerable to extinction (Cribb, 2017). Many of the conservation issues that reptiles experience are the same for almost every animals and plant. So, the threats are habitat loss and change, mortality on roads, pollution, commerce, climate change, introduction of exotic species, diseases (Pough, 2017), human attitudes (Ceríaco, 2012), overharvesting (Tingley *et al*., 2016) and bycatch (Wallace *et al*., 2016)

Climate change affects reptiles by causing declines of populations, reductions in habitat suitability as well as in range sizes and survival (Winter *et al*., 2016). Around 22.1% of reptiles are vulnerable to climate change where this vulnerability is higher in the Neotropical realm and comparing freshwater, terrestrial and marine species, the latter are more vulnerable (Böhm *et al*., 2016). It has been predicted that by 2080, around 39% of lizard species will be extinct because of the effects of climate change (Sinervo *et al*., 2010). The vulnerability is due to habitat specialization, low adaptability and high exposure (Böhm *et al*., 2016; Meng *et al.*, 2016). Meiri *et al*. (2013) show that environmental temperatures have more influence in life-history of reptiles than its body temperature, so changes in global temperatures may have a huge influence on some species. For example, viviparous species are more vulnerable to temperature changes because they are associated with high elevation habitats and in these places, the climate is changing more rapidly (Sinervo *et al*., 2010). But Cattin *et al.* (2016) results' show that species with a polymorphic color, viviparous and/or that live at low latitudes may be more capable of dealing with climate change because they persisted throughout environmental changes in the past. And in the case of species with temperature-dependent sex determination, any change in temperature can affect the sex ratio of species as well phenology alterations (López-Luna *et al*., 2015).

Activities causing loss and alteration of habitat are among others, agriculture, urbanization, mining and logging (Pough, 2017). Reptiles that are specialists, mainly forest specialists, are more vulnerable to habitat fragmentation. This fact may come from the thermoregulatory limitations with morphological specialization and low dispersal (Keinath *et* *al*., 2017). Frishkoff *et al*. (2015) verified that the presence of herpetofauna in modified habitat, in their case deforested habitat, were as well as or better explained by thermal niche than traits.

Roads are associated with urbanization and have various impacts, direct or indirect, on wildlife. In the reptiles' case, the roads are used for thermoregulatory purposes, foraging, nesting and are also crossed when in search of resources (Andrews *et al*., 2015). In all cases, they can be injured or killed by traffic, and such impact in species varies with their dispersal tendency and spatial scale, and frequency of movements. Additionally, roads cause landscape fragmentation that change the physical conditions around them (Andrews *et al*., 2008) and isolate populations, reducing genetic diversity as there is no gene flow (Pough, 2017). Some measures have been made to reduce mortality like using fences or vegetation in roadsides to guide reptiles to crossing structures. And while it's possible to mitigate some road effects, others should be taken into consideration before construction since they have permanent consequences (Andrews *et al*., 2015).

Agriculture is another example of habitat change and in the Afrotropics is considered the greatest threat to reptiles (Meng *et al*., 2016; Tolley *et al*., 2016).

Pollution at which reptiles are subject are metals, herbicides, pesticides and petroleum products (Pough, 2017). Reptiles can be exposed to chemicals that bioaccumulate because many species are carnivorous (Bishop, 2016). The exposure can occur via ingestion and through skin absorption (Weir *et al*., 2015), besides depending on age, size, and sex of reptiles, the contaminant exposure and degree of contamination can differ (Bishop, 2016). However, ecotoxicology studies in this taxon are lacking and had recently taken their first steps as the use of birds as surrogates of reptiles can be inaccurate (Weir *et al*., 2010, 2013).

Verderame & Limatola (2015) studied the effect of an endocrine disruptor chemical used in agriculture, nonylphenol, on lizards. They found that it affects the reproductive cycle of the study species males during mating season and modify secretory activity, which causes limitations on reproductive success of the species.

Another study analysed the effect of a pesticide on a wild lizard population and concluded that it caused oxidative stress, decreased body condition and altered sex ratio (more males) (Mingo *et al*, 2017). Additional effects of diverse contaminants are known, such as genotoxicity (Schaumburg *et al*., 2016), change in thermoregulatory behavior (Carpenter *et al*., 2016), change in physiological processes and size (Neuman-Lee *et al*., 2015). The effects previously quoted are direct nevertheless it's important to take indirect effects into account since changes in biotic and abiotic environments modify animal responses' (Sasaki *et al*., 2016).

Introduced exotic species are harmful to native species causing direct and indirect effects (Pough, 2017). The indirect effects can be competition, the introduction of pathogens and parasites, change in trophic webs with declines or extinctions. And the direct effects are predation, poisoning or hybridization (Kraus, 2015).

Trade of wildlife is a vector to introduce species in non-native ranges. There are two scenarios associated with trade, introduced species are affecting natural ecosystem function while in their native ranges are declining (Gibson & Yong, 2017). Gibson & Yong (2017), present some solutions for this problematic namely the reintroduction of exotic species in their native range, capture these species to breed in captivity, use of introduced populations as research replacements to help on conservation and finally harvest.

Data on declines caused by reptile pet market is underestimated because there is illegal trade and more than 90% of the species are not within CITES and EWRT legislations. But the illegal trade of CITES species nonetheless happens since law and enforcement in such cases is weak. Legislation review is needed as it's possible to trade legally various endemic species and/or that are in risk (Auliya *et al*., 2016a).

Diseases have led many species into high risk of extinction (Sarmiento-Ramírez *et al*., 2014). And species that live in aggregation have more transmission of parasites (Gardner *et al*., 2016). Reptiles are affected by herpesviruses, ranaviruses, fungus and bacteria (Pough, 2017). An example is the sea turtles, that are infected by a fungus that affects their hatching success and the effects are aggravated by environment stressors (Sarmiento-Ramírez *et al*., 2014).

Reptiles are persecuted and considered dangerous, being folklore and misconceptions the major cause for this. However, they are used as food, to make medicines and raw materials (Ceríaco, 2012; Alves *et al*., 2013). In the study of Mendonça *et al* (2014) and Alves *et al*. (2012), reptiles were used as food, as pets, in medicine, as ornaments in commerce and even in magic/religious practices. Furthermore, most of the species were hunted for being considered dangerous and much of the used species are threatened.

In some cases, endangered or endemic species do not have any kind of protection (Meng *et al*., 2016). There are still work to do as only about 45% of all identified species of reptiles are assessed by IUCN, and within this 19% of the species do not have classification since the data on them is insufficient (Tingley *et al*., 2016). More research is needed in order to take conservation actions, having in mind that: tropical regions are greatly threatened by dramatic rates of habitat loss, the data on fossorial reptiles is very scarce and that certain taxa may face an unknown risk of extinction due to lack of information (Böhm *et al*., 2013).

Karst

The Earth has a rich geodiversity that together with biodiversity play an important role in ecosystems (Aqeel *et al*., 2014; Comer *et al*., 2015; Najwer *et al*., 2016; Bailey *et al*., 2017). Some of that geodiversity is represented by carbonate formations undergoing karst processes (karstification) found around 15% of the world (Culver & Pipan, 2009) (Fig.5).

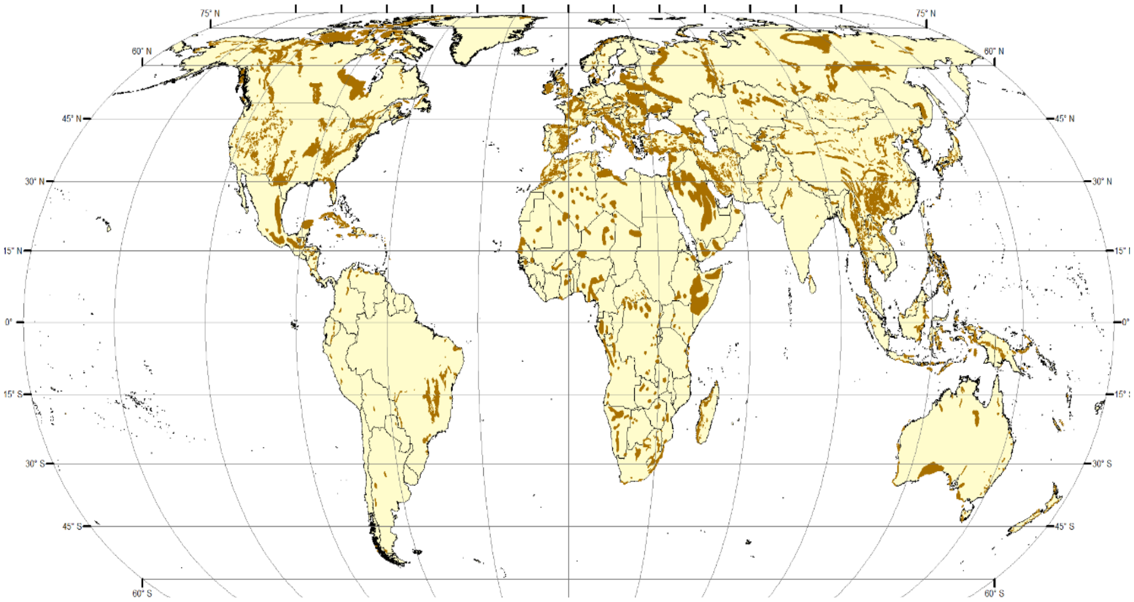

Figure 5 Global distribution of carbonate rocks. (Shapefile retrieved from http://www.sges.auckland.ac.nz/sges_research/karst.shtm, accessed 13 March 2017)

Karst can be described as a special landscape including characteristic hydrology and landforms (Ford & Williams, 2007; Williams, 2008). So, sinking and/or large streams, sinkholes, outcrops, caves, and depressions are found in these areas (Ford & Williams, 2007; Stokes *et al*., 2010).

Karstification occurs in carbonate rocks such as limestone, marble, gypsum, dolostone and halite (Ford & Williams, 2007; Stokes *et al*., 2010). These rocks are very soluble and with the biotic and natural water actions there is the dissolution of the rock, resulting in distinctive surface and subsurface traits. In sum, the karst features result from the interaction of processes in the geochemical and hydrological subsystems included in the karst (Ford & Williams, 2007). The karst systems are divided into three parts: exokarst, epikarst, and endokarst. Exokarst embodies all the characteristics presented in the surface, epikarst is composed of all the openings or fractures in an interval of 10 to 30 metres from surface. For last, all elements found deeper in the underground make the endokarst (Stokes *et al*., 2010).

In these landscapes, the water circulates underground instead of in the surface, thus karst aquifers are very important and are exploited in some places (Gunn, 2004). Around 25% of the world population depends on this water (Ford & Williams, 2007). Despite that there are few hydrogeological studies and the management of this resource is not adequate (Bakalowicz, 2015; Malagò *et al*., 2016).

Cave is defined as a natural opening underground connected to the surface, where a human can enter and that include a zone with permanent and total darkness (Culver & Pipan, 2009; Stokes *et al*., 2010). There are various types of caves according to the rock and processes that form them. So, caves are divided into volcanic, glacier, tectonic, littoral, piping, erosion and the focus of this work are the karstic ones (Gunn, 2004; Romero, 2012), they can also be horizontal, vertical or both.

Darkness is what better describes caves, however, the entrance of a cave allows light to enter. The quantity of light along the cave is classified by three zones (Culver & White, 2005; Reboleira & Correia, 2016):

1 - Entrance zone – where the epigean and hypogean worlds meet. It is here that diversity and environmental variability are greater, the light is more intense and plants can grow.

2 – Twilight zone – here the influence of surface conditions is still visible, although mitigated. The light is indirect and only lower plants are found.

3 – Dark zone – as the name implies, light does not reach this zone, it is totally dark, the conditions are more constant and only adapted organisms inhabit there.

The climate in caves tends to be constant, but seasonal variations around the entrance and heat exchange from inner parts influence it. Then, temperature, relative humidity, and air flow are what also characterize caves (Gunn, 2004) and this ecosystem harbor a great variety of species both subterranean (Reboleira, 2012; Reboleira *et al*., 2013) and superficial (Luo *et al*., 2016).

Although caves are very important features in the karst system due to their definition that they need to have enough space for a human to enter (Stokes *et al*., 2010), caves make up less than 0.01% (Ford & Williams, 2007) of the existing cavities in the karst. Caves are formed in several stages, starting from small fractures and/or openings that are enlarged by water. Then, the water flux in each determines the dissolution rate and with an increase of discharge through those passages they become larger. Eventually, the water ceases and they become filled with air, that is when mineral deposits start to grow. The cave passage can grow by deviation of water to other paths or be destroyed by roof collapse and by surface erosion. If soluble rock exists, new caves can be formed at lower elevations (Audra & Palmer, 2011). It should be noted that while some caves have one or many entrances, others do not have a natural entrance and the only access is artificial (Culver & Pipan, 2009).

Portugal also has karst massifs (Fig. 6) distributed by two major zones in Lusitanian and Algarve basins (Manuppella & Moreira, 1975; Reboleira & Correia, 2016) with limestones or dolomites dated from middle Jurassic and Cretaceous (Thomas, 1985; Cunha, 1990; Pereira *et al*., 2014). Listing them, the most relevant are: Estremenho karst massif, limestone mountain chains of Condeixa-Sicó-Alvaiázere, Outil-Cantanhede massif,

Figure 6 Map of limestones outcrops in Portugal. 1- Boa Viagem; 2- Outil-Cantanhede and Bairrada; 3- Sicó-Condeixa and Alvaiázere; 4- Estremenho; 5- Cesaredas; 6- Montejunto; 7- Lisbon peninsula; 8- Arrábida; 9- Ossa-Estremoz; 10- Adiça-Ficalho; 11- Algarve. Adapted from Reboleira & Correia (2016)

Mealhada limestones, mountains of Boa Viagem, Cesaredas plateau Montejunto, Arrábida and Estremoz-Cano, Adiça-Ficalho and the Algarve (Crispim, 2010; Reboleira *et al*., 2011).

In Portugal, there are more than 3,000 caves (Reboleira *et al*, 2010), from which more than 2,000 are found only in the Estremenho karst massif (Reboleira, 2007). This massif has an area of approximately 900 km^2 (Azerêdo, 2007) and most of it is covered by the Serras de Aire and Candeeiros Natural Park (PNSAC) legally created since 1979 under the Decree-Law 118 of 4th May (ICNF, 2017b).

The Estremenho Massif is morphological divided into three elevated sub-units (ICN & PNSAC, 2004; Carvalho *et al*., 2011):

- Candeeiros mountain;
- Santo António plateau; and
- São Mamede plateau and Aire mountain.

Three depressions originated by big fractures that separate the referred sub-units are respectively Mendiga depression, Mira-Minde polje and Alvados depression.

In this region the climate is a transition between Atlantic and Mediterranean conditions, it is characterized by a humid climate with medium temperatures and water scarcity in summer (ICN & PNSAC, 2004). It also has a diversity of habitats like ephemeral Mediterranean puddles, natural meadows, semi-natural dry grasslands and scrubland facies on calcareous substrates and Mediterranean forests (ICNF, 2017). As for flora, there are 600 species known, but the most represented are the genera *Quercus* that dominate the landscape, there are also pines and olive trees and aromatic plants, such as rosemary (*Rosmarinus officinalis*) (Reboleira, 2007; ICNF, 2017).

The Estremenho massif is important as well as to animals. Due to its habitat heterogeneity, the fauna found here is diverse from subterranean (Reboleira & Enghoff, 2014; Reboleira *et al*., 2015) to superficial species (Loureiro *et al*., 2008; Svensson *et al*., 2012) including some of national importance.

Karst areas are very sensitive due to its unique features, so human activities can cause serious impacts and induce hazardous situations. Alterations made by humans are constructions of dams, deforestation, pollution (Gunn, 2004) and extraction of limestones blocks (Carvalho *et al*., 2011; Carvalho, 2013). It is important to preserve these environments because their resources, namely water, that will be vital in the future. Actions such as education and direct involvement of local people (De Waele *et al*., 2011) will be needed in order to maintain a good management and conservation of this peculiar and sensible realm.

Objectives and thesis structure

This thesis focusses on the herpetofauna of Estremenho karst massif and to respond to the objectives it is divided into four chapters:

- Chapter I – General introduction

Here is approached some aspects of herpetofauna's biology as well as characteristics of the karst ecosystem.

- Chapter II - Distribution of amphibians and reptiles in caves of the Estremenho karst massif (Portugal).

The main goal was to elaborate a distribution map of the occurrence of herpetofauna in caves of the Estremenho massif and to understand if the species found are in caves by accident or intentionally, analyzing the body condition of the animals captured.

This kind of study is important to fill gaps in this area since the distribution of amphibians in karst areas is incomplete. Furthermore, it can contribute to the protection of species and subterranean habitats.

- Chapter III – Evaluation of the effects of water from Estremenho karst massif, in the development and antioxidant defence system of the early life stages of *Hyla arborea*

This chapter aims to study the quality of water bodies of this massif and how it impacts amphibian populations.

The obtained results can be used to take actions of conservation and management.

- Chapter IV – Concluding remarks

Section of the dissertation in which the most important conclusions are highlighted.

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Chapter II

Distribution of Amphibians and Reptiles in Caves of Estremenho Karst Massif (Portugal)

Abstract

Caves are extreme habitats that harbour a variety of animals from invertebrates to mammals. The presence of herpetofauna is not an exception because caves might offer ideal conditions for such species. The Estremenho Karst Massif is a limestone massif in Portugal and it has the major number of caves of the country. Thus, in order to understand their possible relevance to herpetofauna, a survey was made to map amphibian and reptile presence. Three species were found (*Tarentola mauritanica; Salamandra salamandra* and *Pleorodeles waltl*) and among them *P. waltl* shown to use these places for reproduction, only recorded one time in Spain. It was possible to conclude that herpetofauna species can occupy the Estremenho massif caves and even reproduce in them. Nonetheless, more work has to be done in this field with the perspective of conservation of the sites and species.

Introduction

Caves are extreme habitats because of their perpetual darkness, limited energy, nutrient scarcity, constant temperature, high humidity and limited air currents (Culver & Pipan, 2009). However, subterranean habitats can harbour a wide variety of organisms like mammals (Biswas & Shrotriya, 2011; Niemiller *et al*., 2016), amphibians (Ianc *et al*., 2012), reptiles (Esmaeili-Rineh *et al*., 2016), birds (Abantas & Nuñeza, 2014), and invertebrates (Chelini *et al*., 2011; Manenti *et al*., 2013). Organisms found in caves can be obligatory cave dwellers, the troglobionts, non-obligatory cave dwellers, eutroglophiles or subtroglophiles, and accidentals or occasional, the trogloxenes (Sket, 2008).

Many environmental factors lead non-obligatory cave-dwellers into caves. Some may use them as refuge from heat or cold, others as escape from predation, to rest or hibernate, to reproduce (Culver, 2016) and even for diet mineral supplements (Sitienei *et al*., 2011).

Amphibians and reptiles have specific traits of their biology, such as temperature and humidity dependence (Vitt & Caldwell, 2014) and caves offer the conditions needed. In karst areas where the surface water is rare circulating mostly underground (Ford & Williams, 2007; Culver & Pipan, 2009), the hypogean habitat plays an important role in the survival of these species, particularly amphibians (Manenti *et al*.,2009; Köhler *et al*., 2010; Manenti *et al*., 2011; Lunghi *et al*., 2015; Balogová *et al*., 2017).

The occurrence of herpetofauna in caves and associated hypogean habitats is recorded around the world (e.g. Bora *et al*., 2009; Ellis & Pauwels, 2012; Cabauatan *et al*., 2014; Matavelli *et al*., 2015; Weck, 2016).

The Portuguese amphibiofauna includes 19 native species, 7 caudates and 12 anurans (Rebelo *et al*., 2013). The reptiles' native species are 28, 10 snakes, 16 saurians and two freshwater turtles (Cruz *et al*., 2016). From these, 15 amphibians and 19 reptiles can be found in the Estremenho massif territory (Loureiro *et al*., 2008) (Table 1). However, in Portugal, the record of herpetofauna in caves is scarce. Rosa & Penado (2013) reports the reproduction of *Rana iberica* in a subterranean habitat. The other species reported in caves is the *Chioglossa lusitanica* (Gilbert & Malkmus, 1989 *in* Herrero & Hinckley, 2014). Nevertheless, the presence of herpetofauna in these habitats is known by speleologists which encounter them in their caves' exploration (GPS and PNSAC[1] personal communication). Therefore, the aim of this study is to survey the herpetofauna present in caves to help in the knowledge of this area and in the conservation of these species. Furthermore, it is important to perceive the body conditions of the captured animals inside

[1] GPS – Grupo Proteção Sicó: non-profit association that aims to explore, study, protect and preserve the environment and cavities of Condeixa-Sicó-Alvaiázere Limestone Massif - http://www.gps-sico.org/;
PNSAC – Serras de Aire and Candeeiros Natural Park

caves, to understand if their occurrence is accidental or if they actively occupy the subterranean ecosystem.

Table 1 List of herpetofauna species recorded for the Estremenho karst massif

Amphibians	*Pleurodeles waltl*	**Reptiles**	*Mauremys leprosa*
	Salamandra salamandra		*Tarentola mauritanica*
	Triturus boscai		*Anguis fragilis*
	Triturus marmoratus		*Acanthodactylus erythrurus*
	Alytes cisternasii		*Lacerta lepida*
	Alytes obstetricans		*Podarcis hispanica type 2*
	Discoglossus galganoi		*Psammodromus hispanicus*
	Pelobates cultripes		*Psammodromus algirus*
	Pelodytes spp.		*Chalcides bedriagai*
	Bufo bufo		*Chalcides striatus*
	Bufo calamita		*Blanus cinereus*
	Hyla arborea		*Coluber hippocrepis*
	Hyla meridionalis		*Coronella girondica*
	Rana iberica		*Elaphe scalaris*
	Rana perezi		*Macroprotodon cucullatus*
			Natrix maura
			Natrix natrix
			Malpolon monspessulanus
			Vipera latastei

Materials and Methods

Study area

The Estremenho Karst Massif is the biggest karst massif of Portugal. It is located in the central region of the country between the cities of Leiria, Alcobaça, Rio Maior, Torres Novas and Ourém (Fig. 1). A great area of the Estremenho massif is under protection by the Serras de Aire and Candeeiros Natural Park (PNSAC) since 1979 (Carvalho *et al.*, 2011; Carvalho, 2013). This classification is due to its unique geology, geomorphology and diversity of habitats.

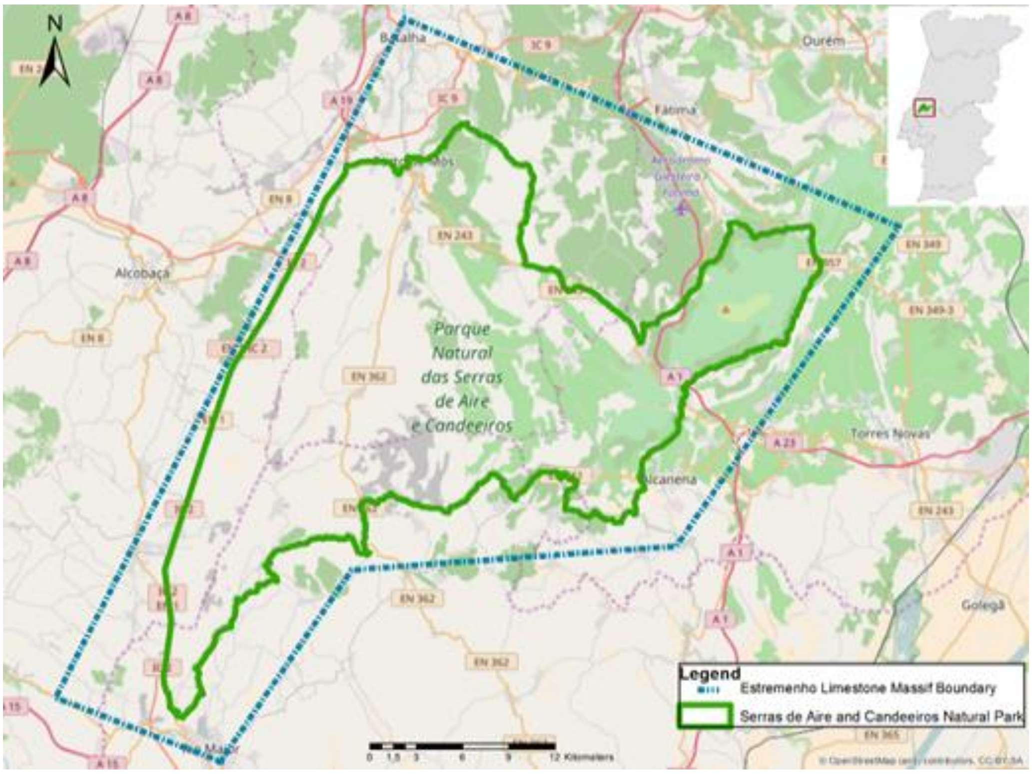

Figure 1 Map representing the boundaries of the study area. Based on Carvalho (2013)

Of all registered caves in Portugal, the Estremenho Massif has the majority with a number superior to 2,000 (source: PNSAC). Within this massif it is possible to find the largest and deepest caves, the Almonda Cave with a length of 10 kilometres, and the Algar do Palopes with 268 metres depth (Reboleira & Correia, 2016).

The karst massif is formed by three major units: Santo António plateau, Candeeiros mountain (615 metres), and São Mamede plateau and Aire mountain (680 metres) (Rodrigues & Fonseca, 2010; Carvalho *et al.*, 2011).

Cave surveys

A total of 11 caves (Table 2) were surveyed in December of 2016. Cave's typology included: Algares (vertical caves), Mines (artificial caves), Grutas (horizontal caves), Lapas (small horizontal caves) and Wells. Caves were chosen based on accessibility conditions in order to understand if the animals use caves willingly (horizontal caves) and not by accident/fall (vertical caves).

Beside mines and wells, the Gruta do Almonda Novo had human intervention. A new entrance was made to facilitate the access to it being protected by a small construction (Fig. 7).

Table 2 Caves visited and respective spatial data. The geographic coordinates (Degrees Decimal Minutes) and altitude (metres).

Site	Cave's Name	Geographic Coordinates	Altitude	Locality	County	Mountain/ Chateau
1	Algar do Pena	N39º27.924 W8º48.405	345	Alcanede	Santarém	Santo António
2	S. Pedro Mine	N39º32.970 W8º50.681	396	Bezerra	Porto de Mós	Candeeiros
3	Sarmento Mine	N39º32.698 W8º50.731	480	Bezerra	Porto de Mós	Candeeiros
4	Lapa dos Morcegos	N39º 32.287 W8º45.103	476	Casas dos Riscos	Porto de Mós	Santo António
5	Well of Gruta das Alcobertas	N39º25.760 W8º55.014	412	Alcobertas	Rio Maior	Candeeiros
6	Gruta das Alcobertas	N39º25.820 W8º54.951	398	Alcobertas	Rio Maior	Candeeiros
7	Gruta do Almonda novo	N39º30.673 W8º36.733	169	Pedrogão	Torres Novas	Aire
8	Gruta do Almonda velho	N39º30.297 W8º36.922	104	Zibreira	Torres Novas	Aire
9	Lapa dos Coelhos	N39º30.291 W8º36.919	113	Zibreira	Torres Novas	Aire
10	Gruta da Nascente do Rio Alviela	N39º26.739 W8º42.729	94	Amiais de baixo	Alcanena	Santo António
11	Gruta Sra. da Luz	N39º20.797 W8º59.323	133	Rio Maior	Rio Maior	Candeeiros

- **Site 1 – Algar do Pena**

Discovered in 1985 by Joaquim Pena in the course of rock removal to sidewalks in Alcanede, this is a vertical cave with the biggest room of its kind known in Portugal (ICNF, 2017b). Opened to public since 1997, this cave has an interpretation centre (CISGAP) siding its underground aspects and its didactic and pedagogic potential. Here the process of speleogenesis and impacts of tourism in these subterranean places are studied (Brandão, 2009).

Figure 2 Room of Algar do Pena. Photos taken by Joana Soares (2016)

- **Sites 2 and 3 – S. Pedro Mine and Sarmento Mine**

These mines are in Bezerra and along with Infelizes, Sul and Assentis are a group of mines referred as Mines of Bezerra. The mines began their function in 1740 but with inconstant activity. The expansion of mining activity occurred between 1885 and 1888 (MPM, 2017), however only in 1920 the application for the granting of industrial exploration was submitted. The coal extraction, namely lignites, possibly started in 1922 and ceased in 1938 due to coal depletion (Brandão, 2017).

Figure 3 a) Entrance of S. Pedro Mine; b) Sarmento Mine

- **Site 4 – Lapa dos Morcegos**

Near Grutas de Santo António in Porto de Mós, this Lapa is a small cave found in a slope of a hill named "Cabeço de Covas". It is completely isolated and is surrounded by rock outcrops and shrub-like vegetation. In 1969 it was made a survey which resulted in the discovery of bone fragments of animals and human skeletal remains (DGPC, 2017d).

Figure 4 Lapa dos Morcegos cave's entrance

- **Site 5 – Well of Gruta das Alcobertas**

A side entrance to Gruta das Alcobertas, it has a gate blocking the entrance of people but not small animals.

Figure 5 Entrance of Well of Gruta das Alcobertas. Retrieved from Cidadania RM, 2017.

- **Site 6 – Gruta das Alcobertas**

Considered a "wonder of nature maybe without rival in Europe" in 1878 (Pinho Leal), this cave in Alcobertas has an extension of 210 metres and in some places reaches 9 metres high. Of archaeological and speleological interest, this cave is classified as national monument (Dec. No. 27 743 of April 6 1934) (Brandão, 2009), because of the discoveries of animals remains, ceramic, stone objects, flint knives and human remains (Araújo & Zilhão, 1991). In 1935 the municipality authorities closed the cave. Forgotten by time and already in a poor state due to vandalism, works to open it to the public started in 1973 (never concluded) degrading the cave even more (Thomas, 1985). Today, this cave is degraded state and visits are only possible with previous appointment.

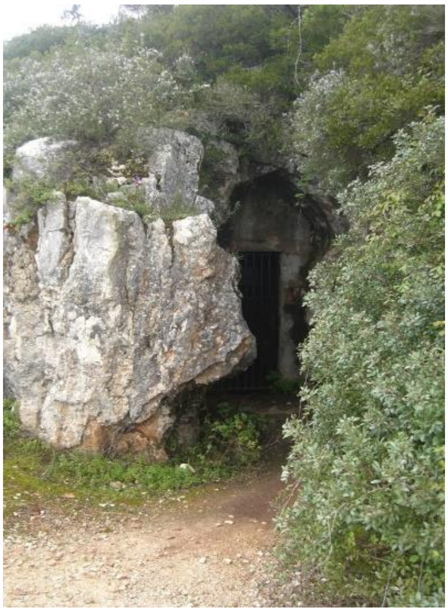

Figure 6 Access to the Gruta das Alcobertas. Retrieved from Cidadania RM, 2017.

- **Site 7 – Gruta do Almonda Novo**

A new entrance baptized as *Entrada do Vale da Serra* opened in 1989 to give access to the galleries upwind of a siphon and that allowed the discover of archaeological artefacts (Zilhão *et al*., 1993).

Found in Pedrógão, this cave along with its natural cavity (Almonda Velho) belongs to a vast net of underground galleries extending for more than ten kilometres making it the biggest karstic system of Portugal so far (NEUA, 2017). And are classified as Public Interest Property (Dec. No. 45/93, DR, I, Serie-B, No. 280 of November 30) because of its heritage value.

Figure 7 Construction that gives access to the entrance of Gruta do Almonda Novo

- **Site 8 – Gruta do Almonda Velho**

Gruta do Almonda Velho ou Gruta da Nascente do Almonda was once a cave filled with water but now it is five metres above the current spring located on the southeast anticlinal edge of the Serra de Aire. It is classified as Public Interest Property (Dec. No. 45/93, DR, I, Serie-B, No. 280 of November 30) due to archaeological importance (Zilhão *et al*., 1990, 1991, 1993; DGPC, 2017a).

Figure 8 Cliff where Gruta do Almonda Velho and Lapa dos Coelhos can be found

- **Site 9 – Lapa dos Coelhos**

Lapa dos Coelhos, also known as Lapa da Bugalheira (DGPC, 2017b), is a small cave located a little above of the entrance of Gruta do Almonda Velho belonging to its karstic complex. This cave was recognized by its archeologically potential in 1988, however the exploration only began in 1997 due to difficult access (Martins, 2007) (Fig 9). The exploration revealed traces of ancient human occupation (Gameiro & Almeida, 2004) dated from Low and Middle Paleolithic (Gameiro, 2008). Due its importance this cave is classified as Public Interest Property (Dec. No. 35.817, DG, I Serie, No. 187 of 1946 August 20) (DGPC, 2017c).

Figure 9 Access to the entrance of Lapa dos Coelhos

- **Site 10 – Gruta da Nascente do Rio Alviela**

Alviela cave is a submerse cave where the main spring of Estremenho emerges, the Alviela spring. It has a dry entrance only submerged in the months of rain (Fig. 10) and the known extension is 130 metres.

Figure 10 Entrance of the Gruta da Nascente do Alviela in charge. ®Olímpio Martins in ICNF, 2017a

- **Site 11 – Gruta da Sra. da Luz II**

In Rio Maior, this cave is also referred to as Gruta dos Vales and is classified as National Monument (Dec. No. 23.743, DG, I Serie, no. 80, of April 6 of 1934) (Araújo & Zilhão, 1991; DGPC, 2017b). It is so classified due to the artefacts (dated from Neolithic to bronze age) found inside and its archaeological importance (Cardoso *et al*., 1996).

Figure 11 a) Cave's entrance of Gruta da Sra. da Luz II seen from inside; b) entrance of the cave during the first excavation (1935-1936). Retrieved from Cardoso *et al*., 1996

Herpetofauna surveys

The survey of animals in caves was done using active search, which consisted in searching the entrance and interior of the cave between rocks and/or anything that could be a den. To standardise the sampling effort, three people carried out the active search during an hour and in small caves it was extended up to the twilight zone.

Specimens were captured by hand using sterile gloves and/or with the help of a net. Then, they were identified, photographed, weighted and measured to posterior analyses of body condition. For the measurements, a slide Vernier caliper was used. The morphometric parameters considered were:

- Weight (in grams);
- Maximum length: measured from the tip of the snout to the end of the tail (in centimetres);
- Length from Snout to Vent: measured from the tip of snout to vent (in centimetres);
- Body diameter: measured after the forelimbs (in centimetres);
- Rear leg: from the toe tip to the insertion of the body (in centimetres)

- Thighbone: length taken from the hind limbs (in centimetres);
- Head: length - from posterior axis of snout to the tip of the jaw - and width – at the widest point of head (in centimetres).

After recording the biometric parameters, the specimens were released on the same spot of capture.

Maps

For the elaboration of the actual map, the ESRI® ArcGIS 10.5 software was used. Shapefile of Serras de Aire and Candeeiros Natural Park were obtained in ICNF (2016).

The Geographic Coordinates were obtained from a Garmin Oregon 550 GPS.

Results

In the 11 horizontal cavities visited in Estremenho massif (Fig. 12) three species were found in a total of 29 captured individuals (16 *Pleurodeles waltl* - Sharp-ribbed Salamander; 1 *Salamandra salamandra*- fire salamander; 12 *Tarentola mauritanica* – Moorish Gecko (Fig.13)). Some individuals were captured and measured (table 3), in the case of *T. mauritanica* it was only possible to capture three as the others were out of reach. In site 8 it was also found the skin shed of a snake which wasn't identified. Furthermore, the presence of one *Salamandra salamandra* in Algar do Pena was reported by the personal of Serras de Aire and Candeeiros Natural Park.

Table 3 Biometric parameters of the captured animals and respective site of capture

Species	Weight (g)	Snout – Vet (cm)	Maximum length (cm)	Body diameter (cm)	Rear leg (cm)	Tigh bone (cm)	Head (cm)	Site
Pleurodeles waltl	1.9	2.9	5.7	-	1.15	0.3	-	2
Pleurodeles waltl	0.3	1.55	3.5		0.6	0.2	-	
Pleurodeles waltl	0.7	0.8	3.7		0.4	0.12	0.7x0.5	
Pleurodeles waltl	0.6	1.4	3.5		0.55	0.2	0.75x0.5	
Pleurodeles waltl	0.4	1.8	3.4		0.5	0.15	0.6x0.4	
Pleurodeles waltl	0.8	2.1	4.4		0.6	0.2	0.8x0.6	
Pleurodeles waltl	0.4	1.75	3.5		1.6	0.17	0.6x0.35	
Pleurodeles waltl	0.3	1.4	3.35		0.5	0.1	0.6x0.4	
Pleurodeles waltl	0.6	2.1	3.9		0.65	0.17	0.8x0.6	
Pleurodeles waltl	0.5	1.5	3.3		0.5	0.17	0.6x0.4	
Salamandra salamandra	52.5	15	23.5	6.8	4.5	1.5	-	3
Tarentola mauritanica	6.6	5.5	12	4.5	2.7	1.2	1.9x0.9	7
Tarentola mauritanica	1.4	4.6	7.8	3.1	2.25	1	1.5x1	
Tarentola mauritanica	4.5	5.4	11.8	4	2.5	1.1	2x1.1	

Between individuals of *P. waltl* except for the biggest individual with 1.9 grams and 5.7 centimetres of length, the mean weight and mean length was of 0.5 grams and 3.6 centimetres respectively. As to *T. mauritanica,* the smaller individual corresponded to a juvenile and the other two although presenting a difference of 2 grams, presented almost the same values in all the measured parameters.

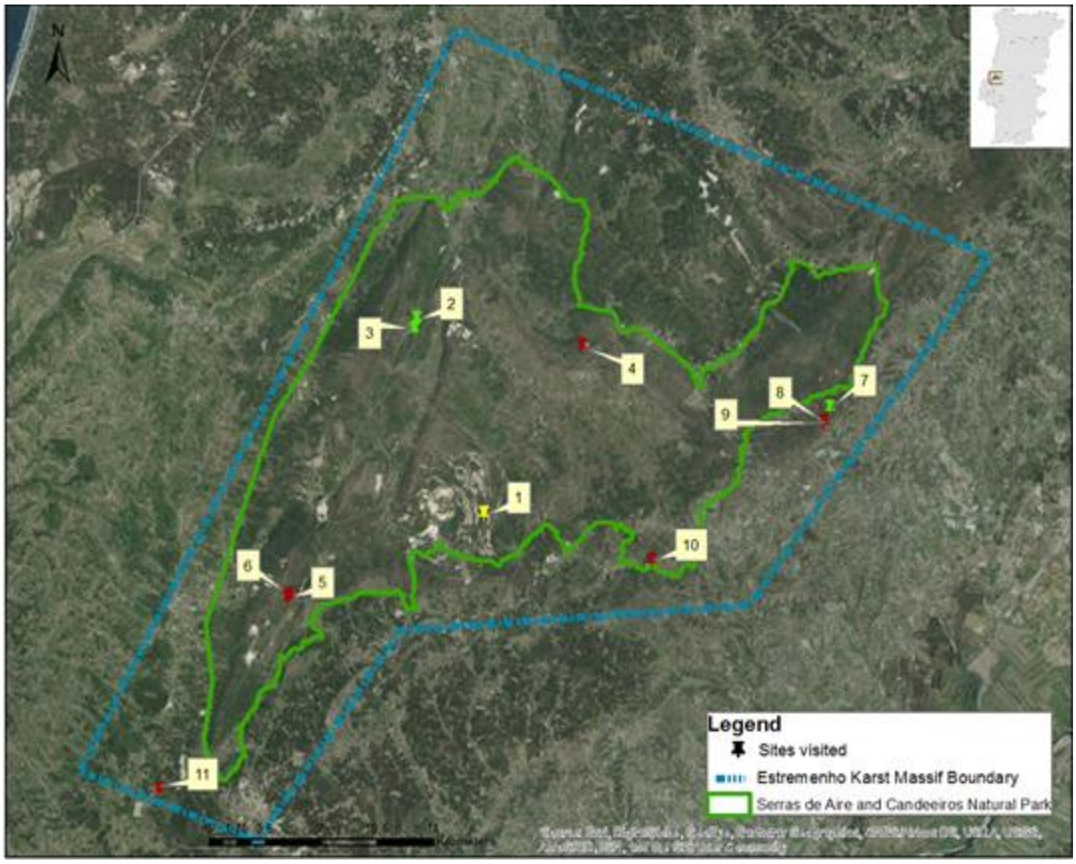

Figure 12 Map with the location of sites visited. Red symbols represent locals where no animals were found; Yellow symbol indicate the site reported to have the *Salamandra salamandra*; Green symbols show the places with record of species.
1 – Algar do Pena; **2** – S. Pedro Mine; **3** – Sarmento Mine; **4** – Lapa dos Morcegos; **5** – Well of Gruta das Alcobertas; **6** – Gruta das Alcobertas; **7** – Gruta do Almonda Novo; **8** – Gruta do Almonda Velho; **9** – Lapa dos Coelhos; **10** – Gruta da Nascente do Alviela; **11** – Gruta da Nossa Senhora da Luz.

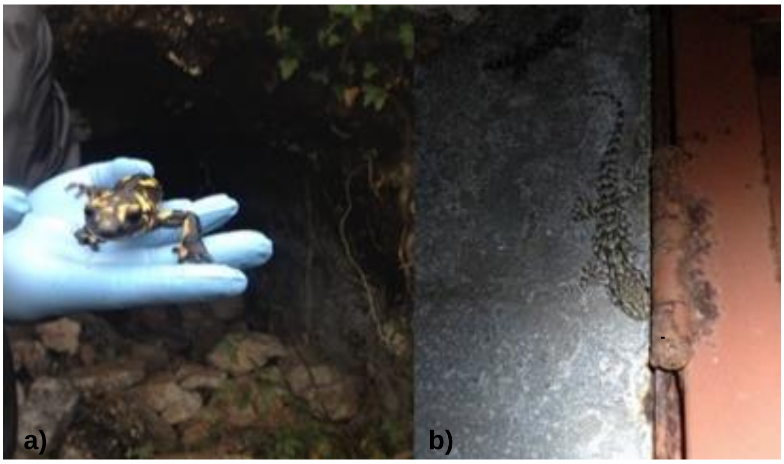

Figure 13 a) *Salamandra salamandra* and b) *Tarentola mauritanica*

Discussion

It is well known that herpetofauna species are frequently found in caves and three species found in Estremenho massif have also been found in caves of other countries (Manenti *et al*., 2009; Montori & Martinez-Silvestre, 2015).

Being a species with predominantly crepuscular and nocturnal habits (Almeida *et al*., 2001), the Moorish Gecko could be using the cave entrance as refuge during the day, leaving at night to feed. Other species of gecko use caves with the same purpose (Ellis & Pauwels, 2012; Esmaeili-Rineh *et al*., 2016). The explanation for the snake presence is the same as gecko, for refuge, particularly during ecdysis (shedding) when it is less active and more vulnerable to predation (King & Turmo, 1997; Tuttle & Gregory, 2009).

The fire salamander is one of the most common amphibian in caves using them as winter refuge (Balogová *et al*., 2017), reproduction and feeding habitats (Manenti *et al*., 2011; Limongi *et al*, 2015). In this study, the salamander was found in the entrance of the mine next to some rocks.

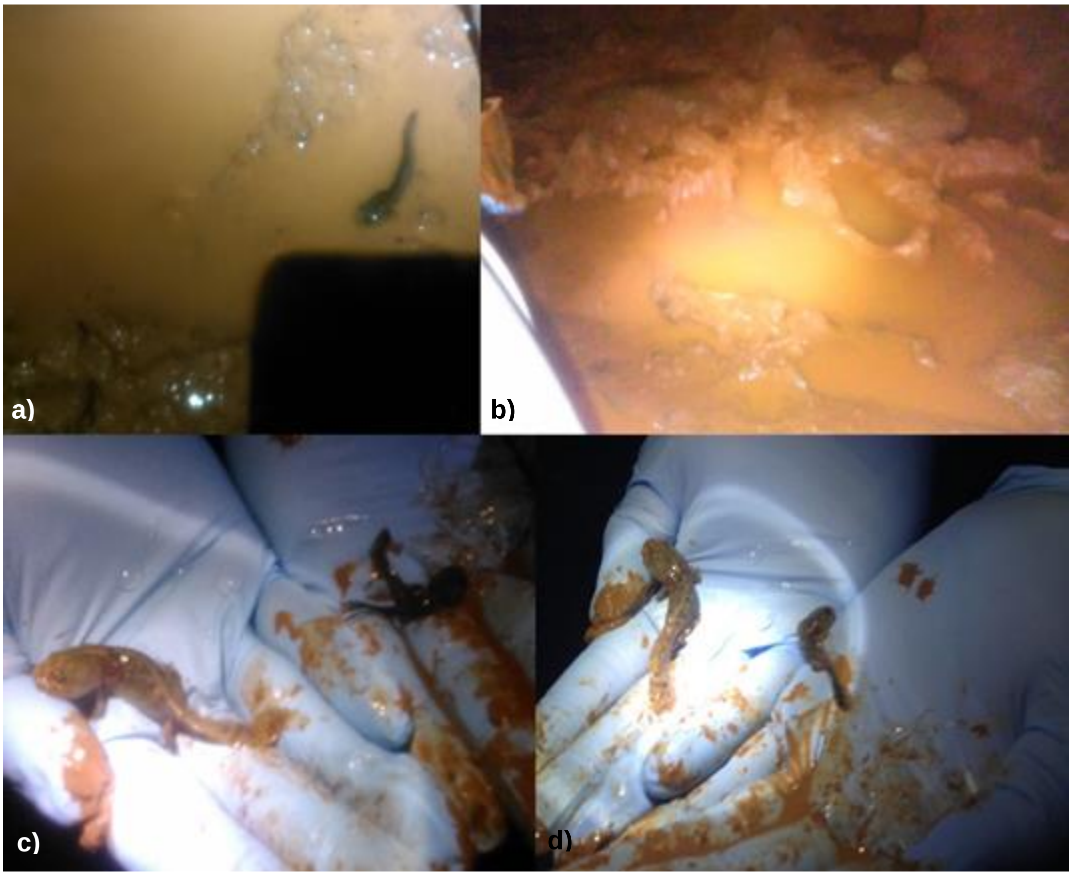

Figure 14 a) *P. waltl* in the puddle where it was found; b) Aspect of the puddles were the specimens were seen; c) and d) Different perspectives of two individuals captured to take biometric parameters.

The presence of the sharp-ribbed salamander must be emphasised. The specimens found were all in the larval stage (Fig. 14) indicating reproduction in the mine. The use of caves by this newt for reproduction purpose was only once reported in Spain (Herrero & Hinckley, 2014). *P. waltl* has a small distribution range, Iberian Peninsula and Morocco, and since its population is declining, the species is classified as near threatened (Beja *et al*., 2009; AmphibiaWeb, 2017). Being a highly water dependent species, when the habitat dries up it takes shelter under cover on land. Therefore, in a limestone realm where water at surface is scarce (Gunn, 2004), cavities are a refuge with ideal humidity and temperature conditions for amphibians (Camp & Jensen, 2007; Balogová *et al*. 2017) and their reproduction (Lunghi *et al*., 2015).

The fact that *P. waltl* was found in a mine has no implications for the organism since the environmental impacts of mining vary, depending on the material mined, techniques used for extraction, geology and geography, and duration. Species can tolerate the presence of contaminants on behalf of the benefits from the absence of anthropogenic activities (Batty, 2005). Such may be the case of *P. waltl* as it is a species tolerant to contamination (Ortiz *et al*., 2004; AmphibiaWeb, 2017) and the major threats to its population are habitat loss, urban development, roadkills, fires and introduced species such as fish and crayfish (*Procambarus clarkii*) (Loureiro *et al*., 2008; Beja *et al*., 2009).

Conclusions

Even though few specimens were surveyed in the caves of Estremenho massif, it was possible to conclude that the herpetofauna use caves even for reproduction. Nonetheless more caves need to be surveyed or at least the same (since they are the only more horizontal) but with more frequency. Other parameters must be evaluated, like the environment features of caves (e.g. temperature, humidity) as well as cave morphology (e.g. maximum height and width, presence of wet walls). Such parameters can be very important to determine the herpetofauna distribution in caves like Ficetola *et al*.(2012) and Lunghi *et al*. (2014) has shown.

The reproduction in a mine has shown the importance of such places for the amphibians, especially to vulnerable species. The next step is to understand to what extent are places important for the survival of amphibians and if they reproduce every year in the same place, or if it is due to dry conditions.

These are research questions that need answers as such places can be essential to the survival of amphibian populations in a region that might face increased aridity according to climate change projections (Giorgi & Lionello, 2008). Consequently, caves and the surface area around them must also be protected including the springs.

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Chapter III

Evaluation of the effects of water from Estremenho karst massif, in the development and antioxidant defence system of the early life stages of *Hyla arborea*

Abstract

The karst landscape has the peculiarity of presenting scarce water resources at the surface, being mostly found underground in a complex hydrological network and serving as water source for many people. The karst system is vulnerable to human activities, such as agriculture, industry, and tourism. Such activities are responsible for the introduction of contaminants in the environment which easily reach the groundwater table and springs and spread over large distances. Although water is scarce, amphibians, which are dependent of water availability, inhabit these habitats. So, the aim of this study was to evaluate the effects of water from the Estremenho karst massif (Portugal) by using embryos of *Hyla arborea* as test subject and studying their initial development and antioxidant defence system as well as damage in cellular membranes through lipid peroxidation determination. The results showed no differences in larval growth nor was reported high number of anomalies or mortality. Nonetheless significant differences were detected in lipid peroxidation between the control and site 2. These results cannot be explained by the analysed pesticides, but may be caused by the higher salinity of this site or by other contaminants not considered in this work. Even so, based on the results the water of Estremenho massif can be considered as not harmful to early life stages of amphibians.

Introduction

Karst landscapes are found around the world (Culver & Pipan, 2009) and present economic, scientific, and cultural values (Gunn, 2004). These landscapes are characterized by different forms created from the dissolution of carbonate rocks and have the peculiarity of presenting no water at the surface (Gunn, 2004; Reboleira & Correia, 2016). The water is found mainly underground in a complex hydrological network (Hartmann, *et al.*, 2014) and is the water source of many people (Ford & Williams, 2007). In sum, the karst system consists of complex hydrodynamic and hydrochemical processes (Chen & Goldscheider, 2014; Lorette *et al*., 2017) that together create its characteristic features. However, these features make the karst system vulnerable to human activities, such as agriculture, industry, and tourism (Gunn, 2004).

Pollution is one of the consequences of human activities, namely agriculture, through the use of fertilizers or pesticides (Neshat, *et al.*, 2015), or even the human use of pharmaceuticals (Reh *et al*., 2013). The contaminants released can easily reach the groundwater table and springs and spread over large distances (Iván & Mádl-Szőnyi, 2017).

Karst massifs have a variety of habitats both subterranean and superficial (Culver, 2016), which are home to diverse species of fauna and flora (Culver & White, 2002; Reboleira & Correia, 2016). Among the animal species found in these places are the amphibians, a group of animals dependent on water or humid places to survive (Vitt & Caldwell, 2014). Therefore, in the karst areas where permanent surface water is rare, all water found by amphibians is of extreme importance. Given the ecology and biology of this group of animals, they are very sensitive to changes in the environment, namely water contamination (Zhou, *et al*., 2008; Klimaszewski *et al*., 2016). The impacts of contaminants on reproduction (Hayes *et al*., 2010; Orton & Tyler, 2015), development (Baker *et al.*, 2013) and survival (Sparling *et al*., 2015) are well known.

Thus, the aim of this study is to evaluate the quality of water of a Portuguese karst massif, the Estremenho, through assessing the effects of exposure to water from study area on an anuran species previously recorded on the region, *Hyla arborea,* and highly dependent of water for reproduction and early life stages development. There are no previous studies with this group of animals, only one study of toxicity on stygofauna (Reboleira *et al*., 2013).

Materials and Methods

Study area

The Estremenho karst massif is found in the Lusitanian Basin of western Portugal (Western Iberian Margin). It is situated between the cities of Leiria, Alcobaça, Rio Maior, Torres Novas and Ourém (Fig. 1).

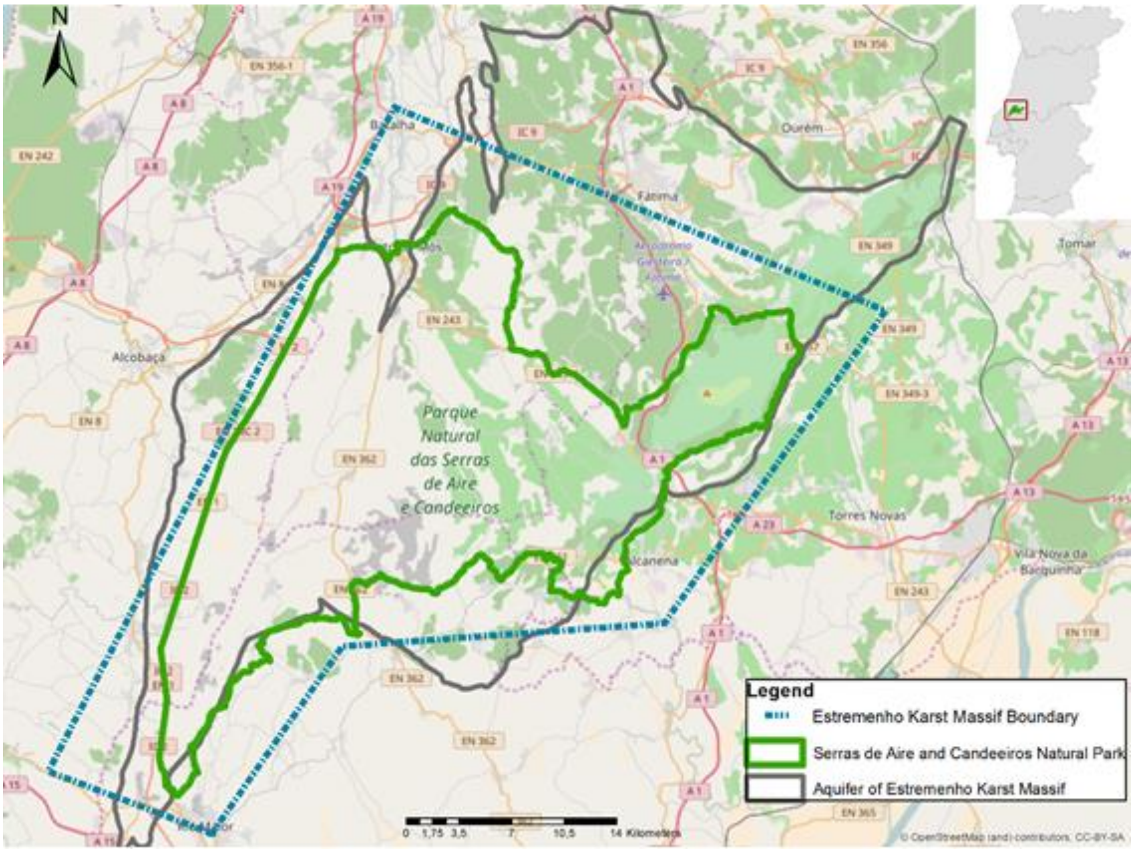

Figure 1 Map representing the boundaries of the Estremenho massif and the area of the aquifer. Blue line based on Carvalho (2013)

This massif is characterized by surface and subsurface karst landforms like uvalas, sinkholes, small karst depressions, poljes and caves (Crispim, 2010a; Rodrigues & Fonseca, 2010), composed of mid Jurassic and Cretaceous limestone or dolomites (Thomas, 1985; Cunha, 1990; Pereira *et al*., 2014). There is no permanent water at surface, however it harbours one of the most important aquifers of Portugal (an area around 770 km^2), being used as local water supply (Carvalho, 2013; Azevedo & Rodrigues, 2015). According to INSAAR (2011) in 2009 about 34% of the continental population was supplied by groundwater, and Calçada (2016) estimates that in the western margin, the Estremenho karst massif supply 15% of the population. This groundwater comes to surface in several temporary springs that appear during periods of flood and five perennial springs. Such perennial springs are Liz and Chiqueda in W side and Almonda, Alviela, and Alcobertas in S/E side (Almeida *et al.*, 2000).

Although of extreme importance, groundwater of the Estremenho Massif is threatened by several factors. There are important highways crossing the region, which contributes to the release of hydrocarbons and metals. Furthermore, there is a high populational density, mostly in Mira de Aire, Minde and Fátima with different activities that produce domestic sewage. Some of the activities are agriculture, animal farming as well as olive mills. There are also industries like textiles, tanneries, wool washing plants, and leather plants (Almeida *et al.*, 1995). But the biggest activity and with irreversible consequences is the rock extraction industry that destroys the epikarst and its functions of infiltration's regulation and storage (Crispim, 2010b).

The diversity of habitats allied with the conservation need of the hydrogeologic resources and its geologic and geomorphologic patrimonial valor, lead to the creation of Serras de Aire and Candeeiros Natural Park (PNSAC) in effect since 1979 (Carvalho *et al.*, 2011; Carvalho, 2013).

Water sampling

Five sites were chosen to analyse the water bodies of the Estremenho Karst Massif. The choice was based on the possible presence of water in that time of the year (3rd May 2017), with possible relevance for amphibian reproduction, and region's representativeness.

Water collection was done in clean 6 L jars (for the assays) and 1.5 L plastic bottles (for chemical analysis) each one properly labelled with day and local of collection. Before collection, some physicochemical parameters were measured and registered using a portable multi-parameter Aquaprobe – Aquaread AP-2000 (Tables 2 and 3).

In laboratory, the water samples for the assays were stored at 4ºC until further use in the exposure assay, while samples for chemical analysis were acidified and refrigerated at 4ºC (metal analysis), or frozen -20ºC (pesticide analysis) until chemical analyses were possible.

The five chosen sites are listed below and in table 1 is the summarized information with the respective spatial data (coordinates taken with a Garmin Oregon 550 GPS).

- **Arrimal Pond (Site 1)**

Also known as small pond of Arrimal it is a sinkhole, a natural enclosed depression with an impermeable bottom due to clay and other sediments. Situated in Mendiga's depression, in the village of Arrimal, Porto de Mós, it gathers water from Mendiga (ICNF, 2017a; NaturalPT, 2017). This pond is used by the populations of Arrimal, Arrabal, Mendiga and Alqueidão as livestock drinking troughs, to irrigation and for domestic use.

Figure 2 *a)* View of Arrimal sinkhole; b) Juvenile specimens of *Rana iberica* found in the site; c) Focus on one juvenile of *R. iberica*

- **Alcobertas spring (Site 2)**

Found in the Santo António Plateau, in the Alcobertas Village (Rio Maior), Alcobertas spring or Olho de Água de Alcobertas is a permanent spring with karstic character (Almeida *et al*., 2000; Azevedo & Rodrigues, 2015). It was used as water supply for people and cattle, but today is used in laundry and watermills, as it´s eutrophication is known.

Figure 3 Alcobertas spring. View from the spring side

- **Gruta do Alviela (Site 3)**

A cave filled with water from the main spring of Estremenho with the same name, Alviela Spring or Olhos de Água do Alviela. Located in the transition between Estremenho Massif and Tagus river basin in Santo António Plateau is the most important karstic spring of the country with an average annual debit of 120 hm^3/year. Until recently it was the main source of water to the capital Lisbon since 1880 (Crispim, 2017.; Almeida *et al.*, 2000; Azevedo & Rodrigues, 2015).

Figure 4 Gruta do Alviela. a) Entrance of cave; b) Local of water sample collection inside the cave of Alviela. Photo a) source: Narciso Azevedo in *www.panoramio.com;* Photo b) by Pedro Carvalho (2012) in (SPE, 2017)

- **Ribeira dos Amiais, next to Poço Escuro (Site 4)**

A small branch of the Alviela River that is at the origin of one of the most interesting fluviokarstic phenomenons of Portugal, the Perda da Ribeira dos Amiais (Loss of the Amiais Stream). The phenomenon consists in a system of losses and resurgences of this stream, basically it disappears in a cave formed by water erosion flowing 250 metres in the subsoil.

Figure 5 Ribeira dos Amiais. a) Local of water collection, on the left side is the wall of Poço Escuro; b) Cave entrance from where the stream reappears at surface.

Then the stream reappears at surface in a valley forming a narrow cannon next to Poço Escuro (Dark Pit), a temporary spring of Alviela River with a levee built in early 20th century to prevent the mixture of waters from the river and stream (Crispim, 2017.; ICNF, 2017b).

- **Almonda Spring (Site 5)**

Initially known as Olho do Moinho da Fonte, this spring is situated in the south-east edge of the Serra de Aire's anticlinal where Targus basin contacts the Estremenho massif and the ground is more permeable. Almonda spring debits 100 cubic hectometres of water per year that is used as water supply to the old factory (Almeida *et al*., 2000; Crispim, 2010b; Azevedo & Rodrigues, 2015; NEUA, 2017).

Figure 6 Almonda spring. a) Dam where water was collect to factory supply; b) Back of the factory.

Table 1 Caves visited and respective spatial data. The geographic coordinates are in Degrees Decimal Minutes and altitude is in metres.

Point code	Water body name	Geographic Coordinates	Altitude	Locality	County
1	Arrimal Pond	N39º29.520 W 8º52.586	329	Arrimal	Porto de Mós
2	Alcobertas Spring	N39º25.585 W8º 54.331	150	Alcobertas	Rio Maior
3	Gruta do Alviela	N39º26.739 W8º42.729	94	Amiais de baixo	Alcanena
4	Ribeira dos Amiais (next Poço Escuro)	N39º26.750 W8º42.778	70	Amiais de baixo	Alcanena
5	Almonda Spring	N39º30.287 W8º36.915	133	Zibreira	Torres Novas

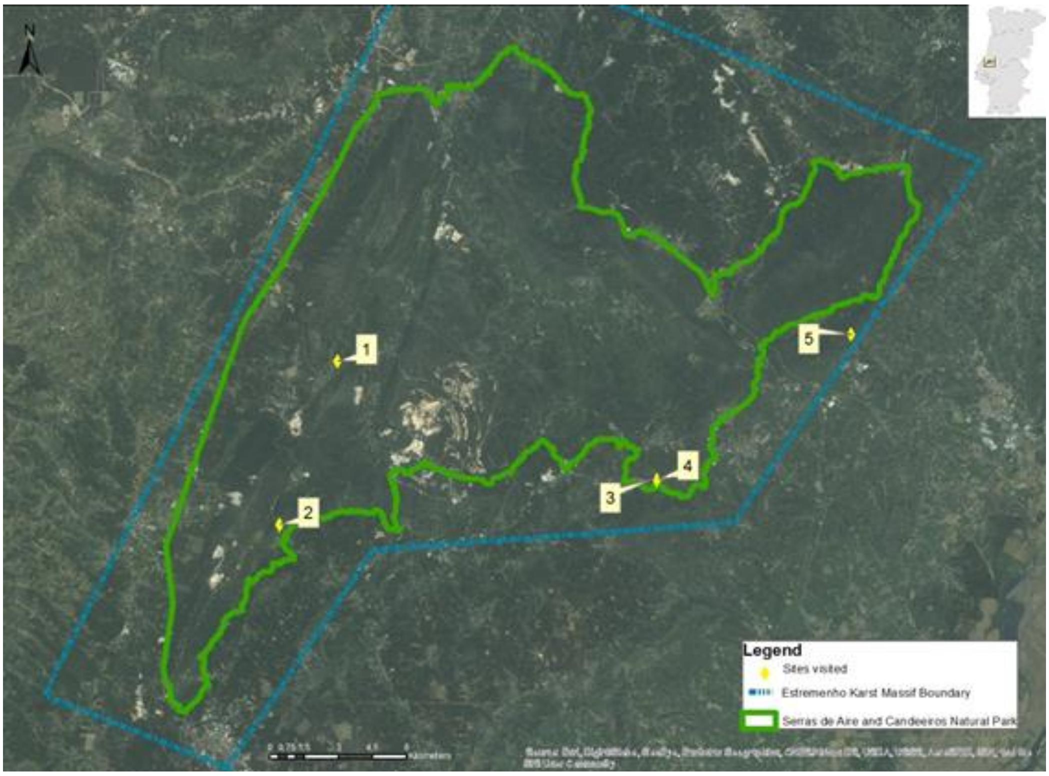

Figure 7 Map with the five points of water sample collection. 1 – Arrimal pond; 2- Alcobertas spring; 3 – Gruta do Alviela; 4 – Ribeira dos Amiais; 5 – Almonda spring

Chemical analyses

Water samples were sent to credited laboratories to analyse its contents of pesticides (Eurofins), FETAX medium was also sent. The pesticides selected for analysis were tebuconazole, dimethomorph, deltamethrin, chlorpyrifos, terbuthylazine, and glyphosate. These pesticides were selected based on sales of plant protection products in Portugal and type (two fungicides, two insecticides, and two herbicides).

Other parameters were measured following the methods given by Aqualytic PC Multidirect photometer system. Values of total alkalinity (method 30), ammonium (method 60), total hardness (method 200), nitrite (method 270) and orthophosphate (method 320) were then obtained.

Test organism

The present study was conducted under the supervision of an accredited expert in laboratory animal science (following Federation of Laboratory Animal Science Associations (FELASA) category C recommendations) and according to the European guidelines on protection of animals used for scientific purposes (directive 2010/63/UE of European Parliament and the Council of European Union).

Figure 8 a) Pond where eggs of *H. arborea* were collected; b) Example of egg masses collected.

Eggs from *Hyla arborea* (Linnaeus, 1758) were collected in a temporary pond in Espinhel, Águeda, Portugal (N40º34.335 W8º29.337) (Fig.8).

The European tree frog (*H. arborea*) is an anuran species listed as least concern both in Portugal (Cabral *et al*., 2005) and in Europe (Kaya *et al*., 2009). Moreover, it is found in the study area and depends on stagnant waters such as reservoirs, lakes, ponds, swamps, and occasionally in ditches and puddles. Sensitive to changes in habitat, the genus *Hyla* is threatened by fragmentation and loss of their suitable habitat, industrial and agricultural contamination, among others (Kaya *et al*., 2009; Loureiro *et al*., 2008). These are the reasons that make this species suitable for carrying out exposure tests with potentially contaminated water.

Bioassays

From the egg masses collected in the field, viable eggs between the 9-12 developmental stages (Gosner, 1960) were sorted out (Fig. 9) and placed into glass containers with FETAX medium (Dawson & Bantle, 1987).

Figure 9 Sorting process of viable eggs from *H. arborea*

The bioassay followed the recommendations from the standard guide for conducting acute toxicity assays with effluents (ASTM, 1997) comprehended five replicates for each sampling site (1, 2, 3, 4 and 5) and for the FETAX control (Fig.10). Each replicate consisted of a 200 mL glass vial with 150 mL of water from the respective site or FETAX control.

For each one 20 *H. arborea* eggs were randomly transferred. Every two days water and FETAX medium were renewed. On a daily basis mortality was checked and dead animals removed. During the assay the temperature was kept constant (20±1ºC) and also the photoperiod ($16h^{L}:8h^{D}$). When all larvae of the FETAX control reached stage 25 (Gosner, 1960) (12-days period) and with help of a macro zoom microscope OLYMPUS SZX9, total body length was measured in 10 tadpoles from each vial and animals were checked for malformations. The larvae were then frozen in liquid nitrogen and stored at -80ºC until further biochemical analysis were possible.

At the beginning and ending of the bioassay, parameters such as dissolved oxygen, pH and conductivity were measured with a multi-parameter Aquaprobe – Aquaread AP-2000. No food was given to the animals throughout the bioassay, since active feeding only starts at stage 25 (Gosner, 1960) which corresponded to the terminus of the assay.

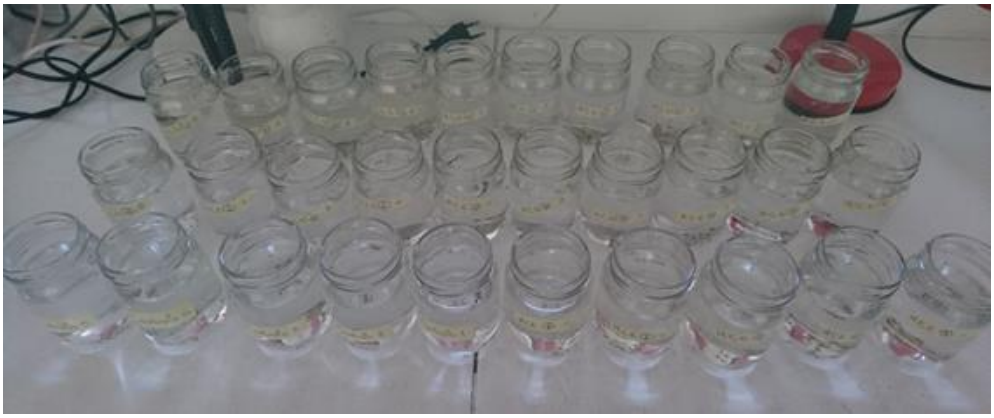

Figure 10 Vials used in the assay, each identified with local of water sample and respective replica.

Biomarkers

Antioxidant defence system was evaluated through the enzymatic determinations of Selenium-dependent and total glutathione peroxidase, glutathione reductase and glutathione S-transferase. Also, lipid peroxidation was determined through the determination of Thiobarbituric Acid Reactive Substances (TBARS). The previously frozen embryos were homogenized in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% TRITON X-100), then the homogenates were centrifuged for 10 min at 10,000 g.

Supernatants were taken and stored in five eppendorfs (one for each determination) at -80ºC (Fig. 11).

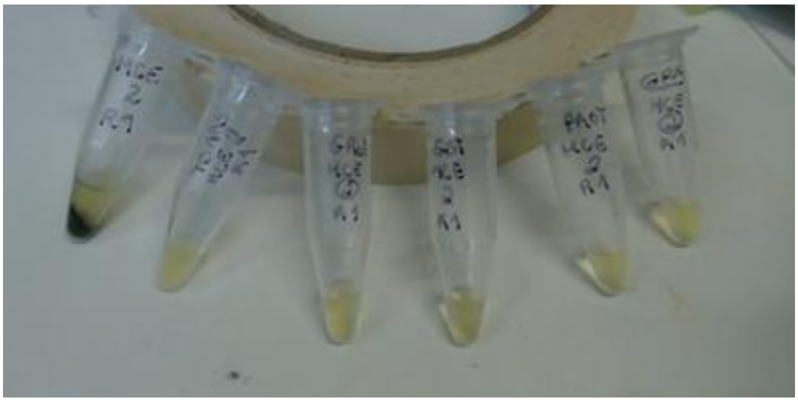

Figure 11 Supernadant division by five eppendorfs, one for each determination (from left to right – Homogenate centrifuged, TBARS, GRed, GST, Protein and GPx).

Glutathione peroxidase (GPx) activity was measured at a wavelength of 340 nm. The reaction consists in the oxidation of NADPH (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$) when glutathione reductase reduces GSSG back to GSH. Hydrogen peroxide (H_2O_2 – 0.255 mM) corresponding to selenium-dependent glutathione peroxidase and cumene hydroperoxide (0.7 mM) corresponding to total glutathione peroxidase was used as independent substrates to study GPx activity. The procedure was made according to Flohé & Günzler (1984).

Glutathione reductase (GRed) activity is also quantified with oxidation of NADPH and according the protocol of Carlberg & Mannervik (1985) it was monitored by spectrophotometry at 340 nm.

Habig *et al*., (1974) was the protocol used to the activity of Glutathione S-Transferase (GST). In this case, GST activity can be monitored by the absorbance increasing at 340 nm. The increment is caused by the formation of a thioeter (molar extinction coefficient of 9.6 mM-1cm-1) derived from catalyse of the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione by GST.

Enzymatic activities were determined in quadruple and were expressed in nmol of substrate hydrolysed per min per mg of sample protein.

TBARS were quantified according to Buege & Aust (1978) to measure lipid peroxidation. Using a spectrophotometer with a wavelength of 535 nm (molar extinction coefficient of 1.56x105 M-^{1}cm-^{1}) it is possible to quantify TBARS in a single determination. Malondialdehyde (MDA) and 2-thiobarbituric acid (TBA) are by-products of lipid peroxidation that react with each other and such is the base of the used methodology. Results are expressed as nmol of MDA equivalents per mg of sample protein.

Protein concentration was done with the method of Bradford (1976) in a wavelength of 595 nm. The biomarkers above described were expressed as function of the protein content.

All methods were adapted to microplates.

Statistical analysis

To test for statistical significant differences between animals exposed to water samples from different sites, data were analysed through parametric one-way analysis of variance (ANOVA) followed by a Tukey test. All data were checked for normality and homogeneity before testing. For rejection of the null hypothesis a level of significance of 0.05 was used. Whenever ANOVA assumptions were not met, a non-parametric Kruskal-Wallis test was performed.

Maps

For the elaboration of maps present is this work ESRI® ArcGIS 10.5 software was used. Layers of Serras de Aire and Candeeiros Natural Park were obtained in ICNF (2016) and the aquifer limit was obtained in SNIRH (2017).

Results

Chemical analyses

In general, physicochemical parameters measured in the water samples and control (table 2) show similar values. The greater differences are in conductivity where control and site 4 show higher values (around 800) while site 1 has a conductivity of 159 µS/cm. Salinity has a value of 2730 mg/L and 1040 mg/L that differ from the remaining sites. Total dissolved solids (TDS) also shows values with great differences among each other, namely between sites 1, 2, and 4 and sites 3 and 5. Being the later above maximum recommendable values.

Table 2 Abiotic parameters measured during the assay. Some of the values present are the means values.

	MECSWQ	*MRV/ MAV*	*Sparling (2010)*	*Control*	*Site 1*	*Site 2*	*Site 3*	*Site 4*	*Site 5*
pH	5.0-9.0	6.5-8.4/4.5-9.0	6.0-7.5	7.56±0.24	7.79±0.11	7.29±0.02	7.33±0.04	7.48±0.05	7.55±0.12
Conductivity (µS/cm)	-	-	150-500	898.75±640.25	159.5±52.5	343±122	368±109	833.25±309.75	298.75±84.25
O_2 (%)	50	-	-	96.1±0.8	95.63±4.12	92.28±5.98	92.88±5.73	95.28±4.33	99.13±0.88
O_2 (mg/L)	5	-	-	8.53±0.08	8.74±0.3	8.54±0.41	8.72±0.34	9.01±0.15	9.39±0.19
Salinity (mg/L)	-	640	-	-	1040	2730	140	530	120
TDS (mg/L)	-	60	-	-	0.05	0.14	290	0.727	267
Total Alkalinity ($CaCO_3$) (mg/L)	-	-	-	82	170	190	325	125	120
Total Hardness ($CaCO_3$) (mg/L)	-	-	-	460	105	145	240	140	85
Phosphate (PO_4) (mg/L)	-	-	-	0.11	0.2	<0.05	<0.05	1.44	<0.05
Ammonium (NH_4^+) (mg/L)	-	-	-	0.1	0.1	<0.02	<0.02	0.09	0.06
Nitrite (NO_2)$^-$ (mg/L)	-	-	-	<0.01	0.02	<0.01	0.02	0.02	<0.01

MECSWQ corresponds to the Minimum Environmental Conditions to Surface Water Quality (MA, 1998).
MRV and **MAV** stands for maximum recommendable values and maximum admissible values of water for irrigation (MA, 1998)

Values of total alkalinity and total hardness are superior in site 3 and inferior in site 5. Phosphate values range from 1.44 mg/L in site 4 and below 0.05 mg/L in sites 2, 3 and 5. As to ammonium values, site 1 has more ammonium in water, 0.1 mg/L, while sites 2 and 3 have less than 0.02 mg/L.

As for the pesticides analysed in the water samples and FETAX medium, the values were below detection limits. Only glyphosate in site 4 was above detection limits but even so the value was below guidelines for water quality.

Table 3 Pesticides analysed in water samples and FETAX medium (control), and respective values of guidelines for water quality.

	CWQPAL	*LC50*	*NOEC*	*Control*	*Site 1*	*Site 2*	*Site 3*	*Site 4*	*Site 5*
Terbuthylazine (µg/L)	-	2200	19	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Tebuconazole (µg/L)	-	4400	12	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Deltamethrin (µg/L)	0.0004	0.26	<0.032	<0.16	<0.16	<0.16	<0.27	<0.18	<0.16
Dimethomorph (µg/L)	-	3400	56	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Chlorpyrifos (µg/L)	0.0035	25	0.14	<0.01	<0.01	<0.01	<0.017	<0.011	<0.01
Glyphosate (µg/L)	65	38000	25000	<0.02	<0.02	<0.02	<0.02	1.8	<0.02

CWQPAL stands for Canadian Water Quality Guidelines for the Protection of Aquatic Life (in http://pesticideinfo.org).
NOEC - No Observed Effect Concentration, highest concentration that did not cause an effect that is statistically significantly different from control over the time period of the test for fishes (IUPAC, 2017).
LC50 is the median lethal concentration in which the chemical concentration in the water will kill 50% of the test animals with a single exposure for fishes (IUPAC, 2017).

Bioassays

At the end of the assay, mortality reach a total of 19 (around 3.12%) animals, being site 5 the one presenting higher mortality (5%) followed by sites 2 and 4 (4%) (table 4). Although in control mortality was not accounted, it was where most abnormalities occurred, a total of 4% and in site 1 there were none (Fig 12). The development of tadpoles represented by their length didn't show statistically significant differences.

Table 4 Number of individuals of *Hyla arborea* in each water sample and respective replicates (R) after the assay, as well the percentage of dead and anomalous animals

	Control	***Site 1***	***Site 2***	***Site 3***	***Site 4***	***Site 5***
R1	20	19	19	18	20	18
R2	20	19	19	19	19	19
R3	20	20	19	20	20	19
R4	20	20	19	20	18	20
R5	20	19	20	20	19	19
Dead	0%	3%	4%	3%	4%	5%
Anomalous	4%	0%	1%	2%	1%	1%

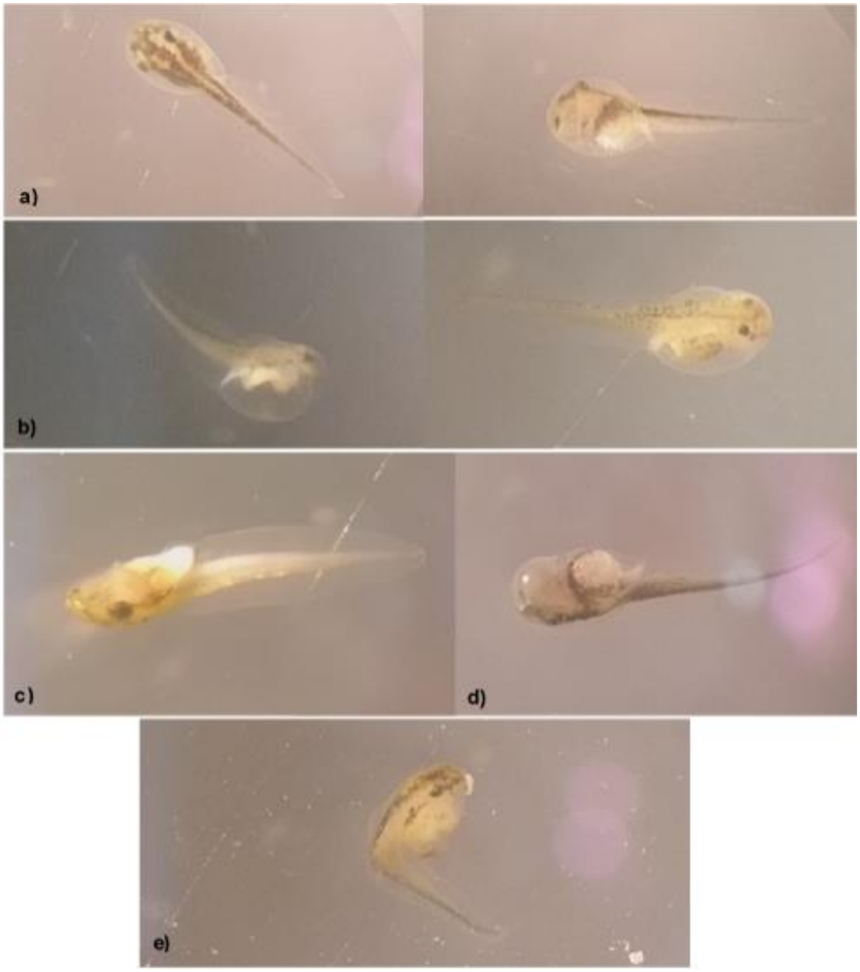

Figure 12 a) Dorsal and ventral view of a *H. arborea* tadpole without abnormalities. b) Lateral and dorsal view of a *H. arborea* tadpole with bubble disease. c), d), and e) Examples of observed malformations.

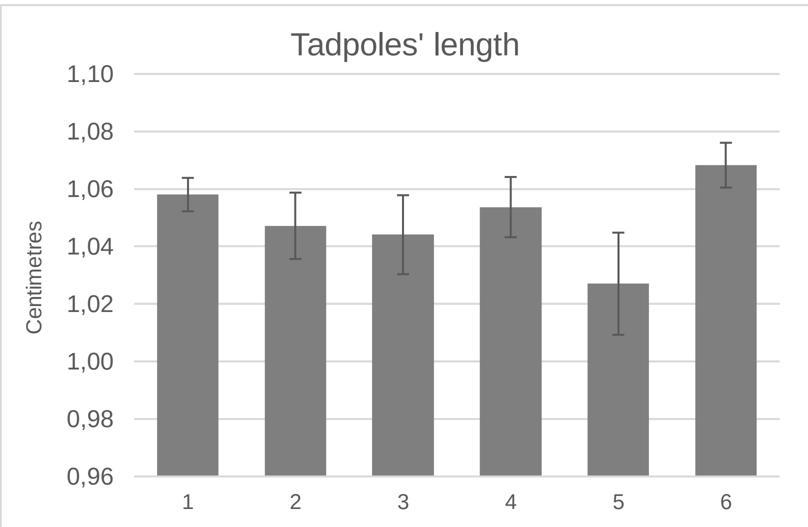

Figure 13 Mean tadpole's length at the end of the assay. Error bars represent standard error.

The tadpoles's length was higher in site 5 and smaller in site 4 (Fig. 13) with a mean size length of 1.07 cm and 1.03 cm.

Biomarkers

Overall the activity of the antioxidant enzymes analysed in tadpoles used in the assay didn't present statistically significant differences ($p<0.05$) between sites, including the FETAX control. Nonetheless, there is a trend, for the animals exposed to water from site 2 and site 5, to present higher enzymatic activities. When considering the Total GPx activity, the animals exposed to site 4 also presented high activity (Fig. 14). Nonetheless, there were no statistically significant differences (GRed: $F=1.879$; $df=5$; $p=0.136$; Selenium-dependent GPx: $F=1.904$; $df= 5$; $p=0.131$; Total GPx: $F=1.007$; $df=5$; $p=0.435$). As for lipid peroxidation (Fig. 15), the highest values were observed in animals exposed to water from site 2, followed by animals from site 4. And significant differences were verified between site 2 and control ($F=3.074$; $df=5$; $p=0.028$).

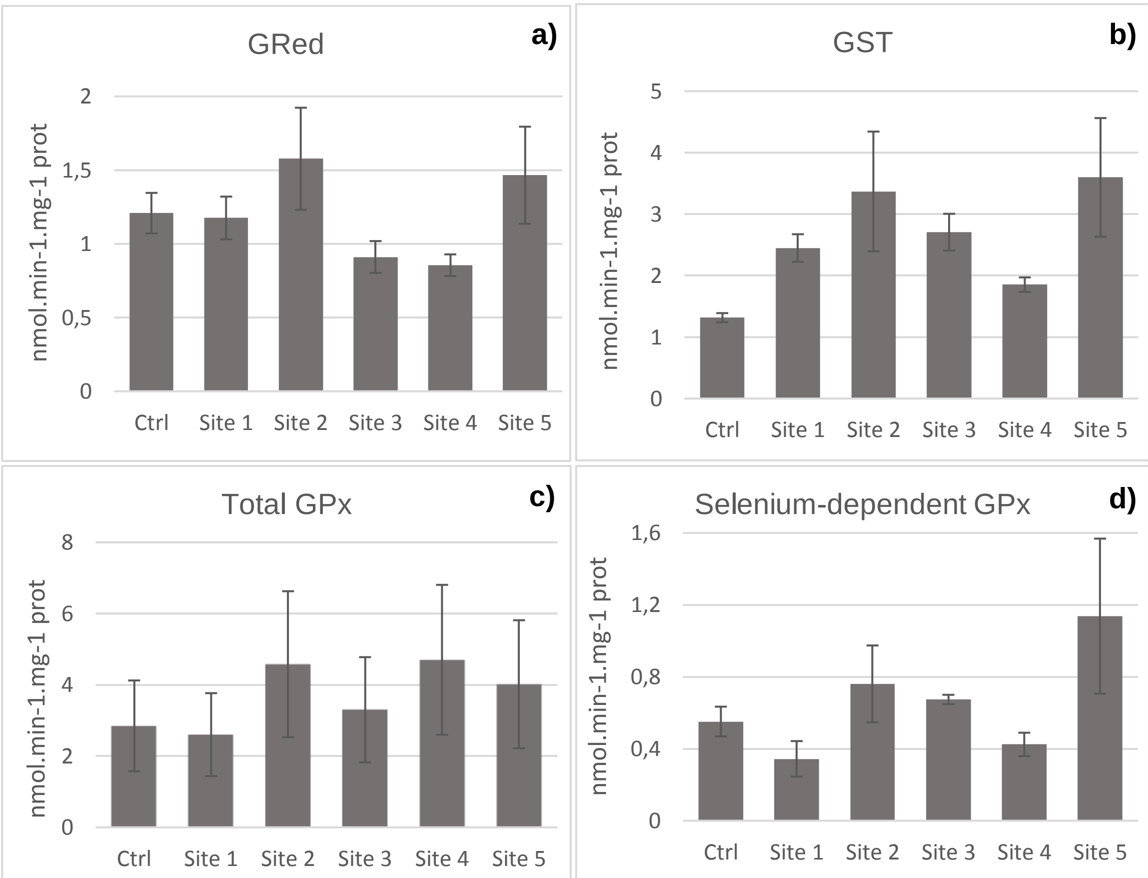

Figure 14 a) Mean glutathione reductase (GRed) activity for the respective sites and control. b) Mean glutathione-S-transferases (GSTs) activity for the respective sites and control. c) Mean total glutathione peroxidase activity for the respective sites and control. d) Mean selenium-dependent glutathione peroxidase activity for the respective sites and control. Error bars represent standard error.

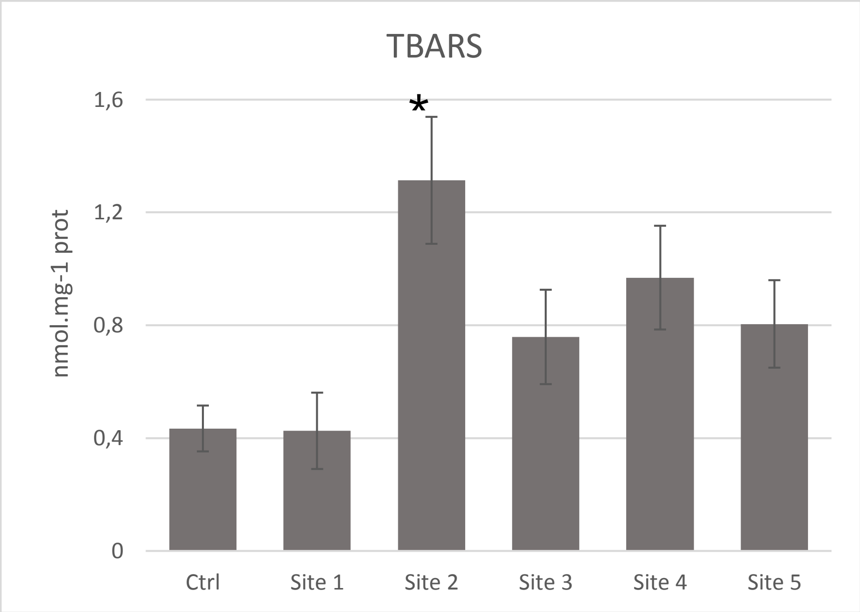

Figure 15 Mean content of thiobarbituric acid reactive substances (TBARS) for the respective sites and control. Error bars represent standard error. "*" represents a statistical significant difference ($p<0.05$) from the control

Discussion

Pollution is a threat to amphibians (Catenazzi, 2015) as it can induce physical and physiological changes in animals (El-Din *et al*., 2012; Willson *et al*., 2012; Henle *et al*., 2017; Zhelev *et al*., 2017). Such changes can affect growth and development of embryos (García-Muñoz *et al*., 2009; Marques *et al*., 2013; Gowda *et al*., 2016; Zhang *et al*., 2017). In this study, there were no significant differences in the growth of the animals exposed to the water of either of the five sites or the control. Ruiz *et al*., (2010) also did not obtain differences in size of larvae exposed to wastewater and reference sites. The same was observed in the study of Bernabò *et al*. (2011) when tadpoles were exposed to an insecticide. However, in the first study (Ruiz *et al*., 2010) a high percentage of abnormalities was observed, which did not occur in the present study. The length of tadpoles was not statistically significant (F=1.749; df=5; p=0.162) between sites and control which, considering the low concentrations of contaminants in the water from the different sites, was expected.

Considering the abiotic parameters assessed, all the sampled sites have limestone as bedrock which is rich in carbonates and bicarbonates, that interact with hydrogen ions, forming natural buffers (Sparling, 2010; Wilson, 2010). Consequently, these waters have high total alkalinity and total hardness. When comparing sites, the values for site 3 were the highest for total alkalinity (325 mg/L) and total hardness (240 mg/L) which enables its classification as a hard water (150-300mg/L) and the remnant sites as moderately hard (75-150mg/L) (Wilson, 2010). This may happen due to the dissolution processes driven by CO_2 – H_2O - $CaCO_3$ and aquatic organic interactions along with seasonal variation (Ford & Williams, 2007; Pu *et al*., 2014; Shaikh *et al*., 2015); and rock type (Gičevski & Hristovski, 2015). The classification of water based on total hardness indicates according to Sparling (2010) that the water found in the 5 sites of the Estremenho massif is appropriate for amphibians for it can stabilise pH and reduce the toxic effects of metals, and favour osmoregulation. Moreover, magnesium and calcium ions present in these waters are involved in bone formation and other physiological processes. When concerning pH, water quality criteria for amphibians includes a pH range of 6.0-7.5, considered as not harmful to most aquatic organisms (Sparling, 2010). The pH measured in this study ranged from 7.29±0.02 to 7.79±0.11, slightly exceeding the no harmful range but within the water quality guidelines (MA, 1998).

Aquatic life stages of amphibians do not have structures to extract oxygen from the atmosphere so the oxygen in water is very important for their survival (Sparling, 2010; Vitt & Caldwell, 2014). Hypoxic conditions can induce certain physiologic responses like lethargy, alterations in blood pH, and in extreme conditions even death. Yet that was not the case in this study as values of dissolved oxygen exceeded the minimum of 50% and 5mg/L stipulated to Portugal by MA (1998) as environmental conditions to surface water quality and also complied all the parameters in the ASTM (1997) standard guide.

In the study area, there are several factors threatening water quality like agriculture, industrial activity and roads that contribute to the release of chemicals and metals to environment (Almeida *et al*., 1995). However, for the six pesticides analysed in this work, although in low concentrations, it is impossible to conclude if some of them are above or below recommended values according to Canadian Water Quality Guidelines for the Protection of Aquatic Life. Since the concentrations of Deltamethrin and Chlorpyriphos are under the detection limits and so the exact values are uncertain, which could be higher or lower than the legal limits. In the case of the chlorpyriphos the detection limits are lower than NOEC for every site. As for deltamethrin, all values are below LC50 except for site 3, which may indicate that the pesticide concentration for this site is lower than the detection limit for it. Because if the value was higher than LC50, it was to be expected mortality, which did not occur. However, it has to be noted that the values of LC50 referred in this work are related to fish since they were the only available values of LC50 to all pesticides selected for this work. Indeed, data of toxicity tests in amphibians is lacking due to three major aspects. There is the assumption that vertebrate species like fish show a comparable sensitivity to that of aquatic life stages of amphibians, and so the risks to aquatic life stages of amphibians can be evaluated using fish data. Also, the animal welfare issues about the use of vertebrates for toxicity tests in some countries, as well as the absence of standard guidelines for such tests in amphibians (Weltje *et al*., 2013).

The results were not expected since other studies (Silva *et al*., 2012a; Silva *et al*., 2012b; Silva *et al*., 2015) detected some of the selected pesticides on waters of the region. So, these results may be explained by the chemical characteristics of the pesticides and their persistence in water (IUPAC, 2017). As some pesticides may accumulate in the sediment or soil and not be available in water. Nevertheless, the low concentrations may explain the low mortality and abnormalities in the bioassay carried out in this work given that these pesticides are known for causing mortality, abnormalities, slow development, and oxidative stress (Bernabò *et al*., 2011; Velisek *et al*., 2015; Radovanović *et al*., 2017; Wagner *et al*., 2017).

Enzymatic activities didn’t show significant differences between different sites including the control, as determined by the one-way ANOVA. Only lipid peroxidation had significant differences between site 2 (Alcobertas spring) and the control. This occurred despite the trend, observed in the animals of the site 2, to present high antioxidant enzymatic activity. This result indicates that some component in the water is causing the increased activity of enzymes. One possible justification for this result is the salinity value, which is the highest of the sampled sites. Indeed, there are some studies showing that salinity can cause increase in antioxidant activity and in lipid peroxidation (Martínez-Álvarez *et al*., 2002; Lushchak, 2011). Other possible justification are the natural environmental factors (Laskowski *et al*., 2010) or the effect of other contaminants not contemplated in this work.

Conclusions

Some of the parameters evaluated in this study were not in accordance with minimum environmental conditions for surface water quality in Portugal (MA 1998). However, the effects of these environmental conditions did not translate in statistically significant alterations of the antioxidant enzymatic activity on *H. arborea*. On the other hand, lipid peroxidation was observed, probably associated with higher values of salinity. Morphological effects were also not reported, both in size as in the number of anomalies and mortality. It is possible to state that the water tested from the Estremenho massif sampled sites reveals that, despite the threat presented by some economical activities in this massif, the water bodies are not impacted in such a way that they would present a threat for the early life stages of amphibians. Nonetheless, considering that different species respond differently to pollution (Güngördü *et al*., 2016) or that this can even occur among populations of the same species (Edge *et al*., 2014), the results obtained here might differ for other species. And it must be considered that pollutants (Polo-Cavia *et al*., 2016) or physicochemical parameters (Troyer & Turner, 2015) may not only cause immediate or visible impacts. They can also affect the behaviour of tadpoles making them more susceptible to predation due to impair of their response to predator cues even at low concentrations.

Future work in this massif is needed, with more chemicals analysed (pesticides and metals) as well in other matrices, like soil and sediment throughout the year. It should also be considered the use of pesticides in the region collecting information among pesticides sellers and through direct inquiries to the farmers. In addition, studies on the effects in other species and in a prolonged period of exposure are needed.

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Chapter IV

Concluding remarks

Karst and its aquifers are vulnerable to human activities such as the construction of dams, deforestation (Gunn, 2004), extraction of limestones blocks (Carvalho *et al*., 2011; Carvalho, 2013) and of course, pollution (Ford & Williams, 2007; Reh *et al*., 2013). Contaminants are easily dispersed through karst aquifers because of its conduit systems and the limited capacity for self-treatment. This contaminant transportation depends as well on the physicochemical properties of the contaminant (Ford & Williams, 2007). Nevertheless, most of the studies on water pollution are on surface waters. But groundwater is an important resource for around 25% of the world population and its preservation is important, as it will be vital in the future. The knowledge about the response of karst to climate and land use changes in the future is limited and it is provable that a decrease in precipitation and increase in temperatures occur (Hartmann *et al*., 2014). However, different models show different results, from increase recharge rates of karst water tables due to increase of extreme events to reduction in mean annual recharge (Pulido-Velazquez *et al*., 2015). Another prediction is the increase of pressure on karst water resources as the population continue to growth along with industrial expansion and a lifestyle with more water demand (Hartmann *et al*., 2014). A fact already seen nowadays (Wada *et al*., 2010), whether by excessive irrigated agriculture (Aeschbach-Hertig & Gleeson, 2012) and by water quality due to pollution (Foley *et al*., 2011).

Hence, in a world where the climate is changing, affecting habitats and so many species whether directly (Loyola *et al*., 2014; Cruz *et al*., 2016) or indirectly (Hallman & Brooks, 2016; Weir *et al*., 2016; Rollins-Smith, 2017), the karst system, if well preserved, can be a refuge to some species. The caves harbour a variety of species from obligatory cave dwellers, non-obligatory cave dwellers and accidentals (Sket, 2008). The climate inside caves tends to be constant in temperature, relative humidity, and air flow (Culver & Pipan, 2009) and such conditions can be ideal to certain groups of animals like amphibians and reptiles. In these animals' case, caves are used as refuge or to complete some part of their life cycle (Manenti *et al*., 2009; Ficetola *et al*., 2013) as it is also reported in the present study. The records of reproduction in caves of Portugal are very rare, but this note and the note of Rosa & Penado (2013), highlight the importance of such places for amphibians in a realm where water is scarce. This is quite relevant if we consider that the herpetofauna is vulnerable to human activities (Herrel & van der Meijden, 2014; Andrews *et al*., 2015; Orton & Tyler, 2015), diseases (Rosa *et al*., 2017), climate changes (Loyola *et al*., 2014; Winter *et al*., 2016), and introduced/exotic species (Bellard *et al*., 2016; Pough, 2017) and that indeed, amphibians are at risk being considered the class of vertebrates most threatened (Catenazzi, 2015). Some of the factors pointed as responsible for the decline of amphibians are consequences of human activities and are well studied throughout the world. Some physiological consequences of the exposure to contaminants are chromosome and DNA damage (Patar *et al*., 2016), damage in organs (Medina *et al*., 2016) immunity impairment (Pochini & Hoverman, 2017) alteration of gastrointestinal microbiota (Zhang *et al*., 2016) and increased risk of predation (Troyer & Turner, 2015; Polo-Cavia *et al*., 2016).

In this dissertation, through the evaluation of the physiological and biochemical effects on *H. arborea* tadpoles exposed to water from five sites from the Estremenho massif it was possible to conclude that for the majority of the sites there are no toxic effects observed. However, for one of the sites, lipid peroxidation was observed, possibly related with salinity. In a long term, this may cause deleterious effects on the animals and compromise their survival. As for the other abiotic parameters measured, some are not in accordance with legislated values, but in levels that did not compromise the animals' overall fitness. Nonetheless, the assessment made of the presence of pesticides in the water raised some concerns as to the suitability of the water for amphibians throughout the year, since, at least for site 4, the presence of glyphosate was detected, and some studies have already showed that it can be hazardous to aquatic animals (Howe *et al*., 2004; Dornelles & Oliveira, 2016).

Considering the relevance of surface water for amphibians, and the scarcity of this resource in the karst, more profound studies are needed to access the quality of water bodies of this region. These should contemplate both chemical analyses along the year, as pollution levels vary with seasons, being more intense in winter and spring (Reh *et al*., 2013), and also different species.

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