Universidade de Aveiro Departamento de Biologia
Ano 2018

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Assessment of the effects of abiotic factors related to climate change on larval stages of *Pelophylax perezi*

Avaliação dos efeitos de fatores abióticos relacionados com alterações climáticas nas fases larvares de *Pelophylax perezi*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Doutor Sérgio Miguel Reis Luís Marques, investigador pós-Doutoramento do Departamento de Biologia e do CESAM (Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro, e do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

Pelo incansável apoio e nunca me deixarem desistir dos meus sonhos, dedico este trabalho aos meus pais.

o júri

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palavras-chave

Rã-verde ibérica, alterações climáticas, stress oxidativo, dano peroxidativo, biomarcadores

resumo

As alterações climáticas são uma preocupação crescente na comunidade científica. As previsões apontam para que estas alterações sejam mais intensas na Península Ibérica, assim como nas regiões montanhosas. As alterações previstas irão ter implicações acentuadas na biota, sendo os anfíbios apontados como uma das classes mais vulneráveis devidos às suas características únicas e a sua alta suscetibilidade a fatores ambientais. Posto isto, o principal objetivo deste estudo foi avaliar os efeitos de fatores abióticos relacionados com as alterações climáticas em larvas de anfíbios oriundos de duas altitudes diferentes (elevada e baixa).

Para alcançar este objetivo foram feitas duas abordagens diferentes. A primeira consistiu em avaliar os efeitos de dois fatores (pH e temperatura) em girinos de ambas as altitudes. O segundo compreendeu avaliar o efeito de diferentes volumes/colunas de água em girinos de altitude elevada, pois estes serão mais propensos a serem afetados pela dessecação. Os parâmetros medidos, para as duas abordagens experimentais, foram morfológicos (tamanho) e fisiológicos (sistema de defesa antioxidante e dano peroxidativo). A nossa abordagem mostrou, no geral, que a temperatura teve maior influência que o pH no tempo de desenvolvimento e no tamanho dos girinos, sendo que os efeitos dependem da origem dos animais (baixa ou elevada altitude). Também os parâmetros fisiológicos sugerem que os animais de altitudes elevadas apresentam um maior stress às temperaturas elevadas. Além disso, o menor volume de água (dessecação) também revelou maior stress para os animais.

No geral, os resultados mais relevantes deste estudo, indicam que as alterações nos fatores abióticos, principalmente na temperatura, tiveram influência no desenvolvimento de *P. perezi* e as populações de altitudes elevadas podem ser mais severamente afetadas num cenário de alterações climáticas em que a temperatura aumente e a dessecação ocorra.

keywords

Iberian Green Frog, climate change, oxidative stress, lipid peroxidation, biomarkers

abstract

Climate change is a growing concern for the scientific community. For the Iberian Peninsula it is predicted that climate change driven alterations will be more profound, as well as for mountain regions. The predictable alterations will have profound implications on biota, being amphibians suggested as one of the most vulnerable classes due to their unique characteristics and high susceptibility to environmental factors. With this in mind the aim of our study was to assess the effects of climate change related abiotic factors in amphibian larvae from two distinct altitudes (low and high).

To achieve this aim two different approaches were made. The first consisted in using a full factorial experimental design to assess the effects of two abiotic factors (pH and temperature) in larvae from both altitudes. The second consisted in assessing the effect of several exposure volumes/water column heights in tadpoles from high altitude which will be more prone to be affected to desiccation effect. The endpoints measured for both approaches were both morphological (size) and physiological (antioxidant defense system and peroxidative damage). From our approach the main results showed that temperature has higher influence than pH in the development time and also in the size in tadpoles, being the effects dependent on the origin (low or high altitude) of the animals. Also, the physiological parameters suggest that animals from high altitudes present higher stress to high temperatures. Furthermore, the scenario of the lowest volume/water column height (desiccation) also revealed high stress for the animals.

Overall, the most relevant results of the present study, indicate that the alteration in abiotic factors, mainly temperature has influence on the development of *P. perezi* and that high altitude populations may be more severely affected in a scenario of climate change were temperature increases and desiccation occurs.

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Chapter I

General Introduction

1.General Introduction

1.1. Climate change

The climate on Earth has changed throughout the years and most of these climate changes are assigned to small variations in orbit of the Earth that cause the fluctuation of the amount of energy from the sun that arrives to our planet. However, presently, 95% of the occurring changes are being related to human activity (NASA, 2018) mainly owed to several factors such as the production of greenhouse gases, by burning fossil fuels like oil, coal and natural gas, by deforestation, land cover and land transformation by humans (Vitousek et al., 1997; WWF, 2018).

One of the most relevant alterations is the increase of temperature. The planet's average temperature has risen 0.9 degrees Celsius since the 19th century but most of this warming occurred in the past 35 years (NOOA National Centers for Environmental Information, 2018). Beyond this rise, the predictions says that the temperature will rise 4ºC until 2100 to Europe (including Iberian Peninsula) (Alcamo et al., 2007; IPCC, 2014). The temperature rising will be more pronounced on north pole with a rise of 9ºC until the end of the century (IPCC, 2014). Another effects of climate change are the rise of sea level (Church and White, 2006), the increase in the frequency of rare events like high temperature and rainfall events (Kunkel et al., 2013), the decreasing of snow cover (the snow cover had a decrease over the past 50 years) (Derksen and Brown, 2012).

Climate change can also affect water flow and nutrients load, as well as cause the decrease of dissolved oxygen concentration, eutrophication, an increase of toxicity or bioavailability of pollutants, a change of the water hardness, conductivity and pH (Niinemets et al., 2017; Whitehead et al., 2009). The increasing of dissolved organic carbon (DOC) can also reduce solar radiation penetration into waterbodies (Niinemets et al., 2017). So dissolved organic carbon, in a certain way, can be considered as an attenuation factor of UV-B on aquatic ecosystems (Peterson et al., 2002).

The extreme events, like heat waves, torrential rains, droughts and winter storms, will be another effect of climate change (Rosenzweig et al., 2001). In Europe, by the end of century, will experiment hot days due to heat waves; the

precipitation will decrease in south of Europe instead of central and northern Europe (Beniston et al., 2007). The decreasing of precipitation will affect the hydrological systems in quantity, it means that the some water bodies (temporary water bodies) may will disappear (IPCC, 2014).

In freshwater systems, the general effects of climate change will be increasing of water temperature, decreasing of the dissolved oxygen levels and increasing of pollutants toxicity (Ficke et al., 2007). The pH of freshwater systems will have changes depending on geological composition of water masses. In some lakes and rivers, the pH will rise due to the increasing of weathering silicates, calcium and magnesium sulphates in their catchment, in other hand the warmer temperatures will decrease the alkalinity of water when enhanced vegetative growth and soil development, because of increased organic acid inputs (Heino et al., 2009; Karst-Riddoch et al., 2005).

Climate warming may have a significant effect on the population and reproductive biology of organisms. Changes in phenology is another consequence of this change, just like changes in geographic range (McCarty, 2001). With the increasing of temperature, the number of icebergs will decrease, and glacial lakes will get larger. The instability in permafrost regions with the defrosting of polar caps will be a possible event, as well as the presence of rock avalanches in mountain regions (IPCC, 2007).

There are plenty of predictions about the effects of this change on our climate. But all the predictions show that climate change will have major effects on biodiversity at local, regional and global scales (Heino et al., 2009).

1.2. Amphibians

Amphibians are a class of vertebrates and are divided into three different orders namely Gymnophiona (caecilians), Urodela (salamanders) and Anura (frogs and toads) (Crump, 2009).

Gymnophiona is an order with individuals with sensory tentacles and limbless bodies (San Mauro et al., 2004). Until 20th century, more precisely in 1968, all individuals of Gymnophiona were included in one single family, the Caeciliidae. Presently there are 9 families belonging to this order, having been proposed by

Wilkinson et al. (2011). The Urodela order comprises tailed amphibians, with four limbs (quadrupedal) and forelimbs and hindlimbs with equal size (Hickman, 2006). This order is composed by three suborders and ten families of living salamanders (Sever, 2003). Anura, the order with more individuals, is and ancient group known for adults without tail, although tail is present in the larval stages (Hickman, 2006). Anurans have a robust body with developed hind limbs to jump and swim (Centro de Fauna Portuguesa, 1991).

All three orders can breathe and absorb water through their skin and are dependent of water for reproduction (National Geographic, 2012). Beyond these characteristics, their naked skin provide mechanical and chemical protection (Marques, 2011).

This taxonomic class have life stories very diversified. Gymnophiona have internal fertilization and eggs are deposited in moist ground near water. In this order, exists species with aquatic larval phase and other species with larval development within the egg. Viviparity is also common in some species of this order (Hickman, 2006). Urodela comprises some aquatic species or terrestrial species. Aquatic species lay their eggs in clusters or in water, and when their hatch produce an aquatic larva having external gills. Completely terrestrial species deposit eggs in grapelike clusters under logs or in excavations in soft moist earth and they present direct development (Bruce, 2003; Hickman, 2006). Usually, Anurans are oviparous (with external fertilization) with aquatic eggs and have an indirect development with a larval stage (tadpole) that suffers metamorphosis into adult form. The eggs have mucoid capsules very permeable with the mainly function of protection of the embryos (Duellman and Trueb, 1986). Anuran larvae are robust with a flat body and the majority of body mass concentrated near the head. They have a thin spine, circled by membranes that help them swim (Maravalhas and Soares, 2017).

1.3. Amphibians and the relevance of climate change

The Amphibians are presently one of the most threatened classes (Fig. 1). In 2008, the latest census done by IUCN shows that 32% of the amphibian species

are threatened or extinct, with more than 40% of amphibian species declining in population.

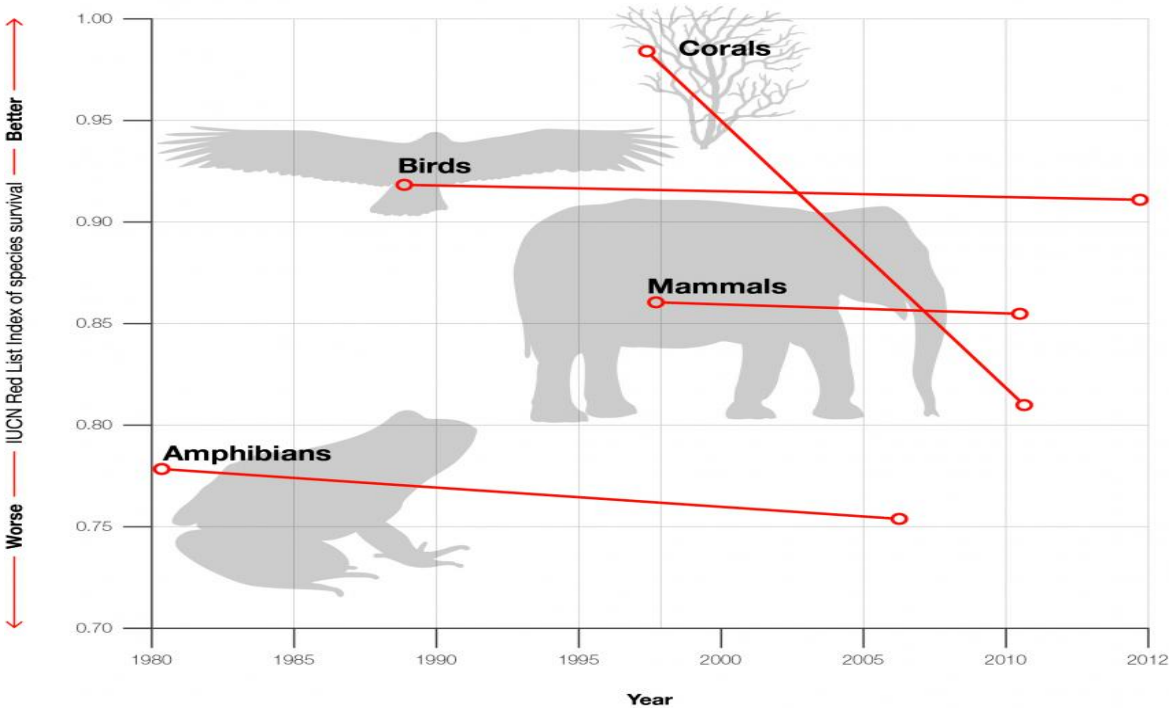

Figure 1: Temporal representation of the most declining classes since 1980 until 2012. Adapted from IUCN (2013).

In the majority of the available studies the causes pointed as responsible for amphibian decline encompass: habitat destruction, habitat alteration, contamination, introduction of invasive species, diseases and climate change (for instance, the increasing of temperature and increasing of UV radiation) (Corn, 2005).

Over the past 30 years, climate change has produced many fluctuations in temperature, influencing distribution and abundance of species (Thomas et al., 2004; Walther et al., 2002). In response to climate change, amphibians can have an early breeding season, in extended dry seasons, but precipitation decrease can also cause the decline and possible extinction of some species of amphibians (Araújo et al., 2006).

Considering that amphibians are ectotherms, their life history is deeply influenced by temperature variation, which can be detrimental for these living beings (Carey and Alexander, 2003; Corn, 2005). The rising of temperature can

affect the amphibians' immunity system, becoming more susceptible to diseases (Raffel et al., 2006), the temperature variation can influence the predator-key dynamics on larval stages of amphibians (Eck et al., 2013). The breading season may change too, causing a late breading season with the increase of temperature (Moore, 1939). UV-B radiation can be an important factor too. A study carried out by Anzalone and co-workers (1998) shows that some amphibian embryos, like *Hyla cadaverina* and *Taricha torosa*, have a higher survival rate with blocked UV-B radiation, this suggests that in their natural habitat they're influenced by radiation. It is possible that these results are transversal to other amphibian species. Nonetheless, there are also studies with other species (*Lithobates pipiens*) suggesting that neither pH, neither UV-B alone have an effect on the survival of embryos, with results pointing to a joint action of both factors (Long et al., 1995).

1.4. Iberian Green Frog (*Pelophylax perezi*)

Pelophylax perezi (Seoane, 1885), also known as Perez's frog, is an amphibian with a fairly wide distribution in Iberian Peninsula, being also found as far as south of France (Ferreira and Crespo, 2003) (Fig. 2). Due to recent phylogenetic studies, this frog changed the taxonomic genus from *Rana* to *Pelophylax* (Loureiro et al., 2010).

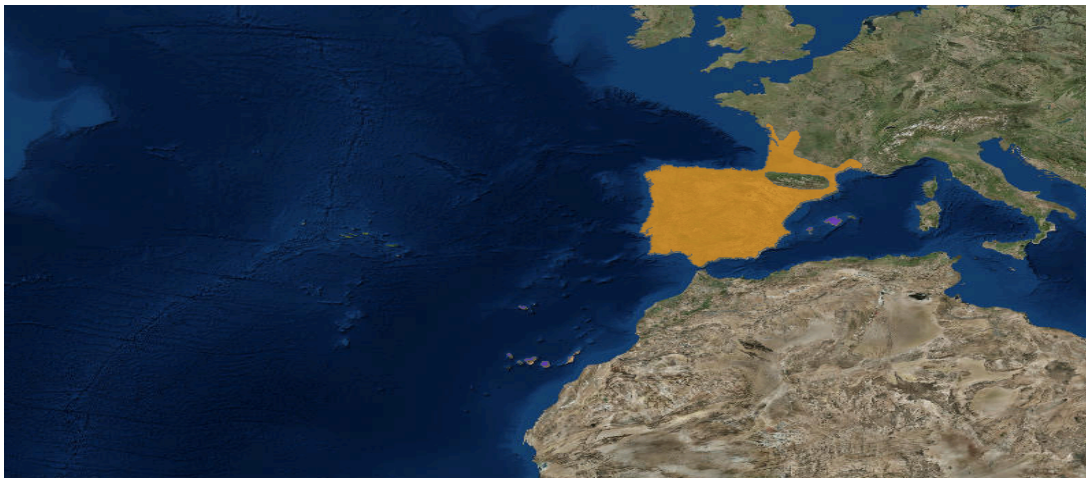

Figure 1: Distribution map of Iberian Green Frog. Adapted from IUCN (2015).

Iberian green frog has a robust body with green and brown coloration (Fig. 3), with black stains and a vertebral line of a lighter green. They present a horizontal

pupil and males have visible vocal bags (near to mouth with grey color) with grey color to call females to mate (Centro de Fauna Portuguesa, 1991).

Figure 2: *Pelophylax perezi* specimen. We can see the green color with the vertebral line lighter and spots in all body. Photo by Diogo Oliveira (2018).

This species is strictly aquatic and occupies all types of water bodies, however they prefer the permanent ones. The reproduction of Iberian Green Frog is made on water, where females can lay an average number of 2309 of eggs. After that, the larval hatching can last over two months (Egea-Serrano, 2014).

The status of conservation of this specie is “least concern”, according to IUCN (2009).

This Anuran specie presents a great deal of ecological plasticity, allowing it to occupy many types of environments, from plains to mountains (Loureiro et al., 2010). However, Iberian green frog, currently, faces many threats to their survival, which include water pollution (Ortiz-Santaliestra et al., 2010), introduction of exotic species on freshwater (J. Cruz et al., 2006) and climate change.

Alcamo and co-workers (2007) forecasted an increase of 4ºC and a decrease of water annual run-off, in the Iberian Peninsula, until the end of the century. The

water decrease in Iberian Peninsula will affect the Iberian amphibians due to their dependence of water (Duarte, 2011).

1.5. Biomarkers

Biomarkers are measurements in body fluids, cells and tissues indicating biochemical or cellular modifications due to a biological response to something external, which may be related to an exhibitor and a chemical toxicant with environmentally harmful effects. It can also be defined as an individual environmental chemical response that indicates a deviation from the normal state (Oost et al., 2003). Biomarkers can be used for prediction and monitoring the response of the organism to an intervention (Atkinson et al., 2001).

Biomarkers, also known as biological markers, can be classified in three types: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. Biomarkers of effect are an indicator of an endogenous component of the body or an imbalance on biological system that is recognized as a damage or disease. A biomarker of susceptibility is when the biological system is plenty sensitive to an exposure to a xenobiotic. Biomarkers of exposure are related to biological events that happen when biological system is subject to an exogenous exposure and the interaction between the endogenous compounds and exogenous compounds (Comittee on Biological Markers of the National Research Council, 1987).

1.5.1. Oxidative stress and antioxidant defense

Oxidative stress results from an imbalance between oxidizing compounds and the performance of antioxidant defense systems. Reactive oxygen species (ROS) are formed in aerobic metabolism, however if there is an alteration affecting the homeostasis of the organism it can result in malignant effects, being abnormal produces of ROS (at rates above normal) one of the consequences (Sies, 1997). These free radicals of oxygen are highly reactive and attack membrane lipids, proteins and DNA (Menon and Rozman, 2007).

The antioxidant defense system has the function of inhibit or reduce the damage done by free radicals. Antioxidant defense system can be enzymatic (Fig. 4) or non-enzymatic. Enzymatic system is made by enzymes and non-enzymatic system drift from dietary source (K. B. F. Barbosa et al., 2010).

Non-enzymatic system include reduced glutathione (GSH), ascorbic acid (vitamin C), vitamin E, uric acid, polyphenols, and others (Menon and Rozman, 2007).

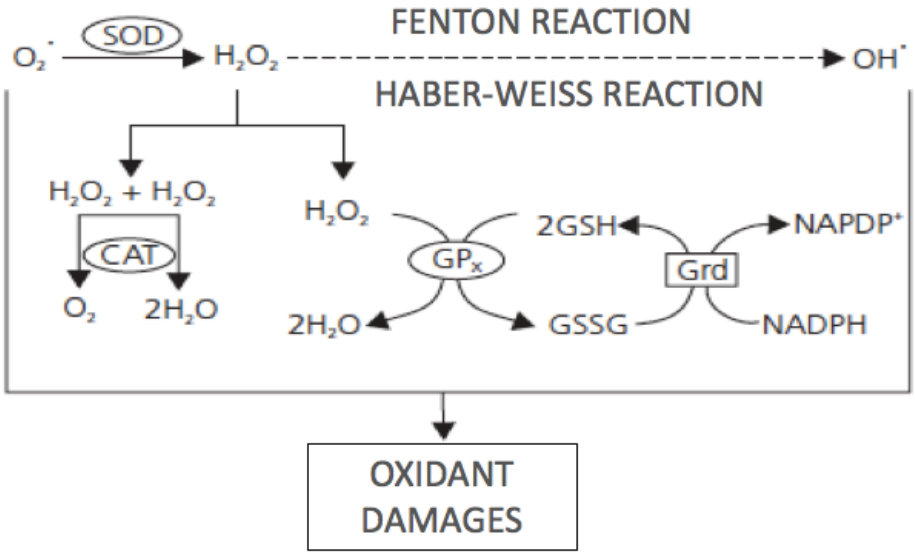

Figure 3: Enzymatic system defense. Adapted from Barbosa et al. (2008).

Some of the enzymes that are part of the enzymatic antioxidant defense system are catalase, glutathione peroxidase (GPx), glutathione reductase (GRed), glutathione S transferase and superoxide dismutase (SOD) (Birben et al., 2012). Catalase decomposes hydrogen peroxide (H2O2) into water and oxygen in the following way: 2H2O2 → 2H2O + O2 (Horozova, Dimcheva, & Jordanova, 2002). In summary, concentrations of hydrogen peroxide is regulated by catalase enzyme, being the main regulator of this chemical compound (Góth et al., 2004).

Another enzyme responsible for catalyzing hydrogen peroxide is Glutathione peroxidase (GPx), which catalyzes the reduction of hydroperoxides (ROOH) by glutathione (GSH) (Tappel, 1978).

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

(GPx reaction formula)

There are two classes of glutathione peroxidase: a selenium dependent GPx, that contains selenium in the form of covalent bound selenocysteine and this GPx is active with both organic hydroperoxides and hydrogen peroxide; proteins that aren't dependent of selenium to catalyze ROOH and have a minimal relation with hydrogen peroxide are selenium independent GPx or Total GPx (Mannervik, 1985).

As for glutathione reductase, the main function is keeping a high level of reduced glutathione (GSH) in cytosol. GRed can work in stress adaptation in a cellular level (Schulz et al., 1978).

$$GSSG + NADPH + H^+ \Leftrightarrow 2GSH + NADP^+$$

(Glutathione reductase formula)

Glutathione S transferases (GST) possess various activities and participate in many types of reactions. The main functions of these enzymes are the detoxication of electrophiles and catalyzing the reduction of organic hydroperoxides (Sherratt and Hayes, 2001; Thérond et al., 2000), detoxication of xenobiotics and their metabolites (Jerina and Bend, 2001).

Superoxide dismutase (SOD) is an enzyme that obliterate the superoxide anions (Kakkar et al., 1984; Scandalios, 1993). There are three types of SOD: the copper/zinc SOD, manganese SOD and iron SOD. This classification is based on the SOD metal cofactor (Bowler et al., 1992).

Lipid peroxidation is known as the free-radical oxidation of polyunsaturated fatty acids and the detection of lipid peroxidation is an evidence that support the implication of reactions with free-radicals (Gutteridge, 1995). Exist several techniques to measure the lipid peroxidation, one of the best known of these techniques is the TBARS assay. This method uses thiobarbituric acid. Thiobarbituric acid reacts with malondialdehyde, formed from the breakdown of polyunsaturated fatty acids (Buege and Aust, 1978).

1.6. Aim of this study and organization of the thesis

In the Iberian Peninsula, according to forecasts, temperatures will increase 4ºC and the annual run-off water will decrease by the end of this century (Alcamo

et al., 2007). The water decrease in Iberian Peninsula will affect the Iberian amphibians because their larvae phases are water dependent (Duarte, 2011). Knowing these predictions, the main aim of this study is to evaluate how tadpoles of *Pelophylax perezi* react to abiotic factors related to climate change, particularly temperature, pH and decrease of water column. In order to achieve these goals, studies with these three factors will be done in tadpoles of Iberian green frog and the antioxidant defense system will be measured through biomarkers to assess how are the reactions to these exposures.

This thesis is composed by three chapters. The first one is the general introduction, where there is a bibliographical compilation on the subjects in general. The chapter two is composed by the experimental assays with *P. perezi* to evaluate how abiotic factors related to climate change affect the tadpoles of Iberian Green Frog. Chapter three is conclusion with final remarks and future perspectives.

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Chapter II

Assessment of abiotic factors related to climate change on larval stage of *Pelophylax perezi*

2. Assessment of abiotic factors related to climate change on larval stage of *Pelophylax perezi*

Abstract

In the past decades, the declining of amphibians has been a concerning topic for scientist. Many reasons were pointed out for this declining, being one of them the climate change. To test the possible influence of climate change on an Iberian amphibian, namely on the most sensitive early life stages, we tested different medium acidity ranges, temperatures and water level on Iberian green frog (*Pelophylax perezi*) larvae. Furthermore, to perceive if responses are different between animals with different altitude origins the assays were conducted with eggs from a low altitude site and also from an high altitude site to achieve the main goal, three assays were made being the first two a full factorial design with pH and temperature tested in tadpoles from different altitudes (low and high), and the third assay with water level. Oxidative stress was measured through antioxidant defense system enzyme (GRed, GST, Total and Selenium Dependent GPx) and the lipid peroxidation was measured through thiobarbituric acid reactive substances (TBARS). Endpoints, like the tadpole's length, mortality and assay duration were annotated too. The results shown that temperature and pH had influence in different ways between tadpoles from high and low altitudes. Tadpoles from high altitude have smaller lengths when exposed to high temperature, instead of tadpoles from low altitudes. In relation to the enzymatic activity, the differences between the tadpoles from different altitudes were few and the both assays have the same peak of LPO on 15ºC and pH 5.5. Regarding the water column height assay, the lowest water level, stimulated the development leading to a shorter time for reaching Gosner stage 25 while simultaneously presenting a smaller, as well as increased lipid peroxidation.

Keywords: Pelophylax perezi, abiotic factors, climate change, TBARS, oxidative stress

2.1. Introduction

In the past years, one of the most discussed topics in the scientific community is climate change. In 2005, there was a consensus on climate change

by the scientific community considering that this change is mainly caused by human activities and one of the major consequences is the heating of Earth's surface (Oreskes, 2005). Also, besides temperature alteration, climate change includes other stressors such as high levels of atmospheric CO^2 and increase in the occurrence and intensity of extreme events like droughts (Barnett et al., 2005). These alterations may have severe implications for various animal groups, including those already threatened. Among the most threatened groups we find amphibians on the top of the list with 40 % of the species threatened (IUCN, 2018). Specifically, due to the relevance that variables such as humidity and temperature have for this group (Dervo et al., 2016), it is expected that climate alterations will have a negative impact in various species. For instance, when considering temperature change, it has been documented as the cause of many modifications in spatial distribution, phenology, abundance, physiological and genetic adaptations on amphibians (Smith et al., 2001). Nonetheless, despite this global temperature rise on our planets' surface, the warming is not spatially uniform, being higher in mountain regions (Bronnimann et al., 2005). Thus, regions with high altitude were classified as sensitive regions to the projected global warming to this century (Rangwala and Miller, 2012). Furthermore, temperature increase may cause the disappearing of temporary water sources or even lakes (Milly et al., 2005). Many lakes and rivers may have increased pH due to an increase of sulfates, base cations and silica, however, when warmer temperatures increase vegetation growth and the soil development in high ecosystems, the alkalinity of soft water can decrease because organic acid inputs can increase (IPCC, 2008). Acidic precipitation is also a concern as it can acidify fresh water (Freda and Dunson, 1986). All of these changes can further affect amphibians, namely in their early life stages because in the majority of the species these stages are entirely aquatic and consequently unable to avoid these stress conditions.

When concerning climate changes and its effects for the Iberian Peninsula, simulations reveal a temperature increase higher than the predicted global mean and a change in precipitation consistent with a short rainy season

(Abrantes et al., 2017; Guiot and Cramer, 2016). If confirmed, such alterations will almost certainly affect amphibians in the Iberian Peninsula.

One way of assessing the effects of environmental stressors on animals is through assessing the activity of antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione reductase (GRed), glutathione S-transferase (GST), and also through lipid peroxidation (LPO). These enzymes have the function of detoxifying reactive oxygen species (ROS) (Regoli et al., 2002).Thus, these parameters indicate the ability of the animals to cope with ROS (Bilham et al., 2018; Schröder and Krutmann, 2005; Valavanidis et. al, 2006) and provide an insight over existent oxidative stress.

Considering the predicted scenario for the Iberian Peninsula, concerning climate change, and also the current vulnerability of amphibian species, it is important to assess the effects of abiotic alterations derived from climate changes in these organisms. Also, it is important to know if populations from different altitudes will have different responses towards these predicted changes. In order provide some answers to the previous questions the main goal of this study was to evaluate how abiotic factors (pH, temperature and water volume) associated to climate change would affect the larval stages of the amphibian *Pelophylax perezi* from low and high altitudes. The growth and the activity of the antioxidant enzymes GPx, GRed, GST, and also the quantification of LPO were used as endpoints.

2.2. Materials & Methods

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).

2.2.1. *Pelophylax perezi*

Pelophylax perezi, also known as Iberian Green Frog, is one of the most common amphibians in the Iberian Peninsula, and has a wide distribution, ranging from low to high altitude areas (Loureiro et. al, 2010). Presently the status of this amphibian species according to IUCN (2015) its “least concern”, and it plays an important role in the ecosystem. Their larval stages (tadpoles) are important in trophic webs, and they are also sensitive to environmental changes (e.g. abiotic factors) (C. Marques et. al, 2018; S. Marques, 2011). Considering this, and its great abundance, this species was selected as test organism.

2.2.2. Collection of egg masses

To carry out this study three different assays were planned. For the assay number 1, the eggs were collected in a low altitude area (7m), “Poço da Cruz” – 40º28’49’’N, 8º47’18’’W. For the second and third assays, egg masses were collected in an area with high altitude (960 m), belonging to Freita Mountain – 40º52’00’’N, 8º15’44’’W.

In both sites, egg masses were collected in stages 8-10 according to Gosner (1960), being placed in closed plastic boxes, filled with water from the collection site, to reduce stress from travel. After arriving to the laboratory, the eggs were individualized from the egg mass to an aquarium with FETAX medium (Dawson and Bantle, 1987).

2.2.3. Experimental design

2.2.3.1. Temperature and pH assay

To assess the effects of temperature and pH on the early life stages of *P. perezi* a full factorial experimental design was planned (Fig. 1) considering three different ranges of temperature (16, 20 and 24$\pm$ 1ºC) and three ranges of pH (5.5; 6.5 and 7.5 $\pm$ 0.05) within each test temperature. For each specific condition there were 5 replicates. For each replicate a 150ml glass flask was filled with

FETAX with the respective pH. Afterwards 20 eggs within stages 8-10 (Gosner, 1960) were placed in each flask. The photoperiod was constant with 16h hours of light and 8 hours of dark (16L:8D). This experimental design was applied for the eggs from both low and high altitudes, tests 1 and 2, respectively.

The pH was measured every day and the adjustment was done with the addition of sulfuric acid (97-98%) to FETAX medium.

	pH 5.5	pH 6.5	pH 7.5
16ºC	5 x 	5 x 	5 x 
20ºC	5 x 	5 x 	5 x 
24ºC	5 x 	5 x 	5 x 

Figure 1: Illustrative scheme of the test 1 and 2.

2.2.3.2. Water column/ volume assay

This assay was carried out to assess the influence of the water column height in tadpoles, since it may vary depending on the amount of rain and also in drought periods. Briefly, for this assay, there were four different water columns height, which corresponded to the volumes 125 ml, 250 ml, 500 ml, 1000 ml. For each medium height there were 3 replicates. In each 60 eggs were placed in a controlled temperature chamber (T = 20 $\pm$ 1ºC), with a controlled photoperiod (16L:8D). Since regions with high altitude are expected to suffer a higher impact with climate changes, this assay was only performed with eggs from high altitude.

2.2.4. Endpoints

Throughout the duration of the tests, mortality and abnormalities in tadpoles were registered daily in each replicate.

The tests ended when tadpoles reached stage 25 according to Gosner (1960). At the end of the test, total body length of the tadpoles was registered, and animals were immediately placed in liquid nitrogen following preservation at -80ºC for further biomarkers' analysis.

2.2.5. Oxidative Stress Biomarkers

Oxidative stress in tadpoles was evaluated by antioxidant enzymes activity, and also lipid peroxidation. The following enzymatic activities were determined: glutathione peroxidase (GPx), glutathione reductase (GRed) and glutathione S transferase (GST). Lipid peroxidation was also measured through thiobarbituric acid reactive substances (TBARs) assay.

Previously preserved tadpoles were homogenized in ice-cold phosphate buffer (50 mM, pH= 7.0 with 0.1 %TRITON X-100). Homogenates were centrifuged at 10,000 G and 4ºC for 10 minutes and supernatants were divided into five aliquots with 200 μL each one. One for each determination (total GPx, selenium dependent GPx, GRed, GSTs and TBARS) and a spare one. Aliquots were stored at -80 ºC until determinations were possible.

Lipid peroxidation was measured by the quantification of TBARS, according to the protocol of Buege and Aust (1978). This methodology is based on the reaction of lipid peroxidation by products with thiobarbituric acid (TBA). The amount of TBARS was measured spectrophotometrically as a single determination, at a wavelength of 535 nm (molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$), and results were expressed as nmol of MDA equivalents per mg of sample protein.

GPx activity was measured according to protocol described in Flohé and Günzler (1984). This method consists in following the oxidation of NADPH, at a wavelength of 340 nm (molar extinction coefficient of 6,22 $mM^{-1}cm^{-1}$), when

GSSG is reduced to GSH by glutathione reductase. GPx activity was monitored using both hydrogen peroxide (0.255 mM) and cumene hydroperoxide (0.7 mM) as independent substrates, corresponding, respectively, to selenium-dependent glutathione peroxidase and total glutathione peroxidase.

GRed activity was determined according the protocol of Carlberg and Mannervik (1985). The activity of this enzyme was determined by spectrophotometry at 340 nm, monitoring the oxidation of NADPH mediated by GRed (molar extinction coefficient of 6,22 $mM^{-1}cm^{-1}$).

GSTs activity was determined according to Habig and co-workers (1974), using spectrophotometer. GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene with glutathione, forming a thioeter (molar extinction coefficient of 9,6$nM^{-1}cm^{-1}$) that can be followed by the increment of absorbance at 340 nm.

Protein concentration of each sample was determined in quadruplicate with spectrophotometer with a wavelength of 595 nm, according to Bradford (1976).

2.2.6. Statistical analysis

To analyze the effect of the exposure to different pH ranges and temperature on the growth, antioxidant enzymes activity and LPO the data was analyzed through two-way analysis of variance (ANOVA). When there was no interaction ($p>0.05$) between the two variables (pH and temperature), differences between groups were analyzed through one-way ANOVA for each factor (pH and temperature) followed by a Tukey test. When interaction existed ($p<0.05$) between pH and temperature, data were analyzed through simple main effects ANOVA followed by a multi-comparison Tukey test.

To test the effect of the water volume/column height on tadpole growth and biochemical parameters the data was analyzed through one-way analysis of variance (ANOVA) followed by a Tukey test.

Before testing, normality and homogeneity were checked up to meet statistical demands (Zar, 1996). Data transformations were done when needed.

2.3. Results

2.3.1. Morphological responses

2.3.1.1. Assay 1 - pH and temperature on tadpoles from low altitudes

The duration of the assays was different according to the different tested parameters. In this assay, the duration was different between temperatures. For the 16ºC temperature the tadpoles reached the 25th Gosner stage after 20 days of exposure, while for the 20ºC the tadpoles reached the same developmental stage after 12 days. For the 24ºC, the tadpoles were in the 25th Gosner stage after only 8 days.

At the end of the assay the total mortality was more relevant for the lowest temperature (16ºC), regardless of the pH (pH 5.5= 13 %; pH 6.5= 18 %; pH7.5= 22%). On the other hand, the animals exposed to 20ºC and 24ºC presented the lowest mortality **(T20ºC** pH5.5 = 13%, pH 6.5= 13%, pH 7.5= 11%; **T24ºC** pH5.5 = 8%, pH 6.5= 14%, pH 7.5= 15%). Comparing different temperatures, the mortality was higher in the test exposed to 16ºC. Regarding to pH, except on 20ºC, the highest range of pH had the higher rate of mortality.

Fairly to tadpoles' length, the two-way ANOVA did not show any interaction between pH and temperature ($p > 0.05$). After that, a one-way ANOVA was done for each factor (pH and temperature) individually followed by a Tukey test.

Considering temperatures, within each pH, on pH 5.5 it was found statistically significant differences on tadpoles' size ($F= 6.228$; d.f. = 2; $p < 0.014$) between tadpoles exposed to 16ºC and tadpoles exposed to 20ºC. In pH 6.5, exists statistically significance differences in tadpoles' size ($F=6.993$; d.f. = 2; $p < 0.016$) between the 16 and 20ºC exposures. As for pH 7.5 significant differences were obtained in total body length between tadpoles exposed to temperature of 16ºC and tadpoles exposed to temperatures of 20 and 24ºC ($F= 16.684$; d.f. = 2; $p < 0.001$) (Fig. 2).

Considering pH within each temperature, for the 16ºC temperature significant differences were obtained in size (F= 7.955; d.f. = 2; p < 0.006) between pH 7.5 and the other two ranges of pH (5.5 and 6.5). For the pH ranges within the 20ºC and 24ºC temperatures no significant differences were found among them (Fig. 2).

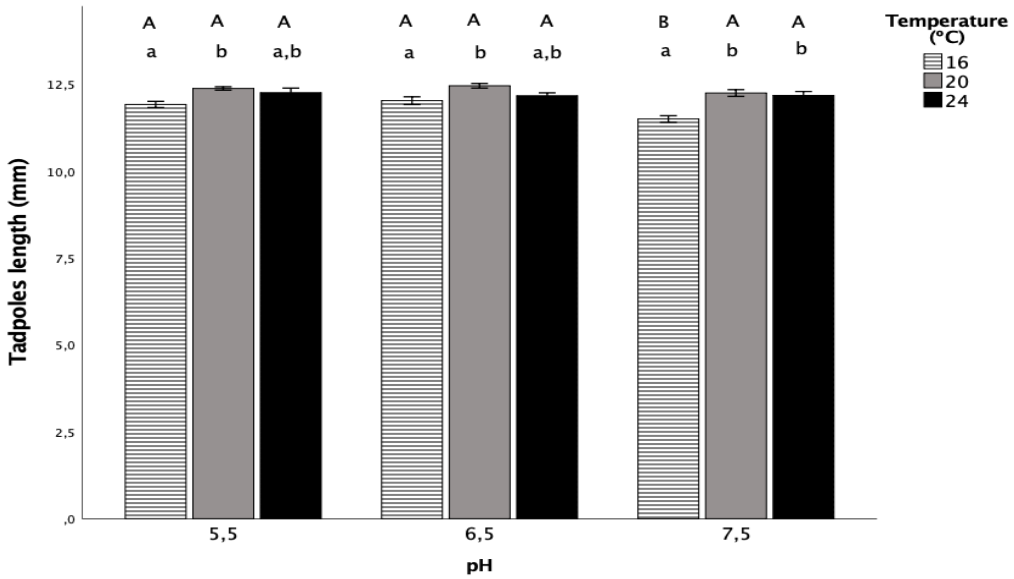

Figure 2: Mean of the size of tadpoles of *Pelophylax perezi* exposed to different pH and different temperatures. Letters “a” and “b” represent significant statistic differences ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically significant differences ($p < 0.05$) between pH for each temperature within pH. Error bars represent standard error.

2.3.1.2. Assay 2 pH and temperature on tadpoles from high altitudes

In this assay, just as it happened in the first assay, the duration of the test was influenced by the different temperatures. The duration of each test for each temperature was equal to assay 1, with tadpoles exposed to 16ºC reaching the 25th Gosner stage after 20 days of exposure, while tadpoles exposed to 20ºC the tadpoles reached the same developmental stage after 12 days. For the 24ºC, the tadpoles were in the 25th Gosner stage after only 8 days.

The mortality results presented the same trend between temperatures. Nonetheless, mortality was much lower on 20ºC and 24ºC (**T20ºC** pH5.5 = 5%,

pH 6.5= 13%, pH 7.5= 7%; **T24ºC** pH5.5 = 1%, pH 6.5= 3%, pH 7.5= 4%) than the first assay. On the other hand, the mortality on the 16ºC was superior than on the same temperature on the first assay (pH 5.5= 21 %; pH 6.5= 29 %; pH7.5= 20%). In relation to pH the highest pH (6.5 and 7.5) had the highest mortality.

Regarding to the length of tadpoles, also in this assay, no interaction between the both factors were found ($p > 0.05$) in two-way ANOVA. After that, a one-way ANOVA was performed for each factor (pH and temperature) followed by a Tukey test.

Considering temperatures within each pH, no statistically significant differences on size were found, for the pH 5.5. However, for the pH 6.5 exposure, tadpoles exposed to 24ºC were significantly different from the tadpoles exposed to 16º and 20ºC ($F= 15.495$; d.f. = 2; $p < 0.001$). Finally, for the pH 7.5 there were significant differences on sizes ($F= 5.361$; d.f. = 2; $p < 0.022$) between the 20ºC and 24ºC exposures (Fig. 3).

Considering pH within each temperature, for the 16ºC and 20ºC exposures there were no statistically significant differences on the size of tadpoles of *P. perezi,* between pHs. However, exposures at 24ºC had significative differences ($F= 5.573$; d.f. = 2; $p < 0.019$) between pH 5.5 and 7.5 (Fig. 3).

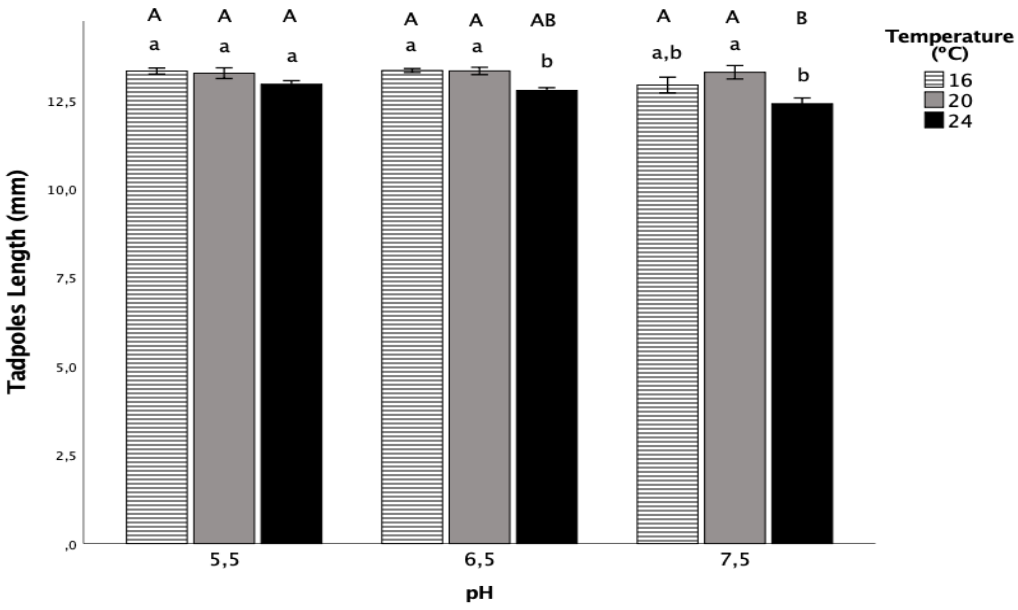

Figure 3: Mean of the size of tadpoles of *Pelophylax perezi* exposed to different pH and different temperatures. Letters “a” and “b” represent significant statistic differences ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically significant differences ($p < 0.05$) between pH for each temperature within pH. Error bars represent standard error.

2.3.1.2. Assay 3 – water volume/ water column

In this assay, when testing different volumes/water column heights at 20ºC, the tadpoles in the lower volume reached the 25th stage after 8 days, while for the remaining volumes (250, 500 and 1000 ml) the time needed for reaching the same stage was 12 days.

The mortality was higher in tadpoles exposed to the lower water volumes (125ml = 11 % and 250 ml=14 %), while the 500 and 1000ml tests had less than half of mortality (500 ml = 5% and 1000 ml = 7%).

As regards as the total body length of tadpoles, for this assay, were found significant differences between the 125ml volume ($F= 15.047$; d.f. = 3; $p <0.001$) and all the other tested volumes, presenting shorter body lengths (Fig. 4).

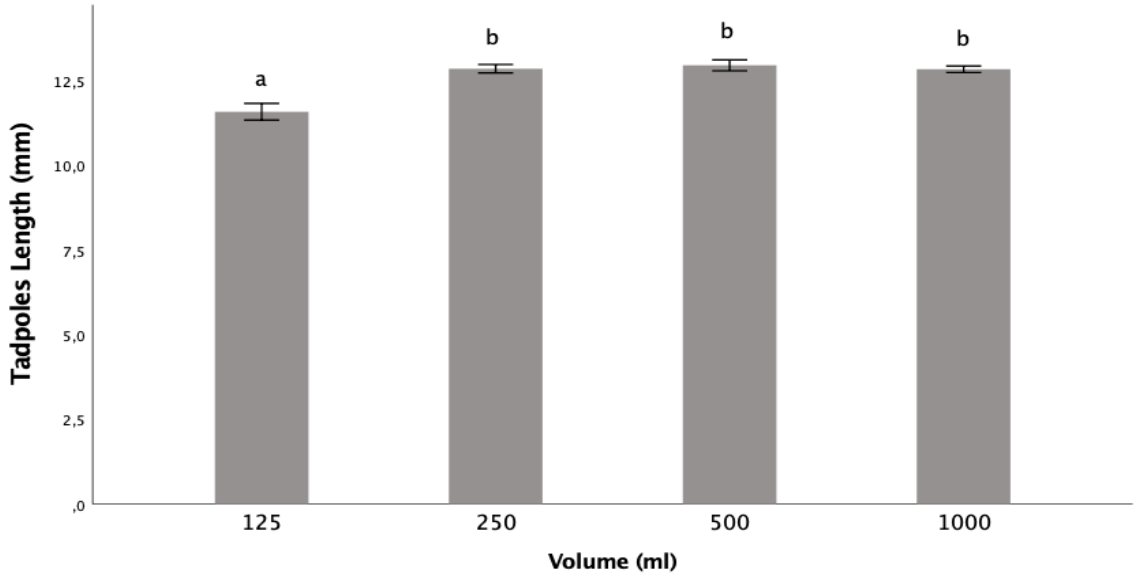

Figure *4*: Mean tadpoles’ size of *Pelophylax perezi* exposed to different water volumes. The different letters, “a” and “b” represent statistically different groups ($p < 0.05$) between different water volumes exposures. Error bars represent standard error.

2.3.2 Physiological responses

2.3.2.1. Assay 1- pH and temperature on tadpoles from low altitudes

The effect of temperature and pH ranges on oxidative stress biomarkers activity (GRed, selenium dependent GPx, total GPx and GSTs) and on lipid peroxidation (TBARS) of tadpoles, as well as interactions between factors were tested by two-way analyses of variance. A significant interaction between both factors was recorded for TBARS ($F= 3.699$; $d.f.= 4$; $p < 0.014$) and for GRed ($F= 3.126$; $d.f.= 4$, $p < 0.026$). Afterwards a simple main effect ANOVA was performed for TBARS and GRed. Relatively to TBARS, significant differences ($F= 5.552$; $d.f.= 2$; $p < 0.020$) were found between the temperature 16ºC and the other temperatures, on pH 5.5 but no other significant differences were recorded for TBARS (Fig. 5). For GRed significant differences were found between activities in the 24ºC and the rest of the temperatures on pH 5.5 ($F=13.133$; $d.f.=2$; $p<0.001$). Also, significant differences were obtained between the GRED activity between pH 5.5 and 6.5 within the 24ºC temperature ($F= 4,924$; $d.f. = 2$; $p< 0,027$) (Fig. 6).

When regarding GST, selenium dependent GPx and total GPx, there were not found any interaction statically significative between pH and temperature, so a one-way ANOVA followed by Tukey test was performed.

Considering the pH within each temperature, the GST activity did not show any statistical differences. Also, for the same enzyme, when considering temperatures within each pH, there were no significant differences between temperatures on pH 5.5 and pH 7.5. However, for the pH 6.5 the GST activity had significant differences ($F= 5.589$; $d.f. = 2$; $p < 0.019$) between the 24ºC exposure and the two other temperatures (16 and 20ºC) (Fig. 7).

For the total GPx activity, when considering pH ranges within each temperature there were significative differences ($F= 11.329$; $d.f. = 2$; $p < 0.002$) between pH 5.5 and the other pHs at 20ºC. When regarding the activity of total GPx, comparing temperatures within each pH range, differences were observed for pH range 5.5 ($F=10.198$; $d.f.=2$; $p < 0.003$) between the 24ºC and the 16ºC

and 20ºC, with the tadpoles exposed to the 24ºC presenting a higher activity (Fig. 8).

The activity of selenium dependent GPx did not reveal any statically significant difference either when analyzing the activity in pHs within temperatures, or in temperatures within each pH range (Fig. 9).

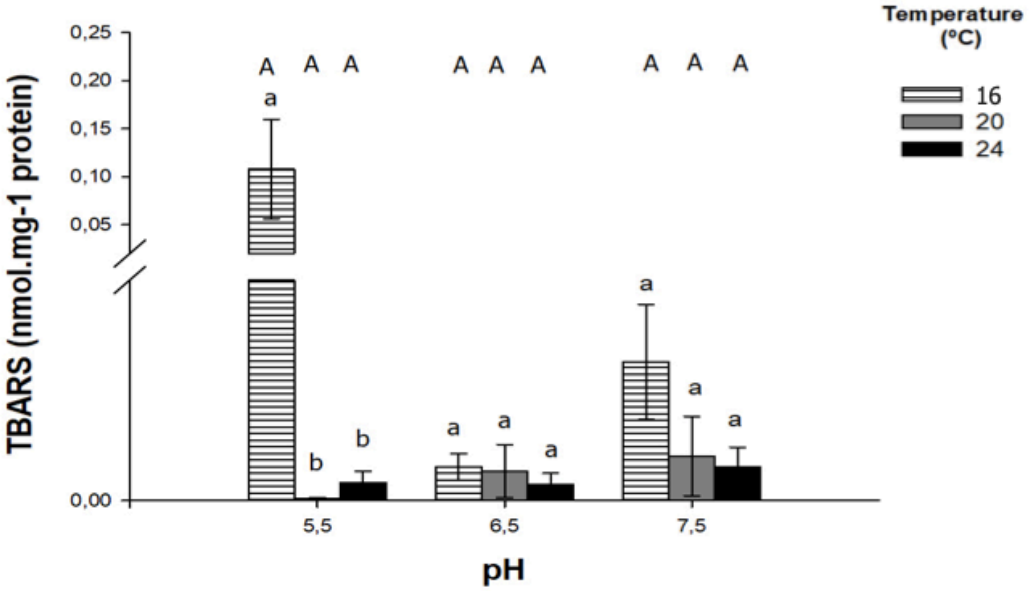

Figure 5: Mean content of thiorbarbituric acid reactive substances (TBARS) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different ($p < 0.05$) groups between pH within each temperature. Error bars represent standard error.

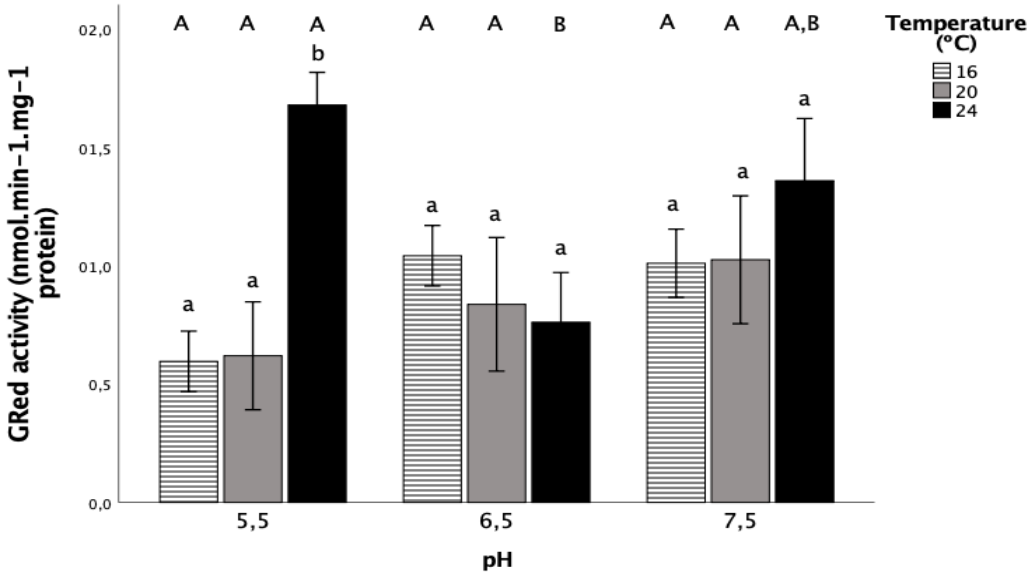

Figure 6: Mean content of Glutathione Reductase (GRed) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

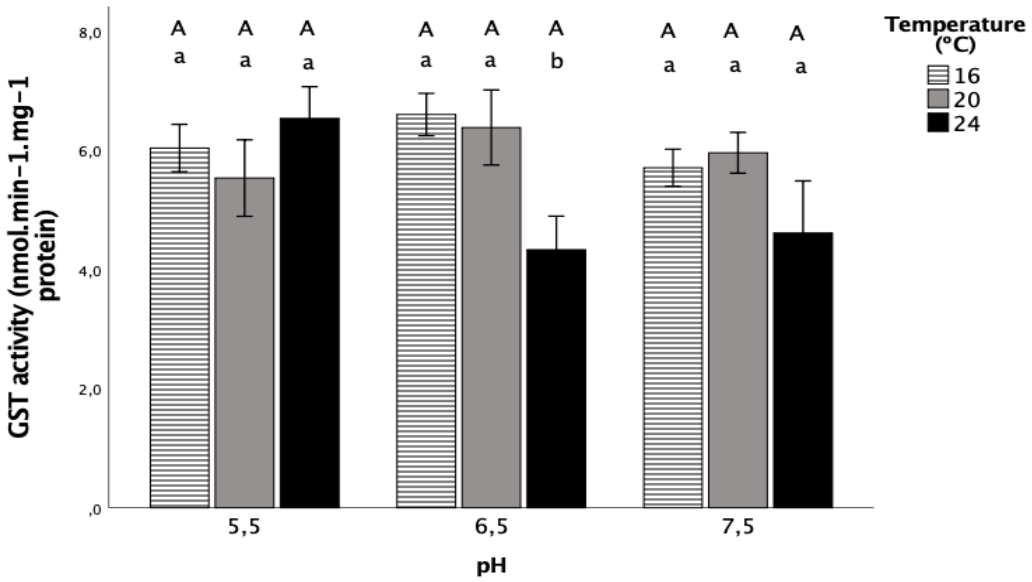

Figure 7: Mean content of Glutathione S Transferase (GST) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

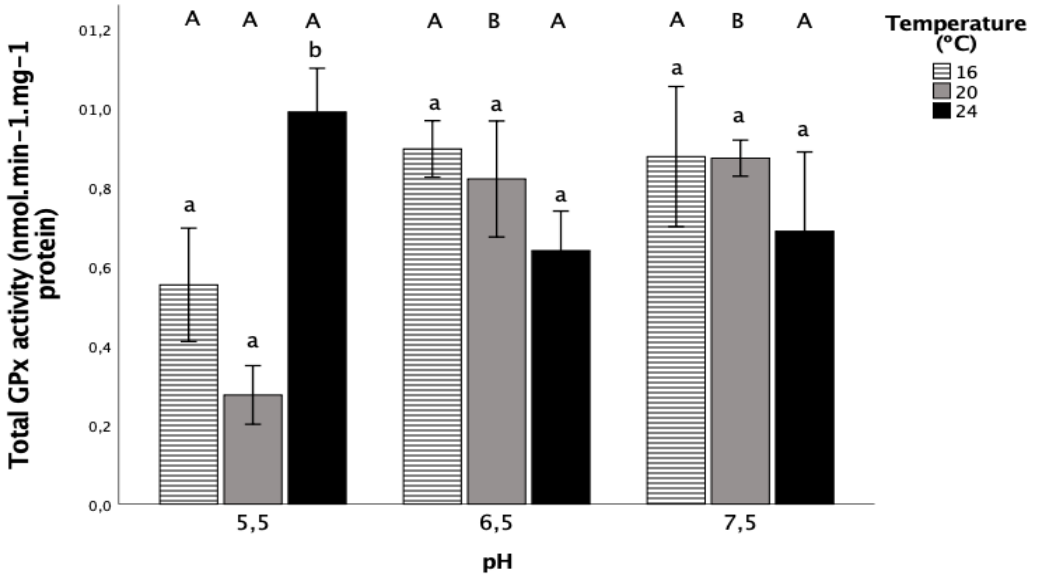

Figure 8: Mean content of Total Glutathione Peroxidase (Total GPx) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

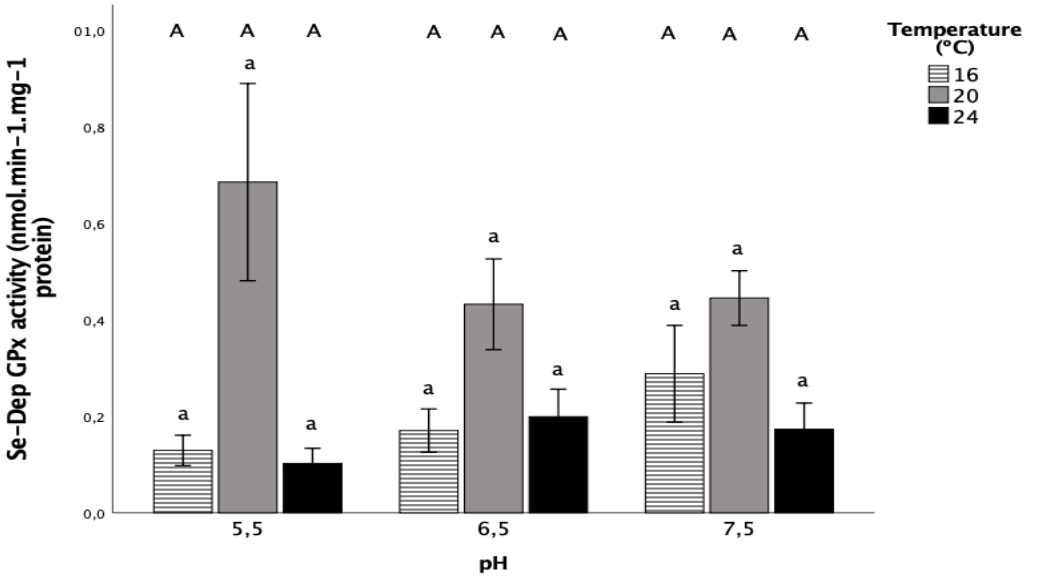

Figure 9: Mean content of Selenium Dependent Glutathione Peroxidase (Selenium Dependent GPx) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

2.3.2.2. Assay 2 - pH and temperature on tadpoles from high altitudes

In this assay, a significant interaction between both factors (pH and temperature) was recorded for TBARS ($F=7.191$; d.f.= 4; $p< 0.001$), GRed ($F= 5.118$; d.f.= 4; $p<0.002$), Total GPx ($F= 4.179$; d.f.=4; $p<0.007$) and Selenium Dependent GPx ($F= 3.396$; d.f.=4; $p<0.019$). Afterwards a simple main effect ANOVA was performed to each one of these biomarkers.

The quantification of thiobarbituric acid reactive substances (TBARS), showed significant differences ($F= 5.249$; d.f.= 2; $p< 0.04$) between pH 5.5 and 6.5 within the 16ºC temperature. Relatively to pH, differences were found on pH 5.5 ($F= 5.741$; d.f.= 2; $p< 0.022$) between 16º and 20ºC; on pH 6.5 the differences were found between 24ºC and the other temperatures ($F= 15.730$; d.f.= 2; $p< 0.001$); the same occurred on pH 7,5, with differences ($F= 25.658$; d.f.= 2; $p< 0.001$) between 24ºC and both the other temperatures (16º and 20ºC) (Fig. 10).

For the GRed activity statistical differences, when comparing temperatures within each pH range, were as follows: for pH 5.5 differences ($F=11.394$; d.f.= 2; $p< 0.002$) were between 24ºC and both the other temperatures (16 and 20ºC); for pH 6,5 the difference ($F= 19.329$; d.f.=2; $p<0.001$) were between temperature 16º and both 20 and 24ºC; and for pH 7.5 the difference was between 16ºC and the 20 and 24ºC ($F= 27.639$; d.f.= 2; $p< 0.001$). When considering the GRed activity comparison between pH ranges within temperatures the only significant difference ($F= 22.525$; d.f.= 2; $p< 0.001$) was between pH 5.5 and both the other pHs for the 20ºC temperature (Fig. 11).

Relatively to the total GPx activity, when comparing temperatures within each pH range there were significant results ($F= 32.336$; d.f.=2; $p<0.001$) between 24ºC and both other temperatures for the pH 5.5. For the pH 6.5 the differences ($F= 6.240$; d.f.= 2; $p<0.014$) were between the 16ºC temperature and the 20 and 24ºC. There were also differences for the pH 7.5 ($F= 23.906$; d.f.=2; $p<0.001$), where the activities were all different between each other. Considering differences between pH within each temperature, the differences were found on T20 ($F= 7.885$; d.f.=2; $p<0.007$) between the pH 5.5 and the others pHs and on

T24 (F= 5.710; d.f.=2; p<0.001) the differences were between pH 5.5 and 7.5 (Fig. 12).

Still in the biomarkers where interaction was observed between variables, the differences found on selenium dependent GPx (F= 4.614; d.f.=2; p<0.033) were only between the temperature of 24º and 16ºC within the pH 5.5 (Fig. 13).

For GST, that did not present any interaction, a one-way ANOVA was performed for each factor.

Considering the pH within each temperature, there were no statistically significant differences in GST's activity. On the other hand, statistical differences (p<0.05) were found between tadpoles exposed to 24ºC and the ones exposed to the other temperatures within the 5.5 pH range (F= 15.679; d.f. = 2; p < 0.001). Also, within the pH 6.5 statistical differences (F= 21.546; d.f. = 2; p < 0.001) were observed between the three temperatures (Fig. 14).

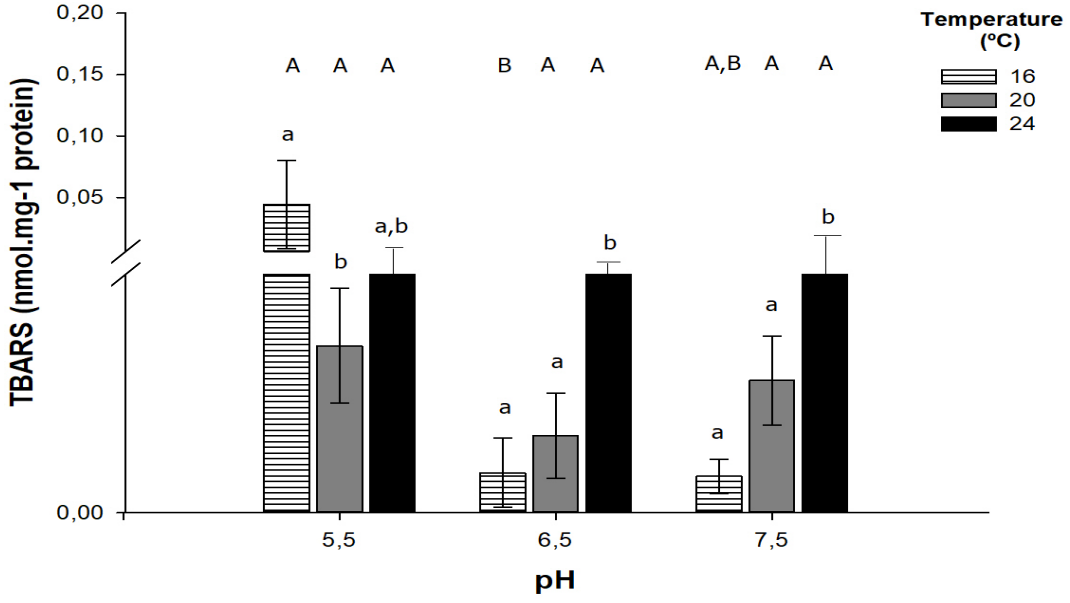

Figure 10: Mean content of thiorbarbituric acid reactive substances (TBARS) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters "a" and "b" represent statistically different groups (p < 0.05) between different temperatures within each pH. Letters "A" and "B" represent statistically different groups (p < 0.05) between pH within each temperature. Error bars represent standard error.

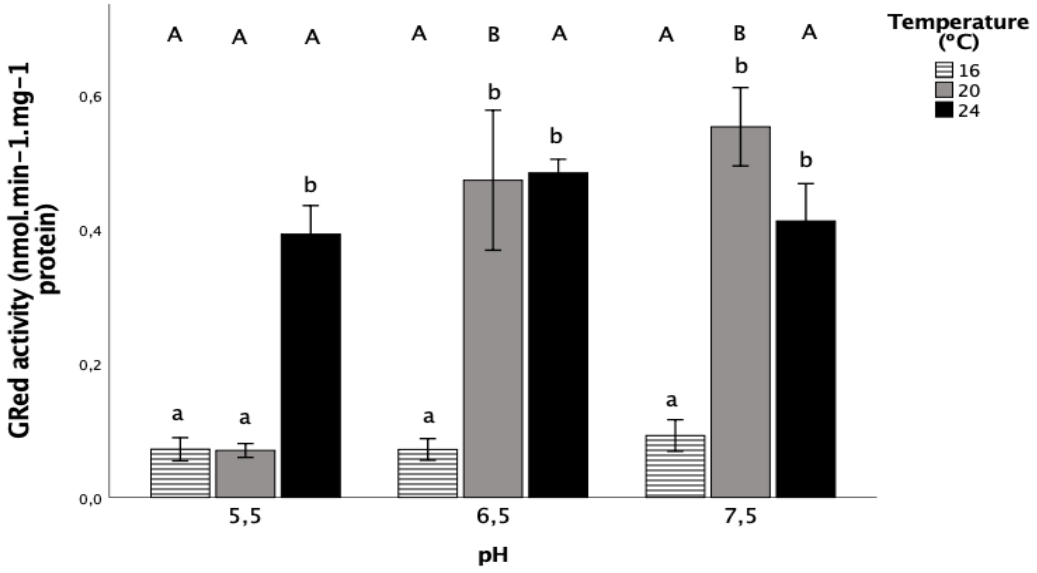

Figure 11: Mean content of Glutathione Reductase (GRed) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

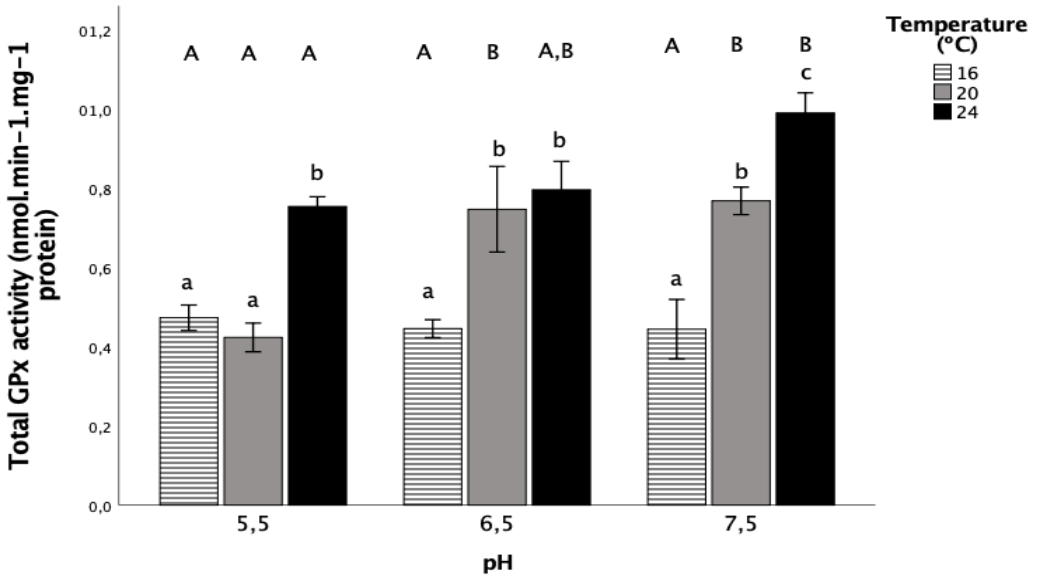

Figure 12: Mean content of Total Glutathione Peroxidase (Total GPx) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

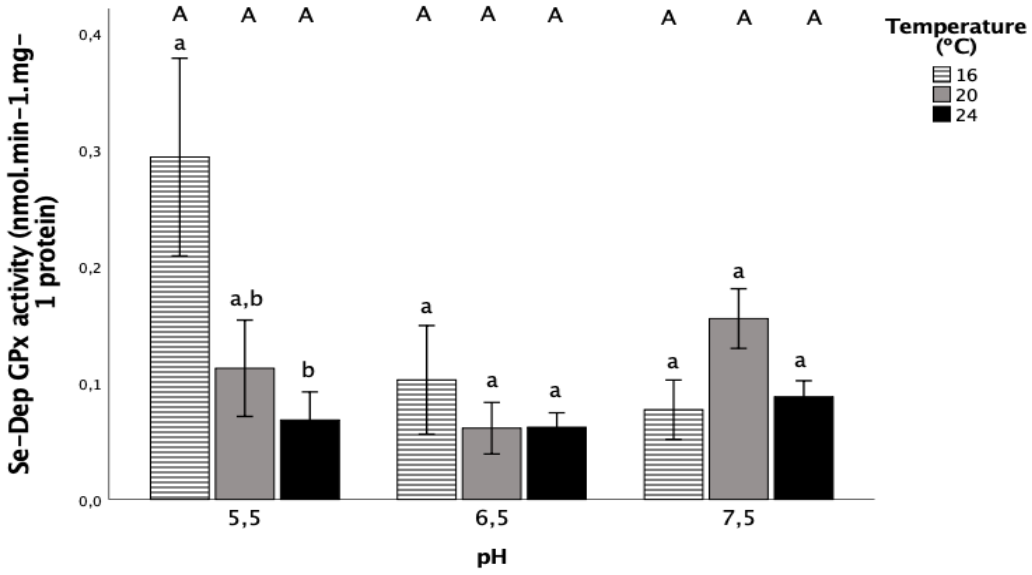

Figure 13: Mean content of Selenium dependent Glutathione Peroxidase (GPx) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a” and “b” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters “A” and “B” represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error

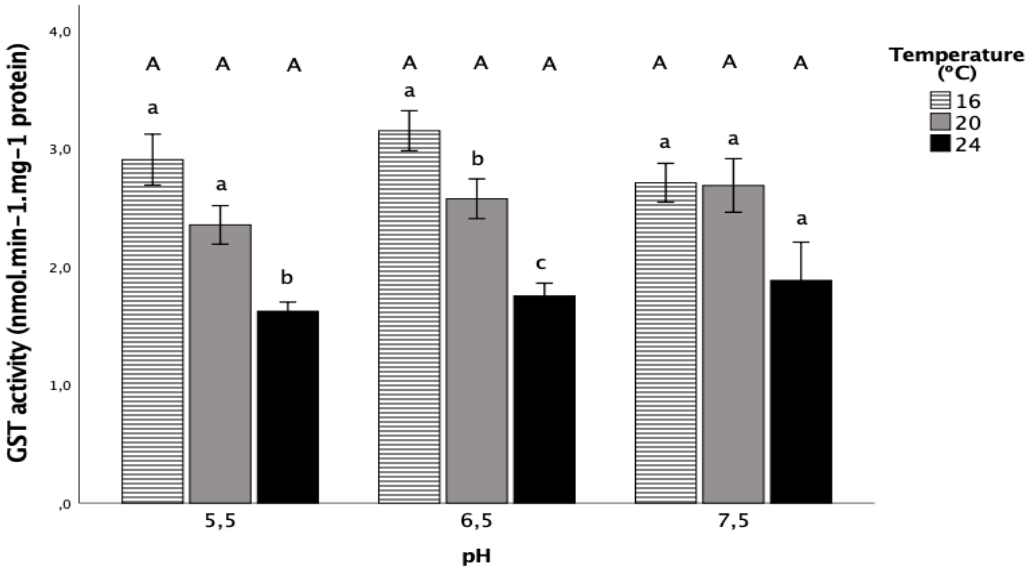

Figure 14: Mean content of Glutathione S Transferase (GST) on tadpoles of *Pelophylax perezi* exposed to different pH and temperatures. Letters “a”, “b” and “c” represent statistically different groups ($p < 0.05$) between different temperatures within each pH. Letters A and B represent statistically different groups ($p < 0.05$) between pH within each temperature. Error bars represent standard error.

2.3.2.3. Assay 3 – water volume/ water column

When considering the exposure to different water volumes the determination of lipid peroxidation through TBARS only revealed significant differences ($F= 787.412$; d.f. = 3; $p < 0.001$) between tadpoles exposed to 125 ml when compared to tadpoles exposed to every other volume, being that tadpoles exposed to 125ml test had greater lipid peroxidation (Fig. 15).

The determination of GST activity (Fig. 16) revealed significant different activity for the 500ml exposure ($F= 19.379$; d.f. = 3; $p< 0.001$). Tadpoles in this volume presented higher activity of this enzyme, when compared to the other tested volumes. As for of the activities of GRed (Fig. 17), total GPx (Fig. 18) and Selenium dependent GPx (Fig. 19), no statistical differences were obtained between tadpoles exposed to different volumes.

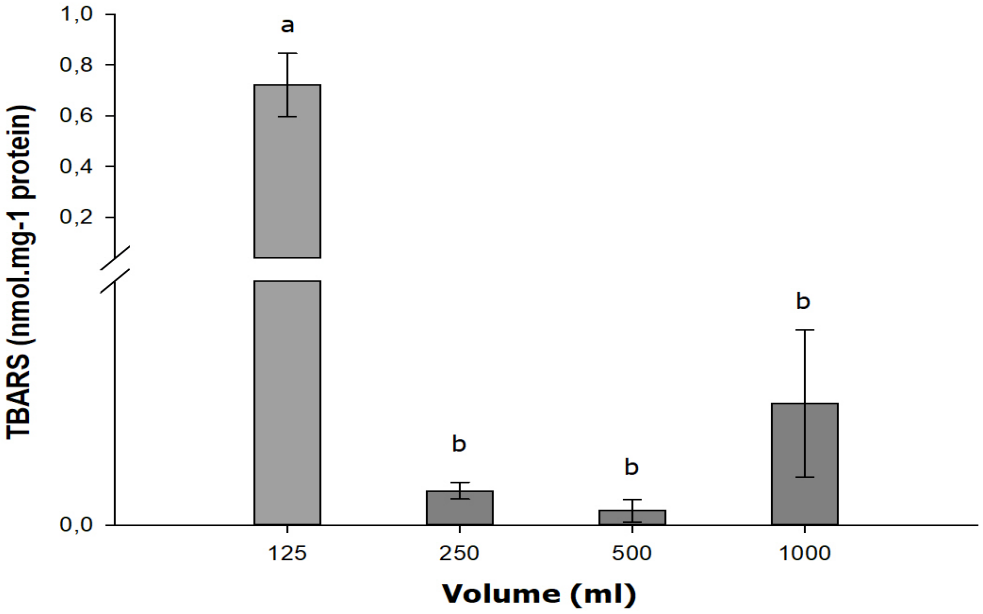

Figure 15: Mean content of thiobarbituric acid reactive substances (TBARS) on tadpoles of *Pelophylax perezi* exposed to different water volumes. The different letters, "a" and "b", represent statistically different groups ($p < 0.05$). Error bars represent standard error.

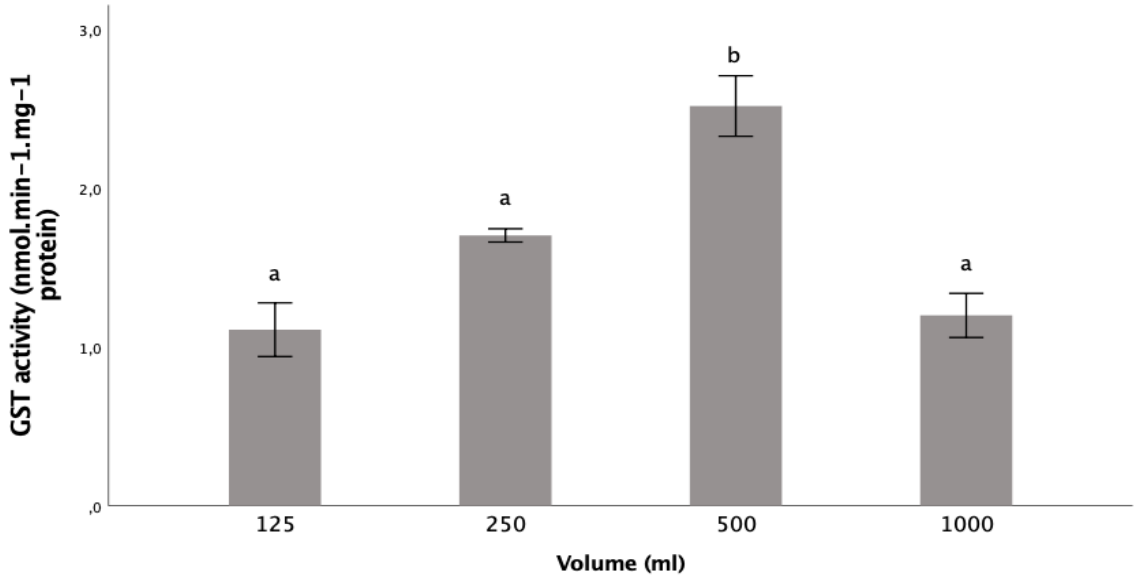

Figure 16: Mean content of Glutathione S transferase (GST) on tadpoles of *Pelophylax perezi* exposed to different water volumes. The different letters, “a” and “b”, represent statistically different groups ($p < 0.05$). Error bars represent standard error.

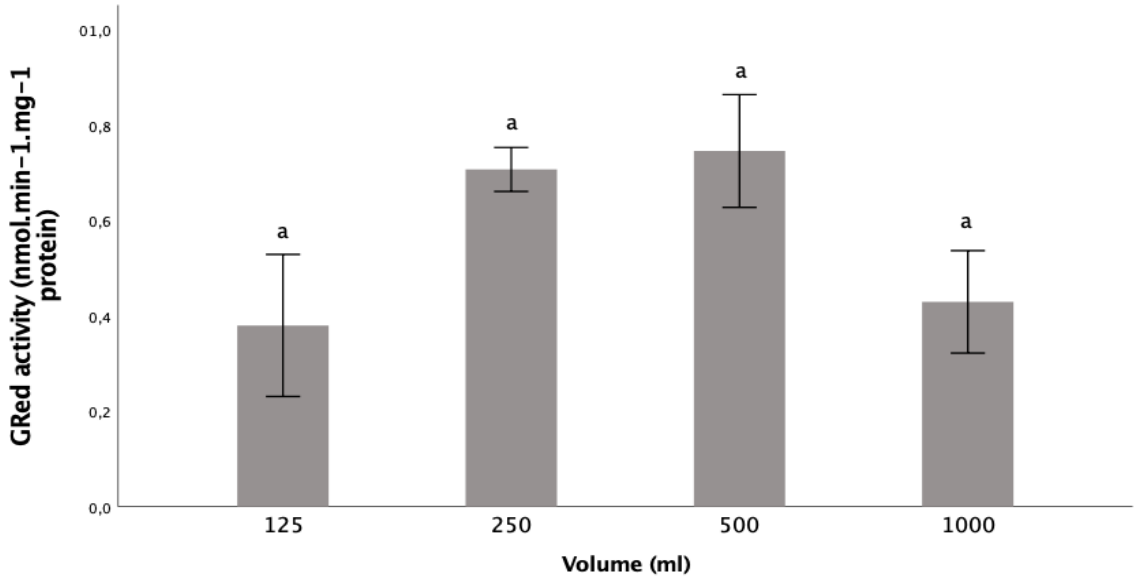

Figure 17: Mean content of Glutathione Reductase (GRed) on tadpoles of *Pelophylax perezi* exposed to different water volumes. The letter “a” represents a unique statistical group. Error bars represent standard error.

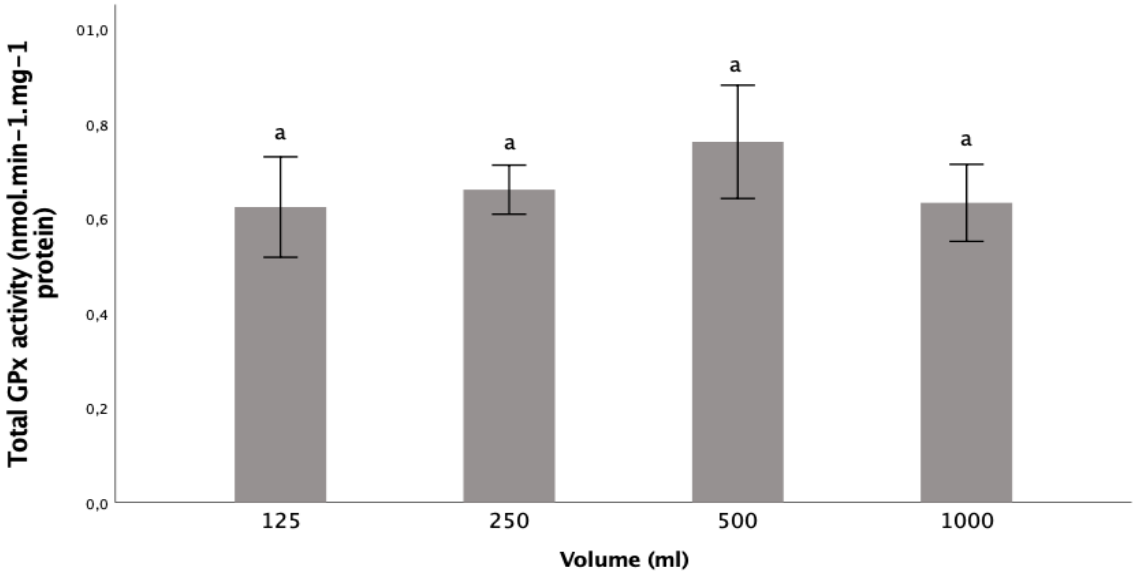

Figure 18: Mean content of Glutathione Peroxidase Total (GPx Total) on tadpoles of *Pelophylax perezi* exposed to different water volumes. The letter "a" represents a unique statistical group. Error bars represent standard error.

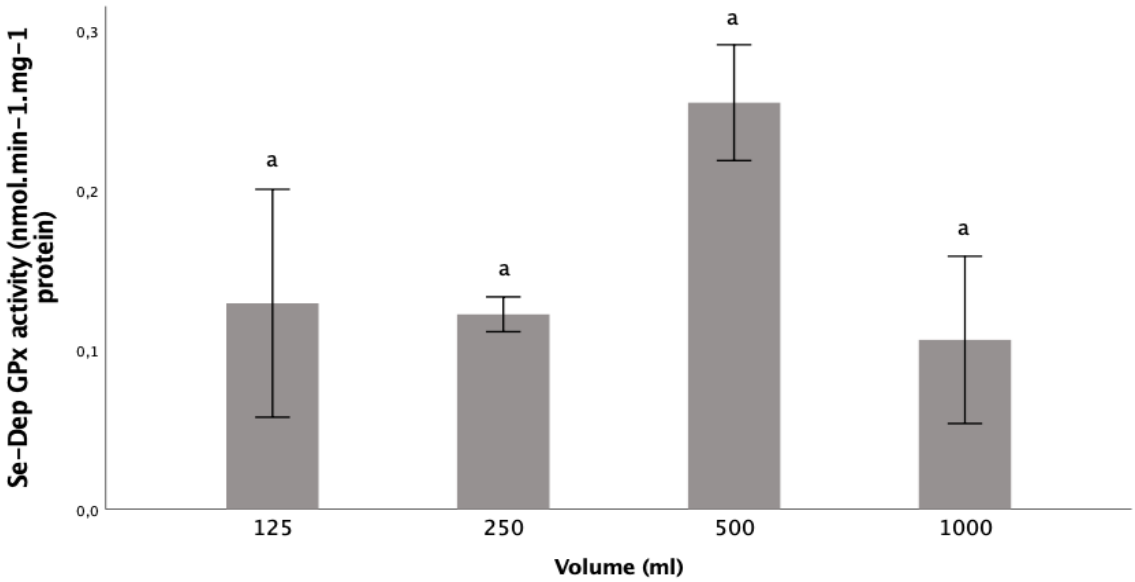

Figure 19: Mean content of Glutathione Peroxidase Selenium Dependent (GPx Selenium Dependent) on tadpoles of *Pelophylax perezi* exposed to different water volumes. The letter "a" represents a unique statistical group. Error bars represent standard error.

2.4. Discussion

In the past decades, the populations of amphibians have been declining, with some species facing extinction and one of the possible factors contributing for this is climate change (Blaustein et al., 2010). According to Guiot and Cramer (2016) and also Abrantes and co-workers (2017) the effects of climate change in the Iberian Peninsula will result in an increase of temperature, higher than the mean global increase, and also an alteration of the precipitation regime resembling a short rainy season. Furthermore, and even if there are still few evidences for freshwater ecosystems, the increase of CO_2 has been contributing for decreasing the pH of some freshwater reservoirs (Weiss et al., 2018). Considering that two of the most relevant parameters for ectotherms, namely amphibians, are temperature (Niehaus et al., 2006) and water pH, and that they will predictably change in the present scenario of climate change, it is important to perceive how they will affect vulnerable groups such as amphibians. A previous study by Marques et al. (2018) just provided a first insight over this topic by focusing on the effects of temperature and larval density in *P. perezi*, however if the acid deposition on water, through the climate change occurs, it may be very harmful to amphibians' species (Home and Dunson, 1995) and it's important not to overlook this factor. Besides that, it is also important to perceive if there is an interaction between pH and temperature in tadpoles of *Pelophylax perezi*. In addition, the water level/volume is a very important factor too, because of the high risk of desiccation of temporary ponds due to high water temperatures and shallowness (Sanuy et al., 2008).

In our pH/temperature experimental assays, tadpoles exposed to the lowest temperature (16ºC) had the highest mortalities in both assays 1 and 2.

Relatively to the development time, it decreased in higher temperature exposures. These results support another study done with this species, where in two ranges of tested temperatures (24 and 28ºC), the tadpoles had a delay in the development time between the both temperatures, being that tadpoles exposed to 28ºC had a faster development (Rodrigues, 2010). Similar results were found in a study with another species of anuran amphibian (*Epidalea calamita*) exposed

to five different ranges of temperatures (22, 24, 26, 28 and 30ºC) and with a rapid development for the animals exposed to high temperatures (Sanuy et al., 2008). Since amphibians are ectothermic higher temperatures will contribute for higher metabolic rates and thus fasts development. The fast development can present some advantages because the larval stages (tadpole stage) are more vulnerable to predation (Brodie and Formanowicz, 1983).

Temperature and pH had no interaction on the length of tadpoles in neither assay 1 or 2. In the assay with tadpoles from low altitudes, the temperature had a higher influence on size than pH. In other words, tadpoles placed on lower temperatures show smaller body length than tadpoles in higher temperatures. On the previous study done by Marques et al. (2018), the results were identical, and individuals exposed to 28ºC presented larger body lengths than the individuals exposed to 16ºC. Nonetheless, the assay with tadpoles collected from high altitudes, tadpoles placed on chambers with high temperatures and high value of pH had smaller size than tadpoles on low pH and low temperatures. A study with the amphibian *Triturus vulgaris*, presented similar results: the individuals exposed to neutral pH and higher temperatures had a smaller length than the individuals exposed to smaller temperatures and acidic pH (Griffithsand and Wijer, 1994). Nevertheless, these results may suggest that the tadpoles from high altitudes can be more susceptible to the increase of temperature, most probably related to the fact that during the breeding season and early stages of development, the temperature in high altitudes is typically low, and the population might not have the mechanisms to enable coping with the stress of higher metabolic rates caused by higher temperatures. Duarte and co-workers (2012) observed that, for two thermally distinct communities (warm community and cool community) of larvae, the critical thermal limits were distinct, with the warm community presenting higher thermal limits and thus coping better with higher temperatures. Besides temperature, in the assay with animals from high altitudes, body length was also affected by pH, namely for the higher temperature (24ºC), with tadpoles presenting smaller sizes for the highest pH. Discordant results were obtained by Freda and Dunson (1986) on American species of anurans (*Hyla andersonii* and *Anaxyrus woodhousii*). In this study, the growth of these two species was reduced

by lower pH. But on this study, the range of pH testes were smaller than our study – pH 3.75; 4.00 and 4.50 to *H. andersonii* and pH 4.10; 4.50 and 6.00 to *A. woodhousii*, which could justify these differences between the two studies.

When regarding the lipid peroxidation results (TBARS) we see that, on assay 1, the tadpoles who presented the major lipid damage were tadpoles exposed to pH 5.5 and 16ºC. In the other exposures the differences were not significant. On assay number 2, we have the same peak of lipid peroxidation on exposures with pH 5.5 and 16ºC. However, and in contrast with the assay 1, significant differences were observed between temperatures within all ranges of pH. Once again it is possible that, being from thermally different areas, the animals from high altitude present less tolerance for high temperatures. Within the pH ranges 6.5 and 7.5, the differences between temperatures had an inversion comparatively to the pH 5.5 exposures. The temperature may mediate the production of ROS through an increase of the metabolism and thus inducing lipid peroxidation (Parihar et al., 1996), which may justify this greater lipid peroxidation on higher temperatures. Nonetheless it doesn't support the higher value of TBARS for the 16ºC. Marques and co-workers (2018), in a study with temperatures on *Pelophylax perezi*, had higher lipid peroxidation on the lowest temperature (16ºC). The peak of lipid peroxidation may be supported by the interaction between the two factors, that is, the two factors conjugated (acidic pH and low temperature) can be detrimental to the *P. perezi* tadpoles and induce a bigger damage. Furthermore, we must take into consideration that at 16ºC the larvae reached the 25th Gosner stage after 20 days, which is a long exposure to the low pH, when compared to the 12 and 8 days for the 20 and 24ºC exposure, respectively, for the same pH.

Relatively to the enzymes activity, on both assays, higher temperatures induced the activities of GRed and total GPx leading to an increased activity. As for GST and selenium dependent GPx, the registered differences indicate a lower activity of these enzymes in response to higher temperatures. Studies with other aquatic organisms show that oxidative stress increased due to the increased of temperatures (Madeira et al., 2013; Verlecar et al., 2007), however, the oposite has also been observed (Lushchak and Bagnyukova, 2006). When considering

pH, for the first assay, a higher activity of GRed and total GPx were found in response to lower pH values. On assay 2, the results for these two enzymes (GRed and total GPx) were contradictory, with a decreased of activity for the lower pH range. GST and selenium dependent GPx did not show any differences between ranges of pH. A study with tadpoles from a south American anuran (*Eupemphix nattereri*) shows an increase of GRed and GST activities in response to a decrease of pH (Freitas and Almeida, 2016).

Regarding the volume assay, the tadpoles were exposed to different water volumes, being the lower volumes the simulation of desiccation. The water volume influenced mortality, with the condition with the lowest volume presenting higher mortality. Thus, for *P. perezi* a low water volume can constitute a relevant stress factor. In a study with *Pelobates cultripes* (another anuran amphibian with a strong presence in Iberian Peninsula), the decreasing of water level reduced the survival of this species (Gomez-Mestre et al., 2013), which was a result similar to obtained in our experiment. Also, for the lowest volume tested tadpoles presented a faster development time comparatively to the other exposures, presenting nonetheless, smaller body sizes. A south American anuran (*Rhinella spinolusa*), exposed to three desiccation regimes presented similar results to our study, with: tadpoles from ponds with high and medium desiccation developing faster and being smaller than individuals in the low desiccation regime (Márquez-García et al., 2009). Also, in a study with the European Common Frog (*Rana temporaria*), the development time speeded up which resulted in an advancement of two days on experimental assays with low water volume (Loman, 1999).

At the biochemical level, the lipid peroxidation was influenced by water volume, with the tadpoles exposed to lower water volumes presenting higher LPO. It is possible that, in an attempt to “escape” the stress condition, tadpoles increase their metabolism to develop faster, which was observed in our assay. If such metabolic increase is not followed by the antioxidant defense system, LPO may occur. Similar results were found in a study with spadefoot toad (*Pelobates cultripes*) larvae, where the faster development in response to pond drying induced a higher lipid damage (Burraco et al., 2017). GST activity was higher on

tadpoles exposed to 500 ml, and no differences were found on the other volumes tested. Besides that, none of the other enzymes (GRed, total GPx and selenium dependent GPx) had significative differences. In the study of Gomez-Mestre et. al (2013), the larval stages of *Pelobates cultripes* with low and high water were tested for antioxidant activity defense system (CAT, SOD and GPx) and LPO was measured too. The results of GPx for this specie shown no differences between the water volumes, just as happened in our study. Also, in that study, for TBARS no differences were found for the early larval stages but after stage 42, the larval stages exposed to low water had higher values of lipid damage.

Overall the results of our assays reveal that changes in the abiotic parameters tested may induce, in some specific conditions, stress for the early life stages of *P. perezi*. The stress conditions are mainly associated with the higher temperatures, or eventually the lower temperature if interacting with low pH. Also, for animals from high altitudes the increase of temperature will present a higher stress than for animals from low altitudes. Such results may indicate that in the case of mean temperature increase in mountain areas of the Iberian Peninsula, some populations of amphibians may suffer deleterious effects from thermal stress, due to a lower thermal tolerance, as observed by Duarte and co-workers (2012).

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Chapter III

Final remarks

3. Final remarks

Climate change is undeniably occurring, and it encompasses several stressors such as temperature changes and elevated CO2 levels, which will cause direct and indirect effects in biota. According to some of the current predictions (Calbó et al., 2012; Guiot and Cramer, 2016) the Iberian Peninsula will have a temperature increase higher than the global mean and will have a decrease in precipitation. Nonetheless the warming will not be spatially uniform, being specially higher in mountain regions (Bronnimann et al., 2005). Undoubtedly, such alterations can affect biota, namely amphibians. Considering this, our study aimed at providing further understanding of the consequences that the alteration of some environmental abiotic parameters may have on amphibians. Our approach consisted in assessing, in a full factorial experimental design, the effects of pH and temperature in the larval stages from both high and low altitude populations. As a result, from our approach we perceived that temperature presented higher influence than pH, namely in development speed and also in size. The trend was for higher temperatures to promote faster development, however, for size, it varied according to the origin of the larvae. Low altitude larvae presented higher lengths in higher temperatures, nonetheless for high altitude larvae the opposite was observed. Also, the higher ranges of pH affected negatively the tadpoles collected from high altitude. The physiological parameters assessed also revealed higher thermal driven stress on larvae from high altitudes when exposed to higher temperatures. To complement the scenario for high altitude we performed also a water volume assay with the larvae, to give an insight of what would be the effect of a desiccation scenario for these larvae. For this assay, the tadpoles that were exposed to 125 ml had faster development and smaller body lengths. Also, on a physiological level, they presented higher values of lipid damage. These results suggest that a scenario of decreased water volume (desiccation) can be prejudicial to *Pelophylax perezi*, as already observed for other amphibian species (Gomez-Mestre et al., 2013; Loman, 1999; Márquez-García et al., 2009; Tejedo and Reques, 1994).

Overall, the most relevant results from this study suggest that in a climate change scenario, mainly through increase of temperature, amphibian populations

from high altitudes will most probably be the ones most affected. Eventually, in extreme situations it is possible for populations of some amphibian species to disappear from high altitudes. Nonetheless, despite the bases provided by this study about how these abiotic factors (pH, temperature, desiccation) will affect the larval stages of *Pelophylax perezi*, more studies are needed to assess how these organisms will react to climate change. There are also other abiotic factors, related to climate change, that may influence this Iberian water frog. Therefore, further studies could be made with the influence of UV-B in *Pelophylax perezi*, as already done with the anuran specie *Rana pipiens* (Long et al., 1995). It would also be interesting to compare the genetic expression between *P. perezi* populations from different altitudes exposed to abiotic factors (Beebee, 2005; Gienapp et al., 2008) to increase the understanding over the mechanisms affected by these factors.

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