Universidade de Aveiro
Ano 2011
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Toxicity interaction of copper and salinity on Perez frog life stages

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, Biodiversidade e Gestão de Ecossistemas realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar e Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Ruth Maria de Oliveira Pereira, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar e do Departamento de Biologia da Universidade de Aveiro.

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o júri

presidente

Prof. Doutor Fernando José Mendes Gonçalves
professor associado com agregação, Departamento de Biologia da Universidade de Aveiro

Doutora Paula Inês Borralho Domingues
Investigadora de Pós-Doutoramento, Departamento de Biologia e CESAM da Universidade de Aveiro

Doutora Isabel Maria Cunha Antunes Lopes (Orientadora)
Investigadora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro

Doutora Ruth Maria de Oliveira Pereira (Co-Orientadora)
Investigadora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro

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keywords Life stage, *Pelophylax perezi*, Metals, Salinity, Interaction

abstract Populations of amphibians are declining worldwide. Among the major causes for such decline are chemical contamination and climate changes (e.g. increase in temperature, salinization of coastal freshwater ecosystems). Actually, the group of amphibians may be very sensitive to these stressors as they possess a thin and permeable skin with no physical protection that allows cutaneous respiration but also the diffusion of chemical agents present in the environment. Furthermore, their biphasic life cycle exposes amphibains both to aquatic, terrestrial and atmospheric contamination, potentiating the period of exposure. Consequently, it is necessary to understand the effects that chemical contamination may pose to this group of organisms and how other factors may influence their sensitivity to chemical stress. Accordingly, the present work intended at evaluating how life stage and the combination with other stressors may influence the toxicity of copper to the Perez´s frog *Pelophylax perezi* (Seoane). To attain this main goal, two specific objectives were delineated: (i) to compare the sensitivity of different life stages, embryos versus tadpoles, to copper (Experimental design 1), and (ii) to evaluate the influence of increased salinity (an indirect effect of climate changes in coastal freshwater lagoons) on the toxicity of copper to embryos and tadpoles of *P. perezi* (Experimental design 2). For this, eggs at Gosner stage 10-11 and tadpoles at Gosner stage 25 were used to carry out 96h exposure assays.

For the first experiment, the two life stages were exposed to a gradient of copper plus a control (FETAX). In the second experiment, embryos and tadpoles were exposed to combinations of copper and NaCl (to simulate an increased salinity) in a complete bifactorial experimental design. In the two experiments the following endpoints were monitored: (i) for embryos, mortality was registered every 24h and at the end of the assay the final body length and malformations rate of surviving larvae were assessed; (ii) for tadpoles mortality and swimming behavior were monitored every 24h. Additionally, at the end of the experimental desing 2 the enzymatic activity, of surviving larvae/tadpoles, was quantified for catalase (CAT), cholinesterase (ChE), glutathione S-transferase (GST) and lactate dehydrogenase (LDH). The obtained results showed that embryos were less sensitive to copper than tadpoles (aproximately 50% of mortality at 1.6 mg/L Cu and LC50=0.93 mg/L Cu, respectively). Furthermore, it was observed that NaCl did not influence the lethal toxicity of copper to tadpoles, but, it significantly reduced the copper toxicity to embryos. Regarding enzymatic responses, a clear and consistent response was not observed for the tested treatments. However, for some copper concentration, the presence of NaCl induced an increase of the activity of CAT, relatively to that observed when orgaisms were exposed solely to copper, both for embryos and tadpoles. Also, in some copper concentrations, the presence of NaCl caused an increase or decrease in the activity of LDH in embryos and tadpoles, respectively. In addition, and contrarirly to what was reported for copper, it was observed that embryos were more sensitive to increased salinity (NaCl) than tadpoles.

The results obtained in the present study, highlighted the need, within the context of ecological risk evaluation, to characterize the sensitivity of different life stages of amphibians to different chemicals and to the combination of diverse stressors.

Palavras-chave Estádio de vida, *Pelophylax perezi*, Metais, Salinidade, Interacção

resumo As populacões de anfíbios estão em declínio a nível mundial. Duas das principais causas para este declínio são a contaminação química e alterações climáticas (e.g. aumento das temperaturas, salinização de zonas costeiras). De facto, os anfíbios podem ser muito sensíveis a estes agentes perturbadores, visto possuírem uma pele fina e permeável, sem protecção física, que permite a respiração cutânea mas também a difusão de agentes químicos presentes no ambiente. Além disso, o seu ciclo de vida bifásico expõe-os a contaminação aquática, terrestre, e atmosférica, potenciando o seu período de exposição. Consequentemente, é necessário compreender os efeitos que a contaminação química pode ter neste grupo de organismos, e de que modo outros factores podem influenciar a sua sensibilidade à perturbação química. Deste modo, o presente estudo pretendeu avaliar a influência do estádio de vida e da presença de outros agentes perturbadores na toxicidade de cobre em rã verde, *Pelophylax perezi* (Seoane). Para atingir este objectivo principal, foram delineados dois objectivos específicos: (i) comparar a sensibilidade de diferentes estádios de vida (embriões verusus girinos) ao cobre (Experiência 1), e (ii) avaliar a influência do aumento de salinidade (efeito indirecto das alterações climáticas em lagoas de água doce costeiras) na toxicidade de cobre para embriões e girinos de *P.perezi* (Experiência 2). Para tal, foram usados ovos no estádio de Gosner 10-11 e girinos no estádio de Gosner 25 para realizar ensaios de toxicicidade com 96h de exposição. Na primeira experiência, os dois estádios de vida foram expostos a um gradiente de cobre mais um controlo (FETAX). Na segunda experiência, os embriões e girinos foram expostos a combinações de cobre e NaCl (para simular um aumento de salinidade) num desenho experimental bifactorial completo. Nas duas experiências foram monitorizadas as seguintes respostas aos agentes perturbadores: (i) para os embriões, a mortalidade foi registada a cada 24h e no final do ensaio o tamanho corporal final e a taxa de malformações nas larvas sobreviventes; (ii) no caso dos girinos, a mortalidade e o comportamento natatório foram monitorizados a cada 24h. Adicionalmente, no final da segunda experiência (em que foi avaliada a influência de NaCl na toxicidade de cobre), foi quantificada a actividade enzimática da catalase (CAT), colinesterase (ChE), glutationa S-transferase (GST) e lactato desidrogenase (LDH) nas larvas (que eclodiram no final do ensaio-96h) e nos girinos. Os resultados obtidos demonstraram que os embriões foram menos sensíveis ao cobre do que os girinos (cerca de 50% de mortalidade na concentração de 1.6 mg/L Cu e LC50=0.93 mg/L Cu respectivamente). Mais ainda, foi observado que o NaCl não influenciou a toxicidade letal do cobre nos girinos, mas reduziu significativamente a toxicidade do cobre nos embriões.

Relativamente às respostas enzimáticas, não foi observado um padrão consistente de repostas aos vários tratamentos. No entanto, em algumas concentrações de cobre, combinadas com NaCl, observou-se que a presença de NaCl induziu a actividade da enzima CAT relativamente ao efeito observado apenas pela presença de cobre. Verificou-se ainda que, em algumas concentrações de cobre, a presença de NaCl induziu uma redução e um aumento da actividade da LDH em girinos e embriões, respectivamente, em comparação com a actividade da enzima em exposições só a cobre. Mais ainda, e contrário ao que foi registado para o cobre, foi observado que os embriões apresentaram uma maior sensibilidade ao aumento da salinidade (NaCl) do que os girinos.

Os resultados obtidos no presente estudo destacam a necessidade de, num contexto das avaliações de risco ecológico, caracterizar a sensibilidade dos diferentes estádios de vida dos anfibios a diferentes químicos e a combinações de de agentes perturbadores.

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Main Introduction

Amphibians Decline and Major Threats

Amphibians are threatened worldwide and their populations are known to have been dramatically declining since 1989 (Houlahan *et al*., 2000; Stuart *el al*., 2004; Beebee and Griffiths, 2005; McCallum, 2007). The Global Amphibian Assessment worldwide project (by the International Union for Conservation of Nature – the oldest and largest global environment network) revealed that almost one-third (32.4%) of the world amphibian species is threatened or extinct (1,896 species) and that at least 43% of all species are declining; thus suggesting that amphibians existence will continue to be threatened in the future. Furthermore, for almost 25% of amphibian species few information is available (e.g. number of individuals, populations, geographic distribution and biology), which does not permit to determine its conservational status (Figure 1). This knowledge gap is of major concern since it is possible that a significant proportion of those species may be threatened as well (Data available in http://www.iucnredlist.org; last visited on July, 4).

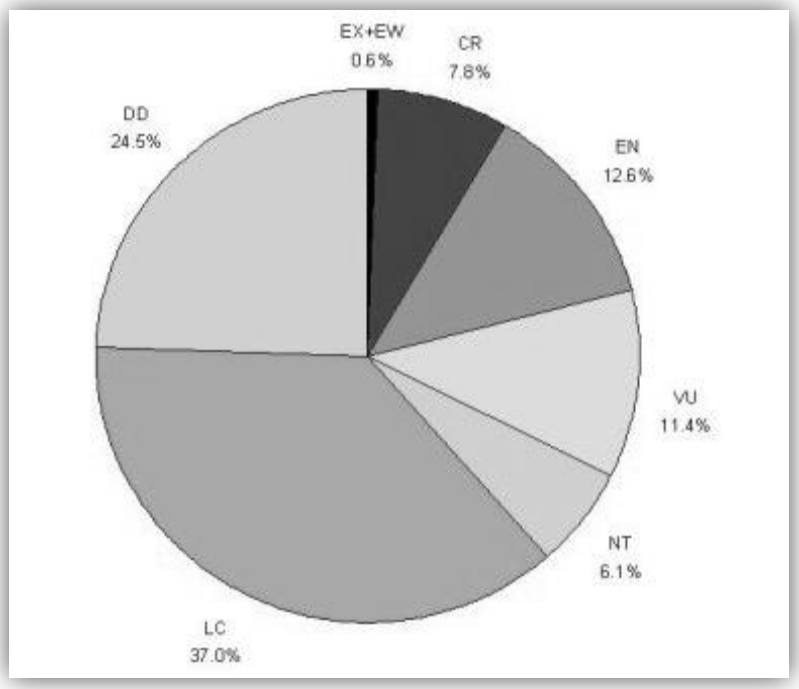

Figure 1 – ***International Union for Conservation of Nature*****-**IUCN Red List Status for amphibian species (2009 IUCN Red List of Threatened Species). EX= Extinct; EW= Extinct in wild; CR= Critically Endangered; ED= Endangered; VU= Vulnerable; NT= Not Threatened; LC= Least Concern; DD= Data Deficient (Copyright by http://www.iucnredlist.org, last visited on: July 4).

Within amphibians, the Order of Anuran is considered under critical danger since it represents almost 90% of amphibian´s diversity, and more than one-third (31.6%) of the species of this taxonomic group is threatened or already extinct (Data available in http://www.iucnredlist.org, last visited on July, 4). Due to these evidences, a growing concern has been observed regarding the worldwide amphibian decline.

This group of organisms is capable of inhabiting a broad range of habitats, ecosystems, and climatic regions (e.g.: deserts, mountains, temperate or tropical regions), which highlights the wide range of environmental adaptations, diversity, and functions in distinct ecosystems where amphibians play a crucial role and where they are abundant components of both aquatic and terrestrial ecosystems.

As Blaustein and Kiesecker (2002) and Beebee and Griffiths (2005) reviewed, there are several major threats to amphibians, at local and also global scale: (i) the direct action of man, like habitat destruction and alteration, exploitation for human uses and introduction of invasive species and, (ii) the indirect effects like increase in UV-B radiation, climate change, anthropogenic contamination, and disease outbreak (Figure 2). Furthermore, some authors proposed that a complex interaction between some of the mentioned causes is also responsible for the global amphibians decline (Kiesecker *et al*. 2001). One example is given by Kiesecker and Blaustein (1995), who observed a synergistic effect between a pathogen and UVB radiation, resulting in increased embryo mortality in nature.

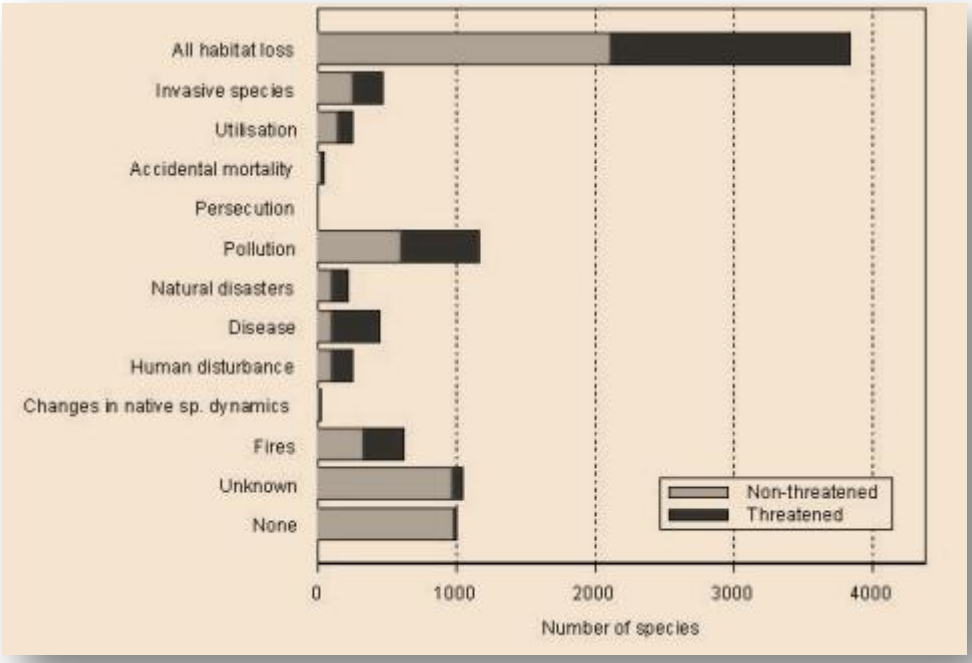

Figure 2 – Major threats identified as responsible for worldwide amphibian decline (Copyright and Information obtained from 2009 IUCN Red List of Threatened Species; **http://www.iucnredlist.org;** last visited on July, 4).

Amphibians as Environmental Indicators and their use in Ecotoxicological Studies

Amphibians play an important ecological role in ecosystems as its different life-stages constitute different trophic levels: the tadpoles are mainly herbivorous, thus primary consumers, while the adults are mainly carnivorous, thus, at least secondary consumers. Also, the amphibians themselves constitute a prey for both aquatic (e.g. fish, macroinvertebrates) and terrestrial (e.g. birds) biota (Hopkins, 2007). Furthermore, as amphibians possess a biphasic life cycle, with aquatic and terrestrial life stages, they play an important role in biomass transfer from these two compartments (Hopkins, 2007). These characteristics and some other (like habitat needs, behavior patterns) contributes to the high sensitivity of amphibians to environmental changes and stressors (LeBlanc and Bain, 1997; Rowe *et al*., 2003). Their biphasic life-cycle with an aquatic and a terrestrial life stage potentiates the exposure to contaminants and perturbations in the two compartments. In addition, it may also facilitate the transference of contaminants

between the two compartments. Furthermore, amphibians possess a typical well vascularised moist skin, with no physical protection, rendering a highly permeable tegument that allows cutaneous respiration but also the diffusion of chemical agents present in the environment (Duellan and Trueb, 1994). All these traits in addition to some behavioral patterns, as habitat selection based on temperature, are responsible for their exposure to many sources and variety of stressors (Bancroft *et al*., 2008).

Due to their high sensitivity to environmental changes, reduced home ranges, and key role in the ecosystem, amphibians are regarded as sentinel organisms, since they can be early indicators of the state of environmental health, occurring changes and ecosystems deterioration (LeBlanc and Bain, 1997; Lefcort 1998; Roy, 2002). Therefore, studies aiming at assessing ecological risk should include the study of stressors in amphibians. Yet, ecotoxicological information regarding this group is still scarce when comparing to other vertebrates. Sparling *et al*. (2010) checked for the number of contaminant-related papers on vertebrates published between 1996 and 2008. They observed that from all the citations examined, 66.7% were for fish, 19.9% for mammals, 8.8% for birds, 3.8% for amphibians, and 0.8% for reptiles. This distribution was found very similar to the one the same authors found in 2000, revealing that during 10 years of research only a small fraction of literature on vertebrate ecotoxicology concerns amphibians. Searches performed on Google Scholar (http://scholar.google.com/) on 17-07-2011 returned the same profile (Figure 3).

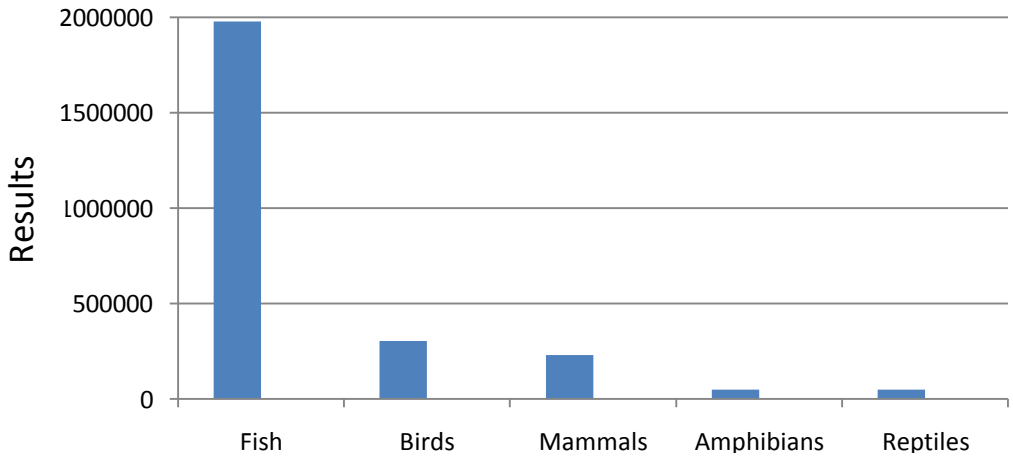

Figure 3 – Added search results for "contamination" and "pollution" by vertebrate class returned by Google Scholar.

Environmental Contamination

As mentioned previously, environmental contamination constitutes one of the major threats to amphibians. Contamination by metals is especially critical due to the several possible sources, its wide distribution, and entry routes in the water bodies. Metals present unique characteristics that potentiate their toxic effects: (i) persistence in the aquatic systems, (ii) moderate solubility, (iii) bioaccumulation ability, and (iv) high toxicity, are some examples (Flemming and Trevors, 1989; Pelgrom *et al*., 1994). Detrimental effects that metals may pose to living organisms range from mortality to several sublethal effects like growth delay, malformations during development, abnormal behavior, or oxidative damage (Lefcort *el al*., 1998; Gaetke and Chow, 2003; Redick and La Point, 2004; Barry, 2011).

Aquatic organisms can absorb and accumulate metals through gills and skin surface as well as by feeding and water consumption (LeBlanc and Bain, 1997). Therefore, among others, metal uptake is influenced by the extent of respiratory surfaces (gills, skin), respiratory and detoxification rates, and body area/volume ratio: some of these factors, organisms are capable of regulate in order to reduce adverse effects of metal contamination. For example, organisms can activate defense mechanisms to decrease metal uptake, such as reducing the membrane permeability or improving the rate of excretion (Valavanidis and Vlachogianni, 2010). Furthermore, organisms are capable of sequestering metals in vacuoles to avoid cell damage, and accumulated them in different tissues (Loumbordis and Wray, 1998).

Among all metals, copper is a widely used metal (e.g. electrical wires, roofing and plumbing, industrial machinery, agriculture) in our society. For this reason, it is extensively mined or extracted as copper sulfides from large open pit mines. These activities greatly increase its concentrations in the environment, leading to severe contamination of aquatic ecosystems. Although being an essential element to several enzymatic activities and constituting the structure of many proteins, excessive concentrations of this metal may be lethal or cause adverse sublethal effects on several classes of organisms and impair population resilience (e.g. Roux *et al*., 1993; Lefcort *el al*., 1998; Redick and La Point, 2004). Furthermore, its toxicity and bioavailability is directly

influenced by several water parameters, and, therefore, factors like temperature (Lemus and Chung, 1999, Macinnes and Calabrese, 1979), pH (Horne and Dunson, 1995), and hardness (Horne and Dunson, 1995). Concentration (Lemus and Chung, 1999), presence of other metals (Pelgrom *et al*., 1994; Lefcort *et al*., 1998), duration of exposure (Lemus and Chung, 1999), and life cycle phase (Greulich and Pflugmacher, 2003) are also important when evaluating the toxicity of copper to biota.

Lethal and sublethal effects of copper have been reported in several biota, for example in crustaceans (Ferrando and Andreu, 1993), algae (Ferrando and Andreu, 1993), fish (Lemus and Chung, 1999), and amphibians (Khangarot and Ray, 1987). Regarding the latter group of organisms, it has been shown by some authors that amphibians, and especially anurans, can be more sensitive than fishes to copper, contrarily to what occurs with some organic contaminants (e.g. carbaryl, permethrin) (Bridges *et al*., 2002). In addition, a broad range of studies comparing toxicities of metals in amphibians concluded that copper was one of the most toxic metals to several species of this Class. For example, Khangarot and Ray (1987) compared the toxicity of a group of seven metals (Silver, Copper, Mercury, Cadmium, Zinc, Nickel, Chromium), and found that copper occupied the third position of the most toxic metals to *Bufo melanostictus* larvae. Similarly, Barry (2011) observed that copper caused adverse effects on the growth and morphology of *Bufo arabicus* tadpoles while zinc at the same concentrations did not caused any significant toxic effects.

Global Changes

Another major factor identified above as being responsible for the worldwide declining of amphibians are global changes. In fact, global changes including environmental and climate alterations can seriously impact ecosystems. Such impacts are presently minimal when compared to pollution or habitat loss, but may become one of the biggest threats to many species in the future, namely to amphibians (Corn, 2005). Increasing of global temperature, UV-B radiations, or salinization of freshwater systems is known to influence survival and population success of several groups of organisms (Häder *et al*., 1998; Sanuy *et al*. 2008).

Within the context of climate changes a global sea-level rise is expected to occur and, thus, expose some coastal low-lying aquatic systems to increased salinity levels (due to seawater intrusions either by surface flooding of through groundwater), constituting an additional perturbation for species inhabiting these ecosystems (IPPC, 2007). Effects of salinity on freshwater organisms include osmotic stress, deficient respiratory functions, growth and development impairment and/or survival reduction (Gomez-Mestre and Tejedo, 2003). For aquatic organisms, the osmotic equilibrium is of major importance for survival. For this reason, salinity levels changes can constitute a major threat for them. This is particularly problematic for amphibians since they have poor ability to regulate osmolarity, because of their high permeability of skin, gills systems and permeability of egg membranes (Boutilier *et al*., 1992). Moreover, few species are capable of tolerating high salinity levels, most of them avoiding brackish waters. An exception is the case of *Bufo calamita* that commonly breed at freshwater bodies, as usual in amphibians, but can tolerate brackish waters (less than 15% seawater (Beebee, 2002; Gomez-Mestre and Tejedo, 2003) or *Rana cancrivora* that typically inhabit brackish waters but may tolerate freshwaters and higher salinity levels (up to 80% of seawater) (Gordon *et al*., 1961).

Combination of environmental stressors

As referred above, several factors have been identified to constitute major threats to amphibians. In fact, most recent works argue that the combination of several factors such as global changes, contamination, and biotic constraints are a major factor contributing for amphibian´s decline (Blaustein and Kiesecker, 2002). Therefore, it is important to understand how the combination of these factors influences their toxicity to amphibians in order to more accurately protect this group of organisms (Ortiz-Santaliestra *et al*., 2010).

Biota is often simultaneously exposed to several stressors, such as the presence of different contaminants and other abiotic parameters (e.g. pH, hardness, temperature, salinity, UV-B radiation) (Blaustein and Kiesecker, 2002). In order to avoid the underestimation of possible interactions, risk evaluations combining the study of different stressors must be carried out to make a more ecologically relevant assessment

of their potential effects (Blaustein and Kiesecker, 2002). Actually, a series of works have already reported that the combination of more than one stressor may potentiate the toxicity of each stressor when compared with the toxicity of each one alone (Hatch and Blaustein, 2000; Macías *et al*., 2007). For example, Macías *et al*. (2007) observed an increased mortality of tadpoles of *Pelophylax perezi* and *Bufo bufo* exposed to combinations of UV-B radiations and nitrite when compared with the effect of each stressor alone. Specifically, metals are highly affected by environmental conditions. Horne and Dunson (1995) found that combinations of copper with high pH levels and lower hardness levels reduced survival of larval amphibians and in general the same pattern was observed with other metals tested in the same work (e.g. aluminum, zinc). Toxicity of copper can be also influenced by salinity levels. Several studies are available on copper-sodium interactions, and apparently toxicity mediation revolves around competition between copper and sodium in transport systems (Daly *et al*., 1990; Handy *et al*. 2002; Pyle *et al*., 2003). However, most studies that reported adverse effects resulting from the combination of metal contamination and changes on salinity were performed mainly on estuarine and marine organisms (Phillips, 1976; MacInnes and Calabrese, 1979; Martins *et al*., 2011) that present a greater ability to regulate the uptake of metals (Philips and Rainbow, 1993). Salinity increase can also influence the toxicity of other contaminants (e.g. fertilizers such as ammonium nitrate) like Ortiz-Santaliestra *et al*. (2010) has observed for *Pelophylax perezi* embryos; the authors found higher mortality levels induced by combinations of salinity increase and ammonium nitrate than by ammonium nitrate alone.

Life Stages Sensitivity

Life cycle of amphibians comprises several stages, with different development periods and distinct environmental requirements (LeBlanc and Bain, 1997). Therefore, to accomplish an accurate identification of amphibian's sensitivity to chemical contamination, it is crucial to understand how these different life stages respond to environmental contamination. Both embryonic and larvae aquatic phases have been proven to be very sensitive to chemical contamination, thus being relevant environmental indicators for aquatic contamination assessment (Haywood *et al.,* 2004; Relyea and Jones, 2009).

The aquatic and terrestrial life stages of amphibians may exhibit different exposure routes to metals: i) Eggs present a semi-permeable jelly coat and can absorb large quantities of aquatic contaminants, for example ammonium nitrate (Hecnar, 1995); ii) larval stages exhibit gills where respiratory changes and ion exchange takes place and can, thus, increase exposure, and a thin semi-permeable skin that can constitute entry routes for contaminants (Henry, 2000); and iii) adults hold an unprotected, fragile and well-vascularized skin and can be exposed to both water and soil contaminants (Loumbourdis and Wray, 1998).

Besides the different contact routes with the medium, each life stage possesses particular characteristics regarding the different environmental perturbation agents. For instance, embryos can be very sensitive to UV-B radiations (Blaustein *et al*., 1998), while larvae can be more susceptible to chemicals present in the water (Berrill *et al*., 1994) and adults can be more exposed to water, soil and air pollution (Loumbourdis and Wray, 1998). Embryos and larvae can be especially sensitive to chemical contamination, as during their development are permanently exposed to the same aquatic systems (where the eggs were layed). This is further exacerbated in the larval stage, by the fact, in most species, this is the longest development aquatic phase which can increase the exposure period of this stage to contaminants. Although less sensitivity is expected from adult organisms, they can show histopathological alterations derived from long-time exposure to contamination (Marques *et al*., 2009) or high concentrations of copper in tissues/organs (Loumbourdis and Wray, 1998) highlighting the fact that these organisms

are capable of accumulating high concentrations of metals and thus being impacted as much as the correspondent early-life stages. Differences in sensitivity between life phases also depend on the toxic agent. For example, Greulich and Pflugmacher (2003) found that the jelly coat did not protect the embryos of *Rana arvalis* from α-cypermethrin exposure while other authors found reduced mortality due to jelly-coat protection to other pesticides such as endosulfan (Berrill *et al*., 1998), suggesting different permeability of the jelly-coat to different chemicals. Taking into consideration the differences in sensitivity to several chemicals that may be found between life stages, it appears to be relevant that for a more accurate prediction of the "throughout life cycle" sensitivity of anuran species, the evaluation of different life stages within ecological risk assessment of contaminants must be carried.

Hypothesis and Objectives

Accordingly to the above mentioned, the main objective of this study was to determine how life stage and the combined exposure to salinity can influence the toxic effects of copper to early life stages of amphibians.

To achieve this main objective, two specific objectives were delineated:

1) to compare copper toxicity between embryonic and larval stages of amphibians;

2) to evaluate the effects of copper and increased salinity, both individually and in combination, in eggs and tadpoles of amphibians.

To attain the objectives proposed, the Perez´s frog (*Pelophylax perezi* Seoane) was used as the model organism. This species was chosen because it is an abundant species, is widely distributed along the Iberian Peninsula and its taxonomic group is commonly used in ecotoxicological studies (Loureiro *et al*., 2008).

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Chapter One

Sensitivity of embryos and tadpoles of Perez´s frog *Pelophylax perezi* (Seoane) to a gradient of copper

Abstract

Metals have been shown to be responsible for survival reduction and developmental disturbances on several life stages of aquatic species. This work aimed at comparing the sensitivity of different life stages (embryos and tadpoles) of the Perez's frog *Pelophylax perezi* (Seoane) to copper. To attain this objective, eggs at Gosner stage 10-11 and tadpoles at Gosner stage 25 were exposed, for a period of 96 h, to a gradient of copper concentrations ranging from 0 to 1.6 mg/L and from 0 to 2.4 mg/L, respectively. The following endpoints were monitored for the embryo assay: survival, malformations, time to hatching, and body length of hatchlings; while for the tadpoles assay the monitored endpoints were: mortality and swimming behaviour.

The 96h-LC_{20}, for embryos survival, was 0.69 mg/L of Cu. Though the 96h-LC_{50} could not be computed, a mortality of approximately 50% was observed at 1.6 mg/L of Cu. The 96h-EC_{20} and 96h-EC_{50} for malformations in hatched larvae, were 0.84 and >1.6 mg/L, respectively. Significant differences were observed in body length in the two highest copper concentrations, but no significant differences were observed in time to hatch, comparatively with the control. For tadpoles, the computed 96h-LC_{50} and 96h-LC_{20} were 0.93 and 0.48 mg/L, respectively. These results revealed that the larvae stage of *P. perezi* was apparently more sensitive to copper than the embryonic stage. Additionally, tadpoles exposed to the highest copper concentrations (1.65 and 2.4 mg/L) exhibited abnormal swimming behaviour. These results suggest that assays with larval stages of amphibian species should be included in ecological risk assessment evaluations. Additionally, exhaustive considerations of embryos relatively to tadpoles' sensitivity should be carried out, to allow the use of scientifically sound safety factors, aiming at avoiding under- or overestimation of risks.

Keywords: Life stage Sensitivity *Pelophylax perezi* Copper

Introduction

Water quality degradation has been identified as one of the most important factors of amphibian populations´ decline, being metal contamination one of the most common (Collins and Storfer, 2003). Metals are naturally found in the majority of aquatic systems in trace levels (ATSDR, 2004). However, their concentrations in the aquatic environment may greatly increase due to anthropogenic activities, namely through chemical-containing runoff of agriculture soils, industrial, commercial and domestic wastes (Flemming and Trevors, 1989).

Among all metals, copper is a widely used metal (e.g. electrical wires, roofing and plumbing, industrial machinery) in our society. For this reason, it is extensively mined or extracted as copper sulfides from large open pit mines. These activities greatly increase its concentrations in the environment, leading to severe contamination of aquatic ecosystems. Copper is and essential metal for organisms, constituting both an enzymatic cofactor and part of the structure of several proteins (Flemming and Trevors, 1989). Despite its clear relevant biological function, it may cause severe toxic effects when present at high concentrations. Its toxicity being dependent on several parameters such as temperature, pH, hardness, dissolved organic matter, presence of other metals (Flemming and Trevors, 1989; Pelgrom *et al*., 1994; Horne and Dunson, 1995; Lefcort *et al*., 1998; Lemus and Chung, 1999) and biological conditionings such the life-cycle stage (Greulich and Pflugmacher, 2003). Regarding the latter parameter, the organisms´ body characteristics, physiological conditions, time and routes of exposure/uptake are often toxicity mediators determined by the organism itself (e.g. area/volume ratio, respiratory and ions exchanges surfaces) (Hodson *et al*., 1979).

Amphibians´ exposure to copper is mainly related with contamination from agriculture fields and the use of fungicides and/or algaecides that contain copper in the form of copper sulphate, copper acetate, and copper carbonate and so on (as reviewed by Flemming and Trevors, 1989). Previous studies reported serious adverse effects (e.g. mortality, reduced body size) of copper on a few species of amphibians (Khangarot and Ray, 1987; Hopkins *et al*., 2000; Redick and La Point, 2004; García-Muñoz *et al*., 2010). For

example, Haywood *et al*. (2004) found a strong negative relationship between increasing copper concentrations and hatching success of embryos of *Xenopus laevis*.

Though the toxicity of copper has been studied for different life stages of several species of amphibians, the comparison of sensitivities of those life stages to this metal is rarely assessed (Khangarot and Ray, 1987; Herkovits and Helguero, 1998; Pérez-Coll and Herkovits, 2006). However, sensitivity of amphibians to contamination appears to be dependent on their life stage, as at each life stage different exposure and uptake routes will exist (Berril *et al*., 1994; Greulich and Pflugmacher, 2003; Edginton *et al*., 2004; Henry, 2000 and citations therein). For example, anuran tadpoles are expected to exhibit a different sensitivity to contaminants comparatively with embryos. The former exhibit a semi-permeable skin that facilitates the uptake of contaminants from the media; exhibit gills where active respiratory functions and ion exchanges occur (Dietz *et al*. 1974); tadpoles often graze in the sediment, ingesting detritus and other particles of the sediment (that may transport adsorbed contaminants) and the larval stage is longer than the embryonic one which can increase its exposure period (Loumbourdis *et al*., 1999).

On the other hand, embryos present a jelly-coat membrane that may function as a barrier to certain compounds but is permeable to others (Berrill *et al*., 1994; Greulich and Pflugmacher, 2003; Edginton *et al*., 2004). In addition, the absence of fully developed organs and body surfaces (during the embryonic stage) that could increase the contact with metals may prevent more serious adverse effects on embryos (Edginton *et al*., 2004).

In the present study, copper toxicity on the Perez's frog *Pelophylax perezi* (Seoane) was evaluated. Embryos and tadpoles were used to compare the sensitivity of both life stages in order to determine which phase is more sensitive, and thus, more suitable for evaluations of copper toxicity in this species. Lethal and sublethal responses were monitored to evaluate the extension of copper toxicity in these organisms.

Material and Methods

Organisms collection

Eggs clutches of Perez's frog *Pelophylax perezi* (Seoane) were collected from a freshwater pond (+40.596364N; -8.695690W) near Aveiro, Portugal, on April, 2011. Several physico-chemical parameters of water were measured in the field during the collection: dissolved oxygen (OXI 330/SET, best nr. 200 232), pH (pH 330/SET-2, best nr. 100 788), conductivity (LF 330/SET, best nr. 300 204), salinity (LF 330/SET, best nr. 300 204), total dissolved salts (LF 330/SET, best nr. 300 204) and temperature (OXI 330/SET, best nr. 200 232) (Table 1).

Table 1 –Values of physico-chemical parameters measured in the water column of the pond where eggs of *Pelophylax perezi* were collected.

Physico-chemical parameter	Value
Dissolved oxygen (mg/L)	8.3
Conductivity (µS/cm)	271
pH	8.5
Salinity	0
Total dissolved salts (mg/L)	127
Temperature (ºC)	17.3

Experimental conditions

Some of the collected eggs were immediately used to carry out the embryos assays, since they were in Gosner developmental stage 10-11 (Gosner, 1960). The remaining eggs were maintained in the artificial medium FETAX (Dawson and Bantle, 1987) with aeration and a 14:10 h (light:dark) photoperiod, at 23±1ºC, until larvae reached the Gosner stage 25. At this stage, were used to perform the tadpoles assay.

Copper concentrations were established by diluting with FETAX stock solutions of copper (II) sulphate pentahydrated ($CuSO_4.5H_2O$, Merck, Darmstadt, Germany). Medium renewal and physico-chemical parameters measurements (pH, dissolved oxygen, conductivity and temperature; using the same equipment described in the previous section) were

performed at 24 h intervals. All the test solutions were daily prepared just before the media renewal.

a) Experiment 1: Embryo Assay

The embryo acute toxicity test was performed according to the ASTM guidelines (ASTM, 1998). The jelly-coat envelope of embryos was left intact to allow a more realistic representation of the natural exposure conditions. For each copper treatment and for the control (FETAX medium), three and four replicates were carried out, respectively. Each replicate consisted of a 10 ml solution placed in 55-mm plastic Petri dishes with 20 embryos at Gosner stage 10-11 (Gosner, 1960). Embryos were exposed to the following copper (Cu) concentrations: 0 (control); 0.05; 0.1; 0.2; 0.4; 0.8 and 1.6 mg/L, for a period of 96h, at 23±1ºC and a photoperiod of 14:10 h (light:dark). These concentrations were obtained by diluting a stock solution at a nominal concentration of 1.6 mg/L of Cu with FETAX.

Several endpoints were monitored. Mortality rate was registered every 24 h and dead individuals were removed to avoid possible microbial contamination of the surviving ones. Hatching rate was verified every day, and, at the end of the experiment, all the surviving organisms were observed to identify its development stage (Gosner, 1960) and the presence of malformations. Surviving hatchlings were fixed in 3% formalin for body length measurements. Hatchling body length was measured from the snout to the tail tip, under a magnifying lens (Leica MS5) at a 10x factor.

b) Experiment 2: Tadpole Assay

The American Society for Testing and Materials document for the testing of larval amphibians was used as the guideline for the tadpole assay (ASTM, 2000).

This assay consisted of three replicates for each copper treatment and for the control in FETAX medium in 500ml plastic recipients. Each replicated contained 250 ml of test solution and ten tadpoles, at Gosner stage 25, which were randomly assigned for each

treatment. The assay was carried out through a period of 96h, under a 14:10h (light:dark) photoperiod cycle and at 23± 1ºC, and with constant aeration.

Tadpoles were exposed to the following copper concentrations: 0; 0.55; 0.79; 1.15; 1.65, and 2.4 mg/L. These concentrations were obtained by diluting a stock solution at a nominal concentration of 100 mg/L of Cu with FETAX. A subsample of organisms was used to assess the initial body length (7.8±0.65 mm) of tadpoles used to initiate the assay, this procedure intended to assure: (i) the homogeneity of tadpole body size and (ii) that possible significant differences on tadpole's sizes, at the beginning of the assay, would not influence the final results.

Similarly to the embryo's assay, media of all treatments were renewed daily and dead individuals were also removed each day. No food was added during the 96h exposure period. Mortality rate was monitored daily and pH, dissolved oxygen (mg/L), and conductivity (mS/cm) were registered every 24h.

Statistical analysis

Mortality rates in both assays were calculated as the mean percentage of dead organisms every 24 h and the total mortality after the 96h period of exposure. The 96-h concentrations inducing 50% and 20% of mortality or malformations (LC50 or 20 and EC50 or 20, respectively) and their 95% confidence limits (CL) were calculated through probit analysis, using the software PriProbit (Sakuma, 1998).

To analyze mortality, malformation, and body size data, and determine differences between the control group and copper treatments the parametric one-way analysis of variance (ANOVA) was used, followed by a Dunnett test (mortality and malformation data were transformed with the square root of the arcsin). Assumptions of normality and homogeneity of data were checked with the Shapiro-Wilks and Bartlett's tests, respectively. The Dunnett test also allowed determining the no observed effect concentration-NOEC and the lowest observed effect concentration-LOEC.

Analyses were performed by using the software SPSS Statistics 19, for Windows (Zar, 1996).

Results

Physico-chemical parameters

Physico-chemical parameters did not show significant variation during the assays. Dissolved oxygen was always above 7.0 mg/L and pH values were always within the interval of 7.91 to 8.32. Conductivity values ranged from 0.5 to 0.6 mS/cm in all the treatments.

Lethal and sublethal effects

a) Experiment 1: Embryo Assay

At the end of the assay, mortality in the control was lower than 10%, as required for validating the test (ASTM, 1998) and has significantly increased in embryos exposed to the highest copper concentration, after 96h of exposure ($F=15.905$; $d.f.=6$; $p=0.000$). Cumulative mortality during the 96h period is presented in Figure 1. After 24h of exposure, mortality was low at the highest concentration of copper but started to increase between the 24h and 48h period of exposure and until the end of the assay, achieving a value above 50% after 96h of exposure. Cumulative mortality, at the end of the assay, in the remaining copper treatments never exceeded 10%.

The LC_{50} value could not be computed, however mortality close to 50% was observed at the highest copper concentration (1.6 mg/L). The LC_{20} computed for cumulative mortality was 0.69 mg/L (as a high mortality was only observed at the highest tested copper concentration, it was not possible to calculate the confidence limits) (Table 3). The no observed effect concentration (NOEC), for mortality, was determined as being 0.8 mg/L and the lowest observed effect concentration as 1.6 mg/L (Figure 4).

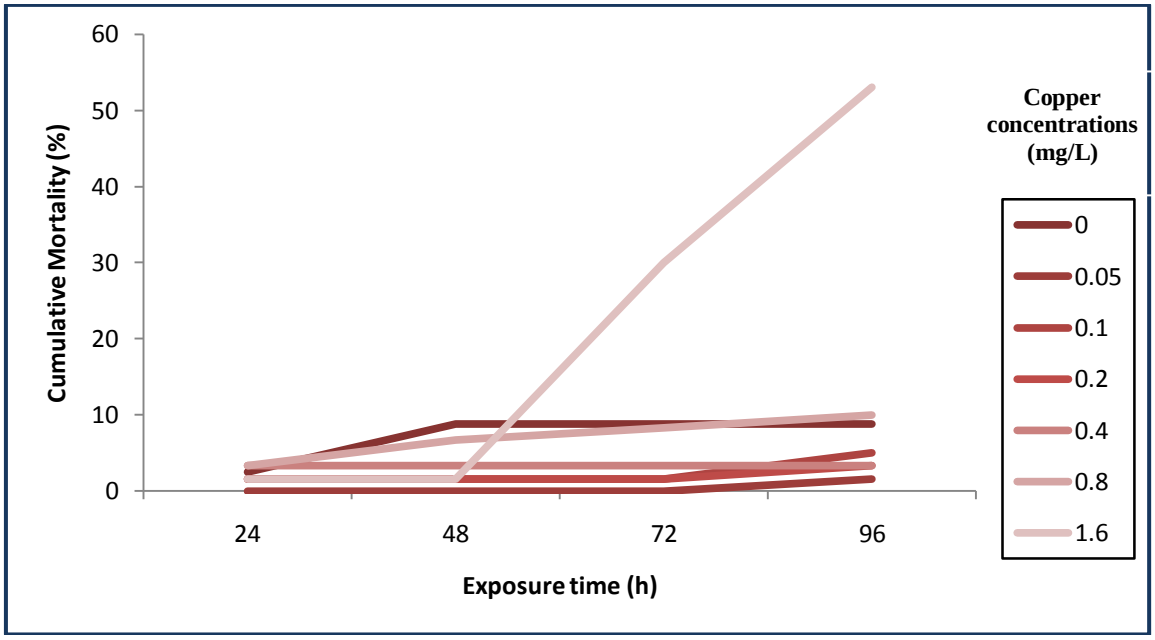

Figure 4 Cumulative mortality (%) of embryos of Pelophylax perezi along a 96h period of exposure to different copper concentrations (mg/L).

Time to hatching was only compared for the organisms that survived until the 96h exposure period. No significant differences in time to hatching were observed between copper concentrations and the control, as embryos, in all the treatments, hatched between the 72h and the 96h ($p>0.05$).

However, at the end of the assay, significant differences on total body length, of the surviving organisms, were found between organisms exposed to copper concentrations and to the control ($F=16.601$; d.f.=6; $p=0.000$). In the two highest copper concentrations, organisms exhibited a significantly smaller body length, comparatively to the control ($p=0.001$) (see Figure 2). Thus, the NOEC for this parameter was determined as being 0.4 mg/L and the LOEC as 0.8 mg/L (Figure 5).

The incidence of malformations in the surviving larvae increased with copper concentrations ($F=15.068$; d.f.=6; $p=0.000$). At the highest copper concentration (1.6 mg/L) almost 40% of the surviving organisms showed some type of malformation. The 96h-EC_{20} and 96h-EC_{50} values (and confidence limits) were 0.84 (0.67-1.07) and > 1.6 mg/L, respectively. The determined NOEC was 0.4 mg/L and the LOEC was 0.8 mg/L of copper (Table 2). The total number of malformations (%) found in each treatment after the 96h, is represented in Table 2.

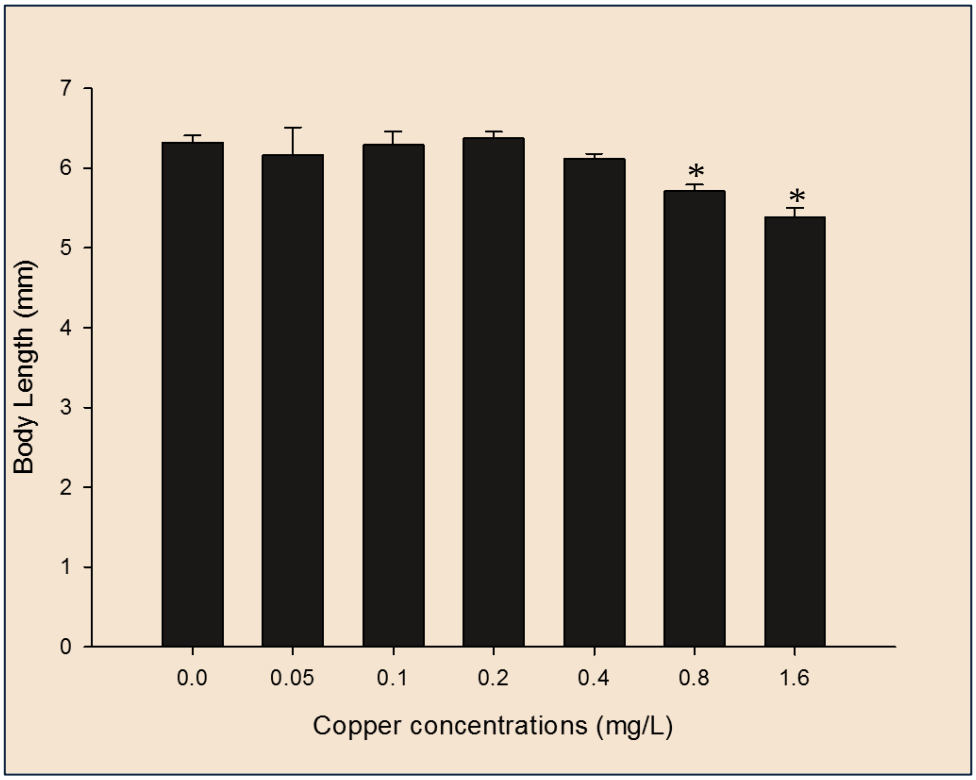

Figure 5 - Mean values of body length (mm), and the respective standard deviation (error bars), of the larvae of *Pelophylax perezi* after being exposed for 96h to different copper concentrations. Asterisks marks significant reduction of body length relatively to the control (p=0.001).

Table 2 – Percentage of malformations in surviving larvae of *Pelophylax perezi* after 96h of exposure to copper. Asterisks mark a significant increase in the rate of occurrence of malformations.

Malformations rate on larvae after hatching (%)		
Copper concentrations (mg/L)	Total of surviving larvae (%)	% of malformations
0	91	0
0.05	98	0
0.1	90	0
0.2	97	0
0.4	97	3.4
0.8	90	24*
1.6	47	39*

The types of malformations observed in the surviving larvae are shown in Figure 6. The malformations most commonly observed were tail curvation and edemas.

No malformations were observed in control groups.

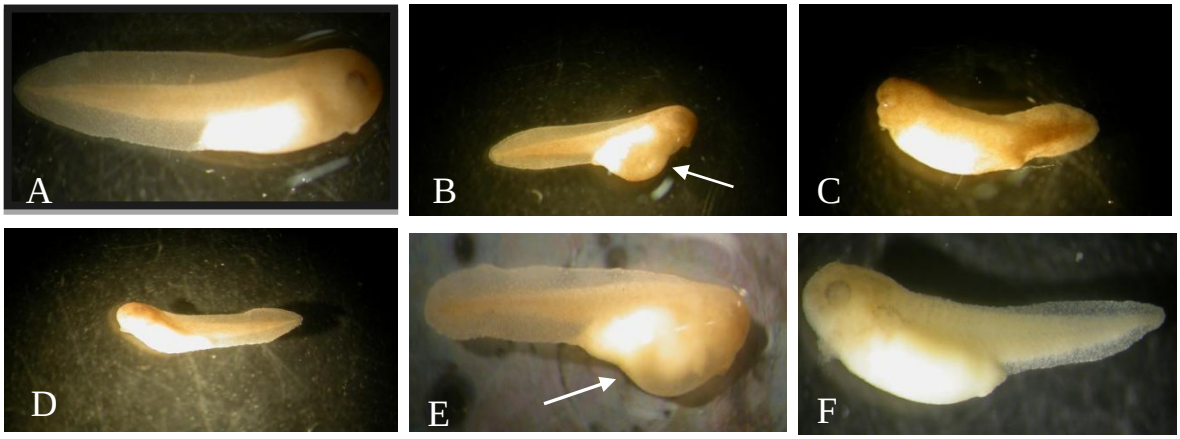

Figure 6 – Lateral view of surviving organisms of *Pelophylax perezi* after 96h exposed to copper concentrations comparing organisms with and without malformations. A) larvae from control treatment (FETAX medium) without malformations; B) larvae expose to 0.8 mg/L Cu with an abdominal edema (arrow indicates it); C) larvae exposed to 0.8 mg/L Cu with reduced tail length D) larvae exposed to 1.6 mg/L Cu with tail deformation; E) larvae exposed to 1.6 mg/L Cu with abdominal edema (arrow indicates it) and tail deformation; F) larvae exposed to 1.6 mg/L Cu with tail deformation.

b) Experiment 2: Tadpole Assay

No mortality was registered in the control groups (FETAX medium). At the end of the assay, all copper treatments caused a significant decrease in survival of tadpoles (%) ($F=9.042$; d.f.=5; $p=0.001$), with the exception of the concentration 0.55 mg/L. Mortality above 20% was observed in the first 24h of exposure, in the four highest copper concentrations and increased thereafter. Cumulative mortality rates (%) during the 96h period are represented in Figure 7.

LC_{20} and LC_{50} values (and respectively 95% confidence intervals) computed after 96h of exposure were 0.48 (0.29-0.62) mg/L and 0.93 (0.75-1.1) mg/L of Cu, respectively (Table 3). The NOEC was determined as being 0.55 mg/L and the LOEC as 0.79 mg/L of Cu.

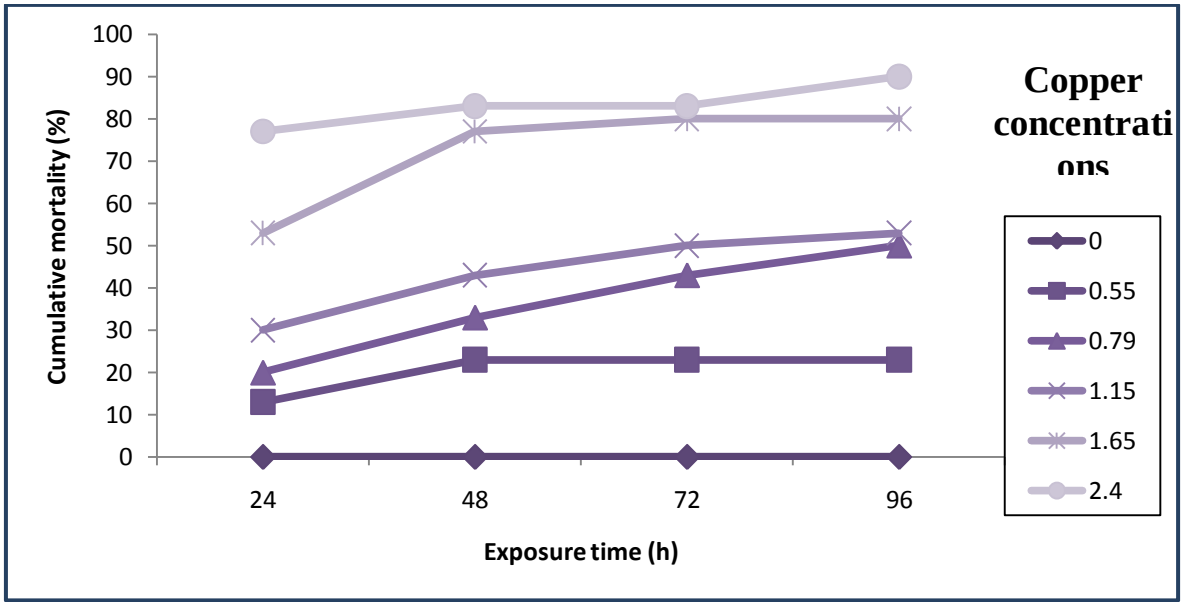

Figure 7 - Cumulative mortality (%) during the 96h exposure to copper concentrations of tadpoles (Gosner stage 25) of *Pelophylax perezi.*

Though it could not be quantified, it was observed that the tadpoles exposed to the two highest copper concentrations exhibited an abnormal swimming behavior. A reduction in swimming activity was observed: tadpoles mainly stayed at the bottom of the recipients, only reacting after receiving an external stimulus, such as being prodded with a plastic pipette. Nevertheless, the movement was always brief and erratic. Comparatively, tadpoles in the control and in the three lowest copper concentrations exhibited normal and fast movements. Copper was more toxic to tadpoles showing a LC_{20} of 0.48 mg/L than to embryos with a LC_{20} of 0.69 mg/L (Table 3). It was not possible to compute the LC50 for embryos but since mortality at the highest copper concentration tested (1.6 mg/L) was higher than 50 % (53.3%), the LC_{50} value expected to embryos is close to 1.6 mg/L, therefore, being higher than that computed for tadpoles 0.93 mg/L of Cu.

Table 3 - Values of LC_{20} and LC_{50} values, with the respective 95% confidence limits, after a 96h period of exposure to copper for embryos and tadpoles of *Pelophylax perezi.*

	96h-LC_{20} (95%CL)	96h-LC_{50} (95%CL)
Embryo Assay	0.69*	*
Tadpole Assay	0.48 (0.29-0.62)	0.93 (0.75-1.1)

Note: The asterisk (*) marks values that could not be computed, for CL (Confidence limits) and LC50 in the embryo assay.

Discussion

Previous experiments already reported adverse effects of copper on a few species of amphibians (Khangarot and Ray, 1987; Haywood *et al*., 2003; Redick and La Point, 2004; Barry, 2011). The results obtained in the present study shows that the sensitivity of embryos and tadpoles of *P. perezi* is within the range of sensitivities observed for other species of amphibians. For tadpoles, values of 96h-LC_{50} for copper within 0.15 mg/l (for *Rana pipiens*) and of 3.96 mg/L (for *Lithobates catesbeianus*); and copper concentrations inducing significant reduction in growth within 34.9 µg/L (for *Bufo arabicus*) and 0.7 mg/l of Cu (for *Xenopus laevis*), were reported in the literature (Landé and Guttman, 1973; Khangarot and Ray, 1987; Lombardi *et al*., 2002; Ossana *et al*., 2010; Barry, 2011). Also, Redick and La Point (2004) observed altered swimming behavior in tadpoles of *R. pipiens* exposed to copper concentrations within the range of 0.036 mg/L and 0.093 mg/L of Cu. For embryos, 96h-LC_{50} values of 0.085 mg/L were reported by Herkovits and Helguero (1998) for *Bufo aerenarum*, while Landé and Guttman (1973) observed that concentration of copper as high as 1.56 mg/L did not exert significant effects on embryos of *R. pipiens*. Also, concentrations of copper higher than 0.5 mg/L were shown to induce limbs malformations in *X. laevis* (Dwyer *et al*., 1997). In the present study, the 96h-LC_{50} for tadpoles of *P. perezi* was 0.96 mg/L of Cu, and concentrations above 1.65 mg/L of copper induced abnormal swimming activities. Furthermore, copper induced approximately 50% of mortality to embryos of *P. perezi*, at a copper concentration of 1.6 mg/L, and a significant percentage of malformations and significant effects on body size occurred at 0.8 mg/L of copper.

Comparative results from the embryo and tadpoles experiments showed a higher sensitivity of tadpoles of *Pelophylax perezi* at stage 25 to copper than the embryonic stage (see Table 3). These results are in line with previous studies, where higher mortalities were also found in anuran tadpoles exposed to other contaminants when compared to their corresponding embryo stage (Landé and Guttman, 1973; Edginton *et al*., 2004; Natale *et al*., 2006). Namely, embryos of *Hypsiboas pulchellus* were more resistant to chromium than the respective tadpoles (Natale *et al*., 2006). Also, Edginton *et al*. (2004) reported a higher sensitivity of tadpoles, at Gosner stage 25, comparatively to

that of embryos in four different anuran species when exposed to a glyphosate-based herbicide (Vision®).

Such differences, in sensitivity to chemicals between embryos and tadpoles, are, most probably, related, with different uptake routes present in each of these life stages. For example, embryos present a jelly-coat membrane that may function as a barrier to certain compounds, reducing the embryo contact with contaminants (Greulich and Pflugmacher, 2003; Edginton *et al.*, 2007; García-Munõz *et al.*, 2009). Nevertheless, embryos are devoid of a protective shell, and some authors reported that this jelly-coat envelope maybe highly permeable to the diffusion of some compounds, namely those that are hydrophilic (as jelly-coat is constituted by glycoproteins, mucoproteins, carbohydrates, and mucopolysaccharides) (Salthe, 1963; Bridges, 2000; Edginton *et al.*, 2004, 2007). In such cases, it has been suggested that a lower embryo sensitivity may be related with the fact that development of target organs is still in an early stage (Edginton *et al.,* 2004). This hypothesis may also explain the observed lower sensitivity of embryos to copper, in the present study.. Gills and cutaneous surfaces are the main entry routes of contaminants during the larval stages. Since gills only appears after Gosner stage 19 and only complete full development between Gosner stages 21-23, direct exposure of these organs to copper occurred for a smaller period during the embryo assay, as organisms only reached stage 21-23 near the end of the assay (at 96h). Therefore, this could also diminish the toxic effect of copper to embryos (Edginton *et al.*, 2004). Furthermore, the fact that the highest mortality occurred after 72h (namely after hatching) of exposure corroborate this hypothesis.

The higher sensitivity demonstrated by tadpoles probably can be due to constant exposure to test solutions since they only have a semi-permeable skin for their protection and metals may enter via dermal surfaces (Duellan and Trueb, 1994). This type of exposure combined with more developed organs can be the main factors contributing to their higher sensitivity. Moreover, some authors defend that nervous system can be more affected at more advanced life-stages (Berrill *et al.*, 1993), since the complete development of central nervous systems only occurs at the final of embryonic development (Gosner, 1960). This could explain the observed abnormal behavior

regarding swimming activity of tadpoles exposed to the two highest copper concentrations. Therefore, the highest sensitivity of tadpoles, relatively to embryos, is an important factor when evaluating toxic effects of a contaminant in amphibian populations since in a large range of species the Gosner stage 25 is the longest larval stage during aquatic development. Evaluations on both life stages are, therefore, of a great importance in risk assessments.

Though the above mentioned, higher sensitivity of embryos compared to tadpoles may also occur. For example, Ortiz-Santaliestra *et al*. (2006) observed that embryos (Gosner stage 13) of *Pelobates cultripes* were more sensitive to ammonium nitrate than latter larval stages (Gosner stages 19, 21 and 24). However, in the same work, authors observed opposite results depending on the tested species: tadpoles of *Bufo calamita* (Gosner stage 21) were more sensitive than early stages testes (Gosner stage 13 and 19) to ammonium nitrate. These differing results highlight the fact that sensitivity range depends upon species, life-stage and contaminant.

Though lethal effects evidenced a higher sensitivity of tadpoles relatively to embryos, it is important to notice that, within a risk assessment scenario, the monitorization of some sublethal effects that were observed in the embryo assay and, which are more expected at environmental relevant concentrations, may be as sensitive as the lethal effects for copper. For example, the registered effects on body size (copper exposed organisms exhibiting a lower body size than the control ones at 0.4 mg/L of Cu) revealed that this parameter was as sensitive as the lethal effects of tadpoles (LC20 of 0.48 mg/L of Cu) to evaluate the toxicity of copper to *P. perezi.* Such a reduction in growth has been associated with a re-allocation of energy, from the investment on growth, to detoxifying mechanisms (Lefcort *et al*., 1998; Haywood *et al*., 2004; Rowe *et al*, 2003). Thus, suggesting that these life stages of amphibians need additional energetic reserves to deal with toxicants in detriment of growth (Haywood *et al*., 2004; Redick and La Point, 2004). Furthermore, the evaluation of effects in this parameter is ecologically relevant as body size may ultimately contribute to reduce survival on anurans. For example, smaller tadpoles can be more predated (Crump, 1984), show inability to compete by resources (Wilbur, 1977) or reach smaller sizes at metamorphosis, thus reducing its fitness (Smith,

1987). Similarly to body size, the evaluation of effects on malformation is ecologically relevant, as it may also have adverse implications in the survival of tadpoles, it may contribute to: an increased vulnerability to predation by impairing the movement and normal escaping behavior; as well it may cause adverse effects on feeding rates of tadpoles (Lefcort, 1998). Additionally, they can lead to the death of individuals if normal development is impaired (Semlitsch, 1988; Greulich and Pflumacher, 2003).

Regarding the tadpoles assay, abnormalities in swimming behavior expectedly, and likewise to malformations and body size in embryos, revealed to be a sensitive endpoint. Also, these observed effects may result in increased predation and feeding depression (Lefcort, 1998), since locomotion activity can be a crucial component of fitness since slower or/and erratic movements of tadpoles could result on abnormal escape behavior and poor competitive ability for resources (Semlitsch and Gibbons, 1988).

Conclusions

The obtained results indicate copper to be more lethal to tadpoles (Gosner stage 25) than embryos (Gosner stage 10-11). It is therefore suggested that ecological risk assessments using solely embryos may not predict with accuracy the impact of contaminants in populations of *P. perezi*, since, for some chemicals (at least copper) it would be underestimated. Therefore, ecotoxicological studies on amphibians should include the evaluation of the adverse impacts on embryos and tadpoles, especially when evaluating metal contamination, due to the wide sensitivity range during its life cycle. Additionally, sub-lethal effects should be evaluated in combination with lethal effects to better predict the real impact on organisms and populations.

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Chapter Two

Salinity and copper interactive effects on the Perez´s frog *Pelophylax perezi* (Seoane)

Abstract

Several works have been focused on metal contamination of freshwater ecosystems and its impact on amphibians. It is expected that in the future, such metal impacted populations of amphibians will have to deal with additional stressors associated with global climate changes, namely increased salinity due to sea level rise.

Accordingly, this study intended to assess the interactive effects of salinity and copper on embryos and tadpoles of the Perez´s frog (*Pelophylax perezi*, Seoane). To attain this objective, single and combined effects of salinity (NaCl) and copper on survival, malformations, body length and biochemical markers (catalase-CAT, cholinesterase-ChE, lactate dehidrogenase-LDH, and glutathione S-transferase-GST) of embryo and larval stages of *P. perezi* (only survival and biochemical markers) were evaluated in a complete bifactorial design. Embryos (Gosner stage 10-11) were exposed to individual and combinations of serial concentrations of copper (0 to 7.4 mg/L) and NaCl (0 to 10.2 g/L). Tadpoles (Gosner stage 25), in the same way, were exposed individually and in combinations to a gradient of concentrations of copper (0 to 2.4 mg/L) and NaCl (0 to 7.4 g/L). Obtained results showed that, as expected (from chapter 1), treatments with solely copper exerted a higher lethal toxicity to tadpoles (90% of mortality at 2.4 mg/L of copper) than to embryos (65% of mortality at 7.4 mg/L of copper). On the contrary, NaCl individually exerted a higher lethal toxicity to embryos (100% of mortality at 6.9 g/L of NaCl) than to tadpoles (50% of mortality at 7.37 g/L of NaCl). Regarding the joint effects of both chemicals, intermediate concentrations of NaCl decreased the lethal effects of copper to embryos and as well the incidence of malformations in the hatched larvae. For tadpoles a different response pattern was observed: NaCl did not induce a significant decrease in copper toxicity.

Regarding enzymatic activities, different responses were registered for embryos and tadpoles. In the former, copper reduced CAT activity, while the combination of the two chemicals induced its activity. For LDH both NaCl solely and its combination with copper induced its activity. For tadpoles, exposure to solely copper induced a reduction in the activity of Che, LDH, and GST, and an induction of CAT. The salt NaCl also induced the activity of CAT. The combination of both chemicals leads to an induction and reduction in

the activities of CAT and LDH, respectively. In conclusion, the obtained results suggest a life-stage dependency on the effects caused by the exposure to the individual chemicals or to its combination. Also, it was observed that a moderated increase in salinity may have a shield effect against copper lethal toxicity for early life-stages of *P. perezi*. Finally, a high variability was observed in the enzymatic responses to exposure to the chemicals individually or in combination.

Keywords: Salinity Copper *Pelophylax perezi* Interaction

Introduction

The global amphibian decline has been recognized since 1989 as a phenomenon of major concern (Wake, 1991; Blaustein, 1994). As such, several works were carried out in order to understand which factors were responsible for such declining. Several causes were identified, namely: habitat loss, diseases, introduction of alien species (either predators or competitors), fires, chemical contamination, and climate change (Blaustein and Kiesecker, 2002; Collins and Storfer, 2003; Beebee and Griffiths, 2005).

More recently, some authors proposed that a complex interaction between some of the mentioned causes is also responsible for the global amphibian decline (Kiesecker *et al*., 2001; Blaustein and Kiesecker, 2002; Collins and Storfer, 2003). However, the majority of studies continued to focus on single stressors affecting amphibian populations, and only a few addressed the effects of combined exposures to more than one stressor (Blaustein *et al*., 2010). As some examples, Kiesecker and Blaustein (1995) observed a synergistic effect between a pathogen and UV-B radiation, which resulted in increased embryo mortality, in three species of amphibians. Also, Releya (2004) evaluated the toxicity of four commercial formulations of pesticides to five species of amphibians. Though the combined pesticides occasionally induced lower effects than each pesticide alone, in general, the results obtained by this author, revealed that the impact of the combination of the four pesticides was similar to that predicted by the total concentration of pesticides in the system.

This type of works, aimed at understanding the combined effects of several stressors, are extremely pertinent and essential, since it is expected that most of the populations of amphibians inhabiting impacted sites are, most probably, exposed to a plethora of abiotic and biotic stressors that may affect them in a variety of ways. Furthermore, the predicted direct and indirect effects of climate change foresee that even those populations exposed to single stressors, may in a near future be exposed to a combination of stressors. The IPCC (2007) projections, within the context of global climate change, predict an increase in global average temperature that will provoke a rise in sea levels (due to thermal expansion or to melting glaciers or ice caps). This will lead to the salinization of low-lying coastal freshwater ecosystems caused by the intrusion of salt

water (either through surface flooding, or though groundwater) (IPCC, 2007). It is, then, expected that this salinization will adversely affect many populations of amphibians inhabiting coastal wetlands, which constitute critical habitats for these species.

With these concerns in mind, some works have already assessed the tolerance of some species of amphibians to increased salinity. For example, Haramura (2007) observed that embryos of *Buergeria japonica* exposed to increasing NaCl concentrations, failed to hatch at a salinity value of 2 (1.57 g/L NaCl). Also, Viertel (1999) reported a survival of 35% in embryos of *Rana temporaria* exposed to a salinity value of 6.2. But, again, most of these works were focused on evaluating the effects, to amphibians, of increased salinity individually.

In natural and actual scenarios, it must be taken into account that some coastal wetlands are already impacted by chemical contamination, and, therefore, species of amphibians inhabiting these areas eventually could have to cope simultaneously with two stressors: increased salinity (e.g. may disrupt the osmotic balance and respiratory functions) and chemicals (e.g. may cause oxidative stress and be an osmoregulatory obstructor) (Mahajan *et al*., 1979; Boutilier *et al*. 1992; Valavanidis and Vlachogianni, 2010).

Therefore, within this context, and aiming to achieve a more accurate protection and conservation of this group of organisms, it is important to understand how "new" stressors, derived from global changes, may influence the toxicity of more commonly perceived stressors.

According to the above mentioned, the present work intended at assessing the influence of increased salinity on the toxicity of copper to different aquatic life-stages of the Perez's frog, *Pelophylax perezi* (Seoane). The metal copper was chosen because it widely used (e.g. electrical wires, roofing and plumbing, industrial machinery) in our society. For this reason, the extraction industry greatly increases its concentrations in the environment, leading to severe contamination of many aquatic ecosystems worldwide. Furthermore, copper is an essential metal for biota, constituting both an enzymatic cofactor and part of the structure of several proteins (Flemming and Trevors, 1989). Despite this fact, copper can be very toxic to organisms at low concentrations, namely for amphibians. For example, Pérez-Coll and Herkovits (2006) found a 24h-LC50 of 0.12mg/L

to embryos of *Bufo arenarum*. Also, concentrations as low as 0.036 mg/L have been shown to cause reduced survival and abnormal behavior on tadpoles of *Rana pipiens* (Redick and La Point, 2004).

Material and Methods

Organisms collection

Egg masses of the Perez's frog, *Pelophylax perezi* (Seoane) were collected in a freshwater pond (+40.596364N; -8.69569W; with 8.0 mg/L of dissolved oxygen, 240µS/cm of conductivity, 154 mg/L of total dissolved salts, and pH value of 8.7) located near the city of Aveiro, Portugal, during May 2011. Some of the collected eggs were immediately used to carry out the assay with embryos, since they were in developmental stages 10-11 determined according to Gosner (1960). The remaining eggs were maintained in FETAX medium (Danson and Bantle, 1987) until hatching and reaching developmental stage 25 (Gosner, 1960). At this time, tadpoles were used to carry out toxicity assays.

Exposure design

Stock solutions of copper (II) sulphate pentahydrated ($CuSO_4.5H_2O$; Merck, Darmstadt, Germany) and sodium chloride (NaCl; Merck, Darmstadt, Germany) were prepared in FETAX medium for embryo and tadpole assays.

a) Experiment 1: Embryo Assay

Eggs of *P. perezi* at Gosner stages 10-11 were exposed to a complete bifactorial design with combinations of seven concentrations of copper (mg/L) and six concentrations of sodium chloride (g/L) and a control constituted by the FETAX medium (Table 4). Stock

solutions of copper (15 mg/L) and NaCl (20.4 g/L) were prepared to obtain the test concentrations by diluting them with FETAX using a dilution factor of 1.5.

Table 4 - Bifactorial design used in the Embryo assay with combinations of copper and sodium chloride during 96h of exposure**.**

	Copper (mg/L)	Cu0	Cu1	Cu2	Cu3	Cu4	Cu5	Cu6	Cu7
NaCl (g/L)		0	0.66	0.99	1.6	2.2	3.3	5.0	7.4
Na0	0	Control	Cu1*Na0	Cu2*Na0	Cu3*Na0	Cu4*Na0	Cu5*Na0	Cu6*Na0	Cu7*Na0
Na1	1.3	Cu*Na1	Cu1*Na1	Cu2*Na1	Cu3*Na1	Cu4*Na1	Cu5*Na1	Cu6*Na1	Cu7*Na1
Na2	2.0	Cu*Na2	Cu1*Na2	Cu2*Na2	Cu3*Na2	Cu4*Na2	Cu5*Na2	Cu6*Na2	Cu7*Na2
Na3	3.0	Cu0*Na3	Cu1*Na3	Cu2*Na3	Cu3*Na3	Cu4*Na3	Cu5*Na3	Cu6*Na3	Cu7*Na3
Na4	4.6	Cu0*Na4	Cu1*Na4	Cu2*Na4	Cu3*Na4	Cu4*Na4	Cu5*Na4	Cu6*Na4	Cu7*Na4
Na5	6.9	Cu0*Na5	Cu1*Na5	Cu2*Na5	Cu3*Na5	Cu4*Na5	Cu5*Na5	Cu6*Na5	Cu7*Na5
Na6	10.2	Cu0*Na6	Cu1*Na6	Cu2*Na6	Cu3*Na6	Cu4*Na6	Cu5*Na6	Cu6*Na6	Cu7*Na6

The assay with the embryos of *P. perezi* was carried out in plastic Petri dishes (55mm of diameter) with 10mL of treatment solution and 10 eggs per dish/replicate. For each treatment two replicates were performed. The jelly coat of eggs was kept intact to guarantee more realistic conditions of exposure, i.e. similar to what occurs in natural scenarios. Organisms were maintained under a 14:10 h (light:dark) photoperiod and at 23 ± 1ºC, during the acute test period of 96h. The test solutions were renewed each 24h and dead individuals removed (to avoid possible microbial contamination of the surviving ones). The following physico-chemical parameters were measured during the assay: dissolved oxygen (OXI 330/SET, best nr. 200 232), pH (330/SET-2, best nr. 100 788), conductivity (LF 330/SET, best nr. 300 204), salinity (LF 330/SET, best nr. 300 204), and dissolved salts (LF 330/SET, best nr. 300 204).

b) Experiment 2: Tadpole Assay

Tadpoles were exposed to a similar complete bifactorial design with combinations of a gradient of five copper concentrations (mg/L) and six sodium chloride concentrations (g/L) plus a control (FETAX medium) (Table 5).

A stock solution of copper with an initial concentration of 100mg/L was prepared with FETAX and all the copper treatments were obtained by dilutions with FETAX using a dilution factor of 1.5x. Sodium chloride concentrations were prepared in the same way, using a dilution factor of 1.5x, from a stock solution of 20g/L of sodium chloride, previously prepared in FETAX. For each treatment, volumes of both stock solutions were combined and diluted with FETAX medium to attain the final volume and concentrations required.

Table 5 - Bifactorial design used in the Tadpole assay, where organisms were exposed to copper, NaCl and a combination of both chemicals during a period of 96h.

	Copper (mg/L)	Cu0	Cu1	Cu2	Cu3	Cu4	Cu5
NaCl (g/L)		0	0.55	0.79	1.15	1.65	2.4
Na0	0	Control	Cu1*Na0	Cu2*Na0	Cu3*Na0	Cu4*Na0	Cu5*Na0
Na1	1.2	Cu*Na1	Cu1*Na1	Cu2*Na1	Cu3*Na1	Cu4*Na1	Cu5*Na1
Na2	1.73	Cu*Na2	Cu1*Na2	Cu2*Na2	Cu3*Na2	Cu4*Na2	Cu5*Na2
Na3	2.37	Cu0*Na3	Cu1*Na3	Cu2*Na3	Cu3*Na3	Cu4*Na3	Cu5*Na3
Na4	3.56	Cu0*Na4	Cu1*Na4	Cu2*Na4	Cu3*Na4	Cu4*Na4	Cu5*Na4
Na5	5.08	Cu0*Na5	Cu1*Na5	Cu2*Na5	Cu3*Na5	Cu4*Na5	Cu5*Na5
Na6	7.37	Cu0*Na6	Cu1*Na6	Cu2*Na6	Cu3*Na6	Cu4*Na6	Cu5*Na6

Tadpoles at Gosner stage 25 were used to carry out the assay. Initial body length of the tadpoles was measured (7.8±0.65 mm) to assure similar sizes in all treatments. The measurements were performed under a magnifying lens (Leika MS5) at a 10x factor.

Tadpoles were exposed in 500 ml plastic vessels, with 250 ml of treatment solution and with constant aeration. Assay was conducted with two replicates per

treatment, each replicated containing ten tadpoles. No food was provided to tadpoles during the 96h exposure period.

All procedures followed the guidelines defined by ASTM (1998, 2000). Test conditions involved a 14:10 h (light:dark) photoperiod and a temperature range of 23±1ºC. The exposure period was 96 h, since the objective was to achieve the effects during an acute period of exposure. Renewals of the test medium were performed every day and dead individuals removed to prevent microbial contamination to the surviving ones.

During the experiments, the following physico-chemical parameters were monitored daily in the test solutions: pH (pH 330/SET-2, best nr. 100 788), dissolved oxygen (mg/l, OXI 330/SET, best nr. 200 232), conductivity (mS/cm, LF 330/SET, best nr. 300 204), and ammonia (mg/L).

Endpoints analysis

Lethal effect

Mortality of embryos and tadpoles was checked every 24h and dead individuals removed to prevent contamination. At the end of the assays (96h), total cumulative mortality was registered for each treatment.

Sublethal effects

1) Behavior and development effects

On Experiment 1 (with embryos), the following sublethal responses were analyzed at the end of the assay: hatching rates and surviving organisms were checked for malformations under a magnifying lens (Leika MS5) at 10x amplification. Afterward, every Petri dish containing the surviving organisms was photographed using a digital camera (Sony DSC-W80) and a millimeter scale to proceed with the measurements of the larvae total body length. Images were analyzed using the software SigmaScan Pro 5. All organisms were measured from the snout to the tail tip.

On Experiment 2, alterations of swimming behavior were monitored as well.

2) Biochemical responses

Surviving organisms from both assays were frozen in liquid Nitrogen and immediately preserved in refrigerate conditions at -80ºC to further evaluation of the enzymatic activities.

Biochemical analyses that were performed consisted in the determination of Catalase (CAT), Glutathione *S*-transferase (GST), Lactate dehydrogenase (LDH) and Cholinesterase (ChE) activities. The enzymatic activities were analyzed in the whole body of the organism. All samples were defrosted on ice, at the day the enzymatic activities were determined.

To Experiment 1, groups of four organisms were homogenized on 0.7 ml potassium phosphate buffer (0.1 M, pH 7.4). For each treatment were prepared three replicates, using a total of four organisms per replicate.

For Experiment 2, groups of two tadpoles were homogenized in 1.2 ml Potassium phosphate buffer (0.1 M, pH 7.4). At least 3 replicates were performed to each treatment. Homogenizations of the samples were performed using a sonifier (Branson S-250A).

After this initial step, the homogenated samples were centrifuged (4ºC, 10.000 g) for 5 and 20 min, respectively for experiment 1 and 2, on a centrifuge Eppendorf, 5810 R. The four enzyme's activity was analyzed directly from the supernatant obtained after centrifugation, using the following methodology:

a) Catalase (CAT, EC 1.11.1.6) activity was determined according to the method described by Clairborne (1985), which, briefly, consists in following the decrease in the absorbance at 240 nm that represents the decomposition of H_2O_2 (which is the substrate for the enzyme) into H_2O and O_2.
b) Cholinesterase (ChE, EC 3.1.1.8) activity was measured using Ellman´s method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996). Acetylthiocholine was used as substrate that is degraded by ChE into acetate and tiocholine. The latter one conjugates with 5,5-dithiobis-2-nitrobenzoic acid (DTNB)

originating a yellow product which can be monitored by the increase of the absorbance at 414 nm.

c) Glutathione S-transferase (GST, EC 2.5.1.18) determination was performed following the method of Habig and Jakoby (1981) and adapted to microplate (Frasco and Guilhermino, 2002). The GST activity is analyze by quantification of the product resulted from the conjugation of reduced 10 mM glutathione (GSH) and 60 mM 1-chloro-2.4-dinitrobenzene (CDNB) as substrate which can be monitored by the increase of absorbance at 340 nm.

d) Lactate dehydrogenase (LDH, EC 1.1.1.27) activity was determined based on the measuring the decrease of absorbance at 340 nm, resulting from NADH oxidation, which was used as substrate, following the method of Vassault (1983).

Enzymatic activities were determined in triplicate for each replicate sample and with three replicates per treatment. The activity of enzymes was expressed as nmol of substrate hydrolyzed per minute per mg of protein. The protein content in the supernatant obtained from centrifuged samples was calculated in quadruplicate using the Bradford method (Bradford, 1976), adapted to microplate, at 595 nm, using bovine γ-globulin as standard.

For determination of GST, LDH, AChE and protein content a Labsystem Multiskan EX microplate reader was used and to measure CAT activity it was used a Jenway 6505 UV/vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK). In Experiment 2 all the four described enzymes were measured while in the first experiment only CAT, ChE and LDH were determined.

Statistical analysis

The lethal and sublethal responses observed in the tested treatments were analyzed by using a two-way analysis of variance (ANOVA) followed by the Tuckey or contrast analysis (when significant interactions were detected), whenever significant differences were detected. Prior to this analysis, mortality data were transformed with the arcsin of the square root of cumulative mortality. These analyses were performed using the software Statistica 6.0, for Windows (Statsoft). Assumptions for normality and

homogeneity of variances were tested with the Shapiro-Wilks and Barttlet tests, respectively.

Results

Physico-chemical parameters

During the assays, values of physico-chemical parameters did not exhibit high variations within treatments. Dissolved oxygen was always above 8 mg/L on embryo assay and above 7.4 mg/L on the tadpole assay. The pH and conductivity values ranged between the intervals 7.4-8.7 and 0.5-19.5 respectively within treatments for both assays. Salinity levels were between 0.3-11.6 on embryo assay and 0-7.9 on tadpole assay and ammonia levels were always between 0.8 and 0.12 mg/L. Variations, within replicates never exceeded 0.56 for pH, 0.037 for salinity and 0.037 mS/cm for conductivity.

Lethal effects

b) Experiment 1: Embryo Assay

No significant mortality (above 10%) was observed in the control groups. The values of cumulative mortality (%) observed at the end of the assay (96h) are represented in Table 6.

Table 6 - Observed cumulative mortality (%) of embryos of *Pelophylax perezi* exposed to individual and combined treatments of copper and NaCl, during a period of 96h.

		Copper treatments (mg/L)							
	%	Cu0 (0)	Cu1 (0.66)	Cu2 (0.99)	Cu3 (1.6)	Cu4 (2.2)	Cu5 (3.3)	Cu6 (5.0)	Cu7 (7.4)
NaCl treatments (g/L)	Na0 (0)	3	10	0	10	10	15	15	65
	Na1 (1.3)	5	0	0	5	0	10	0	25
	Na2 (2.0)	10	5	5	0	0	5	5	15
	Na3 (3.0)	5	0	0	0	0	0	0	20
	Na4 (4.6)	0	5	0	0	5	0	20	45
	Na5 (6.9)	100	100	90	95	95	100	90	100
	Na6 (10.2)	100	100	100	100	100	100	100	100

Treatments with NaCl alone significantly reduced survival (F=828; d.f.=6; p=0.000) of embryos of *P. perezi*, but only at the two highest concentrations of NaCl (contrast analysis: p=0.0002). In the highest concentration of NaCl embryos did not developed and 100% mortality occured within the first 24h. In the second highest concentration of NaCl, the embryos were able to develop during the first 48 to 72h but died just before the end of the assay (96h). The four lowest NaCl concentrations did not negatively affect survival (contrast analysis: p>0.05) (Table 6).

Treatments where embryos of *P. perezi* were exposed solely to copper also exhibited a significant effect on mortality (F=22.6; d.f.=7; p=0.000), but only the highest copper concentration significantly reduced survival (65% of mortality) (contrast analysis: p= 0.000) (Table 6).

In treatments involving exposure to a combination of copper and NaCl, there was a significant interaction between the two chemicals on the observed mortality (F=3.6; d.f.=42; p<0.001) at Cu7Na1, Cu7Na2 and Cu7Na3 treatments (contrast analysis: p<0.000224), which corresponds to the highest copper concentration (7.4mg/L) and three intermediate NaCl concentrations. These combinations have apparently diminished mortality when compared with the same concentration of the metal alone (Cu7Na0)(Table 6). It is also interesting to note that the fourth tested NaCl concentration (4.6 g/L) significantly reduced survival but only when combined with the highest copper concentration (e.g: Cu7Na4) (contrast analysis: p=0.000203).

b) Experiment 2: Tadpole Assay

No mortality was observed in control groups. Observed cumulative mortality (%) at the end of the assay is represented in Table 7.

Treatments with only NaCl cause a significant reduction in tadpole's survival but only at the highest tested concentration (7.37g/L of NaCl) (50% mortality) (F=4.4274; d.f.=6; p=0.001; followed by Tuckey: p= 0.001019).

All treatments where organisms were exposed solely to copper caused a significant increase in mortality (≥20%) (F=15.4746;d.f.=5; p=0.000 followed by Tuckey: p<0.003360).

In treatments combining NaCl and copper, no significantly interaction between the two factors were registered (F=0.3515; d.f.=30; p=0.998). Despite the absence of significant interactions between NaCl and copper the addition of NaCl concentrations, in some copper treatments (e.g. Cu1Na0 versus Cu1Na6, Cu2Na0 versus Cu2Na6; Cu3Na0 versus Cu3Na6, see Table 7) seems to have caused an increase in mortality comparatively to the mortality observed by the metal alone.

Table 7 - Observed cumulative mortality (%) of tadpoles of *Pelophylax perezi*, exposed for 96h, to copper, NaCl and combinations of the two chemicals.

		Copper treatments (mg/L)					
NaCl treatments (g/L)	%	Cu0 (0)	Cu1 (0.55)	Cu2 (0.79)	Cu3 (1.15)	Cu4 (1.65)	Cu5 (2.4)
	Na0 (0)	0	20	50	45	75	90
	Na1 (1.2)	15	30	40	70	70	70
	Na2 (1.73)	0	35	35	60	85	90
	Na3 (2.37)	0	30	50	55	75	80
	Na4 (3.56)	0	55	65	70	75	75
	Na5 (5.08)	0	55	75	100	100	100
	Na6 (7.37)	50	95	100	100	100	100

Sublethal endpoints

Behavior and development effects

a) Experiment 1: Embryo Assay

Malformations

In the control groups no malformations were registered (Table 8 and Figs.8A and 9A).

Exposure to treatments with solely NaCl did not significantly affected the normal development of organisms (F=2.27.; d.f.=4; p=0.07) (Figs.8B and 9C).

Copper treatments alone significantly affected the occurrence of malformations (F=13.14.; d.f.=7; p=0.00). The highest copper concentration (without the addition of NaCl) significantly increased the occurrence of malformations (58%; Tuckey: p=0.000203).

Few treatments (Table 8) combining NaCl and copper caused malformations but in non significant percentages (<35%; F= 1.37; d.f.= 28; p=0.16). However, at the highest copper concentration, the presence of NaCl seems to have reduced the occurrence of malformations (Cu7Na1; Cu7Na3; Cu7Na4) (Table 8).

Table 8 – Malformations occurrence (%) after 96h on surviving organisms of *Pelophylax perezi* exposed to combinations of copper and NaCl. Asterisks represent copper treatments with significantly differences from control. The total number of organisms tested per treatment was 20, inside brackets are depicted the number of surviving organism.

Percentage of Malformations (%) (nr. of surviving organisms)									
	Copper concentrations (mg/L)								
NaCl concentrations (g/L)		Cu0 (0)	Cu1 (0.66)	Cu2 (0.99)	Cu3 (1.6)	Cu4 (2.2)	Cu5 (3.3)	Cu6 (5.0)	Cu7 (7.4)
	Na0 (0)	0 (20)	0 (18)	5 (20)	0 (18)	6 (18)	13 (17)	6 (17)	58* (7)
	Na1(1.3)	0 (19)	0 (20)	0 (20)	6 (19)	0 (20)	0 (18)	0 (20)	25 (15)
	Na2 (2.0)	0 (18)	5 (20)	6 (19)	0 (20)	0 (20)	0 (19)	11 (19)	35* (17)
	Na3 (3.0)	0 (19)	0 (20)	5 (20)	0 (20)	0 (20)	0 (20)	0 (20)	20 (16)
	Na4 (4.6)	0 (20)	6 (19)	10 (20)	5 (20)	0 (19)	10 (20)	7 (16)	20 (11)
	Na5 (6.9)	-	-	100* (2)	100* (1)	100* (1)	-	-	-

The highest tested NaCl concentration (Na5 = 6.9 g/L), for the combinations with copper where 100% mortality did not occur, caused severe damages on the few surviving organisms (see Figures 8B and 9B, 9C). In these cases, malformations were characterized as body abnormal curvature and reduced body length (Figures 9B, 9C). Also, the few organisms that were capable of hatching (<5%) did not show any movement beyond a slight and almost imperceptible tail movement at the end of the assay.

Different copper concentrations caused several adverse effects on the surviving organisms, which were mainly abnormal curvature body, abnormal tail, tail fin and edemas (Figures 9D-H).

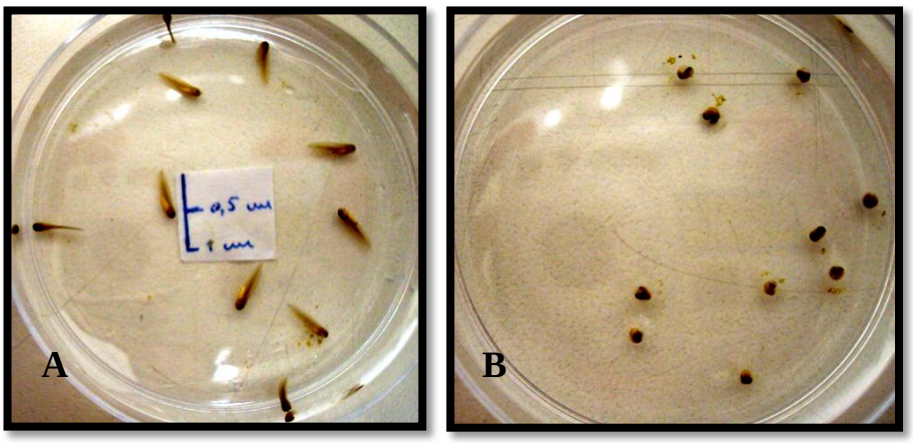

Figure 8 – Global vision of assay conditions of the Experiment 1 after 96h with embryos of *Pelophylax perezi* exposed to copper, NaCl and both combinations; A) Control groups (FETAX medium) ; B) Na5 treatments (6.9g/L NaCl**).**

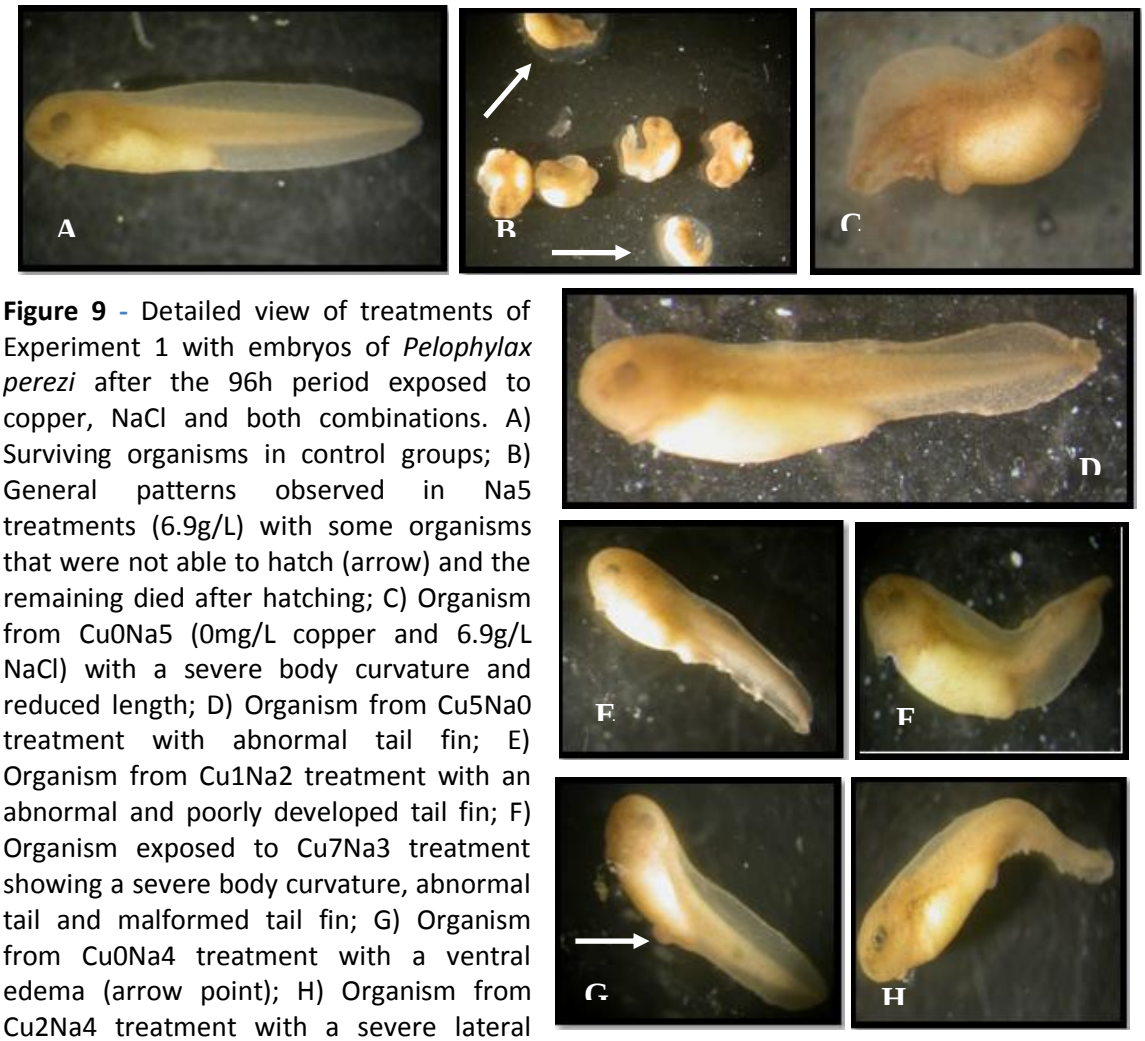

Figure 9 - Detailed view of treatments of Experiment 1 with embryos of *Pelophylax perezi* after the 96h period exposed to copper, NaCl and both combinations. A) Surviving organisms in control groups; B) General patterns observed in Na5 treatments (6.9g/L) with some organisms that were not able to hatch (arrow) and the remaining died after hatching; C) Organism from Cu0Na5 (0mg/L copper and 6.9g/L NaCl) with a severe body curvature and reduced length; D) Organism from Cu5Na0 treatment with abnormal tail fin; E) Organism from Cu1Na2 treatment with an abnormal and poorly developed tail fin; F) Organism exposed to Cu7Na3 treatment showing a severe body curvature, abnormal tail and malformed tail fin; G) Organism from Cu0Na4 treatment with a ventral edema (arrow point); H) Organism from Cu2Na4 treatment with a severe lateral curvature of tail.

Body Length

NaCl treatments did not significantly affect body size ($F= 0.64$; d.f.=4; $p=0.605$). However, it must be highlighted that when performing the statistical analyzes, the two highest NaCl treatments were not included due to the few number of surviving organisms (none organisms in the highest concentration and two organisms in the fifth concentration) and in this last treatment hatchlings showed a smaller body size, mainly due to body malformations

Contrary to NaCl, copper treatments significantly reduced body length at the end of the assay ($F=4.98$; d.f.=7; $p=0.000231$), but only at the two highest concentrations (Cu6= 5 mg/L; Cu7= 7.4 mg/L) (Tuckey: $p<0.001696$)(see Fig.10).

Combined treatments of copper and NaCl did not showed significant interactions (F=0.56; d.f.=28;p=0.952432).

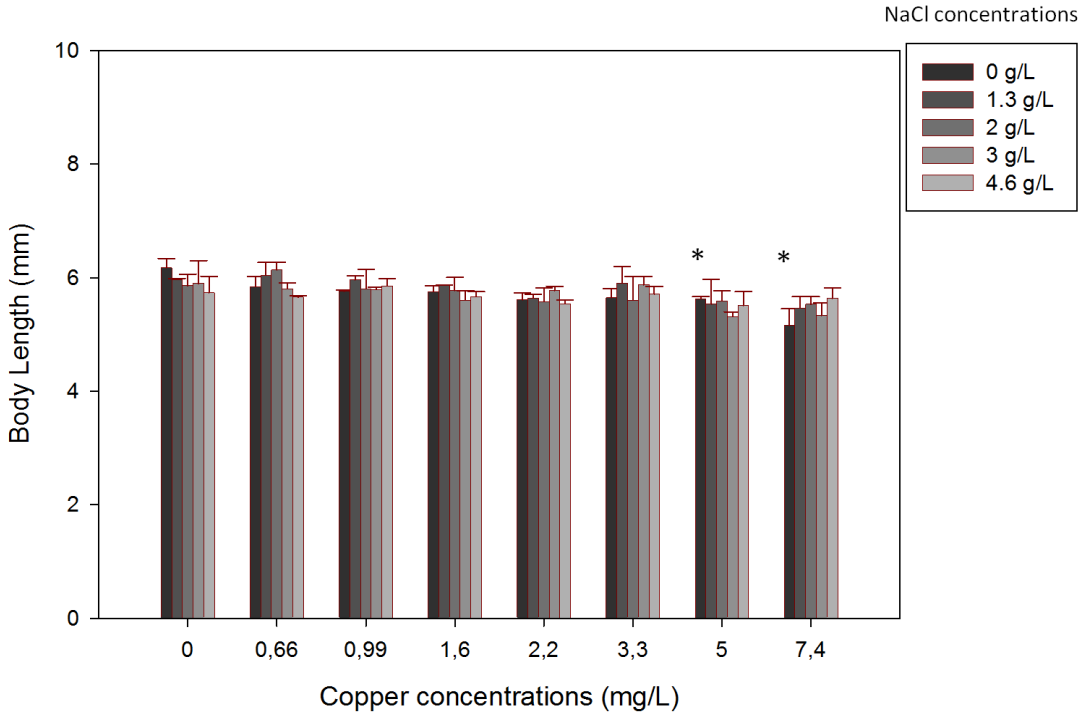

Figure 10 - Body length (mm) (mean value ± standard deviation) of hatchlings of *Pelophylax perezi* after 96h of exposure to combinations of copper and NaCl treatments. Asterisks indicate the treatments of copper individually that were significantly different from the control (p<0.001).

b) Experiment 2: Tadpole Assay

No alterations in swimming activity were observed in tadpoles exposed to treatments with NaCl solely or controls. But, abnormal swimming behavior was observed at the two highest concentrations of copper (solely and combined with NaCl). This abnormal swimming behavior was characterized by erratic movements and only for a few seconds showing poor swimming ability. Moreover, affected larvae were also observed mainly motionlessness staying on the bottom of the test vessels and only reacted if stimulated, which represent a delay on time of response.

Biochemical analyzes

c) Experiment 1: Embryo Assay

Treatments with NaCl did not significantly affected the enzymatic activity of catalase (CAT) (F=0.869; d.f.=4; p=0.486). However the opposite was observed when considering exposure to solely copper (F=11.998; d.f.=7; p=0.00) (see columns with asterisks, Figure 11). The enzyme activity showed a biphasic curve, with a significant decreased at the highest concentrations of copper alone (contrast analysis: p=0.000502) (see columns with asterisks, Figure 11). Further a significant interaction between both factors on CAT activity was recorded (F=1.795; d.f.=28; p=0.023). CAT activity showed an irregular pattern with increasing NaCl concentrations and combination of both chemicals (Figure 11). Despite the lack of consistence to all treatments, the increasing of NaCl concentrations decreased the effects of intermediate concentrations of copper (which inhibited the activity of the enzyme) (see Figure 11).

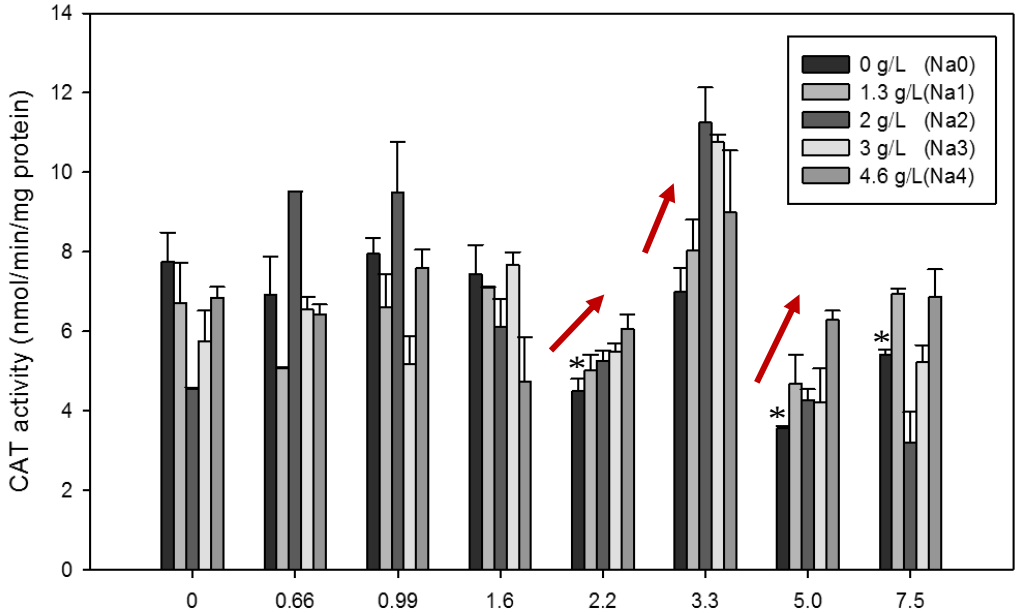

Figure 11 - CAT activity (nmol/min/mg protein) with mean values (and respective standard error) on hatchlings of *Pelophylax perezi after* 96h exposure to combinations of copper and NaCl. Different color columns represent different NaCl treatments**.** Columns with asterisks show significant differences on enzymatic activity of copper treatments Red arrows shows significant interaction of copper and NaCl.

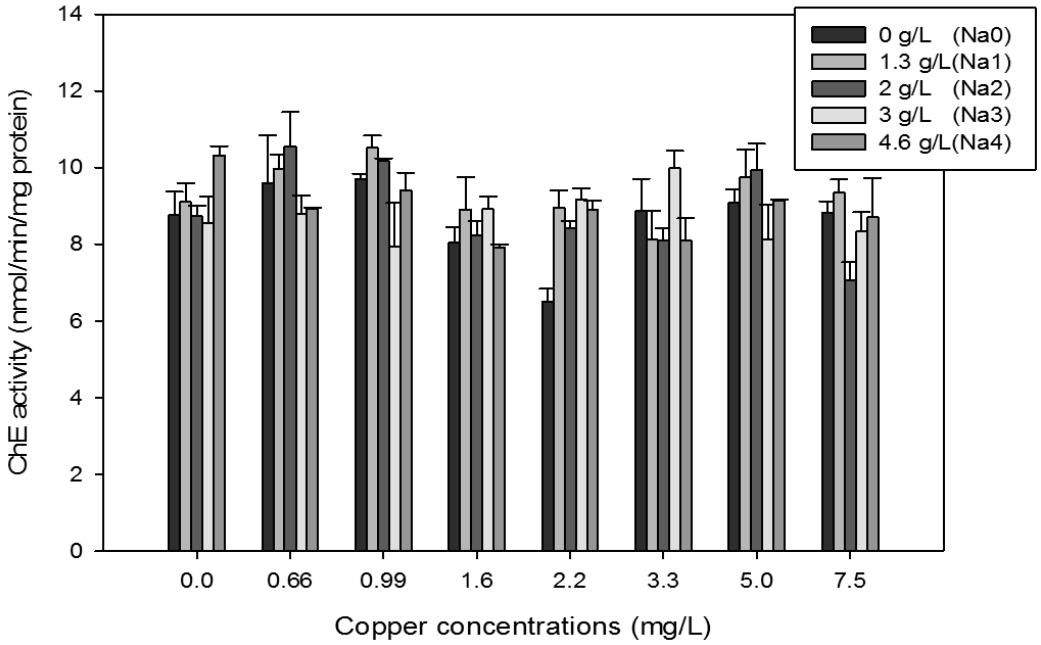

Figure 12 - ChE activity (nmol/min/mg protein) with mean values (and respective standard error) on hatchlings of *Pelophylax perezi* after 96h exposure to combinations of copper and NaCl. Columns with asterisks show significant differences on enzymatic activity of copper treatments. Different color columns represent different NaCl treatments. Red arrows show significant interactions of NaCl and copper.

ChE activity showed similar activities for all NaCl treatments ($F=1.241$; d.f.=4; $p=0.30$). No significant alterations on the enzymatic activity was found for ChE on embryos exposed to copper treatments ($F=1.440$; d.f.=7; $p=0.20$). Also, no significant interaction between both factors was recorded ($F=1.165$; d.f.=28; $p=0.29$).

Though a tendency for an increased in the LDH activity seemed to occur due to exposure to NaCl solely, it was not significantly different between NaCl treatments or the control (F=2.365; d.f.=4; p=0.06). As copper concentrations increase, an initial induction of LDH activity and posterior reduction was observed (F=59.3; d.f.=7; p=0.000; followed by contrast analysis: p<0.000319). A significant interaction between copper and NaCl was observed (F=3.3; d.f.=28; p=0.000017). Combining NaCl and copper concentrations, cause a significant induction of LDH activity, comparatively with treatments with copper alone except at the two highest copper concentrations (contrast analysis: p<0.0354)(Figure 13).

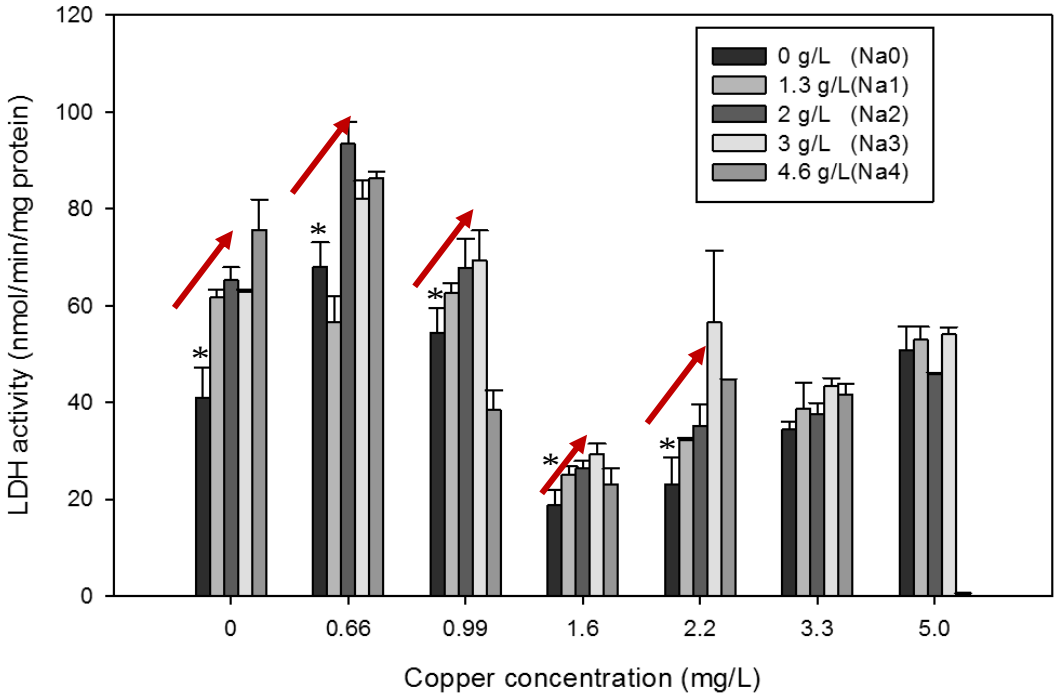

Figure 13 - LDH activity (nmol/min/mg protein) with mean values (and respective standard error) on hatchlings of *Pelophylax perezi* after 96h exposure to combinations of copper and NaCl. Different color columns represent different NaCl treatments. Columns with asterisks show significant differences on enzymatic activity of copper treatments. Different color columns represent different NaCl treatments. Red arrows show significantly interactions of NaCl and copper treatments.

b) Experiment 2: Tadpole Assay

Results obtained from biochemical analyzes (Figure 14) showed significant differences in CAT activity (F=7.638, d.f.=4; p=0.000027) with a variable pattern. Further, copper also exerted significant effects on CAT activity (F=45.298; d.f.=3; p=0.000). The copper concentrations 1.15 and 1.65 mg/L, with sufficient survival organisms for biochemical analysis, caused a significant increase of CAT activity (Contrast analysis: p<0.00174) (Figure 7, asterisks columns). A significant interaction between both factors also occurred (F=6.151; d.f,=12; p=0.000), with combinations of NaCl and copper, at intermediate levels, affecting CAT activity. Addition of NaCl to copper induced the activity of CAT (Figure 14) (contras analysis: p<0.001024).

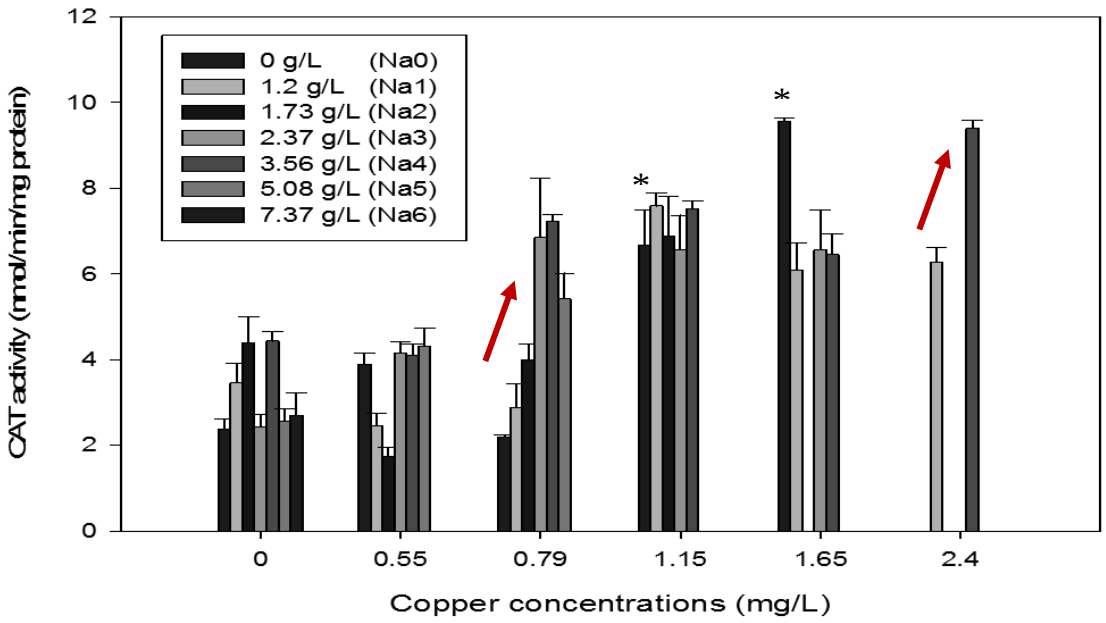

Figure 14 - CAT activity (nmol/min/mg protein) with mean values (and respective standard error bars) on tadpoles of *Pelophylax perezi* after 96h exposure to combinations of copper and NaCl. Columns with asterisks show significant differences on enzymatic activity of copper treatments Different color columns represent different NaCl treatments. Lack of columns represents samples with less than 3 replicates. Red arrows show significantly interactions of NaCl and copper treatments.

GST activity (Figure 15) was significantly affected by copper treatments (F=4.793; d.f.=3; p=0.003957), showing an inhibition with increasing concentrations of copper (Tuckey: p<0.01488). NaCl had a no significant effect on GST activity. Further no significant interaction between both factors was recorded (F=1.802; d.f.=12; p=0.061).

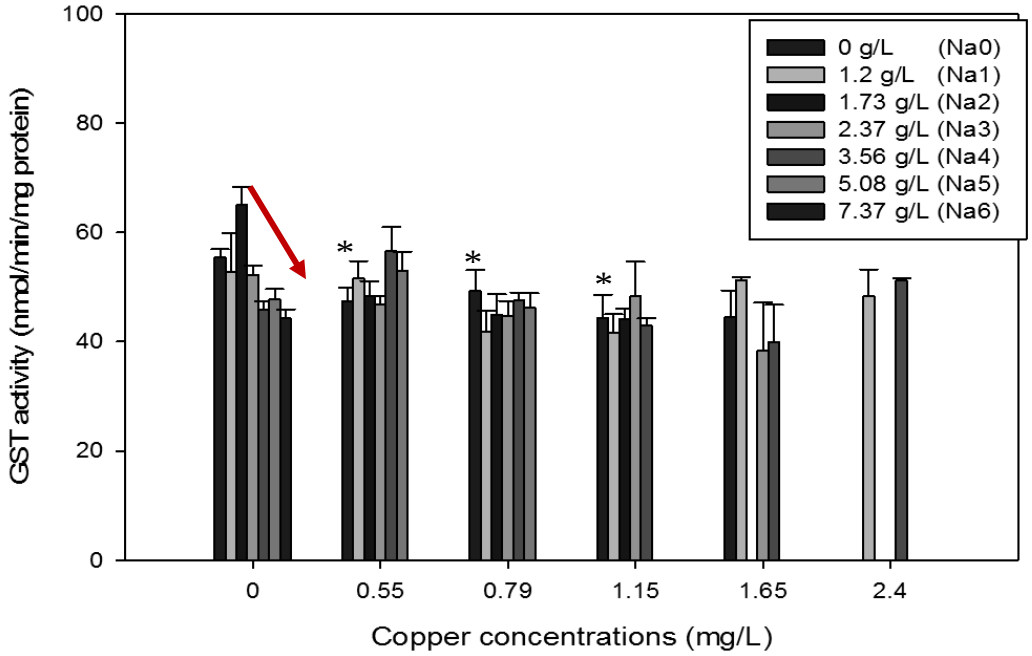

Figure 15 - GST activity (nmol/min/mg protein) with mean values (and respective standard error bars) on tadpoles of *Pelophylax perezi* after 96h exposure to combinations of copper and NaCl. Columns with asterisks show significant differences on enzymatic activity of copper treatments Different color columns represent different NaCl treatments. Lack of columns represents samples with less than 3 replicates. Red arrows show significantly interactions of NaCl and copper treatments.

Regarding the enzymatic activity of ChE (Figure 16) no clear pattern was observed with the increasing copper or NaCl concentrations. However, according to statistical analyses copper significantly affected ChE activity (F=3.633;d.f.=3;p=0.016). At intermediate copper concentrations, ChE activity was inhibited (Figure 16) (Tuckey: p=0.037). NaCl had a no significant effect on this enzyme (F=0.160; d.f.=4; p=0.96), further no significant interaction between both factors has occurred (F=1.002; d.f.= 12; p=0.454).

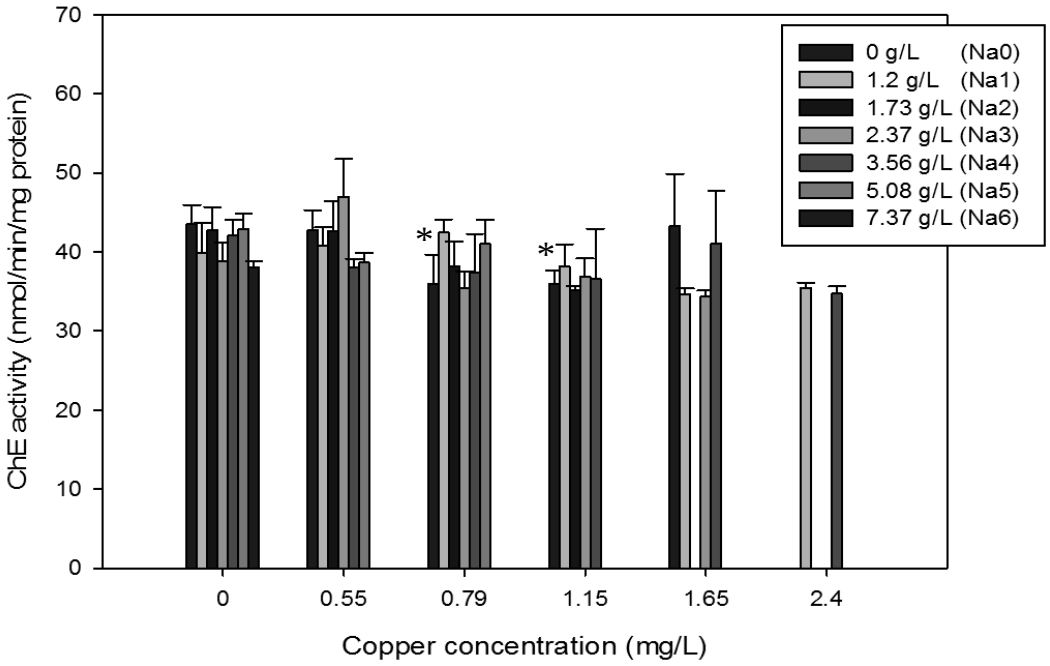

Figure 16 - ChE activity (nmol/min/mg protein) with mean values (and respective standard error bars) on tadpoles of *Pelophylax perezi* after 96h exposure to combinations of copper and NaCl. Columns with asterisks show significant differences on enzymatic activity of copper treatments. Different color columns represent different NaCl treatments. Lack of columns represents samples with less than 3 replicates. Red arrows show significantly interactions of NaCl and copper treatments.

NaCl individually did not affect LDH activity (F=0.547; d.f.=4; p=0.70) but it was significantly affected by copper (F=7.787; d.f.=3; p=0.0001). Activity of LDH suffered a significantly reduction with increasing concentrations of copper (contrast analysis: p <0.001223) (Figure 15, asterisks columns). NaCl in combination with copper had a significantly effect on LDH activity (F=3.526; d.f.=12; p=0.0003) but did not showed any consistent pattern.

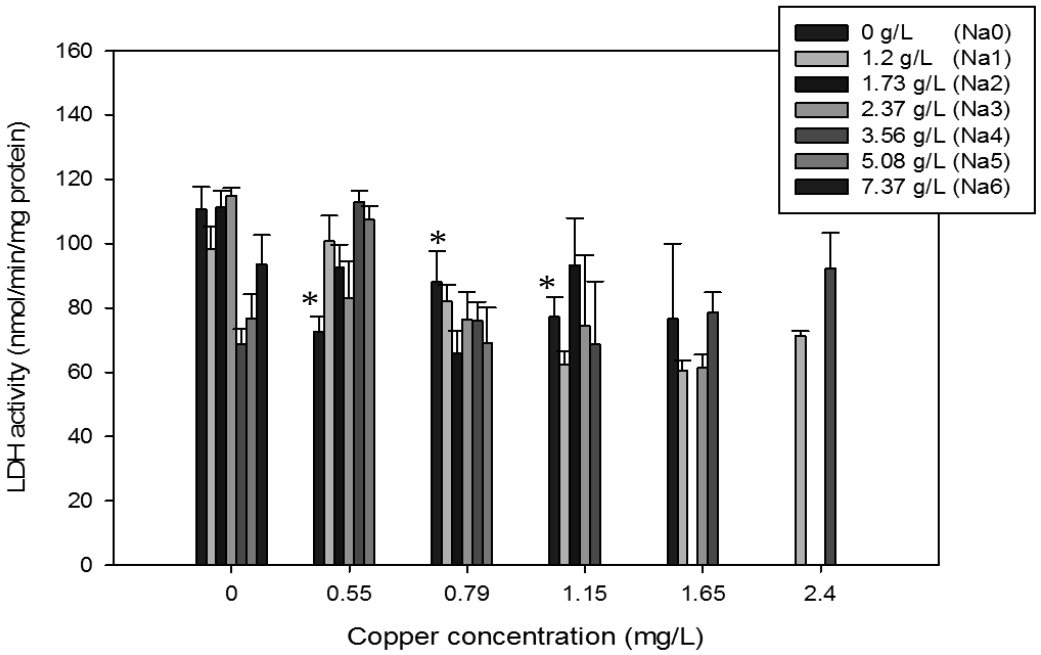

Figure 17 - LDH activity (µmol/min/mg protein) with mean values (and respective standard error bars) on tadpoles of *Pelophylax perezi* after 96h exposure to combinations of copper and NaCl. Columns with asterisks show significant differences on enzymatic activity of copper treatments. Different color columns represent different NaCl treatments. Lack of columns represents samples with less than 3 replicates. Red arrows show significantly interactions of NaCl and copper treatments.

Table 9 - Resume table showing the general pattern observed on enzymatic activity of CAT, ChE, LDH and GST of embryos and tadpoles of *Pelophylax perezi* after 96h exposure to copper, NaCl and combination of both chemicals. Horizontal arrows means no significantly induction or reduction were observed; arrow pointing downwards shows enzymatic activity inhibition; arrow pointing upwards shows enzymatic activity induction.

	EMBRYO ASSAY			TADPOLE ASSAY		
	NaCl	Copper	NaCl*Copper	NaCl	Copper	NaCl*Copper
CAT	→	↘	↗	↗	↗	↗
ChE	→	→	→	→	↘	→
LDH	↗	↘	↗	↘	→	→
GST	----	----	----	↘	→	→

Discussion

Amphibians, in general, have been shown to be highly sensitive to the toxic effects of metals (Haywood *et al*., 2004; García-Muñoz *et al.,* 2009; Barry, 2011); with tadpoles, usually, exhibiting a higher sensitivity than embryos (Lefcort *et al.,* 1998; Haywood *et al.,* 2004; Redick and La Point, 2004). The results obtained in this study (and in chapter 1 of this thesis) support those findings, since tadpoles of *P. perezi*, exposed solely to copper, started to exhibit a significant mortality (≥20%) at 0.55 mg/L of copper, while embryos, even at a concentration of copper as high as 5.0 mg/L, showed a mortality lower than 20%. Also, concentrations inducing growth inhibition in hatched larvae (from the embryo experiment) were higher (5.0 mg/L Cu) than the concentration causing a significant mortality in tadpoles (0.55 mg/L Cu). Despite the low mortality on embryos, the highest copper concentrations significantly induced the occurrence of malformations (58%). The determined activities of the analyzed enzymes did not allow making conclusions about the comparative sensitivities between the two life stages. Opposite responses were observed at the two life stages: a reduction and induction, in CAT activity, was registered, for embryos and tadpoles, respectively. As copper is known to cause oxidative stress, an increase in the activity of CAT was expected, since this is one of the most important enzymes of the cell antioxidant defense system (Dazy *et al.*, 2009), being involved in the deactivation of H_2O_2 (transforming it into H_2O and O2; Apel and Hirt, 2004). However, a reduction in CAT activity on early life stages of amphibians, has also been reported in the literature (Ferrari *et al*. 2008) due to other pro-oxidants compounds (such as pesticides) and due metal exposure on several aquatic organisms (Atli *et al.,* 2006). This reduction has been reported to be associated with high oxidative stress scenarios, where the enzyme may be inhibited by its own substrates or by the direct action of copper, since it can react with –SH groups disrupting the enzyme active-sites (e.g. displacing iron from the enzyme active centre (Stauber and Florence, 1987; Pigeolet *et al*., 1990). The other analyzed enzymes did not allow drawing any conclusion regarding the comparison of sensitivities to copper, between the two tested life stages, since a clear concentration-response relationship was not observed with increasing concentrations of copper. Both an increase and a decrease of enzymatic activities such as the one observed for LDH

represent metabolic stress on aquatic organisms and are a common response (Zhao and Yang, 2008; Sreenivasan *et al*., 2011). The differences between embryos and tadpoles to copper could be related to distinct exposure routes to metal and development stage. Embryos are protected by the jelly-coat that can diminish copper intake and tadpoles only own a thin and permeable skin being at constant direct contact with chemicals. Development of organs is another possibility since in embryo stage they are still poor developed reducing impact of copper (Edginton *et al*., 2004). Gills are main routes for metals intake; however they only starts to develop at Gosner stage 19, thus the exposure period on embryo assay was reduced (Gosner, 1960; Edginton *et al*., 2004).

Opposite results were obtained when embryos and tadpoles were exposed to NaCl solely, i.e., embryos were more sensitive to this salt than tadpoles. A mortality of 100% was observed for embryos exposed to 6.9 mg/L of NaCl, while only 50% of tadpoles died at a higher NaCl concentration (7.37 mg/L of NaCl). Similar results were obtained by Beebee (1985a) that observed salinity tolerance of embryos of *Bufo calamita* up to 4.2 ‰ (NaCl) and of tadpoles up to 7‰ salinity. Actually, if the absence of fully developed organs in the embryonic stage may protect the embryos against copper toxic effects, it can also be the main reason behind the higher toxicity of NaCl to this life stage: in the absence of functional organs capable of dealing with physiological stress of increased salt concentration, embryos must deal with it through active transport of water and ions across the egg membrane (Ortiz-Santaliestra *et al*., 2010). Furthermore, the observed high percentage of malformations in the embryo assay is most probably related with the fact that high salinity levels in the external medium prevent water diffusion into the egg, preventing the membrane to enlarge properly. Thus, inhibiting embryos to develop normally due to lack of space inside the membrane (Gosner and Black, 1957) and impairing survival success. In addition, the differences in sensitivity to NaCl between the two life stages may also be related with the lower sensitivity of tadpoles. This may be explained by the low vascularization of skin in tadpoles, which can reduce loss of water in osmotic stressful environments; also, tadpoles may exhibit another detoxification mechanisms to deal with salt excess that are not developed in the embryos (Duellman and Trueb, 1994). Another possible reason could be related to gill surface: at

developmental stage 25, gills become covered by opercula. Gills constitute entry routes for ions uptake in several aquatic organisms (Piermarini and Evans, 2000). This could have impacted the few embryos that hatched, since hatchlings present external gills. Other authors also found higher sensitivity of embryos to salinity (Viertel, 1999). Though these suggested hypothesis, few is known about physiological mechanisms of response to osmotic stressful environments in amphibians (Gordon *et al*., 1961; Chinathamby *et al.,* 2006; Rios-López, 2008; Wu and Kam, 2009; Ortiz-Santaliestra *et al*., 2010).

In the present study, NaCl only significantly affected the activity of CAT of tadpoles, causing a reduction in its activity at intermediate concentrations. Therefore, the sensitivity of embryos and tadpoles to NaCl could not be ranked according to their biochemical responses, since no significant effects were registered for ChE and LDH and a concentration-response relationship was not observed for CAT (in tadpoles).

Finally, as for the chemicals alone, its combination induced different responses in embryos and tadpoles. Intermediate concentrations of NaCl significantly reduced mortality of embryos induced by copper, suggesting that a slight increase of salinity may contribute to a decrease of copper toxicity to this life stage. These results are in line with works carried out by other authors, who evaluated the effects of Na^+ in the toxicity of copper (Erickson *et al*., 1996; De Schamphelaere and Janssen, 2002; Grossel *et al*., 2007). For example, Erickson *et al.* (1996) observed that the presence of Na^+ reduced the toxicity of copper to fathead minnows. Similar results were observed in *Daphnia magna* by De Schamphelaere and Janssen (2002). These authors examined the effects of Ca2+, Mg2+, Na+ and K+ ions on copper toxicity and found increased 48-h EC_{50} values for copper with higher activities of $Ca2^+$, $Mg2^+$ or Na^+, supporting the concept of competitive binding of these ions and copper for transport sites in membrane cells (Pyle *et al*., 2003). Another reason may be the direct effect of salinity increase on the osmotic stress. In higher levels of salinity, the water tends to flow to external medium and a decrease of inward flow of water, which probably would reduce the intake of toxic ions (Hall and Anderson, 1995). However, this shied effect of NaCl relatively to the toxicity of copper was not observed in tadpoles. Actually, at intermediate concentrations of copper mortality of tadpoles increased with increasing NaCl concentrations. With regards to CAT activity, combinations

of NaCl and copper caused common response embryos and tadpoles where a significantly induction was observed. Since CAT activity is a primary antioxidant enzyme, this induction derived from combination with NaCl can increase the antioxidant efficiency and thus, it can be assumed that somewhat NaCl contributed to reduce toxic effects of copper. This hypothesis is also in accordance with the mortality results on the embryo assay where NaCl at intermediate concentrations was protective against copper toxicity. Concerning possible neurotoxic effects, no statistical differences were found between ChE control and combined treatments on embryos, and for tadpoles the pattern was not full conclusive. The same was observed for GST and LDH activities and thus, it was not possible to deduce a dose-response relationship to these enzymes.

Conclusions

The present work showed that embryos and tadpoles, of *P. perezi*, exhibit opposite sensitivities to copper and to NaCl. Furthermore, it was observed that intermediate concentrations of NaCl exerted a protective effect in the toxicity of copper to embryos, though not to tadpoles. These results highlight the need to better understand the influence of life stage and the interactions between chemicals in their toxicity to organisms, in order to promote a more accurate ecological risk assessment, and thus a better protection of biota.

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General Conclusion

In this work it was shown that different life stages of *P. perezi* may present different tolerances to chemical contamination, namely to copper and NaCl. Tadpoles revealed to be more sensitive to copper than embryos, exhibiting a higher mortality at lower concentrations of this metal. However, it must be noticed that sublethal responses monitored during the 96h exposure of embryos, namely significant growth inhibition and occurrence of malformations, showed significant adverse effects, relatively to the control, at concentrations lower than those exerting significant mortality in tadpoles. These effects may have implications in the future survival of organisms, as a smaller body size and the presence of malformations may, for example, increase their susceptibility to predation.

According, although the assays with larval stages are ethically questionable, the increased sensitivity of tadpoles, to copper, in comparison to embryos suggests that exhaustive considerations of embryos relatively to tadpoles' sensitivity should be carried out, in order to allow the use of scientifically sound safety factors, aiming to avoid under- or overestimation of risk. Furthermore, results obtained in the first chapter of this work suggested that, in addition to mortality, other endpoints must be always monitored during the embryo assay, since some sublethal effects that may occur at lower concentrations may compromise the survival of larvae at latter stages.

The results obtained in the second chapter of this work showed that a certain level of salinity may ameliorate the toxicity of copper, since intermediate concentrations of NaCl reduced the lethal toxicity of copper to embryos of *P. perezi*. For tadpoles, high concentrations of NaCl increase even more the lethal effects already promoted by copper, such fact could result from the enhacement of the toxicity of copper by NaCl or from the addition of effects. However more studies are required to test both hypothesis. Thus, these results suggest that depending on the life stage and on the levels of the two chemicals, NaCl may exert a protective, synergistic or additive influence on copper toxicity. The analyzed biochemical responses for embryos also revealed a shield effect of

NaCl against the adverse effects of copper in CAT and LDH. Again, this protective pattern was not observed with tadpoles.

These results here obtained emphasis the need to carry out integrated studies in order to attain a better and more accurate protection and conservation of amphibians in a worldwide scale. Namely, it is imperative to understand the toxic effects of multiple stressors (e.g. occurring climate changes and already existing chemical contamination) to different life stages of amphibian species.