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EFEITOS DA CONTAMINAÇÃO AMBIENTAL POR MANCOZEBE EM GIRINOS DE *Physalaemus henselii* (PETERS, 1872) (ANURA: LEPTODACTYLIDAE): UMA ESPÉCIE TERMOSSENSÍVEL E DE DISTRIBUIÇÃO RESTRITA

Santa Maria, RS
2020

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Dissertação apresentada ao Programa de Pós-Graduação em Biodiversidade Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Biodiversidade Animal**

Orientador: Tiago Gomes dos Santos

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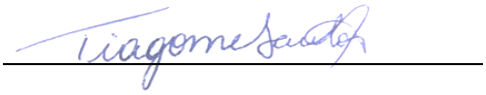

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Por fim, agradeço à minha família e amigos por ficarem ao meu lado durante mais esta jornada.

“The thing about light is...

it never really dies."

Lux - The Lady of Luminosity

RESUMO

EFEITOS DA CONTAMINAÇÃO AMBIENTAL POR MANCOZEBE EM GIRINOS *DE Physalaemus henselii* (PETERS, 1872) (ANURA: LEPTODACTYLIDAE): UMA ESPÉCIE TERMOSSENSÍVEL E DE DISTRIBUIÇÃO RESTRITA

Os habitats nativos experimentam dramáticas taxas de conversão pela expansão da agricultura, resultando em contaminação ambiental e impactos sobre a vida silvestre. O Mancozeb (Mz) é o fungicida de amplo espectro mais vendido no mundo, que afeta negativamente organismos não-alvo. Efeitos negativos sobre a sobrevivência, taxas de crescimento, estresse oxidativo e genotoxicidade foram relatados em animais expostos a Mz. No entanto, pouco se sabe sobre os efeitos de Mz na fauna aquática nativa, uma vez que a maioria dos estudos se restringe a modelos animais estabelecidos, como invertebrados, peixes e ratos. Aqui, testamos os efeitos de Mz em espécie de anfíbio nativo da região Neotropical. Utilizamos girinos de *Physalaemus henselii* para determinar a Concentração Letal de Mancozeb (CL50) e os possíveis efeitos subletais desse fungicida em girinos, utilizando marcadores de estresse oxidativo e alterações redox. Além disso, testamos os efeitos de Mz no máximo térmico crítico (CTmax) e na expressão de proteínas de choque térmico (HSP70). Registramos que a exposição aguda ao fungicida diminuiu a sobrevivência dos girinos. Girinos expostos à concentração subletal de 2mg/L Mz apresentaram menor atividade das enzimas catalase (CAT) e superóxido dismutase (SOD), além de níveis reduzidos de Tióis Proteicos (PSH). Além disso, houve um aumento na atividade glutationa S-transferase (GST) e maior peroxidação lipídica. A exposição também afetou negativamente a fisiologia térmica e a expressão da proteína de choque térmico nos girinos de *Physalaemus henselii*, induzindo um aumento na atividade da HSP70 e reduzindo o máximo térmico suportado pelos girinos. Houve um aumento na expressão de HSP70 em girinos expostos ao Mancozeb e em girinos submetidos a tratamento térmico. Os resultados aqui obtidos demonstraram que o fungicida Mancozeb, mesmo em doses não letais e inferiores às utilizadas em áreas de cultivo, afeta negativamente o funcionamento celular dos girinos. Estes resultados são preocupantes já que atualmente enfrentamos o desafio do aquecimento global em um cenário de acelerada conversão dos campos nativos em lavouras onde os organismos estão expostos a ampla variedade de agroquímicos.

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Palavras-chave: poluição ambiental, ecotoxicidade, ecofisiologia dos anfíbios, estresse oxidativo, contaminação agroquímica, tolerância térmica, CTmax

ABSTRACT

EFFECTS OF ENVIRONMENTAL CONTAMINATION BY MANCOZEBE ON TADPOLES OF *Physalaemus henselii* (PETERS, 1872) (ANURA: LEPTODACTYLIDAE): A THERMOSENSITIVE SPECIES WITH RESTRICTED DISTRIBUTION

Native habitats experience dramatic conversion rates by expanding agriculture, resulting in environmental contamination and impacts on wildlife. Mancozeb (Mz) is a best-selling broad-spectrum fungicide in the world, which negatively affects non-target organisms. Negative effects on survival, growth rates, oxidative stress and genotoxicity have been reported in animals exposed to Mz. However, little is known about the effects of Mz on native aquatic fauna, since most studies are restricted to established animal models, such as invertebrates, fish, and rats. Here, we tested the effects of Mz on an amphibian species in the Neotropical region. We used tadpoles of *Physalaemus henselii* to determine the Lethal Concentration of Mancozeb (LC50) and the possible sublethal effects of this fungicide on tadpoles, using oxidative stress markers and redox alterations. In addition, we tested the effects of Mz on the critical thermal maximum (CTmax) and on the expression of heat shock proteins (HSP70). We recorded that acute exposure to the fungicide decreased the tadpole survival. Tadpoles exposed to a sublethal dose of 2mg/L Mz showed lower activity of catalase (CAT) and superoxide dismutase (SOD) enzymes, in addition to lower protein thiols (PSH) levels. Besides, there was an increase in glutathione S-transferase (GST) activity and greater lipid peroxidation. The exposure also negatively affected the thermal physiology and expression of the heat shock protein in the tadpoles of *Physalaemus henselii*, inducing an increase in the activity of HSP70 and reducing the thermal maximum supported by the tadpoles. There was an increase in HSP70 expression in tadpoles exposed to Mancozeb and in tadpoles subjected to heat treatment. The results obtained here demonstrated that the fungicide Mancozeb, even in non-lethal doses and lower than those used in cultivation areas, negatively affects the cellular functioning of tadpoles. These results are worrisome as we currently face the global warming challenge in a scenario of accelerate conversion of native grasslands to croplands that expose organisms to several types of agrochemicals.

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Keywords: environmental pollution, ecotoxicity, amphibian ecophysiology, oxidative stress, agrochemical contamination, thermal tolerance, CTmax

Introdução geral

Os anfíbios são a classe de vertebrados mais ameaçada, com cerca de 41% das espécies sob alguma categoria de ameaça (Collins e Crump 2009; Hoffmann et al. 2010). Diferentes pressões ambientais afetam os anfíbios, como perda e alteração de habitat (Cushman 2006), doenças (Daszak et al. 2003) e, nas últimas décadas, o uso de fertilizantes e pesticidas (Becker et al. 2007). Os anfíbios são afetados devido à pele permeável e ao ciclo de vida complexo de espécies (desenvolvimento de ovos e larvas no ambiente aquático e vida adulta no ambiente terrestre), o que os torna um bom indicador ambiental (Blaustein 1994; Wake e Vredenburg 2008). Os ambientes aquáticos se tornam um recurso importante para os anfíbios (Crump 2009), e sua contaminação devido ao uso de agroquímicos pode acarretar sérios danos a esses organismos (Wrubleswski et al. 2018; Curi et al. 2019; Lajmanovich et al. 2019). Estudos que verificam os efeitos deletérios da contaminação aquática sobre anfíbios vêm crescendo nos últimos anos, mas permanecem escassos ao considerar o número de produtos químicos disponíveis no mercado e a riqueza de espécies especialmente registrada na região Neotropical. Nos últimos anos, vários impactos negativos sobre os anfíbios foram relatados, principalmente na fase larval, devido à contaminação por agroquímicos. Alterações morfológicas (Lajmanovich et al. 2003), no tempo de desenvolvimento (Svartz e Pérez-Coll 2013), sobre a massa corporal (Mikó et al. 2017), o comportamento (Samarakoon e Pathiratne 2017), bem como alterações em nível celular foram relatadas (por exemplo, aumento da frequência de micronúcleos e estresse oxidativo) (Lajmanovich et al. 2005; Freitas et al. 2017, respectivamente).

A contaminação de ambientes aquáticos por agroquímicos foi identificada como uma importante causa da redução de populações de anfíbios (Davidson 2004). Entre os agroquímicos mais amplamente utilizados no Brasil está o Mancozebe (Mz), o fungicida mais vendido no Brasil (IBAMA 2019) e pertence ao grupo etileno bisditiocarbamato (EBDC). O Mz é um fungicida de amplo espectro usado para proteger muitas frutas, hortaliças e demais culturas contra um amplo espectro de doenças fúngicas, incluindo ferrugem precoce e tardia da batata, mancha das folhas, bolor, casca de maçã por pulverização foliar (Anvisa 2016). Alguns estudos relataram efeitos tóxicos do Mz em organismos não-alvo, como alterações nas taxas de sobrevivência e crescimento, estresse oxidativo e parâmetros de genotoxicidade em

modelos de peixes e invertebrados (Shenoy et al. 2009; Marques et al. 2016; Costa-Silva et al. 2018). Esses efeitos podem ser relacionados com desequilíbrio oxidativo causado pela exposição a Mz durante o desenvolvimento inicial (Costa-silva et al. 2018).

O estresse oxidativo é definido como o desequilíbrio entre os sistemas pró-oxidante e antioxidante. A perturbação pode ocorrer devido ao aumento da exposição a oxidantes ou à diminuição da proteção contra oxidantes (Davies 2000). O aumento de oxidantes nas células causa diversos danos às biomoléculas, como proteínas, lipídios e ácidos nucléicos (Valko et al. 2006). Neste sentido, o Mancozebe atua no aumento de espécies reativas de oxigênio (EROs), provavelmente inibindo a atividade de complexos mitocondriais, reduzindo a eficácia da transferência de elétrons. O vazamento de elétrons durante a transferência é a principal fonte de formação de EROs nas células (Zhang et al., 2003).

A temperatura é outro fator importante para a vida dos anfíbios, já que os ectotérmicos dependem de fontes externas de calor (Angilletta 2009). Padrões de atividade, locomoção, taxas de consumo de oxigênio e trocas gasosas são alguns exemplos de parâmetros afetados pela temperatura em anfíbios (Navas et al. 2008). As espécies têm temperaturas ótimas de desenvolvimento (Topt), com uma faixa de tolerância térmica variando de um mínimo térmico crítico (CTmin) a um máximo térmico crítico (CTmax) (Katzenberger et al. 2012). Esses limites definem a temperatura ambiental na qual o indivíduo pode sobreviver, se desenvolver e reproduzir. Assim, a temperatura desempenha um papel fundamental nos processos bioquímicos e fisiológicos, afetando em última instância a distribuição e a abundância dos organismos (Yu et al. 2012). Nesse contexto, as proteínas de choque térmico (HSPs) são ecologicamente e evolutivamente importantes como adaptação das espécies ao meio ambiente (Parsell e Lindquist 1993; Feder e Hofmann 1999). Proteínas de choque térmico podem ser expressas em condições fisiológicas normais, auxiliar em processos vitais de células (por exemplo, como auxiliares no dobramento de proteínas). Contudo, algumas HSPs são expressas apenas na ação de fatores estressantes (Robert 2003; Castro et al. 2013), como em função do aumento da temperatura, resposta à presença de metais pesados, radiação ultravioleta e processos patológicos (por exemplo, infecções virais e bacterianas), além de doenças autoimunes (Castro et al. 2013). No entanto, informações sobre o efeito de pesticidas na tolerância térmica de espécies neotropicais são praticamente desconhecidas (mas

veja a exceção em Quiroga et al. 2019). Entender a relação entre contaminação ambiental e limites térmicos fisiológicos é cada vez mais importante (Winter et al. 2016) no atual cenário global de uso irrestrito de agroquímicos e de aquecimento global (Tomanek 2010; Lima et al. 2019).

Objetivos

Objetivo geral

Determinar a concentração letal (CL50) assim como os possíveis efeitos subletais do fungicida Mancozebe sobre girinos de *Physalaemus henselii* (Fig. 1), a partir de possíveis mudanças no perfil antioxidante endógeno da espécie, bem como na Tolerância Térmica e sua relação com as proteínas de choque térmico (HSP).

Objetivos específicos

Capítulo 1

Determinar a concentração letal (CL50) de Mancozebe para girinos de *Physalaemus henselii*, bem como determinar o perfil antioxidante endógeno após exposição a concentrações subletais do fungicida.

Capítulo 2

Determinar os efeitos do Mancozebe no máximo térmico crítico (CTmax) em girinos de *Physalaemus henselii*, testando os efeitos da exposição ao Mancozeb e do choque térmico na expressão de proteínas de choque térmico (HSP70).

Figura 1. Adulto e girino de *Physalaemus henselii*. Fonte: © Tiago Gomes e © Brena Gonçalves, respectivamente. A escala para o girino representa 1 cm.

SUBLETHAL CONCENTRATIONS OF MANCOZEB AFFECT SURVIVAL AND OXIDATIVE BALANCE OF *Physalaemus henselii* TADPOLES (ANURA: LEPTODACTYLIDAE)

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Abstract

Native habitats experience dramatic conversion rates due to crop expansion, resulting in environmental contamination and impacts on wildlife. Mancozeb (Mz) is a best-selling broad-spectrum fungicide in the world, which negatively affects non-target organisms. Animals exposed to Mz have been reported to have negative effects on survival, growth rates, oxidative stress and genotoxicity. However, little is known about the effects of Mz on native aquatic fauna, since most studies are restricted to established animal models such as invertebrates, fish and rats. In this study, we tested the effects of Mz on an amphibian species from the Neotropical region. We determined the Mancozeb Lethal Concentration (LC50) in tadpoles of *Physalaemus henselii* and the possible sublethal effects of this fungicide on amphibians, through the use of oxidative stress markers and redox alterations. We recorded that acute exposure to the fungicide decreased the survival rate of tadpoles. Tadpoles exposed to sublethal concentration of 2mg/L Mz showed decreased activity of catalase (CAT) and superoxide dismutase (SOD) enzymes, as well as protein thiols (PSH) levels. In addition, there was an increase in glutathione S-transferase (GST) activity and higher lipid peroxidation. Results obtained here demonstrated that Mancozeb fungicide, even at non-lethal concentrations far lower than those used in croplands, significantly affects the cellular functioning of tadpoles.

Keywords: *ecotoxicity, oxidative stress, environmental pollution, amphibian physiology, agrochemical contamination*

1. Introduction

Amphibians are the most endangered class of vertebrates, with approximately 41% of endangered species (Collins and Crump 2009; Hoffmann et al. 2010). Different environmental pressures affect amphibians, such as habitat loss and alteration (Cushman 2006), disease (Daszak et al. 2003), and, in recent decades, the use of fertilizers and pesticides (Becker et al. 2007). This group is sensitive to environmental changes primarily due to their life cycle, with aquatic eggs and larvae, and due to their permeable skin (Linder et al. 2010). Aquatic environments become an important resource for amphibians (Crump 2009), and contamination due to the use of agrochemicals can lead to serious damage to these organisms (Wrubleswski et al. 2018; Curi et al. 2019; Lajmanovich et al. 2019). As a result of this sensitivity, they are good indicators of environmental health (Blaustein 1994; Wake and Vredenburg 2008; Santos et al. 2015). Their permeable skin presents several defensive functions essential for survival (Clarke 1997). The skin plays an important defensive role against reactive oxygen species (ROS), such that when oxygen concentrations are higher, more absorbed oxygen is consumed (Liu et al. 2010). Maintaining a redox balance is extremely important for amphibians as a changing environment (aquatic to terrestrial) results in higher oxygen exposure and consequently more endogenous formation of ROS (Cavas and Tarhan 2003).

The contamination of aquatic environments caused by agrochemicals has been identified as an important cause of the decline in amphibian populations (Davidson 2004). Over the last few years, as agrochemicals diversify and increase in concentration and use across the globe, contamination rates have also continued to rise. In Brazil in particular, there have been a dramatic weakening of pesticide regulations, most recently with the approval of a record number of pesticides for use in crops (MAPA 2019a,b). Brazil leads the world in the buying of pesticides and in 2019, 382 agrochemicals were registered (MAPA 2019b). Agrochemicals can lead to ecotoxicological damage, mainly in aquatic ecosystems. When applied in indiscriminate form, these chemicals lead to the contamination of water resources (Rose and Carter, 2003; Yadav et al., 2014; Lajmanovich et al. 2015; Attademo et al. 2016; Wrubleswski et al. 2018). The contamination of aquatic ecosystems generates the exposure of non-target organisms to dangerous chemical agents (Sehonova et al. 2018). Damage related to aquatic contamination by agrochemicals has been reported for several invertebrate and vertebrate animal groups, such as morphological,

behavioral and physiological changes (Astiz et al 2009; Panacek et al. 2011; Zhang et al 2013; Quiroga et al. 2019). However, most studies only use animal models already established in science (such as rats, fish and flies), therefore lacking information on native animals. Due to the large number of chemical compounds on the market, the relation between agrochemicals and native fauna remains scarce.

One of the most widely used agrochemicals in Brazil is Mancozeb (Mz), the best-selling fungicide (IBAMA 2019) which belongs to the ethylene bisdithiocarbamate (EBDC) group. Mz is a broad-spectrum fungicide used to protect many fruits, vegetables, and field crops against a wide spectrum of fungal diseases, including early and late blight in potatoes, leaf spots, downy mildew, and apple scabs (Anvisa 2016). Mz is compatible with many systemic fungicides in order to increase efficacy and prevent the development of resistance. Some studies report toxic effects of Mz in non-targets organisms, such as changes in survival and growth rates, oxidative stress and genotoxicity parameters in fishes and invertebrate models (Shenoy et al. 2009; Marques et al. 2016; Costa-Silva et al. 2018). These effects can be attributed to the oxidative imbalance caused by exposure to Mz during initial development (Costa-silva et al. 2018).

Oxidative stress is defined as the imbalance between the pro-oxidant and antioxidant systems. This disturbance may occur due to increased exposure to oxidants or from decreased protection against oxidants (Davies 2000). An increase of oxidants in the cells causes diverse damages to biomolecules like proteins, lipids, and nucleic acids (Valko et al. 2006). Mancozeb acts on the increase of reactive oxygen species, inhibiting the activity of mitochondrial complexes and reducing the effectiveness of electron transfer. Electron leakage during transfer is the main source of ROS formation in cells (Zhang et al., 2003). Other damages related to Mz occur due to its action under NADPH oxidase and xanthine oxidase, increasing the presence of H2O2 (Domico et al., 2007). The sublethal effects of Mancozeb are alarming, especially when considering the lack of information related to ecologically restricted native species that are highly susceptible to contamination. As the demand for extensive agricultural land use remains high, and the grassland to cropland conversion rates increase, there is also a higher level of agrochemicals being utilized (Foley 2005; Costa and Nomura, 2015). The subtropical grasslands of Uruguay, its adjacent Argentina (Entre Ríos Province), and the southernmost Brazilian states of Rio Grande do Sul and Santa Catarina are home to anuran Hensel's Dwarf Frog (*Physalaemus*

henselii Peters, 1872) (Maneyro and Carreira, 2012). This species is naturally influenced by a restricted distribution pattern, specific to grasslands, and a short reproductive cycle, corresponding to the hemispheres colder months (April to September) (Kolenc et al., 2006; Maneyro and Carreira, 2012).

Considering the deleterious effects of Mz on non-target organisms (e.g. changes in redox balance) and its extensive use on regions with physiologically restricted species, we designed this study to characterize the ecotoxicity of Mz on tadpoles of *Physalaemus henselii.* We investigated the Lethal Concentration (LC50) of Mz for this species, as well as the endogenous antioxidant profile after exposure to sublethal fungicide concentrations.

2. Materials and methods

2.1. Collection and maintenance of tadpoles

Tadpoles of *Physalaemus henselii* (300 individuals, stage 26-36; Gosner, 1960) were collected from June to August of 2018 in three ponds located at the municipality of Santa Margarida do Sul, located in the Pampa biome (IBGE, 2019) of Brazil's southernmost state, Rio Grande do Sul (30° 20' 24" S; 54° 04' 48" O, 120m a.s.l.). The pond water temperature during tadpole sampling varied from 6.4ºC to 15.1ºC. After capture, the tadpoles were transferred to tanks with water from the original collection sites and transported to a laboratory in thermal boxes for ongoing screening. Tadpoles were acclimated in a 12:12-h light-dark cycle in tanks with 280L of de-chlorinated tap water at 20ºC from Zebratec® system (pH 7.2 and 400 µS conductivity) and housed under constant aeration for 48h. Tadpoles were fed *ad libitum* every 24h with a mix of spinach and lettuce (proportion of 1:1) (Lajmanovich et al. 2018).

2.2. LC50 determination

Tadpoles were randomly selected and divided into seven groups: control, 1mg/L, 1.5mg/L, 2mg/L, 5mg/L, 10mg/L and 20mg/L. The Mz concentrations were based on literature for larvae of other anuran species (Howard et al. 2002; Shenoy et al. 2009). Treatment groups were performed in triplicate, except for groups of 5mg/L, 10mg/L and 20mg/L which were performed twice in triplicate. Tadpoles were exposed to experimental aquaria (10 animals *per* tank, 30 animals *per* concentration) for 24h, which contained 04 L of de-chlorinated tap water and commercial Mz (Emzeb 800 WP; 80% Mz) solutions, under the same laboratory acclimatization conditions. Exposure

time was determined by the fact that Mz can hydrolyze quickly, resulting in a half-life of less than 2 days (Xu 2000). After 24h, the number of dead individuals was recorded. This study was approved by the Ethics Commission on Animal Use at the Federal University of Pampa (Universidade Federal do Pampa) under process #022/2018.

2.3. Tissue processing

The caudal muscle tissue of control, 1 mg/L, 1.5 mg/L and 2 mg/L groups were collected, homogenized in 20 mM HEPES (pH 7.4) and centrifuged at 20,000g for 30min at 4ºC. The supernatants were isolated for further biochemical determinations.

2.4. Catalase (CAT)

CAT activity was assayed following the clearance of H_20_2 at 240nm for two minutes, following Aebi (1984). The reaction medium consisted of 0.05 M phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H_2O_2, and 0.012% TRITON × 100. CAT activity was expressed as the amount of H_2O_2 degraded *per* minute *per* milligrams of total protein (µmol/min/mg protein).

2.5. Superoxide dismutase (SOD)

SOD activity was measured according to Kostyuk & Potapovich (1989). Quercetin 0.49 mM was used as a superoxide radical sensor in the presence of 7.55 M N,N,N',N'-Tetramethylethane-1,2-diamine (TEMED). The assay medium consisted of 0.025 M phosphate buffer (EDTA 0.1 mM) at pH 10. SOD quantification was expressed by inhibition of superoxide-driven oxidation of quercetin by SOD at 406 nm.

2.6. *Glutathione S-transferase (GST)*

GST activity was measured in the supernatant of the caudal muscle homogenate by the Habig & Jakoby method (1981) which used 1-chloro-2.4-dinitrobenzene (CDNB) as substrate. The reaction medium contained phosphate buffer (0.0025 M EDTA, 0.25 M K_2HPO_4, 0.25 M KH_2PO_4, pH 7.0) containing 100 mM glutathione (GSH). The enzyme activity was assayed by the reaction of the -SH group of GSH with 1-chloro-2,4-dinitrobenzene (CNDB) in the spectrophotometer at 340nm for two minutes. The enzyme activity was expressed as µmol GS-DNB/min/mg protein.

2.7. Determination of Lipid Peroxidation (LPO) and ROS generation

LPO was measured by concentrations of thiobarbituric acid reactive substances (TBARS), according to Ohkawa et al. (1979), based on the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), which can be spectrophotometrically measured at 532 nm. TBARS levels were expressed as nmol TBARS/mg protein. The ROS levels were measured using the fluorescent dye 2,7-dichlorofluorescein-diacetate (DCFDA) as described by Pérez-Severiano et al. (2004). The tadpole caudal tissues were homogenized in 50 mM Tris HCl (pH 7.5) buffer and centrifuged (3000g, 10 min at 4 °C). Supernatants (300–500 µg of protein) were mixed with 0.1 mM 2′,7′-dichlorofluorescein diacetate (DCFH-DA). ROS levels were determined by fluorescence at emission (570 nm) and excitation (545 nm) using dichlorofluorescein (DCF) as standard. Results were expressed as µmol DCF/mg protein.

2.8. Non-Protein Thiols (NPSH) and Protein Thiols (PSH)

NPSH measurement was performed as described by Ellman (1959). A 0.5 mM phosphate buffer (6.8) and 10mM 5,5′-dithio-bis (2-nitrobenzoic acid) was added to the supernatant (DTNB). Color reaction was measured at 412nm. NPSH levels were expressed as nmol NPSH/mg protein. Protein Thiols (PSH) measurement was performed by reaction of -SH group containing species present in the cells with O-phthalaldehyde, as described by Hissin and Hilf (1976).

2.9. Total protein quantification

Protein was determined by the Coomassie blue method using bovine serum albumin as a standard. Absorbance of samples was measured at 595 nm (Bradford,1976).

2.10. Statistical analysis

To verify the normality of the data, the Kolmogorov Smirnov test was applied, while the homogeneity was verified through the Brown-Forsythe test (Li et al., 2015). LC50 was calculated from mortality data using the simple linear regression model between the concentration ratio and the number of dead individuals after 24h of exposure. The Kruskal-Wallis test, followed by the Dunn's test, were used to test possible differences in tadpole survival between groups after 24h exposure. For the parametric data sets, one-way ANOVA was used, followed by the Tukey test to test for

possible differences between groups (CAT, SOD and GST enzyme activity), or t-tests (TBARS, EROs, NPSH and PSH). The tests were considered significant when $p < 0.05$.

3. Results

3.1. Evaluation of Mancozeb lethality

After 24h exposure, dead tadpoles were counted for mortality measurements (Figure 1A). No mortality was observed in the control, 1mg/L, 1.5mg/L and 2mg/L of Mz groups. Statistical estimation of LC_{50} of Mz for *P. henselii* was 3.875 mg/L.

3.1. Mancozeb exposure results in oxidative balance alterations

CAT activity decreased in concentration of 2mg/L when compared with control ($p=0.0002$) (Figure 1B). SOD activity showed a decrease in concentration of 1.5mg/L and 2mg/L when compared with control ($p<0.0001$) (Figure 1C). GST showed a significantly increased activity in tadpoles exposed to 2 mg/L, when compared to control (Figure 1D). Tadpoles exposed to 2mg/L showed increased LPO in relation to control ($p= 0.0005$) (Figure 1E). Although there was a slight upward trend in the production of reactive oxygen species (ROS), no statistically significant changes were observed ($p= 0.4507$) (Figure 1G). This same pattern of slight increase yet with no statistically significant changes also occurred in NPSH levels ($p= 0.3160$) (Figure 1H). PSH levels significantly decreased in 2mg/L ($p=0.0001$) (Figure 1F).

4. Discussion

Our results demonstrated the lethal concentration (LC_{50}) of Mz in *P. henselii* tadpoles, revealing changes in their oxidative stress profile. Several studies demonstrate the toxicity of Mancozeb in non-target organisms, mainly aquatic organisms that are exposed to large amounts of pollutants. However, information about the effect of Mz on native organisms remains scarce, especially in aquatic vertebrates. The LC50 of Mz known has wide variation for other anuran species: 0.2mg/L for *Rana pipiens* (Harris et al. 2000), 0.8mg/L for *Rana sphenocephala* (Howard et al. 2002), 1.4mg/L for *Bufo americanus* (Harris et al. 2000), and 3.97mg/L for *Rhinella arenarum* (Asparch et al. 2019). However, direct comparisons are limited since all studies defined LC50 after 48h or 96h of exposure. In our study we calculated LC50 for 24h exposure, because after this time period the hydrolysis of Mz occurs which can potentially generate more toxic products, such as ethylene

bisisothiocyanate sulfide (EBIS), ethylenethiourea (ETU) and ethyleneurea (EU) (Xu 2000). Among the few available studies for amphibians, skeletal deformities at hatching have been reported in *Rana clamitans* (Harris et al. 1998) and *Bufo americanus* (Harris et al. 2000). It was also verified that *Rana pipiens* have a higher sensitivity to Mz, where the same concentration required to cause malformations in *Bufo americanus* tadpoles can kill all individuals of *Rana pipiens* (Harris et al. 2000). In a later study, changes in growth rate were also found for *R. pipiens* (Shenoy et al. 2009). In addition, biliary epithelium hyperplasia has been reported following Mz contamination of *Pelophylax ridibundus* (Păunescu and Ponepal 2018). Until the current study, information of the Mz effects on Neotropical anurans was restricted to one bufonid species (*R. arenarum*, Asparch et al. 2019). Thus, the LC50 value found in this study for *P. henselii* is extremely relevant because it: i) increases our knowledge on Neotropical leptodactylid anurans, and ii) reveals that the concentration traditionally used in cultivated lands is a thousand times higher than those found in this study with these effects (Atamaniuk et al. 2014). Therefore, we argue that animals in the wild are exposed to Mz for extended periods, being that this chemical is used during warmer seasons for soybean and corn cultivations, and during colder periods for wheat cultivation (ADAPAR 2019).

The exposure of tadpoles to Mz generated a decrease in the activity of CAT and SOD enzymes. Both enzymes are considered an important first line of defense against reactive oxygen species (Asagba et al, 2007; Bansal and Srivastava 2011; Costa-Silva et al. 2018). As expected, our results showed that CAT activity was reflected in SOD activity, since both are linked functionally (Halliwell 1994; Asagba et al. 2007). The activity of these enzymes tends to be upregulated under moderate stress, but when the stress caused generates an excessive accumulation of ROS, it can also generate inhibition in their activities (Rodriguez et al. 2004). In addition, some compounds have the ability to inhibit CAT activity, thus generating a greater accumulation of ROS (Chen et al. 1993; Durner and Klessig, 1995).

Reactive oxygen species (ROS) production was not statistically different between treated tadpoles and control. Furthermore, there was a significant increase in LPO, demonstrating that ROS increase was sufficient enough to induce lipid peroxidation. For amphibians, it is known that glutathione related enzymes play an important role in the detoxification of organic compounds (Czarniewska et al. 2003; Falfushinska et al. 2008). This significant increase in GST can be interpreted as a

mechanism of compensatory detoxification, since Mz negatively affected the CAT and SOD activity (Costa-Silva 2018). This reinforced the importance of GST as an adaptive mechanism for xenobiotic detoxification in amphibians. On the other hand, the substrate used to determine GST activity was CDNB, which has affinity for all GST isoforms (Vidal et al. 2000). Thereby, it is possible that the GST increase can be associated with the LPO increase, caused by the GST-pi isoform that conjugates unsaturated aldehydes produced during the lipid peroxidation process. This regulation is also coordinated with other antioxidants (Keppler et al. 1999; Hayes et al. 2005).

The PSH levels decreased in tadpoles exposed to Mz, while NPSH levels did not show significant variation. Thiols play important roles in various aspects of cellular functions such as enzyme activity, signal transduction, cell division and cell protection against ROS (Haugaard 2006; Yang and Guan 2017). PSH are powerful antioxidants because they have the sulfhydryl radical (-SH), which is quickly oxidized when there are imbalances in the oxidative balance (Mulier et al. 1998). Oxidation of protein thiols may be triggered by increased H2O2 levels, causing transient changes in protein function that are relevant for signal transduction (García-Santamarina et al. 2014; Stöcker et al. 2017). The decrease in PSH concentration can be related with a number of cellular dysfunctions, including changes in enzyme activities, membrane permeability, and energy production (Yang and Guan 2017). The use of biomarkers of effect have been used to elucidate the physiological and biochemical effects related to exposure to a possible stressor. An organism with quantitative changes in biomarkers of effect will suffer loss in fitness (Hook et al. 2014). In tadpoles, developmental time varies from a few weeks to a year (Kolenc et al. 2003; Dastansara et al. 2017). During this period, they require specific needs for healthy development and growth (Haddad et al. 2013; McDiarmid & Altig, 1999). Although tadpoles are considered plastic organisms, meaning that they have a high capacity to adapt to environmental adversities, their compensatory systems demand high energy costs that end up being reflected in the loss of individual fitness (Angilletta, 2009; Bionda et al. 2018). The effects of Mz on the antioxidant profile in *P. henselii* tadpoles are similar to the results obtained in other invertebrate and vertebrate experimental models. Model organisms such as *Drosophila melanogaster* (Common fruit fly) (Saraiva et al. 2018), *Danio rerio* (Zebra fish) (Kayhan et al. 2018; Costa-Silva 2018), and *Rattus norvegicus domestica* (Wistar rat) showed the same changes in antioxidant profile as found in this study. The use of these species are important to understand the mechanism of action and the

effects of toxic compounds (Gad 2016). However, it is often difficult to understand how native species being subjected to the same stressors will respond. It is necessary to expand studies evaluating the risks of contaminants in native vertebrates.

In the current global scenario, human influence on natural systems has been steadily increasing (Wikelski and Cooke, 2006), particularly due to the high conversion rates of native grasslands to croplands (Foley 2005; Staude et al. 2018; Andrade et al 2019). Grasslands provide diverse ecosystem services and are among the world's most species-rich ecosystems (Wilson et al. 2012; Andrade et al. 2015). In Brazil, open and grass-dominated ecosystems are present in all biomes, with predominance in Pampa, Cerrado, and Pantanal. Grasslands are the main vegetation type in southern Brazil, and can be found in the Pampa and Atlantic Forest biomes (IBGE 2019). Approximately 60% of the original grassland area in the state of Rio Grande do Sul was lost due to the conversion of native grasslands to croplands and alien tree plantations (Andrade et al. 2015). These habitat conversions caused damage to local biodiversity and the agrochemicals being used are considered an important factor in the decrease of amphibian species. These agrochemicals contaminate not only the converted region but nearby areas as well (Becker et al. 2007). Although *Physalaemus henselii* is globally classified as a Least Concern species and presents a stable population throughout its distribution (Lavilla et al. 2004), local extinctions of this species along the Uruguayan Pampa have been reported (Kolenc et al., 2006). In fact, *P. henselii* seems to be sensitive to environmental disturbances, as its population has been reported in grasslands with little or no anthropic action (Maneyro and Carreira, 2012). In addition to its limited distribution, *P. henselii* has a short reproductive cycle which corresponds with the colder months of the year, further increasing concern for its conservation (Kolenc et al., 2006; Maneyro and Carreira, 2012).

It is extremely necessary to have actions in place that aim at restricting the use of agrochemicals and implementing less aggressive alternatives or measures that minimize the possibility of aquatic pollution. Amphibians are used as study models in ecotoxicological experiments because of their greater environmental sensitivity (Linder et al. 2010). Once tadpoles are restricted to aquatic environments, they can be exposed to stressors until metamorphosis due to environmental contamination (Brodeur and Candioti, 2017). Moreover, we are concerned about the decrease in amphibian populations, especially that of sensitive species with very restricted distribution and narrow habitat requirements, as is the case of the species studied here.

Physalaemus henselii suffered damage from approximately half of its LC_{50}, which is alarming considering that the LC_{50} found in the study is potentially one thousand times lower than the concentration of Mancozeb used in croplands (Atamaniuk et al. 2014).

5. Conclusion

Herein, we demonstrated by acute exposure that concentrations of Mz fungicide much lower than those used in the environment induced oxidative balance as well as decreased tadpole survival. We also reinforced tadpoles as good models for studies of environmental contamination, promoting the studying of effects on native species and not just laboratory models. For future studies, we recommend that considerations be made as to whether synergistic interactions exist with other cropland pollutants as well as with abiotic factors (e.g. temperature and hydroperiod).

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The CEUA Certificate of Approval was issued under number #022/2018.

Informed consent

All authors declare to be participants in the research and their respective future consequences.

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Figures

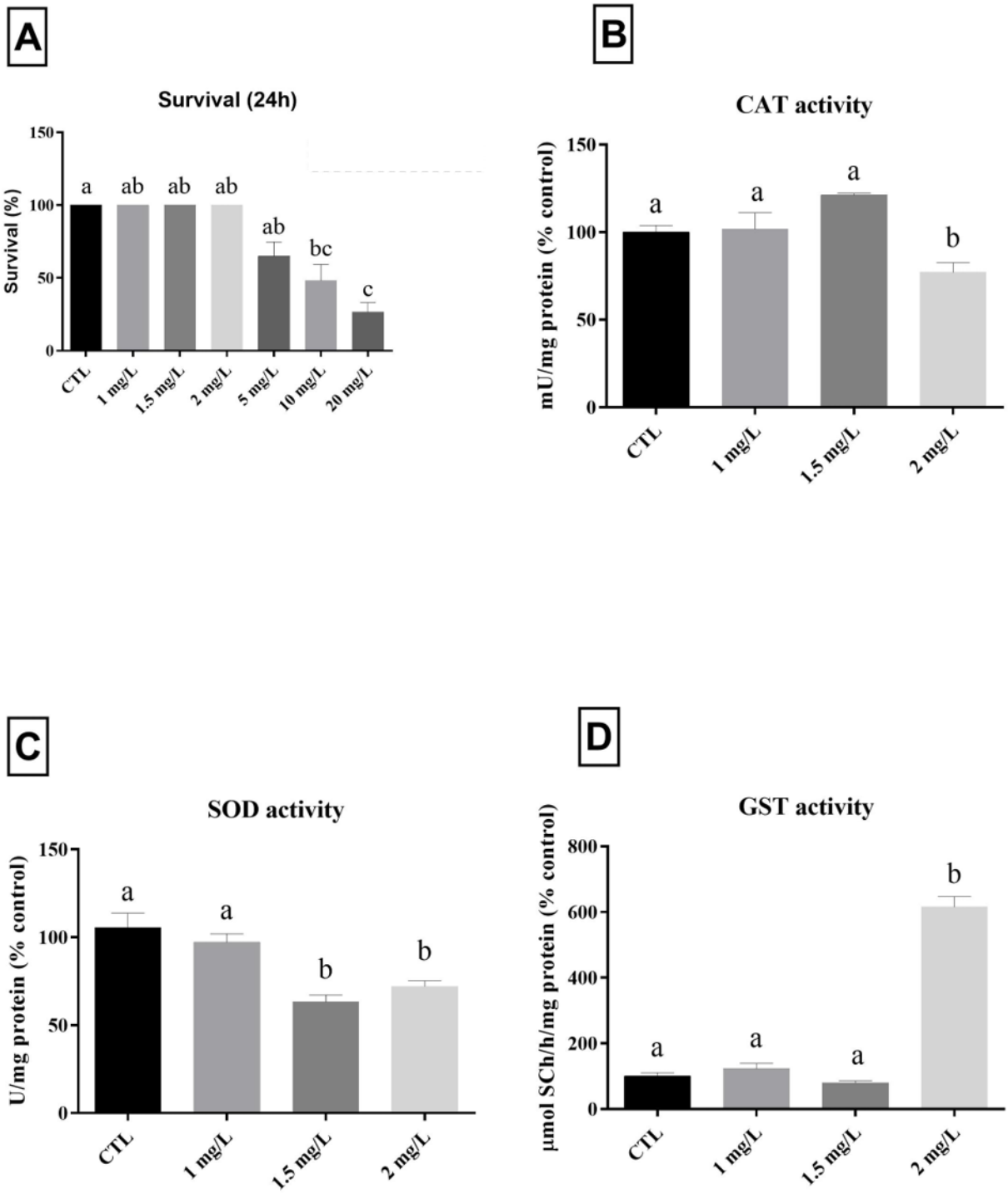

FIGURE 1: Changes in antioxidant enzymes activity and mortality rates of *Physalaemus henselii* tadpoles induced by MZ exposure. (A) Mortality, (B) Catalase (CAT), (C) Superoxide dismutase (SOD), and (D) Glutathione S-transferase (GST).

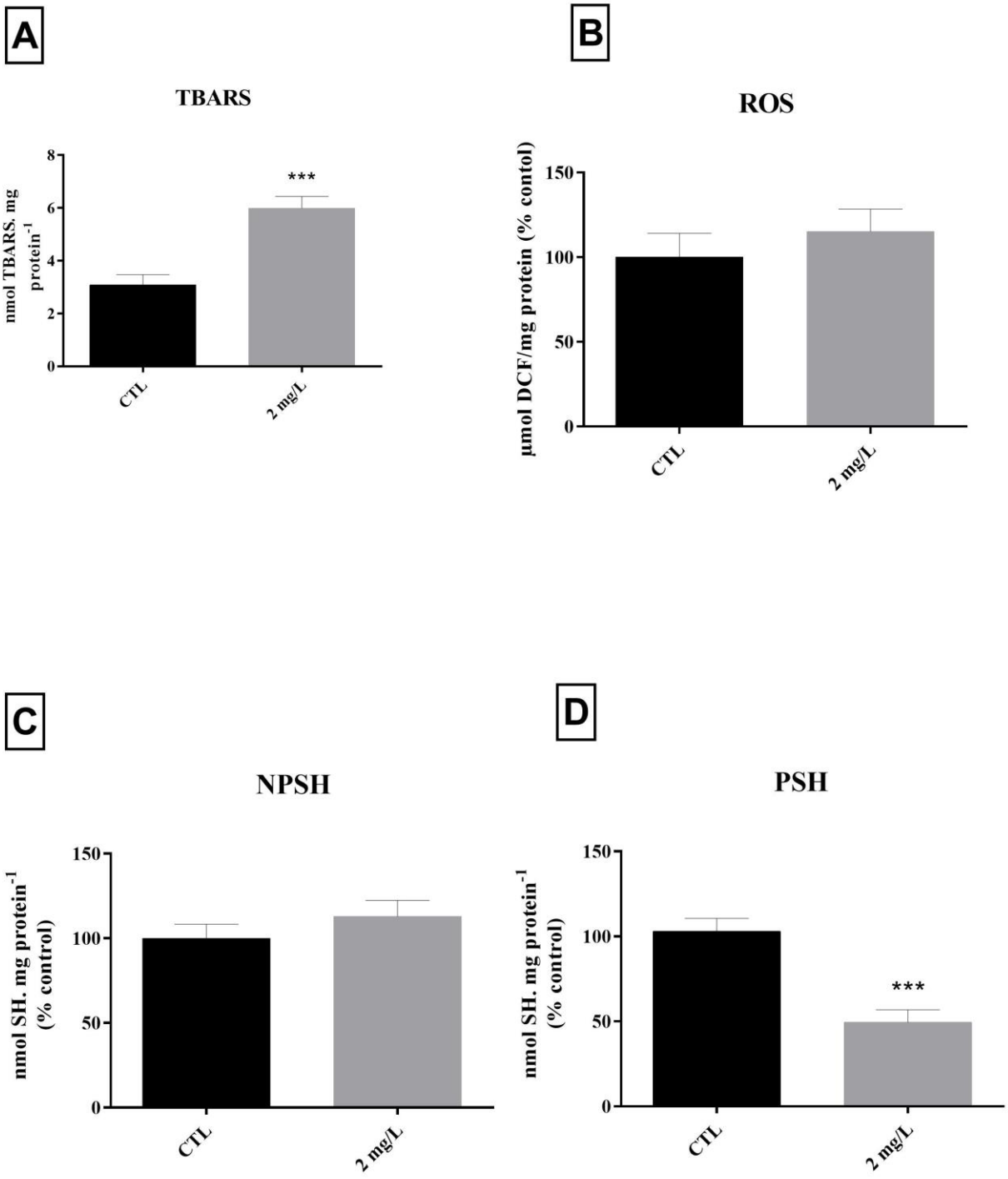

FIGURE 2: Changes in non-enzymatics antioxidants activity in *Physalaemus henselii* tadpoles induced by MZ exposure. (A) Lipid Peroxidation (LPO), (B) Reative Oxygen Species (ROS), (C) Non-Protein Thiols (NPSH), and (D) Protein Thiols (PSH).

CAPÍTULO 2: EFFECTS OF MANCOZEB ON HEAT SHOCK PROTEIN 70 (HSP70) AND ITS RELATIONSHIP WITH THE THERMAL PHYSIOLOGY OF *Physalaemus henselii* (PETERS, 1872) TADPOLES (ANURA: LEPTODACTYLIDAE)

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Abstract

Negative impacts on amphibians have been reported due to contamination by agrochemicals. However, until now, no study has tested the effect of the fungicide Mancozeb (Mz) on thermal tolerance and its relationship with expression of heat shock proteins (HSPs). Mz is a best-selling broad-spectrum fungicide in the world, which negatively affects non-target organisms. Here, we tested for the first time the effects of Mz on critical thermal maximum (CTmax) and on the expression of heat shock protein 70 (HSP70) in an amphibian species from the Neotropical region. We used tadpoles of *Physalaemus henselii* to check whether there would be a difference in CTmax and HSP70 between treated and untreated groups. The sublethal concentration of 2 mg/L of Mancozeb was used. We recorded that the CTmax of the group treated with Mancozeb was lower than the CTmax of the control group. In addition, there was an increase in HSP70 expression in tadpoles exposed to Mancozeb and in tadpoles that underwent heating treatment. However, tadpoles subjected to Mancozeb and heating treatment failed to induce HSP70 protein expression. Our results demonstrated that sublethal doses of Mancozeb fungicide negatively affected the thermal physiology and heat shock protein expression in tadpoles of *Physalaemus henselii* by inducing an increase in HSP70 activity and by reducing the critical thermal maximum supported by tadpoles. It is important to understand the relationship between environmental contamination and physiological thermal limits in our current scenario of high rates of habitat conversion associated with unrestricted use of agrochemicals, as well as the challenger environmental changes induced by the global warming.

Keywords: *ecotoxicity, thermal tolerance, ctmax, agrochemical contamination, neotropical anuran*

1. Introduction

Amphibians have been shown to be a highly susceptible group to pesticide contamination, making them valuable bioindicators of aquatic pollution (Egea-Serrano et al. 2012). Thus, the water quality in the ecosystems is an important factor for survival and healthy development of amphibians (Boyer and Grue 1995; Battaglin et al. 2016). Studies that verify the deleterious effects of aquatic contamination on amphibians have been growing in recent years, but remain scarce when considering the number of chemicals on the market and the richness of amphibian species (especially in the Neotropical region). In recent years, several negative impacts on amphibians have been reported, mainly in the larval phase, due to contamination by agrochemicals. The damages are varied, depending on the chemical class and the amphibian species. Morphological changes (Lajmanovich et al. 2003), developmental time (Svartz and Pérez-Coll 2013), body mass (Mikó et al. 2017), behavior (Samarakoon and Pathiratne 2017), and changes at the cellular level have been reported (e.g. oxidative stress, increased frequency of micronucleus) (Freitas et al. 2017; Lajmanovich et al. 2005, respectively). However, even more obscure information is the effect of pesticides on the thermal tolerance of neotropical species (but see exception in Quiroga et al. 2019).

Temperature is another important factor for amphibian life, as ectotherms rely on external heat sources to maintain temperature (Angilletta 2009). Activity patterns, locomotion, oxygen consumption rates, and gas exchange are some examples of parameters affected by temperature in amphibians (Navas et al. 2008). Species have optimal development temperatures (Topt), with a range of thermal tolerance varying from a critical thermal minimum (CTmin) to a critical thermal maximum (CTmax) (Katzenberger et al. 2012). These limits define the environmental temperature at which the individual can survive, reproduce and develop. In addition, the effects of temperature on biochemical and physiological processes play a key role in the distribution and abundance of organisms (Yu et al. 2012). In particular, heat shock proteins (HSPs) are ecologically and evolutionarily important in adapting species to the environment (Parsell and Lindquist 1993; Feder and Hofmann 1999). These proteins are present in all eukaryotic cells at different basal expression levels, assisting in vital cell processes (e.g. assisting other proteins in protein synthesis and folding) (Gething, 1997). However, certain HSPs are expressed solely under the action of stressors (Robert 2003; Castro et al. 2013), such as due to increased temperature, response to

the presence of heavy metals, metabolic poisons, ultraviolet radiation and pathological processes (e.g. viral infections, bacterial and parasitic diseases, as well as autoimmune diseases) (Castro et al. 2013).

HSPs are assigned to families according to their sequence similarity and molecular mass. Thus, an important family is the HSP70, that consists of several members, which are expressed under normal physiological conditions (in the form of Hses) or in response to stress-causing agents that induce protein denaturation (in the form of HSPs) (Gething, 1997). The relationship between HSPs and thermal tolerance is still scarcely known for Neotropical anurans whose different species can be adapted to occupy from hot until seasonally cold regions (Sunday et al. 2014). The Hensel's Dwarf Frog (*Physalaemus henselii* (Peters, 1872)) is a species whose distribution is restricted subtropical grasslands (Maneyro and Carreira, 2012; Santos et al. 2014). It is found in much of the Uruguayan territory, part of Argentina (Buenos Aires and Entre Ríos), as well as the Brazilian states of Rio Grande do Sul and Santa Catarina (Frost 2020). Throughout this distribution, *Physalemus henselii* is restricted to pristine to few modified grasslands (Maneyro and Carreira, 2012). The reproductive cycle of the species is short-lived, corresponding to cold season in austral region (from April to September) (Kolenc et al., 2006; Maneyro and Carreira, 2008). *Physalaemus henselii* tadpoles present low thermal sensitivity to cold but high thermal sensitivity to heat (Madalozzo 2018). Besides the time of larval development is not known, tadpoles of *Physalaemus henselii* are not found during the hottest period (i.e. most spring and summer) (TGS pers. obs.). The peculiar developmental phenology of *P. henselii* support this species as a potential model for studies on thermal ecology, mainly in the current scenario of global warming as threat to many amphibians (Blaustein et al. 2001; Carey and Alexander 2003; Maxwell et al. 2016; Newbold 2018) in which physiological studies are necessary to better understand the possible effects of climate change (Winter et al. 2016). Herein, our objective was to test the effects of Mancozeb fungicide on tadpole' CTmax, besides to test the effects of Mz exposure and thermal shock on the expression of heat shock protein 70 (HSP70).

2. Materials and Methods

2.1. Collection and maintenance of tadpoles

Sampling occurred in three ponds located in native grasslands from the municipality of Santa Margarida do Sul (30° 20' 24"S; 54° 04' 48" W, 120m a.s.l.), in the Pampa biome (IBGE, 2019) of the Brazilian state of Rio Grande do Sul. The water temperature variation in the ponds was 6.4ºC to 15.1ºC. We collected *Physalaemus henselli* tadpoles (61 individuals between stages 26-36; Gosner, 1960) from June to August of 2018, that correspond to the austral winter. After captured, tadpoles were transferred to tanks with water from the capture sites and transported to laboratory in thermal boxes for posterior triage. Tadpoles were acclimated in 12h-12h light-dark cycles in tanks with 280L of de-chlorinated tap water at 20ºC and housed under constant aeration for 48h. Tadpoles were fed *ad libitum* every 24h with a mix of spinach and lettuce (proportion of 1:1) (Lajmanovich et al. 2018).

2.2. Sample groups for determination of HSP70 protein expression

Tadpoles were randomly selected and divided in the following groups: CTL (samples taken from the 24h acclimated group), MZ (samples taken from the mancozeb exposed group for 24h), Heat (samples taken from the group that underwent the heating test), and MZ + Heat (samples taken from the group that after acclimatization was exposed to mancozeb for 24h and that subsequently was submitted to the heating test).

2.3. Mancozeb exposure

Tadpoles were divided into three groups: control (CTL group), 2mg/L (MZ group) and another 2mg/L (MZ+Heat group). The selected dose is considered sublethal to the tadpoles (See chapter 1). Treatment groups were performed in triplicate. The tadpoles (10 animals per tank, 30 animals per concentration) were placed in aquariums containing 04L of dechlorinated tap water and in the case of the group to be treated, it also contained a dose of 2mg/L of commercial Mz (Emzeb 800 WP). The same laboratorial conditions of acclimations were applied. This study was approved by Ethics Commission on Animal Use of the Federal University of Pampa (Universidade Federal do Pampa) under process #022/2018.

2.4. *Thermotolerance experimental assays*

After 24h exposure, the tadpoles of the 2mg/L Mz treatment (MZ+Heat) and the control (tadpoles that were only acclimated for 24h) (HEAT group) were submitted to the warm-up test to verify possible changes in CTmax. To do this, we use Hutchison's dynamic method. The method consists of the tadpole being exposed to a constant rate of heating until the end point is reached. We used slow heating rate of $\Delta T = 0.05°C\ min^{-1}$. We chose to use the slow rate to simulate natural pond warming rate at subtropical temperatures. The end-point was defined as the point at which tadpoles became immobile and did not respond to external stimuli (8-10 consecutive touches every two seconds using a wooden stick). The experiment was conducted in a large tank (180L) in which the temperature was increased using an immersed heating system controlled by a programmable digital thermostat (Portable Fluid Heaters with Regulation Adjustment, U201431698, Lorenergia, Cordoba, Spain). To keep the water at a uniform temperature inside the tank, we use a submersible pump to recirculate the water. The tadpoles were individually placed in 250ml plastic glasses, filled with 100ml of dechlorinated water at a temperature of 20ºC. The CTmax was recorded using the water temperature at the time the endpoint was reached. We used a Miller and Weber® thermometer (0.1°C accuracy) for temperature measurement. After the determination of CTmax, we transferred the tadpoles to containers with dechlorinated water at 20ºC for recovery. Each tadpole was tested only once and only tadpoles that recovered after 1h were included in subsequent analyzes.

2.5. HSP70 determination

Western blotting was performed according to Martins et al. (2018) with minor modifications. The samples were homogenized in Tris NaF buffer pH 7.0 (50 mM Tris, 1 mM EDTA, 0.1 mM phenyl methyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and protease inhibitor cocktail). The homogenate was then centrifuged at 1.000g for 10 min at 4°C. Then, the supernatant was collected and 10 µL of sample was taken out for protein analysis. In the remaining sample was added 4% SDS stop solution (50 mM Tris, 100 mM EDTA, pH 6.8), 25% glycerol sample (40% glycerol, 25 mM Tris and bromophenol blue, pH 6.8) and β-mercaptoethanol was added to samples to a final concentration of 8%. Then, samples were frozen at −80°C for further analysis. The proteins (30 µg per well) were separated by SDS-PAGE using 15% gels and electrotransferred to nitrocellulose membranes for approximately 3 hours using a GE

Health Care TE22 Mini Tank Transfer System at 4°C. The membranes were blocked with 5% skimmed milk for 1 hour. After, membranes were washed three times in Tris-buffered saline with Tween (TBS-T) containing 100 mM Tris–HCl, 0.9% NaCl, and 0.1% Tween-20, pH 7.5 and incubated overnight (4°C) with primary antibodies anti-rabbit HSP70 Alexafluor 546 (1:500) (Santa Cruz Biotech, Dallas, TX) . Finally, immunoblots were visualized on a Bruker IS4000MM Pro imaging system in a specific wavelength to fluorescence detection (Em: 532nm Ex: 488nm). The densitometric analysis of immunoreactive bands was performed using Scion Image® software. The density of the bands was measured and expressed as a rate (%) of increase in relation to control.

2.6. Statistical analysis

We used t-test to verify the difference in the CTmax of treated group in relation to group control. For the HSP70 expression quantification we used one-way ANOVA, followed by Tukey a posteriori test to test for possible differences between groups. The tests were considered significant when $p < 0.05$.

3. Results

The critical thermal maximum supported by *Physalaemus henselii* decreased for tadpoles exposed to 2mg/L Mancozeb ($p < 0.05$) (Fig. 1): the CTmax for the control group was 36.79ºC whereas it was 34.38ºC for the Mancozeb treated group. The difference in the supported CTmax between these groups was 2.41ºC ($p < 0.05$). HSP70 expression was detected in all analyzed groups at the predictable molecular weight, indicating that the antibody used here is indicated for this species. In both the MZ ($p = 0.0008$) and Heat ($p = 0.0003$) groups there was an increase in protein expression when compared to the control group. The MZ + Heat ($p > 0.05$) group showed similar protein levels reported to the control group (Fig. 2, Fig. 3).

4. Discussion

Our results demonstrated that the sublethal concentration of 2 mg/L of Mancozeb fungicide negatively affected the thermal physiology and heat shock protein expression in tadpoles of *Physalaemus henselii* by inducing an increase in HSP70 activity and by reducing the thermal maximum supported by tadpoles.

Temperature is an important factor for ectotherms as they rely on external heat sources to maintain their body temperature. In addition, environmental temperature affects several vital biological processes. This thermal range modulates important factors of each species such as locomotion, growth, development, growth and fecundity of organisms. The thermal range (CTmin and Cmax) known for tadpoles of *Physalaemus henselii* is 1.30°C and 37.25°C, respectively (Madalozzo et al 2018). This CTmin is considered especially low and close to water crystallization point (Madalozzo et al 2018), but the CTmax reported por *P. henselli* tadpoles can also be considered low if compared to species that breed in warmer seasons (e.g. 41.84ºC for *Rhinella schneideri,* 42.11ºC for *Scinax fuscovarius*) (Oliveira et al. 2015; Madalozzo et al. 2018). In this study, the CTmax for the control group was 36.79ºC, agreeing with the previously reported value (Madalozzo et al. 2018), and seems to be a specific adaptation to colder temperatures, since the *P. henselii* (together *P. fernandezae*) compose the *Physalaemus* species group with the most austral distribution known (Lourenço et al. 2015). Although advantageous (e.g. to reduce possible interspecific competition) (Wilbur and Alford 1985; Pechmann and Semlitsch 1986), it can result in a physiological trade-off since species that acquire greater resistance to cold end up with costs associated with heat resistance (Stillman JH 2006). Here, the CTmax recorded for the group exposed to Mancozeb was 34.38ºC indicating that these costs increased after agrochemical contamination.

Among the adaptations of organisms to temperature increases, the expression of heat shock proteins (HSPs) proves to be an important mechanism (Jin et al. 2019). HSPs are highly conserved molecules that are present in all cellular organisms at baseline levels under non-stressful physiological conditions (Jacob et al. 2017). The expression of HSP is related to the natural levels of thermal stress that species experience in the environment and the temperature fluctuations sufficient to provoke response to thermal stress (Fangue et al. 2006; Huang and Kang 2007; Yu and Wan 2009). However, several stressful stimuli may increase the synthesis of HSPs

(Lindquist, 1986), among them, exposure to agrochemicals (Selvakumar et al. 2005). Among the HSPs, the HSP70 is the most phylogenetically conserved, considered of fundamental importance for protein folding (Daugaard et al. 2007). It is also considered the protein that acts most in response to cell stress, being used as a stress marker (de Pomerai 1996). In our study, HSP70 expression was significantly higher in the MZ and HEAT groups when compared to the control group (CTL). Stable protein conformation occurs due to numerous non-covalent interactions such as hydrogen bridges, van der Wall forces, ionic bonds and hydrophobic interactions. These interactions are sensitive to several ambient conditions, such as temperature rise, which determines denaturation and enzymatic degradation. This degradation causes changes and losses of catalytic activity (Daniel et al. 2006). Studies have shown the adaptive value of HSPs, being important proteins that confer thermal resistance to individuals (Tomanek 2010). When there is stressful stimuli such as sufficient temperature rises to cause protein denaturation, HSP70 influences protein folding, guiding renaturation and preventing protein aggregation through interaction with the exposed hydrophobic parts of the target protein (Mosser and Morimoto, 2004). In addition to conferring thermal resistance, studies suggest that HSP70 has functions in immunological and parasitic processes, both in native and adaptive immunity of organisms (Robert 2003; Marcogliese et al. 2009; Martini et al. 2010).

It was found here that exposure of tadpoles to MZ resulted in increased expression of HSP 70 protein. It has been shown that HSPs protects cells from damage caused by oxidative stress (Bukau and Horwich 1998). In line with this, the exposure toMZ was demonstrated to cause alterations in the oxidative balance of *P. henselii* (see Chapter 1 for details). Thus, fungicide effect recorded in the MZ group was sufficient to induce protein damage and increased HSP synthesis in this group as a protective cellular response. The last group tested, MZ + HEAT, showed no significant difference in relation to the control. It can be inferred that after treatment with 2mg/L Mancozeb, there was an increase in the synthesis of HSPs, as well as the MZ group. However, when submitted to the warm-up test, the HSPs decreased their activities to basal levels as in the control group possibly due to the energy cost related to the higher synthesis of HSPs (Krebs and Feder 1997a and the toxicity presented by an excessive increase of these proteins (Krebs and Feder 1997b). That is, there seems to be a trade-off between the benefits and the cost of producing these proteins. However, the exact mechanisms by wich MZ + HEAT interferes in the cellular machinery involved in the

expression of HSPs need further elucidations.

The results found in our study are innovative for amphibians, since there are no studies so far between the effects of Mancozeb on thermal tolerance and HSPs in anurans. Understanding the relationship between environmental contamination and physiological thermal limits is increasingly important (Winter et al. 2016) in the current global scenario of unrestrained use of agrochemicals and global warming (Foley et al. 2005; Tomanek 2010; Lima et al. 2019). It is a fact that human changes in natural ecosystems have several negative effects on species (Pereira et al. 2010). In recent years, agricultural productivity has grown due to the implementation of new technologies. Among them, the use of agrochemicals (Foley et al. 2005). Mancozeb is the most widely used fungicide in the world, being used in soybean and wheat plantations (IBAMA 2019). The use of Mancozeb is concerning, since the conversion rates of native grasslands to soybean plantations are high (Overbeck te al. 2015; Hasenack et al. 2019). The southern region was responsible for 36% of brazilian productivity including soybean, rice and wheat plantations in 2017 (IBGE, 2019). Grasslands of the Brazilian Pampa biome their vegetation coverage was reduced by 11.4% in 2009 (Weber et al. 2016; Hasenack et al. 2019). With the results obtained, it is possible to predict the damage that the species will suffer from contamination of the aquatic environment by Mancozeb. Contamination by Mancozeb, in addition to negatively affecting the survival rate and altering the cellular functioning of tadpoles, has also been shown to negatively affect thermal physiology and HSP synthesis. The decrease in CTmax supported by the tadpoles after contamination is worrying, as the ability of a species to withstand the increase in temperature can be decisive in its survival in a scenario of global warming (Loeschcke et al., 1997; Thomas et al. 2004; Newbold 2018). The increase in temperature, however small, can affect organisms in several ways (Williams et al. 2003; Nguyen et al. 2011). By the end of this century, the global average temperature is expected to rise between 1.4ºC and 5.8ºC (IPCC 2018), and results suggest that the synergistic effects of land use and climate change will impact reptile and amphibian communities (Newbold 2018). Although amphibians and other organisms have mechanisms such as HSP for thermal protection, in a scenario of environmental contamination and an increase in temperature they will be extremely harmed, mainly under the Brazilian policy characterized by shameful complacency with high rates of habitat loss (Crouzeilles et al. 2017) and by the recent record in legalization of new pesticides (MAPA 2019a, b).

5. Conclusions

In this study we demonstrated that the exposure of *Physalaemus henselii* tadpoles to sublethal doses of the fungicide Mancozeb causes deleterious physiological effects, bringing light on the relationship between environmental pollution and thermal physiology of amphibians. These organisms are extremely sensitive to anthropic changes in natural landscapes, and great concern occurs due to the accelerated rates of conversion of natural habitats for different purposes, mainly for croplands, that expose amphibians to agrochemicals and contamination. In addition, the environmental changes associated to the global warming scenario put the amphibians as one of the most affected groups. Therefore, further studies must explore the relationship between amphibian thermal physiology and environmental contamination, focusing the wide range of agrochemicals used legally and illegally.

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Figures

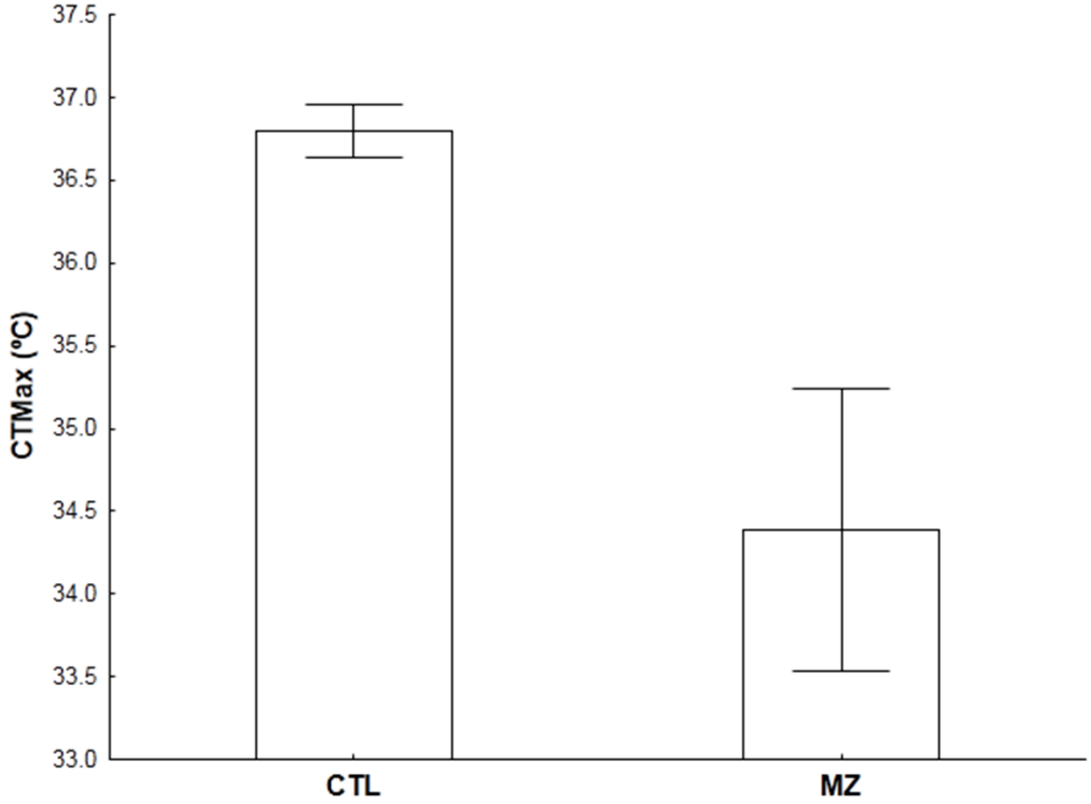

Figure 1. Variation of critical thermal maximum (CTmax) of *Physalaemus henselii* tadpoles at control (CTL) and sub-lethal Mancozeb concentration (MZ).

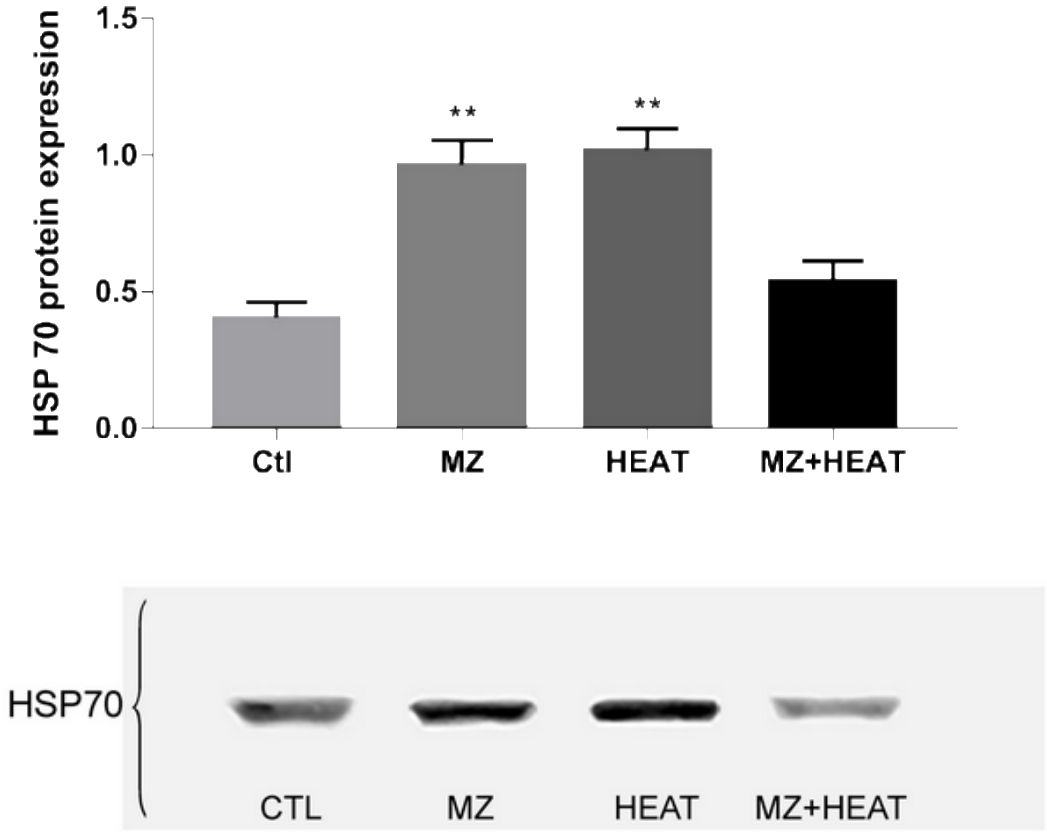

Figure 2. Groups: CTL (control), MZ (exposed to 2mg/L Mancozeb), HEAT (warm-up test), MZ + HEAT (exposed to 2mg/L Mancozeb + warm-up test). **A)** Stress-inducible HSP70 expression for tadpoles of *Physalaemus henselii*. **B)** Western blot showing the presence of Hsp70 in the tail muscle in tadpoles of *Physalaemus henselii*.

Discussão geral

Nosso trabalho quantificou a Concentração Letal (CL50) de Mz em girinos de *Physalaemus henselii*, bem como revelou os impactos negativos de doses subletais sobre o perfil de estresse oxidativo, e sobre a fisiologia térmica e expressão de proteínas de choque térmico.

No atual cenário mundial, o impacto humano sobre os ecossistemas naturais tem aumentado constantemente (Wikelski e Cooke, 2006), como exemplificado pelas alarmantes taxas de conversão dos campos nativos em sistemas agrícolas (Foley 2005; Staude et al. 2018; Andrade et al 2019; Hasenack et al. 2019). Os campos nativos fornecem diversos serviços ecossistêmicos e estão entre os ecossistemas mais ricos em espécies vegetais no mundo (Wilson et al. 2012; Andrade et al. 2015). Aproximadamente 60% dos campos originais no estado do Rio Grande do Sul foram perdidos devido à conversão agrícola (Andrade et al. 2015). Neste cenário, o Pampa possui o maior Índice de Risco de Conservação (IRC) entre todos biomas brasileiros, indicando uma enorme assimetria na proporção entre áreas convertidas e áreas sob alguma categoria de proteção (Overbeck et al. 2015). Essa conversão do habitat causa danos à biodiversidade local (Gaston et al. 2003) e os agroquímicos são considerados um fator importante no contexto da conservação dos anfíbios, pois contaminam não apenas a região convertida, mas também as áreas próximas (Becker et al. 2007; Josende et al. 2014).

Um fator agravante no cenário acima descrito é o franco declínio do Brasil quanto à qualidade das políticas de conservação ambiental. Sem ações de controle sobre a produtividade agrícola, a expansão da agricultura aprofundará significativamente a degradação ambiental (Simões et al. 2020). Assim, questões ambientais explicitamente não são importantes na agenda do governo, que inúmeras vezes demonstrou priorizar os interesses do agronegócio (Ferrante and Fearnside 2019). Um exemplo dramático disso desde o início da atual administração governamental, é a aprovação histórica de dezenas de novos agroquímicos (Ferrante and Fearnside 2019).

O conflito entre a produção de alimentos e a conservação ambiental é um dos maiores desafios da humanidade neste século (Santos et al. 2020). Neste sentido, é necessário adotar medidas para minimizar a poluição ambiental e seus efeitos, como por exemplo, restringir o uso de agrotóxicos e incentivar a pesquisa por meios alternativos de controle menos agressivos a organismos não-alvo, como os anfíbios.

Uma alternativa ao uso de agrotóxicos é uso de controles biológicos, que apresentam altas taxas de eficácia principalmente quando integrados com outros sistemas de manejo de pragas (Junior 2011). De fato, o controle biológico possui diversas vantagens para o produtor agrícola (Filho e Macedo 2011): a) ausência de efeitos colaterais à população; b) alto nível de controle; c) manutenção do controle com baixo custo financeiro após o investimento financeiro inicial; d) não desenvolvimento de resistência aos inimigos naturais.

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