UNIVERSIDADE ESTADUAL PAULISTA
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Programa de Pós Graduação em Biologia Animal

Juliane Silberschmidt Freitas

Influência da temperatura na toxicidade dos herbicidas sulfentrazone (Boral 500SC®) e clomazone (Gamit®) em larvas de anuros *Eupemphix nattereri* (Leiuperidae) e *Rhinella schneideri* (Bufonidae)

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Orientador: Prof. Dr. Eduardo Alves de Almeida

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“Dedico esta tese de Doutorado à minha mãe, que lutou sem esforços para poder estar presente nesse momento. Você está em meu coração.”

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RESUMO

A temperatura é um importante fator ambiental influenciando o metabolismo, desenvolvimento e comportamento de animais de sangue frio. Entretanto, os efeitos indiretos da temperatura em sistemas naturais, tais como o potencial de interação com contaminantes ambientais, ainda são pouco estudados para muitos táxons. As alterações nos padrões de temperatura podem potencialmente alterar a natureza e distribuição de muitos contaminantes no ambiente e, por isso, o maior desafio atual dos ecotoxicologistas está em prever os riscos estabelecidos por essas interações a nível biológico. Os agrotóxicos incluem o principal grupo de agentes tóxicos encontrados em sistemas naturais, sendo grande parte desses compostos provenientes da manutenção de culturas de cana-de-açúcar no controle de plantas invasoras no Brasil. No noroeste paulista do país, muitas espécies de anuros estão distribuídas em áreas comuns à agricultura, com ocorrência reprodutiva restrita à estação chuvosa, a qual coincide com o período de maior contaminação ambiental. O presente trabalho objetivou avaliar a influência da temperatura na toxicidade dos herbicidas sulfentrazone, clomazone e diuron em larvas de anuros, utilizando biomarcadores fisiológicos, bioquímicos e moleculares específicos. O primeiro estudo investigou os efeitos dos herbicidas sulfentrazone (Boral SC500C®) e clomazone (Gamit®) em girinos de *Eupemphix nattereri* e *Rhinella schneideri* submetidos a diferentes condições térmicas (28, 32 e 36 ºC) através da análise das respostas antioxidantes e de peroxidação lipídica. Em um segundo trabalho, objetivamos investigar os potenciais efeitos endócrinos do herbicida diuron e seu metabólito (3,4-DCA) (40 e 200ng/L) em girinos de *Lithobates catesbeianus* submetidos a condições de estresse térmico (28 e 34 ºC), utilizando para isso biomarcadores moleculares e enzimáticos relacionados à atividade da tireoide em anfíbios. Os resultados mostraram que a temperatura foi um importante fator influenciando a toxicidade dos herbicidas em estudo para as diferentes espécies de girinos. Além disso, alterações dos diferentes biomarcadores também foram mediadas pelo efeito isolado da temperatura. A exposição de girinos de *E. nattereri* e *R. schneideri* aos herbicidas clomazone e sulfentrazone provocou alterações na atividade das enzimas antioxidantes, além de aumentos nos níveis de malondialdeido (MDA), indicando ocorrência de estresse oxidativo; entretanto, essas respostas foram diretamente relacionadas às temperaturas de exposição para ambas as espécies. A maioria das enzimas apresentou atividades pronunciadas em temperaturas mais elevadas e em combinação à presença dos contaminantes. Esses dados são provavelmente devido ao aumento da toxicidade de contaminantes em temperaturas mais elevadas como resultado de um incremento das taxas metabólicas desses animais de sangue frio. Os diferentes mecanismos de ativação do sistema antioxidante em *E. nattereri* e *R. schneideri* também sugere que girinos de regiões tropicais devem utilizar diferentes estratégias e mecanismos de adaptação para lidar com os efeitos gerados pela exposição a agrotóxicos em *habitats* onde a temperatura é um fator de influência relevante. Nos resultados de nosso segundo trabalho, encontramos que o herbicida diuron e seu metabólito 3,4-DCA são potentes desreguladores da tireoide em girinos de *L. catesbeianus*, entretanto esses efeitos endócrinos são pronunciados em temperaturas mais elevadas. Além de indução na expressão de genes relacionados ao processo de metamorfose, observamos alterações nos níveis plasmáticos

de triiodotironina (T_3) e uma aceleração na metamorfose de animais expostos à condição combinada de temperatura elevada em presença aos contaminantes, diuron ou 3,4-DCA. De forma geral, nossos resultados sugerem que condições realísticas de temperatura ambiental deveriam ser levadas cada vez mais em consideração em estudos de risco avaliando a exposição de anfíbios a contaminantes ambientais, tais como agrotóxicos. A falta de abordagem dessas variáveis em testes laboratoriais pode levar a interpretações subestimadas relacionadas aos efeitos tóxicos desses compostos em diferentes espécies de anfíbios.

Palavras-chave: Anfíbios, temperatura, agrotóxicos, clomazone, sulfentrazone, diuron, estresse oxidativo, desreguladores endócrinos.

ABSTRACT

Temperature is an important environmental factor influencing the metabolism, development and behavior of cold-blooded animals. However, indirect effects of temperature on natural systems, such as the potential of interaction with environmental contaminants have not been well-characterized for many taxa. Changes in temperature patterns can potentially alter the nature and distribution of many contaminants in the environment, and therefore the current challenge for ecotoxicologists is to predict the risks established by these interactions at biological level. Pesticides are the main group of toxicants present in natural systems and a large part of these compounds are used in sugarcane crops for the control of invasive plants in Brazil. In the northwest of the country, many species of anurans are distributed in areas common to agriculture. Most of them have reproductive activity restricted to the rainy season, which coincides with the period of greatest environmental contamination. This study aimed to investigate the influence of temperature on the toxicity of the herbicides sulfentrazone, clomazone and diuron in anuran larvae, using specific physiological, biochemical and molecular biomarkers. Our first study investigated the effects of the herbicides sulfentrazone (Boral SC500C®) and clomazone (Gamit®) in tadpoles of *Eupemphix nattereri* and *Rhinella schneideri* under different temperatures (28, 32 and 36 ºC), evaluating the antioxidant responses and lipid peroxidation. In our second study, we evaluated the influence of temperature (28 and 34 °C) on the action of diuron and its metabolite 3,4-dichloroaniline (3,4-DCA) on thyroid function and metamorphosis in tadpoles of *Lithobates catesbeianus.* Our results showed that temperature is an important factor influencing toxicity of the studied herbicides for the different tadpole's species. Moreover, studied biomarkers were also changed by isolated effects of temperature. Exposure of *E. nattereri* and *R. schneideri* to the herbicides clomazone and sulfentrazone altered the antioxidant enzymes activities in tadpoles, in addition to increases in malondialdehyde (MDA) levels, indicating the occurrence of oxidative stress; however, these responses were directly related to the temperatures of exposure for both species. Most of the enzymes had their activities more pronounced at higher temperatures and in combination with the presence of contaminants. These data are probably due to the increased toxicity of contaminants at higher temperatures as a result of increased metabolic rates of cold-blooded tadpoles. Differential activation of the antioxidant system in *E. nattereri* and *R. schneideri* suggests that tadpoles of different species may use diverse strategies and adaptive mechanisms to cope with the effects generated by exposure to agrochemicals in habitats where the temperature is a relevant influencing factor. The results of our second study showed that the herbicide diuron and its metabolite 3,4-DCA are potent thyroid deregulators in *L. catesbeianus* tadpoles, however these endocrine effects are more pronounced at higher temperatures. Besides the induction on expression of TH-related genes, we observed changes in plasma levels of triiodothyronine (T3) and an acceleration in the metamorphosis of tadpoles exposed to the combined effects of high temperature and the presence of contaminants, diuron or 3,4-DCA. Overall, our results suggest that realistic environmental temperatures should be increasingly taken into account in risk studies evaluating the exposure of amphibians to environmental contaminants, such as pesticides. The lack of approach of these

variables in laboratory tests can lead to underestimated interpretations of the toxic effects of these and other compounds for different species of amphibians.

Keywords: Amphibians, temperature, pesticides, clomazone, sulfentrazone, diuron, oxidative stress, endocrine disruptors.

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INTRODUÇÃO

1. INTRODUÇÃO

1.1. Ecotoxicologia e declínio das populações de anfíbios

Populações de anfíbios estão sofrendo declínios mundiais ao longo das últimas décadas (Stuart et al., 2004; Wake e Vredenburg, 2008; Hayes et al., 2010; Blaustein et al., 2011). Dados da IUCN (International Union for Conservation of Nature) indicam que aproximadamente 32% das espécies globais estão ameaçadas de extinção e 43% têm populações que já estão em processo de declínio, além das 22% que apresentam dados insuficientes sobre sua situação atual. A redução das espécies sul-americanas tem sido ainda mais rápida do que a média global, independente das simples variações mundiais, espaciais e temporais (Alford et al., 2001; Houlahan et al., 2001). Vários fatores são apresentados para explicar tais quedas, alguns decorrentes direta ou indiretamente das atividades humanas e outros emergentes das mudanças climáticas globais e locais (Blaustein et al., 1994; Blaustein et al., 2011). Podem ser mencionados, por exemplo, a destruição de habitats, infecções fúngicas e bacterianas, o aumento da exposição aos raios ultravioletas e às elevadas temperaturas, e poluição de ambientes aquáticos (Blaustein et al., 1994; Hayes et al., 2010; Blaustein et al., 2011). Pesquisadores acreditam que estamos presenciando um episódio de extinção em massa dos anfíbios, uma vez que a perda e diminuição de espécies estão ocorrendo em taxas sem precedentes e com valores 200 vezes mais altos do que para os demais grupos animais (Roelants et al., 2007; Wake e Vredenburg, 2008; May, 2010).

A poluição ambiental derivada do uso exacerbado de agrotóxicos na agricultura tem sido apontada como um dos principais fatores associados ao declínio de muitas espécies de anfíbios (Todd et al., 2011; Fryday e Thompson, 2012; Hayes et al. 2010). Esses compostos agem provocando efeitos diretos na mortalidade dos indivíduos ou indiretos, como o aparecimento de malformações em girinos, alterações e anormalidades gonadais em adultos, atraso do desenvolvimento, alterações de comportamento, entre outras (Ouellet et al., 1997; Sparling et al., 2001; Davidson e Knapp 2007, McCoy

et al., 2008; Kloas et al., 2009; Blaustein et al., 2011; Peltzer et al., 2013). Embora os agrotóxicos tenham proporcionado benefícios enormes para a sociedade pelo aumento do rendimento agrícola, seus potenciais efeitos em organismos não alvos têm sido grande motivo de preocupação nas últimas décadas (Releya e Jones, 2009). Especialmente para os anfíbios, não é necessária uma avaliação de risco específica para o registro de um novo produto agrícola, até o momento. Entretanto, os efeitos negativos desses compostos são bastante prováveis para o grupo devido à elevada permeabilidade cutânea durante as fases adultas e larvais, sendo as últimas particularmente sujeitas aos efeitos de exposição, uma vez que os girinos são estritamente restritos a ambientes aquáticos e apresentam uma baixa taxa de migração em relação à área de desova (Semlitsch et al. 2000; Tejedo 2003).

Nos últimos três anos, o Brasil vem ocupando o lugar de maior consumidor de agrotóxicos no mundo (ABRASCO - Associação Brasileira de Saúde Coletiva) e assumindo proporções ameaçadoras (Londres, 2011). A cana-de-açúcar é atualmente um dos principais produtos agrícolas que tem se expandido no país, devido principalmente à sua utilização na produção de biocombustíveis (Stefani Margarido et al., 2013). A rápida expansão das lavouras de cana-de-açúcar brasileiras tem contribuído em grande proporção para o uso de agrotóxicos, os quais estão relacionados principalmente ao combate de plantas infestantes (ex. *Cyperus rotundus*) que provocam perdas de até 85% no potencial rendimento da cultura (Kissmann, 1991). O estado de São Paulo, maior produtor nacional de cana (responsável por 60% da produção total), contribui com, aproximadamente, 20% do consumo de agrotóxicos no Brasil, incluindo principalmente as classes dos inseticidas, fungicidas e herbicidas (Abrasco, 2011).

Atualmente, estão registrados pela Anvisa 33 moléculas de herbicidas para uso em culturas de cana-de-açúcar, sendo que a maioria necessita da umidade do solo para melhor absorção pelas sementes das plantas infestantes (Martini e Durigan, 2004). O problema é que esses herbicidas apresentam longo poder residual no ambiente, e como o manejo químico é mais eficaz durante as épocas de chuva, a

contaminação de lagos e corpos d´água próximos às áreas agrícolas geralmente são intensificadas durante esses períodos (Martini e Durigan, 2004; Azania et al., 2009). Em regiões tropicais com estações bem definidas, a ocorrência e reprodução para a maioria das espécies de anuros estão restritas às estações chuvosas (McDiarmid e Altig, 1999; Johansson et al., 2006) e muitas delas estão amplamente distribuídas em regiões de área aberta, incluindo áreas de cultivo (Provete et al., 2011). Por essas razões, há fortes motivos para acreditar que as espécies neotropicais de anfíbios estão sendo gravemente afetadas pelos resíduos agrícolas, principalmente durante a estação de chuvas, que coincide com o período de maior susceptibilidade desses animais aos compostos químicos, durante seu ciclo de vida aquático (Cooke, 1972; Devillers e Exbrayat, 1992; Lefcort et al., 1998).

Herbicidas à base de sulfentrazone e clomazone estão entre os produtos mais utilizados em culturas de cana-de-açúcar no Brasil (Embrapa, 2013), sendo ambos detectados em solo e água de áreas de cultivo no estado de São Paulo (Armas et al., 2005). O herbicida sulfentrazone possui excelente atividade pré-emergente (Hess, 1993; Tomlin, 1994; Rodrigues e Almeida, 2005) e além da cana, está registrado no Brasil para culturas com grande importância comercial como soja, café, citrus e eucalipto. O sulfentrazone (2',4' dicloro-5-(4-difluorometil-4,5-dihidro 3 metil-5-oxo-1H1,2,4-triazol-1-il) metanossulfonanilida) pertence ao grupo das aril-triazolinonas e destaca-se entre os herbicidas com alto potencial de contaminação por possuir um longo efeito residual no solo (Melo et al., 2010; Monquero et al., 2010), com meia-vida estimada entre 110 e 280 dias (Hess, 1993; Rodrigues e Almeida, 2005; Tomlin, 1994). O composto clomazone (2-(2-clorofenil) metil-4-dimetil-3-isoxazolidinona) pertence à classe das isoxazolidinonas e também é bastante empregado em culturas de arroz, mandioca, batata e algodão. Apesar de ser bastante efetivo, o clomazone provoca ampla contaminação ambiental devido sua elevada solubilidade em água (1100 mg L^{-1}), além da meia-vida média de 28 a 84 dias, podendo seus resíduos permanecerem até 130 dias no ambiente (Colby et al., 1989; Zanella et al., 2002). Devido às

propriedades de herbicidas contendo sulfentrazone e clomazone em suas formulações comerciais, junto à utilização de outros compostos persistentes, a cana-de-açúcar tem sido considerada uma das culturas com maior potencial de risco na ocorrência de problemas relacionados à permanência de moléculas de herbicidas no solo e na água por um tempo além do desejável (Blanco et al., 2010), sendo os efeitos tóxicos em anfíbios ainda desconhecidos.

1.2. A influência de fatores abióticos na toxicidade dos xenobióticos

Em sistemas naturais, organismos aquáticos são altamente influenciados por alterações em fatores ambientais, tais como temperatura, pH, salinidade e radiação ultravioleta (Hooper et al., 2013). Os efeitos diretos das variáveis ambientais têm sido caracterizados para diversos táxons, como por exemplo, a influência da temperatura na distribuição de populações de peixes (Harley et al. 2006; Rijnsdorp, 2009; Perry, 2011) e no desenvolvimento larval de anuros (Morand, 1997; Bickford, 2010). Entretanto, muito pouco ainda é conhecido sobre os efeitos indiretos das variações abióticas nos ecossistemas, como o potencial de alterar a toxicidade de compostos químicos no ambiente (Bao et al., 2008; Boxall et al., 2009; Noyes et al., 2009; Rohr et al., 2010; Hooper et al., 2013; Moe et al., 2013). A alteração dos parâmetros abióticos pode modificar significativamente a dispersão, comportamento, biodisponibilidade e o destino dos contaminantes ambientais por uma série de mecanismos (ex. biodegradação, fotólise e oxidação (Boxall et al., 2009). Além disso, eles intervêm diferencialmente nos mecanismos fisiológicos de cada espécie (Gordon, 2003; Raffel et al, 2006), alterando suas respostas biológicas naturais, e tornando os organismos mais susceptíveis à ação dos xenobióticos (Vitousek et al, 1997; Blaustein e Kiesecker, 2002; Moe et al., 2013). Assim, há uma crescente consciência da importância de antecipar os efeitos da poluição química em um panorama de mudanças muito rápidas no

clima, identificando e mitigando seus efeitos naqueles organismos e ecossistemas mais vulneráveis (Noyes et al., 2009).

As alterações nos padrões de temperatura são descritos como um dos principais parâmetros que interferem na toxicidade dos agrotóxicos, por meio da modificação de mecanismos fundamentais de partição ambiental, através de alterações na volatilidade, deposição e degradação (Van den Berg et al., 1999; Noyes et al., 2009). Quando os agrotóxicos estão presentes em comunidades aquáticas, a temperatura desempenha mais do que um papel passivo. Geralmente, à medida que a água é aquecida, as reações químicas e biológicas tornam-se muito mais rápidas e por isso, o efeito de um agente tóxico deve ser mais pronunciado em temperaturas mais elevadas (Middlebrooks, 1973). Por esse motivo, os organismos aquáticos têm sido apontados como principais alvos dos efeitos do aumento de temperatura em áreas influenciadas por atividades agrícolas. Os anfíbios, principalmente no estágio de vida larval, são particularmente vulneráveis a essas interações, as quais têm sido mencionadas ser mais ameaçadoras para o grupo do qualquer outro estressor isolado (Materna et al., 1995; Broomhall, 2002; Broomhall 2004; Rohr e Palmer, 2005). A temperatura afeta praticamente todos os aspectos fisiológicos dos anfíbios, incluindo digestão, visão, locomoção, crescimento e metabolismo (Gatten et al., 1992, Rome et al., 1992). Para esses ectotérmicos, a variação térmica do ambiente é traduzida diretamente na variação do desempenho fisiológico do animal (Fontenot e Lutterschmidt, 2011).

Como mencionado anteriormente, a maioria de espécies de anuros neotropicais se reproduzem durante as estações quentes e chuvosas. No Brasil e em outras regiões tropicais, as temperaturas ambientais podem atingir médias muito elevadas durante o verão. Em poças temporárias, as quais servem como habitat para desenvolvimento de diversas espécies de girinos, as temperaturas médias podem ultrapassar os 40°C (Freitas et al., 2016) devido ao baixo volume de água, tornando as flutuações térmicas muito mais pronunciadas que em qualquer outro sistema aquático. Por isso, oscilações de

temperaturas ambientais deveriam ser consideradas como fator crucial em estudos avaliando os reais riscos de exposição aos agrotóxicos em girinos de regiões neotropicais na natureza.

Apesar do crescente aumento no número de trabalhos explorando os efeitos da variação de temperatura na toxicidade de compostos químicos (Schiedek et al., 2007; Bao et al., 2008; Noyes et al., 2009; Rohr et al., 2011; Moe et al., 2013; Hooper et al., 2013), os dados coletados até o momento não são abrangentes o suficiente para estimar seus verdadeiros riscos para as comunidades biológicas, tampouco avaliam seus potencias efeitos sobre organismos ectotérmicos, os quais apresentam variação inter e intra específica em suas tolerâncias térmicas (Katzenberger et al., 2012). Isso é particularmente preocupante para espécies de anfíbios brasileiras, das quais se tem pouquíssimas informações até o momento.

1.3. Efeitos endócrinos dos agrotóxicos e a influência de fatores ambientais

Muitos dos compostos químicos identificados como desreguladores endócrinos (EDs) são agrotóxicos (Vinggaard et al., 2000; Andersen et al., 2002; Lemaire et al., 2006; Mnif et al., 2011). Compostos que agem como EDs podem interferir na síntese, transporte, metabolismo e eliminação de hormônios, alterando então suas concentrações naturais no organismo (Akhtar, 1996; Cocco, 2002; Leghait et al. 2009; Sugiyama et al., 2005). De um ponto de vista fisiológico, os EDs são substancias naturais ou sintéticas que por meio de exposições ambientais alteram o sistema hormonal e homeostático de um organismo, os quais são responsáveis pela comunicação e resposta ao meio ambiente (Diamnati-Kandarakis et al. 2009; Mnif et al., 2011). Originalmente, as preocupações iniciais sobre a exposição aos EDs estavam quase totalmente relacionados aos distúrbios do sistema endócrino ligados aos aspectos reprodutivos, e muitos compostos passaram a ser denominados como compostos estrogênicos ou androgênicos. Entretanto, se reconheceu posteriormente que alguns compostos poderiam interferir em

outros elementos do sistema endócrino via interação com receptores específicos que não àqueles relacionados aos hormônios sexuais (Philips e Harrison, 1999).

Organismos aquáticos são um dos principais alvos dos EDs no ambiente, principalmente porque muitos desses compostos são utilizados na agricultura como herbicidas, inseticidas ou fungicidas e podem ser lixiviados durante os períodos de chuva, atingindo águas superficiais e uma diversidade de organismos não alvos. O mais preocupante é que esses compostos afetam a saúde dos organismos em concentrações consideradas baixas a nível ambiental, as quais não ocasionam mortalidade direta dos indivíduos, mas provocam uma série de consequências ao sistema endócrino após longas exposições, levando à diminuição da resposta imune, metabolismo energético, habilidade osmorregulatória e função reprodutiva (Mnif et al., 2011). A ação dos EDs em concentrações baixas está associada principalmente ao forte potencial desses compostos em se ligarem a receptores específicos, desencadeando assim respostas endócrinas alteradas ou até mesmo inibindo as mesmas (Mnif et al., 2011). Entre a numerosa lista de agrotóxicos que podem ser citados como EDs, estão: atrazina, carbaril, diazinon, dieldrin, endosulfan, glifosato, malation e diuron (Soto et al., 1994; Cocco, 2002; Manabe et al., 2006).

Diuron (3-(3,4-diclorofenil)-1, 1-dimetiluréia) é um dos agrotóxicos encontrados com maior frequência em ecossistemas de água doce (e.g. Pesce et al., 2008; Schuler e Rand, 2008; Stork et al., 2008; Morin et al., 2009). É um herbicida pré-emergente aplicado para controle de plantas infestantes em culturas de citros, algodão, frutas e cana-de-açúcar, sendo que sua persistência no solo pode durar de 1 mês até 1 ano (Okamura et al., 2003). Uma vez liberado no ambiente, o diuron é degradado via aeróbica (Ellis e Camper, 1982) e anaeróbica (Attaway et al., 1982) através da degradação microbiana. Seu principal produto de biodegradação, 3,4-dicloroanilina (3,4-DCA), é também altamente persistente no solo, água e lençóis freáticos e tem sido mencionado por exibir maior toxicidade que seu composto

original. Assim, a poluição das águas e do solo pelo diuron tem se tornado um problema ainda mais sério devido à formação do 3,4-DCA no ambiente (Giacomazzi e Cochet, 2004).

Estudos prévios têm apontado o herbicida diuron como um potente desregulador endócrino em diversos organismos não alvo, como caramujos, peixes, anfíbios e lagartos (Nebeker e Schuytema, 1998; Schuytema e Nebeker 1998; Lavado et al., 2004; Giacomazzi e Cochet, 2004; Cardone et al., 2008). Por exemplo, estudos com tilápia do Nilo (*Oreochromis niloticus*) mostraram que fêmeas expostas ao herbicida diuron e seus metabólitos apresentaram um evidente aumento na produção de ovócitos vitelogênicos após 1 mês de exposição (Pereira et al., 2016). Os efeitos anti-androgênicos do diuron e seus metabólitos também foram observados em machos após o mesmo período de exposição, evidenciados pela diminuição dos hormônios testosterona e 11-ketotestosterona, além da diminuição do índice gonadossomático em adultos (IGS) (Pereira et al., 2015). Em anfíbios, o diuron é capaz de alterar o desenvolvimento de pelo menos quatro espécies de anuros, embora em concentrações muito mais elevadas que aquelas detectadas no ambiente (Schuytema e Nebeker, 1998).

Entre os potenciais efeitos causados pelos desreguladores endócrinos, está o potencial de certos agrotóxicos alterarem o funcionamento normal da glândula tireoide em vertebrados (Goto et al., 2006; Sharma e Patiño, 2008; Brande-Lavridsen et al., 2010; Hooper et al., 2013). Por exemplo, a produção de hormônios da tireoide pode ser inibida por alguns agrotóxicos como fipronil, mancozebe, ioxynil, manebe, pentacloronitrobenzeno, prodiamina, entre outros (Akhtar et al., 1996; Cocco, 2002; Sugiyama et al., 2005; Leghait et al., 2009). A exposição aos compostos que desregulam a tireoide são de especial preocupação para girinos porque o processo de metamorfose é totalmente dependente e regulado por hormônios tireoidianos (THs), os quais promovem a remodelação de larvas aquáticas em adultos tetrápodes. Isso significa que alterações estruturais e funcionais de tecidos larvais durante o processo de

metamorfose poderiam estar relacionadas ao mau funcionamento da glândula, e poderiam, portanto, ser utilizados como relevantes parâmetros refletindo exposição aos EDs em anfíbios (Miyata e Ose, 2012).

Deve-se considerar, entretanto, que os EDs estão sob influencia de uma variedade de fatores abióticos em sistemas naturais, tais como a temperatura, a qual é considerada variável chave afetando a metamorfose de girinos como resultado de um mecanismo adaptativo (Denver et al., 1998; Walsh et al., 2008). Durante estágios de vidas iniciais, a exposição aos desreguladores da tireoide deve prejudicar a capacidade de girinos em responder adequadamente ao estresse gerado pelo aumento de temperatura ambiental. Assim, estágios de vida mais precoces devem ser particularmente sensíveis ao duplo estresse gerado pela presença de desreguladores da tireoide junto às alterações da temperatura ambiental, uma vez que o sistema dependente da tireoide em girinos ainda não está completamente formado, mas é crucial para o desenvolvimento normal de anuros adultos (Hooper et al., 2013). Até o momento, os dados disponíveis na literatura não são suficientes e tampouco evidenciam claramente a diversidade de agrotóxicos capaz de alterar o funcionamento da tireoide em anfíbios, especialmente considerando os mecanismos de ação usados pelos diferentes contaminantes ambientais durante os estágios iniciais de desenvolvimento. A maioria dos estudos tem avaliado o potencial desses compostos em inibir a metamorfose e danos ao tecido tireoidiano em anfíbios. Entretanto, poucos são direcionados em entender os reais riscos ecológicos causados pela exposição aos EDs sob um importante cenário de alteração dos padrões abióticos. Assim, faz-se urgente e necessário um incremento de estudos investigando os potenciais efeitos endócrinos dos diversos agroquímicos liberados em ambientes utilizados por anfíbios como habitats, dos quais a maioria não se tem nenhuma informação toxicológica registrada para o grupo.

1.4. Indicadores de contaminação aquática

Sabe-se que o metabolismo de certas classes de agrotóxicos, assim como de outros tipos de poluentes, resulta no aumento da produção de espécies reativas de oxigênio (ERO), como os ânions superóxido ($O_2^{\bullet-}$), peróxidos de hidrogênio (H_2O_2) e radicais hidroxil (•OH), os quais podem provocar diversos danos às biomoléculas devido às suas elevadas reatividades (Fridovich, 1986; Davies et al., 1987; Imlay e Linn, 1988). Sob condições normais, os sistemas de defesa antioxidantes protegem as células contra os efeitos citotóxicos das ERO (Jones et al., 2010). Em organismos aquáticos, esses sistemas de defesa são representados pelas enzimas antioxidantes, tais como a superoxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e glicose-6-fosfato desidrogenase (G6PDH) (Fig. 1) e por antioxidantes não enzimáticos, tais como aminoácidos, ácido ascórbico (vitamina C), retinol (vitamina A) e tocoferol (vitamina E) (Martinez-Alvarez et al., 2005; Grim et al., 2010). No entanto, a taxa de ERO pode ser substancialmente aumentada em condições adversas, e quando a produção das ERO excede a sua taxa de decomposição por meio dos sistemas antioxidantes, ocorre uma perturbação do estado redox da célula e é desencadeado então o estresse oxidativo, o qual pode agravar os danos celulares sob diferentes alvos (Almeida et al., 2005; Lushchak, 2011).

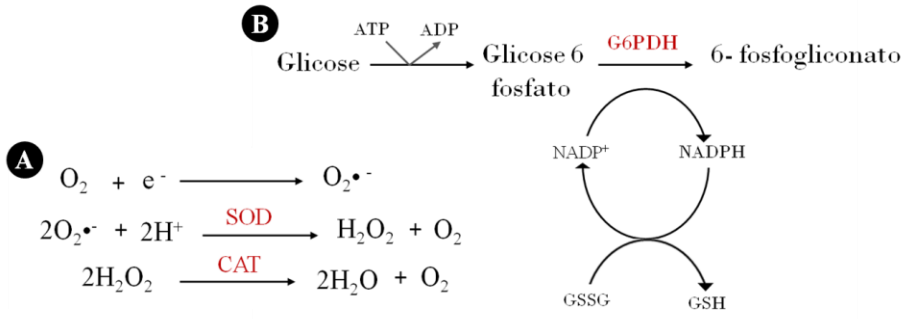

Fig. 1: Representação esquemática da atividade de algumas enzimas antioxidantes. A: superóxido dismutase (SOD) e catalase (CAT). O superóxido ($O_2^{\bullet-}$) é formado pela redução do oxigênio molecular, e é considerado uma espécie reativa de oxigênio. A SOD catalisa a dismutação do superóxido ($O_2^{\bullet-}$) em oxigênio (O_2) e peróxido de hidrogênio (H_2O_2), ao passo que a CAT decompõe o superóxido ($O_2^{\bullet-}$) em água e oxigênio. B:

Representação da atividade da glicose-6-fosfato desidrogenase (G6PDH) no ciclo das pentoses. Primeiramente ocorre desidrogenação enzimática da glicose-6-fosfato pela G6PDH para formação de 6- fosfoglicono-d -lactona, a qual é hidrolisada para a forma ácida livre 6- fosfogliconato por uma lactonase específica, sendo que o NADP+ é o receptor de elétrons. A ação da G6PDH é importante para manter os níveis de NADPH intracelulares, o qual participa na redução da glutationa oxidada (GSSG) em glutationa reduzida (GSH), um importante peptídeo protegendo as células contra os danos oxidativos.

A avaliação do *status* antioxidante dos organismos, bem como o grau de lesões oxidativas, podem indicar potenciais efeitos de exposição aos xenobióticos e por isso esses biomarcadores têm sido amplamente empregados para avaliação da toxicidade de diversas classes de agrotóxicos em animais aquáticos, incluindo girinos (Zazeri Leite et al., 2010; Lajmanovich et al., 2011; Stefani Margarido et al., 2013). Da mesma forma, análises de enzimas de biotransformação, tais como as enzimas de fase II glutationa-*S*-transferases (GST), as quais podem designar o grau de atividade de detoxificação do organismo contra compostos exógenos potencialmente tóxicos, são recomendadas como sensíveis biomarcadores de contaminação ambiental (Van der Oost et al., 2003). Uma vez envolvidas na conjugação de xenobióticos a constituintes endógenos, tais como a glutationa reduzida (GSH), as GST também agem diretamente no estado redox das células contribuindo no processo contra danos oxidativos, uma vez que esses conjugados têm geralmente uma toxicidade reduzida e são mais facilmente excretados do organismo (Fig. 2).

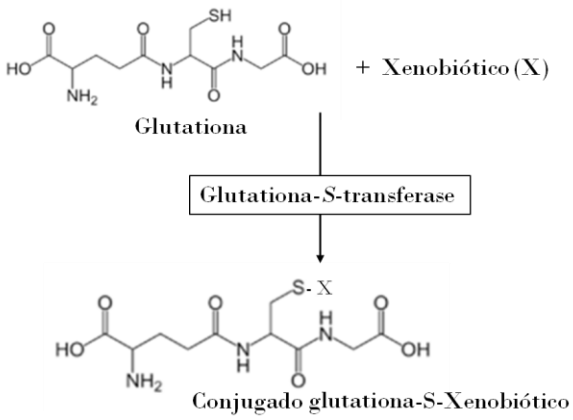

Fig. 2: Ação de enzimas da família glutationa-*S*-transferase (GST), as quais agem na fase II no processo de detoxificação, o qual envolve a conjugação de metabólitos produzidos durante a fase I com compostos endógenos (glutationa), reduzindo então a probabilidade desses compostos se ligarem a outras macromoléculas.

Além das enzimas antioxidantes, outras enzimas denominadas esterases, tais como acetilcolinesterases (AChE) e carboxilesterases (CbE), também são importantes biomarcadores utilizadas em estudos de avaliação à exposição aos agrotóxicos. AChE é uma importante enzima que atua na hidrólise da acetilcolina, um éster liberado na fenda sináptica quando o impulso nervoso é transmitido de uma célula para outra (Bainy et al. 2006) (Fig. 3). Quando AChE é inibida, a acetilcolina é consequentemente acumulada, levando a alterações na função nervosa que pode ser letal para o organismo (Peakall, 1996). As CbE, por sua vez, catalisam a liberação de ácidos graxos de cadeia curta endógenos ou exógenos, os quais estão envolvidos no metabolismo dos xenobióticos, auxiliando assim sua detoxificação (Maxwell 1992; Hyne e Maher 2003). As atividades das CbE também são mencionadas por estarem envolvida em mecanismos de resistência e defesa contra intoxicação por organofosforados (Wheelock et al. 2008). Alguns agrotóxicos, especialmente organofosforados e carbamatos são tipicamente conhecidos por inibir a atividade da AChE (ex. van der Oost et al. 2003; Zazeri Leite et al., 2010). Entretanto, outros tipos de praguicidas surfactantes e inclusive metais, têm sido relatados como elementos que podem alterar a atividade de ambas AChE e CbE em animais aquáticos, como os anfíbios (Bainy et al., 2006; Attademo et al., 2007; Lajmanovich et al., 2010; Modesto e Martinez et al., 2010; Zazeri Leite et al., 2010).

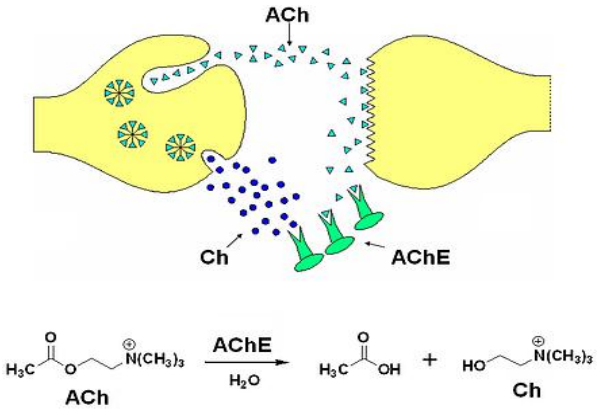

Fig. 3: Transmissão neuronal da acetilcolina e sua hidrólise pela acetilcolinesterase (AChE). Fonte: Faculdade de Farmácia, Universidade do Porto - http://tabunga.wixsite.com/tabun/alvo-e-mecanismo-de-acao.

Dessa forma, a determinação de parâmetros de estresse oxidativo, enzimas de biotransformação e atividade das esterases são considerados bons indicadores para a avaliação de estresse fisiológico as quais os animais estão submetidos quando expostos a poluentes ambientais, tais como os herbicidas (Modesto e Martinez, 2010; Zazeri Leite et al., 2010; Stefani Margarido et al., 2013). Porém, é sabido que esses parâmetros são significativamente alterados em função de variações na temperatura do ambiente em animais aquáticos, podendo ser, portanto, consideradas ferramentas relevantes para a investigação das relações entre os efeitos dos herbicidas sob diferentes condições térmicas (Lushchak e Bagnyukova, 2006; Hooper et al., 2013; Moe et al., 2013).

Indicadores moleculares (DNA, RNA e proteínas) também são ferramentas interessantes para estudos em ecotoxicologia porque eles podem identificar a presença de poluentes em concentrações muito baixas, induzindo diferentes tipos de efeitos metabólicos e subletais em espécies alvo, antes que eles causem danos claramente visíveis em consequência de sua acumulação. Os efeitos subletais podem ser identificados, por exemplo, na ativação, inativação, superexpressão de genes ou na interferência de diferentes rotas metabólicas em resposta à presença dos xenobióticos (Quintero e Zafra, 2016). Assim, alterações específicas na expressão de genes elegidos podem ser mensuradas após o período de estresse submetido, indicando possíveis alterações fisiológicas como consequência da mudança na atividade celular modulada por tais genes. Adicionalmente, as alterações na expressão de genes de relevante interesse podem servir de alerta precoce em estudos de avaliação de risco em espécies nativas das quais não se tem informações disponíveis.

Numerosos genes-alvo são utilizados para identificação de respostas desencadeadas por diferentes poluentes. Uma das categorias inclui genes associados às respostas endócrinas (Quintero e Zafra, 2016). Por exemplo, a indução na expressão da vitelogenina, uma glicoproteína que age como precursor na

formação de vitelo, em machos juvenis é útil para estimar a exposição a compostos estrogênicos e podem agir como alarme precoce da exposição desses animais às substancias anti-androgênicas em ambientes aquáticos (Fort et al., 2015). Em anfíbios, a avaliação de genes relacionados à atividade da tireoide, tais como os receptores nucleares *trα* e *trβ* e a atividade das enzimas deiodinases (*dio1, dio2* e *dio3*), também são altamente eficientes na identificação de poluentes desreguladores da glândula, os quais podem levar a consequências como alterações das produções dos hormônios tireoidianos e no tempo de metamorfose (Zoeller, 2007; Tietge et al., 2013). Além dos poluentes ambientais, estudos recentes têm também mostrado que a expressão desses e outros genes relacionados ao processo de metamorfose em anuros é fortemente influenciada por variações nas temperaturas ambientais (Mochizuki et al., 2012; Hammond et al., 2015). A figura 4 representa como a alteração na expressão de genes associados à tireoide pode gerar uma resposta celular especifica alterada.

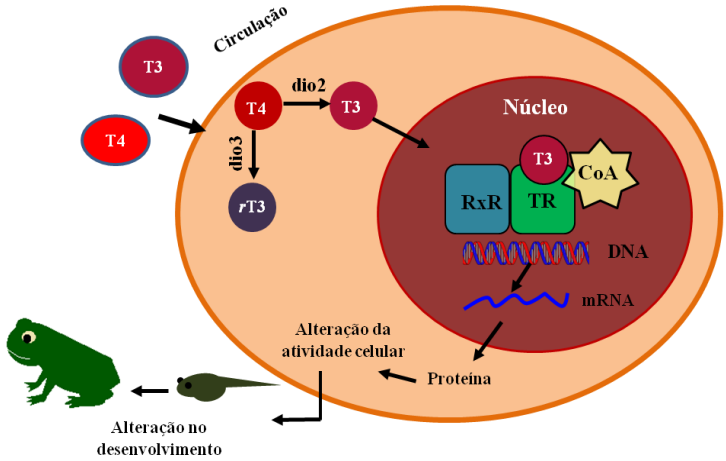

Fig. 4: Figura representando a ação das enzimas deiodinases e dos receptores da tireoide durante a atividade celular. Os hormônios triiodotironina (T_3) e tiroxina (T_4) são incorporados para o interior da célula por meio de transportadores. As concentrações plasmáticas de T_3 são reguladas principalmente pelas deiodinases II (dio2) e III (dio3). A dio2 gera a forma ativa do hormônio da tireoide, o T_3, via deiodinação do T_4. Em contraste, a dio3 inativa o T_3 (rT_3), impedindo que o T_4 seja ativado. A atividade das deiodinases pode, portanto, alterar substancialmente a sinalização hormonal da tireoide em uma célula. Em presença da forma ativa do hormônio, os receptores da tireoide (TR) formam um heterodímero com um receptor retinóide (RxR), o qual se liga especificamente ao T_3. A mudança conformacional faz com que o complexo formado pelo TR recrute proteínas Co-ativadoras (Co-A), o qual pode ser uma RNA polimerase, que irá ativar a transcrição de genes associados, alterando então a atividade celular. Assim, alterações na expressão das deiodinases ou dos receptores da tireoide resultam em uma atividade celular alterada, a qual é refletida morfologicamente no desenvolvimento dos organismos.

OBJETIVOS

2. OBJETIVOS

2.1. Objetivo – Projeto desenvolvido no Brasil

Esse trabalho teve como objetivo geral avaliar a toxicidade dos herbicidas sulfentrazone (Boral 500SC®) e clomazone (Gamit®) em larvas dos anuros *Eupemphix nattereri* (Leiuperidae) e *Rhinella schneideri* (Bufonidae) em diferentes gradientes de temperatura, a fim de identificar os riscos associados à utilização de herbicidas em condições realísticas de regiões neotropicais, onde a temperatura é considerada um importante estressor ambiental.

Objetivos específicos:

Em diferentes temperaturas, foram avaliados:

- A capacidade dos herbicidas em alterar o sistema de defesa antioxidante de girinos, por meio de análises das enzimas antioxidantes: superóxido dismutase (SOD), catalase (CAT), glicose 6-fosfato desidrogenase (G6PDH), bem como níveis de peroxidação lipídica (MDA) e de glutationa total (*t*GSH).
- O efeito dos herbicidas sobre as enzimas de biotransformação de Fase II, utilizando para isso a avaliação da atividade da glutationa S-transferase (GST).
- O efeito dos herbicidas na atividade enzimática das esterases, acetilcolinesterase (AChE) e carboxilesterase (CbE).
- As taxas de crescimento e desenvolvimento nas espécies propostas após os tratamentos por meio de análises morfológicas.
- Inferir a sensibilidade das diferentes espécies frente à contaminação por herbicidas, por meio de análises comparativas dos biomarcadores enzimáticos e morfológicos estabelecidos.

2.2. Objetivo – Projeto de Pesquisa no Exterior

O objetivo desse trabalho foi avaliar a influência da temperatura na ação do herbicida diuron e seu metabólito de biodegradação 3,4-DCA como desregulador da tireoide e na metamorfose de girinos de *Lithobates catesbeianus.*

Objetivos específicos:

Em diferentes temperaturas, foram avaliados:

- Efeitos no crescimento e desenvolvimento de girinos expostos ao diuron e ao 3,4-DCA.
- Efeitos nas concentrações de hormônios da tireoide, por meio das medidas das concentrações de 3,5,3'-triiodotironina (T_3) plasmática.
- Possíveis alterações na expressão de genes relacionados ao desenvolvimento e metamorfose em girinos: enzimas deiodinases (*dio2, dio3*), receptores da tireoide (*trα, tr*β) e dos fatores de transcrição (*klf9* e *thibz*).
- Inferir sob quais condições ambientais os efeitos tóxicos do diuron e do 3,4-DCA são pronunciados.

METODOLOGIA E RESULTADOS

3. METODOLOGIA E RESULTADOS

Os resultados referentes ao presente estudo estão organizados em 4 capítulos, onde terão suas respectivas metodologias e resultados descritos separadamente de acordo com seus objetivos. Os manuscritos estão em acordo com as normas das revistas selecionadas para posterior submissão ou daquelas em que já foram publicados, em cumprimento com as normas do Programa de Biologia Animal, Instituto de Biociências, Letras e Ciências Exatas, UNESP/IBILCE.

Capítulo 1: "Antioxidant Defense System of Tadpoles (*Eupemphix nattereri*) Exposed to Changes in Temperature and pH."

Os resultados abordados nesse capítulo são referentes aos resultados de um experimento piloto, realizado previamente aos experimentos de exposição aos agroquímicos, onde objetivamos o melhor compreendimento das condições ambientais as quais os girinos das espécies em estudo estariam aptos a sobreviver sob condições experimentais de laboratório. Posteriormente, realizamos análises bioquímicas e obtivemos resultados interessantes relacionados às respostas antioxidantes. Esses resultados foram publicados no periódico *Zoological Science* (doi: http://dx.doi.org/10.2108/zs150075).

Capítulo 2: "Influence of temperature on the antioxidant responses and lipid peroxidation of two species of tadpoles (*Rhinella schneideri* and *Eupemphix nattereri*) exposed to the herbicide sulfentrazone (Boral 500SC®)"

Capítulo gerado como resultado do projeto de pesquisa "Influência da temperatura na toxicidade dos herbicidas Boral 500SC® e Gamit® em larvas de anuros *Eupemphix nattereri* (Leiuperidae) e *Rhinella schneideri* (Bufonidae)" (FAPESP 2013/16948-3). Este trabalho será submetido ao periódico *Comparative Biochemistry and Phisiology - Part C: Toxicology & Pharmacology* (ISSN: 1532-0456).

Capítulo 3: "Combined effects of temperature and clomazone (Gamit®) on biochemical stress responses and B-esterase activity of tadpoles from *Eupemphix nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae)"

Capítulo gerado como resultado do projeto de pesquisa "Influência da temperatura na toxicidade dos herbicidas Boral 500SC® e Gamit® em larvas de anuros *Eupemphix nattereri* (Leiuperidae) e *Rhinella schneideri* (Bufonidae)" (FAPESP 2013/16948-3). Este trabalho será submetido ao periódico *Environmental Science and Pollution Research* (ISSN 1614-7499).

Capítulo 4: "Influence of Temperature on the Thyroidogenic Effects of Diuron and Its Metabolite 3,4-DCA in Tadpoles of the American Bullfrog (*Lithobates catesbeianus*)"

Capítulo gerado como resultado do projeto de pesquisa "Disrupting effects of Diuron and metabolite 3,4-DCA (3,4 - dichloroaniline) on tadpoles of *Lithobates catesbeianus* (Anura: Ranidae) exposed to thermal stress", desenvolvido durante o período de "Doutorado Sanduíche" (FAPESP 2015/03545-3) em parceria com a Universidade da Califórnia, Riverside – UCR, sob supervisão do Prof. Dr. Daniel Schlenk. Os resultados dessa pesquisa foram publicados no periódico *Environmental Science and Technology* (doi: 10.1021/acs.est.6b04076).

REFERÊNCIAS BIBLIOGRÁFICAS

4. Referências Bibliográficas

Alford, R.A.; Dixon, P.M.; Pechmann, J.H.K. 2001. Global amphibian population declines. *Nature* 412: 499-500.

Akhtar, N.; Kayani, S.A.; Ahmad, M.M.; Shahab, M. 1996. Insecticide-induced changes in secretory activity of the thyroid gland in rats. *J Appl Toxicol* 16: 397-400.

Almeida, E.A; Bainy, A.C.D.; Dafre, A.L.; Gomes, O.F. 2005. Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. *J Exp Mar Biol Ecol* 318: (21): 30.

Andersen, H.R.; Cook, S.J.; Waldbillig, D. 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*. *Toxicol Appl Pharmacol* 179: 1-12.

Anvisa; UFPR. 2012. Seminário de mercado de agrotóxico e regulação. Brasília: ANVISA. Acesso em: 11 de abril de 2012.

Armas, E.D; Monteiro, R.T.R.; Amâncio, A.V.; Correa, R.M.L.; Guercio, M.A. 2005. Uso de agrotóxicos em cana-de-açúcar na bacia do rio Corumbataí e o risco de poluição hídrica. *Quim Nova* 28(6): 975-982.

Associação Brasileira de Saúde Coletiva. 2012. Grupo Inter GTs de Diálogos e Convergências da ABRASCO Comissão Executiva do Dossiê Rio de Janeiro, World Nutrition.

Attademo, A.M.; Peltzer, P.M.; Lajmanovich, R.C.; Cabagna, M.; Florenza, G. 2007. Plasma B-esterase and glutathione S-transferase activity in the toad *Chaunus schneideri* (Amphibia, Anura) inhabiting rice agroecosystems of Argentina. *Ecotoxicology* 16:533–539.

Attaway, H.H.; Camper, N.D.; Paynter, M.J.B. 1982. Anaerobic microbial degradation of diuron by pond sediment. *Pest Biochem Physiol* 17: 96–101.

Azania, C.A.M.; Azania, A.A.P.M.; Pizzo, I.V.; Schiavetto, A.R.; Zera, F.S.; Marcari, M.A.; Santos, J.L. 2009. Manejo químico de Convolvulaceae e Euphorbiaceae em cana-de-açúcar em período de estiagem. *Planta Daninha* 27(4): 841-848.

Bainy, A.C.D.; Medeiros, M.H.G.; Mascio, P.D.; Almeida, E.A. 2006. In vivo effects of metals on the acetylcholinesterase activity of the *Perna perna* mussel's digestive gland. *Biotemas* 19: 35-39.

Bao, V.W.W.; Koutsaftis, A.; Leung, K.M.Y. 2008. Temperature-dependent toxicities of chlorothalonil and copper pyrithione to the marine copepod *Tigriopus japonicus* and *Dinoflagellate pyrocystis lunula*. *Aust J Ecotox* 14:45-54.

Bickford, D.; Howard, S. D.; Ng, D. J. J.; Sheridan, J. A. 2010. Impacts of climate change on the amphibians and reptiles of Southeast *Asia Biodivers Conserv* 19 (4): 1043−1062.

Blanco, F.M.G.; Velini, E.D.; Batista-Filho, A. 2010. Persistência do herbicida sulfentrazone em solo cultivado com cana-de-açúcar. Bragantia, Campinas 69 (1): 71-75.

Blaustein, A.R.; Hoffman, P.D.; Hokit, D.G.; Kiesecker, J.M.; Walls, S.C.; Hays, J.B. 1994. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? Proceedings of the National Academy of Sciences of the USA, 91: 1791-1795.

Blaustein, A.R.; Han, B.A.; Relyea, R.A.; Johnson, P.T.J.; Julia C. Buck, J.C.; Gervasi,S.S.; Kats, L.B. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Ecol Conserv Biol* 1223:108-119.

Blaustein, A.R.; Kiesecker, J.M. 2002. Complexity in conservation: Lessons from the global decline of amphibian populations. *Ecology Letters* 5:597-608.

Boxall, A.B.A.; Hardy, A.; Beulke, S.; Boucard, T.; Burgin, L.; Falloon, P.D. et al. 2009. Impacts of Climate Change on Indirect Human Exposure to Pathogens and Chemicals from Agriculture. *Environ Health Perspect* 17:508-514.

Brande-Lavridsen, N.; Christensen-Dalsgaard, J.; Korsgaard, B. 2010. Effects of Ethinylestradiol and the Fungicide Prochloraz on Metamorphosis and Thyroid Gland Morphology in *Rana temporaria*. *Open Zool J* 3: 7-16.

Broomhall, S. 2002. The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability to predation in *Litoria citropa* tadpoles. *Aquat Toxicol* 61(3–4):243–50.

Broomhall, S.D. 2004. Egg temperature modifies predator avoidance and the effects of the insecticide endosulfan on tadpoles of an Australian frog. *J Appl Ecol* 41(1): 105–13.

Cardone, A.; Comitato, R.; Angelini, F. 2008. Spermatogenesis, epididymis morphology and plasma sex steroid secretion in the male lizard *Podarcis sicula* exposed to diuron. *Environ Res* 108: 214–223.

Cocco, P. On the rumors about the silent spring. Review of the scientific evidence linking occupational and environmental pesticide exposure to endocrine disruption health effects. *Cad Saúde Pública* 18: 379-402.

Colby, S.R.; Lym, R.G.; Hill, E.R.; Mc Avoy, W.J.; Kitchen, L.M.; Prasad, R. 1989. Herbicide Handbook of the Weed Science Society of America. 6th ed. *Illinois.*

Cooke, A. S. 1972. The effect of DDT, dieldrin and 1,4-D on amphibian spawn and tadpoles. *Environ Pollut* 3: 51-68.

Davidson, C.; Knapp, K.A. 2007. Multiple stressors and amphibian declines: dual impacts of pesticides and fish on yellow-legged frogs. *Ecol Appl* 17:587-597.

Diamanti-Kandarakis, E.; Bourguignon, J.P.; Giudice, L.C.; Hauser, R.; Prins, G.S.; Soto, A.M.; Zoeller, R.T.; Gore, A.C. 2009. Endocrine-disrupting chemicals; An Endocrine Society scientific statement. *Endocr Rev* 30: 293-342.

Davies, K.J.A.; Lin, S.W.; Pacifici, R.E. 1987. Protein damage and degradation by oxygen radicals. IV. Degradation of denatured protein. *J Biol Chem* 262: 9914-9920.

Devillers, J.; Exbraya, J. M. 1992, Ecotoxicity of chemicals to amphibians. In Handbook of *Ecotox Data* 1: 351.

Denver, R.J.; Mirhadi, N.; Phillips, M. 1998. Adaptive plasticity in amphibian metamorphosis: Response of *Scaphiopus hammondii* tadpoles to habitat desiccation. *Ecology* 79: 1859–1872.

Ellis, P.A.; Camper, N.D. 1982. Aerobic degradation of diuron by aquatic microorganisms. *J Environ Sci Health B* 17: 277–289.

Embrapa. 2013. Empresa Brasileira de Pesquisa Agropecuária. Plantas Daninhas. Disponível em: http://www.agencia.cnptia.embrapa.br/gestor/cana-de-acucar/arvore/CONTAG01_52_711200516718.

Fontenot, C.L.; Lutterschmidt, W.I. 2011. Thermal selection and temperature preference of the aquatic salamander, *Amphiuma tridactylum. Herp Conserv Biol* 6(3):395–399.

Fort, D.,J.; Mathis, M.; Fort, C.E.; Fort, H.M.; Bacon, J.P. 2015. Application of endocrine disruptor screening program fish short-term reproduction assay: Reproduction and endocrine function in fathead minnow (*Pimephales promelas*) and killifish (*Fundulus heteroclitus*) exposed to Bermuda pond sediment. *Environ Toxicol Chem* 34: 1283-1295.

Freitas, J.S.; Kupsco, A.J.; Diamante, G.; Felicio, A.A.; Alves de Almeida, E.; Schlenk, D. 2016. Influence of temperature on the thyroidogenic effects of Diuron and its metabolite 3,4-DCA in tadpoles of the American bullfrog (*Lithobates catesbeianus*). *Environ Sci Technol* acs.est.6b04076. doi:10.1021/acs.est.6b04076

Fridovich, I. 1986. Superoxide dismutases. Advances in Enzymology and Related Areas of Molecular Biology 58:61–97.

Fryday, S.; Thompson, H. 2012. Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. EFSA Supporting Publications: EN-343-348.

Gatten, R.E.; Miller, K.; Full, R.J. 1992. Energetics at rest and during locomotion. In: Feder, M.E., Burggren, W.W. (Eds.), Environmental Physiology of the Amphibians. University of Chicago Press, Chicago, pp. 314–377.

Giacomazzi, S.; Cochet, N. 2004. Environmental impact of diuron transformation: a review. *Chemosphere* 56: 1021–1032.

Gordon, C.J. 2003. Role of environmental stress in the physiological response to chemical toxicants. *Environ Res* 92: 1–7.

Goto, Y.; Kitamura, S.; Kashiwagi, K.; Oofusa, K.; Tooi, O.; Yoshizato, K.; Sato, J.; Ohta, S.; Kashiwagi, A. 2006. Suppression of amphibian metamorphosis by bisphenol A and related chemical substances. *J Health Sci* 52: 160–168.

Grim, J.M.; Miles, D.R.B.; Crockett, E.L. 2010. Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. *J Exp Biol* 213: 445–452.

Hess, D.F. 1993. Herbicide effects on plant structure, physiology, and biochemistry. In: Altman, J. (Ed.) Pesticide Interactions in Crop Production. London: CRC Press p. 13-34.

Hammond, S. A.; Veldhoen, N.; Helbing, C. C. 2015. Influence of temperature on thyroid hormone signaling and endocrine disruptor action in *Rana (Lithobates) catesbeiana* tadpoles. *Gen Comp Endocrinol* 219: 6−15.

Harley, C.D.G.; Hughes, A.R.; Hultgren, K.M.; Miner, B.G.; Sorte, C.J.B.; Thornber, C.S.; Rodriguez, L.F.; Tomanek, L.; Williams, S.L. 2006. The impacts of climate change in coastal marine systems. *Ecol Lett* 9 (2): 228−241.

Hayes, T.B.; Falso P.; Gallipeau S.; Stice, M. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. *J Exp Biol* 213: 921-933.

Hooper, M.J.; Ankley, G.T.; Cristol, D.A.; Maryoung, L.A.; Noyes, P.D.; Pinkerton. K.E. 2013. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. *Environ Toxicol Chem* 32:32–48.

Houlahan, J.E.; Findlay, C.S.; Meyer, A.H.; Kuzmin, S.L.; Schmidt, B.R. 2001. Global amphibian population declines. *Nature* 412, 499: 500.

Hyne, R.V.; Maher, W.A. 2003. Invertebrate biomarkers: links to toxicosis that predict population decline. *Ecotoxicol Environ Saf* 54: 366–374.

Imlay, J.A.; Linn, S. 1988. DNA damage and oxygen radical toxicity. *Science* 240:1302–1309.

Johansson, M.; Piha, H.; Kylin, H.; Merila, J. 2006. Toxicology of six pewsticides to common frog (*Rana temporaria*) Tadpoles. *Environ Toxicol Chem* 12 (25): 3164 – 3170.

Jones, L.; Gossett, D.R.; Banks, S.W.; Mccallum, M.L. 2010. Antioxidant Defense System in Tadpoles of the American Bullfrog (*Lithobates catesbeianus*) Exposed to Paraquat. *J Herpet* 44(2): 222–228.

Katzenberger, M.; Tejedo, M.; Duarte, H.; Marangoni, F.; Francisco Beltrán, J.F. 2012. Tolerância e sensibilidade térmica em anfíbios. *Revista da Biologia* 8: 25-32.

Kissmann, K.G. 1991. Plantas Infestantes e Nocivas. 1. Ed. São Paulo: Basf, 606: 67-94.

Kloas, W.; Lutz, I.; Springer, T.; Krueger, H.; Wolf, J.; Holden, L.; Hosmer, A. 2009. Does Atrazine Influence Larval Development and Sexual Differentiation in *Xenopus laevis*? *Global Change Biol* 17: 657–666.

Lajmanovich, R.C.; Peltzer, P.M.; Junges, C.M.; Attademo, A.M.; Sanchez, L.C.; Basso, A. 2010. Activity levels of B-esterases in the tadpoles of 11 species of frogs in the middle Paraná River

floodplain: Implication for ecological risk assessment of soybean crops. *Ecotoxicol Environ Saf* 73:1517–1524.

Lajmanovich, R.C.; Attademo, A.M.; Peltzer, P.M.; Junges, C.M.; Cabagna, M.C. 2011. Toxicity of Four Herbicide Formulations with Glyphosate on *Rhinella arenarum* (Anura: Bufonidae) Tadpoles: B-esterases and Glutathione S-transferase Inhibitors. *Arch Environ Contam Toxicol* 60:681–689.

Lavado, R.; Thibaut, R.; Raldúa, D.; Martín, R.; Porte, C. 2004. First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicol Appl Pharmacol* 196: 247– 257.

Lefcort, H.; Meguire, R.A.; Wilson, L.H.; Ettinger, W.F. 1998, Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. *Arch Environ Contam and Toxicol* 35: 447-456.

Leghait, J.; Gayrard, V.; Picard-Hagen, N.; Camp, M.; Perdu, E.; Toutain, P.L.; Viguié, C. 2009. Fipronil-induced disruption of thyroid function in rats is mediated by increased total and free thyroxine clearances concomitantly to increased activity of hepatic enzymes. *Toxicology* 255: 38-44.

Lemaire, G.; Mnif, W.; Mauvais, P.; Balaguer, P.; Rahmani, R. 2006. Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. *Life Sci.* 79: 1160-1169.

Londres, F. 2011. Agrotóxicos no Brasil: um guia para ação em defesa da vida. – Rio de Janeiro: AS-PTA – Assessoria e Serviços a Projetos em Agricultura Alternativa. 190 p.

Lushchak, V.I.; Bagnyukova, T.V. 2006. Temperature increase results in oxidative stress in goldfish tissues.1. Indices of oxidative stress. *Comp Biochem Phys* Part C 143: 30–3.

Lushchak, V.I. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicol* 101(1): 13-30.

Manabe, M.; Kanda, S.; Fukunaga, K.; Tsubura, A.; Nishiyama, T. 2006. Evaluation of the estrogenic activities of some pesticides and their combinations using MtT/Se cell proliferation assay. *Int J Hyg Environ Health* 209: 413-421.

Martinez-Alvarez, R.; Morales, A.; Sanz, A. 2005. Antioxidant defences in fish: biotic and abiotic factors. *Rev Fish Biol Fish* 15: 75–88.

Martini, G.; Durigan, J.C. 2004. Influência do teor de água na superfície do solo sobre a eficácia e seletividade do flazasulfuron, na cultura de cana-de-açúcar. *Planta Daninha* 22(2):259-267.

Materna, E.J.; Rabeni, C.F.; Lapoint, T.W. 1995. Effects of the synthetic pyrethroid insecticide, esfenvalerate, on larval leopard frogs (*Rana spp.*). *Environ Toxicol Chem* 14(4): 613–22.

May, R.M. 2010. Ecological science and tomorrow's world. *Philos Trans Royal Soc B* 365: 41–47.

Maxwell, D.M. 1992. Detoxification of organophosphorus compounds by carboxylesterase. In: Chambers JE, Levi PE (eds) Organophosphates: chemistry, fate and effects. Academic Press, San Diego, p 183.

McCoy, K.A.; Bortnick, L.J.; Campbell, C.M.; Hamlin, H.J.; Guillette, J.R.; Mary, C. 2008. Agriculture Alters Gonadal Form and Function in the Toad *Bufo marinus. Environ Health Persp* 116: 1526–1532.

McDiarmid, R.W.; Altig, R. 1999. Tadpoles, the Biology of Anuran Larvae. The University of Chicago Press, Chicago. p. 444.

Melo, C.A.D.; Medeiros, W.N.; Tuffi Santos, L.D.; Ferreira, F.A.; Ferreira, G.L.; Paes, F.A. S.V.; Reis, M.R. 2010. Efeito residual de sulfentrazone, isoxaflutole e oxyfluorfen em três solos. *Planta Daninha* 28(4): 835-842.

Middlebrooks, E.J.; Gaspar, M.J.; Gaspar, R.D.; Reynolds, J.H.; Porcella, D.B. 1973. "Effects of Temperature on the Toxicity to the Aquatic Biota of Waste Discharges - A Compilation of the Literature". *Reports*.

Mnif, W.; HAssine A.I.H.H.; Bouaziz, A.; Bategi, A.; Thomas, O.; Roig, B. 2011. Effect of Endocrine Disruptor Pesticides: A Review. *Int J Environ Res Public Health* 8: 2265-2303.

Mochizuki, K.; Goda, T.; Yamauchi, K. 2012. Gene expression profile in the liver of Rana catesbeiana tadpoles exposed to low temperature in the presence of thyroid hormone. *Biochem Biophys Res Commun* 420 (4): 845−850.

Modesto, K.A.; Martinez, C.B.R. 2010. Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus. Chemosphere* 78: 294–299.

Moe, S.J.; Schamphelaere, K.; Clements, W.H.; Sorensen, M. T.; Van Den Brink, P.J.; Liess, M. 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. *Environ Toxicol Chem* 32(1): 49–61.

Monquero, P.A.; Da Silva, P.V.; Ortiz, F.G.; Orzari, I.; Oliveria, C. T. 2010. Lixiviação e persistência dos herbicidas sulfentrazone e Imazapic. *Planta Daninha* 28(1): 185-195.

Morand, A.; Joly, P.; Grolet, O. 1997. Phenotypic variation in metamorphosis in five anuran species along a gradient of stream influence. *C R Acad Sci Ser* III 320 (8): 645−652.

Morin, S.; Bottin, M.; Mazzella, N.; Macary, F.; Delmas, F.; Winterton, P.; Coste, M. 2009. Linking diatom community structure to pesticide input as evaluated through a spatial contamination potential (Phytopixal): A case study in the Neste river system (South-West France). *Aquat Toxicol* 94: 28–39.

Myers, N.; Mittermeier, R.A.; Mittermeier, C.G.; da Fonseca G.A.B.; Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

Miyata, K.; Keiko, O. 2012. Thyroid Hormone-disrupting Effects and the Amphibian Metamorphosis Assay. *J Toxicol Pathol* 25: 1–9.

Nebeker, A.V.; Schuytema, G.S. 1998. Chronic effects of the herbicide diuron on freshwater cladocerans, amphipods, midges, minnows, worms, and snails. *Arch Environ Contam Toxicol* 35: 441–446.

Noyes, P.D.; McElwee, M.K.; Miller, H.D.; Clark, B.W.; Van Tiem, L.A.; Walcott, K.C.; Erwin, K.N.; Levin, E.D. 2009. The toxicology of climate change: Environmental contaminants in a warming world. *Environ Int* 35: 971–986.

Ouellet, M.;.Bonin, J.H.; Rodrigues, J.; DeGranges, J.; Lair, S. 1997. Hindlimb deformities (Ectromelia, Ectrodactyly) in free-living anurans from agricultural habitats. *J Wildl Dis* 33: 95-104.

Okamura, H.; Aoyama, I.; Ono, Y.; Nishida, T. 2003. Antifouling herbicides in the coastal waters of western Japan. *Mar Pollut Bull* 47: 59–67.

Peakall, D.B. 1996. Disrupted patterns of behavior in natural populations as an index of ecotoxicity. *Environ Health Perspect* 104(2): 331–335.

Peltzer, P.M.; Junges, C.M.; Attademo, A.M.; Basso, A.; Grenon, P.; Lajmanovich, R.C. 2013. Cholinesterase activities and behavioral changes in *Hypsiboas pulchellus* (Anura: Hylidae) tadpoles exposed to glufosinate ammonium herbicide. *Ecotoxicology* 22:1165–1173.

Peltzer, P.M.; Lajmanovich, R.C.; Attademo, A.M. et al. 2013. Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (*Trachycephalus typhonius*) tadpoles. *Ecotoxicol Environ Saf* 98:142–151.

Pereira, T. S. B.; Boscolo, C. N. P.; Silva, D. G. H.; da Batlouni, S. R.; Schlenk, D.; Almeida, E. A. 2015. Anti-androgenic activities of diuron and its metabolites in male Nile tilapia (*Oreochromis niloticus*). *Aquat Toxicol* 164: 10115.

Pereira, T.S.B.; Boscolo, C.N.P.; Felizieo, A.A.; Batlouni, S.R.; Schlenk, D.; Almeida, E.A. 2016. Estrogenic activities of diuron metabolites in female Nile tilapia (*Oreochromis niloticus*). *Chemosphere* 146: 497-502.

Perry, R. I. Potential impacts of climate change on marine wild capture fisheries: an update. *J Agric Sci* 2011, 149: 63−75.

Provete, D.C.; Garey, M.V.; Silva, F.R.; Rossa-Feres, D.C., 2011. Anuranfauna from Northwestern region of the state of São Paulo: species list and taxonomic key for adults. *Biota Neotropica* 11: 377-391.

Phillips, P.; Harrison, P. 1999. Overview of endocrine disrupters issue. Issues in environmental science and technology. Endocrine disrupting chemicals. The Royal Sciety of Chemistry.

Quintero, W.V.; Zafra, G. 2016. Use of Molecular Biomarkers in Studies Of Aquatic Environmental Impact. *Journ Indust Poll Cont* ISSN: 0970-2083.

Raffel, T.; Rohr, J.; Kiesecker, J.; Hudson, P. 2006. Negative effects of changing temperature on amphibian immunity under field conditions. *Funct Ecol* 20: 819-828.

Relyea, R.A.; Jones, D.K.. 2009. The toxicity of Roundup original Maxh to 13 species of larval amphibians. *Environ Toxicol Chem* 28(9): 2004–2008.

Rijnsdorp, A. D.; Peck, M.A.; Engelhard, G. H.; Mollmann, C.; Pinnegar, J. K. 2009. Resolving the effect of climate change on fish populations. ICES *J Mar Sci* 66 (7): 1570−1583.

Rodrigues, N.B.; Almeida, F.S. 2005. Guia de herbicidas. 5.ed. Londrina. 592 p.

Roelants, K.; Gower, D.J.; Wilkinson, M.; Loader, S.P.; Biju, S.D.; Guillaume, K.; Moriau, L.; Bossuyt, F. 2007. Global patterns of diversification in the history of modern amphibians. *Proc Nat Acad Sci* USA 104: 887-892.

Rohr, J.R.; Palmer, B.D. 2005. Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. *Environ Toxicol Chem* 24:1253-1258.

Rohr, J.R.; Timothy M.; Terhenn, S.E.; Stieha, T. 2010. Will climate change reduce the effects of a pesticide on amphibians?: partitioning effects on exposure and susceptibility to contaminants. *Global Change Biol* 17: 657–666.

Rome, L.C.; Stevens, E.D.; John-Alder, H.B., 1992. The influence of temperature and thermal acclimation on physiological function. In: Feder, M.E., Burggren, W.W. (Eds.), *Environmental physiology of the amphibians.* University of Chicago Press, Chicago.

Semlitsch, R.D.; Bridges, C.M.; Welch, A.M. 2000. Genetic variation and a fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl. *Oecologia* 125:179–185.

Sharma, B.; Patiño, R. 2008. Exposure of *Xenopus laevis* Tadpoles to Cadmium Reveals Concentration-dependent Bimodal Effects on Growth and Monotonic Effects on Development and Thyroid Gland Activity. *Toxicol Sci* 105(1): 51–58.

Schiedek, D.; Sundelin, B.; Readman, J.W.; Macdonald, R.W. 2007. Interactions between climate change and contaminants. *Mar Pollut Bull* 54: 1845–1856.

Schuler, L.J.; Rand, G.M. 2008. Aquatic risk assessment of herbicides in freshwater ecosystems of South Florida. *Arch Environ Contam Toxicol 54: 571–583.*

Schuytema, G.S.; Nebeker, A.V. 1998. Comparative Toxicity of Diuron on Survival and Growth of Pacific Treefrog, Bullfrog, Red-Legged Frog, and African Clawed Frog Embryos and Tadpoles. *Arch Environ Contam Toxicol* 34: 370–376.

Soto, A.M.; Chung, K.L.; Sonnenschein, C. 1994. The pesticides endosulfan; toxaphene; and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ Health Perspect* 102*:* 380-383.

Sparling, D.W.; Fellers, G.M.; McConnell, L.S. 2001. Pesticides and amphibian population declines in California, USA. *Environ Toxicol Chem* 20:1591– 1595.

Stefani Margarido, T.C.; Felício, A.A.; Rossa-Feres, D.C., Almeida, E.A. 2013. Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. *Mar Environ Res* p.1-7.

Stuart, S.N.; Chanson, J.S.; Cox, N.A.; Young, B.E.; Rodrigues, A.S.L.; Fischman, D.L; Waller R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1783–1786.

Sugiyama, S.; Shimada, N.; Miyoshi, H.; Yamauchi, K. 2005. Detection of thyroid system disrupting chemicals using *in vitro* and in vivo screening assays in *Xenopus laevis. Toxicol Sci* 88: 367-374.

Stork, P.R.; Bennett, F.R.; Bell, M. 2008. The environmental fate of diuron under a conventional production regime in a sugarcane farm during the plant cane phase. *Pest Manag Sci* 64: 954–963.

Tejedo, M. 2003. El declive de los anfibios. La dificultad de separar las variaciones naturales del cambio global. *Munibe Suplement* (16): 20–43.

Tietge, J.E.; Degitz, S.J.; Haselman, J.T.; Butterworth, B.C.; Korte, J.J.; Kosian, P.A.; Lindberg-Livingston, A.J.; Burgess, E.M.; Blackshear, P.E.; Hornung, M.W. 2013. Inhibition of the thyroid hormone pathway in *Xenopus laevis* by 2-mercaptobenzothiazole. *Aquat Toxicol* 126: 128−136.

Todd, B.D.; Bergeron, C.M.; Hepner, M.J.; Hopkins, W.A. 2011. Aquatic and terrestrial stressors in amphibians: A test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. *Environ Toxicol Chem* 30: 2277–2284.

Tomlin, C. 1994. Pesticide Manual. 10. ed. Cambridge: British Crop Protection Council and The Royal Society of Chemistry, 1994. 1341 p.

Van den Berg, F.; Kubiak, R.; Benjey, W.G.; Majewsjki, M.S.; Yates, S.R.; Reeves, G.L.; Smelt, J.H.; Van der Linden, A.M.A. 1999. Emission of pesticides in the air. In: Fate of Pesticides in the Atmosphere, Implications for Environmental Risk Assessment, van Dijk, H.F.G., van Pul, W.A.J., de Voogt, P. (eds.). Kluwer Academic Publishers, Dordrecht/Boston/London. *Water, Air, Soil Pollution* 115: 195-218.

Van Der Oost, R.; Beyer, J.; Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers. in environmental risk assessement: a review. *Environ Toxicol Pharmacol* 13(2): 57149.

Vitousek, P,M,; Mooney, H.A.; Lubchenco, J,; Melillo, J.M. 1997. Human domination of Earth's ecosystems. *Science* 277: 494–499.

Vinggaard, A.M.; Hnida, C.; Breinholt, V.; Larsen, J.C. 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity *in vitro*. *Toxicol In Vitro* 14: 227-234.

Wake, D.B.; Vredenburg, V. T. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *PNAS* 105(1):11467.

Walsh, P.T.; Downie, J.R.; Monaghan, P. 2008. Temperature-mediated morphology changes during metamorphic climax in the African clawed frog, *Xenopus laevis*. *J Therm Biol* 33:244–249.

Wheelock, C.E.; Phillips, B.M.; Anderson, B.S.; Miller, J.L.; Miller, M.J.; Hammock, B.D. 2008. Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). *Rev Environ Contam Toxicol* 195:117–178.

Zanella, R.; Primel, E.G.; Machado, S.L.O.; Gonçalves, F.F.; Marchezan, E. 2002. Monitoring of the herbicide clomazone in environmental water samples by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection. *Chromatographia* 55: 573– 577.

Zazeri Leite, P.; Stefani Margarido, T.C.; Lima, D.; Rossa-Feres, D.C.; Almeida, E.A. 2010. Esterase inhibition in tadpoles of *Scinax fuscovarius (*Anura, Hylidae) as a biomarker for exposure to organophosphate pesticides. *Environ Sci Pollut Res* 17:1411–1421.

Zoeller, R. T. 2007. Environmental chemicals impacting the thyroid: targets and consequences. *Thyroid* 17 (9): 811−817.

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Antioxidant Defense System of Tadpoles (*Eupemphix nattereri*) Exposed to Changes in Temperature and pH

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Amphibians are highly susceptible to environmental changes, mainly at the larval stage during which they are restricted to small and ephemeral aquatic habitats, which are subject to large fluctuations of abiotic parameters, such as temperature and pH. Consequently, tadpoles experience changes in biochemical, physiological, and molecular processes related to the maintenance of homeostasis, which may lead them to an oxidative stress state. In the present study, we investigated the effects of stress caused by changes in temperature and pH on the antioxidant enzymes catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione-S-transferase (GST) in tadpoles of *Eupemphix nattereri*. The results show that changes in temperature and pH conditions induce an antioxidant response in tadpoles. GST and GR showed temperature-dependent activities; GST activity was higher in tadpoles exposed to 28°C, whereas GR exhibited increased activity in response to 28°C and 36°C. At 32°C, both GST and GR had the lowest activity. CAT was induced by treatments with acidic (pH 5.0) and alkaline (pH 8.5) pH. Tadpoles exposed to acidic pH also had increased GR activity. The G6PDH was not changed in either experiment. Our data demonstrate that *E. nattereri* possesses an efficient antioxidant defense system for coping with the damaging effects of heat and acidity/alkalinity conditions in water. The alterations in antioxidant enzymes are probably a result of immediate physiological adaptation of individuals in response to increased production of ROS under environmental stress conditions.

Key words: amphibians, temperature, pH, biomarkers, oxidative stress

INTRODUCTION

Aquatic organisms are constantly exposed to anthropogenic and natural stressors, which may alter the survival and physiological mechanisms of adaptation of the animals. Amphibians are strongly susceptible to environmental changes, mainly at the larval stage when they are restricted to small and ephemeral aquatic habitats, which periodically dry and refill with fresh precipitation. In these areas, fluctuations in abiotic parameters, such as pH and temperature, occur daily as a consequence of changes in the photosynthetic rate of plants, the release of pollutants, and variations in the incidence of sunlight (Pierce, 1993; Robertson-Bryan, 2004; Wang et al., 2002). Thus, these animals may experience changes in biochemical, physiological, and molecular processes related to the maintenance of homeostasis (Madeira et al., 2013). For example, changes in environmental conditions can lead to oxidative stress in organisms by increased production of reactive oxygen species (ROS) (Ahmed, 2005; Halliwell and Gutteridge, 2007).

Reactive oxygen species are physiologically produced in aerobic organisms by cells as a by-product of the metabolism of molecular oxygen, and play an important role in maintaining homeostasis and cellular activity (Cadenas, 1989; Halliwell and Gutteridge, 2007). Approximately 0.1–0.2% of the oxygen consumed by aerobic cells is converted into ROS during normal cell respiration due to the uncompleted reduction of molecular oxygen in the electron transport chain (Fridovich, 2004). However, many environmental stressors can increase the amount of ROS produced by organisms and cause oxidative stress, leading to increases in the levels of oxidative damage to lipids, proteins, DNA and carbohydrates (Halliwell and Gutteridge, 1999; Toyokuni, 1999; Abele and Puntarulo, 2004). To counteract the damage caused by ROS, cells possess antioxidant defense systems (Van Der Oost et al., 2003). In aquatic animals, the antioxidant system is represented by enzymatic antioxidants, such as the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione *S*-transferases (GST), glucose-phosphate-dehydrogenase (G6PDH) (Livingstone, 2001) and glutathione-dependent enzymes (GSH), and non-enzymatic defenses, such as amino acids, ascorbic acid (vitamin C), retinol (vitamin A) and tocopherol (vitamin E) (Martínez-Álvarez et al., 2005; Grim et al., 2010). Enzymatic antioxidants represent a significant fraction of the cellular antioxidant response, and have been widely used as biomarkers in environmental

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monitoring (e.g., Livingstone, 2001; Ahmed, 2005; Ahmad et al., 2008; Velkova-Jordanoska et al., 2008; Vinagre et al., 2012; Madeira et al., 2013).

Alterations in oxidative stress biomarkers have been observed in aquatic organisms exposed to a wide variety of environmental stressors on varying temporal and spatial scales. Changes in temperature, for example, promote an increase in oxidative stress in several aquatic animals, such as fish (Parihar and Dubey, 1995; Lushchak and Bagnyukova, 2006; Bagnyukova et al., 2007a, b; Vinagre et al., 2012; Madeira et al., 2013), amphibians (Bagnyukova et al., 2003), bivalves (Winston et al., 1991; Abele et al., 2002), crustaceans (Wang et al., 2009) and other organisms (Verlecar et al., 2007; Bocchetti et al., 2008). Temperature is known to affect all living organisms, but this parameter critically influences ectotherms, as they depend on environmental temperature to achieve the appropriate physiological status. Furthermore, metabolic processes are stimulated by increased temperature (Lushchak, 2011), leading to an increase in oxygen consumption that can result in increased production of ROS (Lushchak, 2011), despite the decrease in the solubility of oxygen in water as temperature increases. Changes in environmental pH are also known to be potent stressors in aquatic organisms; currently, there is evidence that a change in the acidity/alkalinity of the water can induce oxidative stress in these organisms (Wang et al., 2009; Mai et al., 2010). For example, exposure to pH levels of 9.3 and 5.6 promote oxidative stress in salt water shrimp (*Litopenaeus vannamei*), as detected by increased expression of genes of CAT, GPx (gluthatione peroxidase) and thioredoxin (Wang et al., 2009). Other studies have also shown that exposure of tilapia (*Oreochromis niloticus*) to acute acidity (pH 5.3) induces lipid peroxidation, damages DNA, and promotes apoptosis and cell necrosis (Mai et al., 2010). This occurs because pH affects the ability of organisms to regulate the basic processes that sustain life, especially the exchange of respiratory gases and salts in water (Robertson-Bryan, 2004). These changes may cause an osmotic imbalance and thus an increase in ROS production in these animals (Ellis, 1937; Westfall, 1945; Boyd, 1990).

The indirect impacts of changes in environmental variables on amphibian populations have been addressed by some authors, including deficiencies in the immune system and the development of emerging diseases (Pierce, 1985; Pierce, 1993; Harvell et al., 2002; Kilpatric et al., 2010). In larval stages, these studies have been mainly directed at the comparative study of tolerance among species, as well as the effects on survival and behavior (ex. Pough et al., 1983; Gibbs et al., 2001; Eggert, 2004; Wells, 2007; Blaustein et al., 2010). To date, few studies have clarified the role of antioxidant defense in amphibian larval stages under conditions of environmental stress.

Considering the presented points, this study aimed to investigate the effects of stress caused by changes in temperature and pH on biomarkers of oxidative stress in tadpoles of *Eupemphix nattereri*, through the analysis of the antioxidant enzymes catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione-S-transferase (GST). This species is one of the most frequently observed in the Cerrado region of southeastern and southern Brazil (Provete et al., 2011), and also occurs on the plains of Bolivia and Paraguay. These tadpoles occupy a benthic habitat that occurs primarily in temporary ponds (Rossa-Feres and Nomura, 2006), where abiotic parameters vary daily. We hypothesize that changes in temperature and pH should stimulate the antioxidant defense system of the tadpoles in response to stress conditions due to changes in their metabolic rates. This is the first time to our knowledge that the effects of temperature and pH have been evaluated using antioxidant enzyme biomarkers in a neotropical species of frogs, which have suffered population reduction at much faster rates than the overall average (Alford et al., 2001; Houlahan et al., 2001).

MATERIALS AND METHODS

Test organisms

Spawns of the anuran *Eupemphix nattereri* (Leptodactylidae) were collected in temporary ponds in non-agricultural areas in the region of São José do Rio Preto, São Paulo, Brazil (20°47′07.05″ S, 49°02′42.09″ W) during the rainy period. After hatching, the larvae were maintained in the laboratory under ideal conditions of temperature, pH and oxygen (28°C, pH 7.5–8.0). Tadpoles at development stages 29–30 (Gosner, 1960), which correspond to the stages when the animal has no legs but the limb buds have developed, were selected for the experiments. In this phase of larval development, the relationship between growth and differentiation is positively correlated; the differentiation rate increases after stage 32 (Smith-Gill and Berven, 1979; Caramaschi and Jim, 1983). The animals were collected under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

Tadpole exposure to different temperature and pH

Temperature and pH data were first recorded in five different locations (20°42′25.200″ S, 49°18′39.816″ W; 20°43′8.724″ S, 49°18′41.796″ W; 20°22′07.8″ S, 49°17′53.8″ W; 20°46′15.0″ S, 49°18′34.7″ W; 20°45′06.2″ S, 49°17′53.8″ W) where tadpoles of the target species were found to record the natural conditions. For this, data loggers submerged in the five ponds were configured to record temperatures every 30 minutes for 10 days and a Multiparameter Analyzer Horiba U-10 was used to record the pH. The minimum and maximum temperatures and pH recorded in each pond are summarized in Table 1. The values recorded in the field were used as the basis for laboratory studies, which have pH and temperature conditions controlled.

To test the effects of temperature change, tanks containing 7 L of dechlorinated water were set to three thermal levels, corresponding to 28°C, 32°C and 36°C. The values were established based on average summer temperatures of São José do Rio Preto (average temperature of 27.5°C, reaching a maximum of 35°C; data provided

Table 1. Maximum and minimum values of temperature and pH data recorded in field studies. Data were recorded in five different ponds where *E. nattereri* tadpoles were registered. Temperature values were taken every 30 minutes by data loggers submerged in water for 10 days. pH data was taken in each pond using Multiparameter Analyzer Horiba U-10.

Field record	Temperature (°C)		pH	
	Minimum	Maximum	Minimum	Maximum
Pond I	22.81	42.16	6.87	9.39
Pond II	23.29	38.89	7.63	9.87
Pond III	23.77	41.34	6.68	9.28
Pond IV	23.48	36.40	5.75	7.44
Pond V	23.67	35.97	7.44	8.43

by the Brazilian Institute of Geography and Statistics/IBGE and confirmed by field studies (Table 1)), which correspond to the period of occurrence of most frogs in the region. Differences in temperature between different groups were also adjusted according to the current changes recorded by the Intergovernmental Panel on Climate Change (increases from 1.1 to 4.6°C). The temperatures of the tanks were controlled by thermostats coupled to regulating thermometers. Each experimental group consisted of three replicates with six tadpoles in each tank (n = 18). Initially, the tadpoles were placed in aquariums and their temperatures adjusted gradually (during the first day) to avoid the influence of thermal shock on the physiology of the animals. The temperature of each tank was recorded three times daily, and the temperature varied by a maximum of 0.5°C. The tanks were kept under constant aeration and were maintained under the experimental conditions for 7 days, at which point the animals were collected for biochemical analyses.

For the experiments with different pH, tanks containing 7 L of dechlorinated water were set to three pH levels: 5.0; 7.0; and 8.5. In this experiment, the tanks were maintained under constant temperature (27.5°C) and aeration. The pH was then raised to 8.5 by the gradual addition of 1 mol/L of sodium chloride HCl, and reduced to 5.0 by adding 1 mol/L HCl. The pH was adjusted and monitored daily using a pH electrode attached to a pH meter (Oaklon® ISO 9001). Each experimental group consisted of three replicates with six tadpoles in each tank (n = 18) and were maintained for seven days, at which point the animals were collected for biochemical analyses (Table 2).

The water was not changed during the experimental period; however excrement and food scraps were removed every two days, when the tadpoles were fed. The values of ammonia and oxygen dissolved in the water were measured at the beginning and end of the experiment, and they were not significantly different. After both experiments, tadpoles were collected and euthanized in liquid nitrogen and subsequently stored in a freezer at –80°C. The stage of development, weight and head–tail length of all animals were recorded. This work was approved by the Ethics Committee on Animal Use in Research of the São Paulo State University (CEUA-IBILCE/UNESP No. 086/2013).

Preparation of samples

For the enzymatic measurements, the tadpoles were entirely and individually homogenized in a ratio of 1/4 (weight/volume) in a cold (4°C) 20 mM Tris-HCl homogenization buffer (TrisHCl 20 mM, EDTA 1 mM, DL-dithiothreitol (DTT) 1 mM, sucrose 0.5 M, KCL 0.15 M, phenylmethylsulfonyl fluoride (PMSF) 1 mM), pH 7.4, and centrifuged for 30 min at 9168 g and 4°C. The supernatant fraction was collected and centrifuged again for 1 h at 50,000 g. The second supernatant fraction was collected and stored at –80°C for subsequent evaluations.

Table 2. Experimental design, indicating numbers of tadpoles (*Eupemphix nattereri*) used in the treatments.

Variable	Treatment	Number of replicates	*N* tadpoles/ replicate	*N* tadpoles/ treatment
pH	5.0	3	6	18
	7.0	3	6	18
	8.5	3	6	18
				Total: 54
Temperature	28°C	3	6	18
	32°C	3	6	18
	36°C	3	6	18
				Total: 54

Biochemical analyzes

The analysis of CAT was performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes. The activities of GR, GST and G6PDH were performed on a Victor TM X3 microplate reader (Perkin Elmer®). The activity of all enzymes was evaluated at the respective temperatures (28°C, 32°C and 36°C) for the treatments with thermal stress. For experiments with different pH levels, enzymatic analyses were performed at a constant temperature (27°C).

CAT activity was measured using the method described by Beutler (1975), which monitors the rate of decomposition of hydrogen peroxide by the enzyme at 240 nm for 1 min. Specific activity was expressed as U mg of protein^{-1} using a molar extinction coefficient of 0.071 mM^{-1} cm^{-1}. The assays were performed using Tris-HCl buffer (1 M, pH 8.0) with 5 mM EDTA and 10 mM H_2O_2 as the substrate.

GST activity was measured using the method described by Keen et al. (1976), which monitors the formation of the conjugate of 1-chloride-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) catalyzed by GST in the sample for 1 min at 340 nm. Specific activity was expressed as U.mg of protein^{-1} using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. The final volume of the assay was 110 µL, which contained a potassium phosphate buffer (0.2 M, pH 6.5), 1 mM CDNB (dissolved in 1.0 mL of absolute ethanol), 1 mM GSH and the sample.

GR activity was measured based on the Carlberg and Mannervik (1985) methodology, in which the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) is monitored for 2 min at 340 nm in the presence of the substrate glutathione disulfide (GSSG). This method involves the reduction of GSSG to GSH by GR through the oxidation of NADPH. The final volume of the assay was 100 µL, which contained a potassium phosphate buffer (0.1 M, pH7.5) with 2 mM GSSG (dissolved in buffer), 0.1 mM NADPH (dissolved in NaHCO3 0.1% (v/v)), 0.15mM GSH and the sample. Spe-

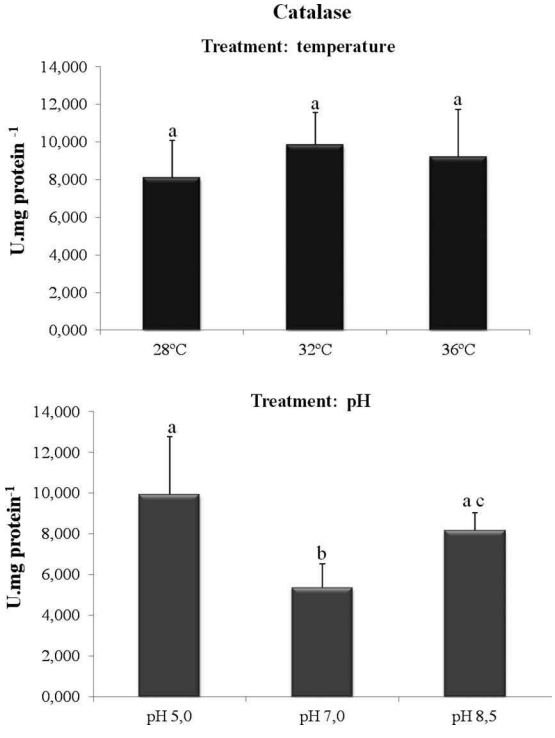

Fig. 1. Catalase activity in tadpoles of *Eupemphix nattereri* exposed to 28°C, 32°C and 36°C (first graph) and pH 5.0, 7.0 and 8.5 (second graph). Different letters indicate significant differences between the groups (P < 0.05).

cific activity was expressed as U.mg of protein^{-1} using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

The enzymatic assay of G6PDH (EC 1.1.1.49) was based on the Glock and McLean (1953) methodology, which measures the formation of NADPH at 340 nm for 1 min. The assay consists of the reduction of $NADP^+$ to NADPH by the G6PDH using glucose-6-phosphate (G6P) as the substrate. The assay had a final volume of 205 µL, which contained Tris-HCl buffer (0.1 M, pH 7.4), $MgCl_2$, $NADP^+$, G6P and the sample. The reference blank did not contain G6P. Specific activity was expressed as mU.mg of protein^{-1} using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

The quantification of proteins was performed using the Bradford assay (1976) with Coomassie Brilliant Blue G-250 in an acidic solution. The absorbance values were determined at 595 nm, and the results were compared to the analytical curve prepared with bovine serum albumin (BSA) as the standard.

Statistical analysis

The statistical analysis was conducted using R software version 2.11.1 (R Development Core Team, 2010). We evaluated if the activities of the enzymes CAT, GST, GR and G6PDH were altered by temperature and pH. The presence of outliers was evaluated and the normality of the data was checked using the Shapiro-Wilk test. Statistical differences were identified using one-way ANOVA followed using the Tukey post hoc test. The association of relevant variables was studied by Pearson correlation. To assess whether there was a difference between the stages of development in animals in the different treatments, the G test was performed with Yates' correction (Sokal and Rohlf, 1995). Values of $P < 0.05$ were considered as a reference to assign statistical significance.

RESULTS

All tadpoles survived the treatments with temperature and pH, showing that the acidic (pH 5.0) and alkaline (pH 8.5) conditions were not lethal to this species at the tested levels. Similarly, temperatures ranging from 28°C to 36°C did not cause mortality in individuals within the same period of seven days. However, both experimental conditions led to changes in parameters of oxidative stress in the animals.

The activity of CAT was not altered by treatment with temperature ($P = 0.22$). However, changes in CAT activity were observed after treatments with varying pH ($P < 0.001$). The lowest enzyme activity was observed at pH 7.0 and the highest activity at pH 5.0. An increase in CAT activity was also observed for the treatments at pH 5.0 and 8.5, when these were compared individually with the treatment at pH 7.0. There was no difference in enzyme activity between the treatments at pH 5.0 and 8.5. The P values were 0.0009 (pH 5.0 vs. pH 7.0), 0.011 (pH 8.5 vs. pH 7.0) and 0.078 (pH 5.0 vs. pH 8.5), respectively (Fig. 1).

Unlike CAT, changes in GST activity were observed only in the experiment with different temperatures ($P = 0.042$). The variation in pH did not cause alterations in the activity of this enzyme ($P = 0.086$). Animals exposed to 28°C showed higher enzyme activity than those exposed to 32°C. Animals exposed to 36°C showed no change in GST activity compared to those exposed to other temperatures. The P values between treatments were, respectively, 0.035 (28°C × 32°C), 0.24 (32°C × 36°C) and 0.58 (28°C × 36°C) (Fig. 2).

Both treatments caused changes in GR activity ($P < 0.001$). Animals exposed to 28°C and 36°C showed an increase in GR activity compared to those exposed to 32°C ($P = 0.01$ and $P = 0.02$, respectively). No changes were observed between the treatments at 28°C and 36°C ($P = 0.94$). An increase in GR activity was observed in animals

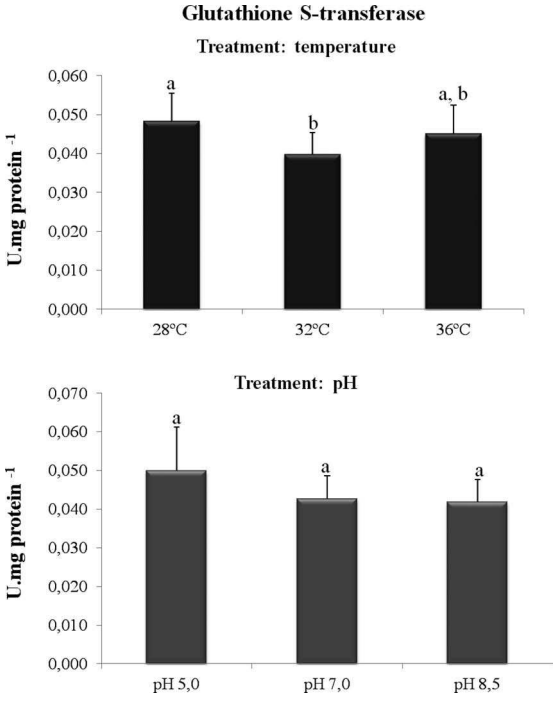

Fig. 2. Glutathione S-transferase activity in tadpoles of *Eupemphix nattereri* exposed to 28°C, 32°C and 36°C (first graph) and pH 5.0, 7.0 and 8.5 (second graph). Different letters indicate significant differences between the groups ($P < 0.05$).

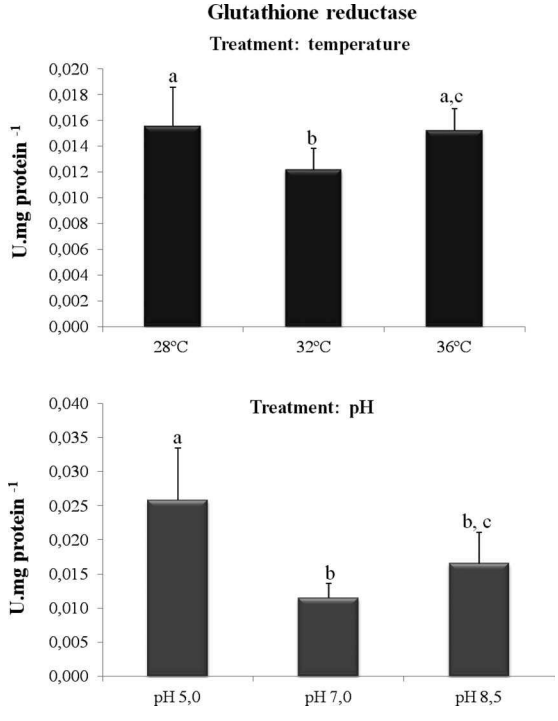

Fig. 3. Glutathione reductase activity in tadpoles of *Eupemphix nattereri* exposed to 28°C, 32°C and 36°C (first graph) and pH 5.0, 7.0 and 8.5 (second graph). Different letters indicate significant differences between the groups ($P < 0.05$).

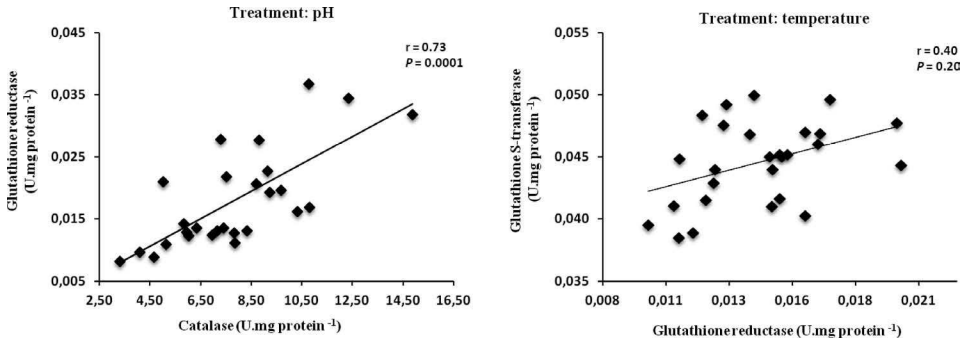

Fig. 4. Pearson correlation between significant covariates. Left: correlation between catalase and glutathione reductase activity following treatment with different pHs. Right: correlation between glutathione reductase and glutathione-S-transferase activity in the treatment with different temperatures.

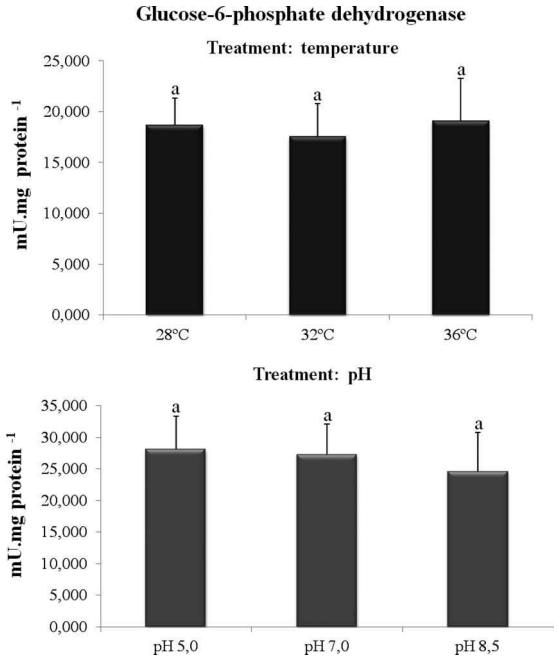

Fig. 5. Glucose-6-phosphate dehydrogenase activity in tadpoles of *Eupemphix nattereri* exposed to 28°C, 32°C and 36°C (first graph) and pH 5.0, 7.0 and 8.5 (second graph). Same letters indicate no significant differences between these groups ($P > 0.05$).

Table 3. Developmental stage of tadpoles (*Eupemphix nattereri*) registered after the experiments with different pH and temperatures. Developmental stages were determined following Gosner (1960).

Variable	Treatment	*N* tadpoles	Stage of development					
			stg30	stg31	stg32	stg33	stg34	stg35
pH	5.0	18	0	7	10	1	0	0
	7.0	18	6	8	3	1	0	0
	8.5	18	2	6	8	2	0	0
Tempara-ture	28°C	18	0	0	6	6	5	1
	32°C	18	0	1	6	5	5	1
	36°C	18	0	1	3	6	6	2

exposed to pH 5.0 compared to those exposed to pH 7.0 and pH 8.5 ($P = 0.001$ and $P = 0.003$, respectively). There was no difference in GR activity between treatments at pH 7.0 and pH 8.5 ($P = 0.13$) (Fig. 3). Correlation analysis showed that GR activity was positively correlated with CAT in the pH experiments and there was no correlation between the activity of GR and GST in the temperature experiments (Fig. 4). G6PDH activity was not changed in either experiment. The *P* values for G6PDH were $P = 0.54$ and $P = 0.36$ for the temperature and pH experiments, respectively (Fig. 5).

At the end of the experiments, the tadpoles were classified into six stages of development (stages 30–35), according to the Gosner (1960) classification scale. Tadpoles exposed to different temperature showed no changes in their stages of development ($P = 0.903$). Similarly, tadpoles exposed to acid and alkaline pH (pH 5.0 and 8.5, respectively) did not present changes in their stages of development compared to those tadpoles exposed to pH 7.0 ($P = 0.637$). Data showing the number of tadpoles in each stage of development for the experiments at different temperatures and pHs levels are shown in Table 3.

DISCUSSION

The results presented in this study showed that antioxidant enzymes are sensitive to changes of pH and temperature in tadpoles of *E. nattereri*. It has been established that different stressful conditions are followed by increased production of ROS in aquatic organisms, suggesting that the changes in the antioxidant enzymes activity are related to the adaptation of organisms to several environmental stressors (Lushchak, 2011). Studies have also shown that the expression of biomarkers triggered by environmental conditions is usually species-specific. Thus, antioxidant enzymes do not exhibit the same behavior for different species and groups, even when they are taxonomically related (Vinagre et al., 2012; Madeira et al., 2013). Our data are of great relevance for environmental monitoring and studies on the impact of environmental variables for amphibians, especially for neotropical anurans, for which the scientific literature contains only very limited data at present.

CAT activity was not affected by temperature in this study. Unlike this result, some studies have shown that CAT is very sensitive to temperature changes in aquatic organisms, although this response varies among the species. For example, in juvenile fish *Dicentrarchus labrax*, which have optimum physiological performance between 20°C and 25°C, CAT activity was increased by nearly seven-fold after exposure of the animals to 28°C, followed by depletion of this enzyme (Person-Le Ruyet et al., 2004; Vinagre et al., 2012). In contrast, there was a decrease in CAT activity in *Diplodus vulgaris* after exposure to temperatures of 28°C and CAT activity was not altered in *Diplodus sargus*, (Person-Le Ruyet et al., 2004; Vinagre et al., 2012), as seen in tadpoles in this study. The data presented in this study show that CAT was not sensitive to thermal stress within the range of 28°, 32° and 36°C in tadpoles. However, it has

been shown that, in some cases, CAT unresponsiveness may result from the inhibition caused by a large fluctuation in superoxide radicals (Filho, 1996).

Unlike thermal stress, treatment with pH caused changes in CAT activity. An increase in CAT activity was observed in animals exposed to pH 5.0 and 8.5 when compared to those exposed to pH 7.0. CATs are enzymes found in places such as peroxisomes and erythrocytes, which promote the reduction of H_2O_2 to H_2O and O_2. At 25°C, pH 7.0 has been described as the point at which the concentrations of hydrogen and hydroxyl ions (OH-) are balanced (each at 10^{-7} mol/L) and therefore, the generation of ROS and subsequent expression of antioxidant enzymes must be lower at this pH. Thus, changing the pH to more acidic or basic levels tend to cause increased ROS production and changes in the activity of antioxidants enzymes, such as CAT. Studies have shown that changes in CAT gene expression were induced in shrimp (*L. vanneni*) maintained under alkaline (pH 9.3) and acid (pH 5.6) stress, although there were differences in the expression response with respect to the duration of induction and the different pH treatments (Wang et al., 2009). In our data, the increase in CAT activity in tadpoles may be a result of the proliferation of peroxisomes in response to stress generated during acute exposure to conditions of acid and alkaline pH, which may have led the animals to overproduce ROS.

Changes in GST activity were observed only in animals exposed to thermal stress. Glutathione S-transferases are a family of multifunctional dimeric enzymes, which are involved in detoxification of xenobiotics, protection against oxidative damage, and in the intracellular transport of endogenous and exogenous hormones and metabolites of chemicals in several aquatic organisms (Eaton and Bammler, 1999; Sheehan et al., 2001; Frova, 2006; Blanchette et al., 2007; Goto et al., 2009). However, changes in GST activity were also reported in animals exposed to thermal stress conditions, such as species of saltwater fish (*Diplodus vulgaris*, *Diplodus sargus*, *Dicentrarchus labrax*, *Gobius niger* and *Liza ramada*), showing that this enzyme also acts to protect organisms against the stress generated by changes in environmental temperature. In *D. vulgaris*, for example, increasing the thermal gradient is accompanied by a progressive increase in the activity of GST (Madeira et al., 2013). In our results, animals exposed to 32°C showed the lowest activity of GST, while those exposed to 28°C had significantly higher values. It was also observed that GST activity was not altered by treatment with a higher temperature (36°C). GR activity showed similar behavior; however, both temperatures 28°C and 36°C resulted in increased activity of this enzyme. Although there are no data reporting the thermal tolerance and optimum temperature for *E. nattereri* in the environment, we believe that the lower antioxidant activity at temperatures around 32°C are the result of better adaptation of the species to this thermal range, which coincides with a period of higher occurrence of the species in the environment. Since ectotherms do not regulate their body temperature independently of environmental temperature variations, an offset in temperature 4°C above or below the optimal average may stimulate the antioxidant defense system in tadpoles as a response mechanism to changes in metabolic rates.

An increase in GR activity was also observed in animals exposed to pH 5.0 compared to those exposed to pH 7.0 and pH 8.5. Previous studies have shown that acid exposure can cause several physiological responses in aquatic organisms, including excessive ROS production and alteration in antioxidant enzyme systems (Vuorinen et al., 2003; Wang et al., 2009; Mai et al., 2010). In fact, one of the main mechanisms that may cause oxidative stress in amphibians involves changes in the metabolism as a result of a decrease in pH (Pierce, 1993). Previous studies have shown that the increased production of ROS as result of acid exposure in rainbow trout, Pacific white shrimp and freshwater prawn may lead to widespread cell damage in organisms as a result of lipoperoxidation and genotoxicity (Witters et al., 1996; Cheng and Wang, 2001; Wang et al., 2009). GR is an enzyme responsible for maintaining levels of GSH by reducing GSSG, using electrons from NADPH (Eriyamremu et al., 2008). In tadpoles, changes in GR activity were previously reported in animals exposed to organochlorine and carbamate pesticides as a result of changes in GSH levels, which could be altered by the presence of ROS in the cellular environment (Ferrari et al., 2009; Ferrari et al., 2011). In this study, it was observed that the induction of GR in animals exposed to acid pH was similar, and was likely a response of excess production of ROS to low pH conditions, which may have altered the GSH levels in the organisms. Moreover, the increase in GR activity in animals exposed to stress conditions suggests the great capacity of regeneration of GSH in this neotropical anuran.

The activity of G6PDH was unchanged in our experiments on temperature and pH. G6PDH is a cytosolic enzyme that participates in the pentose phosphate pathway, maintaining the level of the NADPH. In contrast, the electrons of NADPH are used by GR to reduce GSSG to GSH, so a positive balance between GR and G6PDH activities is expected (Stefani Margarido et al., 2013). However, the increase in GR activity in animals exposed to thermal and pH stress was not reflected in the G6PDH activity, which did not vary in any experiments. These results suggest that G6PDH is not responsive to thermal and pH stress tested under these experimental conditions.

The application of antioxidant enzymes as biomarkers in assessing stress in animals exposed to different environmental conditions has been employed with great success in several groups of aquatic animals, such as estuarine crab, *Chasmagnathus granulata* (Oliveira et al., 2005), brown mussel, *Perna perna* (Almeida et al., 2005), gastropod mollusk, *Littorina littorea* (Pannunzio and Storey, '98), Chinese shrimp, *Fenneropenaeus chinensis* (Jiang et al., 2009) and European seabass, *Dicentrarchus labrax* (Vinagre et al., 2012). It has been reported that the responses of these biomarkers in different species may reveal some mechanisms that make a specific animal more or less tolerant to variations in abiotic parameters in their natural environment and may also show what strategies these organisms have developed to cope with different stress conditions (Almeida and Mascio, 2011). This is especially true for amphibians, which have evolved differing sensitivities to different environmental conditions (Pierce, 1993). For example, some species such as *Rana virgatipes* and *Hyla andersoni* appear to be ecologically specialized for naturally acidic habitats (Gosner and

Black, 1957; Freda and Dunson, 1985), while other species simply do not survive. In this study, we reported that the antioxidant enzymes of tadpoles showed great sensitivity to changes in temperature and pH, suggesting that the assessment of oxidative parameters is of great relevance to studies of tolerance between different species of amphibians exposed to different natural systems. Since these animals are adapted to deal with such variations in environmental conditions, it is reasonable to expect that the tadpoles have intricate mechanisms to respond to changes in temperature and pH. Hence, small changes in the activity of these enzymes may indicate an important pathway used by the tadpoles to adapt to environmental fluctuations, preventing against oxidative stress.

Regarding the development of individuals, studies have shown that the developmental stage of tadpoles is commonly altered by abiotic factors (Pierce and Montgomery, 1989; Morand et al., 1997; Buchholz and Hayes, 2000). For amphibians, temperature and pH act as controlling factors for many physiological processes, including the rate of oxygen consumption, transportation, water balance, survival and development (Freda and Dunson, 1985; Cummins, 1989; Rome et al., 1992). In tadpoles, exposure to elevated temperatures promotes a reduction in time of metamorphosis for many species (Morand et al., 1997), such as *Scaphiopus couchii* and *Spea multiplicata* (Buchholz and Hayes, 2000). In addition, the exposure of tadpoles to acidic pH can inhibit growth and development and lead to many other sublethal effects (Freda and Dunson, 1985; Cummins, 1989). We observed that, in *E. nattereri*, the temperature and pH treatments caused no changes in the survival and developmental stage of individuals. However, we know that these characteristics may change if the animals are exposed to extreme conditions or longer periods of exposure, which were not tested in this study.

Our study suggests a possible way to describe how the stress caused by changes in temperature and pH affects the antioxidant system of tadpoles. The variation in the response of antioxidant enzymes can be considered an immediate adaptive response to physiological changes in tadpoles exposed to stress conditions, protecting the organism against possible increases in ROS production and avoiding oxidative stress. Since it is predicted that changes in global abiotic variables, such as frequency of heat waves and changes in acidity-alkalinity of aquatic environments, will increase in coming years, it is urgently necessary to understand how these changes affect amphibian species at the individual and population levels. This is the first time to our knowledge that antioxidant responses have been demonstrated as effective biomarkers in tadpoles for monitoring the physiological effects of environmental change.

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REFERENCES

Abele D, Heise K, Pörtner HO, Puntarulo S (2002) Temperature dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J Exp Biol 205: 1831–1841

Abele D, Puntarulo S (2004) Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp Biochem Physiol A 138: 405–415

Ahmad I, Maria VL, Oliveira M, Serafim A, Bebianno MJ, Pacheco M, Santos MA (2008) DNA damage and lipid peroxidation vs. protection responses in the gill of *Dicentrarchus labrax L.* from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Sci Total Environ 406: 298–307

Ahmed RG (2005) Is there a balance between oxidative stress and antioxidant defense system during development? Medical J of Islamic World Acad of Sci 15(2): 55–63

Alford RA, Dixon PM, Pechmann JHK (2001) Global amphibian population declines. Nature 412: 499–500

Almeida EA, Bainy ACD, Dafre AL, Gomes OF, Medeiros MHG Di Mascio P (2005) Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and resubmersed. J Exp Marin Biol Ecol 318: 21–30

Almeida EA, Mascio PD (2011) Hypometabolism: Strategies of Survival in Vertebrates and Invertebrates, 39-55 ISBN: 978-81-308-0471-2 Editors: Anna Nowakowska and Michał Caputa

Bagnyukova TV, Danyliv SI, Zin'ko OS, Lushchak VI (2007a) Heat shock induces oxidative stress in rotan *Perccottus glenii* tissues. J Therm Biol 32: 255–260

Bagnyukova TV, Luzhna LI, Pogribny IP, Lushchak VI (2007b) Oxidative stress and antioxidant defenses in goldfish liver in response to short-term exposure to arsenite. Environ Mol Mutagen 48: 658–665

Bagnyukova TV, Storey KB, Lushchak VI (2003) Induction of oxidative stress in *Rana ridibunda* during recovery from winter hibernation. J Therm Biol 28: 21–28

Beutler E (1975) Red Cell Metabolism: A Manual of Biochemical Methods. Grune & Stratton, New York

Blanchette B, Feng X, Singh BR (2007) Marine glutathione S-transferases. Mar Biotechnol 9: 513–542

Blaustein AR, Walls SC, Bancroft BA, Lawler JJ, Searle CL, Gervasi SS (2010) Direct and Indirect Effects of Climate Change on Amphibian Populations. Diversity 2: 281–313

Bocchetti R, Lamberti CV, Pisanelli B, Razzetti EM, Maggi C, Catalano B, et al. (2008) Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams. *Tapes philippinarum*, and mussels, *Mytilus galloprovincialis*, from Adriatic Sea. Mar Environ Res 66: 24–26

Boyd CE (1990) Water quality in ponds for aquaculture. Alabama Agricultural Experiment Station, Auburn University, Auburn, Alabama. Birmingham Publishing Co., Birmingham, Alabama. p 482

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Bioch 72: 248–254

Buchholz DR, Hayes TB (2000) Larval period comparison for the spadefoot toads *Scaphiopus couchii* and *Spea multiplicata* (Anura: Pelobatidae) Herpetologica 56(4): 455–468

Cadenas E (1989) Biochemistry of oxygen toxicity. Annu Rev Biochem 58: 79–110

Caramaschi U, Jim J (1983) Observações sobre hábitos e desenvolvimento dos girinos de *Phyllomedusa vaillanti* (Amphibia, Anura, Hylidae). Rev Brasil Biol 43: 261–268

Carlberg I, Mannervik B (1985) Glutathione-Reductase. Methods Enzymol 113: 484–490

Cheng W, Wang CH (2001) The susceptibility of the giant freshwater prawn *Macrobrachium rosenbergii* to *Lactococcus garvieae* and its resistance under copper sulfate stress. Dis Aquat Organ 47: 137–144

Cummins CP (1989) Interaction between the effects of pH and density on growth and development in *Rana temporaria* L. tadpoles. Funct Ecol 3: 45–52

Eaton DL, Bammler TK (1999) Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol Sci 49: 156–164

Eggert C (2004) Sex determination: the amphibian models. Reprod Nutr Dev 44: 539–549

Ellis MM (1937) Detection and measurement of stream pollution. Bull US Bur Fish 48: 365–437

Eriyamremu GE, Osagie VE, Omoregie SE, Omofoma CO (2008) Alterations in glutathione reductase, superoxide dismutase, and lipid peroxidation of tadpoles (*Xenopus laevis*) exposed to Bonny Light crude oil and its fractions. Ecotox Environ Saf 71: 284–290

Ferrari A, Lascano CI, Anguiano OL, Pechen de D'Angelo AM, Venturino A (2009) Antioxidant responses to azinphos methyl and carbaryl during the embryonic development of the toad *Rhinella (Bufo) arenarum* Hensel. Aquat Toxicol 93: 37–44

Ferrari A, Lascano CI, Pechen de D'Angelo AM, Venturino A (2011) Effects of azinphos methyl and carbaryl on *Rhinella arenarum* larvae esterases and antioxidant enzymes. Comp Biochem Physiol Part C 153: 34–39

Filho DW (1996) Fish antioxidant defences – a comparative approach. Braz J Med Biol Res 29: 1735–1742

Freda J, Dunson WA (1985) Field and laboratory studies of ion balance and growth rates of *Ranid* tadpoles chronically exposed to low pH. Copeia 1985: 415–423

Fridovich I (2004) Mitochondria: are they the seat of senescence? Aging Cell 3: 13–16

Frova C (2006) Glutathione transferases in the genomics era: new insights and perspectives. Biomol Eng 23: 149–169

Gibbs JP, Breisch AR (2001) Climate warming and calling phenology of frogs near Ithaca, New York, 1900–1999. Conserv Biol 15: 1175–1178

Glock GE, McLean P (1953) Further studies on the properties and assay of glucose 6´phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem J 55: 400–408

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183–190

Gosner KL, Black IH (1957) The effects of acidity on the development and hatching of New Jersey frogs. Ecology 38: 256–62

Goto S, Kawakatsu M, Izumi S, Urata Y, Kageyama K, Ihara Y, Koji T, Kondo T (2009) Glutathione S-transferase pi localizes in mitochondria and protects against oxidative stress. Free Radical Biol Med 46: 1392–1403

Grim JM, Miles DRB, Crockett EL (2010) Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. J Exp Biol 213: 445–452

Halliwell B, Gutteridge JMC (1999) Oxidative stress. In "Free Radicals in Biology and Medicine 3rd ed" Ed by B Halliwell, JMC Gutteridge, Oxford University Press, New York, pp 246–350

Halliwell B, Gutteridge JMC (2007) Reactive species can be poisonous. In "Free Radicals in Biology and Medicine 4th ed" Ed by B Halliwell, JMC Gutteridge, Oxford University Press, New York, pp 440–487

Harvell CD, Mitchell C, Ward J, Altizer S, Dobson A, Ostfeld R, Samuel M (2002) Climate warming and disease risks for terrestrial and marine biota. Science 296: 2158–2162

Houlahan JE, Findlay CS, Meyer AH, Kuzmin SL, Schmidt BR (2001) Global amphibian population declines. Nature 412: 499–500

Jiang H, Li F, Xie Y, Huang B, Zhang J, Zhang J, et al. (2009) Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* response to hypoxic stress. Proteomics 9: 3353–3367

Keen JH, Habig WH, Jakoby WB (1976) Mechanism for the several activities of the glutathione S-transferases. J Biol Chem 251: 6183–6188

Kilpatric AM, Briggs CJ, Daszak P (2010) The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends Ecol Evol 25: 109–118

Ling RW, Vanamberg JP, Werner JK (1986) Pond acidity and its relationship to larval development of *Amystoma maculatumi* and *Rana sylvatica* in upper Michigan. J Herpetol 20: 230–236

Livingstone DR (2001) Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42: 656–666

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 10: 13–30

Lushchak VI, Bagnyukova TV (2006) Effects of different environmental oxygen levels on free radical processes in fish. Comp Biochem Physiol Part B 144: 283–289

Madeira D, Narciso L, Cabral HN, Vinagre C, Diniz MS (2013) Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comp Biochem Physiol Part A 166: 237–243

Mai WJ, Yan JI, Wangs L, Zheng Y, Xin Y, Wang W (2010) Acute acidic exposure induces p53-mediated oxidative stress and DNA damage in tilapia (*Oreochromis niloticus*) blood cells. Aquat Toxicol 100: 271–281

Martínez-Álvarez R, Morales A, Sanz A (2005) Antioxidant defences in fish: biotic and abiotic factors. Rev Fish Biol Fish 15: 75–88

Morand A, Joly P, Grolet O (1997) Phenotypic variation in metamorphosis in five anuran species along a gradient of stream influence. Académie des sciences, Sciencies de la vie 320: 645–652

Oliveira UO, Araújo ASR, Belló-Klein A, Silva RSM, Kucharski LC (2005) Effects of environmental anoxia and different periods of reoxygenation on oxidative balance in gills of the estuarine crab *Chasmagnathus granulata.* Comp Biochem Physiol Part B 140: 51–57

Pannunzio TM, Storey KB (1998) Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod, *Littorina littorea*. J Exp Mar Biol Ecol 221–277

Parihar MS, Dubey AK (1995) Lipid peroxidation and ascorbic acid status in respiratory organs of male and female freshwater catfish *Heteropneustes fossilis* exposed to temperature increase. Comp Biochem Physiol Part C 112: 309–313

Person-Le Ruyet J, Mahé K, Bayon N, Delliou H (2004) Effects on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax.* Aquaculture 237: 269–280

Pierce BA (1985) Acid Tolerance in Amphibians. BioSci 35: 239–243

Pierce BA (1993) The effects of acid precipitation on amphibians. Ecotoxicol 2: 65–77

Pierce BA, Montgomery J (1989) Effects of short-term acidification on growth rates of tadpoles. J Herpetol 23: 97–102

Pough FH, Taigen TL, Stewart ML, Brussard PF (1983) Behavioral modification of evaporative water loss by a Puerto Rican frog. Ecology 64: 244–252

Provete DC, Garey MV, Silva FR, Rossa-Feres DC (2011) Anuran-fauna from Northwestern region of the state of São Paulo: species list and taxonomic key for adults. Biota Neotrop 11: 377–391

R Development Core Team (2010) R: a language and environment for statistical computing. R Foundation for Statistical Comput-

ing. Vienna, Austria. Available at: www.r-project.org
Robertson-Bryan, Inc (2004) PH Requirements of Freshwater Aquatic Life: a Technical Memorandum. Robertson-Bryan, Inc.; Elk Grove, CA
Rome LC, Stevens ED, John-Alder HB (1992) The influence of temperature and thermal acclimatation on physiological function. In "Environmental Physiology of the Amphibians" Ed by ME Ferder, WM Burggren,University of Chicago Press, Chicago
Rossa-Feres DC, Nomura F (2006) Characterization and taxonomic key for tadpoles (Amphibia: Anura) from the northwestern region of São Paulo State, Brazil. Biota Neotrop 6(1): http://www.biotaneotropica.org.br/v6n1/pt/abstract?identification-key+bn00706012006
Sheehan D, Meade G, Foley VM, Dowd CA (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J 360: 1–16
Smith-Gill SJ, Berven KA (1979) Predicting amphibian metamorphosis. Am Naturalist 113: 563–585
Sokal RR, Rohlf FJ (1995) Biometry: The Principles and Practice of Statistics in Biological Research. 3rd ed. W.H. Freeman & Co, New York
Stefani Margarido TC, Felício AA, Rossa-Feres DC, Almeida EA (2013) Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. Mar Environ Res 1–7
Toyokuni S (1999) Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 49: 91–102
Van Der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessement: a review. Environ Toxicol Phar 13: 57–149
Velkova-Jordanoska L, Kostoski G, Jordanoska B (2008) Antioxidative enzymes in fish as biochemical indicators of aquatic pollution. Bulg J Agric Sci 14: 235–237
Verlecar XN, Jena KB, Chainy GB (2007) Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chem-Biol Interact 167: 219–226
Vinagre C, Madeira D, Narciso L, Cabral HN, Diniz M (2012) Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax.* Ecol Indic 23: 274–279
Vuorinen PJ, Keinanen M, Peuranen S (2003) Reproduction, blood and plasma parameters and gill histology of vendace (*Coregonus albula* L.) in long-term exposure to acidity and aluminum. Ecotox Environ Saf 54: 255–276
Wang WN, Wang AL, Chen L, Liu Y, Sun RY (2002) Effects of pH on survival, phosphorus concentration, adenylate energy charge and Na^+–K^+ ATPase activities of *Penaeus chinensis* Osbeck juveniles. Aquat Toxicol 60: 75–83
Wang WN, Peng Wang JZ, Ying Zheng TTT, Liu Y, Mai WJ, Wang AL (2009) Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. Comp Biochem Physiol Part C 150: 428–435
Wells KD (2007) The ecology and behavior of amphibians. Chicago: University of Chicago Press
Westfall BA (1945) Coagulation anoxia in fishes. Ecology 26: 283–287
Winston GW (1991) Oxidants and antioxidants in aquatic animals. Comp Biochem Physiol Part C 100: 173–176
Witters HE, Van Puymbroeck S, Stouthart AJHX, Wendelaar Bonga SE (1996) Physi-cochemical changes of aluminium in mixing zones: Mortality and physiological dis-turbances in brown trout (*Salmo trutta* L). Environ Toxicol Chem 15: 986–996

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Influence of temperature on the antioxidant responses and lipid peroxidation of two species of tadpoles (*Rhinella schneideri* and *Eupemphix nattereri*) exposed to the herbicide sulfentrazone (Boral 500SC®)

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Abstract

Amphibians can experience large temperature fluctuations in their habitats, especially during the larval stage, when tadpoles are restricted to small and ephemeral ponds. Changes in water temperature can alter development, metabolism and behaviour of cold-blooded animals but also the toxicokinects of chemicals in the environment. In Brazil, pesticides application is intensified during the rainy season, which is the period of reproduction for many amphibian species. In this study, we evaluated the influence of temperature (28, 32, and 36ºC) on the toxicity of the herbicide sulfentrazone (Boral®SC) in tadpoles of *Eupemphix nattereri* and *Rhinella schneideri* after three and eight days, by analysis of oxidative stress biomarkers. Exposure of tadpoles to sulfentrazone changed the antioxidant enzymes activities and induced lipid peroxidation with temperature-associated responses. Catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PDH) were impaired by combined effect of temperature and sulfentrazone in both species. G6PDH was increased in most groups exposed to sulfentrazone at 36ºC. Biotransformation enzyme glutathione-*S*-transferase (GST) was also altered by temperature and sulfentrazone, with more evident alterations in *E. nattereri*. *t*GSH contents were changed following the treatments, showing a different pattern for the studied species. Lipid peroxidation was particularly induced in tadpoles of *E. nattereri*, indicating greater sensibility of this species to the effects of sulfentrazone combined to the different temperatures. Our study showed that temperature modulates biochemical responses in tadpoles exposed to sulfentrazone with a species-specific pattern. These findings imply that the effects of abiotic factors should be taken into account to evaluate the real risks of exposure of amphibians to commonly used pesticides.

Keywords: Amphibians, sulfentrazone, temperature, biochemical biomarkers, oxidative stress.

1. INTRODUCTION

Increased agriculture activity in Brazil has caused extensive impact on several natural ecosystems and non-target organisms, especially due to the intensified use of pesticides. Brazil is the largest consumer of pesticides in the world, and sugarcane is one of the main crops contributing to the expansion of cultivated areas in recent years (UNICA, 2015). In south-eastern Brazil, the state of São Paulo is responsible for the usage of approximately 20% of all Brazilian pesticides and has the highest rates of conversion of natural habitats to agricultural land (IPT, 2000). Several species of amphibians have been recorded in this area (Bernarde and Kokubum, 1999; Prado et al., 2008; Provete et al., 2011) and many of them are distributed in areas common to agriculture practice. Considering the accelerated expansion of sugarcane in Brazil, mainly due to its use in biofuel production, there is a great concern that several amphibian species are threatened by exposure to local pesticides.

Sulfentrazone (2',4'-dichloro-5-(4-difluoromethyl-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl) methanesulfonanilide) is an herbicide widely used in sugarcane crops in Brazil and other areas of the world. It is also applied in crops of great commercial importance, such as soy, coffee, citrus and eucalyptus (Embrapa, 2013). Sulfentrazone belongs to the aryl triazolinones group and is considered a potential contaminant due to its long residual effect (110–280 days) in soil (Hirata, 2010; Melo et al., 2010). Following application to soil, sulfentrazone can runoff into surface water, potentially leading to negative effects to aquatic organisms. Concentrations of sulfentrazone found in freshwater environments such as rivers, lagoons or even temporary ponds used by amphibians as a transitory environment during larval development, are still unknown due to a lack of studies in the literature. However, evidence has shown that sulfentrazone has been found in both soil and water in agriculture areas of Sao Paulo (Dutra De Armas et al.,

2005). Concentrations of this and other herbicides may be particularly high in small and ephemeral ponds used by amphibians as habitats, since they are formed by flooding of soil during the rainy season.

Larval stages of anurans are especially susceptible to pesticides and other chemical compounds (Greulich and Pflugmacher, 2003) because tadpoles are strictly aquatic and have high skin permeability (Yan et al., 2008). In tropical areas with well-defined seasons, the reproduction of most amphibian species, and the subsequent occurrence of tadpoles, is restricted to the rainy season due to the availability of water (McDiarmid et al., 1999; Provete et al., 2011). It is also during this season that the contamination of lakes and streams as result of herbicide application is usually increased, especially due to the use of pre-emergent herbicides, such as sulfentrazone, which are better absorbed in moist soils. In Brazil, the rainy season occurs predominantly during the summer and is accompanied by the highest temperature averages of the year. Due to the small volume of water in ephemeral ponds, the water temperature is easily elevated, increasing the average temperatures and the daily thermal fluctuations. Previous studies have shown that the water temperature can exceed 40°C during the summer in ponds where several species of tadpoles have been found in Brazil (Freitas et al., 2016). Therefore, it should be considered that neotropical tadpoles inhabiting agricultural areas are not only threatened by pesticide exposure, but also by the interaction effects of these substances and the environmental temperature variations.

Temperature is mentioned as one of the main abiotic factors influencing the toxicity of chemical compounds in the environment (Hooper et al., 2013; Noyes et al., 2009). Chemical and biological reactions become faster in warmer waters, and therefore, the effect of a toxic agent may be more pronounced at higher temperatures (Middlebrooks et al., 1973). For poikilothermic

animals, thermal variation of the environment is directly translated into the variation of the physiological performance (Fontenot and Lutterschmidt, 2011). Temperature affects virtually all physiological aspects of amphibians, including digestion, vision, locomotion, growth and metabolism (Rome, 2007; Gatten et al., 1992). Thus, changes in environmental temperature can interfere with many natural biological responses in different species, making the organisms more susceptible to the action of xenobiotics (Blaustein et al., 2010; Blaustein and Kiesecker, 2002; Moe et al., 2013). Previous studies have shown that temperature plays an important role in the toxicity of several pesticides for amphibians (Boone and Bridges, 1999; Freitas et al., 2016; Hammond et al., 2015). For example, the mortality of tadpoles of *Rana clamitans* exposed to the insecticide carbaryl was increased at lower concentrations when animals were exposed to higher temperatures (Boone and Bridges, 1999). Exposure of tadpoles of the American bullfrog (*Lithobates catesbeianus*) to the herbicide diuron and its metabolite 3,4-DCA also showed that thyroidogenic effects of both compounds were more pronounced in animals exposed at higher temperatures (Freitas et al., 2016).

Despite the increasing number of studies exploring the effects of temperature on the toxicity of chemical compounds (Bao et al., 2008; Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009; Rohr and Palmer, 2005; Schiedek et al., 2007), the data collected to date are not comprehensive enough to routinely support integrated risk assessments for biological communities. Cold-blooded animals, such as amphibians, have inter- and intra-specific variations in their thermal tolerances (Katzenberger et al., 2012), so studying the toxicity of a chemical at one standard temperature may not be adequate to estimate negative effects to different species in environmental systems. Biomarkers of oxidative stress have been widely used to assess negative effects of pesticides on amphibians (e.g., Dornelles and Oliveira, 2014;

Gripp et al., 2017; Stefani Margarido et al., 2013), since they may provide valuable information about amphibian health status (Peltzer et al., 2013). They are also relevant to assess the effects of thermal stress on poikilotherms, since temperature is an important factor affecting antioxidant enzymes in these organisms (e.g., Bagnyukova et al., 2003; Madeira et al., 2013; Vinagre et al., 2012). So far, no studies have investigated the effects of sulfentrazone on tadpoles and very few studies report its effects in non-target organisms, especially considering temperature variations. Therefore, this study aimed to investigate the effects caused by the interaction between temperature and the herbicide sulfentrazone in two species of tadpoles in Brazil, *Eupemphix nattereri* and *Rhinella schneideri*, using oxidative stress markers. For this, we evaluated enzymes involved in antioxidant responses, including activities of superoxide dismutase (SOD), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), the biotransformation enzyme glutathione-S transferase (GST) and levels of total glutathione (*t*GSH). Lipid peroxidation was also evaluated by measuring the levels of malondialdehyde (MDA). We hypothesized that a temperature increase may enhance the effects of sulfentrazone; with alterations to antioxidant defence systems, and induction of lipid peroxidation as a response of increased generation of reactive oxygen species (ROS) by stimulation of the metabolic processes in both species. Also, sulfentrazone may cause distinct effects in different tadpole species, indicating that they can be distinctly susceptible to negative effects caused by this herbicide.

2. MATERIAL AND METHODS

2.1. Animals

Laboratory tests were conducted using two species of tadpoles, since species- or family-level sensitivity has been increasingly considered for use in laboratory tests evaluating toxicity of pesticides to amphibian populations (Egea-Serrano et al., 2012; Relyea, 2005; Shinn et al., 2008;

Snodgrass et al., 2008). *Eupemphix nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) are common anuran species with a wide geographical distribution in the northwest of São Paulo, Brazil. They can be found in different habitats, including pastures and sites of intense agriculture (Provete et al., 2011). Tadpoles of the selected species have different habitats in water bodies, with *E. nattereri* preferring benthic habitats and *R. schneideri* neustonic habitat (Prado et al., 2008; Provete et al., 2011).

2.2. Tadpole collection and acclimation

Spawns of the anurans *E. nattereri* and *R. schneideri* were collected in temporary ponds in non-agricultural areas in the region of Sao Jose do Rio Preto, Sao Paulo, Brazil (20°47′07.05″ S, 49°02′42.09″ W). After hatching, the larvae were maintained in the laboratory under ideal conditions of temperature (28°C) and pH (7.5–8.0) in aerated aquariums, until they reached stages 27–30 (Gosner, 1960), just before the development of legs. The animals were collected under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

2.3. Experimental design

2.3.1. Temperature treatment

Aquariums containing dechlorinated water were heated to three different temperatures: 28, 32 and 36ºC. Temperatures were maintained by autoregulating thermostats in 5 L aquariums placed in a 100 L “water bath”. The temperature of the tanks and individual aquariums had their values recorded daily to observe possible variations during the experimental period. Temperature values used in this study were based on Freitas et al. (2016), which recorded the water

temperature in 5 natural ponds in which tadpoles of *R. schneideri* and *E. nattereri* are frequently found. These data were recorded during the summer, which coincides with the rainy season and reproduction for most neotropical frogs.

2.3.2. Chemical treatments

Tadpoles of *E. nattereri* and *R. schneideri* were exposed to 0.01, 0.05 and 0.1 mg/L of sulfentrazone (2',4'-dichloro-5-(4-difluoromethyl-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl) methanesulfonanilide), using the commercial herbicide Boral®500SC. Tadpoles of each species were exposed separately to each concentration at temperatures of 28, 32 and 36ºC for three and eight days. Concentrations of sulfentrazone were based on the product concentration recommended for use in the field (Martinez et al., 2010) and in studies reporting possible concentrations in water after leaching that may be available in aquatic habitats (Thorngren et al., 2016). The experimental groups consisted of five replicates (n=5) containing six tadpoles in each aquarium. The tadpoles were placed into the aquariums, and the water temperature was gradually adjusted during the first day to avoid thermal shock. Then, sulfentrazone was added at nominal concentrations as described above. The herbicide was dissolved in a stock solution of 1 mL of water and then added to the aquariums. A control group containing no contaminants was provided for each temperature and experiment duration. Aquariums were constantly aerated at a pH of 8.00 ± 0.4 and using a light-dark cycle of 12-12 h. The water was renewed every two days to ensure the original concentration of the contaminant and the dissolved oxygen, pH and ammonia were quantified for the same period. No significant variations were observed in these parameters among treatments during the experiments (dissolved oxygen 6.5–7.1 mg/L, pH 7.2–8.3). The animals were fed every two days with commercial feed for tropical fish in

minimum quantities (200 mg) avoiding accumulation in the aquariums. At the end of the experiment, the tadpoles were removed from the tanks and euthanized by direct immersion in liquid nitrogen. All the procedures were performed in accordance with the Ethics Committee on Animal Use in Research of the São Paulo State University (CEUAIBILCE/UNESP No. 086/2013).

2.4. Analysis

2.4.1. Morphological analysis

Effects on growth (snout-vent length (SVL) and body weight) and development (Gosner, 1960) were evaluated individually in tadpoles from both species after three and eight days of exposure at different temperatures.

2.4.2. Preparation of samples

For the enzymatic measurements, two tadpoles of each replicate (n=10) were entirely and individually homogenized at a ratio of 1:4 (weight:volume) in cold (4°C) 20 mM Tris-HCl homogenization buffer (TrisHCl 20 mM, EDTA 1 mM, DL-dithiothreitol (DTT) 1 mM, sucrose 0.5 M, KCL 0.15 M, phenylmethylsulfonyl fluoride (PMSF) 1 mM), pH 7.4, and centrifuged for 30 min at 9000 g at 4°C. The supernatant fraction was collected and centrifuged again for 1 h at 50,000 g. The second supernatant fraction was collected and stored at -80°C for subsequent evaluation.

Lipid peroxidation was assessed by the measurements of MDA levels. For this analysis, two tadpoles of each replicate (n=10) were entirely homogenized at a ratio of 1:3 (weight:volume) in 0.1 M Tris-HCl buffer, pH 8.0. After homogenization, 300 µL of

thiobarbituric acid (TBA, Sigma-Aldrich, Germany) diluted in 0.4% HCl were added to the sample. The mixture was heated at 90°C for 40 min, and the reaction product was extracted with 1.0 mL of n-butanol (Sigma-Aldrich, USA). After this procedure, the n-butanol fraction was injected (10 µL) into a High-Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan). The chromatogram was monitored for 10 min and peaks were identified and quantified using LAB Solutions 5.71 software (Shimadzu Corporation).

For *t*GSH analysis, two tadpoles of each replicate (n=10) were entirely and individually homogenized at a ratio of 1:9 (weight:volume) in a cold (4°C) solution of perchloric acid (PCA) 0.5 M and centrifuged for 5 min at 15,000 g at 4°C. Supernatants were collected and neutralized with a solution of potassium phosphate 0.1 M (0.1 M, pH 7.0) and then centrifuged at 15,000 g for 2 min at 4°C.

2.4.3. Antioxidant enzyme measurements

Analyses of CAT were performed in a Thermo Evolution 300 spectrophotometer with a dual beam, and capacity for seven cuvettes. The activities of GST, G6PDH and SOD were performed on a Victor TM X3 microplate reader (Perkin ElmerR). The activity of all enzymes was evaluated at the respective temperatures (28, 32 and 36°C) at which the animals were subjected to treatments.

CAT activity was measured using the method described by Beutler (1975), which monitors the rate of decomposition of hydrogen peroxide by the enzyme at 240 nm for 1 min. Specific activity was expressed as U mg of protein^{-1}. The assays were performed using Tris-HCl buffer (1 M, pH 8.0) with 5 mM EDTA and H_2O_2 as the substrate.

GST activity was measured using the method described by (Keen et al., 1976), which monitors the formation of the conjugate of 1-chloride-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) catalysed by GST in the sample for 1 min at 340 nm. Specific activity was expressed as U mg of protein^{-1}. The final volume of the assay was 110 µL, which contained potassium phosphate buffer (0.2 M, pH 6.5), 1 mM CDNB (dissolved in 1.0 mL of absolute ethanol), 1 mM GSH, and the sample.

The enzymatic assay of G6PDH (Glock and McLean, 1953) measured the formation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for 1 min. The assay consists of the reduction of NADP+ to NADPH by the G6PDH using glucose-6-phosphate (G6P) as the substrate. The assay had a final volume of 205 µL, which contained Tris-HCl buffer (0.1 M, pH 7.4), $MgCl_2$, NADP+, G6P and the sample. The reference blank did not contain G6P. Specific activity was expressed as mU mg of protein^{-1}.

SOD activity was measured using SOD Assay Kit-WST (Sigma, Aldrich) by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase activity, and is inhibited by SOD. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity was quantified by measuring the decrease in the colour development at 440 nm.

2.4.4. Determination of total GSH levels (tGSH)

*t*GSH contents were assessed by the Tietze (1969) protocol, adapted by Sies (2003). The total amount of GSH was measured based on the reaction between GSH and DTNB (5,5′-

Dithiobis(2-nitrobenzoic acid)), which produces the conjugate GS-TNB and the yellow TNB (5-thio-2-nitrobenzoic acid) anion that can be detected at 412 nm. The rate of TNB production is proportional to the amount of GSH in the extract. Thus, the more GSH in the sample, the more TNB is formed, which is monitored at 412 nm for 2 min. A recycling reaction catalysed by glutathione reductase in the presence of NADPH was also coupled to the assay, regenerating 2GSH from the GSSG formed previously by the reaction with DTNB. This enables a continuous production of TNB, which is proportional to the total GSH (GSH + GSSG) in the sample. The concentration in μmol GSH g^{-1} of tissue in the sample was calculated based on a standard curve with known amounts of GSH, obtained in the assay by the change in absorbance per minute (ΔA). Analyses of GSH were performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes.

2.4.5. MDA measurements

MDA levels were evaluated following the method described by Almeida (2003) using a high performance liquid chromatography (HPLC) system (ESA, USA) coupled to a UV-Vis detector (526, ESA, USA) to analyse the product formed between malonaldehyde (MDA) and TBA. The chromatographic column used in analysis was a Shimadzu C18 column (150 x 4.6 mm, 5 μm), using 50 mM potassium phosphate solution at pH 7.0 as the mobile phase, with 40% methanol, and at a flow rate of 1.0 mL/min.

2.4.6. Protein quantification

Proteins were quantified using the Bradford (1976) protocol with Coomassie Brilliant Blue G-250 in an acidic solution. The absorbance values were determined at 595 nm, and the results

were compared to the analytical curve prepared with bovine serum albumin (BSA) as the standard.

2.4.7. Statistical analysis

The presence of outliers was evaluated and the normality and homogeneity of the data was checked by the Shapiro-Wilk and Levene's tests, respectively. Two-way analysis of variance (ANOVA 2-way), followed by the Tukey post hoc test were used to identify statistical differences (antioxidant enzyme activity, MDA levels and effects on growth – length and weight). This test was used to evaluate the individual effects of temperature (28, 32 and 34°C) and chemical treatments (concentrations of sulfentrazone at 0.01, 0.05 and 0.1 mg/L), and the interaction effects between the temperature and chemical treatments. Non-exposed tadpoles at 28°C were assumed as the control group for comparison of interaction effects of temperature and chemical exposure, since the animals were acclimatized at this temperature and this group had no influence of either thermal stress or chemical exposure. To assess whether there were differences between the stages of development of the tadpoles, the G test was performed with Yates' correction (Sokal, 1995). Statistical analysis was conducted using R version 2.11.1 (R Development Core Team, 2010) and STATISTICA (StatSoft). Values of $P<0.05$ were considered as a reference to assign statistical significance (Zar, 1999).

3. RESULTS

3.1. Effects on tadpole mortality, growth and development

Mortality was not observed in any of the experimental groups for either species. Effects on growth (snout-vent length (SVL) and weight) were assessed at the end of the treatments. For

E. nattereri, biometric analysis showed no changes on growth and length of tadpoles for any treatment after three days of exposure (Table S1). Stages of development were also unchanged after three days for any treatment (P=0.47) (Table S3). After eight days of exposure, untreated tadpoles maintained at 36°C presented higher weight (P=0.043) and body length (P=0.016) than those maintained at 28°C (Table S1). Chemical treatments caused no effects on weight or body length of tadpoles at any other temperature. Stage of development was accelerated in tadpoles exposed to 0.05 mg/L at 32°C (stages Gs31–36) compared to the control group at the same temperature (stages Gs29–34) (P=0.020) after eight days. This group presented 20% of tadpoles at Gs35 and 30% at Gs36, while any tadpoles of control overtook Gs34 (Table S4).

In *R. schneideri*, temperature had no single effect on the weight and length of tadpoles for both experimental periods. Sulfentrazone also had no effects on weight (P=0.298) or length (P=0.764) of tadpoles after three days of exposure at any temperature (Table S2). After eight days, no effects on weight were observed; however, tadpoles exposed to 0.1 mg/L at 32°C were larger than animals of the control group assessed at 28°C (P=0.034) and those exposed to the same concentration (0.1 mg/L) at 28°C (P=0.009) and 36°C (P=0.01) (Table S3). Stages of development were unaffected by treatments in *R. schneideri* (P=0.60) (Table S5 and S6).

3.2. Antioxidant enzyme and lipid peroxidation

E. nattereri

Temperature had isolated effects on antioxidant enzymes in tadpoles of *E. nattereri* not exposed to sulfentrazone. G6PDH activity was lower in animals of the control group at 32°C than the control animals at 28°C after three days (P=0.007). Similarly, *t*GSH was lower in animals maintained at 32°C than those kept at 28°C (P=0.017) (Fig. 1). GST was increased in

animals maintained at 36°C compared to the control group at 28°C for both experimental periods of three (P=0.005) and eight days (P=0.034) (Fig. 2). After eight days, SOD activity was diminished in tadpoles of the control group at 32°C compared to control at 28°C (P=0.013) (Fig. 2). CAT activity was not affected by temperature (P>0.05).

Treatment with sulfentrazone at different thermal conditions also changed the antioxidant activity of the enzymes in *E nattereri* tadpoles. After three days of exposure, treatment with sulfentrazone at 28°C increased CAT activity in animals exposed to 0.1 mg/L compared to the control at the same temperature (P=0.012). GST activity was induced in animals exposed to all concentrations of sulfentrazone at 28°C (P=0.036, P<0.001, P<0.001, respectively). *t*GSH levels were also elevated in tadpoles exposed to 0.05 mg/L compared to the control group (P=0.043). SOD and G6PDH activities were unchanged following sulfentrazone treatments at 28°C (P>0.05). At 32°C, animals exposed to 0.01 mg/L of sulfentrazone had lower CAT activity than those exposed to 0.1 mg/L (P<0.001). Interaction effects with temperature also diminished CAT activity in tadpoles exposed to 0.01 mg/L at 32°C compared to the control at 28°C (P=0.0002). SOD was decreased in tadpoles exposed to 0.05 mg/L of sulfentrazone at 32°C compared to the control group at the same temperature (P=0.039). Increases in G6PDH activity were observed in animals treated with sulfentrazone at 0.01 and 0.1 mg/L at 32°C compared to the respective control (P=0.020; P=0.00013, respectively), with higher induction of G6PDH in the highest concentration. An increase in GST was only found in animals treated at the higher concentration at 32°C (P=0.0001) after three days. At 36°C, treatments with sulfentrazone had no effects on CAT or SOD activities, or on *t*GSH levels. Animals treated with 0.1 mg/L at 36°C presented an increase on G6PDH activity compared to control animals at the same temperature (P<0.001), and to the control at 28°C (P=0.002), showing an interaction effect at 36°C. An interactive effect

was also observed for GST (P=0.005), which was increased in all groups treated at 36°C compared to the control at 28°C (P<0.001) (Fig. 1).

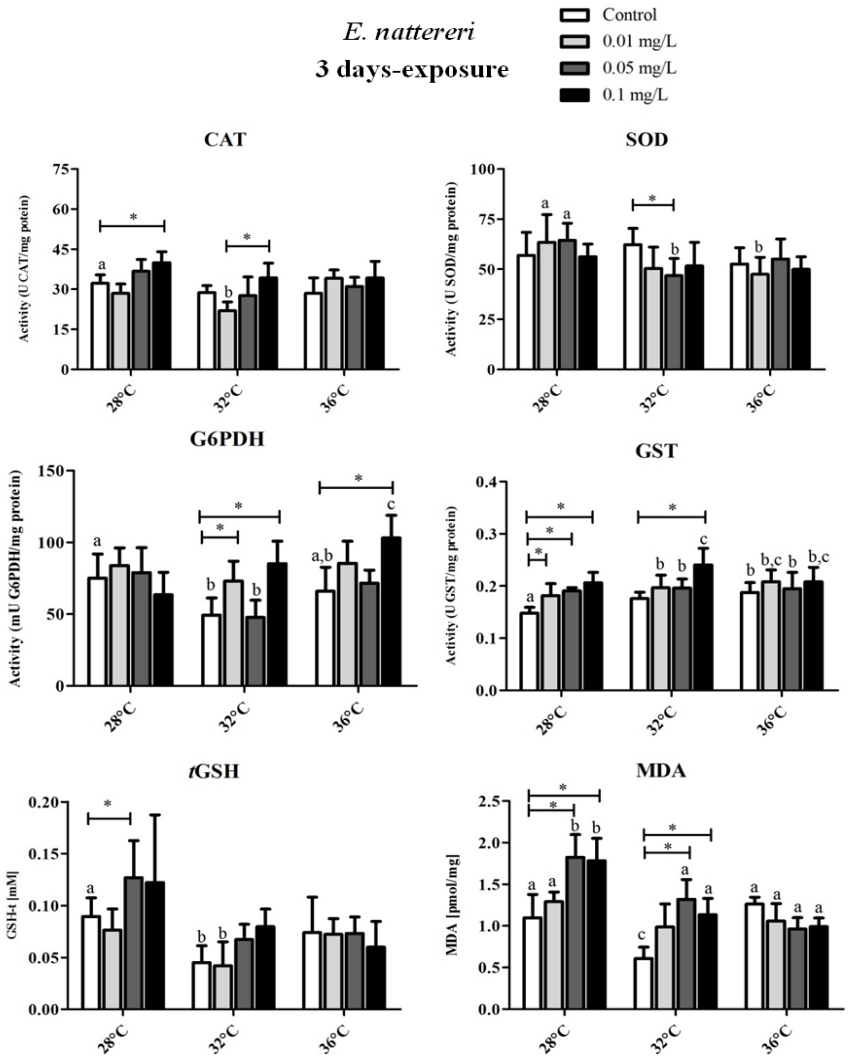

Figure 1: Antioxidant enzymes (SOD, CAT, GST and G6PDH) activity and *t*GSH and MDA levels in tadpoles of *E. nattereri* exposed to sulfentrazone at different temperatures (28°C, 32°C and 36°C) after three days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05). Standard deviations are represented in bars.

After eight days of exposure, sulfentrazone caused no effects on antioxidant enzymes of animals treated at 28°C, except for G6PDH that had its activity increased 2.6-fold in tadpoles

exposed to 0.05 mg/L compared to the control group (P=0.00012) (Fig. 2). At 32°C, G6PDH activity was increased in animals treated at concentrations of 0.01, 0.05 and 0.1 mg/L (P=0.020; P=0.0042; P=0.00012, respectively), with a 2.5-fold increase for the higher concentration. An increase in G6PDH activity was also observed for animals treated at 0.01 and 0.1 mg/L at 32°C compared to control at 28°C (P=0.001, P=0.0001, respectively). Similarly, GST activity was induced by exposure to sulfentrazone in animals treated at all concentrations (P=0.00012, P=0.049, P=0.00012, respectively). An interaction effect of sulfentrazone and temperature also increased GST activity in animals exposed to 0.01 and 0.1 mg/L at 32°C compared to the control at 28°C (P<0.001). *t*GSH levels were also augmented in tadpoles exposed to the highest concentration at 32°C compared to the control at the same temperature (P=0.009). No effects were observed in the activities of SOD or CAT at 32°C (P>0.05). At 36°C, G6PDH had a similar response to that observed in the treatments at 32 °C, with increased activity in animals exposed to all concentrations of sulfentrazone (P=0.00024, P=0.00017, P=0.00012). An interactive effect of sulfentrazone and the higher temperature was detected in the G6PDH activity of animals exposed to 0.1 mg/L at 36°C compared to the control at 28°C (P=0.0001). Tadpoles treated with 0.1 mg/L of sulfentrazone at 36°C also had an increase on GST activity compared to the control at the same temperature (P=0.0001) and to the control at 28°C (P=0.0001), showing evidence for an interaction effect at 36°C. In contrast to that observed at 32°C, *t*GSH levels were decreased in animals exposed to 0.05 mg/L of sulfentrazone at 36°C compared to its respective control (P=0.017). Effects of sulfentrazone were not found for SOD or CAT activities at 36°C (P>0.05) (Fig. 2).

Lipid peroxidation was induced in tadpoles exposed to sulfentrazone with temperature-dependent responses for both experimental periods in tadpoles of *E. nattereri* (Figs. 1 and 2).

Single effects of temperature were also detected in untreated tadpoles. Animals maintained at 32ºC presented the lowest levels of MDA compared to the control at 28ºC (P=0.021) and 36ºC (P<0.001) after three days (Fig. 1). After eight days, untreated animals maintained at 36ºC had the highest levels of MDA (P<0.001) (Fig. 2).

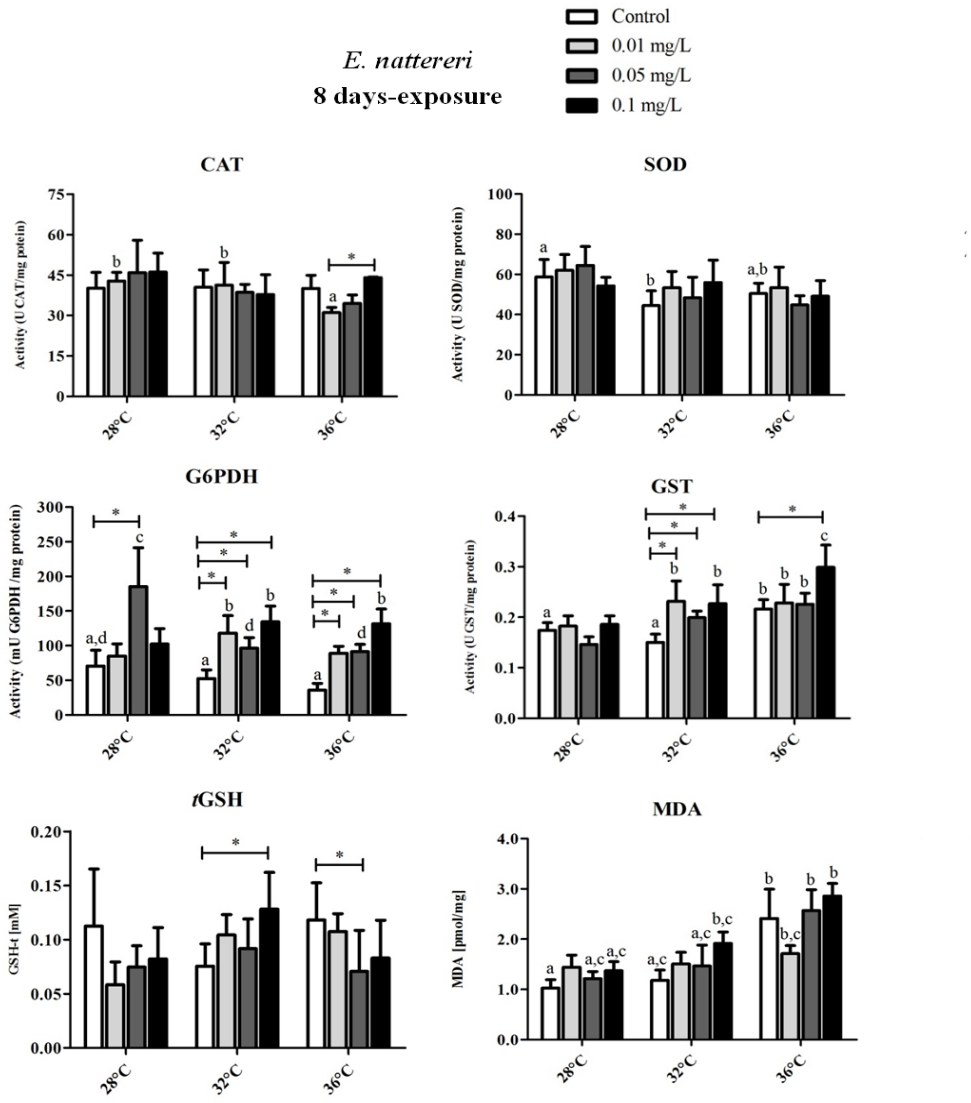

Figure 2: Antioxidant enzymes (SOD, CAT, GST and G6PDH) activity and *t*GSH and MDA levels in tadpoles of *E. nattereri* exposed to sulfentrazone at different temperatures (28°C, 32°C and 36 °C) after eight days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05). Standard deviations are represented in bars.

Tadpoles of *E. nattereri* exposed to 0.05 and 0.1 mg/L of sulfentrazone at 28ºC and 32ºC had increased MDA levels compared to the control groups at the respective temperatures ($P<0.001$) after three days of exposure (Fig. 1). No changes were found in animals treated at 36ºC, and no interaction effects were detected after three days ($P>0.05$) (Fig. 1). After eight days, sulfentrazone had no effect on MDA levels at 28ºC ($P>0.05$). However, exposure of animals to 0.1 mg/L at 32ºC increased the levels of MDA compared to untreated animals at 28ºC ($P=0.0086$). Also, interaction effects of sulfentrazone treatments at 36ºC increased MDA in tadpoles of all groups compared to controls at 28ºC ($P<0.001$) (Fig. 2).

R. schneideri

Single effects of temperature were observed in antioxidant responses of unexposed tadpoles of *R. schneideri* to sulfentrazone (Figs. 3 and 4). After three days, CAT activity was decreased in control animals maintained at 32°C and 36°C compared to those at 28°C ($P<0.001$). G6PDH was 2-fold higher in animals maintained at 36°C than the control group at 28°C ($P=0.012$). *t*GSH also increased 2-fold in tadpoles of the control group at 36°C ($P=0.006$) (Fig. 3). After eight days, CAT ($P=0.41$), SOD ($P=0.97$) and *t*GSH ($P=0.54$) were unchanged by the single effect of temperature; however, G6PDH had a 4.5-fold increase in tadpoles maintained at 36°C compared to the control group at 28°C ($P<0.001$). GST activity was higher in control animals at 32°C after eight days ($P=0.048$) (Fig. 4).

The exposure of *R. schneideri* to sulfentrazone for three and eight days promoted changes in enzyme activity with temperature-associated responses (Figs. 3 and 4). Treatments with sulfentrazone at 28°C decreased the activities of CAT ($P=0.001$) and GST ($P=0.041$) in tadpoles treated with 0.05 mg/L after three days. CAT was also lower in tadpoles exposed to 0.1 mg/L

than the control group at 28°C (P=0.05). G6PDH and *t*GSH had no changes after exposure to sulfentrazone at this temperature (P>0.05). Significant temperature-associated increases of G6PDH activity were observed in all groups treated at 32°C compared to control animals at the same temperature (P<0.001). Increases up to 4-fold in G6PDH were also observed as result of an interaction between sulfentrazone and temperature at 32°C compared to untreated animals at 28°C (P<0.001). SOD activity was increased by the interaction effect of the higher concentration of sulfentrazone at 32°C compared to the control at 28°C (P=0.0006). No effects of sulfentrazone on GST (P=0.9), CAT (P=0.102) and *t*GSH (P=0.16) were observed for treatments at 32°C after three days. Treatments at 36°C decreased CAT activity in all concentrations compared to the control at 28°C (P<0.001). In contrast, SOD and G6PDH activities were increased following exposure to 0.05 and 0.1 mg/L at 36°C compared to the control at 28°C (P<0.01). No effects of sulfentrazone were observed on GST and tGSH at 36°C after three days (P>0.05) (Fig. 3).

After eight days of exposure, sulfentrazone treatments at 28°C did not alter antioxidant response of the animals, except for *t*GSH levels, which were increased in tadpoles exposed to 0.05 mg/L (P=0.001). The activity of the enzymes CAT, SOD and G6PDH were also not altered following the treatments at 32°C (P>0.05). In addition, no differences were observed in *t*GSH levels at this temperature (P>0.05). GST was the only enzyme altered by sulfentrazone at 32°C, with a 1.5-fold decrease in animals exposed to 0.1 mg/L (P=0.024). Also, animals exposed to 0.01 mg/L at 32°C had higher GST activity than the same treatment at 28°C (P=0.006). The highest levels of CAT were observed in tadpoles exposed to all concentrations of sulfentrazone at 36°C after eight days. G6PDH activity was decreased in tadpoles treated with 0.05 and 0.1 mg/L of sulfentrazone at 36°C compared to the control at the same temperature (P=0.002, P=0.0001, respectively). However, the interaction effect of the higher temperature and

sulfentrazone promoted a pronounced increase of G6PDH (increases up to 4.5-fold) in comparison to the other treatments at 28 and 32°C (P<0.001). SOD, GST and *t*GSH had no changes after eight days of treatments at 36°C (P>0.05) (Fig. 4).

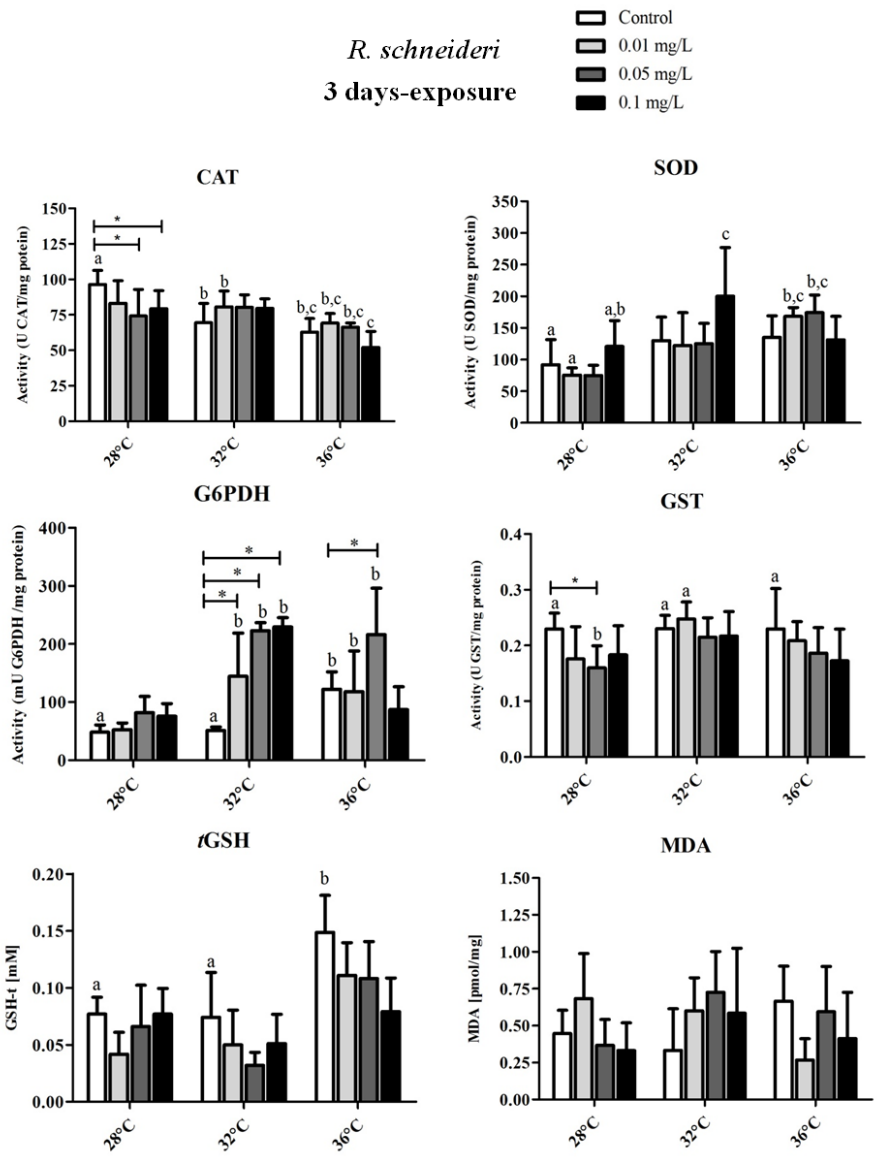

Figure 3: Antioxidant enzymes (SOD, CAT, GST and G6PDH) activity and *t*GSH and MDA levels in tadpoles of *R. schneideri* exposed to sulfentrazone at different temperatures (28°C, 32°C and 36 °C) after three days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05). Standard deviations are represented in bars.

Unlike *E. nattereri*, temperature had no isolated effects on lipid peroxidation in *R. schneideri* tadpoles. Furthermore, exposure to sulfentrazone at any temperature and concentration changed MDA levels in this species (P>0.05) (Figs. 3 and 4).

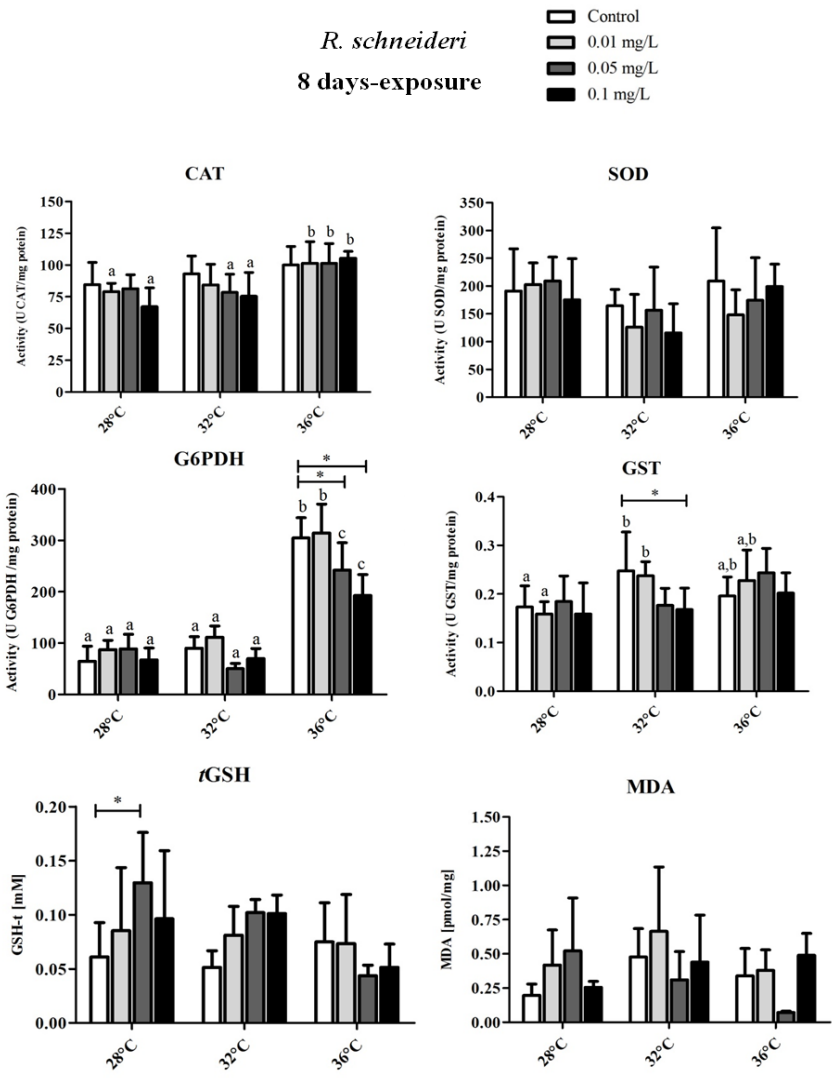

Figure 4: Antioxidant enzymes (SOD, CAT, GST and G6PDH) activity and *t*GSH and MDA levels in tadpoles of *R. schneideri* exposed to sulfentrazone at different temperatures (28°C, 32°C and 36 °C) after eight days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05). Standard deviations are represented in bars.

4. DISCUSSION

Several environmental variables can directly or indirectly affect the health of organisms in a variety of taxa (Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009; Tu et al., 2012). Changes in environmental temperatures are of particular concern because they may not only change the toxicity of chemical compounds, but also the physiological responses of cold-blooded organisms and their ability to deal with contaminants in the environment (Hooper et al., 2013). Antioxidant responses are expected to be affected by changes in environmental temperature in these organisms, since heat exposure can dramatically increase ROS production as result of an increased oxygen consumption (Lushchak, 2011). However, there is a lack of data clarifying how antioxidant systems of aquatic organisms may alter with high temperatures in contaminated environments. Amphibians are one of the most vulnerable vertebrate groups to effects of combinations of environmental stressors in their habitats. Surprisingly, studies on amphibians are scarce. In the current study, we showed that antioxidant enzymes in tadpoles of *E. nattereri* and *R. schneideri* had their responses altered by single and combined exposures to sulfentrazone and temperature treatment. Temperature-associated responses of the antioxidant enzymes and MDA levels in tadpoles exposed to sulfentrazone indicate that temperature plays a relevant role in the toxicity of this herbicide in tadpoles of both studied species. The different patterns found in the measured variables in *E. nattereri* and *R. schneideri* support the concept that expression of biomarkers driven by environmental conditions may be species-specific, even for taxonomically related groups. According to previous studies (Gripp et al., 2017; Harley et al., 2006; Lushchak, 2011), we suggest that such alterations to antioxidant defence systems are associated with the adaptation mechanisms used by these organisms to deal with increased ROS production in animals exposed to stressful environmental conditions.

The antioxidant defence system is crucial for counteracting damage caused by ROS in aerobic organisms (van der Oost et al., 2003), and enzymatic antioxidants represent a significant fraction of the cellular antioxidant response (Madeira et al., 2013; Velkova-Jordanoska et al., 2008; Vinagre et al., 2012). Single effects of temperature promoted changes in antioxidant enzymes of tadpoles not exposed to sulfentrazone. After three days, thermal treatment increased GST activity in tadpoles of *E. nattereri* maintained at 36°C compared with those maintained at 28°C. Levels of *t*GSH and G6PDH activity were lower in control tadpoles at 32°C than at 28°C. In *R. schneideri,* GST was unaffected by temperature after three days; however, CAT activity was lower in animals kept at 32°C than at 28°C. *t*GSH and G6PDH activities were higher in tadpoles at 36°C than 28°C. After eight days, temperature also displayed changes to antioxidant responses for both species. In *E. nattereri,* GST was also higher in animals of the control group at 36°C; however, G6PDH activity was normalized after a longer period, and no more changes were observed in this enzyme or in *t*GSH levels. SOD activity, which was not altered after three days in non-exposed animals, was lower in tadpoles of *E. nattereri* at 32°C after eight days. For *R. schneideri*, G6PDH activity was also higher in tadpoles maintained at 36°C after eight days, but no more effects were observed in *t*GSH levels or CAT activity. GST activity was also higher in animals maintained at 32°C than those at 28°C without contaminant. Previous studies have shown that temperature can change antioxidant response and cause oxidative stress in several aquatic organisms, such as fish (Bagnyukova et al., 2003; Lushchak and Bagnyukova, 2006; Madeira et al., 2013; Parihar et al., 1997; Vinagre et al., 2012), amphibians (Bagnyukova et al., 2003; Freitas and Almeida, 2016), bivalves (Abele et al., 2002), crustaceans (Wang et al., 2009) and other organisms (Bocchetti et al., 2008; Verlecar et al., 2007). For example, CAT activity was increased in the juvenile fish *Dicentrarchus labrax* exposed to a temperature higher (28°C)

than the optimum temperature for this species (20–25°C). This response was opposite in *Diplodus vulgaris,* where a depletion of CAT was observed (Vinagre et al., 2012). GST activity was also increased at higher temperatures in the saltwater fish *D. vulgaris* (Madeira et al., 2013), showing that this enzyme also acts to protect organisms against the stress generated by changes in environmental temperature. As mentioned before, increases in ROS production are consequences of increased oxygen consumption caused by a metabolic process stimulated by temperature changes. However, these responses may vary in different species, depending on their optimum environmental temperature and the thermal gradient at which their antioxidant system has the best performance.

Exposure to sulfentrazone caused temporal- and temperature-dependent responses in antioxidant enzymes and MDA levels for both species. Antioxidant enzymes also had different patterns following exposure to different concentrations of sulfentrazone. In tadpoles of *E. nattereri,* sulfentrazone-treated tadpoles presented several alterations on biochemical responses after three days of exposure; however, most of them were more evident at 28 and 32°C, including MDA levels, which were increased in tadpoles exposed to sulfentrazone at both temperatures. At 36°C, only G6PDH and GST activities were altered following sulfentrazone treatments after three days. On the other hand, sulfentrazone effects were more pronounced at higher temperatures after eight days of exposure. For the longer period, changes in SOD, G6PDH, GST and *t*GSH were detected in animals treated at 32 and 36°C, alongside the increase in MDA levels, which were more evident at 36°C. For *R. schneideri*, the responses of some biochemical parameters exhibited different changes compared to *E. nattereri.* In this species, most of the antioxidant enzymes had activities altered after three days of exposure, with more evident responses at higher temperatures (32 and 36°C). After longer exposure, no alterations

were observed in antioxidant enzymes in animals treated at 28°C. CAT, SOD and G6PDH had their activities normalized at 32°C and increased activity of CAT and G6PDH was only evident in tadpoles exposed to sulfentrazone at 36°C after eight days. Unlike observed in *E. nattereri*, no alterations in MDA levels were observed in *R. schneideri* tadpoles after exposure to sulfentrazone at any temperature for both periods. The results observed in *E. nattereri* and *R. schneideri* in our study showed that the behaviour of antioxidant enzymes in tadpoles exposed to sulfentrazone vary in different species depending on the temperature of exposure. Also, these responses can be different at different exposure durations, probably due to differential stimulation of the antioxidant system in an attempt to adapt the animals to such adverse conditions during an extended period. However, varied responses could also be the result of a prolonged effect of the chemical in combination with thermal stress, which could trigger further damage to the antioxidant defence system during an extended period.

Previous studies have shown that interactive effects of heat stress and pesticide exposure may impair the antioxidant defence system of some aquatic animals (Kaur et al., 2011; Tu et al., 2012). For example, the exposure to the insecticide deltamethrin combined to the heat stress treatment caused a significant decrease in the activity of CAT in the liver, kidney and gills, and an increase of GST and glutathione peroxidase (GPx) in the liver and kidney in the saltwater fish *Channa punctata* (Kaur et al., 2011). Activity of CAT was also decreased in the black tiger shrimp (*Penaeus monodon*) exposed to deltamethrin at 34°C after four days, but not at 24 and 29°C (Tu et al., 2012). According to Sekine et al. (1996), increasing or decreasing the temperature can influence toxicity of several pollutants. Synergistic effects of water temperature and herbicides have been reported for some species of fish (Moore and Waring, 2001; Tarja et al., 2003). These integrated effects may impair the antioxidant system, stimulating or inhibiting

enzyme activity in specific tissues and organisms. An increase in antioxidant enzyme activity has been reported to be a general response of aquatic organisms, such as fish and amphibians, exposed to environmental contaminants (Gripp et al., 2017; Jones et al., 2010; Paola M. Peltzer et al., 2013; Stephensen et al., 2002) as an activation process to neutralize the impacts of ROS (Lopez-Torres et al., 1993). On the other hand, decreases in antioxidant enzymes should occur when the stress condition is exceeding and cannot be compensated by the antioxidant system anymore (Kaur et al., 2011). In agreement with previous studies, the variation in antioxidant responses observed in tadpoles exposed to sulfentrazone at different thermal gradients confirms that temperature is an important factor influencing the activity of many antioxidant enzymes in aquatic organisms exposed to environmental contaminants. This could in turn affect all physiological functions and the ability of these animals to adapt to adverse conditions. These results are especially relevant for amphibians, since, until now, there has been no information on how the integrated effects of chemicals and temperatures could affect these biochemical parameters for different species.

G6PDH and GST activity were the most evident biomarkers jointly affected by sulfentrazone and thermal stress. G6PDH activity was notably altered in tadpoles exposed to sulfentrazone at higher temperatures, with a pronounced increase for both species. G6PDH is an important cytosolic enzyme that participates in the first step of the pentose phosphate pathway, maintaining the levels of NADPH in the cells (Kletzien et al., 1994). NADPH in turn reduces oxidized glutathione (GSSG) in glutathione (GSH), which is an important tripeptide that protects cells against oxidative damage (Almeida et al., 2011). Thus, cells with decreased G6PDH may be especially sensitive to oxidative stress. Previous studies with *E. nattereri* have shown that G6PDH activity was increased in tadpoles exposed to a metabolite of the insecticide fipronil

(fipronil sulfide) after seven days (Gripp et al., 2016). In our study, G6PDH activity was not only increased by sulfentrazone treatment, but also by the interaction effects of chemical and heat stress. This is probably due to an increased demand of NADPH in the cells in an attempt to reduce oxidant effects caused by these combined stress conditions. Conversely, depletion of G6PDH was observed in tadpoles of *R. schneideri* exposed to higher concentrations of sulfentrazone at 36°C after eight days when compared to the control at the same temperature. This behaviour of G6PDH in tadpoles of *R. schneideri* is probably a result of the intensified effect generated at 36°C, which was not compensated by the increased activity of this enzyme. It could impair other antioxidant NADPH-dependent factors, leading organisms to have greater vulnerability to dealing with environmental stressors.

*t*GSH levels were also modulated following treatments with sulfentrazone at different temperatures for both species. Glutathione (GSH) is the predominant nonprotein sulfhydryl in cells and is also an integral part of the antioxidant system (Almeida et al., 2011; Pastore et al., 2003; Sies, 2003). GSH content has been mentioned as a protective mechanism that aquatic organisms adopt in the initial phases of exposure to xenobiotics (Stephensen et al., 2002). It is a primordial electron donor in reductive processes for synthesis and degradation of proteins, formation of deoxyribonucleotides and for reduction of H_2O_2 and organic peroxides (Almeida et al., 2011). Studies have previously shown that GSH content is a relevant biomarker in studies evaluating the combined effects of temperature and chemical exposure for aquatic organisms. Fish (*Channa punctata*) pre-exposed to deltamethrin followed by heat stress had an increase in the GSH content in the liver and a decrease in the gills, indicating the different vulnerability of different tissues to the toxicants (Kaur et al., 2011). Higher *t*GSH levels were also reported in gills and hepatopancreas in the marine shrimp *Penaeus monodon* exposed to deltamethrin at the

highest tested temperature (34°C) than those at 29°C and 24°C (Tu et al., 2012). *t*GSH levels in the black rockfish *Sebastes schlegeli* exposed to copper were also highly influenced by environmental temperature, with higher levels of *t*GSH in different tissues of fish exposed to the metal at elevated temperatures (Min et al., 2014). In our study, the *t*GSH content had a temperature-associated response in tadpoles of *E. nattereri* and *R. schneideri,* with differential behaviour for different periods of exposure. In *E. nattereri*, levels of *t*GSH were higher in animals exposed to sulfentrazone at 28°C after three days, whereas it was increased at 32°C and decreased at 36°C after eight days. The opposite was observed for *R. schneideri*, where *t*GSH levels were increased only in tadpoles maintained at 36°C without contaminant after three days. After eight days, tadpoles exposed to 0.05 mg/L of sulfentrazone at 28°C presented the highest levels of *t*GSH. These varied responses could be in part due to the different needs of tadpoles in dealing with an oxidative stress induced by adverse conditions of temperature and chemical exposure in a pathway using GSH as a co-factor in the antioxidant response. Changes in GSH levels may also interfere in the formation of conjugates between glutathione and exogenous compounds in a reaction catalysed by glutathione-*S*-tranferases (Boyland and Chasseau, 1969), which is an essential aspect on the metabolism of xenobiotics. Increased rates of conjugate reaction, as part of the chemical metabolism, could lead to GSH depletion in the cells, increasing susceptibility to lipid peroxidation and oxidative stress (Almeida et al., 2011).

Interactive effects of thermal stress and sulfentrazone increased MDA levels only in tadpoles of *E. natereri*, suggesting that lipid peroxidation was particularly induced in this species. The isolated effect of temperature also changed the levels of MDA in tadpoles of *E. nattereri*. Animals unexposed to sulfentrazone at 32°C presented the lowest levels of MDA after three days, while, after eight days, these levels were 2-fold higher in tadpoles of the control at

36°C. Indeed, thermal stress has been reported to induce lipid peroxidation in fish (Chien and Hwang, 2001; Parihar et al., 1997), which is also evident in the findings of the present study. However, these effects can be enhanced when thermal stress is combined to chemical exposure, as observed previously in the fish *Channa punctata* exposed deltamethrin at different temperatures (Kaur et al., 2011). In our study, sulfentrazone-exposed tadpoles had lipid peroxidation induced in animals treated with higher concentrations at 28 and 32°C after three days. After eight days, MDA was higher in all groups treated at 36°C, suggesting that sulfentrazone effects could be masked by heat effects in *E. nattereri*, since lipid peroxidation had already been stimulated by temperature in longer periods of exposure. Induced lipid peroxidation has been previously reported in different species of tadpoles exposed to several pesticides such as fipronil, atrazine, glyphosate and quinclorac (Dornelles and Oliveira, 2014; Gripp et al., 2017). However, data available in the literature do not clarify how temperature fluctuations and consequently thermal stress in natural systems can potentiate damage on lipid membranes by exposure to pesticides. Lipid peroxidation is considered a major negative effect of oxidative stress as a consequence of excessive ROS generation in cells (Ojha et al., 2011). Increased levels of MDA in tadpoles of *E. nattereri* exposed to sulfentrazone and thermal stress indicates an oxidative stress condition for this species resulting from the exposure of animals to both interaction effects and temperature effects only. In addition, the lack of alterations in MDA levels in *R. schneideri* added to the antioxidant responses, which were normalized after longer exposure, suggests that this species may be less sensitive to the stress generated by the tested conditions in the field.

Acceleration of development was observed in tadpoles of *E. nattereri* exposed to 0.05 mg/L of sulfentrazone at 32°C after eight days of exposure. No alterations in metamorphosis

were observed in tadpoles of *R. schneideri* for either period. Amphibian larvae have the ability to accelerate metamorphosis in response to deteriorating conditions in the environment, such as the presence of chemicals, as well as alterations of temperature (Brande-lavridsen et al., 2010; Morand et al., 1997; Murata and Yamauchi, 2005; Paola M. Peltzer et al., 2013). Exposure to elevated temperatures usually reduces the time needed to complete metamorphosis for many amphibian species (Morand et al., 1997). This mechanism can provide benefits for the species, such as escaping mortality or toxic effects of contaminated habitats. However, it may also incur costs, such as reduced juvenile and adult survival, alterations to physiological performance and higher vulnerability to external predators (Denver et al., 1998; Goater et al., 1993; Smith, 1987). Although changes in development have not been observed in the other tested experimental conditions for *E. nattereri* and *R. schneideri*, it is reasonable to expect developmental alterations if tadpoles are exposed to higher concentrations or longer periods of exposure, which were not addressed in the present study.

In summary, we found that temperature can alter the toxic effects of sulfentrazone on certain biochemical biomarkers, importantly impairing antioxidant defence and causing oxidative stress in tadpoles. Differential activation of the antioxidant system in *E. nattereri* and *R. schneideri* suggests that tadpoles of different species may use diverse strategies and adaptive mechanisms to cope with the effects generated by exposure to agrochemicals in habitats where the temperature is a relevant influencing factor. The efficiency of the antioxidant mechanisms is crucial for the health of these organisms, since the imbalance in ROS content can cause several negative consequences, such as damage to cellular constituents and regulation of cellular process, impairing adaptation to internal and environmental changes. Such alterations could in turn result in extensive consequences at population levels, and this is particularly worrying for

native species in Brazil, where tadpoles usually experience large temperature fluctuations in their habitats. Given the highly variable results of sulfentrazone at different temperatures and in different species, it is clear that more studies need to be conducted to arrive at a general consensus of the impacts of thermal stress in the toxicity of this and other pesticides on amphibians.

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REFERENCES

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205, 1831–1841. doi:10.1016/j.ecolind.2011.04.007

Almeida, E.A., 2003. Avaliação de Variações Bioquímicas em Moluscos bivalves em Resposta ao Estresse Ambiente (Ph.D. thesis). Universidade de São Paulo – Instituto de Química, São Paulo, Brasil.

Bagnyukova, T. V., Storey, K.B., Lushchak, V.I., 2003. Induction of oxidative stress in Rana ridibunda during recovery from winter hibernation. J. Therm. Biol. 28, 21–28. doi:10.1016/S0306-4565(02)00031-1

Bao, V.W.W., Koutsaftis, A., Leung, K.M.Y., 2008. Temperature-dependent toxicities of chlorothalonil and copper pyrithione to the marine copepod Tigriopus japonicus and dinoflagellate *Pyrocystis lunula*. Australas. J. Ecotoxicol. 14, 45–54.

Bernarde, P.S., Kokubum, M.N.D.C., 1999. Anurofauna do município de Guararapes, Estado de São Paulo, Brasil (Amphibia: Anura). Acta Biol. Leopoldensia.

Beutler, E., 1975. Red Cell Metabolism: A Manual of Biochemical Methods. Grune & Straton, New York.

Blaustein, A.R., Kiesecker, J.M., 2002. Complexity in conservation: Lessons from the global decline of amphibian populations. Ecol. Lett. 5, 597–608. doi:10.1046/j.1461-0248.2002.00352.x

Blaustein, A.R., Walls, S.C., Bancroft, B.A., Lawler, J.J., Searle, C.L., Gervasi, S.S., 2010. Direct and indirect effects of climate change on amphibian populations. Diversity 2, 281–313. doi:10.3390/d2020281

Bocchetti, R., Lamberti, C.V., Pisanelli, B., Razzetti, E.M., Maggi, C., Catalano, B., Sesta, G., Martuccio, G., Gabellini, M., Regoli, F., 2008. Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams, *Tapes philippinarum*, and mussels, Mytilus galloprovincialis, from Adriatic sea. Mar. Environ. Res. 66, 24–26. doi:10.1016/j.marenvres.2008.02.013

Boone, M.D., Bridges, C.M., 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*) . Environ. Toxicol. Chem. 18, 1482–1484. doi:10.1002/etc.5620180720

Boyland, E., Chasseaud, L.F. 1969. The Role of Glutathione and Glutathione S-Transferases in Mercapturic Acid Biosynthesis. Advances in Enzymology and Related Areas of Molecular Biology. 32, 173-219.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–54.

Brande-lavridsen, N., Christensen-dalsgaard, J., Korsgaard, B., 2010. Effects of Ethinylestradiol and the fungicide Prochloraz on metamorphosis and thyroid gland morphology in *Rana temporaria*. Open Zool. … 3, 7–16. doi:10.2174/1874336601003010007

Chien, L.T., Hwang, D.F., 2001. Effects of thermal stress and vitamin C on lipid peroxidation and fatty acid composition in the liver of thornfish *Terapon jarbua*. Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 128, 91–97. doi:10.1016/S1096-4959(00)00299-2

De Almeida, E.A., Humberto Silva, D.G., Dias Bainy, A.C., Freitas, F.P., Motta, F.D., Gomes, O.F., Gennari De Medeiros, M.H., Di Mascio, P., 2011. Evaluation of Glutathione Status in Aquatic Organisms. Oxidative Stress Aquat. Ecosyst. 381–388. doi:10.1002/9781444345988.ch28

Denver, R., Mirhadi, N., Phillips, M., 1998. Adaptive plasticity in amphibian metemorphosis: Responses of *Scaphiopus hammondii* tadpoles to habitat desiccation. Ecology 79, 1859–1873. doi:10.1890/0012-9658(1998)079[1859:APIAMR]2.0.CO;2

Dornelles, M.F., Oliveira, G.T., 2014. Effect of atrazine, glyphosate and quinclorac on biochemical parameters, lipid peroxidation and survival in bullfrog tadpoles (*Lithobates catesbeianus*). Arch. Environ. Contam. Toxicol. 66, 415–429. doi:10.1007/s00244-013-9967-4

Dutra De Armas, E., Rosim Monteiro, R.T., Valler Amâncio, A., Lopes Correa, R.M., Guercio, M.A., 2005. Uso de agrotóxicos em cana-de-açúcar na bacia do rio Corumbataí e o risco de poluição hídrica. Quim. Nova 28, 975–982. doi:10.1590/S0100-40422005000600008

Egea-Serrano, A., Relyea, R.A., Tejedo, M., Torralva, M., 2012. Understanding of the impact of chemicals on amphibians: A meta-analytic review. Ecol. Evol. 2, 1382–1397. doi:10.1002/ece3.249

Embrapa., 2013. Empresa Brasileira de Pesquisa Agropecuária. Plantas Daninhas. Disponível em:http://www.agencia.cnptia.embrapa.br/gestor/cana-de-acucar/arvore/CONTAG01_52_711200516718.

Fontenot, C.L., Lutterschmidt, W.I., 2011. Thermal selection and temperature preference of the aquatic salamander, *Amphiuma tridactylum*. Herpetol. Conserv. Biol. 6, 395–399.

Freitas, J.S., Almeida, E.A., 2016. Antioxidant Defense System of Tadpoles (*Eupemphix nattereri*) Exposed to Changes in Temperature and pH. Zoolog. Sci. 33, 186–194. doi:10.2108/zs150075

Freitas, J.S., Kupsco, A.J., Diamante, G., Felicio, A.A., Alves de Almeida, E., Schlenk, D., 2016. Influence of temperature on the thyroidogenic effects of Diuron and its metabolite 3,4-DCA in tadpoles of the American bullfrog *(Lithobates catesbeianus*). Environ. Sci. Technol. acs.est.6b04076. doi:10.1021/acs.est.6b04076

Gatten, R. E., Miller, K., Full, R. J., 1992. Energetics at rest and during locomotion. In Environmental Physiology of the Amphibians (ed. M. E. Feder and W. W. Burggren), pp. 314–377. Chicago: The University of Chicago Press.

Glock, G.E., McLean, P., 1953. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J. 55, 400–8.

Goater, C.P., Semlitsch, R.D., Bernasconi, M. V, Goater, C.P., Semlitsch, R.D., Bernasconi, M. V, 2016. Nordic Society Oikos Effects of Body Size and Parasite Infection on the Locomotory Performance of Juvenile Toads , *Bufo bufo* Published by : Wiley on behalf of Nordic Society Oikos Stable URL : http://www.jstor.org/stable/3545205 REFERENCES Linked referenc 66, 129–136.

Gosner, K., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183–190.

Greulich, K., Pflugmacher, S., 2003. Differences in susceptibility of various life stages of amphibians to pesticide exposure. Aquat. Toxicol. 65, 329–336. doi:10.1016/S0166-445X(03)00153-X

Gripp, H.S., Freitas, J.S., Almeida, E.A., Bisinoti, M.C., Moreira, A.B., 2017. Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae). Ecotoxicol. Environ. Saf. 136, 173–179. doi:10.1016/j.ecoenv.2016.10.027

Hammond, S.A., Veldhoen, N., Helbing, C.C., 2015. Influence of temperature on thyroid hormone signaling and endocrine disruptor action in *Rana (Lithobates) catesbeiana* tadpoles. Gen. Comp. Endocrinol. 219, 6–15. doi:10.1016/j.ygcen.2014.12.001

Harley, C.D.G., Hughes, A.R., Hultgren, K.M., Miner, B.G., Sorte, C.J.B., Thornber, C.S., Rodriguez, L.F., Tomanek, L., Williams, S.L., 2006. The impacts of climate change in coastal marine systems. Ecol. Lett. 9, 228–241. doi:10.1111/j.1461-0248.2005.00871.x

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M., 2006. Monograph Pesticide Mixtures , Endocrine Disruption , and Amphibian Declines : Are We Underestimating the Impact ? 40. doi:10.1289/ehp.8051

Hirata, S., 2010. Lixiviação e persistência dos herbicidas sulfentrazone e imazapic Leaching and Persistence of Sulfentrazone and Imazapic 28, 185–195. doi:10.1590/S0100-83582010000100022

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32, 32–48. doi:10.1002/etc.2043

IPT, Instituto de Pesquisas tecnológicas do estado de São Paulo, 2000. Diagnóstico da situação

atual dos recursos hídricos e estabelecimento de diretrizes técnicas para a elaboração do plano da Bacia de São José dos Dourados e Relatório número 40675 e Minuta. CR Room.

Jones, L., Gossett, D., Banks, S., McCallum, M., 2010. Antioxidant defense system in tadpoles of the American bullfrog (*Lithobates catesbeianus*) exposed to paraquat. J. Herpetol. 44, 222–228. doi:10.1670/07-243.1

Katzenberger, M., Tejedo, M., Duarte, H., Marangoni, F., Beltrán, J.F., 2012. Tolerância e sensibilidade térmica em anfíbios. Rev. da Biol. 8, 25–32. doi:10.7594/revbio.08.05

Kaur, M., Atif, F., Ansari, R.A., Ahmad, F., Raisuddin, S., 2011. The interactive effect of elevated temperature on deltamethrin-induced biochemical stress responses in *Channa punctata* Bloch. Chem. Biol. Interact. 193, 216–224. doi:10.1016/j.cbi.2011.06.011

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for the several activities of the glutathione S-transferases. J. Biol. Chem. 251, 6183–8.

Lopez-Torres, M., Perez-Campo, R., Cadenas, S., Rojas, C., Barja, G., 1993. A comparative study of free radicals in vertebrates-2. Nonenzymatic antioxidants and oxidative stress. Comp. Biochem. Physiol. B-Biochemistry Mol. Biol. 105, 757–763.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. doi:10.1016/j.aquatox.2010.10.006

Lushchak, V.I., Bagnyukova, T. V., 2006. Temperature increase results in oxidative stress in goldfish tissues. 1. Indices of oxidative stress. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 143, 30–35. doi:10.1016/j.cbpc.2005.11.017

Madeira, D., Narciso, L., Cabral, H.N., Vinagre, C., Diniz, M.S., 2013. Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 166, 237–243. doi:10.1016/j.cbpa.2013.06.008

Martinez, C.O., Silva, C.M.M.S., Fay, E.F., Abakerli, R.B., Maia, A.H.N., Durrant, L.R., 2010. Microbial degradation of sulfentrazone in a Brazilian rhodic hapludox soil. Brazilian J. Microbiol. 41, 209–217. doi:10.1590/S1517-83822010000100030

Mcdiarmid, Roy.W and Altig, R., 1999. Tadpoles: The Biology of Anuran Larvae, McDiarmid, Altig. Chicago.

Melo, C., Medeiros, W., Tuffi Santos, L.D., FERREIRA, F., Ferreira, G., Paes, F.A.S.V., Reis, M.R., 2010. Residual effect of sulfentrazone, isoxaflutole and oxyfluorfen in three soils. Planta Daninha 28, 835–842.

Middlebrooks, E.J., Gaspar, M.J., Gaspar, R.D., Reynolds, J.H., 1973. Effects of Temperature on the Toxicity to the Aquatic Biota of Waste Discharges - A Compilation of the Literature.

Min, E., Baeck, S., Kang, J.-C., 2014. Combined effects of copper and temperature on Hematological constituents in the Rock fish, *Sebastes schlegeli*. J. fish Pathol. 27, 57–65. doi:10.7847/jfp.2014.27.1.057

Moe, S.J., De Schamphelaere, K., Clements, W.H., Sorensen, M.T., Van den Brink, P.J., Liess, M., 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ. Toxicol. Chem. 32, 49–61. doi:10.1002/etc.2045

Moore, A., Waring, C.P., 2001. The effects of a synthetic pyrethroid pesticide on some aspects of reproduction in Atlantic salmon (*Salmo salar L.*). Aquat. Toxicol. 52, 1–12. doi:10.1016/S0166-445X(00)00133-8

Morand, A., Joly, P., Grolet, O., 1997. Phenotypic variation in metamorphosis in five anuran species along a gradient of stream influence. Comptes Rendus l'Academie des Sci. - Ser. III 320, 645–652. doi:10.1016/S0764-4469(97)85698-3

Morey, S., Reznick, D., Fish, U.S., Service, W., Avenue, N.E., 2017. Effects of Larval Density on Postmetamorphic Spadefoot Toads (*Spea hammondii*) Author (s): Steven Morey and David Reznick Published by : Wiley Stable URL : http://www.jstor.org/stable/2679876 REFERENCES Linked references are available on JSTOR for this 82, 510–522.

Murata, T., Yamauchi, K., 2005. Low-temperature arrest of the triiodothyronine-dependent transcription in *Rana catesbeiana* red blood cells. Endocrinology 146, 256–264. doi:10.1210/en.2004-1090

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: Environmental contaminants in a warming world. Environ. Int. 35, 971–986. doi:10.1016/j.envint.2009.02.006

Ojha, A., Yaduvanshi, S.K., Srivastava, N., 2011. Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues. Pestic. Biochem. Physiol. 99, 148–156. doi:10.1016/j.pestbp.2010.11.011

Parihar, M.S., Javeri, T., Hemnani, T., Dubey, A.K., Prakash, P., 1997. Responses of superoxide dismutase, glutathione peroxidase and reduced glutathione antioxidant defenses in gills of the freshwater catfish (*Heteropneustes fossilis*) to short-term elevated temperature. J. Therm. Biol. 22, 151–156. doi:10.1016/S0306-4565(97)00006-5

Pastore, A., Federici, G., Bertini, E., Piemonte, F., 2003. Analysis of glutathione: Implication in redox and detoxification. Clin. Chim. Acta 333, 19–39. doi:10.1016/S0009-8981(03)00200-6

Peltzer, P.M., Lajmanovich, R.C., Attademo, A.M., Junges, C.M., Cabagna-Zenklusen, M.C., Repetti, M.R., Sigrist, M.E., Beldoménico, H., 2013. Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (*Trachycephalus typhonius*) tadpoles. Ecotoxicol. Environ. Saf. 98, 142–51. doi:10.1016/j.ecoenv.2013.09.010

Prado, V.H.M. Do, Borges, R.E., Silva, F.R. Da, Tognolo, T.T., Rossa-Feres, D.D.C., 2008. Amphibia, Anura, Hylidae, *Phyllomedusa azurea*: Distribution extension. Check List 4, 55–56.

Provete, D.B., Garey, M.V., Silva, F.R. Da, Rossa-Feres, D.D.C., 2011. Anurofauna do noroeste paulista: lista de espécies e chave de identificação para adultos. Biota Neotrop. 11, 377–391. doi:10.1590/S1676-06032011000200036

Relyea, R.A., 2005. The lethal impacts of roundup and predatory stress on six species of North American tadpoles. Arch. Environ. Contam. Toxicol. 48, 351–357. doi:10.1007/s00244-004-0086-0

Rohr, J.R., Palmer, B.D., 2005. Aquatic Herbicide Exposure Increases Salamander Desiccation Risk Eight Months Later in a Terrestrial Environment. Environ. Toxicol. Chem. 24, 34.

Rome, L.C., 2007. The effect of temperature and thermal acclimation on the sustainable performance of swimming scup. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 362, 1995–2016. doi:10.1098/rstb.2007.2083

Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. Mar. Pollut. Bull. 54, 1845–1856. doi:10.1016/j.marpolbul.2007.09.020

Sekine, M., Nakanishi, H., Ukita, M., 1996. Study on fish mortality caused by the combined effects of pesticides and changes in environmental conditions Pyridaphenthi ~ 86, 259–264.

Shinn, C., Marco, A., Serrano, L., 2008. Inter- and intra-specific variation on sensitivity of larval amphibians to nitrite. Chemosphere 71, 507–514. doi:10.1016/j.chemosphere.2007.09.054

Sies, H., 2003. Assay of Glutathioine Glutathione Disulfide and Glutathione Miced Disulfides in Biological Samples 77, 10.

Smith, D.C., 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology 68, 344–350. doi:10.2307/1939265

Snodgrass, J.W., Casey, R.E., Joseph, D., Simon, J.A., 2008. Microcosm investigations of stormwater pond sediment toxicity to embryonic and larval amphibians: Variation in sensitivity among species. Environ. Pollut. 154, 291–297. doi:10.1016/j.envpol.2007.10.003

Sokal, R. and R.J., 1995. Biometry: The Principles and Practices of Statistics in Biological Research: New York.

Stefani Margarido, T.C., Felício, A.A., de Cerqueira Rossa-Feres, D., Alves de Almeida, E., 2013. Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. Mar. Environ. Res. 91, 61–67. doi:10.1016/j.marenvres.2013.02.001

Stephensen, E., Sturve, J., Förlin, L., 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 133, 435–442. doi:10.1016/S1532-0456(02)00129-1

Tarja, N., Kirsti, E., Marja, L., Kari, E., 2003. Thermal and metabolic factors affecting bioaccumulation of triazine herbicides by rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. 18, 219–226. doi:10.1002/tox.10118

Thorngren, J.L., Harwood, A.D., Murphy, T.M., Huff Hartz, K.E., Fung, C.Y., Lydy, M.J., 2016. Fate and risk of atrazine and sulfentrazone to non-target species at an agriculture site. Environ. Toxicol. Chem. n/a-n/a. doi:10.1002/etc.3664

Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27, 502–522. doi:10.1016/0003-2697(69)90064-5

Tu, H.T., Silvestre, F., Meulder, B. De, Thome, J.P., Phuong, N.T., Kestemont, P., Thi, H., Silvestre, F., Meulder, B. De, Thome, J.P., Thanh, N., Kestemont, P., 2012. Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (*Penaeus monodon*). Chemosphere 86, 83–91. doi:10.1016/j.chemosphere.2011.09.022

van der Oost, R., Beyer, J., Vermeulen, N.P.., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149. doi:10.1016/S1382-6689(02)00126-6

Velkova-Jordanoska, L., Kostoski, G., Jordanoska, B., 2008. Antioxidative enzymes in fish as biochemical indicators of aquatic pollution. Bulg. J. Agric. Sci. 14, 235–237.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2007. Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. Chem. Biol. Interact. 167, 219–226. doi:10.1016/j.cbi.2007.01.018

Vinagre, C., Madeira, D., Narciso, L., Cabral, H.N., Diniz, M., 2012. Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecol. Indic. 23, 274–279. doi:10.1016/j.ecolind.2012.04.009

Wang, W., Zhou, J., Wang, P., Tian, T., Zheng, Y., Liu, Y., Mai, W., Wang, A., 2009. Comparative Biochemistry and Physiology , Part C Oxidative stress , DNA damage and antioxidant enzyme gene expression in the Paci fi c white shrimp , *Litopenaeus vannamei* when exposed to acute pH stress. Comp. Biochem. Physiol. Part C 150, 428–435. doi:10.1016/j.cbpc.2009.06.010

Yan, D., Jiang, X., Xu, S., Wang, L., Bian, Y., Yu, G., 2008. Quantitative structure-toxicity relationship study of lethal concentration to tadpole (*Bufo vulgaris formosus*) for organophosphorous pesticides. Chemosphere 71, 1809–15. doi:10.1016/j.chemosphere.2008.02.033

Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall.

Supplementary material

Influence of temperature on the antioxidant responses and lipid peroxidation of two species of tadpoles (*Rhinella schneideri* and *Eupemphix nattereri*) exposed to the herbicide sulfentrazone (Boral 500SC®)

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Table S1: Mean values of the body weight, snout-vent length and stage of development of tadpoles from *E. nattereri* exposed to sulfentrazone at different temperatures

Table S2: Mean values of the body weight, snout-vent length and stage of development of tadpoles from *R. schneideri* exposed to sulfentrazone at different temperatures

Table S3: Table summarizing stage of development of tadpoles from *E. nattereri* after 3-days exposure to sulfentrazone

Table S4: Table summarizing stage of development of tadpoles from *E. nattereri* after 8-days exposure to sulfentrazone

Table S5: Table summarizing stage of development of tadpoles from *R. schneideri* after 3-days exposure to sulfentrazone

Table S6: Table summarizing stage of development of tadpoles from *R. schneideri* after 8-days exposure to sulfentrazone

Table S1: Mean values (±S.E.M) of the body weight, snout-vent length (SVL) and the effects on the stage of development (*N*: non-affected, +: acceleration on development) in tadpoles of *E. nattereri* exposed to sulfentrazone (Boral®SC) during 3 and 8 days at different temperatures (ANOVA-2-way, Tukey test, $P < 0.05$).

	28°C				32°C				36°C			
3 DAYS	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**
Weight	0.176 ± 0.04	0.180 ± 0.04	0.173 ± 0.04	0.167 ± 0.03	0.162 ± 0.05	0.181 ± 0.03	0.158 ± 0.06	0.144 ± 0.05	0.177 ± 0.05	0.198 ± 0.06	0.189 ± 0.05	0.189 ± 0.05
Length	1.00 ± 0.09	0.98 ± 0.28	0.98 ± 0.12	0.98 ± 0.06	0.96 ± 0.11	1.02 ± 0.06	0.094 ± 0.12	0.091 ± 0.11	0.98 ± 0.11	1.02 ± 0.05	0.89 ± 0.29	0.97 ± 0.08
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*
8 DAYS												
Weight	0.127 ± 0.04 a	0.175± 0.04	0.139 ± 0.02	0.167 ± 0.04	0.129 ± 0.05	0.147 ± 0.04	0.159 ± 0.06	0.135 ± 0.01	0.187 ± 0.04 b	0.154 ± 0.02	0.184 ± 0.04	0.171 ± 0.03
Length	0.94 ± 0.11	0.98 ± 0.07	0.98 ± 0.05	1.02 ± 0.07	0.89 ± 0.13	0.84 ± 0.26	0.98 ± 0.08	0.96 ± 0.05	1.03 ± 0.04	0.93 ± 0.06	0.96 ± 0.06	1.0 ± 0.09
Stage	*N*	*N*	*N*	*N*	*N*	*N*	+	*N*	*N*	*N*	*N*	*N*

Table S2: Mean values (±S.E.M) of the body weight, snout-vent length (SVL) and the effects on the stage of development (*N*: non-affected, +: acceleration on development) in tadpoles of *R. schneideri* exposed to sulfentrazone (Boral®SC) during 3 and 8 days at different temperatures (ANOVA-2-way, Tukey test, $P < 0.05$).

	28°C				32°C				36°C			
3 DAYS	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**
Weight	0.063 ± 0.02	0.057 ± 0.01	0.050 ± 0.01	0.053 ± 0.01	0.064 ± 0.03	0.069 ± 0.01	0.050 ± 0.01	0.066 ± 0.01	0.060 ± 0.01	0.064 ± 0.01	0.062 ± 0.01	0.058 ± 0.01
Length	0.68 ± 0.10	0.74 ± 0.05	0.67 ± 0.05	0.68 ± 0.07	0.73 ± 0.12	0.77 ± 0.09	0.70 ± 0.06	0.71 v 0.07	0.67 ± 0.07	0.65 ± 0.10	0.75 ± 0.08	0.72 ± 0.10
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*
8 DAYS												
Weight	0.074 ± 0.02	0.083 ± 0.03	0.060 ± 0.02	0.056 ± 0.01	0.075 ± 0.02	0.066 ± 0.01	0.078 ± 0.02	0.065 ± 0.02	0.065 ± 0.02	0.076 ± 0.02	0.057 ± 0.01	0.058 ± 0.02
Length	0.75 ± 0.06 a	0.82 ± 0.05	0.71 ± 0.09	0.72 ± 0.11 a	0.83 ± 0.10	0.76 ± 0.11	0.74 ± 0.26	0.86 ± 0.09 b	0.73 ± 0.12	0.75 ± 0.12	0.74 ± 0.06	0.72 ± 0.04 a
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*

Table S3: Table summarizing stage of development (Gosner, 1960) of tadpoles from *E. nattereri* after 3-days exposure to sulfentrazone. Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development									
		stg30	stg31	stg32	stg33	stg34	stg35	stg36	stg37	stg38	stg39
28°C	Control	0	0	0	5	3	2	0	0	0	0
	0.01mg/L	0	0	2	3	2	3	0	0	0	0
	0.05 m/L	0	0	2	4	0	4	0	0	0	0
	0.1 mg/L	0	0	2	5	0	3	0	0	0	0
32°C	Control	0	1	2	2	4	0	0	0	0	0
	0.01mg/L	0	0	1	5	4	0	0	0	0	0
	0.05 m/L	0	2	1	2	3	2	0	0	0	0
	0.1 mg/L	1	0	4	4	1	0	0	0	0	0
36°C	Control	0	0	3	2	2	3	0	0	0	0
	0.01mg/L	0	0	0	2	3	4	1	0	0	0
	0.05 m/L	0	0	1	1	4	2	0	0	0	2
	0.1 mg/L	0	0	2	1	0	3	0	1	0	3

Table S4: Table summarizing stage of development (Gosner, 1960) of tadpoles from *E. nattereri* after 8-days exposure to sulfentrazone. Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development										
		stg29	stg30	stg31	stg32	stg33	stg34	stg35	stg36	stg37	stg38	stg39
28°C	Control	3	0	0	1	3	1	1	0	0	1	0
	0.01mg/L	0	0	4	2	1	2	1	0	0	0	0
	0.05 m/L	0	0	0	7	1	0	0	2	0	0	0
	0.1 mg/L	0	0	0	2	2	3	0	3	0	0	0
32°C	Control	1	2	0	1	3	2	0	0	0	1	0
	0.01mg/L	2	0	0	0	4	3	1	0	0	0	0
	0.05 m/L	0	0	3	2	0	0	2	3	0	0	0
	0.1 mg/L	0	0	0	2	2	2	2	2	0	0	0
36°C	Control	0	0	0	0	0	6	2	2	0	0	0
	0.01mg/L	0	0	0	2	0	2	2	4	0	0	0
	0.05 m/L	0	0	0	0	0	2	2	0	6	0	0
	0.1 mg/L	0	0	0	0	6	0	2	2	0	0	0

Table S5: Table summarizing stage of development (Gosner, 1960) of tadpoles from *R. schneideri* after 3-days exposure to sulfentrazone. Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development							
		stg28	stg29	stg30	stg31	stg32	stg33	stg34	stg35
28°C	Control	0	0	0	2	3	3	1	1
	0.01mg/L	0	0	0	1	3	4	1	1
	0.05 m/L	1	0	0	1	5	0	3	0
	0.1 mg/L	0	0	0	6	1	0	3	0
32°C	Control	0	0	2	2	1	1	2	1
	0.01mg/L	0	0	1	3	0	0	4	2
	0.05 m/L	0	0	0	5	3	1	1	0
	0.1 mg/L	0	0	1	2	2	3	2	0
36°C	Control	0	0	0	3	3	1	2	1
	0.01mg/L	0	0	2	1	1	1	2	2
	0.05 m/L	0	0	0	2	2	1	3	2
	0.1 mg/L	0	0	0	1	4	1	2	1

Table S6: Table summarizing stage of development (Gosner, 1960) of tadpoles from *R. schneideri* after 8-days exposure to sulfentrazone. Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development							
		stg28	stg29	stg30	stg31	stg32	stg33	stg34	stg35
28°C	Control	0	1	2	1	3	2	1	0
	0.01mg/L	2	0	0	3	3	2	0	0
	0.05 m/L	0	1	1	4	4	0	0	0
	0.1 mg/L	2	1	2	2	2	1	0	0
32°C	Control	0	0	0	4	4	1	0	1
	0.01mg/L	0	0	0	3	3	2	2	0
	0.05 m/L	1	2	1	2	3	0	1	0
	0.1 mg/L	1	0	1	3	3	1	1	0
36°C	Control	1	1	1	4	2	1	0	0
	0.01mg/L	3	0	1	2	2	2	0	0
	0.05 m/L	0	3	2	2	2	1	0	0
	0.1 mg/L	0	0	1	3	4	1	1	0

Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Eupemphix nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles

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Abstract

Temperature is an important factor influencing the toxicity of chemicals in aquatic environments. Amphibians from neotropical areas can experience large temperature fluctuation in their habitats, especially during the larval stage, when tadpoles are restrict to small and ephemeral ponds. If located in agricultural areas, ponds can be contaminated by several pesticides and therefore there is a great concern that tadpoles are being affected by the combined effects of temperature and local pesticides in natural systems. Clomazone is one of the most used herbicides in sugar cane and rice crops in Brazil and it can cause extensive contamination due to its high solubility in water. In this study, we evaluated the effects caused by the exposure to clomazone (Gamit®) (0.01, 0.05, 0.1 mg/L^{-1}) at different temperatures (28, 32 and 36 °C) on biochemical stress responses and esterase activities in tadpoles of *Eupemphix nattereri* and *Rhinella schneideri* after three and eight days. Results showed that temperature was an important factor inducing biochemical response in tadpoles exposed to clomazone. Isolated effects of temperature also induced antioxidant enzymes activities at higher temperatures, especially for *E. nattereri*. Catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PDH) had their activities increased in tadpoles of *E. nattereri* exposed to combined effects o clomazone and higher temperatures, with more evident responses after three days. The biotransformation enzyme glutathione-*S*-transferase (GST) was also increased by exposure to clomazone at 32 and 36 °C. SOD was the only enzyme increased at 28 °C. In *R. schneideri*, clomazone failed to alter antioxidant enzymes at 28 °C for both periods, excepted by G6PDH, which had the activity decreased after eight days. SOD and GST activities were also increased by clomazone at higher temperatures in *R. schneideri* after three days, but all enzymes had their activities returned to the control levels after eight days. *t*GSH contents were unchanged in *E. nattereri*, but it was decreased in all groups treated at higher temperatures after both periods in *R. schneideri*. Lipid peroxidation was induced by clomazone after longer exposure in *E. nattereri* and *R. schneideri* at 32 and 36 °C, but not at 28 °C. Esterase analysis also showed that AChE was not sensitive to clomazone and thermal stress conditions, and most treatments impaired CbE activity with different behavior to the studied species. Our results evidenced that temperature modulate the effects of clomazone on biochemical response of tadpoles. This implies that tadpoles from tropical areas may present differential responses in their physiological mechanism linked to antioxidant defence to deal with temperature fluctuations and agrochemicals presence in their habitats. Thus, we highlight the need to consider the abiotic factors in further studies assessing pesticides impact in amphibian species.

Keywords: Temperature, pesticides, clomazone, neotropical tadpoles, oxidative stress, esterase.

INTRODUCTION

Temperature is an important variable that has direct impacts for aquatic ectotherms, such as amphibians. Changes on environmental temperature can alter behavior, metabolism, development, digestion and vision of amphibians, resulting in altered physiological performance in the environment (Murata and Yamauchi 2005; Rome 2007). Indeed, temperature can also interfere in the amphibian's responses triggered by environmental contaminants in natural systems (Noyes et al. 2009; Hooper et al. 2013), and one of the current challenges in environmental toxicology is to understand how abiotic factors, such as temperature fluctuations can interfere on the effects of chemical compounds in aquatic organisms.

Amphibians can experience large temperature fluctuations in their habitats, especially during the larval stages, in which they are restricted to small and ephemeral ponds, where the water occupies small volumes and then can be rapidly heated due to sun incidence (Wilbur 1990). In tropical areas, the water of these habitats can reach elevated temperatures (up to 40 °C) during the summer, which is the period of reproduction for many amphibian species (Freitas et al. 2016). Higher water temperature diminishes the availability of dissolved oxygen, accelerates ventilation and the metabolism, enhancing the oxygen demand of cold-blooded animals (Kaur et al. 2011), and possibly increasing the susceptibility to the effects of xenobiotics (Blaustein et al. 2010; Moe et al. 2013). Gradual change in temperature can be physiologically compensated by the organism, however a rapid change disturbs homeostasis and may becomes a stress (Kaur et al. 2011), especially in the presence of environmental pollutants.

In Brazil, open areas used by amphibians for breeding are being gradually invaded by agriculture. After intense periods of rain, soils of agriculture areas can be flooded forming ephemeral ponds used by amphibians to spawn. However, rainwater usually carries several

agrochemicals, such as insecticides and herbicides from soil to the water fraction by leaching, making them available to tadpoles, and often at higher concentrations than those found in rivers and lakes.

The rapid expansion of sugarcane plantations due to the increased biofuel demand has contributed significantly to the use of pesticides in Brazil (Kissmann, 1991). Clomazone (2-(2-clorofenil) metil-4-dimetil-3-isoxazolidinone) is an herbicide widely used in sugarcane crops, but also widely applied in rice, potato and cotton plantations. In 2009, clomazone ranked among the top ten herbicides most widely used in Brazil (IBAMA, 2010). Although very effective, clomazone causes extensive environmental contamination due to its high solubility in water (1100 $mg.L^{-1}$), besides a half-life that can reach 84 days (Colby et al., 1989; Zanella et al., 2002). This herbicide is often applied in sugarcane culture from September to March, corresponding to the spring/summer period of the year. This period coincides with the higher rain incidence in some areas, such as the northwest region of the São Paulo state, in which 36 species of anuran were registered (Bernarde and Kokubum 1999; Prado et al. 2008; Provete et al. 2011). Therefore, the intensive use of clomazone in sugarcane during the reproductive period of anurans in the northwest of São Paulo State would represent a risk for the anuran populations due to runoff of this herbicide to the aquatic environment, especially considering the influence of the higher temperatures recorded in this period of the year.

Environmental contaminants can lead organisms to oxidative stress, a condition that is also triggered by increases of temperature (Almeida and Mascio 2011; Vinagre et al. 2012; Madeira et al. 2013; Stefani Margarido et al. 2013; Dornelles and Oliveira 2014; Freitas and Almeida 2016; Gripp et al. 2017). Studies have indicated that changes on environmental temperature can alter physiological stress response in aquatic organisms, and that ROS production is usually increased at higher temperatures as a consequence of an increased metabolism which leads to

higher oxygen consumption (Lushchak and Bagnyukova 2006; Almeida and Mascio 2011). Moreover, it is also known that temperature can also modulate neurotoxicity of acetylcholinesterase (AChE) inhibitors (Beauvais et al. 2002; Durieux et al. 2011). Previous studies have shown that clomazone cause suppression of the antioxidant enzyme catalase (CAT) and enhance lipid peroxidation levels in fish (*Rhamdia quelen*) (Crestani et al. 2007), besides activating the activity of biotransformation enzyme glutathione-*S*-tranferase (GST) in *Prochilodus lineatus* (Pereira et al. 2013). Moreover, clomazone was also reported as an inhibitor of AChE in brain and muscles of *P. lineatus* (Pereira et al. 2013), suggesting a neurotoxic effect of this herbicide despite at relatively high concentration (10 $mg.L^{-1}$). However, the implication of temperature elevation on oxidative stress parameters and AChE inhibition caused by clomazone is not yet known, especially for amphibians. In fact, the toxic effects of clomazone for amphibians are almost unknown, especially considering studies with field species. There is only a recent study showing that acute exposure (96hrs) to clomazone changed the hepatic response such as, melanomacrophages contents, accumulation of eosinophils and occurrence of lipidosis in the hepatocytes in tadpoles of the American bullfrog (*Lithobates catesbeianus*) at environmental concentrations (0.5 $mg.L^{-1}$) (Oliveira et al. 2016).

Considering these points, this study aimed to investigate the effects of the clomazone-based herbicide (Gamit® 500) in two species of tadpoles *Eupemphix nattereri* and *Rhinella schneideri* under different thermal conditions (28, 32 and 36 °C). Both species have wide geographical distribution in Brazil, including sites of intense agriculture practice and sugar cane plantation (Provete et al. 2011). *E. nattereri* has the current population trend designed as decreasing and its major threats is the spread of intensive agriculture in the Cerrado biome. *R. schneideri* is generally a common and increasing species, although its range appears to be contracting in some areas from South America (Stuart et al., 2008). In this study, oxidative stress

parameters, such as the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), glutathione-S transferase (GST), and the levels of total glutathione (*t*GSH) and lipid peroxidation were evaluated in whole organism. The activities of AChE and carboxylesterase (CbE) were also assessed to evaluate effects of clomazone on tadpoles under different temperatures. We hypothesize that temperature rise in combination with clomazone exposure may enhance the oxidative stress responses and that temperature may interfere on esterase inhibition by clomazone in tadpoles, with a species-specific pattern.

METHODOLOGY

Tadpoles' collection and acclimatation

Spawns of both species were collected in temporary ponds in non-agricultural areas in the region of Sao Jose do Rio Preto, Sao Paulo, Brazil (20°47′07.05″ S, 49°02′42.09″ W). After hatching, larvae were acclimatized and maintained in the laboratory under ideal condition of temperature, pH and oxygen (28°C, pH 7.5–8.0) until they reached stages 27-30 (Gosner 1960). Both species were collected under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

Experimental design

Temperature treatment

Aquariums (5L) containing dechlorinated water were immersed in three different tanks (100L) in a "water bath" system. Tanks had the water heated to three different temperatures, 28, 32 or 36 ºC and maintained by autoregulating thermostats. Temperature of the tanks and individual aquariums were recorded three times a day to observe possible variations during the

experimental period. Temperature range selected in this study was based on field studies conducted by Freitas et al. 2016, where water temperature of five ponds were recorded during the summer in areas where *E. nattereri* and *R. schneideri* are often found in Brazil.

Chemical exposure

Tadpoles of *E. nattereri* and *R. schneideri* were exposed separately to 0.01, 0.05 and 0.1 $mg.L^{-1}$ of nominal concentrations of clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) from the commercial herbicide Gamit® 500 (Gamit 500 CE, FMC Corporation, Philadelphia, EUA). Clomazone concentrations used in this experiment were based on those detected in areas of sugarcane and rice cultivation in Brazil (0.2 to 0.4 $mg.L^{-1}$) (Zanella et al. 2002; Dutra De Armas et al. 2005; Primel et al. 2005). Animals were exposed to each concentration at temperatures of 28, 32 and 36 ºC during three and eight days. A control group containing no contaminants was provided for each temperature and experiment duration. Each experimental group consisted of five replicates (n=5) containing eight tadpoles in each aquarium (n=40 pseudoreplicates). First, tadpoles were placed in aquariums and the water temperature was gradually adjusted to avoid thermal shock during the first 24 hours. Then, concentrations of clomazone dissolved in a stock solution of water (final volume of 1 mL) were added into the aquariums. Aquaria were constantly aerated at a pH of 8.00 ± 0.26 and using a light-dark cycle of 12-12 h. Previous studies have demonstrated that clomazone concentrations are unchanged after four days in the water (Oliveira et al. 2016). Thus, the water and contaminant were renewed every two days to ensure the original concentrations of clomazone and also dissolved oxygen and ammonia. No significant variations on these parameters were observed among treatments during the experiments (dissolved oxygen 6.06-7.04 mg/L, pH 8.12-8.46). Animals were fed every two days with commercial food for tropical fish in minimum quantities (200 mg) avoiding

accumulation into the aquariums. After three and eight days, tadpoles were removed from the tanks and euthanized in liquid nitrogen. All the procedures were done in accordance with the Ethics Committee on Animal Use in Research of the São Paulo State University (CEUAIBILCE/UNESP No. 086/2013).

Analysis

Morphological analysis

Effects on growth (snout−vent length (SVL) and body weight) and development (Gosner, 1960) were evaluated individually in tadpoles of *E. nattereri* and *R. schneideri* after three and eight days of exposure at different temperatures.

Preparation of samples

For the enzymatic measurements, two tadpoles of each replicate (n=10) were entirely and individually homogenized in a ratio of 1/4 (weight/volume) in a cold (4 °C) homogenization buffer (Tris-HCl 20 mM, EDTA 1 mM, DL-dithiothreitol 1 mM, sucrose 0.5 M, KCL 0.15 M, phenylmethylsulfonyl fluoride 1 mM), pH 7.4, and centrifuged for 30 min at 9000 g and 4°C. The supernatant fraction was collected and centrifuged again for 1 h at 50.000 g. The second supernatant fraction was collected and stored at –80°C for subsequent evaluations.

Lipid peroxidation was assessed by the measurements of MDA levels. For this analysis, other two tadpoles of each five replicate (n=10) were entirely homogenized in a ratio of 1:3 (w:v) in 0.1 M Tris-HCl buffer, pH 8.0. After homogenization, 300 µL of thiobarbituric acid (TBA, Sigma-Aldrich, Germany) diluted in 0.4% HCl were added to the sample. The mixture was heated at 90 ^{o}C for 40 minutes and the reaction product was extracted with 1.0 mL of n-butanol (Sigma-Aldrich, USA). After this procedure, the n-butanol extracted fraction was injected (10

µL) into a High-Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan). Chromatogram was monitored during 10 min and peaks were identified and quantified using LAB Solutions 5.71 software (Shimadzu Corporation).

For *t*GSH analysis, other two tadpoles of each replicate (n=10) were entirely and individually homogenized in a ratio of 1/9 (weight/volume) in a cold (4 °C) solution of perchloric acid (PCA) 0.5 M and centrifuged during 5 min at 15.000 g and 4 °C. Supernatants were collected and neutralized with a solution of potassium phosphate 0.1M , pH 7.0 and posteriorly centrifuged at 15.000 g during 2 min at 4 °C.

AChE and CbE were assessed in tadpoles entirely homogenized in a ratio 1:4 (weight/volume) in a cold (4 °C) solution of Tris HCl 0,1 mol.L^{-1} pH 8,0 and centrifuged at 10.000 g during 30 min at 4 °C. The enzymes were analyzed in the supernatant fraction.

Biochemical analysis

Antioxidant enzymes

Analyses of CAT were performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes. The activities of GST, G6PDH and SOD were performed on a Victor TM X3 microplate reader (Perkin ElmerR). The activity of all enzymes was evaluated at the respective temperatures (28, 32 and 36 °C) at which the animals were subjected for the treatments.

CAT activity was measured using the method described by Beutler (1975), which monitors the rate of decomposition of hydrogen peroxide (H_2O_2) by the enzyme at 240 nm for 1 min. Specific activity was expressed as U mg of $protein^{-1}$. The assays were performed using Tris-HCl buffer (1 M, pH 8.0) with 5 mM EDTA and H_2O_2 as the substrate.

GST activity was measured using the method described by Keen et al. 1976, which monitors the formation of the conjugate of 1-chloride-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) catalyzed by GST in the sample for 1 min at 340 nm. Specific activity was expressed as U.mg of protein^{-1}. The final volume of the assay was 110 μL, which contained a potassium phosphate buffer (0.2 M, pH 6.5), 1 mM CDNB and 1 mM GSH and the sample.

The enzymatic assay of G6PDH (Glock and McLean 1953) measured the formation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for 1 min. The assay consists of the reduction of $NADP^+$ to NADPH by the G6PDH using glucose-6-phosphate (G6P) as the substrate. The assay had a final volume of 205 μL, which contained Tris-HCl buffer (0.1 M, pH 7.4), MgCl2, NADP+, G6P and the sample. The reference blank did not contain G6P. Specific activity was expressed as mU.mg of protein^{-1}.

SOD activity was measured using SOD Assay Kit-WST (Sigma, Aldrich) by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase activity, and is inhibited by SOD. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity was quantified by measuring the decrease in the color development at 440 nm. Specific activity was expressed as U.mg of protein^{-1}.

Determination of GSH levels

Total GSH contents were assessed by Tietze (1969) protocol, adapted by Sies (2003). Total amount of GSH was measured based on the reaction between GSH and DTNB (5,5′-Dithiobis(2-nitrobenzoic acid)), which produces the conjugate GS-TNB and the yellow TNB (5-thio-2-

nitrobenzoic acid) anion that can be detected at 412 nm. The rate of TNB production is proportional to the GSH in the extract. Thus, the more GSH in the sample, the more TNB was formed, which was monitored at 412nm during 2 minutes. A recycling reaction catalyzed by glutathione reductase in presence of NADPH was also coupled to the assay, regenerating 2GSH from the GSSG previously formed by the reaction with DTNB. This enables a continuous production of TNB, which is proportional to the total GSH (GSH + GSSG) in the sample. The μmol GSH g^{-1} of tissue in the sample was calculated based on a standard curve with known amounts of GSH, obtained in the assay by the change in absorbance per minute (ΔA). Analyses of GSH were performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes.

Lipid peroxidation

MDA levels were evaluated following the method described by Almeida (2003) using a high performance liquid chromatography (HPLC) system (ESA, USA) coupled to a UV-Vis detector (526, ESA, USA) to analyze the product formed between malonaldehyde (MDA) and TBA. The chromatographic column used in the analyzes was a Shimadzu C18 column (150 x 4.6 mm, 5μm), using 50 mM potassium phosphate solution at pH 7.0 as the mobile phase, with 40% methanol, and at a flow rate of 1.0 mL/min.

Protein quantification

Proteins were quantified using the Bradford (1976) with Coomassie Brilliant Blue G-250 in an acidic solution. The absorbance values were determined at 595 nm, and the results were compared to the analytical curve prepared with bovine serum albumin (BSA) as the standard.

AChE and *CbE* activity

Analyses of AChE and CbE were performed on a Victor TM X3 microplate reader (Perkin Elmer) following the methodology of Ellman et al. 1961. This method measure the formation of a thiol derivative produced by the action of the enzyme on the substrate, which react with the DTNB producing a yellow compound, which is monitored at 412 nm at 25ºC. Acetylthiocholine was used as substrate for analysis of AChE and phenylthioacetate for analysis of CbE. Specific activities were expressed as U/mg protein^{-1}.

Statistical Analysis

Statistical analysis was conducted using the softwares R version 2.11.1 (R Development Core Team, 2010) and STATISTICA (StatSoft). Normality and homogeneity of the data was checked by the Shapiro-Wilk and Levene's test, respectively. Two-way analysis of variance (ANOVA 2-way) followed by the Tukey post hoc test were used to identify statistical differences (antioxidant enzymes activity, MDA levels, esterase activities and effects on growth – length and weight). This test evaluated the individual effects of temperature (28, 32 and 36 °C) and chemical treatments (clomazone - 0.01, 0.05 and 0.1 mg.L^{-1}) and also the interacted effects of the temperature and clomazone. Non-exposed tadpoles at 28 °C were assumed as the control group for comparison of interacted effects, since all animals were previously acclimatized at this temperature and this group had no influence of either thermal stress or chemical exposure. G-test (likelihood ratio test) with Yates' correction (Sokal 1995) was performed to assess effects on development of tadpoles. Values of $P<0.05$ were considered as a reference to assign statistical significance (Zar 1999).

RESULTS

Growth and development

No effects of clomazone and temperature treatments were detected on the body weight and SVL of tadpoles of *E. nattereri* and *R. schneideri* for both three and eight days (P>0.05) (Table S1 and S2). Development of *R. schneideri* was also unaffected by any treatment (P>0.05) (Table S5 and S6). However, tadpoles of *E. nattereri* exposed to 0.01 $mg.L^{-1}$ of clomazone at 36 °C had the development accelerated compared to the control at 28 °C after eight days of exposure (P=0.048) (Table S4). For *E. nattereri*, tadpoles were classified between stages 27-39 (Gosner 1960) and the amount of animals at each Gosner stage is summarized in Table S1. It was also observed that in the end of the experiment, the control group (28 °C) presented tadpoles distributed at younger stages of development (Gs27-Gs33) compared to those treated with 0.01 $mg.L^{-1}$ at 36 °C, which presented 60% of tadpoles distributed between Gs35-Gs39. The frequency of tadpoles from both species classified at each Gosner (1960) stage is summarized at Tables S3, S4, S5 and S6.

Antioxidant enzymes, MDA levels and tGSH

The activity of the antioxidant enzymes, MDA and *t*GSH contents were altered following treatments with clomazone at different temperatures for both species. Also, temperature had single effects in tadpoles non-exposed to the herbicide clomazone.

Eupemphix nattereri

All studied enzymes had their activities changed by the isolated effect of temperature in tadpoles of *E. nattereri* after three days (Fig. 1). Tadpoles unexposed to clomazone had CAT, SOD, G6PDH and GST activities increased in animals maintained at higher temperatures (32 and

36 °C) compared to those at 28 °C (P=0.004, 0.0001, 0.0001, 0.0001, respectively) (Fig. 1). After eight days, the same pattern was observed for SOD, with increased activity at 36 °C (P=0.019) (Fig. 2B). CAT was increased only in the control group at 32 °C after eight days (P=0.033) (Fig. 2A), and in an opposite way, GST was decreased in the control at 36 °C compared to the those at 28 °C (P=0.0002) (Fig. 2D). G6PDH also presented a lower activity in tadpoles of the control at 36 °C compared to those at 32 °C (P=0.006).

At 28 °C, clomazone treatments only increased SOD activity at the highest concentration after three days (P=0.05, P=0.04) (Fig. 1 B). No other effects on the enzymes activities were observed at 28 °C. The combined effects of temperature and clomazone increased CAT, SOD, G6PDH and GST in tadpoles treated at 32 and 36 °C compared to the control at 28 °C after three days (P<0.05) (Fig. 1). At 32 °C, animals exposed to 0.01 and 0.05 $mg.L^{-1}$ had an increase on CAT activity comparing to animals at 28 °C (P=0.01, P<0.001) (Fig. 1A). However, tadpoles exposed to 0.1 $mg.L^{-1}$ at 32 °C showed a decrease on CAT activity compared to the control at the same temperature (P=0.006) (Fig. 1A). SOD and GST were increased in all groups treated at 32 and 36 °C after three days (P<0.05) (Fig. 2A). G6PDH had its activity rised in all groups treated at 32 °C compared to control at 28 °C (P=0.01, P=0.03, P=0.001), in addition to an increased G6PDH activity in tadpoles exposed to 0.01$mg.L^{-1}$ at 36 °C (P=0.0004) (Figs. 2C and D).

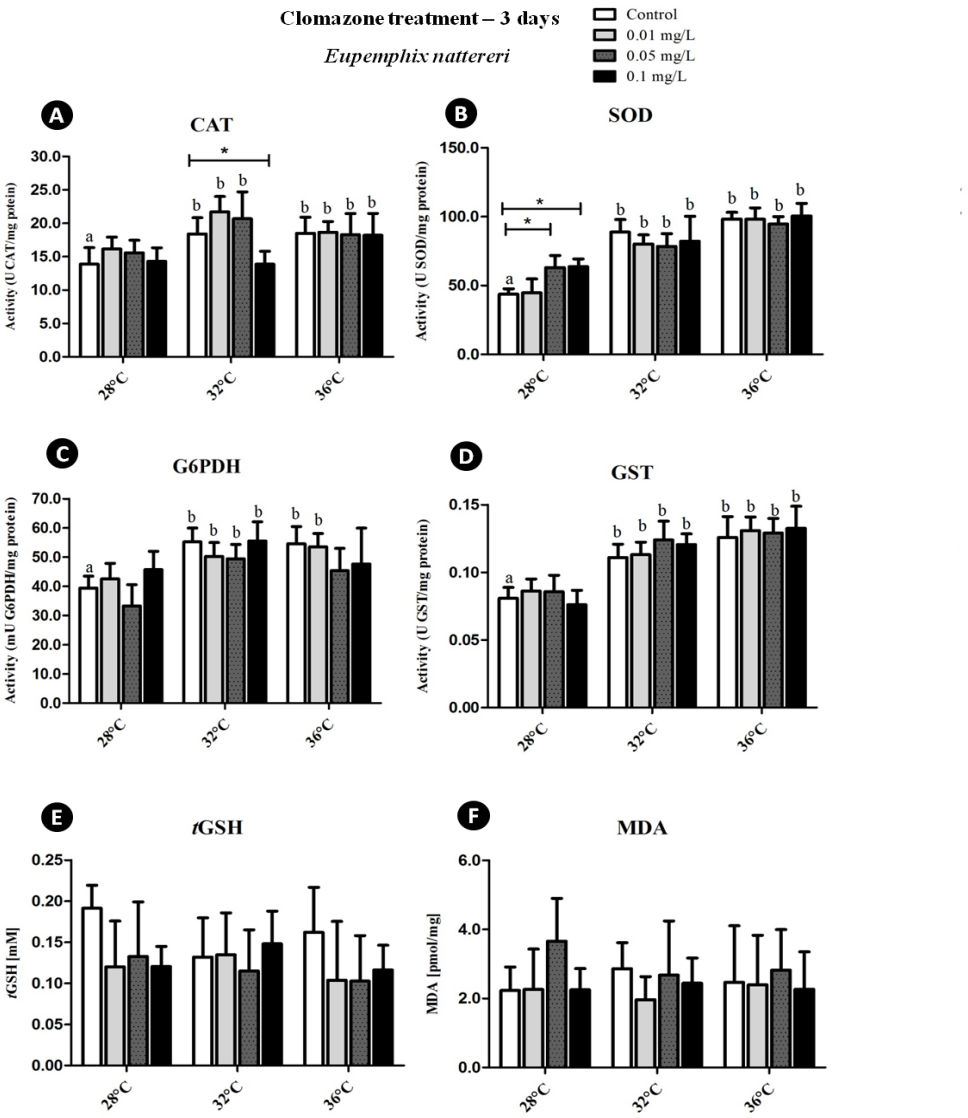

Figure 1: Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *E. nattereri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36°C) after three days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05).

After eight days, no effects of clomazone were observed at 28 °C (Fig. 2). SOD had its activity increased by the interacted effects in tadpoles exposed to 0.1 $mg.L^{-1}$ of clomazone at 32 °C (P= 0.003) and 0.05 $mg.L^{-1}$ at 36 °C (P<0.001), compared to the control at 28 °C (Fig. 2B). No effects of clomazone were observed on G6PDH and GST activities at 28 and 32 °C after this

period (P>0.05) (Figs. 2C and D). However, animals treated with 0.01 $mg.L^{-1}$ of clomazone at 36 °C decreased both G6PDH (P=0.017) and GST (P=0.006) activities compared to the control at 28 °C (Figs. 2C and D). GST activity had also a different response in animals exposed to 0.1 $mg.L^{-1}$ at 36 °C, with an increased activity compared to the control at the same temperature (36 °C) (P=0.005) (Fig. 2D).

*t*GSH levels were not altered in tadpoles of *E. nattereri* by clomazone exposure at any temperature for both experimental periods (P>0.05) (Figs. 1 and 2). MDA levels were also not altered by treatments after three days in tadpoles of *E. nattereri* (Fig. 1F). After eight days, no effects on lipid peroxidation were evidenced in animals treated at 28 and 32 °C. However, MDA level was increased in non-exposed tadpoles at 36 °C (P=0.001) and also in those treated with 0.1 $mg.L^{-1}$ at 36°C (P=0.1 mg/L^{-1}) after longer exposure (Fig. 2E).

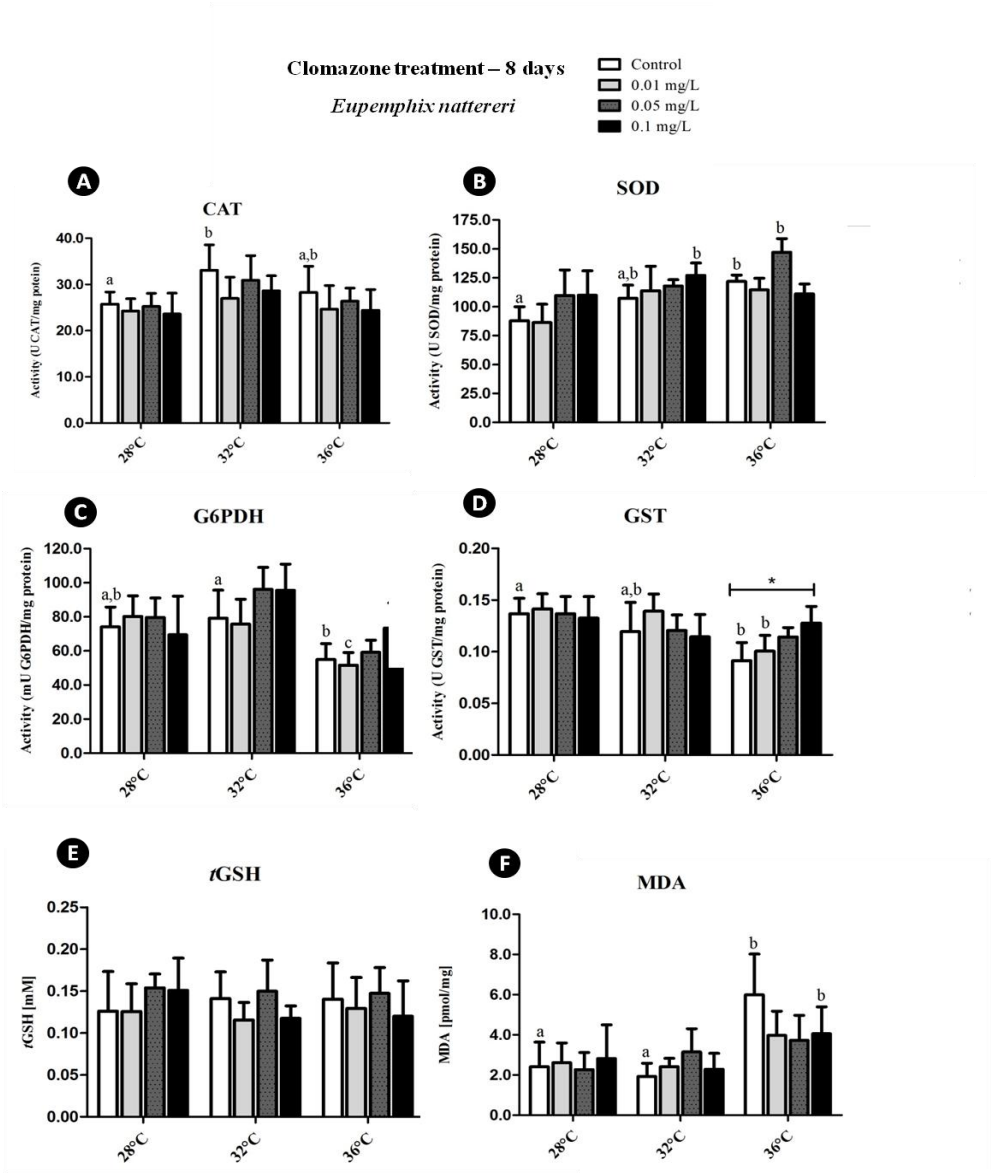

Figure 2: Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *E. nattereri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36°C) after eight days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05).

Rhinella schneideri

Temperature rise only increased activity of SOD (P=0.046) and GST (P=0.04) in non-exposed tadpoles of *R. schneideri* after three days (Figs. 3B and C). No other changes on

antioxidant enzymes were observed as result of isolated thermal stress. After eight days, temperature had no isolated effects on enzymes activities (Fig. 4).

Clomazone caused no alterations in the activity of antioxidant enzymes in tadpoles treated at 28 and 32 °C after three days, excepted by SOD, which had its activity increased in animals exposed to 0.01 and 0.1 $mg.L^{-1}$ at 32 °C (P=0.00, P=0.0003) compared to the control at 28 °C. At 36 °C, GST activity was enhanced in tadpoles exposed to 0.1 $mg.L^{-1}$ compared to the control at the same temperature and at 28 °C (P=0.011) (Fig. 3D). SOD also had its activity increased in all groups treated with clomazone at 36 °C compared to the control at 28 °C (P=0.014, P=0.010, P=0.0001), showing an interactive effect at higher temperatures (Fig. 3B). CAT and G6PDH were not changed following treatments with clomazone at any temperature and concentration after three days (P>0.05) (Fig. 3A and B). After eight days of exposure, no changes were observed in the antioxidant enzymes response for any treatment, excepted by G6PDH which had its activity decreased in tadpoles exposed to 0.01 $mg.L^{-1}$ at 28 (P=0.0001) and 32 °C (P=0.046) compared to the control at 28 °C (Fig. 4).

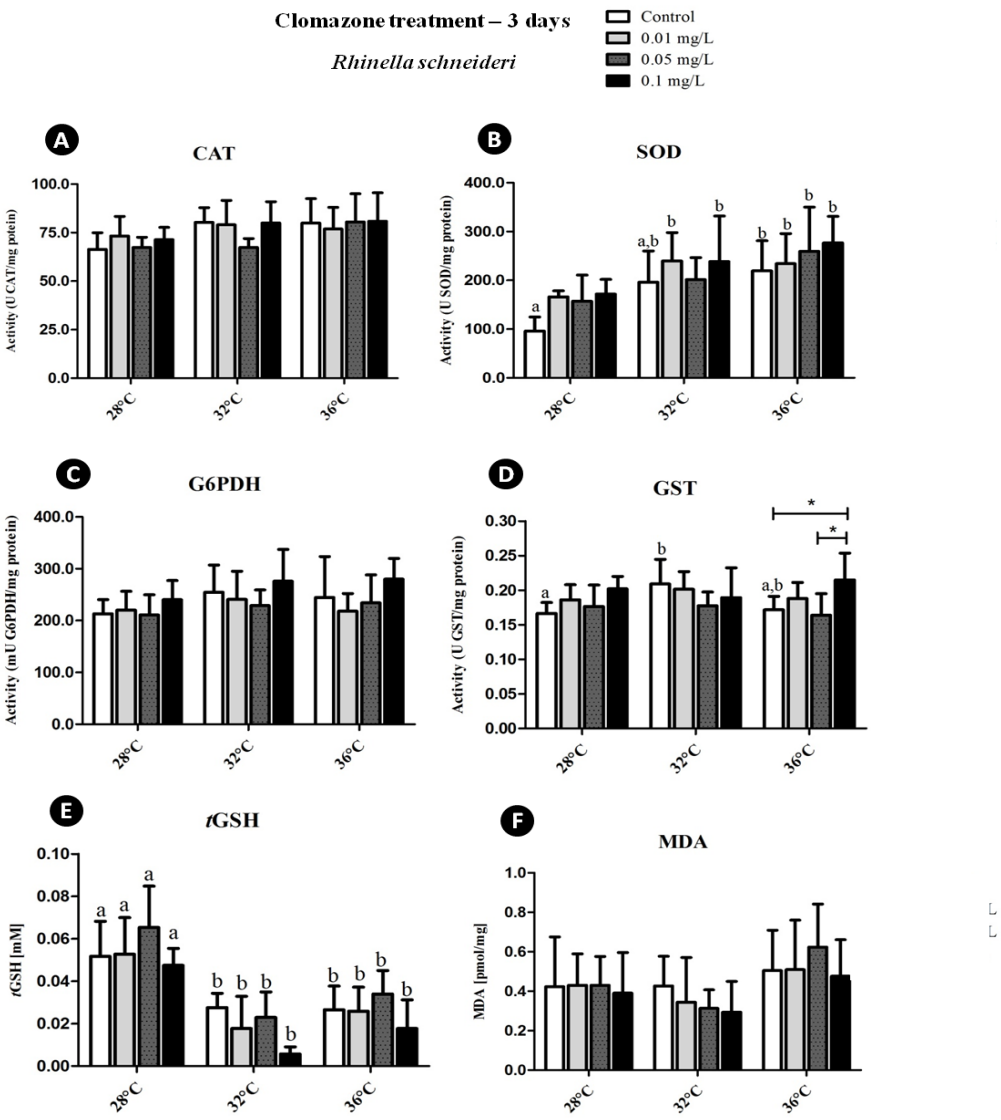

Figure 3: Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *R. shneideri* exposed to clomazone (Gamit®500) at different temperatures (28, 32 and 36°C) after three days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05).

*t*GSH levels were lower in all groups treated with clomazone at 32 °C and 36 °C compared to those at 28 °C, after three and eight days of exposure (P<0.05) (Figs. 3E and 4E). After eight days, tadpoles of *R. schneideri* treated with 0.01 and 0.05 mg.L^{-1} at 32 °C also had

lower *t*GSH levels than those from the control at the same temperature (P=0.003, P=0.005, respectively) (Fig. 4E).

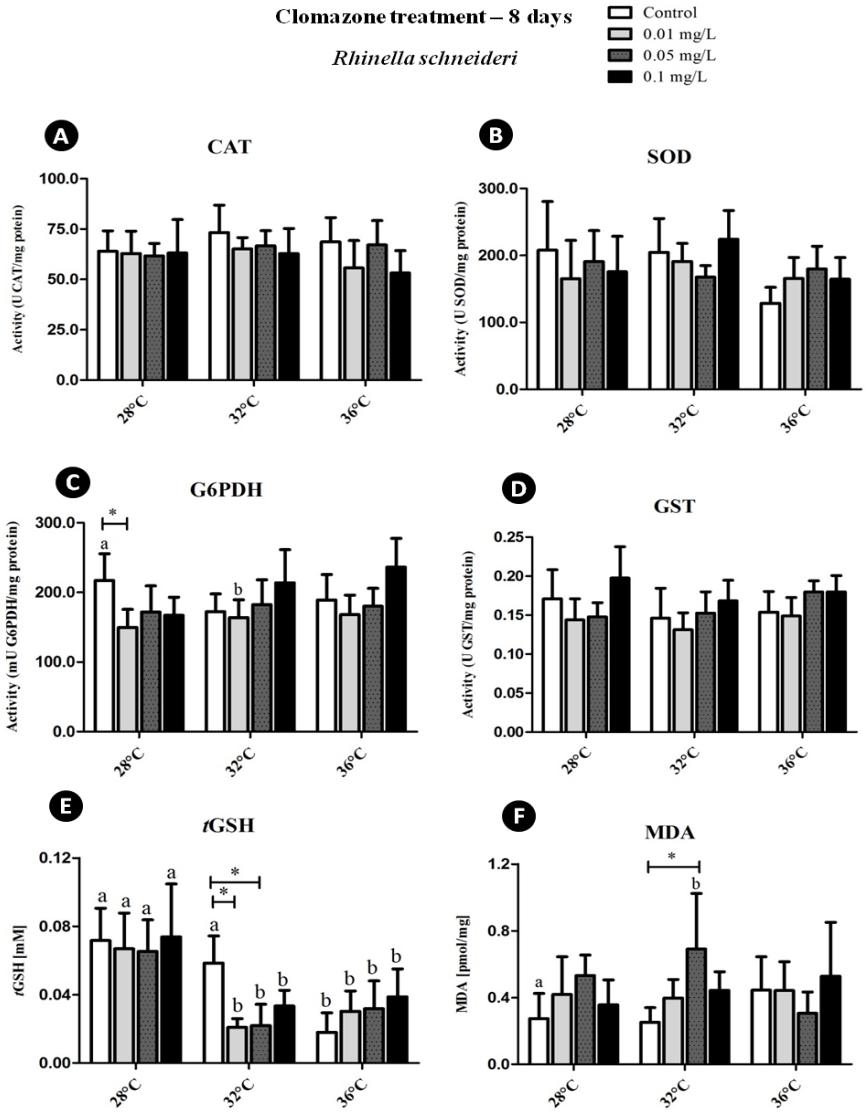

Figure 4: Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *R. schneideri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36°C) after eight days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05).

Similarly to *E. nattereri*, no changes on MDA were observed after three days of treatments in tadpoles of *R. schneideri* (P>0.05) (Fig 3E). After eight days, tadpoles exposed to 0.05 $mg.L^{-1}$ of clomazone at 32 °C had its MDA increased compared to the controls at 32 (P=0.003) and 28 °C (P=0.006) (Fig. 4E). No alterations on MDA levels were observed at 28 and 36 °C after the longer exposure.

AChE and CbE activities

AChE activity was unchanged following clomazone exposures in tadpoles of *E. nattereri* after both periods (P>0.05) (Fig. 5). CbE was lower in animals exposed to 0.1 $mg.L^{-1}$ at 36 °C than at 28 °C after three days (P=0.019), but no differences were observed comparing to the controls. After eight days, CbE was increased in *E. nattereri* exposed to 0.01 $mg.L^{-1}$ of clomazone at 28 °C (P=0.007) and to 0.1$mg.L^{-1}$ at 32 °C (P=0.005) compared to their respective controls (Fig. 5). In *R. schneideri*, tadpoles exposed to 0.1 $mg.L^{-1}$ at 36 °C presented lower activity of AChE than those exposed to the same concentration at 32 °C (P=0.033) after three days. No differences on AChE in *R. schneideri* were observed after eight days (P>0.05). CbE was higher in tadpoles of *R. schneideri* exposed to 0.1 $mg.L^{-1}$ at 28 °C compared to the control after three days (P=0.0003), and no relevant differences were noticed after eight days (Fig. 6).

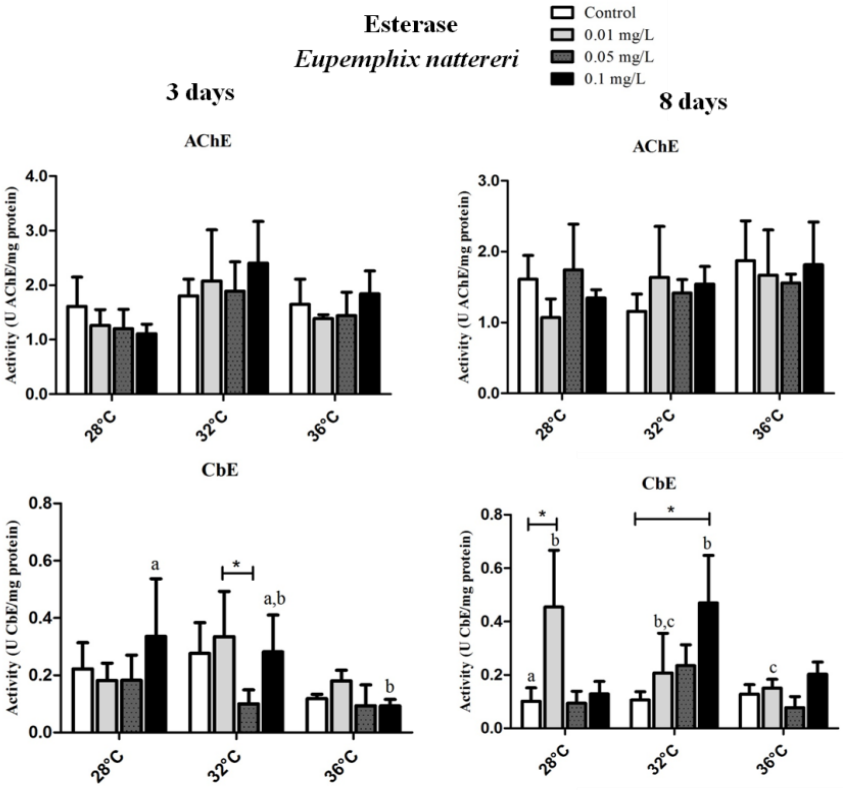

Figure 5: Acetylcholinesterase (AChE) and carboxylesterase (CbE) activities in tadpoles of *E. nattereri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36°C) after three and eight days. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (ANOVA-2-way followed by Tukey, P <0.05).

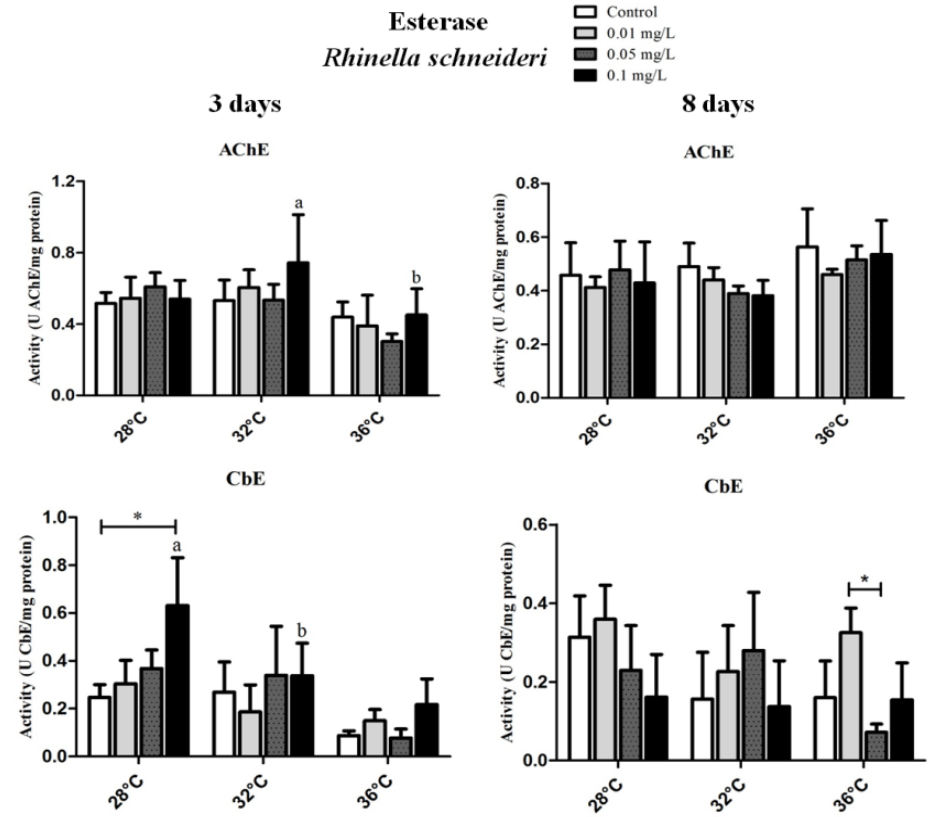

Figure 6: Acetylcholinesterase (AChE) and carboxylesterase (CbE) activities in tadpoles of *R. schneideri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36°C) after three and eight days. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures

Discussion

Results of the present study indicated that temperature is an important factor affecting the toxicity of the herbicide clomazone in tadpoles at field concentrations, evidenced by alterations in the antioxidant defence enzymes and lipid peroxidation status with temperature-associated responses. The use of pesticides in agricultural areas has been pointed as one of the main factors contributing to decline of local amphibian populations (Hayes et al. 2010). Brazil is the richest country in amphibian biodiversity. Surprisingly, the toxicity of most agrochemicals used in crops with high commercial importance, such as sugarcane and rice, is still unknown for this group of animals. In addition, bioassays approaching responses of different native species, which usually have different mechanisms to cope with stressful conditions in natural systems, are particularly scarce. Considering that tadpoles are often exposed to pesticides in environments where water temperatures have drastic fluctuations, it is urgent and necessary to understand how different local species are dealing with the presence of these compounds in habitats influenced by thermal stress, thus estimating the real risks for neotropical amphibians.

Tadpole's development was unaltered in *R. schneideri* after exposure to clomazone at different temperatures. In *E. nattereri*, tadpoles exposed to 0.01 $mg.L^{-1}$ at 36 °C presented more advanced stages than those from the control at 28 °C after eight days. It is already known that changes of temperature (Morand et al. 1997) and the presence of chemicals (Brande-lavridsen et al. 2010; Peltzer et al. 2013b; Freitas et al. 2016) in the environment can alter the development of frogs. Acceleration on metamorphosis process is considered an acclimative mechanism used by different species for dealing with adverse conditions in stressed environments (Hooper et al. 2013). Although this may bring benefits, accelerated metamorphosis compromise many associated traits, such as body size, age of reproduction and fecundity (Semlitsch 1988), which result in adults with altered physiological performance and not competitive enough to survive in

nature (Denver 1997). This study indicated that combined exposure to clomazone and higher temperatures can accelerate the development of *E. nattereri*, however more studies with longer exposures are recommended to clarify the effects of clomazone on metamorphosis of tadpoles.

Our results showed that clomazone affects oxidative stress and esterase activities in tadpoles of *E. nattereri* and *R. schneideri*, and that temperature had a strong influence on these parameters for both species. Isolated effects of temperature were also evident for both species considering the studied biochemical parameters. This was particularly evidenced in *E. nattereri*, which had all enzyme activities augmented with temperature increase in non-exposed tadpoles after three days. In *R. schneideri*, temperature rise only augmented SOD and GST activities after three days, and no other changes were observed after longer exposure. Studies have shown that temperature rise can increase the activity of many enzymes in the organisms, including antioxidant enzymes in both vertebrate and invertebrate, with an opposite effect at low temperatures (Almeida and Mascio 2011). However, in aquatic species such as amphibians, the temperature directly affects the enzyme kinetics and metabolism, but also influences oxygen solubility in water, consequently changing oxygen availability (Almeida and Mascio 2011). Changing environmental temperatures to those outside the optimal temperature range in aquatic ectotherms may causes a disturbance in antioxidant status of animals, which can be reflected in antioxidant defence system. The increased activity of antioxidant enzymes in tadpoles at higher temperatures observed in our study are probably a consequence of increased metabolic rates, which resulted in a higher oxygen consumption and consequently ROS production.

GST activity was changed by both temperature and clomazone effects in both species, but GST was mostly increased by the interaction of higher temperature and clomazone exposure. Enzymes of the glutathione-*S*-transferase (GSTs) family act in phase II of the detoxification process, which involves the conjugation of xenobiotics with endogenous compounds (Di Giulio

et al. 1989). Higher GST activity were previously observed in toad *Rhinella schneideri* plasma samples from agricultural areas of Argentine, suggesting this enzyme as an important biomarker to assess pesticides effects in field studies with amphibians (Attademo et al. 2007). Previous studies with the herbicide clomazone have reported an increased activity of GST in fish (*P. lineatus*) exposed to 5 and 10 $mg.L^{-1}$ after 96 hours (Pereira et al. 2013), in accordance to the increased GST observed in this study in *E. nattereri* and *R. schneideri*, although these responses to clomazone were strictly associated to the higher temperatures. The sensibility of GST to environmental temperatures have been already reported in aquatic organisms exposed to thermal stress, such as in saltwater fish (*Diplodus vulgaris*, *Diplodus sargus*, *Dicentrarchus labrax*, *Gobius niger* and *Liza ramada*) (Vinagre et al. 2012; Madeira et al. 2013) and tadpoles of *E. nattereri* (Freitas and Almeida 2016). Studies investigating the effects of elevated temperature in fish (*Channa punctata*) exposed to deltamethrin also showed that GST in liver and kidney was particularly induced in animals exposed to the combined effect of the chemical and heat stress (Kaur et al. 2011). In contrast, negative modulation of GST was reported in the Antarctic fish *Notothenia rossii* after six days of heat stress (8 °C) compared to those at 0 °C (Machado et al. 2014). The augmented GST activity observed in our and other studies may be associated with an adaptation of the organism to stressful conditions in the environment, which have been previously pointed to occur in response to many organic compounds (van der Oost et al. 2003).

SOD was the only enzyme changed by exposure to clomazone at 28 °C in *E. nattereri*, with an increased activity in tadpoles exposed to the highest concentration. SOD was also influenced by heat stress for both species, an effect that was particularly pronounced after shorter exposure. After eight days, SOD activity was returned to control levels for all treatments in *R. schneideri*. SOD is already known to be altered by exposure to some pesticides in aquatic organisms, such as methyl parathion, atrazine and DDC (diethyldithiocarbamate) (Nwani et al.

2010; Lushchak 2011; Abhijith et al. 2016), but its activity can be also enhanced by changes on environmental temperature in aquatic organisms (Hsu and Chiu 2009; Nakano et al. 2014; Vinagre et al. 2014; Walters et al., 2016). Studies with salmon (*Oncorhynchus kisutch*) showed that SOD was significantly increased in thermal stressed fish after 17.5 h, but the activity had returned to the basal levels after 48 h (Nakano et al. 2014). Otherwise, SOD activity was observed to decrease in fish of *Notothenia coriiceps* and *Notothenia rossii* exposed to heat stress after three and six days compared to those exposed for one day (Machado et al. 2014). In agreement with the literature data, our findings suggest that increased SOD activity might neutralize the harmful effects of superoxide radicals generated as a result of thermal stress and also by the combined effect of heat stress and clomazone exposure; however its activity may be compensated by adaption mechanisms used by *R. schneideri* after longer periods.

Heat treatments induced CAT activity in all groups treated at 32 and 36° C in *E. nattereri* after three days. However, CAT was decreased in tadpoles exposed to higher concentrations of clomazone at 32° C when compared to the control at the same temperature, showing that clomazone affected CAT activity depending on the water temperature. CAT activity was unchanged in *R. schneideri* for any treatment. Previous studies have showed opposite results for CAT in different species of fish exposed to the combined effects of temperature and presence of pesticides (Kaur et al. 2011; Tu et al. 2012). For example, the saltwater fish *Channa punctata* had CAT activity decreased in animals exposed to the insecticide deltamethrin in combination with heat stress (Kaur et al. 2011). Activity of CAT was also decreased in the black tiger shrimp (*Penaeus monodon*) exposed to deltamethrin at 34 °C after four days, but not at 24 and 29 °C (Tu et al. 2012). Similarly to CAT, G6PDH activity was also increased in tadpoles of *E. nattereri* exposed to clomazone at 32 and 36 °C after three days. However a decrease on G6PDH was observed after eight days in animals from the control and those exposed to 0.01 $mg.L^{-1}$ of

clomazone at 36 °C. Decreased G6PDH was also observed in tadpoles of *R. schneideri* exposed to 0.01 $mg.L^{-1}$ at 28 and 32 °C. G6PDH and CAT are important enzymes for redox metabolism. Altered CAT activity may impair the capacity of cells to catalyze the decomposition of hydrogen peroxide (H_2O_2). Increased CAT activity observed in animals exposed to the combination of clomazone and high temperatures may be a consequence of increased H_2O_2 production in organisms exposed to stressful conditions, in an attempt to neutralize the injuries caused by the excess of this compound in the cells. Otherwise, decreased CAT activity should compromise the ability of the organisms to act against ROS damages. Changes on G6PDH activity are also important to organisms because this enzyme participates of the pentose phosphate pathway recycling NADPH, an important reducing agent used to maintain GSH levels in the cells. Decreased G6PDH activity can result in an diminished supply of GSH, which is an important tripeptide that protects cells against oxidative damage (Almeida et al., 2011). Therefore, balanced activities of both CAT and G6PDH are required for a good cellular performance linked to antioxidant response, and alterations in their functioning can make the animals more vulnerable to ROS effects and consequently increasing chances of oxidative stress.

*t*GSH levels were unchanged in tadpoles of *E. nattereri* following the treatments with clomazone and temperature. In *R. schneideri*, higher levels of *t*GSH were observed in the groups exposed to 28 °C after three and eight days. After three days, clomazone had no direct effects on *t*GSH and the temperature rise masked effects of clomazone, since all groups maintained at 32 and 36 °C had their *t*GSH levels decreased, including the controls, indicating an increased GSH demand due to temperature increase. However, after eight days, clomazone diminished *t*GSH in animals exposed to 0.01 and 0.05 $mg.L^{-1}$ at 32 °C compared to the control at the same temperature, indicating direct effects of clomazone on *t*GSH at this thermal gradient in *R. schneideri*. Endogenous GSH plays a role in cells as an antioxidant, as a co-factor of glutathione

peroxidase (GPx), and participates in the reduction of peroxides, with concomitant formation of oxidized glutathione disulfide (GSSG) (Almeida et al. 2011). GSH is central to the detoxification of ROS and has been mentioned as one of the protective mechanisms that aquatic organisms adopt in the initial phases of exposure to xenobiotics, being also an important co-factor for GST during phase II biotransformation (Stephensen et al. 2002). Thermal stress can potentially alter GSH contents in fish (Machado et al. 2014; Nakano et al. 2014), but also the presence of chemicals combined to temperature effects (Kaur et al. 2011; Min et al. 2014). For example, GSH levels was higher in the Antarctic fish *N. rossii* exposed to 8 °C than to 0 °C (control temperature) (Machado et al. 2014). GSH levels were also higher in the black rockfish *Sebastes schlegeli* exposed to different concentrations of cooper at 23 and 28 °C than at 18 °C (Min et al. 2014). Fish of *Channa punctata* pre-exposed to deltamethrin followed by heat stress had an increase in the GSH content in the liver and decrease in gills, indicating the different vulnerability of the tissues to the toxicants following GSH pathway (Kaur et al. 2011). Furthermore, some studies have shown that fluctuations in GSH in organisms exposed to some chemicals can be accompanied by variations in GST, which conjugates GSH to various xenobiotic compounds (Sheweita et al., 1998). In our study, contents of *t*GSH had a temperature-associated response in tadpoles of *R. schneideri,* with differential response after three and eight days of exposure, however alterations on GSH contents were not associated to GST activity for any of the species. These results can be due to total amounts of both oxidized and reduced glutathione measured in this study, which was not separately related to GST. However, we observed that temperature was an important factor controlling GSH levels in the cells of tadpoles, with differential responses for different species probably due to different pathways using GSH as co-factor in the antioxidant response.

Peroxidation of membrane components are caused when enzyme activity is not compensated by the excessive amount of ROS produced in response to stress. Pesticides may induce oxidative stress, leading to the generation of free radicals and causing lipid peroxidation (LPO) (Kehrer 1993; Sevgiler et al. 2004). In agreement to this, clomazone exposure has been previously mentioned to induce LPO in liver, brain and muscle tissues of the piava *Leporinus obtusidens* (Miron et al. 2008). In *R. quelen*, exposure to clomazone (0.5 or 1.0 $mg.L^{-1}$) also increased TBARS production in liver after exposures of 12 to 192 h, with differential responses in brain, where these levels were decreased after 96 and 192 h (Crestani et al. 2007). Increases on environmental temperature were also associated to increased (Nakano et al. 2014) or decreased (Machado et al. 2014) LPO in fish. Our study showed that MDA levels were most altered by temperature than exposure to clomazone in *E. nattereri*, which had their MDA increased for both control and $0.1mg.L^{-1}$ clomazone-treated tadpoles at 36 °C after three days. For *R. schneideri*, no changes were observed after three days, but LPO was induced in tadpoles exposed to 0.05 $mg.L^{-1}$ at 32 °C after longer exposure. The lack of variation on MDA in the other experimental groups can be related to the activities of antioxidant enzymes, which were mostly upregulated in animals exposed to combination of higher temperatures and clomazone exposure, potentially protecting the cells against lipid peroxidation.

Cholinesterases such as AChE and CbE are known to be inhibited by pesticides such as organophosphates and carbamates (van der Oost et al. 2003; Oruç and Usta 2007). However, AChE activity was also reported to be inhibited by other pesticides, such as the herbicide clomazone (Miron et al. 2008) and the insecticide endosulfan (Dutta and Arends 2003). Previous studies have shown that clomazone (5 and 10 $mg.L^{-1}$) act as a potent brain-inhibitor in fish of *P. lineatus* and *R. quelen* (5, 10 and 20 $mg.L^{-1}$) after 96 h of exposure (Miron et al. 2005; Pereira et al. 2013). Subsequent studies also showed that *R. quelen* was sensitive to lower concentrations of

clomazone, presenting inhibition of brain and muscle AChE when exposed to 0.5 and 1.0 $mg.L^{-1}$ in different experimental periods (Crestani et al. 2007). In the opposite way, muscle tissue of the fish *Leporinus obtusidens* showed AChE activity increased until 65% after exposure to the herbicide clomazone (0.5 $mg.L^{-1}$), indicating that herbicides can inhibit or activate AChE; however, the mechanisms that lead to activation are still unknown. In the present study, we observed that AChE was unchanged following clomazone treatments in *E. nattereri.* In *R. schneideri*, AChE was lower in tadpoles exposed to 0.1 $mg.L^{-1}$ at 36 °C compared to those exposed to the same concentration at 32 °C after three days. No other alterations on AChE were observed. CbE activity is also mentioned to be altered by different pesticides in tadpoles (Attademo et al. 2007; Lajmanovich et al. 2010; Leite et al. 2010). This enzyme is involved in the metabolism of xenobiotics and endogenous or exogenous short-chain fatty acids (Hyne and Maher 2003; Wheelock et al. 2008). Ours results showed that CbE was sensitive to clomazone at tested conditions, with diminished activity in animals exposed to the combined effects of the chemical at higher temperatures for both species after three days. After eight days, CbE changes were more pronounced in *E. nattereri* tadpoles. B-esterase enzymes have been recommended as important biomarkers evaluating amphibians exposure to pesticides in contaminated areas (e.g. Attademo et al. 2007; Lajmanovich et al. 2010; Leite et al. 2010; Peltzer et al. 2013a). However, studies relating the effects of other pesticides than organophosphate and carbamates in tadpoles are still scarce, especially considering autoctone species from Brazil.

In summary, we showed that temperature is an important factor inducing changes on biochemical responses in tadpoles of *E. nattereri* and *R. schneideri* exposed to the herbicide clomazone. For the first time, we reported the negative effects of clomazone on the biochemical stress response in amphibians. However, more pronounced effects could be observed in higher concentrations of clomazone, as observed in the previous studies with fish. Antioxidant enzymes

are important integrants of the antioxidant defence system, acting in the organisms to minimize the effects of ROS, which are generated during the biotransformation of the xenobiotics (van der Oost et al. 2003). Therefore, disturbance in the activity of these enzymes can interfere in the redox homeostasis of tadpoles, resulting in oxidative damage of cellular components. Different from the results observed in fish studies, clomazone had not pronounced effects on AChE at studied concentrations and temperatures. However, clomazone alone or in combination with higher temperature impaired CbE activity in *E. nattereri* and *R. schneideri*, which has not previously been reported for aquatic organisms exposed to this compound. Based on the studied biomarkers, *R. schneideri* showed better adaptability to the tested conditions, since most of the enzymes had their activities returned to the control levels after longer exposure. The discrepancies in physiological responses of *E. nattereri* and *R. schneideri* also support that the susceptibility and ecological risk of amphibians populations to exposure to environmental contaminants and temperatures may be particularly varied among species. Also, we strongly suggest that fluctuations on environmental temperature should be consider in further studies assessing environmental risks of pesticides for amphibian communities, especially considering tropical species.

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REFERENCES

Abhijith BD, Ramesh M, Poopal RK (2016) Responses of metabolic and antioxidant enzymatic activities in gill, liver and plasma of *Catla catla* during methyl parathion exposure. J Basic Appl Zool 77:31–40. doi: 10.1016/j.jobaz.2015.11.002

Almeida EA De, Mascio P Di (2011) 3 . Hypometabolism and antioxidative defense systems in marine invertebrates. Hypometabolism Strateg Surviv Vertebr Invertebr 661:39–55.

Attademo AM, Peltzer PM, Lajmanovich RC, et al (2007) Plasma B-esterase and glutathione S-transferase activity in the toad *Chaunus schneideri* (Amphibia, Anura) inhabiting rice agroecosystems of Argentina. Ecotoxicology 16:533–539. doi: 10.1007/s10646-007-0154-0

Beauvais SL, Cole KJ, Atchison GJ, Coffey M (2002) Factors affecting brain cholinesterase activity in bluegill (*Lepomis macrochirus*). Water Air Soil Pollut 135:249–264. doi: 10.1023/A:1014778109657

Bernarde PS, Kokubum MNDC (1999) Anurofauna do município de Guararapes, Estado de São Paulo, Brasil (Amphibia: Anura). Acta Biol. Leopoldensia 21:89–97.

Blaustein AR, Walls SC, Bancroft BA, et al (2010) Direct and indirect effects of climate change on amphibian populations. Diversity 2:281–313. doi: 10.3390/d2020281

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–54.

Brande-lavridsen N, Christensen-dalsgaard J, Korsgaard B (2010) Effects of Ethinylestradiol and the fungicide Prochloraz on metamorphosis and thyroid gland morphology in *Rana temporaria*. Open Zool … 3:7–16. doi: 10.2174/1874336601003010007

Crestani M, Menezes C, Glusczak L, et al (2007) Effect of clomazone herbicide on biochemical and histological aspects of silver catfish (*Rhamdia quelen*) and recovery pattern. Chemosphere 67:2305–2311. doi: 10.1016/j.chemosphere.2006.09.070

De Almeida EA, Humberto Silva DG, Dias Bainy AC, et al (2011) Evaluation of Glutathione Status in Aquatic Organisms. Oxidative Stress Aquat Ecosyst 381–388. doi: 10.1002/9781444345988.ch28

Denver RJ (1997) Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Horm Behav 31:169–79. doi: 10.1006/hbeh.1997.1383

Di Giulio RT, Washburn PC, Wenning RJ (1989) Biochemical responses in aquatic animals: a

review of determinants of oxidative stress. Environ Toxicol Chem 8:1103–1123.

Dornelles MF, Oliveira GT (2014) Effect of atrazine, glyphosate and quinclorac on biochemical parameters, lipid peroxidation and survival in bullfrog tadpoles (*Lithobates catesbeianus*). Arch Environ Contam Toxicol 66:415–429. doi: 10.1007/s00244-013-9967-4

Dos Santos Miron D, Crestani M, Shettinger MR, et al (2005) Effects of the herbicides clomazone, quinclorac, and metsulfuron methyl on acetylcholinesterase activity in the silver catfish (*Rhamdia quelen*) (Heptapteridae). Ecotoxicol Environ Saf 61:398–403. doi: 10.1016/j.ecoenv.2004.12.019

Durieux EDH, Farver TB, Fitzgerald PS, et al (2011) Natural factors to consider when using acetylcholinesterase activity as neurotoxicity biomarker in Young-Of-Year striped bass (*Morone saxatilis*). Fish Physiol Biochem 37:21–29. doi: 10.1007/s10695-010-9412-9

Dutra De Armas E, Rosim Monteiro RT, Valler Amâncio A, et al (2005) Uso de agrotóxicos em cana-de-açúcar na bacia do rio Corumbataí e o risco de poluição hídrica. Quim Nova 28:975–982. doi: 10.1590/S0100-40422005000600008

Dutta HM, Arends DA (2003) Effects of endosulfan on brain acetylcholinesterase activity in juvenile bluegill sunfish. Environ Res 91:157–162. doi: 10.1016/S0013-9351(02)00062-2

Freitas JS, Almeida EA (2016) Antioxidant Defense System of Tadpoles (*Eupemphix nattereri*) Exposed to Changes in Temperature and pH. Zoolog Sci 33:186–194. doi: 10.2108/zs150075

Freitas JS, Kupsco AJ, Diamante G, et al (2016) Influence of temperature on the thyroidogenic effects of Diuron and its metabolite 3,4-DCA in tadpoles of the American bullfrog (*Lithobates catesbeianus*). Environ Sci Technol acs.est.6b04076. doi: 10.1021/acs.est.6b04076

Glock GE, McLean P (1953) Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem J 55:400–8.

Gosner K (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Gripp HS, Freitas JS, Almeida EA, et al (2017) Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae). Ecotoxicol Environ Saf 136:173–179. doi: 10.1016/j.ecoenv.2016.10.027

Hayes TB, Falso P, Gallipeau S, Stice M (2010) The cause of global amphibian declines: a

developmental endocrinologist's perspective. J Exp Zool 213:921–33. doi: 10.1242/jeb.040865

Hooper MJ, Ankley GT, Cristol DA, et al (2013) Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. Environ Toxicol Chem 32:32–48. doi: 10.1002/etc.2043

Hsu CY, Chiu YC (2009) Ambient temperature influences aging in an annual fish (Nothobranchius rachovii). Aging Cell 8:726–737. doi: 10.1111/j.1474-9726.2009.00525.x

Hyne R V., Maher WA (2003) Invertebrate biomarkers: Links to toxicosis that predict population decline. Ecotoxicol Environ Saf 54:366–374. doi: 10.1016/S0147-6513(02)00119-7

Kaur M, Atif F, Ansari RA, et al (2011) The interactive effect of elevated temperature on deltamethrin-induced biochemical stress responses in *Channa punctata* Bloch. Chem Biol Interact 193:216–224. doi: 10.1016/j.cbi.2011.06.011

Keen JH, Habig WH, Jakoby WB (1976) Mechanism for the several activities of the glutathione S-transferases. J Biol Chem 251:6183–8.

Kehrer JP (1993) Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol 23:21–48. doi: 10.3109/10408449309104073

Lajmanovich RC, Peltzer PM, Junges CM, et al (2010) Activity levels of B-esterases in the tadpoles of 11 species of frogs in the middle Parana River floodplain: Implication for ecological risk assessment of soybean crops. Ecotoxicol Environ Saf 73:1517–1524. doi: 10.1016/j.ecoenv.2010.07.047

Leite PZ, Margarido TCS, de Lima D, et al (2010) Esterase inhibition in tadpoles of *Scinax fuscovarius* (Anura, Hylidae) as a biomarker for exposure to organophosphate pesticides. Environ Sci Pollut Res 17:1411–1421. doi: 10.1007/s11356-010-0326-y

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101:13–30. doi: 10.1016/j.aquatox.2010.10.006

Lushchak VI, Bagnyukova T V. (2006) Temperature increase results in oxidative stress in goldfish tissues. 2. Antioxidant and associated enzymes. Comp Biochem Physiol - C Toxicol Pharmacol 143:36–41. doi: 10.1016/j.cbpc.2005.11.018

Machado C, Zaleski T, Rodrigues E, et al (2014) Effect of temperature acclimation on the liver antioxidant defence system of the Antarctic nototheniids *Notothenia coriiceps* and *Notothenia rossii*. Comp Biochem Physiol Part - B Biochem Mol Biol 172–173:21–28. doi:

10.1016/j.cbpb.2014.02.003

Madeira D, Narciso L, Cabral HN, et al (2013) Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comp Biochem Physiol Part A Mol Integr Physiol 166:237–243. doi: 10.1016/j.cbpa.2013.06.008

Min E, Baeck S, Kang J-C (2014) Combined effects of copper and temperature on Hematological constituents in the Rock fish, *Sebastes schlegeli*. J fish Pathol 27:57–65. doi: 10.7847/jfp.2014.27.1.057

Miron D dos S, Pretto A, Crestani M, et al (2008) Biochemical effects of clomazone herbicide on piava (*Leporinus obtusidens*). Chemosphere 74:1–5. doi: 10.1016/j.chemosphere.2008.09.070

Moe SJ, De Schamphelaere K, Clements WH, et al (2013) Combined and interactive effects of global climate change and toxicants on populations and communities. Environ Toxicol Chem 32:49–61. doi: 10.1002/etc.2045

Morand A, Joly P, Grolet O (1997) Phenotypic variation in metamorphosis in five anuran species along a gradient of stream influence. Comptes Rendus l'Academie des Sci - Ser III 320:645–652. doi: 10.1016/S0764-4469(97)85698-3

Murata T, Yamauchi K (2005) Low-temperature arrest of the triiodothyronine-dependent transcription in *Rana catesbeiana* red blood cells. Endocrinology 146:256–264. doi: 10.1210/en.2004-1090

Nakano T, Kameda M, Shoji Y, et al (2014) Effect of severe environmental thermal stress on redox state in salmon. Redox Biol 2:772–776. doi: 10.1016/j.redox.2014.05.007

Noyes PD, McElwee MK, Miller HD, et al (2009) The toxicology of climate change: Environmental contaminants in a warming world. Environ Int 35:971–986. doi: 10.1016/j.envint.2009.02.006

Nwani CD, Lakra WS, Nagpure NS, et al (2010) Toxicity of the herbicide atrazine: Effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa Punctatus* (Bloch). Int J Environ Res Public Health 7:3298–3312. doi: 10.3390/ijerph7083298

Oliveira CR de, Fraceto LF, Rizzi GM, et al (2016) Hepatic effects of the clomazone herbicide in both its free form and associated with chitosan-alginate nanoparticles in bullfrog tadpoles. Chemosphere 149:304–313. doi: 10.1016/j.chemosphere.2016.01.076

Oruç EÖ, Usta D (2007) Evaluation of oxidative stress responses and neurotoxicity potential of

diazinon in different tissues of *Cyprinus carpio.* Environ Toxicol Pharmacol 23:48–55. doi: 10.1016/j.etap.2006.06.005

Peltzer PM, Junges CM, Attademo AM, et al (2013a) Cholinesterase activities and behavioral changes in *Hypsiboas pulchellus* (Anura: Hylidae) tadpoles exposed to glufosinate ammonium herbicide. Ecotoxicology 22:1165–1173. doi: 10.1007/s10646-013-1103-8

Peltzer PM, Lajmanovich RC, Attademo AM, et al (2013b) Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (*Trachycephalus typhonius*) tadpoles. Ecotoxicol Environ Saf 98:142–151. doi: 10.1016/j.ecoenv.2013.09.010

Pereira L, Fernandes MN, Martinez CBR (2013) Hematological and biochemical alterations in the fish *Prochilodus lineatus* caused by the herbicide clomazone. Environ Toxicol Pharmacol 36:1–8. doi: 10.1016/j.etap.2013.02.019

Prado VHM Do, Borges RE, Silva FR Da, et al (2008) Amphibia, Anura, Hylidae, Phyllomedusa azurea: Distribution extension. Check List 4:55–56.

Primel EG, Zanella R, Kurz MHS, et al (2005) Poluição das águas por herbicidas utilizados no cultivo do arroz irrigado na região central do Estado do Rio Grande do Sul, Brasil: Predição teórica e monitoramento. Quim Nova 28:605–609. doi: 10.1590/S0100-40422005000400010

Provete DB, Garey MV, Silva FR Da, Rossa-Feres DDC (2011) Anurofauna do noroeste paulista: lista de espécies e chave de identificação para adultos. Biota Neotrop 11:377–391. doi: 10.1590/S1676-06032011000200036

Rome LC (2007) The effect of temperature and thermal acclimation on the sustainable performance of swimming scup. Philos Trans R Soc Lond B Biol Sci 362:1995–2016. doi: 10.1098/rstb.2007.2083

Semlitsch RD (1988) time and size ar metamorphosis related to adult fitness.pdf. Ecology 69:184–192.

Sevgiler Y, Oruç EÖ, Üner N (2004) Evaluation of etoxazole toxicity in the liver of *Oreochromis niloticus*. Pestic Biochem Physiol 78:1–8. doi: 10.1016/j.pestbp.2003.09.004

Sies H (2003) Assay of Glutathioine Glutathione Disulfide and Glutathione Miced Disulfides in Biological Samples. 77:10.

Sokal R and RJ (1995) Biometry: The Principles and Practices of Statistics in Biological Research: New York

Stefani Margarido TC, Felício AA, de Cerqueira Rossa-Feres D, Alves de Almeida E (2013) Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. Mar Environ Res 91:61–67. doi: 10.1016/j.marenvres.2013.02.001

Stephensen E, Sturve J, Förlin L (2002) Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comp Biochem Physiol - C Toxicol Pharmacol 133:435–442. doi: 10.1016/S1532-0456(02)00129-1

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 27:502–522. doi: 10.1016/0003-2697(69)90064-5

Tu HT, Silvestre F, Meulder B De, et al (2012) Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (*Penaeus monodon*). Chemosphere 86:83–91. doi: 10.1016/j.chemosphere.2011.09.022

van der Oost R, Beyer J, Vermeulen NP. (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149. doi: 10.1016/S1382-6689(02)00126-6

Vinagre C, Madeira D, Mendonça V, et al (2014) Effect of increasing temperature in the differential activity of oxidative stress biomarkers in various tissues of the Rock goby, *Gobius paganellus*. Mar Environ Res 97:10–14. doi: 10.1016/j.marenvres.2014.01.007

Vinagre C, Madeira D, Narciso L, et al (2012) Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecol Indic 23:274–279. doi: 10.1016/j.ecolind.2012.04.009

Wheelock CE, Phillips BM, Anderson BS, et al (2008) Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). Rev Environ Contam Toxicol 195:117–178. doi: 10.1007/978-0-387-77030-7_5

Wilbur HM (1990) Coping with Chaos: Toads in ephemeral ponds. Trends Ecol Evol 5:37. doi: 10.1016/0169-5347(90)90043-D

Zanella R, Primel EG, Machado SLO, et al (2002) Monitoring of the herbicide clomazone in environmental water samples by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection. Chromatographia 55:573–577. doi: 10.1007/BF02492903

Zar JH (1999) Biostatistical Analysis. Prentice Hall

Supporting Information

Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Eupemphix nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles

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Table S1: Mean values of the body weight, snout-vent length and stage of development of tadpoles from *E. nattereri* exposed to clomazone at different temperatures

Table S2: Mean values of the body weight, snout-vent length and stage of development of tadpoles from *R. schneideri* exposed to clomazone at different temperatures

Table S3: Table summarizing stage of development of tadpoles from *E. nattereri* after 3-days exposure to clomazone

Table S4: Table summarizing stage of development of tadpoles from *E. nattereri* after 8-days exposure to clomazone

Table S5: Table summarizing stage of development of tadpoles from *R. schneideri* after 3-days exposure to clomazone

Table S6: Table summarizing stage of development of tadpoles from *R. schneideri* after 8-days exposure to clomazone

Table S1: Mean values (±S.E.M) of the body weight, snout-vent length (SVL) and the effects on the stage of development (*N*: non-affected, +: acceleration on development) in tadpoles of *E. nattereri* exposed to clomazone (Gamit®) during three and eight days at different temperatures (ANOVA-2-way, Tukey test, $P < 0.05$).

	28°C				32°C				36°C			
3 DAYS	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**
Weight	0.102± 0.03	0.107± 0.04	0.103 ± 0.04	0.114 ± 0.02	0.121 ± 0.03	0.101 ± 0.03	0.103 ± 0.03	0.124 ± 0.02	0.136 ± 0.04	0.103 ± 0.05	0.103 ± 0.03	0.123 ± 0.04
Length	0.784 ± 0.08	0.795 ± 0.12	0.745 ± 0.07	0.803 ± 0.11	0.846 ± 0.06	0.816 ± 0.07	0.763 ± 0.08	0.835 ± 0.08	0.867 ± 0.08	0.773 ± 0.15	0.812 ± 0.08	0.804 ± 0.09
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*
8 DAYS												
Weight	0.085± 0.02	0.129± 0.03	0.147 ± 0.06	0.089 ± 0.03	0.118 ± 0.05	0.111± 0.06	0.110 ± 0.04	0.102 ± 0.04	0.115 ± 0.04	0.145 ± 0.05	0.130 ± 0.05	0.126 ± 0.04
Length	0.792 ± 0.04	0.817 ± 0.02	0.908 ± 0.12	0.785 ± 0.09	0.845 ± 0.13	0.815 ± 0.12	0.752 ± 0.25	0.815± 0.08	0.793 ± 0.14	0.913 ± 0.08	0.883 ± 0.10	0.845± 0.10
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	+	*N*	*N*

Table S2: Mean values (±S.E.M) of the body weight, snout-vent length (SVL) and the effects on the stage of development (*N*: non-affected, +: acceleration on development) in tadpoles of *R. schneideri* exposed to clomazone (Gamit®) during three and eight days at different temperatures (ANOVA-2-way, Tukey test, $P < 0.05$).

	28°C				32°C				36°C			
3 DAYS	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**	**Control**	**C1**	**C2**	**C3**
Weight	0.047 ± 0.01	0.044 ± 0.009	0.041 ± 0.01	0.039 ± 0.007	0.049 ± 0.01	0.046 ± 0.01	0.040 ± 0.009	0.042± 0.005	0.045 ± 0.01	0.057 ± 0.01	0.040 ± 0.009	0.041 ± 0.008
Length	0.656 ± 0.08	0.636 ± 0.09	0.644 ± 0.05	0.619 ± 0.06	0.685 ± 0.06	0.601 ± 0.09	0.648 ± 0.14	0.651 ± 0.05	0.648 ± 0.03	0.723 ± 0.06	0.651 ± 0.06	0.665 ± 0.03
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*
8 DAYS												
Weight	0.056 ± 0.01	0.058 ± 0.01	0.062 ± 0.02	0.044 ± 0.01	0.063 ± 0.02	0.066 ± 0.02	0.048 ± 0.02	0.061 ± 0.02	0.046 ± 0.01	0.056 ± 0.02	0.057 ± 0.01	0.069 ± 0.01
Length	0.713 ± 0.05	0.724 ± 0.07	0.718 ± 0.09	0.660 ± 0.12	0.726 ± 0.09	0.746 ± 0.07	0.730 ± 0.09	0.647± 0.09	0.716 ± 0.07	0.721 ± 0.06	0.729 ± 0.06	0.724 ± 0.04
Stage	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*

Table S3: Table summarizing stage of development (Gosner, 1960) of tadpoles from *E. nattereri* after 3-days exposure to clomazone (Gamit®). Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development									
		stg27	stg28	stg29	stg30	stg31	stg32	stg33	stg34	stg35	stg36
28°C	Control	2	0	0	2	4	1	0	0	1	0
	0.01mg/L	1	0	1	3	0	2	2	0	0	1
	0.05 m/L	2	2	1	1	1	1	0	0	1	1
	0.1 mg/L	2	0	1	0	2	2	0	0	1	2
32°C	Control	0	1	0	2	1	2	2	0	2	0
	0.01mg/L	0	1	1	2	2	3	0	0	0	1
	0.05 m/L	4	1	0	1	3	0	0	0	0	1
	0.1 mg/L	0	1	0	2	2	1	3	0	0	1
36°C	Control	0	1	1	0	4	1	0	2	0	1
	0.01mg/L	2	1	0	3	1	0	3	0	0	0
	0.05 m/L	2	1	1	2	1	0	1	1	0	1
	0.1 mg/L	1	0	2	1	0	2	2	0	0	2

Table S4: Table summarizing stage of development (Gosner, 1960) of tadpoles from *E. nattereri* after 8-days exposure to clomazone (Gamit®). Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development												
		stg27	stg28	stg29	stg30	stg31	stg32	stg33	stg34	stg35	stg36	stg37	stg38	stg39
28°C	Control	3	0	1	1	1	2	2	0	0	0	0	0	0
	0.01mg/L	0	0	0	2	0	4	0	2	0	1	0	0	1
	0.05 m/L	0	0	1	1	1	3	0	1	0	0	0	3	0
	0.1 mg/L	0	2	3	3	0	1	1	0	0	0	0	0	0
32°C	Control	0	0	1	2	2	0	1	1	1	0	0	2	0
	0.01mg/L	1	2	2	0	1	1	1	0	0	0	0	0	2
	0.05 m/L	0	0	1	3	0	3	1	0	0	0	0	0	2
	0.1 mg/L	0	0	1	2	1	1	2	1	0	0	0	0	2
36°C	Control	0	1	2	2	1	0	1	0	1	0	1	0	1
	0.01mg/L	0	1	0	1	2	0	0	0	1	1	1	0	3
	0.05 m/L	0	1	2	2	0	0	0	0	0	3	0	0	2
	0.1 mg/L	1	0	0	1	2	1	3	0	0	2	0	0	0

Table S5: Table summarizing stage of development (Gosner, 1960) of tadpoles from *R. schneideri* after 3-days exposure to clomazone (Gamit®). Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development						
		stg29	stg30	stg31	stg32	stg33	stg34	stg35
28°C	Control	0	0	4	4	2	0	0
	0.01mg/L	0	0	3	4	3	0	0
	0.05 m/L	2	0	3	2	3	0	0
	0.1 mg/L	1	2	2	5	0	0	0
32°C	Control	0	0	4	3	3	0	0
	0.01mg/L	1	1	3	3	1	1	0
	0.05 m/L	0	0	1	6	3	0	0
	0.1 mg/L	0	0	2	4	3	1	0
36°C	Control	0	0	5	3	2	0	0
	0.01mg/L	0	0	2	3	2	1	2
	0.05 m/L	0	0	4	6	0	0	0
	0.1 mg/L	0	1	4	3	2	0	0

Table S6: Table summarizing stage of development (Gosner, 1960) of tadpoles from *R. schneideri* after 8-days exposure to clomazone (Gamit®). Numbers represent the amount of tadpoles classified in each Gosner (1960) stage.

Treatments		Stage of development							
		stg28	stg29	stg30	stg31	stg32	stg33	stg34	stg35
28°C	Control	0	1	2	1	3	2	1	0
	0.01mg/L	2	0	0	3	3	2	0	0
	0.05 m/L	0	1	1	4	4	0	0	0
	0.1 mg/L	2	1	2	2	2	1	0	0
32°C	Control	0	0	0	4	4	1	0	1
	0.01mg/L	0	0	0	3	3	2	2	0
	0.05 m/L	1	2	1	2	3	0	1	0
	0.1 mg/L	1	0	1	3	3	1	1	0
36°C	Control	1	1	1	4	2	1	0	0
	0.01mg/L	3	0	1	2	2	2	0	0
	0.05 m/L	0	3	2	2	2	1	0	0
	0.1 mg/L	0	0	1	3	4	1	1	0

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Influence of Temperature on the Thyroidogenic Effects of Diuron and Its Metabolite 3,4-DCA in Tadpoles of the American Bullfrog (*Lithobates catesbeianus*)

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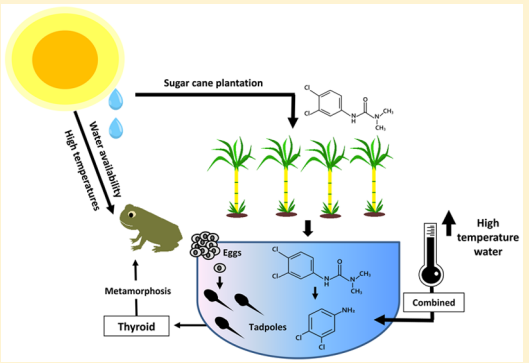
Supporting Information

ABSTRACT: Temperature is a key variable affecting the timing of amphibian metamorphosis from tadpoles to tetrapods, through the production and subsequent function of thyroid hormones (TH). Thyroid function can be impaired by environmental contaminants as well as temperature. Tadpoles can experience large temperature fluctuations in their habitats and many species are distributed in areas that may be impacted by agriculture. Diuron is a widely used herbicide detected in freshwater ecosystems and may impact endocrine function in aquatic organisms. We evaluated the influence of temperature (28 and 34 °C) on the action of diuron and its metabolite 3,4-dichloroaniline (3,4-DCA) on thyroid function and metamorphosis in tadpoles of *Lithobates catesbeianus*. Exposure to both compounds induced more pronounced changes in gene expression and plasma 3,3′,5-triiodothyronine (T_3) concentrations in tadpoles treated at higher temperature. T_3 concentrations were increased in tadpoles exposed to 200 ng/L of diuron at 34 °C and an acceleration of metamorphosis was observed for the same group. Transcriptomic responses included alteration of thyroid hormone induced bZip protein (*thibz*), deiodinases (*dio2*, *dio3*), thyroid receptors (*trα*, *trβ*) and Krüppel-like factor 9 (*klf9*), suggesting regulation by temperature on TH-gene expression. These results suggest that environmental temperature should be considered in risk assessments of environmental contaminants for amphibian species.



INTRODUCTION

Temperature is an important environmental factor regulating the development, metabolism, and behavior of poikilothermic or cold-blooded animals.[1] Changes in environmental temperature have been observed to affect distributions of fish populations[2−4] and time needed to complete metamorphosis for many amphibian species.[5,6] However, indirect effects of temperature such as the potential for interactions with environmental pollutants have not been well-characterized.[7] Since thermal variation of the environment can be translated directly to physiological performance,[8] the toxic effects of chemicals may be more pronounced at higher temperatures.[9]

Amphibians, especially during the larval stage of life, are particularly vulnerable to these interactions since they often inhabit small ephemeral ponds. Water temperature is elevated by water loss, increasing the mean temperature and the daily thermal variation of bodies of water.[10] If located in agricultural areas, exposure to pesticides may occur in combination to elevations in temperature. This is especially true in tropical areas of the world, where the temperatures can reach very high averages during the summer. In southeastern Brazil, the state of São Paulo is the largest producer of sugar cane and is responsible for the usage of approximately 20% of all Brazilian pesticides.[11] In this area, as in other tropical regions of the world, intensive agricultural activity and the use of pesticides coincides with the rainy season, which is the hottest season and the period of reproductive activity for many local species of amphibians due to the greater availability of water.[12,13]

Diuron (3-(3,4-dichlorophenyl)-1, 1-dimethylurea) is one of the most frequently detected pesticides in freshwater ecosystems.[14,15] It is widely used for sugar cane cultivation in Brazil,[16] which is used for the production of sugar and ethanol.[17] Following applications to soil, diuron can undergo

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runoff to surface water,[18] potentially leading to negative effects to aquatic organisms.[19] Endocrine disrupting effects of diuron have been reported in many nontarget organisms such as snails, fish, frogs, and lizards.[15,20−24] In vertebrates, the endocrine system can be affected by different chemicals leading to alteration in hormone levels, including circulating thyroid hormones such as 3,3′,5-triiodothyronine (T_3) and thyroxine (T_4), which could negatively affect metabolism rates, growth and development.[25] Exposures to thyroid-disrupting chemicals are of special concern to tadpoles because frog metamorphosis is regulated by thyroid hormones (TH), promoting the remodeling of the aquatic larvae into an adult tetrapod.[26] Previous studies have shown that at least four species of tadpoles had growth rates and development affected by exposure to diuron, although at concentrations much higher than normally observed in field applications.[22] Although diuron affects metamorphosis, it is still unknown if this herbicide impacts the thyroid gland at environmentally relevant concentrations. Considering that exposure to pesticides has been considered one of the main causes contributing to the decline of amphibian populations worldwide,[27,28] and taking into account the intense use of diuron in agriculture, the evaluation of effects caused by this herbicide at environmentally relevant concentrations could be of great importance for a better understanding of the potential risks of diuron to amphibian populations.

Although the potential of some pesticides to inhibit metamorphosis and to damage the thyroid gland in amphibians has been examined, few studies have evaluated the effects caused by endocrine disruptors under different thermal gradients. Since tadpoles are often exposed to a range of temperatures in their natural systems, this study aimed to investigate the effects caused by interaction between temperature and the known endocrine disruptor diuron and its metabolite 3,4-DCA in tadpoles of *Lithobates catesbeianus*, the American bullfrog, using end points for evaluating disrupting effects linked to thyroid activity. In this study we assessed genes involved in metamorphic pathways, including thyroid hormone receptors (*trα* and *trβ*), deiodinases (*dio2* and *dio3*), TH-induced bZip protein (*thibz*) and the transcriptor factor Krüppel-like factor 9 (*klf9*) and the relationship of their expression to TH levels. We also investigated the integrated effects on the growth and development of the tadpoles, showing how the metamorphosis process can be affected. We hypothesized that temperature increases should enhance the endocrine effects of diuron and 3,4-DCA, with alterations on thyroid-related gene expression and circulating thyroid hormone levels.

MATERIALS AND METHODS

Animals. *Lithobates catesbeianus* (Anura, Ranidae) is an amphibian species native from North America that was introduced in South America,[29,30] and is commonly used as an experimental model in biomedical research and studies on ecotoxicology. Tadpoles of *L. catesbeianus* (American Bullfrog) were provided from Agrobusiness Ranaville (São Roque, Brazil). After transport, the animals were maintained in laboratory under controlled conditions of temperature (26.5 °C), pH, (7.5−8.0) and constant aeration for acclimatization until they reached the prometamorphic stage, which is the period of appearance of the hindlimbs to the forelimbs. This stage of development was chosen because the amount of spontaneously released thyroid stimulating hormone (TSH) is higher in prometamorphic and climactic tadpoles than in early premetamorphic larvae, juvenile and adult frogs.[31] The larval period of this particular anuran species can extend over 2−3 years,[32] and so the temperature is considered a key regulator in the postembryonic developmental program.[32]

Experimental Design. *Temperature Treatment.* Aquariums containing dechlorinated water were heated to two different temperatures, 28 and 34 °C. Temperatures were maintained by autoregulating thermostats in 5 L aquaria placed in a 100 L "water bath". The temperature of the tanks and individual aquaria had their values recorded daily to observe possible variations during the experimental period. Temperature values used in this study were based data obtained after monitoring water temperature in five natural ponds in which tadpoles are frequently found in northwest Brazil (Supporting Information (SI) Figure S1) (see SI Table S1 for coordinates), recorded during the summer (December, 2014 to January, 2015), which coincides with the rainy season and reproduction for most species of neotropical frogs.

Chemicals. All chemicals used were of analytical grade and purchased from Sigma-Aldrich Chemical (St. Louis, MO).

Exposures. Tadpoles of *L. catesbeianus* were exposed to 40 ng/L and 200 ng/L of herbicide diuron (3-(3,4-dichlorophenyl)-1, 1-dimethylurea) or its metabolite 3,4−DCA (3,4−dichloroaniline) at temperatures of 28 and 34 °C. Exposure concentrations were based on previous studies showing a maximum value of 40 ng/L in surface waters of Central Valley rivers in northern California[15] and an arbitrary 5-fold higher concentration. Tadpoles were exposed to each concentration at temperatures of 28 and 34 °C for 7 days. The experimental groups consisted of five replicates (n = 5) containing three tadpoles in each aquarium (15 animals in total). The tadpoles were placed in aquariums and the water temperature was gradually adjusted during the first day to avoid thermal shock. Then, the chemicals were added at nominal concentrations as described above. Both contaminants were dissolved in a stock solution of 1 mL acetone and then added (concentration lower than 0.0001%) into the aquariums. Control groups also received the same volume of acetone to avoid ambiguous interpretation of the results due to possible solvent effects. Aquaria were constantly aerated at a pH of 8.00 ± 0.26 and using a light-dark cycle of 12−12 h. The water was renewed every 2 days to ensure the original concentration of the compounds and the dissolved oxygen, pH and ammonia were quantified for the same period. No significant variations on these parameters were observed among treatments during the experiments (dissolved oxygen 6.1−7.1 mg/L, pH 7.5−8.0). Measurements of diuron and 3,4-DCA were made as described below. The animals were fed every 2 days with commercial feed for tropical fish in minimum quantities (200 mg). At the end of the experiment, the tadpoles were removed from the tanks and the blood was collected from the tail vein in order to measure thyroid hormones, using the local anesthetic lidocaine (50 mg/g). Subsequently, the animals were anesthetized with a lethal dose of benzocaine (100 mg/L, Sigma, Aldrich) and the liver of each animal was removed and immediately immersed in RNAlater (Sigma, Aldrich), a reagent used to stabilize RNA, and stored at −80 °C until further analysis. All the procedures were done in accordance with the Ethics Committee on Animal Use in Research of the São Paulo State University (CEUA-IBILCE/UNESP No. 119/2015).

Analysis and Measured Parameters. Morphological Analysis. Effects on growth (snout−vent length (SVL), tail

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length and total body weight) and development stages were evaluated in the tadpoles after 7 days of exposure to the chemicals. Stages of development of individual tadpoles were evaluated according to Gosner (1960). SVL and weight measurements were used to calculate the condition factor of tadpoles (Condition factor (k) = 100.(weight/SVL^3).

Thyroid Hormones. Blood from two animals of each replicate was collected and pooled due to the small size of the tadpoles, in order to obtain sufficient amounts of blood for the analyses. Individual syringes and needles were heparinized to prevent clotting. Tubes containing blood were centrifuged at 2000g for 15 min and the plasma was subsequently collected and frozen at −80 °C until further analysis. Total 3,3′,5′-triiodothyronine (T_3) was measured by enzyme immunoassay (ELISA) kit (Sigma, Aldrich), ranging from 0.0−7.5 ng/mL. The quality criteria for the application of kits produced for human plasma was verified by testing the values in a serial dilution. The limit of detection for this kit was 0.0078 ng/mL. The levels of T_3 (ng/mL) were normalized with the protein concentration of each sample and the final concentration of T_3 was presented in ng/mg. We also performed immunoassay analyzes to detect total thyroxine (T_4) using ELISA kit produced for human plasma (Sigma, Aldrich) ranging from 0 to 25 μg/dL, but the sensitivity of the method was insufficient to measure T_4 levels in *L. catesbeianus* at this stage of development. In this assay the limit of detection was 0.438 μg/dL, obtained from serial standard dilutions.

Gene Expression. RNA Isolation and Reverse Transcription. Total RNA from liver tissue was extracted from two animals of each replicate (n = 10) using Promega Kit (SV Total RNA Isolation System). Total RNA was suspended in RNase-free water and the concentrations were determined using the NanoDrop 1000 Spectrophotometer (NanoDrop Technologies Inc.). After RNA extraction, total cDNA was synthesized using Promega "Reverse Transcription System" according to Reserve Transcription Protocol. First, aliquots containing 1 μg of total RNA were incubated at 70 °C for 10 min. Then, cDNA was synthesized in a 20 μL reverse transcription reaction by adding 4 μL $MgCl_2$ (25 mM), 2 μL Reverse Transcription 10× Buffer, 2 μL dNTP Mixture (10 mM), 0.5 μL Recombinant Rnasin Ribonuclease Inhibitor, 15u AMV Reverse Transcriptase (High Conc.) and 0.5 μg Random Primer. The reaction was incubated at room temperature for 10 min, and then incubated at 42 °C for 15 min. After, the samples were heated at 95 °C for 5 min, then incubated at 0−5 °C for 5 min and stored at −20 °C until further analysis.

Real-Time Quantitative RT-PCR. RT-PCR was performed using primers designed to protein coding regions of the partial cDNAs for deiodinase enzymes (*dio2* and *dio3*), thyroid hormone receptors (*trα* and *trβ*), TH-induced bZip protein (*thibz*) and Krüppel-like factor 9 (*klf9*). Gene ribosomal protein *rpl8* was used as normalizing gene, based on previous studies that have effectively employed this gene as control in analyzes evaluating expression of genes associated with thyroid signaling pathway in tadpoles.[33,34] All primers were synthesized by Integrated DNA Technologies (Coralville, IA) and they were designed based on cDNA sequences from *Lithobates catesbeianus* (*trα*, *trβ*, *dio2*, and *dio3*) and *Pelophylax nigromaculatus* (*thibz* and *klf9*) (SI Table S2). Specificity of the primer sets was confirmed by sequencing PCR products, as well as by melt-curve analysis during the quantitative PCR runs.

Quantitative RT-PCR was conducted in 20 μL reactions. Each reaction contained 6.0 μL nuclease-free water (Sigma, Aldrich), 10.0 μL iTaq SYBR green Supermix (Bio-Rad, Hercules, CA), 1.0 μL each of forward and reverse primers (50 mM), and 2.0 μL of reverse-transcribed cDNA sample. Each sample was run in duplicate in optically clear 96-well plates. PCR conditions were 3 min at 95 °C, and 40 cycles of 10s at 95 °C and 30s of 55 °C and the products were subject to melting curve analysis from 65 to 85 °C in 0.5 °C increments (2−5 s/step) with continuous fluorescence measurement. Thermal cycling was selected according to Bio-Rad protocol, which suggests the cycles and temperatures based on Syber Green One-Step kit assay. After RT-PCR runs, gel electrophoresis of RT-PCR products was performed to confirm single amplicons.

The plates were analyzed in "iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad)" and the data analyzed in MyiQ5 qRT-PCR system software. Duplicate data obtained for each sample were averaged and gene expression data was normalized in order to obtain gene quantification analysis. Comparative C_T method (ΔΔCT) was used to determine relative gene expression, considering fold-differences between samples for measuring changes in the expression level of each target gene. Relative fold differences were calculated considering the expression levels of the target genes as 1 in the control group (animals unexposed to contaminants at 28 °C). Thus, fold changes were analyzed comparing the variation on gene expression of each treated group in relation to the control group.

Chemical Analyses. Water samples (10 mL) from the experimental groups were taken before adding the tadpoles into the aquaria at the beginning of exposures and prior to each water renewal for the measurement of the concentrations of diuron and 3,4-DCA. The values were based on the average of the five replicates used for each group. Concentrations were measured by HPLC system (Shimadzu Corporation, Kyoto, Japan), which consisted of one CBM20A communication bus module, two LC20AD-XR pumps, one DGU20A3R degassing unit, one SIL20AC-XR autosampler, one CTO20AR column oven, and one SPDM20A photodiodearray (PDA) detector. Fifty microliters of the filtered (0.22 μm) water were directly injected into the system, and the compounds were separated by a Shimadzu Shim-Pack XR-ODS column (2.0 × 100.0 mm, 2.2 μm particle size, 8 nm pore size). The PDA detector was set at 200−600 nm for all analytes, which were quantified at 250 nm. Acetonitrile and water (40:60, v/v) were used as the mobile phase that was isocratically pumped at a flow rate of 0.5 mL/min. The column oven temperature was set to 40 °C. Chromatogram was monitored during 5 min and peaks were identified and quantified using LAB Solutions 5.71 software (Shimadzu Corporation). Calculations were based on a calibration curve previously constructed by injecting authentic standards into the HPLC system (10−1000 ng/L).

Statistical Analysis. The presence of outliers was evaluated and the normality of the data was checked by the Shapiro-Wilk test. Two-way analysis of variance (ANOVA 2-way) followed by the Tukey post hoc test were used to identify statistical differences (gene expression, T_3 levels and effects on growth) among the groups exposed to combined effect of temperature with Diuron or 3,4-DCA. This test was used to evaluate the individual effects of temperature (28 and 34 °C) and chemical treatments (Diuron or 3,4-DCA) and also the interaction effects between the temperature and each contaminant individually. The association of relevant variables within each contaminant was assessed by Pearson correlation, considering

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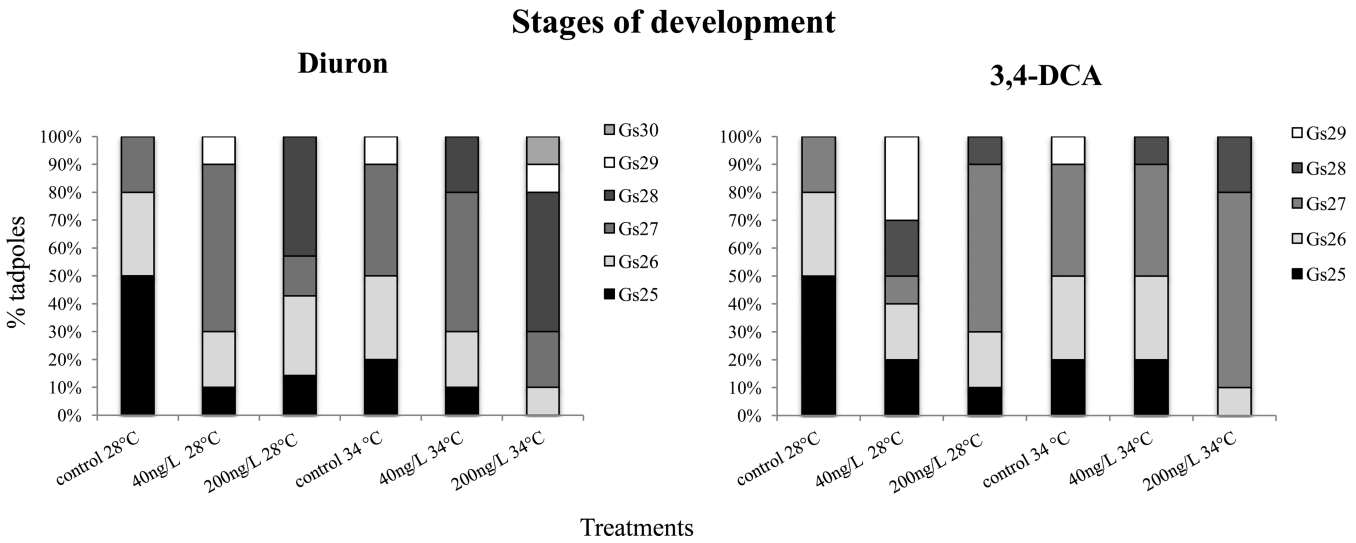

Figure 1. Developmental stages of tadpoles (*L. catesbeianus*) recorded after the treatments with diuron and 3,4-DCA at different temperatures (28 and 34 °C). Data represent the percentage of tadpoles in each stage of development (n = 10) (Gs25, Gs26, Gs27, Gs28, Gs29, and Gs30 according to Gosner, 1960). Figure shows an acceleration of development stage of tadpoles exposed to 200 ng/L of diuron or 3,4-DCA at 34 °C compared to control at 28 °C (G-test, likelihood ratio test, $P < 0.05$). Control groups are the same for both compounds.

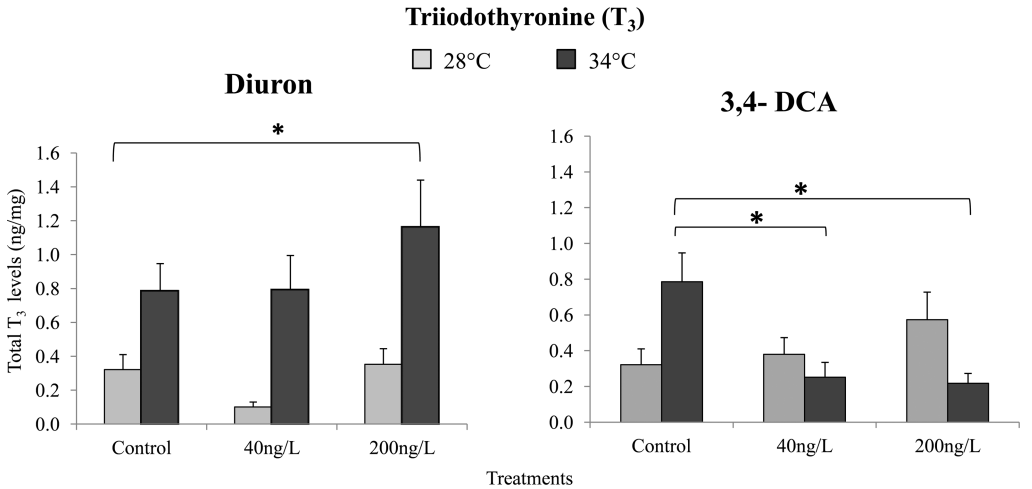

Figure 2. Total triiodothyronine (T_3) levels in plasma of *L. castebeianus* exposed to diuron and 3,4-DCA after 7 days. Data are averages (ng/mg) (+ S.E.M) (n = 10) for total T_3 concentrations. * indicates significant differences between indicated groups (ANOVA-2-way followed by Tukey, $P < 0.05$). Control groups are the same for both compounds.

data from control and treated groups at both temperatures. To assess whether there were differences between the stages of development of the tadpoles, the G test (likelihood ratio test) was performed with Yates' correction (Sokal and Rohlf 1995). Statistical analysis was conducted using the softwares R version 2.11.1 (R Development Core Team, 2010) and STATISTICA (StatSoft). Values of $P < 0.05$ were considered as a reference to assign statistical significance.[35]

RESULTS

Chemical Analysis in Water. Measured concentrations of diuron and 3,4-DCA (40 and 200 ng/L; mean ± SD) in water were 41.07 ± 14.26, 230.56 ± 55.39 ng/L (diuron), and 48.48 ± 7.82, 193.56 ± 11.94 ng/L (3,4-DCA).

Effects on Tadpole Mortality, Growth and Development. Mortality was not observed in any of the experimental groups. Effects on growth (snout−vent length (SVL), tail length and weight) were assessed at the end of the treatments. Temperature alone (without diuron or 3,4-DCA exposure) did not affect the weight of tadpoles (P = 0.347). Diuron also had no effects on the weight of animals exposed at 28 °C ($P > 0.05$). However, an effect was observed with diuron between tadpoles treated with 40 ng/L (P = 0.008) and 200 ng/L at 34 °C (P = 0.0324). 3,4-DCA treatments had no effects on weight of tadpoles (P = 0.260) and neither had an interaction with temperature (P = 0.152). Length and condition factors of tadpoles was also unchanged by all treatments with diuron ($P > 0.05$) or 3,4-DCA ($P > 0.05$) (SI Table S3).

Tadpoles were classified at six different stages of development (according to Gosner (1960) scales: Gs25, Gs26, Gs27, Gs28, Gs29, and Gs30) and the percent of animals at each Gosner stage is summarized in Figure 1. We observed that at the end of the experiment, the control group maintained at 28 °C presented tadpoles distributed at the three younger stages of development, with 50% of the tadpoles at Gs25, 30% at Gs26 and 20% at Gs27. No effect of temperature was observed

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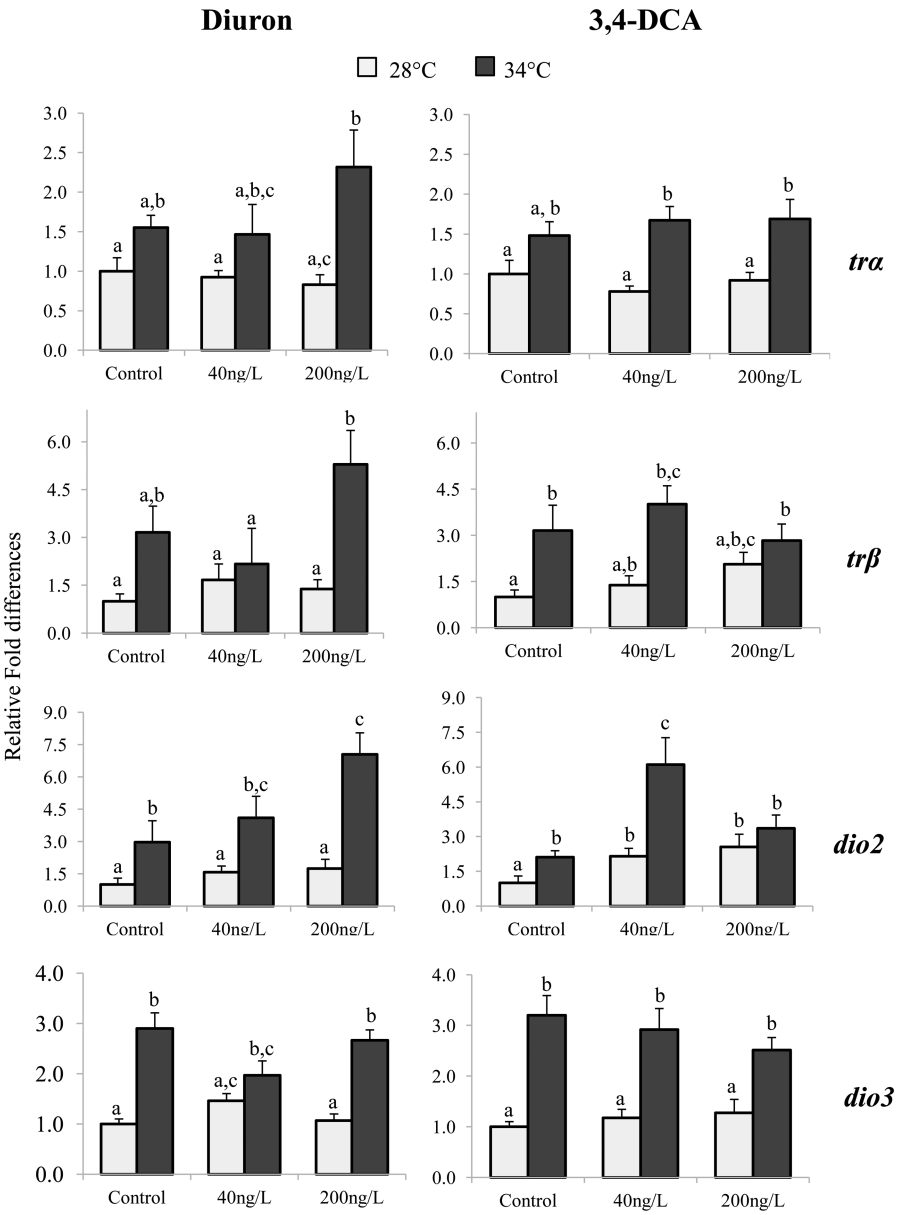

Figure 3. Effects of diuron and 3,4-DCA at different temperatures (28 and 34 °C) on the relative abundance of thyroid hormone receptors (*trα*, *trβ*) and deiodinases (*dio2*, *dio3*) mRNA in tadpoles of *L. catesbeianus* after 7-days exposure. Data are presented as fold change relative to control 28 °C (n = 10). Different letters between the groups indicate statistical significance (ANOVA 2-way, Tukey, $P < 0.05$).

between control groups at 28 and 34 °C (P = 0.492). Treatment with both compounds diuron (P = 0.252) or 3,4-DCA (P = 0.159) did not alter the development of animals at 28 °C. However, interaction between diuron and higher temperature caused an acceleration of the development of animals (P = 0.0033). Treatment with 200 ng/L of diuron at 34 °C showed a significant decrease in the frequency of tadpoles at stages Gs25 (decrease of 100%) and Gs26 (decrease of 20%) compared to control at 28 °C. In addition, 50% of the tadpoles reached the Gs28 stage and 10% reached the stages Gs29 and Gs30 in this group. A similar interaction between 3,4-DCA and higher temperature was also detected (P = 0.0083). For the group exposed to 200 ng/L of 3,4-DCA at 34 °C, a reduction of 20% was observed in tadpoles at the Gs26 stage compared to the control at 28 °C, in addition to an increase of 50% of tadpoles at the Gs27 stage and 20% at the Gs28 stage (Figure 1).

T_3 Analysis. Changes in T_3 levels were not observed between tadpoles of control groups at different temperatures (P = 0.35), as shown in Figure 2. Treatment with diuron or 3,4-DCA also had no effects on T3 levels at 28 °C (P = 0.165). However, an interaction with diuron was observed with the higher temperature ($P < 0.001$). Tadpoles exposed to 200 ng/L of diuron at 34 °C had their T_3 levels increased compared to the control group at 28 °C (P = 0.007). In contrast, tadpoles exposed to 40 and 200 ng/L of 3,4-DCA at 34 °C presented a decrease in T_3 levels compared with the control group at the same temperature (P = 0.017 and P = 0.009, respectively for 40 and 200 ng/L), but no difference was detected compared to the control at 28 °C (P = 0.985) (Figure 2).

Gene Expression. The exposure of tadpoles to diuron and 3,4-DCA for 7 days promoted changes on gene expression with temperature-associated responses (Figure 3 and 4). Temperature also had isolated effects on expression of some genes.

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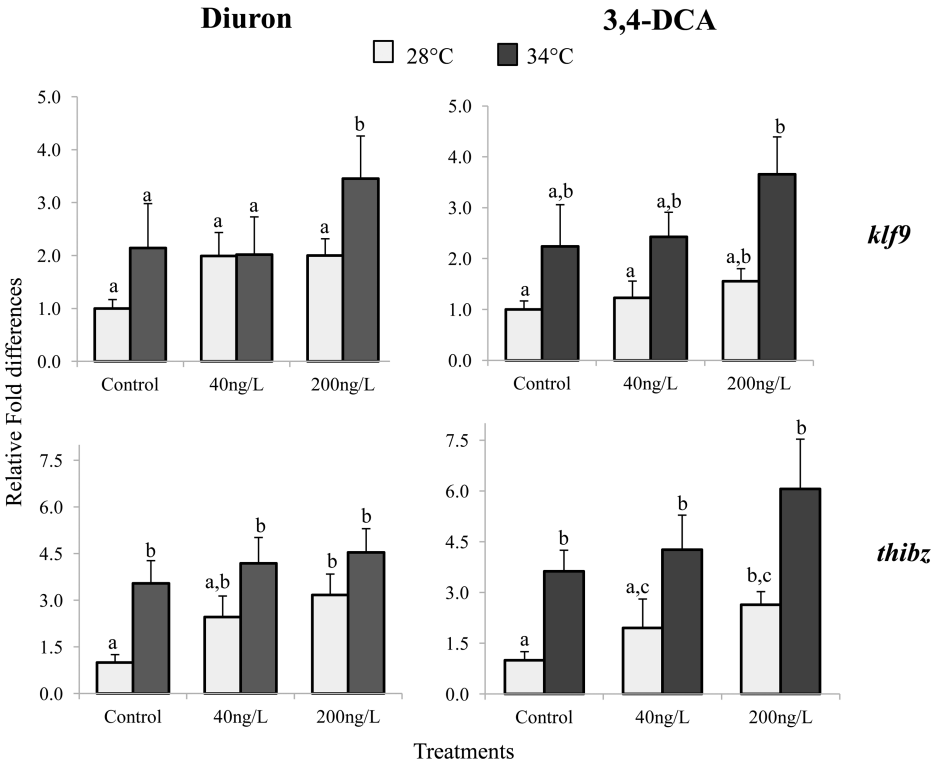

Figure 4. Effects of diuron and 3,4-DCA on the relative abundance of Krüppel-like factor 9 (*klf9*) and thyroid hormone-induced bZip protein (*thibz*) mRNA in tadpoles of *L. catesbeianus* after 7-days exposure at different temperatures (28 and 34 °C). Data are presented as fold change relative to control 28 °C (n = 10). Different letters between the groups indicate statistical significance (ANOVA 2-way, Tukey, $P < 0.05$).

Animals of the control group maintained at 34 °C showed an increased expression of *trβ*, *dio2*, *dio3*, and *thibz* compared with the control at 28 °C (P = 0.012, P = 0.005, P = 0.0001, P = 0.003, respectively).

Exposure to diuron at 28 °C failed to alter the expression of the targeted genes ($P > 0.05$) (Figure 3 and 4), with the exception of *thibz* which had a 3-fold increased expression in tadpoles exposed to 200 ng/L (P = 0.014) (Figure 4). However, a significant interaction of diuron with higher temperature was observed on the expression of the target genes ($P < 0.001$). Significant temperature-associated increases of *trα* and *trβ* expression was observed in animals exposed to 200 ng/L of diuron at 34 °C compared to animals of control group assessed at 28 °C (P = 0.01) (Figure 3). Transcripts of *trβ* had a 5-fold increase compared to the control group ($P <$ 0.001). Increased *dio2* mRNA was observed in all groups kept at 34 °C ($P < 0.001$) with a pronounced increase of up to 7-fold in animals exposed to 200 ng/L of diuron (P = 0.0001) (Figure 3). *Dio3* transcripts were also increased in all groups treated at 34 °C compared to the control group at 28 °C ($P <$ 0.001, P = 0.008, and $P < 0.001$, respectively to control, 40 and 200 ng/L) (Figure 3). Increases in *thibz* mRNA were also observed in all groups submitted to higher temperature in combination with diuron (P = 0.0006 and P = 0.0001). Expression of *klf9* was increased only in animals exposed to the 200 ng/L of diuron at 34 °C (P = 0.031) (Figure 4).

Treatments with 3,4-DCA at 28 °C did not change expression of *trα* (P = 0.99), *trβ* (P = 0.14), *dio3* (P = 0.89) or *klf9* (P = 0.72); however, an increased expression of *dio2* mRNA was observed in tadpoles exposed to both concentrations at 28 °C (P = 0.008 and P = 0.001) and *thibz* transcripts were increased in animals exposed to 200 ng/L at 28 °C (P = 0.035) (Figure 3 and 4). At 34 °C, all genes examined had altered expression as a result of the interaction with 3,4-DCA. Animals exposed to both concentrations of 3,4-DCA had higher *trα* and *trβ* mRNA at 34 °C compared to animals assessed at 28 °C (P = 0.020 and 0.038, respectively) (Figure 3). *Dio2* expression was also increased in all groups exposed to higher temperature (P = 0.005, P = 0.0002, respectively for control 34 °C and 200 ng/L), but a marked increase of up to 6-fold was noticed in tadpoles exposed to 40 ng/L (P = 0.0001) (Figure 4). Similar to diuron treatments, the levels of *dio3* and *thibz* mRNA were increased in all animals kept at 34 °C compared with animals exposed at 28 °C ($P < 0.005$) (Figures 3 and 4). Transcripts of *klf9*, were increased only in animals exposed to 200 ng/L of 3,4-DCA at 34 °C (P = 0.008) (Figure 4).

Correlations. The expression of *dio2* and *dio3* was compared to total T_3 concentrations in animals exposed to both compounds and temperatures, since deiodinase enzymes regulate the activity of thyroid hormone. Transcripts of *dio2* and *dio3* were positively correlated (r = 0.504 and P = 0.0018; r = 0.411 and P = 0.0012, respectively) with T_3 levels in animals exposed to diuron. Expression of *trβ* and *klf9* mRNA were also submitted to Pearson correlation, since it has been reported that *klf9* influences TH/TR signaling by regulating the expression of *trβ* in amphibians. A strong positive correlation (r = 0.682 and $P < 0.001$) between *trβ* and *klf9* was observed in animals undergoing diuron treatment and a moderate positive correlation (r = 0.405 and $P < 0.001$) was showed in animals exposed to 3,4-DCA (SI Figure S2).

DISCUSSION

Developmental stages of tadpoles are commonly altered by abiotic factors,[6] as well as environmental contaminants.[36,37]

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Temperature is a key variable affecting the timing of larval transition in frogs and the exposure to elevated temperatures usually reduce the time needed to complete metamorphosis for many species,[6] such as *Scaphiopus couchii* and *Spea multiplicata*.[38] Accelerated metamorphosis is considered to be an acclimative mechanism for dealing with temperature stressed environments.[7] Several commonly used pesticides have been identified to disturb thyroid function in amphibians, especially when exposure occurs during larval stages and metamorphosis.[39−41] However, few studies have considered the combined effects of environmental temperature on pesticide toxicity. In the current study, diuron and its metabolite 3,4-DCA changed the expression of thyroid associated genes and T_3 plasma concentrations in prometamorphic tadpoles of *L. catesbeianus* maintained at different temperatures. Our results showed that both compounds had more pronounced effects at higher temperature. In fact, previous studies have shown that temperature plays an important role on chemical toxicity to amphibians. For example, mortality of tadpoles of *R. clamitans* exposed to insecticide carbaryl occurs faster at lower concentrations when animals are exposed to higher temperatures.[42] At a molecular level, both increases and decreases on expression of TH-induced genes in tadpoles of *L. catesbeianus* maintained at cold water (5 °C) in the presence of the antimicrobial agent triclosan and T_3 (10 nM) were only evident upon temperature shifts.[43] Our findings were in accordance with previous studies indicating that temperature influences frog development alone and may have interactions with pesticides such as diuron.

Effects of temperature increase on animals unexposed to diuron or 3,4-DCA were observed only with molecular measurements. Stage of development of tadpoles and T_3 concentration in plasma were not altered by the single effect of temperature and alterations in these end points were associated with the combined effects to the compounds. Transcripts of *trβ*, *dio2*, *dio3*, and *thibz* were increased in animals maintained at 34 °C without diuron/3,4-DCA. Indeed, previous studies using tadpoles of *L. catesbeianus* have shown that some genes involved in metamorphosis, including *trα* and *trβ*, *dio2*, and *dio3*, *thibz*, and *rlk1*, are sensitive to environmental temperature.[43,44] Drastic reduction of *trβ* mRNA observed previously in liver of tadpoles exposed to low temperatures (5 °C) suggests that a temperature-sensitive step may exist in the intracellular TH signaling pathway between T_3 and thyroid receptors (TRs).[44] *Dio3* mRNA was also reported to be increased in tadpoles of *L. catesbeianus* previously injected with T_3 (10 pmol/g) after shifting the animals from cold temperatures (5 °C) to the typical husbandry temperatures (24 °C).[43] Other studies using tadpoles of the same species exposed to low temperature (5 °C) indicate that *dio3* expression is abolished by exposure to cold.[44] In agreement with those studies, our results showed that transcripts of both thyroid receptors and deiodinase genes were increased at high temperatures, indicating that in *L. catesbeianus* the temperature effects on TH-associated genes may be primordial in order to maintain the TH balance in plasma when tadpoles are subjected to thermal stress.

In general, diuron had no effect in tadpoles exposed at 28 °C. Acceleration of metamorphosis was observed only in animals exposed to combined effect of diuron at high temperature (34 °C). In addition, concentrations of T_3 in plasma were 3-fold higher in tadpoles exposed to 200 ng/L of diuron at 34 °C, which could in turn explain the acceleration of metamorphosis in this treated group, since metamorphosis in frogs is regulated by THs. It is already known that some toxicants that target the thyroid have the ability to change circulating levels of TH, altering the relationship between TH biosynthesis and elimination.[45] For example, in rats prochloraz altered thyroid function[46] and increased levels of T_3 in tadpoles of *Rana temporaria*.[39] Increased levels of T_4 were also observed in salamanders *Ambystoma tigrinum* exposed to 75 and 250 μg/L of the herbicide atrazine.[41] Interestingly, we observed in this study that the effects of diuron on T_3 levels in the concentrations studied were only significant at higher temperature. TH regulates growth and development in vertebrates and invertebrates, but also controls metabolism and maintenance of body temperature in endotherms.[47,48] However, mechanisms of TH regulation on the physiological responses in ectotherms, such as frogs, in response to changes in their thermal environment are still underexplored. Recent studies with zebrafish (*Danio rerio*) have found that the actions of TH are temperature-specific and T_3/T_4 probably regulate thermal acclimation in ectotherms.[49] They suggest that TH elicits a positive or negative response depending on the actual temperature and thermal history of the animal.[49] According to Little et al. (2013),[49] increased concentrations of total T_3 in plasma may be a response to thermal acclimation of tadpoles to higher temperatures in the environment, and their adaptability may be compromised by diuron. However, further studies are needed to confirm this possibility.

T_3 plasma concentrations are regulated by deiodinases; iodothyronine deiodinases II (Dio2) and III (Dio3).[50] Dio3 catalyzes the inactivation of thyroid hormone by inner ring deiodination of thyroxine (T4) and T3 to inactive metabolites, 3,3′,5′-triiodothyronine and 3,3′-diiodothyronine, respectively.[50] In contrast, Dio2 enhances the synthesis of T_3 through T_4 biosynthesis.[50] Thus, the expression of *dio3* is upregulated when levels of T_3 are increased, in order to increase T_3 clearance, while *dio2* expression is slightly downregulated, decreasing T_3 production.[50] Our results showed that *dio2* mRNA expression was raised by the increase in temperature in tadpoles, and that this effect was enhanced (7.4-fold increase compared to controls maintained at the lower temperature) when animals were exposed the highest concentration of diuron. Consistent with the increase of *dio2* mRNA, T_3 concentrations were also increased in animals maintained at the higher temperature. However, transcripts of *dio3* were also raised by temperature in animals exposed to diuron.

Increases in *dio2* mRNA should lead to enhanced T_3 levels, which in turn upregulate *dio3* which should diminish T_3 concentrations. The increase observed in both *dio2* and *dio3* due to temperature rise was unexpected, but may be a kinetic/temporal issue of rapid response of the thyroid T_3.[50] We hypothesize that temperature combined with diuron induces the production of T_3 by increasing *dio2* expression, and that the subtle increase in T_3 concentration could trigger the expression of *dio3* as a secondary response in order to balance T_3 levels.

Transcripts of *trα* and *trβ* were unchanged following chemical treatment at 28 °C. However, increased mRNA levels were observed for both receptors when animals were exposed to 34 °C in the presence of diuron (200 ng/L), especially for *trβ* which had a 5-fold increase compared to the control group. TRs are transcription factors whose activity is regulated by the binding of thyroid hormone.[37] The increase in TRs expression seen in this study for animals exposed to diuron at the higher temperature could make them more responsive to TH, which

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Table 1. Summary of the Main Outcomes Studied Showing the Effects Caused by Temperature Alone (Control 28°C and Control 34°C) or in Combination with Exposure to Diuron or Its Metabolite 3,4-DCA at Different Concentrations

outcomes		control		diuron				3,4-DCA			
				28 °C		34 °C		28 °C		34 °C	
		28 °C	34 °C	40 ng/L	200 ng/L	40 ng/L	200 ng/L	40 ng/L	200 ng/L	40 ng/L	200 ng/L
development		0	0	0	0	0	+	0	0	0	+
T3 levels		0	0	0	0	0	+	0	0	0	-
deiodinases	*dio2*	0	+	0	0	+	+	+	+	+	+
	dio3	0	+	0	0	+	+	0	0	+	+
thyroid Receptors	*trα*	0	0	0	0	0	+	0	0	+	+
	trβ	0	+	0	0	0	+	0	0	+	+
transcription factors	*Klf9*	0	0	0	0	0	+	0	0	0	+
	thibz	0	+	0	+	+	+	0	+	+	+

in turn could explain the acceleration of development observed for those animals exposed to diuron at 34 °C. Environmental chemicals may bind to TRs activating or inhibiting the action of endogenous T_3, or affecting TR function by interfering with coactivators or signaling mechanisms. Indeed, some chemicals can change the affinity of TRs for thyroid response elements (TREs) in the promoter regions of specific target genes.[45] These interactions may alter the normal ability of TRs to bind to TH and therefore affect the physiological responses dependent upon T_3 levels.

Other transcription factors, such as Krüppel-like factors (KLFs), may be induced by TH/TR signaling. In amphibians, Klf9 influences TH/TR regulating the expression of *trβ* and acting as an accessory transcription factor.[51,52] *Klf9* gene expression is induced by TH through direct TR binding to regions upstream of the *klf9* locus in *Xenopus* and rodents.[53] Our results showed a positive correlation between *trβ* and *klf9* mRNA in animals treated with diuron. For both genes, the expression was increased in animals exposed to combined effect of high temperature and concentration of the diuron. T_3-regulated transcription factors involved in tadpole development also include the basic leucine zipper protein (bZip).[54] During amphibian metamorphosis, TH/bZip is expressed in different tissues,[55] suggesting an important role of this gene in several organs during metamorphosis.[56] In our study, *thibz* was the only gene that had its expression altered by exposure to diuron at 28 °C. The exposure to high temperature also resulted in increased expression of *thibz*. Since this transcription factor plays a fundamental role in the TH-signaling pathway during amphibian metamorphosis, the variation of the temperature response may indicate how transcription factors can interfere directly or indirectly the expression of a cascade of other genes involved in metamorphosis when tadpoles are exposed to high temperatures in environments influenced by thyroid disruptors.

Similar to diuron, exposure to 3,4-DCA at 200 ng/L also accelerated the stages of development in tadpoles treated at 34 °C. 3,4-DCA is the principal metabolite generate by aerobic degradation of diuron in the environment[57,58] and it has been reported to exhibit higher toxicity than the parent compound.[59,60] Although 3,4-DCA presented tadpoles at advanced stages (Gs26, Gs27, Gs28) at 34 °C, diuron enhanced tadpole metamorphosis with treated animals presenting later stages of development (Gs26, Gs27, Gs28, Gs29, Gs30). This is consistent with the increased T_3 concentrations that occurred only in animals exposed to diuron and maintained at 34 °C. Plasma concentrations of T_3 had no changes in animals exposed to 3,4-DCA compared to control group at 28 °C, but a decrease was observed to both concentrations compared to untreated animals at 34 °C.

As with diuron, transcripts of *dio2* and *dio3* were also increased by temperature in animals exposed to 3,4-DCA. However, no such increase was observed in T_3 levels. The upregulation observed for *dio3* was higher than *dio2* in animals exposed to 3,4-DCA, with agrees with the significant decrease in T_3 concentrations in animals exposed to this compound at the higher temperature. The kinetics of the interactions is unclear, but the results indicate 3,4-DCA may cause a more rapid increase of T_3 which is countered through negative feedback through *dio3*. Without measurements of T_3 at early time points, this hypothesis is highly speculative and requires additional conformational studies. Thyroid hormone receptors had their expression increased by treatments with the metabolite at 34 °C. Unlike that observed for diuron, transcripts of *trβ* were increased in all groups treated with DCA at 34 °C, including untreated animals. Positive correlations were also observed between *trβ* and *klf9* transcripts in animals treated with 3,4-DCA, which strengthens the association between *kfl9* and *trβ* on TH regulation in amphibians. *Thibz* was the only gene upregulated for both compounds at 28 °C and temperature increase caused similar response on its expression in animals exposed to diuron and 3,4-DCA. Indeed, tissue-specific activation or suppression of TH-target genes in amphibians are highly influenced by exposure to environmental pollutants[52] and also several temperature-sensitive processes may be involved in the TH-signaling pathway.[43,44]

In summary, we found that temperature is an important factor influencing the toxicity of diuron as a thyroid disruptor in tadpoles of *L. catesbeianus*. Also, the degradation of diuron to other degradates such as 3,4-DCA in the environment does not minimize the deleterious effects of this compound for tadpoles, especially when it is associated with changes in temperature. Our results showed that both compounds changed the expression of TH-response genes in liver of tadpoles with upregulation at higher temperature. Metamorphosis was also accelerated in tadpoles exposed to combined effect of higher temperature and diuron or 3,4-DCA (Table 1). Most native amphibians in Brazil and other neotropical regions are subject to temperature increases in their habitats. This study emphasized that conducting toxicity tests at one temperature may underestimate the effects of environmental contaminants on amphibian populations and therefore more studies are warranted to better understand the interactions of environmental factors and contaminants.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04076.

Further information provides coordinates of the water bodies (SI Table S1), primers used in qPCR (SI Table S2), temperature recorded in field studies (SI Figure S1), mean values of the weight and length of tadpoles (SI Table S3), and Pearson correlation between significant covariates (SI Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Murata, T.; Yamauchi, K. Low-temperature arrest of the triiodothyronine-dependent transcription in *Rana catesbeiana* red blood cells. *Endocrinology* **2005**, *146* (1), 256−264.

(2) Harley, C. D. G.; Hughes, A. R.; Hultgren, K. M.; Miner, B. G.; Sorte, C. J. B.; Thornber, C. S.; Rodriguez, L. F.; Tomanek, L.; Williams, S. L. The impacts of climate change in coastal marine systems. *Ecol. Lett.* **2006**, *9* (2), 228−241.

(3) Rijnsdorp, A. D.; Peck, M. a; Engelhard, G. H.; Mollmann, C.; Pinnegar, J. K. Resolving the effect of climate change on fish populations. *ICES J. Mar. Sci.* **2009**, *66* (7), 1570−1583.

(4) Perry, R. I. Potential impacts of climate change on marine wild capture fisheries: an update. *J. Agric. Sci.* **2011**, *149* (SupplementS1), 63−75.

(5) Bickford, D.; Howard, S. D.; Ng, D. J. J.; Sheridan, J. A. Impacts of climate change on the amphibians and reptiles of Southeast Asia. *Biodivers. Conserv.* **2010**, *19* (4), 1043−1062.

(6) Morand, A.; Joly, P.; Grolet, O. Phenotypic variation in metamorphosis in five anuran species along a gradient of stream influence. *C. R. Acad. Sci., Ser. III* **1997**, *320* (8), 645−652.

(7) Hooper, M. J.; Ankley, G. T.; Cristol, D. A.; Maryoung, L. A.; Noyes, P. D.; Pinkerton, K. E. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. *Environ. Toxicol. Chem.* **2013**, *32* (1), 32−48.

(8) Fontenot, C. L.; Lutterschmidt, W. I. Thermal selection and temperature preference of the aquatic salamander. *Amphiuma tridactylum. Herpetol. Conserv. Biol.* **2011**, *6* (3), 395−399.

(9) Middlebrooks, E. J.; Gaspar, M. J.; Gaspar, R. D.; Reynolds, J. H. *Effects of Temperature on the Toxicity to the Aquatic Biota of Waste Discharges - A Compilation of the Literature*, 1973

(10) Wilbur, H. M. Coping with Chaos: Toads in ephemeral ponds. *Trends Ecol. Evol.* **1990**, *5* (2), 37.

(11) Stefani Margarido, T. C.; Felício, A. A.; de Cerqueira Rossa-Feres, D.; Alves de Almeida, E. Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. *Mar. Environ. Res.* **2013**, *91*, 61−67.

(12) Prado, V. H. M. Do; Borges, R. E.; Silva, F. R. Da; Tognolo, T. T.; Rossa-Feres, D. D. C. Amphibia, Anura, Hylidae, *Phyllomedusa azurea*: Distribution extension. *Check List* **2008**, *4* (1), 55−56.

(13) Provete, D. B.; Garey, M. V.; Silva, F. R. Da; Rossa-Feres, D. D. C. Anurofauna do noroeste paulista: lista de espécies e chave de identificação para adultos. *Biota Neotrop.* **2011**, *11* (2), 377−391.

(14) Morin, S.; Bottin, M.; Mazzella, N.; Macary, F.; Delmas, F.; Winterton, P.; Coste, M. Linking diatom community structure to pesticide input as evaluated through a spatial contamination potential (Phytopixal): A case study in the Neste river system (South-West France). *Aquat. Toxicol.* **2009**, *94* (1), 28−39.

(15) Schlenk, D.; Lavado, R.; Loyo-Rosales, J. E.; Jones, W.; Maryoung, L.; Riar, N.; Werner, I.; Sedlak, D. Reconstitution studies of pesticides and surfactants exploring the cause of estrogenic activity observed in surface waters of the San Francisco Bay Delta. *Environ. Sci. Technol.* **2012**, *46* (16), 9106−9111.

(16) Lourencetti, C.; de Marchi, M. R. R.; Ribeiro, M. L. Determination of sugar cane herbicides in soil and soil treated with sugar cane vinasse by solid-phase extraction and HPLC-UV. *Talanta* **2008**, *77* (2), 701−709.

(17) Goldemberg, J.; Coelho, S. T.; Guardabassi, P. The sustainability of ethanol production from sugarcane. *Energy Policy* **2008**, *36* (6), 2086−2097.

(18) Lamoree, M. H.; Swart, C. P.; Van Der Horst, A.; Van Hattum, B. Determination of diuron and the antifouling paint biocide Irgarol 1051 in *Dutch marinas* and coastal waters. *J. Chromatogr. A* **2002**, *970* (1−2), 183−190.

(19) Mhadhbi, L.; Beiras, R. Acute toxicity of seven selected pesticides (alachlor, atrazine, dieldrin, diuron, pirimiphos-methyl, chlorpyrifos, diazinon) to the marine fish (turbot. *Water, Air, Soil Pollut.* **2012**, *223* (9), 5917−5930.

(20) Cardone, A.; Comitato, R.; Angelini, F. Spermatogenesis, epididymis morphology and plasma sex steroid secretion in the male lizard Podarcis sicula exposed to diuron. *Environ. Res.* **2008**, *108* (2), 214−223.

(21) Pereira, T. S. B.; Boscolo, C. N. P.; Silva, D. G. H.; da Batlouni, S. R.; Schlenk, D.; Almeida, E. A. de. Anti-androgenic activities of diuron and its metabolites in male Nile tilapia (*Oreochromis niloticus*). *Aquat. Toxicol.* **2015**, *164*, 10115.

(22) Schuytema, G. S.; Nebeker, A. V. Comparative toxicity of diuron on survival and growth of Pacific treefrog, bullfrog, red-legged frog, and African clawed frog embryos and tadpoles. *Arch. Environ. Contam. Toxicol.* **1998**, *34* (4), 370−376.

(23) Wu, H.; Gao, C.; Guo, Y.; Zhang, Y.; Zhang, J.; Ma, E. Acute toxicity and sublethal effects of fipronil on detoxification enzymes in juvenile zebrafish (*Danio rerio*). *Pestic. Biochem. Physiol.* **2014**, *115*, 9−14.

(24) López-Doval, J. C.; Poquet, M.; Muñoz, I. Sublethal effects of the herbicide diuron on the freshwater snail. *Physella acuta.* **2011**, *29* (331), 205−216.

(25) Heimeier, R. A.; Shi, Y. B. Amphibian metamorphosis as a model for studying endocrine disruption on vertebrate development: Effect of bisphenol A on thyroid hormone action. *Gen. Comp. Endocrinol.* **2010**, *168* (2), 181−189.

(26) Miyata, K.; Ose, K. Thyroid Hormone-disrupting Effects and the Amphibian Metamorphosis Assay. *J. Toxicol. Pathol.* **2012**, *25* (1), 1−9.

(27) Hayes, T. B.; Case, P.; Chui, S.; Chung, D.; Haeffele, C.; Haston, K.; Lee, M.; Mai, V. P.; Marjuoa, Y.; Parker, J.; et al. Monograph Pesticide Mixtures, Endocrine Disruption, and Amphibian Declines: Are We Underestimating the Impact? 2006, *40* (April).

(28) Stuart, S. N.; Stuart, S. N.; Chanson, J. S.; Cox, N. A.; Young, B. E.; Rodrigues, A. S. L.; Fischman, D. L.; Waller, R. W. Status and Trends of Amphibian Declines and Extinctions Worldwide. 2013, *1783* (2004).

DOI: 10.1021/acs.est.6b04076
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(29) Catesbeiana, R.; River, C.; Role, T.; Author, L. C.; Source, S. J. K.; Society, E.; Url, A. S.; Linked, U. T. C. R. Bullfrog (*Rana Catesbeiana*) Invasion of a California River: The Role of Larval Competition. *Ecology* **2015**, *78* (6), 1736−1751.
(30) Stumpel, A. H. P. Successful reproduction of introduced bullfrogs *Rana catesbeiana* in northwestern Europe: a potential threat to indigenous amphibians. *Biol. Conserv.* **1992**, *60* (1), 61−62.
(31) Suzuki, S.; Fujikura, K. Circulating thyroglobulin in tadpoles and adult frogs of *Rana catesbeiana*. *Gen. Comp. Endocrinol.* **1994**, *94* (1), 72−77.
(32) Cecil, S. G.; Just, J. J. Survival rate, population density and development of a naturally occurring anuran larva (*Rana catesbeiana*) http://www.jstor.org/stable/1443221?seq=1#page_scan_tab_contents (accessed April 15, 2016).
(33) Fort, D. J.; Rogers, R. L.; Gorsuch, J. W.; Navarro, L. T.; Peter, R.; Plautz, J. R. Triclosan and anuran metamorphosis: No effect on thyroid-mediated metamorphosis in *Xenopus laevis*. *Toxicol. Sci.* **2010**, *113* (2), 392−400.
(34) Navarro-Martín, L.; Lanctôt, C.; Jackman, P.; Park, B. J.; Doe, K.; Pauli, B. D.; Trudeau, V. L. Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frogs (*Lithobates sylvaticus*) tadpoles. I: Chronic laboratory exposures to VisionMax®. *Aquat. Toxicol.* **2014**, *154*, 278−290.
(35) Zar, J. H. *Biostatistical Analysis*; Prentice Hall, 1999.
(36) Kuiper, G. G. J. M.; Kester, M. H. a; Peeters, R. P.; Visser, T. J. Biochemical mechanisms of thyroid hormone deiodination. *Thyroid* **2005**, *15* (8), 787−798.
(37) Shi, Y. B.; Wang, J.; Puzianowska-Kuznicka, M. Thyroid hormone receptors: Mechanisms of transcriptional regulation and roles during frog development. *J. Biomed. Sci.* **1996**, *3* (5), 307−318.
(38) Buchholz, D. R.; Hayes, T. B. Larval Period Comparison for the Spadefoot Toads Scaphiopus couchii and *Spea multiplicata* (Pelobatidae: Anura) on JSTOR http://www.jstor.org/stable/3893113?seq=1#page_scan_tab_contents (accessed April 15, 2016).
(39) Brande-lavridsen, N.; Christensen-dalsgaard, J.; Korsgaard, B. Effects of Ethinylestradiol and the fungicide Prochloraz on metamorphosis and thyroid gland morphology in *Rana temporaria*. *Open Zool. J.* **2010**, *3*, 7−16.
(40) Tietge, J. E.; Degitz, S. J.; Haselman, J. T.; Butterworth, B. C.; Korte, J. J.; Kosian, P. A.; Lindberg-Livingston, A. J.; Burgess, E. M.; Blackshear, P. E.; Hornung, M. W. Inhibition of the thyroid hormone pathway in *Xenopus laevis* by 2-mercaptobenzothiazole. *Aquat. Toxicol.* **2013**, *126*, 128−136.
(41) Larson, D. L.; McDonald, S.; Fivizzani, A. J.; Newton, W. E.; H, S. J. Effects of the Herbicide Atrazine on *Ambystoma tigrinum* Metamorphosis: Duration, Larval Growth, and Hormonal Response on JSTOR http://www.jstor.org/stable/10.1086/515999?seq=1#page_scan_tab_contents (accessed April 15, 2016).
(42) Boone, M. D.; Bridges, C. M. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). *Environ. Toxicol. Chem.* **1999**, *18* (7), 1482−1484.
(43) Hammond, S. A.; Veldhoen, N.; Helbing, C. C. Influence of temperature on thyroid hormone signaling and endocrine disruptor action in *Rana (Lithobates) catesbeiana* tadpoles. *Gen. Comp. Endocrinol.* **2015**, *219*, 6−15.
(44) Mochizuki, K.; Goda, T.; Yamauchi, K. Gene expression profile in the liver of Rana catesbeiana tadpoles exposed to low temperature in the presence of thyroid hormone. *Biochem. Biophys. Res. Commun.* **2012**, *420* (4), 845−850.
(45) Zoeller, R. T. Environmental chemicals impacting the thyroid: targets and consequences. *Thyroid* **2007**, *17* (9), 811−817.
(46) Ghisari, M.; Bonefeld-Jorgensen, E. C. Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells. *Mol. Cell. Endocrinol.* **2005**, *244* (1−2), 31−41.
(47) López-Alcaide, S.; Macip-Ríos, R. *Effects of Climate Change in Amphibians and Reptiles. Biodiversity Loss in a Changing Planet*; Grillo, O., Y. G., Venora, Eds.; InTech: Rijeka, 2011, 163−184.
(48) Silva, J. E. Thyroid hormone control of thermogenesis and energy balance. *Thyroid* **1995**, *5* (6), 481−492.
(49) Little, A. G.; Kunisue, T.; Kannan, K.; Seebacher, F. Thyroid hormone actions are temperature-specific and regulate thermal acclimation in zebrafish (*Danio rerio*). *BMC Biol.* **2013**, *11* (1), 26.
(50) Bianco, A.; Kim, B. Deiodinases: Implications of the local control of thyroid hormone action. *J. Clin. Invest.* **2006**, *116* (10).257110.1172/JCI29812
(51) Kanamori, a; Brown, D. D. The regulation of thyroid hormone receptor beta genes by thyroid hormone in *Xenopus laevis*. *J. Biol. Chem.* **1992**, *267* (2), 739−745.
(52) Machuca, I.; Tata, J. R. Autoinduction of thyroid hormone receptor during metamorphosis is reproduced in Xenopus XTC-2 cells. *Mol. Cell. Endocrinol.* **1992**, *87* (1−3), 105−113.
(53) Denver, R. J.; Ouellet, L.; Furling, D.; Kobayashi, A.; Fujii-Kuriyama, Y.; Puymirat, J. Basic Transcription Element-binding Protein (BTEB) Is a Thyroid Hormone-regulated Gene in the Developing Central Nervous System: EVIDENCE FOR A ROLE IN NEURITE OUTGROWTH. *J. Biol. Chem.* **1999**, *274* (33), 23128−23134.
(54) Denver, R. J. The molecular basis of thyroid hormone-dependent central nervous system remodeling during amphibian metamorphosis. *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol. Endocrinol.* **1998**, *119* (3), 219−228.
(55) Denver, R. J.; Pavgi, S.; Shi, Y. B. Thyroid hormone-dependent gene expression program for Xenopus neural development. *J. Biol. Chem.* **1997**, *272* (13), 8179−8188.
(56) Ikuzawa, M.; Shimizu, K.; Yasumasu, S.; Iuchi, I.; Shi, Y. B.; Ishizuya-Oka, A. Thyroid hormone-induced expression of a bZip-containing transcription factor activates epithelial cell proliferation during Xenopus larval-to-adult intestinal remodeling. *Dev. Genes Evol.* **2006**, *216* (3), 109−118.
(57) Attaway, H. H.; Camper, N. D.; Paynter, M. J. B. Anaerobic microbial degradation of diuron by pond sediment. *Pestic. Biochem. Physiol.* **1982**, *17* (1), 96−101.
(58) Ellis, P. A.; Camper, N. D. Aerobic degradation of diuron by aquatic microorganisms. *J. Environ. Sci. Health, Part B* **1982**, *17* (3), 277−289.
(59) Giacomazzi, S.; Cochet, N. Environmental impact of diuron transformation: A review. *Chemosphere* **2004**, *56* (11), 1021−1032.
(60) Da Rocha, M. S.; Arnold, L. L.; Dodmane, P. R.; Pennington, K. L.; Qiu, F.; De Camargo, J. L. V; Cohen, S. M. Diuron metabolites and urothelial cytotoxicity: In vivo, in vitro and molecular approaches. *Toxicology* **2013**, *314* (2−3), 238−246.

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5. CONSIDERAÇÕES FINAIS E CONCLUSÃO GERAL

Os resultados provenientes dessa pesquisa de doutorado nos permitem concluir que:

- Alterações nos padrões abióticos, tais como mudanças na temperatura e pH ambiental, as quais tendem a se tornar fatores de estresse para anfíbios, alteram o sistema de defesa antioxidante de girinos como resposta adaptativa imediata às alterações fisiológicas provocadas por tais condições. Os resultados mostrando os efeitos isolados dessas variáveis ambientais em espécies endêmicas brasileiras são de grande relevância, pois auxiliam em um melhor entendimento sobre os diferentes mecanismos fisiológicos usados por esses organismos para superar períodos de exposição a condições ambientais muito variáveis, as quais girinos estão corriqueiramente submetidos.

- A temperatura é um importante fator ambiental induzindo efeitos tóxicos mais expressivos dos herbicidas sulfentrazone e clomazone em girinos de *E. nattereri* e *R. schneideri.* De um modo geral, observamos que as enzimas antioxidantes têm suas atividades aumentadas em girinos expostos ao efeito combinado de exposição aos herbicidas a temperaturas elevadas. Esses dados condizem com nossa hipótese de que efeitos tóxicos de compostos ambientais devem ser pronunciados em temperaturas mais altas devido à estimulação nas taxas metabólicas de cada espécie. Em resposta a um possível aumento na produção das ERO, as enzimas antioxidantes foram estimuladas com o aumento de temperatura, especialmente em girinos expostos aos compostos. Similar resposta foi observada para os níveis de MDA, os quais foram utilizados como indicadores de peroxidação lipídica em nosso estudo. Entretanto, essas respostas podem variar dependendo das concentrações, tempo de exposição e das espécies em estudo.

- Essa foi a primeira vez que os efeitos adversos em consequência à exposição ao herbicida sulfentrazone foram noticiados para anfíbios. Da mesma forma, foi mostrado pela primeira vez que a exposição ao herbicida clomazone pode prejudicar o sistema de defesa

antioxidante e provocar peroxidação lipídica em anuros, além de perturbar a atividade da CbE, especialmente quando essas exposições estão associadas a diferentes gradientes térmicos.

- As diferentes espécies de girinos estudadas apresentaram respostas diversificadas aos mesmos contaminantes e condições ambientais, confirmando dados da literatura, onde a resposta espécie-especifica de organismos não-alvos expostos a diferentes xenobióticos tem sido observada. Esses dados sugerem uma maior abrangência de estudos envolvendo espécies nativas em investigações de risco como resultado de contaminação ambiental por agrotóxicos em anfíbios.

- O herbicida diuron e seu metabólito 3,4-DCA são potentes desreguladores da tireoide em anfíbios, sendo esses efeitos evidenciados pela primeira vez na literatura. Entretanto, a ação desses compostos é altamente influenciada pela temperatura ambiental, sendo elas pronunciadas em temperaturas mais elevadas. Tais afirmações foram obtidas a partir dos resultados observados, tais como a superexpressão de genes relacionados à atividade tireoidiana e aumento na produção de T_3, os quais levaram a uma aceleração na metamorfose de girinos expostos ao efeito combinado. Os resultados desse trabalho inferem a necessidade de se considerar a temperatura ambiental em estudos identificando potenciais desreguladores endócrinos em girinos, principalmente em regiões de clima quente.

6. OUTRAS PRODUÇÕES CIENTÍFICAS

Artigos científicos publicados durante o período de desenvolvimento do projeto de doutorado (Mar/2013 a Mar/2016) e em parceria com outros grupos de pesquisa que estão, de alguma forma, relacionados ao objeto de estudo:

- 2014: Morphological effects of bacterial compounds on the testes of *Eupemphix nattereri* (Anura).

- 2015: Morphological and histochemical studies of Bidder's organ in *Rhinella schneideri* (Amphibia: Anura) males.

- 2016: Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae).

- 2016: Time- and Oil-Dependent Transcriptomic and Physiological Responses to Deepwater Horizon Oil in Mahi-Mahi (*Coryphaena hippurus*) Embryos and Larvae.

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Morphological effects of bacterial compounds on the testes of *Eupemphix nattereri* (Anura)

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Abstract

Amphibians are susceptible to environmental pollutants and these compounds influence the development, morphology, physiology, behavior, and reproduction of these animals. *Escherichia coli* is common in aquatic habitats of frogs and could also damage their reproductive activity. Thus, our objective was to evaluate the effects of *E. coli* lipopolysaccharide on the testes of *Eupemphix nattereri* after 2, 6, 12, and 24 hours. As in other anurans, the testes of *E. nattereri* are paired structures and spermatogenesis into the locule is cystic, with mast cells in the interstitial region as well as testicular melanocytes. The administration of lipopolysaccharide decreased the absolute locular volume after 12 h while increasing the interstitial volume. In addition, lipopolysaccharide treatment decreased the absolute volume of all cell types in animals analyzed after 12 h. The amount of mast cells in the interstitial region increased after 12 h of inoculation. Acute exposure to lipopolysaccharide clearly alters testicular morphology, decreasing the volume of both locular and germ cells. However, acute treatment did not impair spermatogenesis and after 24 h, the treatment effects were minimized. Thus, this study was the first to demonstrate that lipopolysaccharide is a potential agent for causing damage to the testes of anurans.

Keywords

Anura; germ cells; LPS; mast cells; testis

Introduction

Aquatic contaminants can contribute in different ways to the decline of amphibian populations (Hayes et al., 2010) by slowing down larval growth and development,

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promoting changes in morphology, physiology, and behavior, as well as reducing reproductive output (Carey & Bryant, 1995). Amphibians have a wide range of reproductive modes, mostly dependent on water (Duellman & Trueb, 1994).

The reproductive system of anurans consists of a pair of rounded testes, usually whitish-yellow, associated with ventral kidneys through the gonadal mesentery (Oliveira & Franco-Belussi, 2012). The testes of some frog species, including *Eupemphix nattereri*, have a dark coloration due to visceral melanocytes (Franco-Belussi et al., 2009). Testicular morphology varies among anurans and there is no description available in the literature about the germ cells in *E. nattereri*. This study provides a morphological description of the germ cells whose classification was based on Oliveira et al. (2003), in order to evaluate the effects of lipopolysaccharide (LPS) on germ cells.

Eupemphix nattereri (Steindachner, 1863) occurs in open areas in Brazil, eastern Paraguay, and Bolivia and has an average 4 cm snout-vent length (Frost, 2011). It often lives in environments polluted by human activity and is subject to contamination by pathogens or chemicals from sewage effluents, which often contains *E. coli*. This organism is associated with zoonotic diseases and can cause health problems in wildlife. In iguanas, Sylvester et al. (2014) isolated a serotype of *E. coli* and established the profile of antimicrobial resistance. Adults and larvae of frogs are also infected by *E. coli*, however, the means by which this occurs are unclear (Gray et al., 2007). The LPS of this bacterium is known to promote apoptosis of testicular germ cells in mice (Kajihara et al., 2006) and has been reported to directly influence the reproductive output of anurans (Alford & Richards, 1999; Bonneaud et al., 2003).

Administration of LPS to animals mimics systemic infection and inflammation, and it is known to promote short-term injuries in germ cells, inhibit testicular steroidogenesis and disrupt spermatogenesis in mammals in the short-term (O'Bryan et al., 2000; Kajihara et al., 2006). The long-term effects on spermatogenesis were not examined in this study. In contrast, studies on how bacterial compounds affect the testes of frogs are scarce.

LPS is also responsible for activating immune cells, such as mast cells. These cells secrete a variety of products (e.g., histamine, proteases, leukotrienes, and lipid mediators), which contribute to the inflammatory process (Shakoory et al., 2004), and activate neutrophils as well (Benjamin et al., 2002). Mast cells are intimately associated with melanocytes in frogs, both in the skin and visceral organs, such as the heart (Baccari et al., 1998). Mast cells also modulate the inflammatory process induced by LPS from *E. coli* (Silva et al., 2011) and can be employed as indicators of inflammatory effects in different tissues.

To understand the effects of LPS-induced inflammation, as well as morphological and functional interaction between the architectural components of the testes (e.g., seminiferous versus interstitial compartment, and the dynamics of germ cysts versus stromal cells involved in defense mechanisms) it is necessary to detail the general morphology and spermatogenesis of *E. nattereri*.

In this paper, we evaluated the effects of LPS on the testes of *Eupemphix nattereri*, considering that LPS from *E. coli* is used for modeling the systemic inflammation, and that LPS also damages spermatogenesis in mammals (Kajihara et al., 2006). The spermatogenesis in *E. nattereri* is also described. Thus, it is hypothesized that *E. coli* LPS alters the testicular morphology of *E. nattereri*, reducing locular absolute volume while increasing interstitial volume. LPS is also a potent activator of mast cells, which trigger inflammatory responses. Therefore, we believe that the mast cell population in the testis should increase after LPS treatment.

Material and methods

Animals

We used 30 adult *Eupemphix nattereri* males collected in temporary ponds during the breeding season (December 2011 to January 2012) in southeastern Brazil (20°47′07.05″S; 49°02′42.09″W). All captures of animals were made with the permission of the Brazilian Institute of Environment (RAN-IBAMA #18573-1). The handling of animals and all experimental procedures were approved by the Committee on Ethics and Animal Experimentation of the São Paulo State University (CEUA-IBILCE/UNESP #038/2011) and followed the recommendations of the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee).

Animals remained in terraria (28 cm × 21 cm × 15 cm) with 5 cm of soil at the bottom, food and water at room temperature, and daylight regime (27 ± 0.5°C and 14 h:10 h light/dark) for 7 days prior to experiments. After acclimation, the animals were transferred to sterilized terraria of the same size but without soil to prevent secondary infections.

LPS from E. coli *treatment*

The animals of all treatment groups were intraperitoneally inoculated with a single dose of 18 mg/kg LPS from *Escherichia coli* (Serotype 127:B8, Sigma, St. Louis, MO; adapted from Flores Quintana & Ruas de Moraes, 2001) diluted in 0.02 ml of sterile saline solution adjusted to amphibian osmolarity (60% of mammalian osmolality). Then, the animals were divided into four experimental groups with varying periods of LPS exposure ($N = 5$ animals per group): 2 hours (LPS2h group, hereafter), 6 hours (LPS6h group, hereafter), 12 hours (LPS12h group, hereafter), and 24 hours (LPS24h group, hereafter) (adapted from O'Bryan et al., 2000). We adopted these experimental periods to observe how germ cells respond immediately (2 and 6 hours) and later (12 and 24 hours) to LPS administration. For the control group, we injected five animals with the same amount of sterile saline solution without LPS and analyzed after 2 hours (CONT2h) and 24 hours (CONT24h) after injection. After the experiments, we deeply anesthetized the animals with a Benzocaine dose (1 g/l of water). Testes were removed and weighed. To determine the gonadosomatic index, testis weight was divided by the weight of the animal.

Germ cell quantification

We fixed fragments of the testes with Karnovsky fixative solution (0.1 M Sörensen phosphate buffer, phosphate buffer pH 7.2 with 5% paraformaldehyde, and 2.5% glutaraldehyde) for 24 hours at 4°C. Subsequently, samples were washed with water, dehydrated in alcoholic series and embedded into historesin (Leica Historesin embedding kit; Leica Microsystems, Wetzlar, Germany). Sections of 2 μm were obtained in microtome (RM 2265, Leica Microsystems, Heerbrugg, Switzerland) and stained with Hematoxylin-Eosin. The area occupied by each germinative cyst was measured in 25 micrographs using a 20× objective per animal. For quantifying the locular area, we evaluated 10 micrographs using a 10× objective per animal using Image-Pro Plus v. 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA). Absolute volume of germ cells, locular and interstitial regions was determined by multiplying the relative area values by testes weight, based on the determination that 1 mg tissue has a volume of approximately 1 mm^3, according to Vilamaior et al. (2006). Collagen was detected according to the Picrosirius method while the reticular fibers present in the testicular interstitium were detected following the Gomori's Reticulin method. The morphological description of the germ cells was performed while their classification was based on cellular phenotypes observed in the anuran *Scinax fucovarius* (Oliveira et al., 2003), in order to evaluate effects of LPS in germ cells. For statistical analysis we used a nested model, where animal identity was nested within treatment and histological sections were nested in animal identity.

Mast cell analysis

Mast cell quantification was conducted using 10 histological fields of testes per animal, stained with toluidine blue in borax. These cells were indicative of inflammatory effects in the testes. To analyze this data we also used a nested model, where animal identity was nested within treatment and histological sections were nested within animal identity.

Statistical analysis

We tested whether the volume occupied by the locular region and each germ cell type (spermatogonia, spermatocyte, spermatid, and spermatozoon; response variables) decreased with time of exposure to LPS (predictor variable). We also tested if mast cells (response variable) increased with time following LPS administration (predictor variable). We transformed the data using square root to meet the assumptions of normality and homogeneity of variance. Subsequently, the response variables were compared between treatments using a nested ANOVA, followed by a post hoc Tukey test. We tested for a correlation between the locular and interstitial area and the area of the germ cells. All analyses were conducted in R v. 2.11.1 (R Development Core Team, 2010).

Results

The testes of *Eupemphix nattereri* are paired, ovoid structures, with intense pigmentation that can be observed externally (fig. 1A). Testes weigh on average 14.36 ± 2.11 (SE) mg and have a mean gonadosomatic index of 0.173 ± 0.02 (SE). Neither the gonadosomatic index nor testes weight changed with LPS treatment ($F_{4,20} = 0.103$; $P = 0.98$). Germ cells are arranged in cysts within seminiferous locules (fig. 1B). The interstitial region, the space between locules, contains connective tissue with a large amount of somatic and immune cells (fig. 1B, C, D). There are also visceral melanocytes that give the organ a dark coloration (fig. 1B, C, D).

In the interstitial region, we observed collagen and reticular fibers that sustain the seminiferous locules while acting as a testicular support network (fig. 2). The

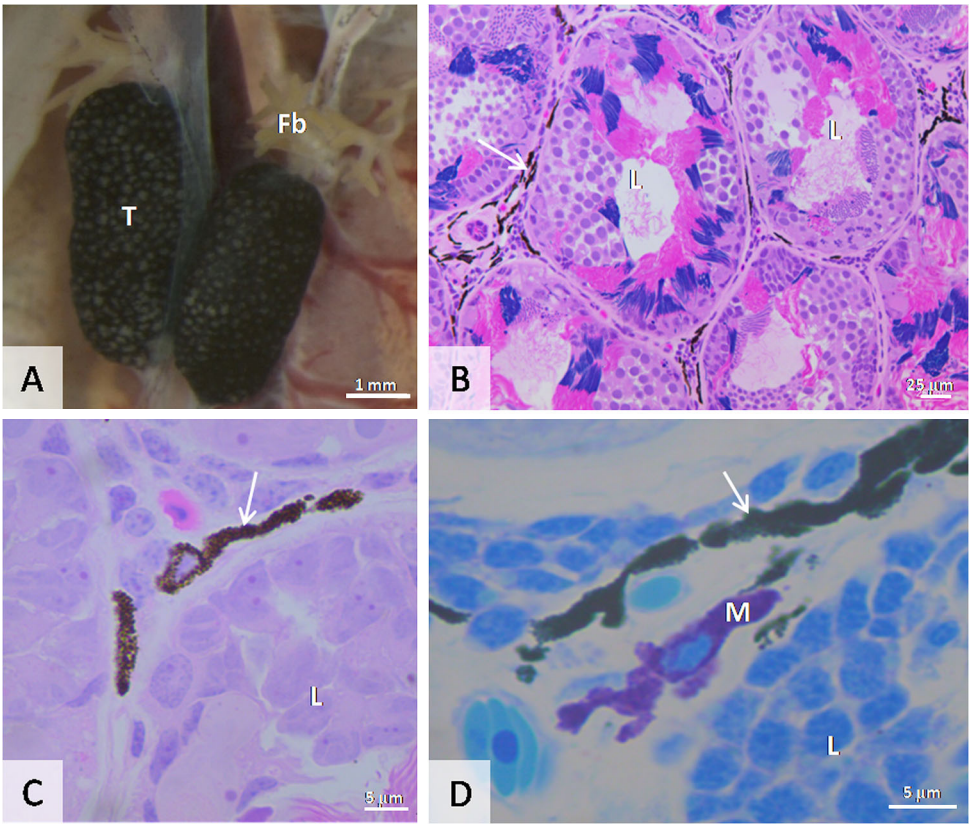
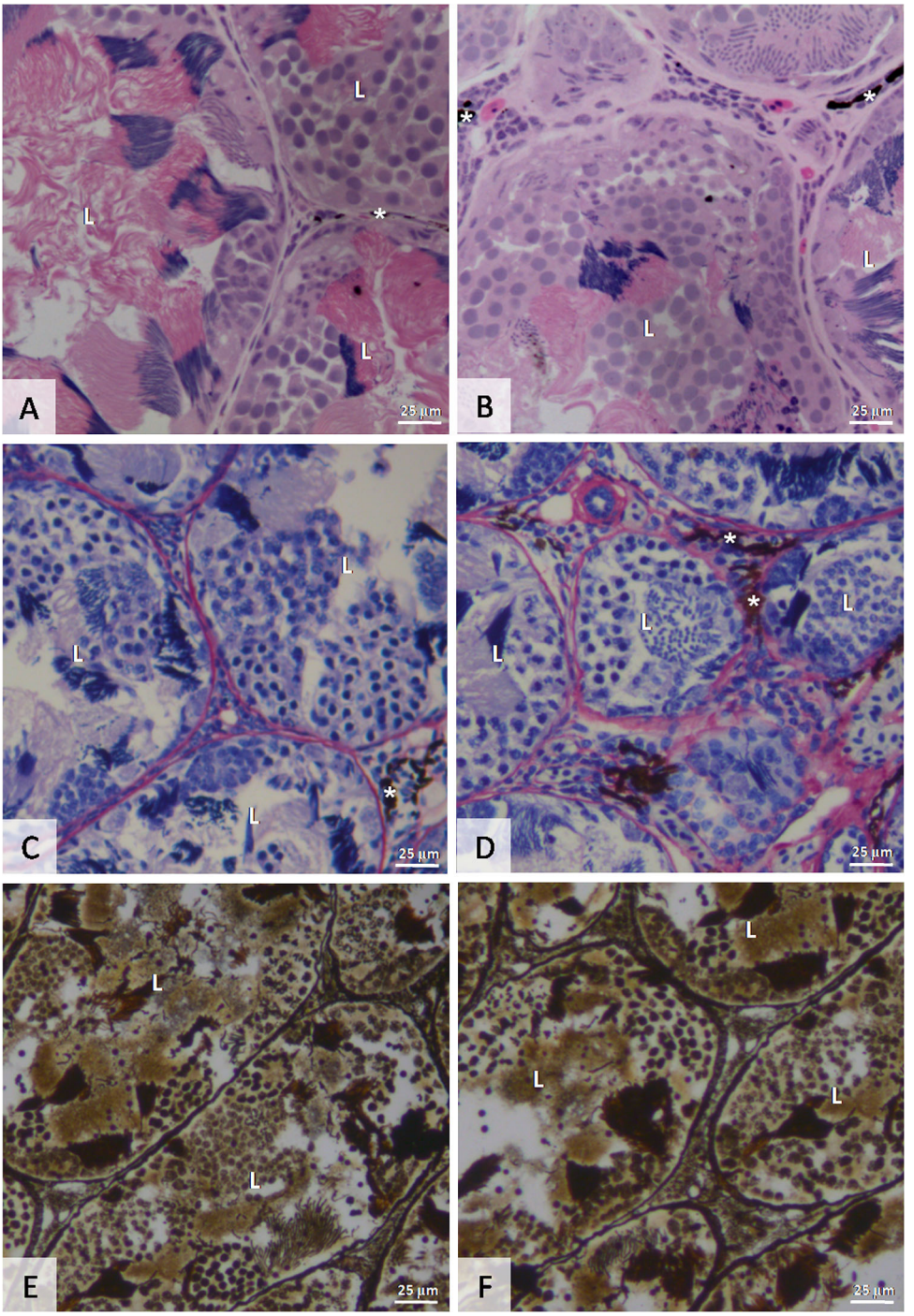


Figure 1. Anatomical and histological morphology of testes of *Eupemphix nattereri*. Testes (T) showing the pigmentation on the organ's surface (A). The testes have a locular arrangement (B), with visceral melanocytes (arrow) in the interstitial region (C). Mast cells (M) also occur among seminiferous locules (D). Other abbreviations: Fb: fatty bodies; L: seminiferous locules. Staining: B and C, Hematoxylin-eosin; D, Toluidine blue. Scale bars: 1 mm (A), 25 μm (B) and 5 μm (C-D). This figure is published in colour in the online version.


A
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collagen fiber bundles are arranged parallel, near the seminiferous locules, with similar diameter. In addition, reticular fibers are mainly found in the basal lamina (fig. 2C, D). In *E. nattereri*, these fibers bundles have varying diameters, being thinner in portions of contact between the locules than in other interstitial areas (fig. 2E, F).

Spermatogenesis in Eupemphix nattereri

The primary spermatogonia are the initial germ cells of spermatogenesis. After several mitotic and meiotic divisions, these cells become mature sperm that are released into the lumen (fig. 3). We identified all developmental stages within the seminiferous locule of *E. nattereri* testes, and, germ cells were organized in spermatogenic cysts by Sertoli cells.

The spermatogenesis occurs within the locules with the spermatogonial cells, which are voluminous, irregularly-shaped cells located in the periphery of the seminiferous loci (fig. 3A). Spermatocytes I are originated from spermatogonia after cell differentiation, without cell division. Spermatocytes I are spherical, large cells that underwent meiosis and originate morphologically identical cells, but with half the diameter of their predecessors, called spermatocytes II (fig. 3B, C). Spermatocytes occupy greater volume than seminiferous locules (about 60%). The spermatids I undergo cell elongation by contracting the nucleus during the second meiotic division and begin to be arranged in bundles supported by Sertoli cell extensions, becoming spermatids at this stage (fig. 3D). Spermatids II have less cytoplasmic volume and more condensed chromatin than spermatids I (fig. 3E). During spermiogenesis, spermatids differentiate into spermatozoa. These cells are at the last maturation stage, and their nuclei are extremely compact, due to chromatin condensation and cytoplasmic reduction. At this developmental stage, the cells are no longer organized in cysts with Sertoli cells. They are no longer organized into bundles but are free in the lumen. There are two morphologically distinct regions in the fully mature sperm: the head and the flagellum (fig. 3F). The spermatozoon bundle occupies the second largest volume into the seminiferous locule (about 20% of seminiferous volume). The cell types that occupy the biggest volume of the seminiferous locule are spermatocytes I and II (about 60%). Spermatids I and II occupy about 10% and spermatogonia less than 10%.

Figure 2. Locular arrangement of testes of *Eupemphix nattereri*. Histological sections of testes showing the arrangement of locule (A, B), collagen (C, D) and reticular (E, F) fibers in testicular framework. Treatment with LPS affects seminiferous locule size and arrangement of the fibers. A, C and E represent animals of control group. B, D and F demonstrate animals treated with LPS for 12 hours. Abbreviation and symbol: L, seminiferous locules; *, melanocytes in interstitial region. Staining: A and B, Hematoxylin-eosin; C and D, Picrosirius method; E and F, Gomori's Reticulin. Scale bars: 25 μm (A-F). This figure is published in colour in the online version.

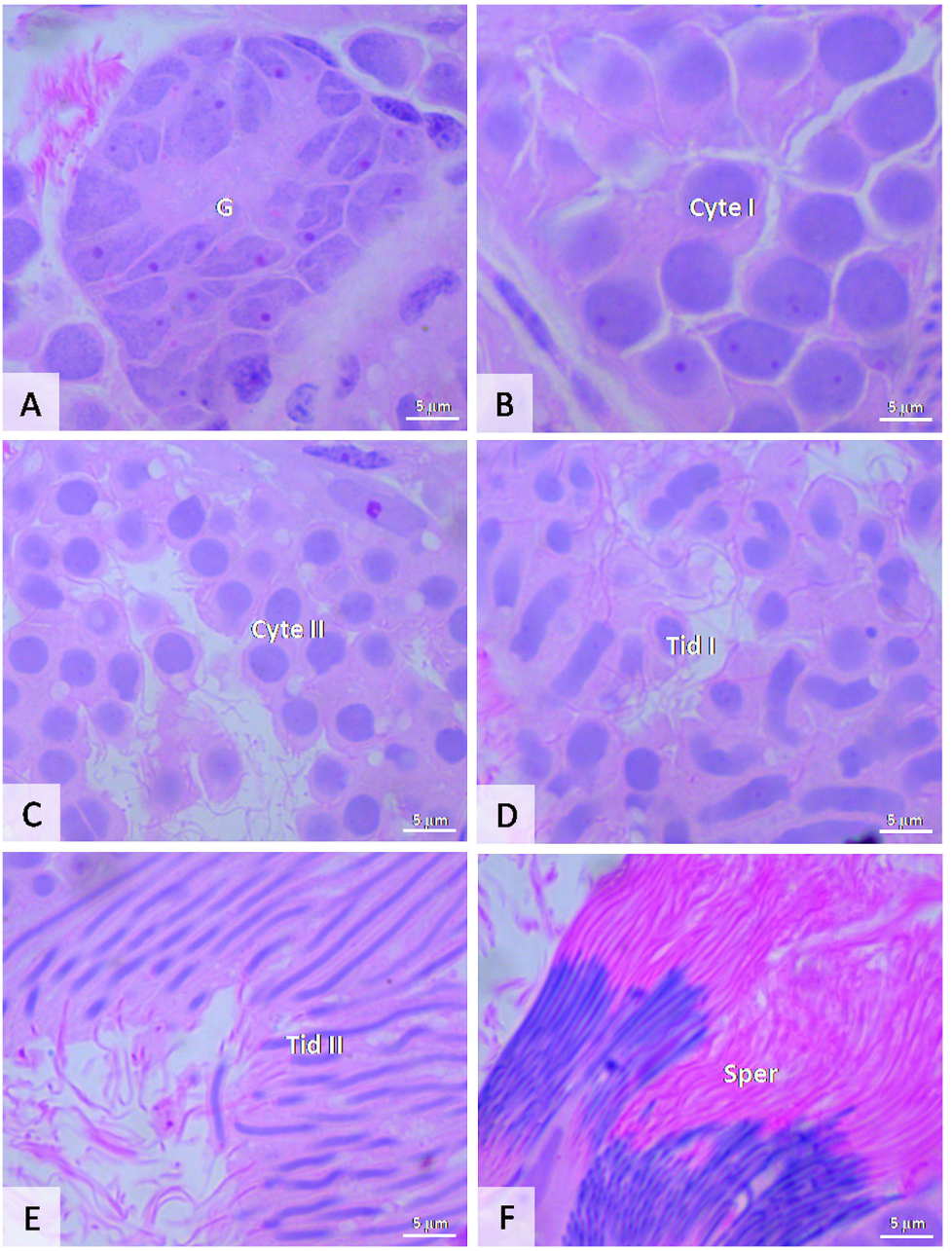

Figure 3. Morphology of germ cell types composing spermatogenesis in *E. nattereri*. (A) Spermatogonia cyst (G). (B) Spermatocytes I cyst (Cyte I). (C) Spermatocytes II cyst (Cyte II). (D) Spermatids in cellular elongating cyst (Tid I). (E) Spermatids II cyst (Tid II). (F) Spermatozoon bundle (Sper). Staining: Hematoxylin-Eosin. Scale bars: 5 μm (A-F). This figure is published in colour in the online version.

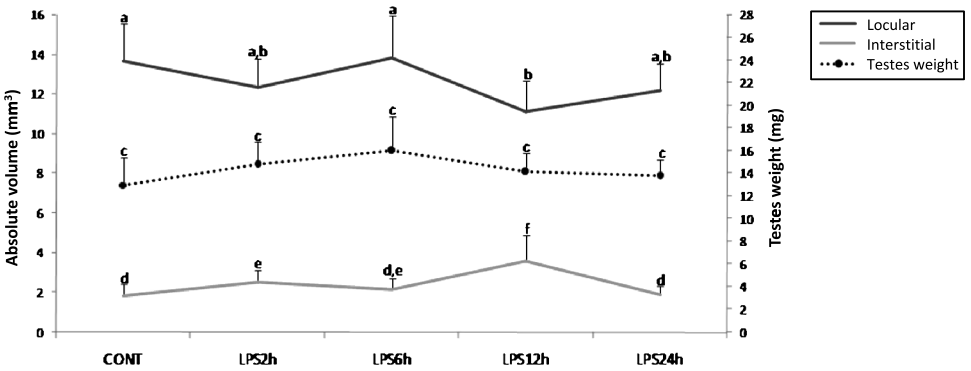

Figure 4. Effects of LPS in locular and interstitial volumes. Testes weight did not vary with treatment. Different letters represent significant differences for a given cell type of the experimental groups. CONT indicates Control group; LPS 2h, 6h, 12h and 24h indicate animals analyzed after 2, 6, 12, and 24 hours of LPS administration, respectively.

Effects of LPS on germ cells

LPS induced changes in the testicular tissue and reduced the volume occupied by the locule and all germ cell types (figs. 4, 5). The volume occupied by seminiferous locules diminished after 12 h ($F_{4,600} = 252.81$; $P < 0.0001$). However, interstitial volume increased after 2 h, and 12 h of treatment ($F_{4,600} = 1872.56$; $P < 0.0001$; fig. 4). In LPS24h, neither locular, nor interstitial changes in volume were observed ($F_{4,600} = 252.81$; $P = 0.167$; $F_{4,600} = 1872.56$; $P = 0.279$; fig. 4). This variation in the locular and interstitial volume is linear and inversely correlated.

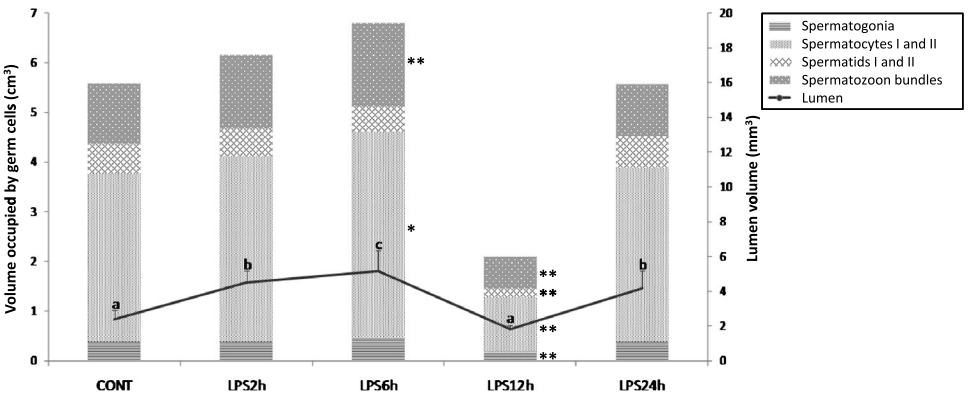

Figure 5. Germ cells and lumen volume occupied after LPS administration. Variation is observed at 12 hours of treatment when cells respond to LPS. A variance analysis was performed by Nested-ANOVA and results are indicated by asterisks: $^{*}P < 0.05$; $^{**}P < 0.001$. Different letters represent significant post hoc differences for a given lumen volume of experimental groups. CONT indicates Control group; LPS 2h, 6h, 12h and 24h indicate animals analyzed after 2, 6, 12, and 24 hours of LPS administration, respectively.

The volume of spermatogonia decreased about 50% after 12 hours of LPS administration ($F_{4,600} = 23.66$; $P < 0.0001$). The volume of spermatocytes and spermatids was also reduced by 70% ($F_{4,600} = 97.66$; $P < 0.0001$; $F_{4,600} = 24.89$; $P < 0.0001$, respectively) after 12 h of treatment. However, spermatocyte volume increased about 20% in LPS6h ($F_{4,600} = 97.66$; $P < 0.0001$). The volume occupied by spermatozoon bundles increased 30% after 6 hours (LPS6h; $F_{4,600} = 43.05$; $P < 0.0001$), reducing by about 50% after 12 h of treatment ($F_{4,600} = 43.05$; $P < 0.0001$; fig. 5) when compared to the control group.

In the above analyses we observed effects of treatment and variation in the animals within each experimental group. In all volumes analyzed (e.g., locule, lumen, interstitial and germ cells) we observed variation in animals of the same group. However, variation due to treatment was higher when compared with the variation among the experimental groups. Therefore, we confirmed the effects of the treatments with LPS on testicular morphology in *E. nattereri*.

The network of collagen and reticular fibers was also affected by the LPS treatment. After 12 h of LPS administration with the decrease of the locular region, collagen fibers were retracted and appeared spread in the interstitial region (fig. 2C, D). The reticular fibers retracted, as well, following the locular decrease (fig. 2E, F).

Mast cell quantification

Mast cells are found in the interstitial region of the testes, associated with testicular melanocytes (fig. 1D). The occurrence of mast cells increased as exposure time to LPS increased (fig. 6).

The amount of mast cells in the testes of animals tripled after 12 h (LPS12h) of treatment, compared with the control group ($F_{4,225} = 21.32$; $P < 0.0001$; fig. 6). The amount of mast cells decreased by half after 24 h of treatment (LPS24h), compared to LPS12h ($F_{4,225} = 21.32$; $P < 0.0001$; fig. 6). The amount of mast cells

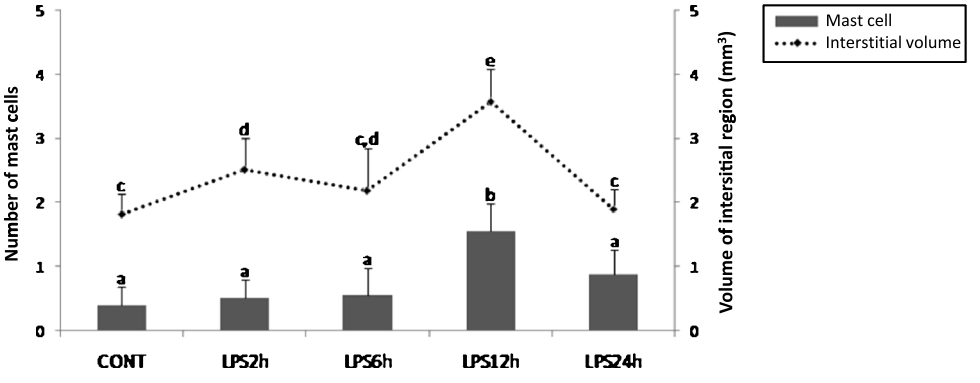

Figure 6. Amount of mast cells in the testis of *E. nattereri*. Different letters represent significant post hoc differences in mast cells or interstitial volume of experimental groups. CONT indicates Control group; LPS 2h, 6h, 12h and 24h indicate animals analyzed after 2, 6, 12, and 24 hours of LPS administration, respectively.

did not change after 2 h, 6 h, and 24 h of LPS administration ($F_{4,225} = 21.32.71$; $P = 0.51$; $P = 0.35$; $P = 0.09$, LPS2h, LPS6h, LPS24h, respectively) if compared to the control group. The increase of mast cells was correlated positively with the increase of interstitial area in LPS12h (fig. 6).

Discussion

Injections of LPS from *Escherichia coli* reduce the locular volume and also the volumes of spermatogonia, spermatocytes, spermatids, and spermatozoa in testes of *Eupemphix nattereri* after 12 hours of administration. External variables, such as environmental conditions, are important factors related to production of germ cells and volume occupied by germ cysts (Santos et al., 2011), while germ cell occurrence can also be altered by inflammatory processes.

Spermatogenesis in anurans is cystic, in which spermatocysts contain cells at the same stage of differentiation (Clermont, 1972; Báo et al., 1991; Santos et al., 2011; Oliveira & Franco-Belussi, 2012). The collagen fibers that occur in the testicular interstitium possess proteoglycans and are flexible and resistant. Reticular fibers are composed by glycoproteins that confer stability to the tissue (Ushiki, 2002). The treatment with LPS affects both germ cells and testicular interstitial components.

A decrease in all cell types was observed. This fact is related to the influence of LPS since cyst development is constant (Lofts, 1974). However, there was a decrease in the volume occupied by spermatogonia after 12 h of exposure to LPS. Moreover, there was also a decrease in spermatocytes, spermatids, and spermatozoa due to the continuity of spermatogenesis with the decrease in spermatogonia. Spermatocytes and spermatozoa increased in the LPS6h group, but decreased again in LPS12h. We hypothesize that this increase is a protection mechanism of germ cells, where the germ cell production is accelerated to generate viable gametes before the inflammation causes irreversible damage. Spermatocytes and spermatozoon bundles occupied a larger volume in the locular region. In the first case, the spermatocytes comprise the growth period of germ cells while in the spermatozoon bundles, the cells are elongated and occupy the second largest volume. These cell types belong to the meiotic and spermiogenic phases and they persist for a long period during spermatogenesis (Segatelli et al., 2009). Therefore, the increase of germ cells is observed only in cell types that occupied a large volume in the seminiferous locule.

All germ cells decreased after 12 hours of LPS administration. However, the area of spermatogonia, spermatocytes, spermatids and spermatozoon bundle increased after 24 hours, compared to the animals analyzed after 12 hours in our study. There is an increase of spermatocytes after 24 hours of LPS administration in mice (Liew et al., 2007). The results suggest that the main effect after 24 hours of LPS administration is the delay of meiosis, with spermatocytes arrested at the leptotene/zygotene. In rats, the numbers of prematurely released spermatocytes and round spermatids were observed within the lumen of the seminiferous tubules in

the LPS-treated groups at days 3 and 7 (Liew et al., 2007). However, the decrease of germ cells in frogs occurred early, after 12 h of LPS administration.

The reduction of germ cells after 12 h (more than 50% in volume) is directly related to the decrease in the locular area. The reduction of germ lineages within the seminiferous tubules occurs also in rabbits, after seven days of exposure to LPS 50 μg/kg (Collodel et al., 2012). Conversely, other studies did not find short-term changes (3 to 6 hours) in the organization of seminiferous tubules in rats treated with a high dose of LPS (5 mg/kg). However, there was evidence of degradation of the seminiferous epithelium after 12 hours (O'Bryan et al., 2000). In mice, all the LPS-treated groups demonstrated large apoptotic cells (e.g., spermatogonia, spermatocytes, and spermatids) in seminiferous tubule with a peak at 24 h after the injection (Kajihara et al., 2006). Therefore, the pronounced acute effects of a single dose of LPS on spermatogenesis in rats had only minor consequences for spermatogenic production on the longer term (Liew et al., 2007). For anurans, we observed a marked acute effect of LPS on spermatogenesis in the short time group (about 12 h treatment), and although the spermatogenesis process is distinct, this finding is related to mammals. Thus, LPS administered intraperitoneally seems to affect germ cells in various animals, and the time of responses of germ cell types are related to the dose of the substance.

Mast cells were observed in the interstitial region of testes. A number of immune cells are found in mammalian testes that protect the germinal epithelium (e.g., macrophages, mast cells, and lymphocytes; Nistal et al., 1984; Gaytan et al., 1989; Hedger & Meinhardt, 2000). Mast cells are key mediators in the immune response by secreting mediators, such as cytokines, histamine, and heparin that trigger inflammatory processes (Metcalfe, 2008). Therefore, these cells count increases after LPS administration. The number of mast cells increases in the testes of mammals when the spermatogenesis is abnormal, resulting in male infertility (Densmore & Green, 2007). A large increase (about 3× more) was observed in the mast cells of animals analyzed after 12 h of LPS administration. There was also a decrease in the seminiferous locule and germ cells in this experimental group. On the other hand, the interstitial region increased after 12 h from the administration of LPS. Therefore, it is hypothesized that the increase of mast cells is due to infiltration of inflammatory exudates and immune cells in the interstitial region, which is also increased.

In the testes of ectotherms there are also melanocytes in addition to immune cells (Oliveira & Franco-Belussi, 2012). These cells contain melanin that is responsible for photoprotection, thermoregulation, and has also bactericidal action (Riley, 1997; Franco-Belussi & Oliveira, 2011). In mammals, cutaneous melanocytes suggest a potential role as sentinel and perhaps even antigen-presenting cells due the morphology (e.g., large and dendritic cells) and location (e.g., superficial layers of skin; Plonka et al., 2009). LPS effects are observed in testicular melanocytes of anurans too. These pigment cells occur in testes and are responsive to anti-inflammatory α-melanocytes stimulating hormone suggesting that melanocytes participate in the

defense of germinative epithelium (Franco-Belussi et al., 2013). Regarding contamination of frogs with *E. coli*, the means by which it happens are not known; however, it has been reported that anurans are suitable hosts for this bacterium (Gray et al., 2007).

Acute exposure to LPS clearly alters testicular morphology, decreasing both locular and germ cells volumes. However, in the acute treatment, there was no impairment of spermatogenesis, since after 24 h treatment effects are minimized. Concurrently, the number of mast cells in the testicular tissue increased, demonstrating an inflammatory response against LPS administration. Furthermore, the interstitial region is increased by infiltration of inflammatory exudate, followed by decreasing locular volume. Thus, it has been demonstrated for the first time that LPS is a potential agent causing damage to testes in anuran.

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References

Alford, R.A. & Richards, S.J. (1999) Global amphibians declines: a problem in applied ecology. *Ann. Rev. Ecol. System.*, 30, 133-165.

Baccari, G.C., Matteo, A.P.L., Gentile, M. & Minucci, G.M.S. (1998) In situ characterization of mast cells in the frog *Rana esculenta*. *Cell Tissue Res.*, 292, 151-162.

Báo, S.N., Dalton, G.C. & Oliveira, S.F. (1991) Spermiogenesis in *Odontophrynus cultripes* (Amphibia, Anura, Leptodactylidae): ultrastrutural and cytochemucal studies of proteins using E-PTA. *J. Morphol.*, 207, 303-314.

Benjamin, C.F., Silva, J.S., Fortes, Z.B., Oliveira, M.A., Ferreira, S.H. & Cunha, F.Q. (2002) Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. *Infec. Immun.*, 70, 3602-3620.

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B. & Sorci, G. (2003) Assessing the cost of mounting an immune response. *Am. Natura.*, 161, 367-379.

Carey, C. & Bryant, C.J. (1995) Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environ. Health Perspect.*, 103, 13-17.

Clermont, Y. (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.*, 52, 198-236.

Collodel, C., Castellini, C., del Vecchio, M.T., Cardinali, R., Geminiani, M., Rossi, B., Spreafico, A. & Moretti, E. (2012) Effect of a bacterial lipopolysaccharide treatment on rabbit testis and ejaculated sperm. *Reprod. Dom. Anim.*, 47, 372-378.

Densmore, C.L. & Green, D.E. (2007) Diseases of amphibians. *ILAR Journal*, 48(3), 235-254.

Duellman, W.E. & Trueb, L. (1994) *Biology of Amphibians*. McGraw-Hill, New York.

Flores Quintana, C. & Ruas de Moraes, F. (2001) Respuesta inflamatória a la inoculación de LPS em pacu (*Piaractus mesopotamicus*) suplementados con cromo. *Ver. Ict.*, 9, 13-19.

Franco-Belussi, L., Castrucci, A.M.L. & Oliveira, C. (2013) Responses of melanocytes and melanomacrophages of *Eupemphix nattereri* (Anura: Leiuperidae) to Nle^4, D-Phe^7-α-melanocyte stimulating hormone and lipopolysaccharides. *Zool.*, 116, 316-324.

Franco-Belussi, L. & Oliveira, C. (2011) Lipopolysaccharides induce changes in the visceral pigmentation of *Eupemphix nattereri* (Anura: Leiuperidae). *Zool.*, 114, 298-305.

Franco-Belussi, L., Zieri, R., Santos, L.R.S., Moresco, R.M. & Oliveira, C. (2009) Pigmentation in anuran testes: anatomical pattern and variation. *Anat. Rec.*, 292, 178-182.

Frost, D.R. (2011) Amphibian species of the World: an online reference. Version 5.5 (31 January, 2011). Electronic database accessible at http://research.amnh.org/vz/herpetology/amphibia/, American Museum of Natural History, New York, USA. (Last access Dec. 2012).

Gaytan, F., Carrera, G., Pinilla, L., Aguilar, R. & Bellido, C. (1989) Mast cells in the testis, epididymis and accessory glands of the rat: effects of neonatal steroid treatment. *J. Androl.*, 10, 351-358.

Gray, M.J., Rajeev, S., Miller, D.L., Schmutzer, A.C., Burton, E.C., Rogers, E.D. & Hickling, G.L. (2007) Preliminary evidence that American bullfrogs (*Rana catesbeiana*) are suitable hosts for *Escherichia coli* O157:H7. *Applied Environ. Microbiol.*, 73, 4066-4068.

Hayes, T.B., Falso, P., Gallipeau, S. & Stice, M. (2010) The cause of global amphibian decline: a developmental endocrinologist's perspective. *J. Exp. Biol.*, 213, 921-933.

Hedger, M.P. & Meinhardt, A. (2000) Local regulation of T cell numbers and lymphocyte-inhibiting activity in interstitial tissue of the adult rat testis. *J. Reprod. Immunol.*, 48, 69-80.

Kajihara, T., Okagaki, R. & Ishihara, O. (2006) LPS-induced transient testicular dysfunction accompanied by apoptosis of testicular germ cells in mice. *Med. Molec. Morphol.*, 39, 203-208.

Liew, S.H., Meachem, S.J. & Hedger, M.P. (2007) A stereological analysis of the response of spermatogenesis to a acute inflammatory episode in adult rats. *J. Androl.*, 28, 176-185.

Lofts, B. (1974) Reproduction. In: *Physiology of the Amphibia*, *Vol. 2*, pp. 107-218. Academic Press, New York.

Metcalfe, D.D. (2008) Mast cells and mastocytosis. *Blood*, 112, 946-956.

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. (2011) *Guide for the Care and Use of Laboratory Animals*. 8th ed. National Academies Press, Washington, DC, USA. Available from: http://www.ncbi.nlm.nih.gov/books/NBK54050/

Nistal, M., Santamaria, L. & Paniagua, R. (1984) Mast cells in the human testis and epididymis from birth to adulthood. *Acta Anat.*, 119, 155-160.

O'Bryan, M.K., Schlatt, S., Phillips, D.J., de Kretser, D.M. & Hedger, M.P. (2000) Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. *Endocrinol.*, 141, 238-246.

Oliveira, C. & Franco-Belussi, L. (2012) Comparative morphology of anuran testes. In: Y. Nemoto & N. Inaba (Eds.) *Testis: Anatomy, Physiology and Pathology*, pp. 111-126. Nova Science Publishers.

Oliveira, C., Vicentini, C.A. & Taboga, S.R. (2003) Structural characterization of nuclear phenotypes during *Scinax fuscovarius* spermatogenesis (Anura, Hylidae). *Caryologia*, 56, 75-83.

Plonka, P.M., Passeron, T., Brenner, M., Tobin, D.J., Shibahara, S., Thomas, A., Slominski, A., Kadekaro, A.L., Hershkovitz, D., Peters, E., Nordlund, J.J., Abdel-Malek, Z., Takeda, K., Paus, R., Ortonne, J.P., Hearing, V.J. & Schallreuter, K.U. (2009) What are melanocytes really doing all day long. . . ?. *Experim. Dermatol.*, 18, 799-819.

R Development Core Team. (2010) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org (accessed on 20 July 2013).

Riley, P.A. (1997) Melanin. *Intern. J. Biochem. Cell Biol.*, 29, 1235-1239.

Santos, L.R.S., Franco-Belussi, L. & Oliveira, C. (2011) Germ cell dynamics during the annual reproductive cycle of *Dendropsophus minutus* (Anura, Hylidae). *Zool. Science*, 28, 840-844.

Segatelli, T.M., Batlouni, S.R. & França, L.R. (2009) Duration of spermatogenesis in the bullfrog (*Lithobates catesbeianus*). *Theriogenology*, 72, 894-901.

Shakoory, B., Fitzgerald, S.M., Lee, S.A., Chi, D.S. & Krishnaswamy, G. (2004) The role of human mast cell-derived cytokines in eosinophil biology. *J. Interf. Cytok. Researc.*, 24, 271-281.

Silva, P.S., Girol, A.P. & Oliani, S.M. (2011) Mast cells modualte the inflamatory process in endontoxin-induced uveits. *Molecular Vision.*, 17, 1310-1319.

Sylvester, W.R.B., Amadi, V., Hegamin-Younger, C., Pinckney, R., Macpherson, C.N.L., McKibben, J.S., Bruhl-Day, R., John-Sylvester, K.D. & Hariharan, H. (2014) Occurrence of antibiotic resistant *Escherichia coli* in green iguanas (*Iguana iguana*) in Grenada, West Indies. *Inter. J. Vet. Med. Res. Rep.* DOI:10.5171/2014.260412.

Ushiki, T. (2002) Collagen fibers, reticular fibers and elastic fibers. A comprehensive understanding from a morphological viewpoint. *Arch. Histol. Cytol.*, 65, 109-126.

Vilamaior, P.S.L., Taboga, S.R. & Carvalho, H.F. (2006) The postnatal growth of the rat ventral prostate in Wistar rats: a stereological and morphometrical study. *Anat. Rec.*, 288, 885-892.

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Morphological and histochemical studies of Bidder's organ in *Rhinella schneideri* (Amphibia: Anura) males

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Abstract
Bufonids have an organ that produces female germ cells in both sexes, known as the Bidder's organ (BO). In males, BO is located in the anterior pole of the testis and it has been compared to a rudimentary ovary. It has been demonstrated that in some species the bidderian follicles can accumulate vitellogenin in males, while in other species, the development of follicles is inhibited by the differentiation of the corresponding gonad. This study describes the anatomical, histochemical and ultrastructural aspects of the BO in males of the neotropical frog *Rhinella schneideri* during the breeding season. A topographic model has also been built using three-dimensional (3D) reconstruction. BO has an irregular shape with lobes varying in size and number. There is no physical barrier between the BO and male gonads and, for this reason, female cells are in intimate association with seminiferous locules. Histologically, two distinct regions are observed in the BO: the cortex, and medulla. In the cortex, bidderian oocytes are surrounded by follicle cells forming bidderian follicles, which are in pre-vitellogenic stage. The ooplasm of bidderian oocytes is rich in cytoplasmic organelles. Microvilosities are formed in the oolemma, where the follicular cells are juxtaposed with oocytes, and amorphous extracellular material is deposited. Lipofuscin and myelin bodies occur in the medulla as a result of the cellular degradation. Pigmented cells were also detected in the medullar region. The oogonias observed in the BO periphery, and the significant amount of degenerating oocytes in the cortex, showed the renewal capacity of BO cells in *R. schneideri* males. The BO anatomical pattern in *R. schneideri* is similar to that observed for most species of bufonids. This work describes for the first time specific aspects related to the morphological description with emphasis on architecture, morphometry and histochemistry.

Keywords: *Anura, morphology, histochemistry, Bidder's organ,* Rhinella schneideri

Introduction

Bufonids (except some *Dendrophryniscus*) are characterized for the presence of a structure called Bidder's organ (BO), a rudimentary ovary capable of producing female germ cells (Spengel 1876; McDiarmid 1971; Petrini & Zaccanti 1998). BO develops early during the larval life in both sexes and before the differentiation of the gonads (Beccari 1925; Vitale-Calpe 1969; Petrini & Zaccanti 1998). The anterior portion of the gonadal primordium develops into the BO and the posterior portion differentiates into either ovary or testis (Petrini & Zaccanti 1998). In some species such as *Bufo bufo*, *B. ictericus* and *B. vulgaris*, BO is present in adults of both sexes (Ponse 1927; Farias et al. 2002; Falconi et al. 2007). However, this organ remains in *B. marinus* and *B. lentiginosus* adult males only (King 1908; Brown et al. 2002).

In males, BO is located at the cranial portion of the testis and has been used as a character in systematic studies of the group (Duellman & Trueb 1999). Studies have suggested that BO development is inhibited by the differentiation of the corresponding gonad (Tanimura & Iwasawa 1986; Duellman & Trueb 1999). In fact, some authors (e.g. Orr 1986; Tanimura & Iwasawa 1986) reported that the BOs develop into a functional ovary only after removing testes or blocking gonads experimentally (Brown et al. 2002).

Histological analyses have demonstrated that the BO is composed of bidderian follicles at several stages of development in male toads (Farias et al.

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2002). However, the degree of development of the bidderian follicles can vary among the different species. In some species such as *B. ictericus*, *B. marinus* and *B. japonicus formosus*, the males have only previtellogenic follicles in their BO (Tanimura & Iwasawa 1986; Brown et al. 2002; Farias et al. 2002). However, vitellogenic follicles were detected in males of *Rhinella arenarum* under normal conditions (Scaia et al. 2011). The production of female germ cells by the BO may represent a morphological reproduction strategy in bufonids. For many authors, BO is a vestigial structure that may become functional under certain circumstances (Vitale-Calpe 1969; Pancak-Roessler & Norris 1991; Duellman & Trueb 1999). The ability to produce the succeeding generation is an essential feature to maintain the species in the environment (Tanimura & Iwasawa 1986; Duellman & Trueb 1999). Bufonids are particularly interesting and important to the study of sex differentiation.

BO differentiation has been previously described by several authors (Beccari 1925; Vitale-Calpe 1969; Petrini & Zaccanti 1998; Falconi et al. 2007), but the morphological aspects of the BO in adults remain scarce for many bufonids. In adulthood, the reproductive organs are at their developmental peak, which facilitates the investigation of the reproductive traits of different species. For example, the BO morphology of *Rhinella schneideri*, a member of a clade restricted to South America, is still unknown. This species is widely distributed in South America, occupying even disturbed areas (Cochran 1955), which are often affected by a variety of compounds that interfere with the growth of germinal tissue. Information about the morphology of reproductive structures is relevant in designing efficient strategies for conservation of anuran species, understanding how the biotic and abiotic factors can interfere with their normal reproduction. For bufonids, this is especially true due to the presence of the BO producing female cells in males. Thus, this study aimed at evaluating BO morphological, histochemical and ultrastructural aspects in *R. schneideri* males during the breeding season, using routine histochemical techniques for light and electron microscopy. In addition, a topographic model of the BO was built using three-dimensional (3D) reconstruction methods.

Materials and methods

Animals collected

Fifteen *Rhinella schneideri* adult males were collected from permanent ponds and wetlands in a non-agricultural area, in southeastern Brazil (20° 47′07.05″; 49°02′42.09″W) during the reproductive season, at night. The animals were transported to the laboratory, immediately anaesthetized, and euthanized with benzocaine (5 g/l water). Male gonads were weighed, measured, and analysed to confirm that toads were at the same stage of sexual development. All experimental procedures were authorized by the Ethics Committee of the São Paulo State University (Protocol #040/2011 CEUA) and followed the NIH Guide for the Care and Use of Laboratory Animals.

Histological and histochemical analyses

The toads were dissected exposing the BO for macroscopic analysis and photo documentation under a dissecting microscope (Leica MZ16), coupled to an image capture system (Image Manager – IM50).

For histological analysis, the BO and testes were removed and fixed for 24 h in a Karnovsky fixative solution (0.1M Sörensen buffer phosphate, phosphate buffer pH 7.2, containing 5% paraformaldehyde and 2.5% glutaraldehyde), at 4°C. Subsequently, the material was dehydrated in increasing ethanol series and embedded in glycol methacrylate resin (Historesin Leica®). Sections of 2 μm were obtained with a microtome (RM 2265, Leica, Switzerland) and stained with haematoxylin–eosin, Gömori trichrome, and silver ion impregnation. BO general morphology was observed and described under a microscope (Leica DM4000 B) coupled with an image capture system (Leica DFC 285). The distinct bidderian follicles were quantified to assess the occurrence of the oocytes (young follicles, late follicles and follicles in degeneration). A total of 250 histological sections of the germinal epithelium were analysed using the Image Pro-plus software v. 6.0, through manual counting. The G-test of goodness-of-fit and the Yates correction (Sokal & Rohlf 1995) were used to test the difference in the amount of cells. This test was implemented with the code provided by Prof. Peter Hurd, available at http://www.psych.ualberta.ca/~phurd/cruft/g.test.r. All analyses used the R v. 2.11.1 software (R Development Core Team 2010).

For histochemical analysis of lipofuscin detection, sections were incubated in Schmorl's solution, composed of 75 ml of 1% ferric chloride, 10 ml of potassium ferricyanide, and 15 ml of distilled water, for 15 min. Then, sections were immersed in 1% neutral red, followed by 1% eosin.

Three-dimensional reconstruction

The tissue mass containing the BO, testes, and fat bodies was embedded in historesin. The 3-µm thick serial sections were stained with haematoxylin–eosin. Images were captured and processed using the Reconstruct 3.2 software (Build 743; Digivision-SIS, San Diego, CA). After image alignment, regions containing BO, testes and fat body were isolated in each image and later processed to obtain the interface limit of each section, generating a 3D model. The model allows an improved visualization of the topographical anatomy of the organ, as well as its limits and connections with male gonad.

Ultrastructural analysis

Tissue samples were fixed in 3% glutaraldehyde and 0.25% tannic acid in Millonig buffer pH 7.3, for 2 h at 25°C. Posteriorly, samples were post-fixed in 1% osmium tetroxide diluted in the same buffer for 1 h, dehydrated in acetone, and embedded in Araldite (Cotta-Pereira et al. 1976). Ultrathin sections (50–75 nm) were contrasted with 2% uranyl acetate for 20 min (Watson 1958), and lead citrate in 1N sodium hydroxide solution (Venable & Coggeshall 1965) for 8 min, and examined under a Leo-Zeiss EM – 906 electron microscope operating at 80 kV.

Results

BOs are paired structures, located in the cranial portion of *Rhinella schneideri* testes (Figure 1A). Male toads have well-developed testes with smaller yellow–brown BO weighing on average 0.05 g (± 0.002 g). The 3D model showed that the BO shape is irregular, with projections of different sizes (Figure 2). Some projections are adhered to the testes, so that the bidderian tissue is associated with testicular tissue (Figure 2D). There is no physical barrier between the BO and male gonads. Thus, the bidderian oocytes are close to seminiferous locules with spermatocysts at different stages (Figure 1B). Externally, the organ is covered by a thin capsule of connective tissue (Figure 3B).

Histological arrangement showed two distinct regions of the BO: the cortex and medulla (Figure 3A–D). The centrally located medulla is smaller than the cortex (Figure 3C). It receives the blood vessels and has abundant collagen fibers synthesized by fibroblast (Figure 3C–E). Lipofuscin granules are detected in the same region, and pigment cells occur dispersed in the tissue (Figure 3E,F).

Most of the cell population in the medullar region is represented by somatic cells, which have highly basophilic nuclei, due to chromatin compaction, well-defined nucleoli, and are relatively small and highly electron-dense when compared to germ cells. Somatic cells surrounding oocytes are called follicular cells because they constitute the follicular layer of the bidderian oocytes (Figure 4C). Follicular cells are mostly flat, but may be round when attached to oocytes in advanced developmental stages. These cells differ not only in shape, but also in the degree of electron density, both in the nucleus and cytoplasm (Figure 4G). Dark and clear follicular cells are associated with the oocytes by cytoplasmic communications while their boundary is easily visualized due to differences in colour (Figure 4G). Nonetheless, all other features and structures are shared. They have many organelles, such as

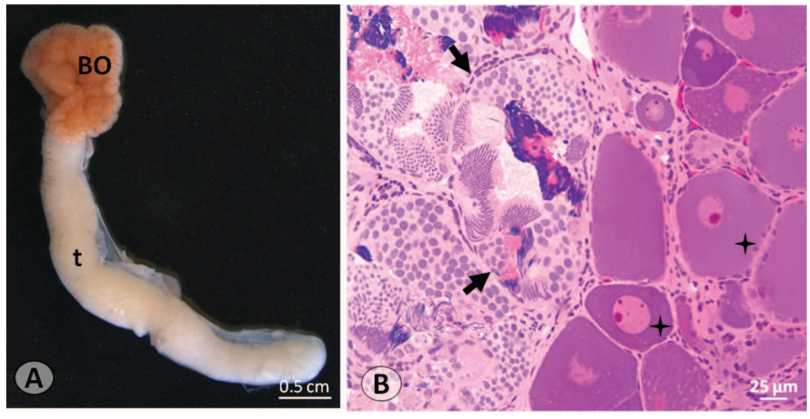

Figure 1. BO of *Rhinella schneideri* male. (A) BO macroscopic aspect showing its localization in the cranial portion of the testis (t). (B) Histological section of the region between BO and testis. Seminiferous locules (arrow) are in direct contact with bidderian oocytes (✦). Staining: haematoxylin–eosin.

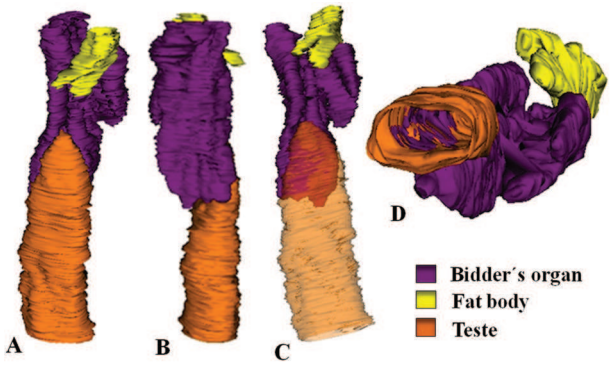

Figure 2. Three-dimensional reconstruction (3D) of the complex formed by BO, testes, and fat body. (A, B) Ventral and dorsal view of the BO and testes, respectively. (C) BO overview through the transparency of the testes. Irregular lobes with different sizes can be seen in the 3D model. (D) Internal view of the complex, showing the arrangement of testicular and bidderian tissue.

mitochondria, well-developed endoplasmic reticulum, and some vesicles (Figure 4D,E). Follicular cells are attached to the follicular layer by focal adhesions, connecting the cytoplasm of the two cells (Figure 4D,E).

Follicles of different stages are found in the BO cortex. In general, bidderian follicles are large structures with a diameter ranging from 68.763 to 172.394 μm and mean area varying from 3108.2 to 16,887.354 μm^2. Bidderian follicles have an either oval or rounded acidophilus nucleus with well-delimited nuclear membrane, which consists of many nuclear pore complexes (Figure 4B). Furthermore, the nucleus has one or more well-defined nucleoli with intense basophilia and uniform electron density (Figures 4B and 5E,F).

The ooplasm of bidderian oocytes is rich in mitochondria and other well-developed organelles, such as endoplasmic reticulum and Golgi complex. There are also many lipid droplets, which should participate in yolk metabolism (Figure 4B). Microvilosities are formed in the oolema as a result of oocyte development, where follicular cells are juxtaposed with oocytes (Figure 4D,E). Amorphous extracellular material is deposited along the microvilosities to the subsequent formation of the zona pellucida (Figure 4E). This material is moderately electron-dense and mainly composed of proteins and proteoglycans. Electron microscopy also revealed myelinic bodies that originated from the degradation of intracellular material (Figure 4F).

Oogonias were observed near the germinal layer at the periphery of the organ (Figures 4A and 5A,B). They have irregular and elliptical shape, very lobed and slightly acidophilic nucleus and abundant nuage material. The nucleus has one or more small, highly basophilic and uniformly electron-dense nucleoli (Figure 4A). Oogonias are separated from the cortex by the connective tissue and can be arranged into nests of multiple cells (Figures 5A,B). Prophase oocytes (POs) originate from the oogonias mitoses and are compartmentalized into asynchronous nests in the BO periphery (Figure 5C). Unlike oogonia, these round cells have spherical nucleus, with moderate chromatin compaction and a slight increase in cytoplasmic basophilia, but the nucleus remains quite basophilic. After that, POs are individualized and originate young follicles (Figure 5D).

The atretic and degenerating oocytes, also found in the BO, were the most common cell type in the organ (68.9% of the bidderian oocytes; Figure 6). Degenerating oocytes are larger cells (± 172.394 μm average diameter) and have nuclear disintegration with acidophilic and impregnated areas in the cytoplasm (Figure 5F). At this stage, the follicle cells surrounding oocytes are less flattened and partially invade the oocyte membrane. Subsequently, somatic cells invaginate towards the interior of the oocyte and promote follicular atresia in advanced stages of degeneration (Figure 5G).

Discussion

The general morphology and anatomy of the BO in *Rhinella schneideri* males are similar to that found in other species of bufonids, such as *Bufo ictericus*, *B. marinus*, *Bufo japonicus* (Moriguchi et al. 1991; Tanimura & Iwasawa 1992; Farias et al. 2002) and *Bufo woodhousii* (Pancak-Roessler & Norris 1991). In these species, the BO is a small organ located in the cranial portion of the testes and has the typical morphology of an undeveloped ovary with pre-vitellogenic oocytes in successive developmental stages. However, distinctive features, which have not been reported for other species, such as pigment cells, lipofuscin granules and the 3D arrangement of the BO were observed.

In most male bufonids, the presence of vitellogenic oocytes in the BO is known to occur only after castration, which suggests that testes are necessary to suppress vitellogenin accumulation (Pancak-Roessler & Norris 1991; Zaccanti 1994; Brown et al. 2002). *B. marinus* male frogs exposed to agricultural sites also exhibited some bidderian follicles in early or late vitellogenesis because agricultural compounds act as endocrine disruptors, interfering with the normal development of the reproductive organs in males (McCoy et al. 2008). However, there is little information regarding the morphological aspects of BO in bufonids under natural conditions. In *R. arenarum*, for example,

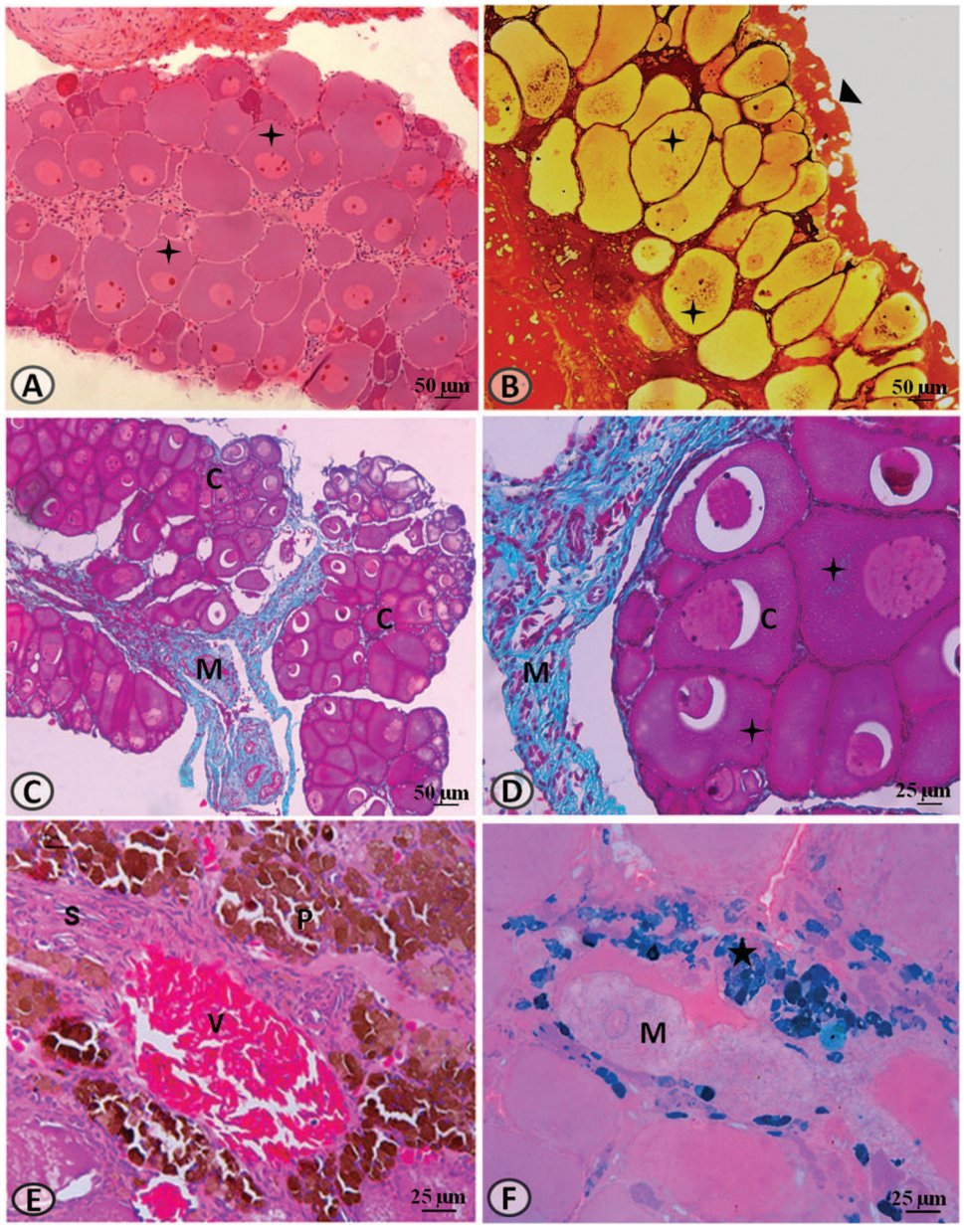

Figure 3. Histological sections of the BO of *Rhinella schneideri*. (A) Bidderian follicles (+) disposed in the cortical region in several development stages. (B) A thin capsule of connective tissue covers the BO externally (arrowhead); (+) bidderian follicles. (C, D) Cortical (C) and medullar (M) regions in the BO. Bidderian follicles (+) in several developmental stages are distributed in the cortex. Medulla (M), rich in collagen fibres (in blue), is observed using the Gömori trichrome technique. (E) Blood vessel (V), pigment cells (P) and somatic cells (S) found in the BO medulla. (F) Lipofuscin granules (star) are also detected in the medullar region (M). Staining: haematoxylin–eosin (A, F), Gömori trichrome (C, D), silver ion impregnation (B) and acidic ferrocyanide (F).

vitellogenic oocytes were reported in the BO of males (Scaia et al. 2011). We believe that the development of oocytes must be linked to the physiological status of the animals and, thus, the development stages of bidderian oocytes could vary naturally among bufonids species. Nevertheless, it is a consensus that oogenesis is not completed in the BO of males.

Although the bidderian oogenesis is interrupted during the pre-vitellogenic stage in *R. schneideri* males, it was noticed that bidderian oocytes have high cell activity. This can be inferred based on the large amount of organelles, such as mitochondria, Golgi complexes, and well-developed endoplasmic reticulum in the ooplasm. Moreover, large, prominent nucleoli and many micronucleoli occur in the

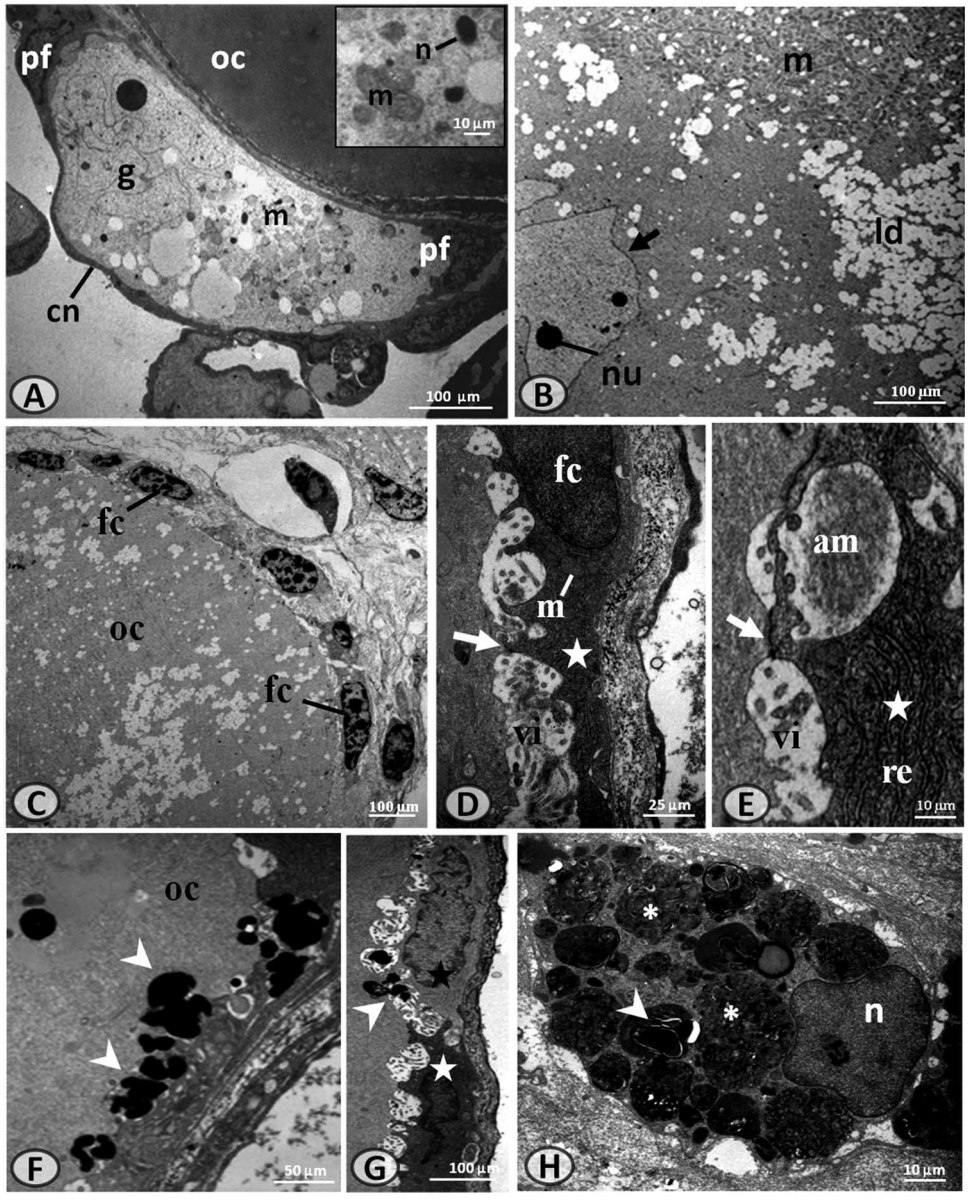

Figure 4. Electron micrographs of the BO. (A) Single oogonium involved by connective tissue (cn) in the peripheral region of the organ. The nucleus is very lobed and the nucleolus is uniformly electron-dense (nu). Nuage material (n) within the oogonia is mainly associated with mitochondria (m). pf, prefollicle cell. (B) Bidderian oocytes showing the nuclear pore complexes delimiting the nuclear membrane (arrow). Cytoplasm contains many mitochondria (m) and lipid droplets (ld). (C) Bidderian follicle, formed by oocytes (oc) surrounded by many follicular cells (fc). (D, E) Region of adhesion between the oocyte and follicular cells (fc). Focal adhesion (white arrow) can be seen in this region, along the microvilosities (vi) in the periphery of oocytes. Amorphous material (am) is accumulated along the villi. Endoplasmic reticulum (re) is observed in cytoplasm of follicle cell. (F) Myeloid bodies (arrowhead) in the periphery of oocyte. (G) Follicular cells with distinct electron density in the nucleus and cytoplasm. Dark (white star) and clear follicular cell (dark star) communicate through cytoplasmic connections (white arrow). (H) Pigmented cells, showing the large nucleus (n) and heterogeneous granules in the cytoplasm (*).

nucleus, indicating intense metabolic activity. The presence of many nucleoli formed by amplified ribosomal DNA genes is common to amphibians (Brown & Dawid 1968; Amaldi et al. 1973; Scheer & Dabawall 1985). The deposition of amorphous extracellular material was observed in the surroundings of oocytes, within the microvilosities. This material is rich in glycoproteins and essential for the formation of the zona pellucida. Amorphous material has also been reported in *R. icterica* BO, but covering the entire oocyte membrane (Farias et al. 2002) and not deposited inside of the microvilosities as in *R. schneideri*. Degenerated and atretic oocytes abound in the BO, probably due to the lack

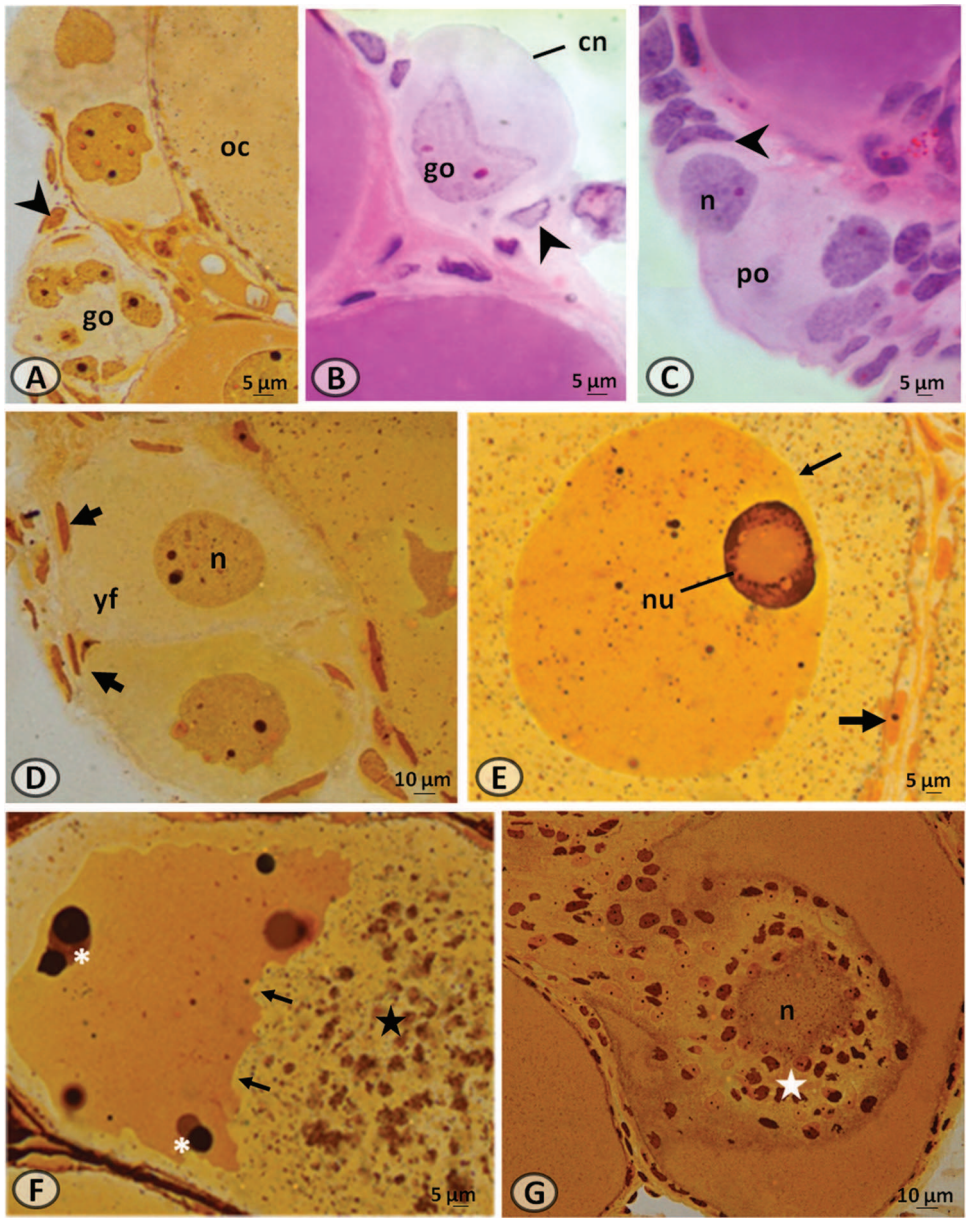

Figure 5. Different developmental stages of germ cells in the BO. (A) Nest of oogonia (go) accompanied by prefollicle cell (arrowhead); oc, oocyte. (B) Simple oogonia (go), separated from the bidderian epithelium by connective tissue (cn). (C) Prophase oocytes (po), organized into nest; n, nucleus. (D) Young follicles (yf) surrounded by a few follicular cells (arrow), constituting the new follicular structures. Cytoplasm and nucleus are distinctly delimited now. (E) Follicles with increased volume of the nucleolus (nu) and nucleus (thin arrow), which have well-delimited aspect. Flattened follicular cells (large arrow) surrounding these oocytes. (F, G) Oocytes in degeneration. (F) The nucleus loses its spherical form (thin arrow), and nuclear material is accumulated in the ooplasm (star). Nucleoli are more fragmented (*). (G) Oocyte in advanced degeneration, showing the invagination of somatic cells (white star) into the oocyte. The nucleus (n) is almost disintegrated. Staining: silver ion impregnation (A, D–G) and heamatoxylin–eosin (B, C).

of stimuli to develop the oocytes. Some authors show that the end of development of bidderian oocytes in males is marked by the degeneration of large previtellogenic oocytes (Zaccanti & Gardenghi 1968; Zaccanti et al. 1971; Brown et al. 2002; Farias et al. 2002). Atretic oocytes are also identified in the BO of other bufonids, such as *B. marinus* (Brown et al. 2002) and *R. arenarum* (Scaia et al. 2011). The absence of a barrier between the BO and the testes can cause the interruption of oocyte development because hormonal regulation in male testes may influence the BOs, contributing to the degeneration of feminine cells. Plasma androgens, such as testosterone, may block ovarian or Bidder estradiol

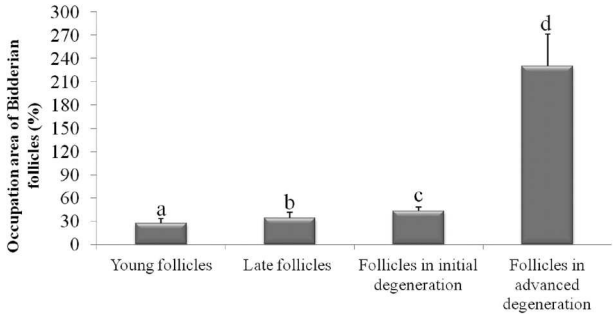

Figure 6. Area occupied by bidderian follicles in *Rhinella schneideri* males. The figure shows the area occupied by different oocytes in the BO. Oocytes in late degeneration are the most common cells in the BO. Different letters indicate statistical significance ($P < 0.05$). Statistical values: $P < 0.0001$ (a–d).

production and/or denying estradiol to the BOs by certain processes, as implied by BO atrophy after administration of testosterone (Deb & Chatterjee 1963). Thus, the presence of testes may in some way inhibit the BO from collecting enough estradiol to take part in vitellogenesis (Calisi 2005). Echeverria (1990) also reports that the degradation of old oocytes in the BO permits a qualitative levelling with the production of young oocytes. In *R. schneideri*, even in the presence of intense oocyte degeneration, oogonia were constant in the BO, showing cell renewal capacity.

A great amount of blood vessels, collagen fibres and reticular fibres is observed in the medullar region. In addition, pigmented cells are observed in the same BO region of *R. schneideri.* These structural components and pigmented cells are also reported in the BO of other species, such as *B. ictericus* (Farias et al. 2002). Generally, pigmented cells in amphibians are associated to an extracutaneous pigmentary system composed of melanin-containing cells in various tissues and organs (Agius & Agbede 1984; Moresco & Oliveira 2009; Franco-Belussi et al. 2011; Oliveira & Franco-Belussi 2012). Some species of frogs have intense pigmentation in male gonads while others have no pigment cells (Zieri et al. 2007; Franco-Belussi et al. 2009, 2011; Moresco & Oliveira 2009). The functional role of the pigment cells in these organs has not been defined yet, although several hypotheses have been proposed (Gallone et al. 2002), including cytoprotective functions (Fenoglio et al. 2005; McGraw 2005). In general, the presence of pigment cells in the BO of bufonids is not well reported; however, it may be considered an adaptive characteristic of the different species to the environment.

Many lipofuscin granules were also found in the medulla and around the bidderian oocytes. Histochemical analysis to detect lipofuscin has not yet been performed on this organ. The presence of these granules indicates the occurrence of autophagocytosis and renovation of intracellular components. Lipofuscin is an intralysosomal, polymeric substance, primarily composed of cross-linked protein residues, resulting from iron-catalysed oxidative processes. Lipofuscin accumulation in postmitotic cells is inevitable because it is not degradable and cannot be removed via exocytosis (Pickford 1953). Although lysosomal degradation is imperfect in all cells, only postmitotic and slowly dividing cells accumulate lipofuscin. Actively proliferating cells, both *in vivo* and *in vitro*, efficiently dilute lipofuscin during successive divisions (Terman 2001). However, lipofuscin accumulates when the proliferation of normal mitotic active cells is inhibited (Jahani et al. 1985; Terman & Brunk 1998). Bidderian oocytes frequently divide in the BO, but oogenesis inhibition causes follicle degeneration in males. The interruption of oocyte development and the high number of atretic oocytes may have been the contributing factor for lipofuscin accumulation around the cell and in the medulla.

The results show that the BO of *R. schneideri* males is similar to a young ovary and has successive germ cells, including pre-vitellogenic oocytes at different developmental stages. Although the BO is known as a vestigial structure in males, we demonstrated that its cells have intense activity and that it is not appropriate to designate the BO as a rudimentary ovary. Furthermore, we believe that both the morphology and developmental stage of bidderian oocytes may vary among bufonids according to the physiological and adaptive characteristics of each species. The reproductive biology of amphibians brings valuable information that could be used in conservation plans, mainly for neotropical species, which have been widely threatened in recent years.

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References

Agius C, Agbede SA. 1984. An Electron microscopical study on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. Journal of Fish Biology 24:471–488. doi:10.1111/jfb.1984.24.issue-4.

Amaldi F, Lava-Sanchez PA, Buongiorno-Nardelli M. 1973. Nuclear DNA content variability in *Xenopus laevis*: A redundancy regulation common to all gene families. Nature 242:615–617. doi:10.1038/242615a0.

Beccari N. 1925. Ovogenesi larvale, organi di Bidder e differenziamento dei sessi nel *Bufo viridis*. Italian Journal of Anatomy and Embryology 22:483–549.

Brown D, Dawid B. 1968. Specific gene amplification in oocytes: Oocyte nuclei contain extrachromosomal replicas of the genes for ribosomal RNA. Science 160:272–280. doi:10.1126/science.160.3825.272.

Brown FD, Del Pino EM, Krohne G. 2002. Bidder's organ in the toad *Bufo marinus*: Effects of orchidectomy on the morphology and expression of lamina-associated polypeptide 2. Development, Growth & Differentiation 44:527–535. doi:10.1046/j.1440-169X.2002.00665.x.

Calisi RM. 2005. Variation in Bidder's organ volume is attributable to reproductive status in *Bufo woodhousii*. Journal of Herpetology 39:656–659. doi:10.1670/05-05N.1.

Cochran DM. 1955. Frogs of southeastern Brazil. Bulletin of the United States National Museum 206:1–423. doi:10.5479/si.03629236.206.1.

Cotta-Pereira G, Rodrigo FG, David-Ferreira JF. 1976. The use of tannic acid-glutaraldehyde um the study of elastic related fibers. Stain Technology 51:7–11.

Deb C, Chatterjee A. 1963. Histochemical studies on the nature of Bidder's organ in toad (*Bufo melanostictus*). Endokrinologie 44:291.

Duellman WE, Trueb L. 1999. Biology of amphibians. New York: McGgraw-Hill. 405 pp.

Echeverria DD. 1990. Organogénesis y ciclos estacionales del organo de Bidder em las larvas los juveniles de *Bufo arenarum* (Anura: Bufonidae). Cuaderno de Herpetología 5:1–9.

Falconi R, Dalpiaz D, Zaccanti F. 2007. Morphological aspects of gonadal morphogenesis in *Bufo bufo* (Amphibia, Anura): Bidder's organ differentiation. The Anatomical Record 290:801–813. doi:10.1002/ar.20521.

Farias CF, Carvalho-e-Silva SP, Brito-Gitirana L. 2002. Bidder´s organ of *Bufo ictericus*: A light and electron microscopy analysis. Micron 33:673–679.

Fenoglio C, Boncompagni E, Fasola M, Gandini C, Comizzoli S, Milanesi G, Barni S. 2005. Effects of environmental pollution on the liver parenchymal cells and Kupffer-melanomacrophagic cells of the frog *Rana esculenta*. Ecotoxicology and Environmental Safety 60:259–268. doi:10.1016/j.ecoenv.2004.06.006.

Franco-Belussi L, Santos LRS, Zieri R, Oliveira C. 2011. Visceral pigmentation in four *Dendropsophus* species (Anura: Hylidae): Occurrence and comparison. Zoologischer Anzeiger 250:102–110. doi:10.1016/j.jcz.2010.12.001.

Franco-Belussi L, Zieri R, Santos LRS, Moresco RM, Oliveira C. 2009. Pigmentation in anuran testes: Anatomical pattern and variation. The Anatomical Record 292:178–182. doi:10.1002/ar.20832.

Gallone A, Guida G, Maida I, Cicero R. 2002. Spleen and liver pigmented macrophages of *Rana esculenta L.* A new melanogenic system? Pigment Cell Research 15:32–40. doi:10.1034/j.1600-0749.2002.00052.x.

Jahani M, Gracy RW, Cathepsin B. 1985. Activity in human skin fibroblasts from young, old and premature aging syndromes. Federation Proceedings 44:876–886.

King HD. 1908. The structure and development of Bidder's organ in *Bufo lentiginosus*. Journal of Morphology 19:439–468. doi:10.1002/(ISSN)1097-4687.

McCoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette LJ, Mary C. 2008. Agriculture Alters Gonadal Form and Function in the Toad *Bufo marinus*. Environmental Health Perspectives 116:1526–1532. doi:10.1289/ehp.11536.

McDiarmid R. 1971. Comparative morphology and evolution of frogs of the Neotropical genera *Atelopus, Dendrophryniscus, Melanophryniscus*, and *Oreophrynella*. Bulletin and Contributions in Science of the Natural History Museum of Los Angeles County 12:1–66.

McGraw KJ. 2005. The antioxidant function of many animal pigments: Are there consistent health benefits of sexually selected colourants? Animal Behavior 69:757–764.

Moresco RM, Oliveira C. 2009. A comparative study of the extracutaneous pigmentary system in three anuran amphibian species evaluated during the breeding season. South American Journal of Herpetology 4:1–8. doi:10.2994/057.004.0101.

Moriguchi Y, Tanimura A, Iwasawa H. 1991. Annual changes in the Bidder's organ of the toad *Bufo japonicus formosus*: Histological observation. Science Reports of Niigata University Series D Biology 28:11–17.

Oliveira C, Franco-Belussi L. 2012. Melanic pigmentation in ectothermic vertebrates: Occurrence and function. In: Ma X-P, Sun X-X, editors. Melanin: Biosynthesis, Functions and Health Effects. New York: Nova Science Publishers. pp. 213–226.

Orr RT. 1986. Biologia dos vertebrados. 5th ed. São Paulo: Roca. 508 pp.

Pancak-Roessler MK, Norris DO. 1991. The effects of the orchidectomy and gonadrotopins on steroidogenesis and oogenesis em Bidder´s organ of the toad *Bufo woodhousii*. Journal of Experimental Zoology 260:323–336. doi:10.1002/jez.1402600307.

Petrini S, Zaccanti F. 1998. The effects of aromatase and 5-α-reductase inhibitors, antiandrogen, and sex steroids on Bidder's organs development and gonadal differentiation in *Bufo bufo* tadpoles. The Journal of Experimental Zoology 280:245–259. doi:10.1002/(ISSN)1097-010X.

Pickford GW. 1953. Fish endocrinology. A study of the hypophysectomized male killifish, *Fundulus heteroclitus* (L.). Bulletin of the Bingan Oceanographic Collection 14:5–41.

Ponse K. 1927. Les hypotheses concernant la signification del' organe de Bidder du Crapaud. Ibrd 96:777–778.

R Development Core Team. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna. Available: http://www.r-project.org. Accessed Nov 2013 05.

Scaia MF, Regueira E, Sassone AG, Volonteri MC, Ceballos NR. 2011. The Bidder's organ of the toad *Rhinella arenarum* (Amphibia, Anura). Presence of steroidogenic enzymes.

Journal of Experimental Zoology 315:439–446. doi:10.1002/jez.691.

Scheer V, Dabawall MC. 1985. Functional organization of the amphibian oocyte nucleus. Developmental Biology 1:385–430.

Sokal RR, Rohlf FJ. 1995. Biometry: the principles and practice of statistics in biological research. 3rd. New York: W.H. Freeman.

Spengel JW. 1876. Das Urogenitalsystem der Amphibien. I. Teil. Der anatomische Bau des Urogenitalsystems. Arbeiten aus dem Zoologisch-Zootomischen Institut in Würzburg 3:51–114.

Tanimura A, Iwasawa H. 1986. Development of gonad and Bidder's organ in *Bufo japonicus formosus*: Histological observation. Science Reports of Niigata University Series D Biology 23:11–21.

Tanimura A, Iwasawa H. 1992. Ultrastructural observations of the ovary and Bidder's organ in young toad, *Bufo japonicus formosus*. Science Reports of Niigata University Series D Biology 29:27–33.

Terman A. 2001. Garbage catastrophe theory of aging: Imperfect removal of oxidative damage? Redox Report 6:15–26. doi:10.1179/135100001101535996.

Terman A, Brunk UT. 1998. Ceroid/lipofuscin formation in cultured human fibroblasts: The role of oxidative stress and lysosomal proteolysis. Mechanisms of Ageing and Development 104:277–291. doi:10.1016/S0047-6374(98)00073-6.

Venable JH, Coggeshall R. 1965. A simplified lead citrate stain for use in electron microscopy. The Journal of Cell Biology 25:407–408. doi:10.1083/jcb.25.2.407.

Vitale-Calpe R. 1969. The fine structure of the organ of bidder in the newly differenciate male of *Bufo arenarum*. Zeitschrift fiir Anatomic und Entwicklungsgeschichte 129:1–13. doi:10.1007/BF00521951.

Watson ML. 1958. Staining of tissue sections for electron microscopy with heavy metals. The Journal of Cell Biology 4:475–478. doi:10.1083/jcb.4.4.475.

Zaccanti F. 1994. Early sex differentiation of germ cells in the embryos of vertebrates. In: Dallai R, editor. Sex origin and evolution. Modena: Mucchi. pp. 167–181.

Zaccanti F, Di Grande F, Gardenghi G. 1971. Bidder's organ development and gonad differentiation in young post metamorphic *Bufo bufo* toads. Monitore Zoologico Italiano (N.S.) 5:11–23.

Zaccanti F, Gardenghi G. 1968. Osservazioni citometriche su ovociti ovarici e bidde-riani di *Bufo Bufo* (L.) durante il ciclo sessuale annuale. Bolletino di Zoologia 35:165–175. doi:10.1080/11250006809436898.

Zieri R, Taboga SR, Oliveira C. 2007. Melanocytes in the testes of *Eupemphix nattereri (*Anura, Leiuperidae): Histological, estereological and ultrastructural aspects. The Anatomical Record 290:795–800. doi:10.1002/ar.20553.

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Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae)

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ABSTRACT

Amphibians are very sensitive to environmental change and pollution because they have both aquatic and terrestrial life cycle stages and high skin permeability. Particularly during the larval stages, when these animals are restricted to small, transient ponds, exposure to high concentrations of pesticides is inevitable in agricultural areas. Given that pesticide application increases during the summer, which coincides with the reproductive season and the occurrence of most neotropical tadpoles in their natural environment, strong indications exist that tadpoles are developing in contaminated ponds. Fipronil is one of the primary insecticides used in sugarcane cultivation in Brazil, and little is known about its toxic effects on non-target organisms such as tadpoles. The purpose of this study was to evaluate the effects of fipronil and its metabolites on oxidative stress in *Eupemphix nattereri* tadpoles after exposure in water and sediment at concentrations of 35, 120 and 180 μg kg^{-1}. We assessed the activities of the antioxidant enzymes glutathione S-transferase (GST), glucose 6-phosphate dehydrogenase (G6PDH) and catalase (CAT) and lipid peroxidation (malondialdehyde, MDA). The results showed that fipronil has an inherent capacity to cause oxidative stress in tadpoles, as evidenced by a decrease in CAT activity and an increase in lipid peroxidation levels at all concentrations tested. Fipronil sulfone also produced elevated MDA levels at two of the tested concentrations and increased G6PDH activity in tadpoles exposed to the highest concentration of this metabolite but did not affect MDA levels. Our data showed that fipronil and its degradation products promoted oxidative stress in *Eupemphix nattereri* tadpoles exposed to environmentally relevant concentrations and could lead to a decrease in the long-term physiological performance of these animals, leading to detrimental effects at the population level.

1. Introduction

The intensification of agricultural activity in Brazil has caused extensive environmental impacts on many ecosystems, especially due to intensive pesticide use. Brazil is the largest consumer of pesticides in the world, with São Paulo being responsible for approximately 20% of the national consumption. In northwest São Paulo, the principal crop is sugarcane, and the cultivated area has increased in recent years (UNICA, 2015). Sugarcane cultivation requires a large amount of pesticides, especially the herbicides diuron, tebuthiuron and glyphosate and the insecticides carbofuran and fipronil (Bicalho et al., 2010; Peret et al., 2010). In Brazil and other tropical regions of the world, application of these pesticides is generally intensified during the rainy season, which coincides with the reproductive period of most amphibian species. Because many amphibians are distributed in areas common to agriculture practice, there is a great concern that many species are being affected by exposure to local pesticides.

The phenylpyrazole fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfonyl)pyrazole-3-carbonitrile) is among the most commonly used insecticides, which acts directly on the γ-aminobutyric acid (GABA) chloride channels in insects to disrupt neuronal signalling (Gunasekara and Troung, 2007). GABA antagonists such as fipronil are known to cause hyperactivity, convulsions and death in fish (Beggel et al., 2012). In addition, recent evidence suggests

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that fipronil also induces reactive oxygen species (ROS) production in cells, which can lead to increased lipid peroxidation and oxidative stress (Ki et al., 2012; Margarido et al., 2013; Möhler et al., 2004).

Under the aerobic conditions and sandy soils that characterize regions of sugarcane cultivation, fipronil has a half-life of 122–128 days. According to the Groundwater Ubiquity Score (GUS) index, fipronil and its degradation products have a low affinity for water, moderate mobility in soil and a sorption coefficient (Koc) of 803. Fipronil sulfone and fipronil sulfide also have low mobility and Koc values of 2511 and 3981, respectively. Fipronil has high affinity for natural organic matter present in water and sediments and is more susceptible to photodegradation than hydrolysis, except under alkaline conditions (Gunasekara and Troung, 2007; Gustafson, 1989).

In general, pesticides may be transported by drift, precipitation and runoff and may be found in different environmental matrices such as soil, surface water, and sediment, thus affecting various non-target organisms (Edwards, 1993; Hoerger et al., 2014; LeNoir et al., 1999; Moreira et al., 2010; Peret et al., 2010; Rand et al., 1995). Once in the environment, pesticides can be degraded, generating by-products that can be more or less toxic than the original compound (Cheyns et al., 2010; Gunasekara and Troung, 2007; Katagi, 2004; Muneer et al., 1999). Fipronil degradation gives rise to several products, especially fipronil sulfide and fipronil sulfone, which are generated by reduction and oxidation reactions, respectively. The concentrations of fipronil, fipronil sulfone and fipronil sulfide in the soil of São José do Rio Preto, São Paulo, Brazil were recently shown to range between 35 and 180 μg kg^{-1} (de Toffoli et al., 2015). These soils undergo flooding during the rainy season, forming temporary ponds that are used by anurans for reproduction. Due to their proximity to the cultivation areas, fipronil concentrations in these ponds can be higher than those commonly found in most permanent aquatic environments, such as rivers and lakes, thereby posing a substantial risk for local anuran species. However, to our knowledge, data regarding fipronil concentrations in small temporary ponds formed close to agriculture areas are not described in the scientific literature.

Amphibians are known to be more sensitive to pesticide contamination during the larval stage than as adults because tadpoles are strictly aquatic and have higher skin permeability (Yan et al., 2008). Once in water, fipronil rapidly partitions between the water column and the sediment (Maul et al., 2008; Tingle et al., 2003), posing a risk to benthic organisms such as *Eupemphix nattereri* tadpoles, which use the sediment and organic matter as a food source. However, studies on how fipronil affects tadpoles are still limited. Many studies have found that exposure of tadpoles to pesticides such as atrazine, glyphosate, quinclorac and fipronil in laboratory assays may alter the antioxidant response and may trigger oxidative stress (e.g., Dornelles and Oliveira, 2016; Margarido et al., 2013). Margarido and collaborators showed that fipronil impairs the antioxidant defense system in *Scinax fuscovarius* tadpoles, increasing their susceptibility to oxidative stress. However, no studies have investigated the effects of fipronil metabolites. Additionally, the study by Margarido et al. (2013) considered only fipronil dissolved directly in water and used a commercial formulation (Regent® 800WG). Due to the low water solubility of fipronil, it is possible that its association with aquatic sediment contributes more to its toxicity, especially towards benthic species that feed by foraging through the sediment. Therefore, the present study aimed to evaluate the effect of fipronil and its metabolites, fipronil sulfone and fipronil sulfide, at different environmental concentrations found in soils near sugarcane crops. To monitor oxidative stress, we measured malondialdehyde (MDA) levels to indicate lipid peroxidation and the activities of the antioxidant enzymes catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH) and glutathione S-transferase (GST, also a phase II biotransformation enzyme) in *Eupemphix nattereri* tadpoles, one of the most common benthic tadpole species found in the northwest region of São Paulo State, Brazil.

2. Material and methods

2.1. Test organisms

Spawn of the anuran *Eupemphix nattereri* (Leiuperidae) were collected from temporary ponds in the São José do Rio Preto region in northwest São Paulo State, Brazil (20° 47' 07.05"S, 49° 02' 42.09" W), during the rainy season (November–February). The larvae were kept in the laboratory under ideal temperature (28 °C), pH (7.5–8.0) and oxygen (~5 mg/L) conditions until they reached stages 29–33 (0.081 ± 0.018 g) just before the development of legs (Gosner, 1960). The animals were collected under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), and this work was approved by the Ethics Committee on Animal Use in Research of the São Paulo State University (CEUA-IBILCE/UNESP nº 086/2013).

2.2. Preparation of fipronil and its metabolites in the water and the sediment

Prior to exposure, the concentrations of fipronil and its metabolites in the water and the sediment were measured in aquariums in the absence of tadpoles to better understand the dispersion and availability of the compounds in the sediment and water. For this experiment, fipronil, fipronil sulfide and fipronil sulfone were tested at three concentrations: 35, 120 and 180 μg kg^{-1}. For the preparation of the experimental concentrations (35, 120 and 180 μg kg^{-1}), 7, 24 and 36 μg of each compound (fipronil, fipronil sulfide or fipronil sulfone), from stock solutions (72 mg L^{-1}**)** were diluted in acetone and then separately added to 200 g of soil. Water (1 L) was added to aquariums only after the acetone had evaporated. The soil was collected from non-agricultural areas of Sao Jose do Rio Preto, SP-Brazil, cleaned and washed twice with water and acetone, and used in the experiments only after the solvents had totally evaporated. No traces of fipronil or any of its metabolites were detected in the soil samples after the clean-up procedure. The concentrations of fipronil and the metabolites that were added to the soil were selected based on a recent study that found similar concentrations in soils close to agricultural areas in the region of São José do Rio Preto, Brazil (de Toffoli et al., 2015). The aquariums were agitated to disperse the compounds into the aqueous phase and placed on a horizontal surface to allow the soil to settle. Control aquariums with the solvent alone were also prepared using the same procedure for comparison. After 24 h, when the soil fraction had completely settled, water and soil were collected for chemical analysis. Chemical analysis of soil and water were also performed after 7 days after experimental exposure to the compounds in all aquariums containing tadpoles.

2.3. Chemical analysis

Fipronil and its metabolites were extracted from the water and sediment samples according to the method described by de Toffoli et al. (2015), which involves solid-phase extraction (SPE) on C18 cartridges (Supelco Analytical, Bellefonte, Pennsylvania, USA) for the water samples and liquid-liquid extraction (acetone/dichloromethane) for the sediment. To monitor the recovery rate, all water and sediment samples were spiked with the surrogate diazinon d-10 (40.0 μg L^{-1}). Deuterated d-10 phenanthrene (1.0 mg/L) was also used as an internal standard, before separation on C18 SPE cartridges. The cartridges were conditioned with 3.0 mL of a 3: 1 solution of hexane/isopropanol (Sigma-Aldrich, Germany) followed by 0.5 mL of methanol (Merck - SupraSolv®, Germany) and finally 1.0 mL of ultrapure water. With the aid of a manifold system (Agilent Technologies, USA), 400.0 mL of sample that had been pre-filtered through 0.7-μm glass fibre (Sartorius Stedim Biotech, Germany) was percolated through the cartridge and then extracted, and analytes were eluted with 3.0 mL of ethyl acetate

(Sigma-Aldrich, Mexico). The eluate was dried under commercial nitrogen in a drying block (SL - 1625 Solab, Brazil) and resuspended in a total volume of 1000 μL (800 μL of ethyl acetate and 200 μL of internal standard). The sediment samples were extracted via sonication. Dried sediment (20.0 g) and anhydrous sodium sulfate (10.0 g) (Sigma-Aldrich, Germany) were dissolved in 50.0 mL of 1: 1 acetone/dichloromethane (Macron Fine Chemicals, USA), and the mixture was sonicated for 15 min (USC-1600A, Unique, Brazil). The supernatant was transferred to vials for subsequent filtration through 0.7-μm glass fibre, dried in a gas drying block under commercial nitrogen and resuspended in a total volume of 1000 μL (800 μL of ethyl acetate and 200 μL of internal standard). Fipronil and its derivatives were then quantified on a gas chromatograph, model 7890A, coupled to mass spectrometer, model 5975C (Agilent Technologies, Santa Clara, California, USA) (GC-MS), using an HP-5MS column (5% phenyl - 95% polydimethylsiloxane, 30 m×0.25 mm×0.25 μm) from Agilent Technologies, USA, following laboratory QA/QC protocols.

2.4. Exposure of tadpoles to fipronil and its metabolites

E. nattereri tadpoles were separately exposed to 35, 120 and 180 μg kg^{-1} of the insecticide fipronil ($C_{12}H_4Cl_2F_6N_4OS$) or its metabolites fipronil sulfide ($C_{12}H_4Cl_2F_6N_4S$) and fipronil sulfone ($C_{12}H_4Cl_2F_6N_4O_2S$). For each treatment, amounts corresponding to the studied concentrations of the compounds (7, 24 and 36 μg) were added to aquariums containing 200 g of soil and 1.0 L of dechlorinated water, exactly as described in item 2.2. Tadpoles were exposed to each concentration at constant temperature of 26 °C for 7 days. Each experimental group consisted of four replicates containing eight tadpoles in each aquarium (n=4 replicates; 32 pseudo-replicates), totalling 384 tadpoles. Control groups also received the same volume of acetone to avoid ambiguous interpretation of the results due to possible solvent effects, and water was added to the aquariums only after total evaporation of the solvent. The aquariums were maintained under constant aeration, pH (8.0 ± 0.3) and photoperiod (12-h light-dark cycle) during the experiment. The water was not changed during the experiment, but excrement and food scraps were removed every two days, when the tadpoles were fed. The levels of ammonia and dissolved oxygen in the water were measured at the end of the experiment, and tadpoles were collected, immediately frozen in liquid nitrogen and subsequently stored at −80 °C.

2.5. Preparation of tadpole protein extracts for biochemical analyses

For enzymatic analysis, the tadpoles were individually and thoroughly homogenized (1:4, w-v) in Tris–HCl buffer, pH 7.5, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, China) as a protease inhibitor, and then centrifuged (9,000*g*) at 3.0 °C for 20 min. The supernatant was then centrifuged again at 50,000*g* and 3.0 °C for 60 min. The resulting supernatant was collected and used to measure GST, CAT and G6PDH activity.

Lipid peroxidation was assessed by measuring MDA levels. Animals (~100 mg) were homogenized at a 1:3 (w:v) ratio in 0.1 M Tris–HCl buffer, pH 8.0. After homogenization, 300 μL of thiobarbituric acid (TBA, Sigma-Aldrich, Germany) diluted in 0.4% HCl was added to the sample. The mixture was heated at 90 °C for 40 min, and the reaction products were extracted with 1.0 mL of *n*-butanol (Sigma-Aldrich, USA). The *n*-butanol-extracted fraction (50 μL) was injected into a High-Performance Liquid Chromatography (HPLC) system.

2.6. Biochemical analysis

GST activity was measured following the method of Keen et al. (1976), adapted for a microplate reader (Victor-X3, Perkin Elmer, Singapore). The reaction system contained 0.2 M phosphate buffer, pH 6.5, 1 mM 1-chloro-2,4-dinitrobenzene (CNDB, Sigma-Aldrich, Germany), and 1 mM reduced Glutathione (GSH, Sigma-Aldrich, Japan), and the sample was monitored for 1 min at 340 nm. G6PDH activity was also measured in a microplate reader following the method of Glock and McLean (1953) by measuring the reduction of NADP to NADPH at 340 nm, with glucose-6-phosphate as the substrate. CAT activity was measured using the spectrophotometric method described by Beutler (1975), which quantifies the rate of hydrogen peroxide decomposition by the enzyme by measuring the decrease in absorbance at 240 nm using a spectrophotometer (Thermo Evolution 300, Madison, WI USA). Protein concentrations were determined using the Bradford method (1976) adapted for a microplate reader at a wavelength of 595 nm. Bovine serum albumin served as the standard.

MDA levels were determined as described by Almeida (2003) using HPLC (ESA, Chelmsford, Massachusetts, USA) coupled to a UV–vis detector (526, ESA, Chelmsford, Massachusetts, USA) to analyse the product formed by the reaction of MDA and TBA. A Shimadzu C18 column (150×4.6 mm, 5 μm) was used for the analysis, with a mobile phase of 50 mM potassium phosphate (pH 7.0) in 40% methanol at a flow rate of 1.0 mL/min.

2.7. Statistical analysis

Statistical analyses were performed using R software version 2.11.1 (stats package) (R Development Core Team, 2010). Differences in the activities of GST, G6PDH and CAT and MDA levels were evaluated in all groups after exposure to fipronil and its metabolites. Outliers were assessed, and the normality and homogeneity of data were verified by the Shapiro–Wilk and Levene's test, respectively. For parametric data, significant differences were assessed using one-way ANOVA followed by Tukey's post hoc test, and nonparametric data were evaluated using the Kruskal–Wallis test followed by an additional multiple comparison test of the means. P values < 0.05 were considered statistically significant.

3. Results

3.1. Analysis of water and sediment

After 24 h (aquariums without tadpoles), the lower concentration of fipronil (35 μg kg^{-1}) was found in a greater proportion in the water than in the soil. The same occurred for all concentrations of the metabolites of fipronil sulfone and fipronil sulfide. Concentrations of fipronil and its metabolites in sediment and water after 24 h are shown in Fig. 1. After 7 days of exposure, we found that fipronil, fipronil sulfone and fipronil sulfide were all preferentially deposited in the sediment in all aquariums containing tadpoles. For fipronil, fipronil sulfone and fipronil sulfide, after 7 days, the percentages of analyte adsorbed in the sediment ranged from 58% to 77%, 72% to 85%, and 95% to 98%, respectively, of the amount initially added to the aquariums.

3.2. Biochemical analysis

All of the tadpoles survived in all of the treatments, indicating that at the concentrations studied, fipronil and its metabolites are not lethal to tadpoles after 7 days of exposure. GST activity was unchanged in tadpoles exposed to fipronil and its metabolites (Fig. 2A). Compared with the control group, G6PDH activity was higher only in tadpoles exposed to the highest fipronil sulfide concentration (P=0.02), but no significant changes were observed for the other compounds (Fig. 2B). However, tadpoles exposed to fipronil sulfone at 35 μg kg^{-1} had higher G6PDH activity than those exposed to fipronil at the same concentration. CAT activity was unaltered in tadpoles exposed to fipronil and its metabolites at the lower concentrations (P=0.36). On the other hand, decreased CAT activity was detected in tadpoles exposed to 120 μg kg^{-1} of fipronil (P=0.01), and increased CAT activity was observed at the

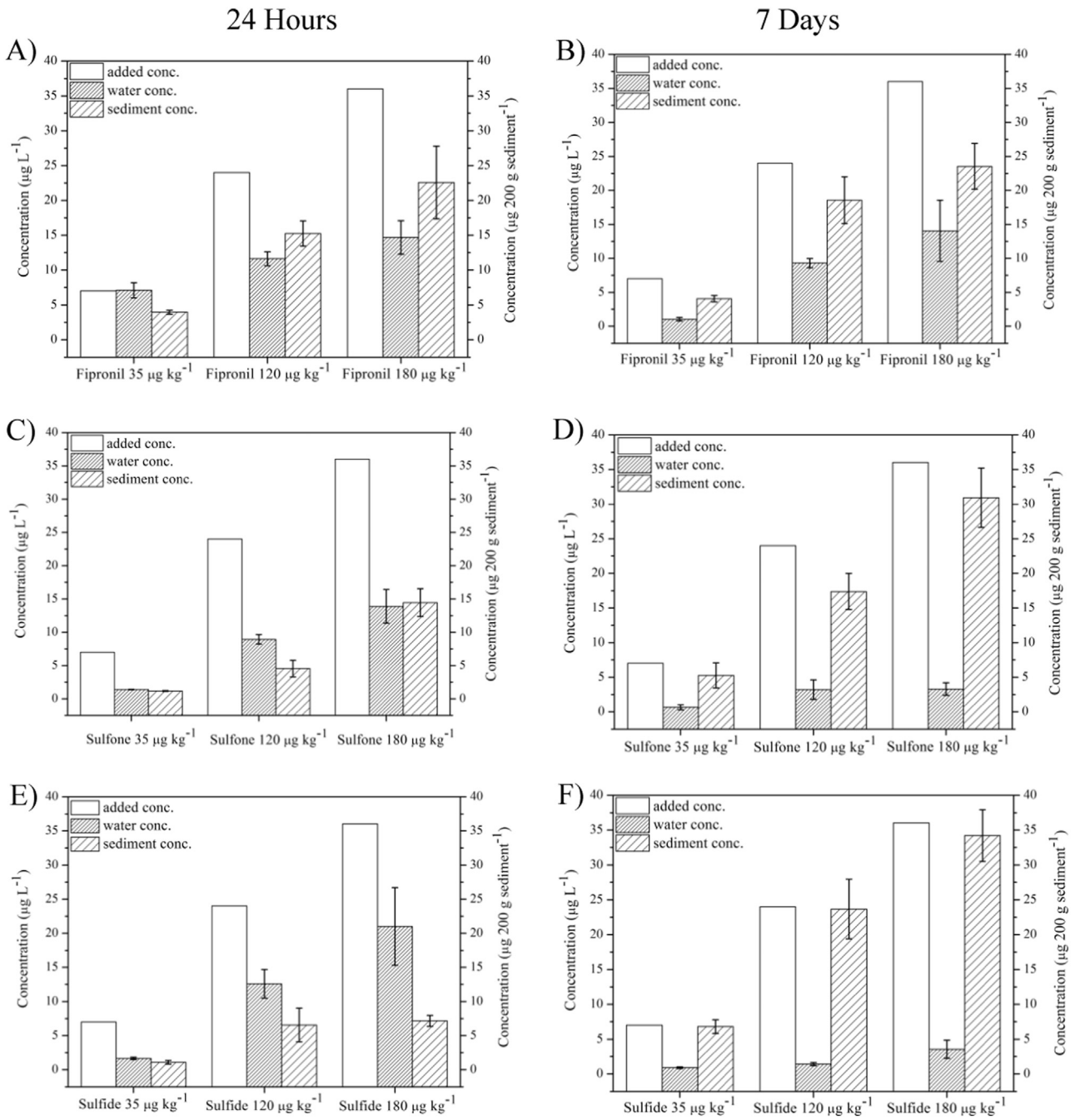

Fig. 1. Levels added to aquariums and concentrations found in the water (μg L^{-1}) and sediment (μg 200 g $sediment^{-1}$) after exposure for 24 h (aquariums without tadpoles) and 7 days to three concentrations – 35.0, 120.0 and 180 μg kg^{-1} (aquariums containing tadpoles). A) Fipronil, 24 h; B) Fipronil, 7 days; C) Sulfone, 24 h; D) Sulfone, 7 days; E) Sulfide, 24 h; F) Sulfide, 7 days.

highest concentration of fipronil sulfide (P=0.02) (Fig. 2C).

Lipid peroxidation was increased in tadpoles exposed to fipronil at all concentrations tested ($P < 0.001$) compared to the control group (Fig. 3). For tadpoles exposed to fipronil sulfone, MDA levels increased upon exposure to 35.0 μg kg^{-1} ($P < 0.001$) and 180.0 μg kg^{-1} (P=0.03) but to a lesser extent than in the fipronil group. Tadpoles exposed to fipronil sulfide showed no alterations in MDA levels at any of the concentrations studied.

4. Discussion

Although most pesticides have selective toxicity against specific organisms, some can also be harmful to non-target organisms, depending on the concentration and route of exposure (Woo et al., 2009). Pesticides applied to soils for agricultural practices can exert toxic effects on tadpoles during the rainy seasons because the intense rain can form temporary ponds that can be used by anurans for reproduction and spawning. Fipronil has a relatively low solubility in water, although adjuvant compounds present in commercial formulations can increase its water solubility. However, due to its low solubility in water, fipronil is expected to be associated with the sediment in water environments, and its effects could be more pronounced for benthic organisms that inhabit and feed within sediments, such as *E. nattereri* tadpoles. The chemical analysis of water and sediment conducted in this study after 24 h showed that although the compounds present moderate lipophilicity, the adsorption of these compounds in the sediment occurs slowly because after 24 h, the compounds were preferentially present in water, with the exception of

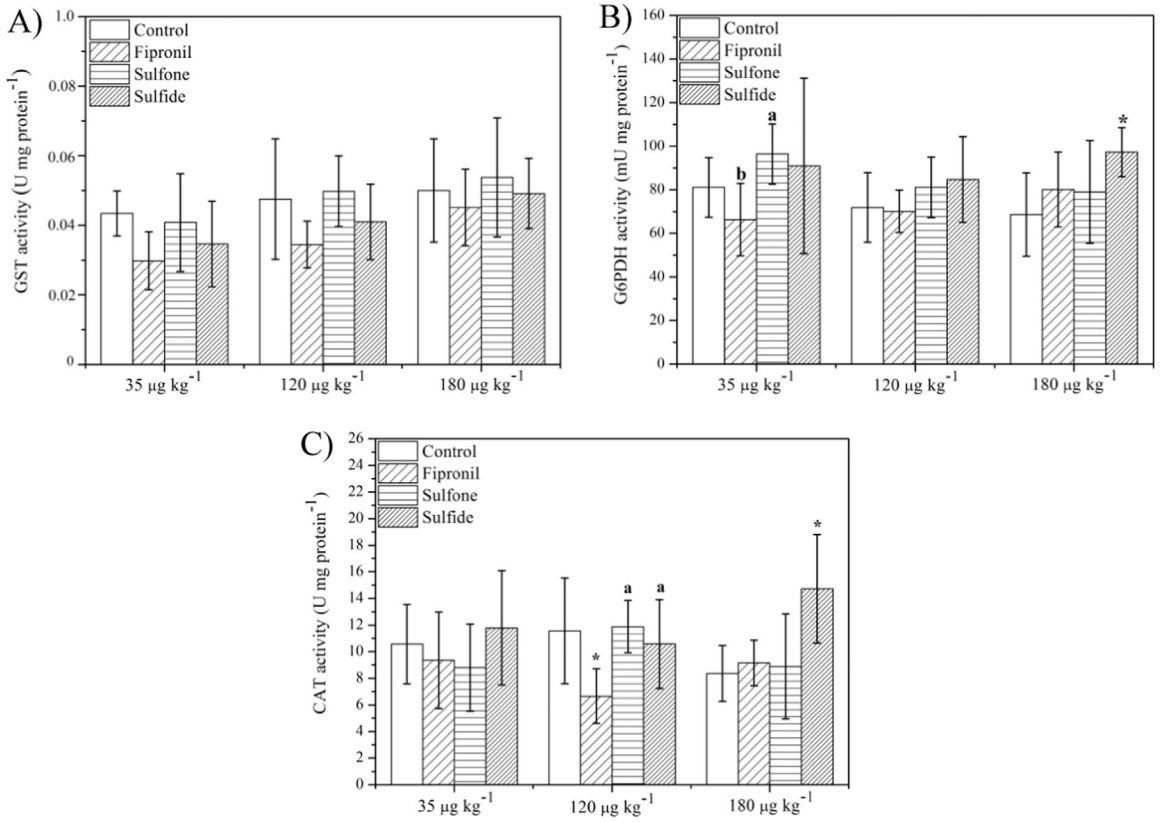

Fig. 2. Enzymatic activity in the control, fipronil, sulfone and sulfide groups at the indicated concentrations. *, significant difference ($P < 0.05$) compared to the corresponding control group; a, significant difference ($P < 0.05$) compared to the fipronil group with the same concentration; b, significant difference ($P < 0.05$) compared to the sulfone group with the same concentration. A) GST activity; B) G6PDH activity; C) CAT activity.

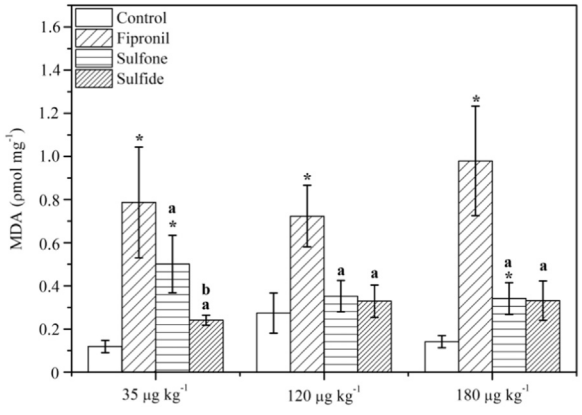

Fig. 3. MDA concentrations (pmol mg^{-1}) in the control, fipronil, sulfone and sulfide groups at the indicated concentrations. *, significant difference ($P < 0.05$) compared to the corresponding control group; a, significant difference ($P < 0.05$) compared to the fipronil group with the same concentration; b, significant difference ($P < 0.05$) compared to the sulfone group with the same concentration.

fipronil at the lower concentration (35 μg kg^{-1}). Water and sediment analyses performed after 7 days showed that all compounds were predominantly found in the sediment and were more available for benthic organisms, confirming the high lipophilicity of these compounds.

The results presented in this study showed that although fipronil and its metabolites altered the activity of some antioxidant enzymes, these alterations were variable, and the enzymes did not exhibit dose-dependent changes in response to fipronil and its metabolites in *E. nattereri* tadpoles. Nevertheless, these alterations in antioxidant enzymes suggest a modulation in response to increased ROS generation due to pesticide exposure. Increased levels of MDA caused by exposure to fipronil and fipronil sulfone also indicate an oxidative stress condition resulting from exposure of the tadpoles to these contaminants. It has been shown that stress conditions eventually cause an increase in ROS production in aquatic organisms, suggesting that changes in antioxidant enzyme activities are associated with the adaptation of these organisms to environmental changes (Lushchak, 2011; Tomanek, 2014).

CAT and G6PDH activity increased in organisms exposed to the higher concentration of the sulfide metabolite, which could be related to the lack of increased MDA levels in this group. CAT activity was also increased in animals exposed to the intermediate concentration (120 μg kg^{-1}) of fipronil sulfide. However, a decrease in CAT activity was observed in tadpoles exposed to 120 μg kg^{-1} of fipronil, indicating a possible inhibitory effect of fipronil on this enzyme and a consequent increase in the susceptibility of the tadpoles to oxidative stress, which could be related to the high lipid peroxidation level in this group. Previous studies have shown that CAT activity may be inhibited in animals exposed to different pesticides (Margarido et al., 2013; Peltzer et al., 2013; Sun et al., 2014), including the insecticide fipronil, which inhibited CAT activity in the fish *Cyprinus carpio* after exposure to 0.65 mg L^{-1} after 7, 30 and 40 days (Clasen et al., 2012). In contrast, *Scinax fuscovarious* tadpoles exposed to 5, 20 and 100 μg L^{-1} of a commercial formulation of fipronil (Regent 800WG) exhibited no alterations in CAT activity after 5, 10 and 14 days of exposure (Margarido et al., 2013). These data indicate that CAT may be altered by fipronil in different ways in different organisms, and also that fipronil metabolites are able to alter the activity of this enzyme, causing the animals to be more vulnerable to deleterious effects caused ROS.

The enzyme G6PDH was increased only in tadpoles exposed to the metabolite sulfide at higher concentration. Exposure to fipronil did not alter G6PDH activity at any concentrations. G6PDH is an important cytosolic enzyme that participates of the first step of the pentose

phosphate pathway. It is an important source of NADPH and an important co-factor for antioxidant enzymes and numerous other biosynthetic reactions (Kletzien et al., 1994). A previous study with *S. fuscovarious* tadpoles also showed that fipronil did not alter the activity of G6PDH (Margarido et al., 2013). These results suggest that the metabolite fipronil sulfide may act by changing the G6PDH activity of tadpoles by a different pathway from that of the original compound, fipronil. Additionally, the increase in G6PDH observed in our study could also be a response to the increased demand of NADPH in the cells in an attempt to reduce the oxidant effects caused by the contaminant.

GST activity was unchanged by contaminants in this study. GSTs are a family of multifunctional dimeric enzymes involved in phase II reactions of biotransformation of several xenobiotics and also protect animals against oxidative damage because some isoforms have peroxidase activity. They are also responsible for the intracellular transport of endogenous and exogenous chemicals (Blanchette et al., 2007; Eaton and Bammler, 1999; Frova, 2006; Goto et al., 2009; Sheehan et al., 2001). The lack of variation in GST activity in tadpoles contrasts with previous findings by Margarido et al. (2013), in which a significant decrease in GST activity was observed in *S. fuscovarius* tadpoles exposed to the commercial formulation of fipronil (Regent®800WG). This discrepancy could be due to the commercial formulation of fipronil, which contains unknown adjuvant compounds that likely trigger different responses in tadpoles that are not triggered by the active ingredient, fipronil. In addition, the presence of adjuvants in the commercial formulation (Regent®800WG) could have increased the water solubility of fipronil and thus increased its bioavailability. Nevertheless, the lack of alteration of GST activity could also indicate that fipronil and its metabolites are not metabolized by this enzyme, and therefore, GST is not affected by these contaminants. In fact, Roques et al. (2012) demonstrated that rats treated with fipronil and fipronil sulfone presented an increase in the expression of CYP3A1, CYP2B2, glucuronosyltransferase and sulfotransferase transcripts, suggesting the involvement of these enzymes in fipronil and fipronil sulfone metabolism.

The lack of evident antioxidant responses in tadpoles exposed to fipronil and the sulfone metabolite may explain the higher susceptibility of these organisms to oxidative stress, as evidenced by increased MDA levels. All groups treated with fipronil exhibited increased levels of MDA at the concentrations studied. In contrast, fipronil sulfone and sulfide did not exacerbate oxidative stress at high concentrations, though the sulfone metabolite had an effect at the lowest concentration tested. In combination with the lack of response of the antioxidant enzymes in animals exposed to fipronil, the prominent increase in MDA levels induced by fipronil suggests that tadpoles exposed to the original compound may be more susceptible to lipid membrane damage than those exposed to the metabolites. Lipid peroxidation is considered a major negative effect of oxidative stress as a consequence of excessive ROS generation in cells. Cells employ various mechanisms to counteract oxidative stress and repair damaged macromolecules, and the primary defense is provided by enzymatic antioxidants (Ojha et al., 2011). Several studies have shown that MDA levels can be increased in aquatic organisms exposed to different pesticides such as organochlorines, organophosphates and carbamates, which makes MDA an important biomarker for different conditions of environmental stress (e.g., Slaninova et al., 2009; Uchendu et al., 2012). MDA levels have also been used in bioassays of amphibians to assess deleterious effects triggered by pesticide exposure. For example, *Lithobates catesbeianus* tadpoles exposed to atrazine, glyphosate and quinclorac exhibited an increase in MDA levels (Dornelles and Oliveira, 2014). Increased MDA levels in tadpoles exposed to fipronil in our study suggest that this compound increases ROS generation in the animal and that larval antioxidant defense systems are not sufficiently robust to protect against ROS increases after 7 days of fipronil exposure, leading these organisms to suffer the deleterious effects caused by lipid peroxidation.

Because amphibians are among the most sensitive vertebrates that can be used in ecotoxicity tests and are also the main group threatened by the overuse of agriculture pesticides, knowledge of the toxicity and physiological response to many pesticides evaluated by different bioassays is always valuable. In addition, the use of biomarkers such as antioxidant enzymes and lipid peroxidation is an important tool to assess the sensitivity of a species in its environment because the toxicity of and response to different compounds are species-specific. *E. nattereri* is an endemic Brazilian species, and our data have shown the potential of the insecticide fipronil to cause oxidative stress during the larval stage. These results imply that recent increases in the cultivation area for sugarcane can promote changes in the normal defense system used by these animals to survive in contaminated areas, potentially leading to a reduction in their adaptive response and to subsequent population decline.

In summary, our study showed how exposure to fipronil and its metabolites at environmentally relevant concentrations can impair the antioxidant system and establish oxidative stress in *E. nattereri* tadpoles. The variations of antioxidant enzymes may alter physiological performance during the larval development of amphibians in environments contaminated by fipronil because these enzymes are crucial pathways that protect the organisms against possible increases in ROS production and avoid oxidative stress. We also observed that exposure to the original fipronil compound increased MDA levels, suggesting that fipronil has an increased capacity to induce oxidative stress in *E. nattereri* tadpoles. Because the effects of fipronil metabolites were less pronounced at the concentrations studied, it is possible that fipronil degradation in the environment can decrease its toxicity. Nevertheless, additional studies are necessary to better address the toxicity of fipronil in comparison to its degradation metabolites, paying particular attention to the response of other key endpoints of tadpole development and health, such as hormonal changes, malformation, mortality and genotoxicity. These data are of great relevance for studies that monitor the effects of pesticides, such as the insecticide fipronil, on amphibian populations, especially neotropical species, which have very limited data in the literature.

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References

Almeida, E.A., 2003. Avaliação de Variações Bioquímicas em Moluscos bivalves em Resposta ao Estresse Ambiente (Ph.D. thesis). Universidade de São Paulo – Instituto de Química, São Paulo, Brasil.

Beggel, S., Werner, I., Connon, R.E., Geist, J.P., 2012. Impacts of the phenylpyrazole insecticide fipronil on larval fish: time-series gene transcription responses in fathead minnow (*Pimephales promelas*) following short-term exposure. Sci. Total Environ. 426, 160–165. http://dx.doi.org/10.1016/j.scitotenv.2012.04.005.

Beutler, E., 1975. Red Cell Metabolism: A Manual of Biochemical Methods. Grune & Straton, New York.

Bicalho, S.T.T., Langenbach, T., Rodrigues, R.R., Correia, F.V., Hagler, A.N., Matallo, M.B., Luchini, L.C., 2010. Herbicide distribution in soils of a riparian forest and neighboring sugar cane field. Geoderma 158, 392–397. http://dx.doi.org/10.1016/j.geoderma.2010.06.008.

Blanchette, B., Feng, X., Singh, B.R., 2007. Marine glutathione S-transferases. Mar. Biotechnol. 9, 513–542. http://dx.doi.org/10.1007/s10126-007-9034-0.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

Cheyns, K., Mertens, J., Diels, J., Smolders, E., Springael, D., 2010. Monod kinetics rather than a first-order degradation model explains atrazine fate in soil mini-columns: implications for pesticide fate modelling. Environ. Pollut. 158, 1405–1411. http://dx.doi.org/10.1016/j.envpol.2009.12.041.

Clasen, B., Loro, V.L., Cattaneo, R., Moraes, B., Lópes, T., de Avila, L.A., Zanella, R., Reimche, G.B., Baldisserotto, B., 2012. Effects of the commercial formulation

containing fipronil on the non-target organism Cyprinus carpio: implications for rice-fish cultivation. Ecotoxicol. Environ. Saf. 77, 45–51. http://dx.doi.org/10.1016/j.ecoenv.2011.10.001.

de Toffoli, A.L., Da Mata, K., Bisinoti, M.C., Moreira, A.B., 2015. Development, validation, and application of a method for the GC-MS analysis of fipronil and three of its degradation products in samples of water, soil, and sediment. J. Environ. Sci. Health B 50, 753–759. http://dx.doi.org/10.1080/03601234.2015.1058091.

Dornelles, M.F., Oliveira, G.T., 2014. Effect of atrazine, Glyphosate and Quinclorac on biochemical parameters, lipid peroxidation and survival in bullfrog tadpoles (*Lithobates catesbeianus*). Arch. Environ. Contam. Toxicol. 66, 415–429. http://dx.doi.org/10.1007/s00244-013-9967-4.

Dornelles, M.F., Oliveira, G.T., 2016. Toxicity of atrazine, glyphosate, and quinclorac in bullfrog tadpoles exposed to concentrations below legal limits. Environ. Sci. Pollut. Res. Int. 23, 1610–1620. http://dx.doi.org/10.1007/s11356-015-5388-4.

Eaton, D., Bammler, T.K., 1999. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol. Sci. 49, 156–164. http://dx.doi.org/10.1093/toxsci/49.2.156.

Edwards, C.A., 1993. The impact of pesticides on the environment. In: Pimentel, D., Leman, H. (Eds.), The Pesticide Question: Environment, Economic and Ethics. Springer Verlag Science, New York, 13–46.

Frova, C., 2006. Glutathione transferases in the genomics era: new insights and perspectives. Biomol. Eng. 23, 149–169. http://dx.doi.org/10.1016/j.bioeng.2006.05.020.

Glock, G.E., McLean, P., 1953. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J. 55, 400–408. http://dx.doi.org/10.1042/bj0550400.

Gosner, K.L., 1960. A simplified table for staging anuran ambryos and larvae with notes on identification. Herpetologica 16, 183–190.

Goto, S., Kawakatsu, M., Izumi, S., Urata, Y., Kageyama, K., Ihara, Y., Koji, T., Kondo, T., 2009. Glutathione S-transferase pi localizes in mitochondria and protects against oxidative stress. Free Radic. Biol. Med. 46, 1392–1403. http://dx.doi.org/10.1016/j.freeradbiomed.2009.02.025.

Gunasekara, A., Troung, T., 2007, Environmental Fate of Fipronil. Report. Environmental Monitoring Branch, Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, CA..

Gustafson, D.I., 1989. Groundwater ubiquity score: a simple method for assessing pesticide leachability. Environ. Toxicol. Chem. 8, 339–357. http://dx.doi.org/10.1002/etc.5620080411.

Hoerger, C.C., Akhtman, Y., Martelletti, L., Rutler, R., Bonvin, F., Grange, A., Arey, J.S., Kohn, T., 2014. Spatial extent and ecotoxicological risk assessment of a micropollutant-contaminated wastewater plume in Lake Geneva. Aquat. Sci. 76, 7–19. http://dx.doi.org/10.1007/s00027-013-0315-6.

Katagi, T., 2004. Photodegradation of pesticides on plant and soil surfaces. Rev. Environ. Contam. Toxicol. 182, 1–195.

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for the several activities of the glutathione S-transferases. J. Biol. Chem. 251, 6183–6188.

Ki, Y.W., Lee, J.E., Park, J.H., Shin, I.C., Koh, H.C., 2012. Reactive oxygen species and mitogen-activated protein kinase induce apoptotic death of SH-SY5Y cells in response to fipronil. Toxicol. Lett. 211, 18–28. http://dx.doi.org/10.1016/j.toxlet.2012.02.022.

Kletzien, R.F., Harris, P.K., Foellmi, L.A., 1994. Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J. 8, 174–181.

LeNoir, J.S., McConnell, L.L., Fellers, G.M., Cahill, T.M., Seiber, J.N., 1999. Summertime transport of current-use pesticides from California's central valley to the Sierra Nevada Mountain range, USA. Environ. Toxicol. Chem. 18, 2715–2722. http://dx.doi.org/10.1002/etc.5620181210.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. http://dx.doi.org/10.1016/j.aquatox.2010.10.006.

Margarido, T.C., Felício, A.A., de Cerqueira Rossa-Feres, D., de Almeida, E.A., 2013. Biochemical biomarkers in Scinax fuscovarius tadpoles exposed to a commercial formulation of the pesticide fipronil. Mar. Environ. Res. 91, 61–67. http://dx.doi.org/10.1016/j.marenvres.2013.02.001.

Maul, J.D., Brennan, A.A., Harwood, A.D., Lydy, M.J., 2008. Effect of sediment-associated pyrethroids, fipronil, and metabolites on Chironomus tentans growth rate, body mass, condition index, immobilization, and survival. Environ. Toxicol. Chem. 27, 2582–2590. http://dx.doi.org/10.1897/08-185.1.

Möhler, H., Fritschy, J.M., Crestani, F., Hensch, T., Rudolph, U., 2004. Specific GABA(A) circuits in brain development and therapy. Biochem. Pharmacol. 68, 1685–1690. http://dx.doi.org/10.1016/j.bcp.2004.07.025.

Moreira, S.M., Moreira-Santos, M., Rendón-von Osten, J., da Silva, E.M., Ribeiro, R., Guilhermino, L., Soares, A.M.V.M., 2010. Ecotoxicological tools for the tropics: sublethal assays with fish to evaluate edge-of-field pesticide runoff toxicity. Ecotoxicol. Environ. Saf. 73, 893–899. http://dx.doi.org/10.1016/j.ecoenv.2010.04.007.

Muneer, M., Theurich, J., Bahnemann, D., 1999. Formation of toxic intermediates upon the photocatalytic degradation of the pesticide diuron. Res. Chem. Intermed. 25, 667–683. http://dx.doi.org/10.1163/156856799×00220.

Ojha, A., Yaduvanshi, S.K., Srivastava, N., 2011. Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues. Pestic. Biochem. Physiol. 99, 148–156. http://dx.doi.org/10.1016/j.pestbp.2010.11.011.

Peltzer, P.M., Lajmanovich, R.C., Attademo, A.M., Junges, C.M., Cabagna-Zenklusen, M.C., Repetti, M.R., Sigrist, M.E., Beldoménico, H., 2013. Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (*Trachycephalus typhonius*) tadpoles. Ecotoxicol. Environ. Saf. 98, 142–151. http://dx.doi.org/10.1016/j.ecoenv.2013.09.010.

Peret, A.M., Oliveira, L.F., Bianchini, I., Seleghim, M.H., Peret, A.C., Mozeto, A.A., 2010. Dynamics of fipronil in oleo lagoon in Jataí Ecological Station, São Paulo-Brazil. Chemosphere 78, 1225–1229. http://dx.doi.org/10.1016/j.chemosphere.2009.12.060.

Rand, G.M., Wells, P.G., McCarty, L.S., 1995. Introduction to aquatic toxicology. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment. Taylor & Francis, Boca Raton, FL.

R Development Core Team, 2010. R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. Available at: ⟨www.r-project.org⟩.

Roques, B.B., Lacroix, M.Z., Puel, S., Gayrard, V., Picard-Hagen, N., Jouanin, I., Perdu, E., Martin, P.G., Viguié, C., 2012. CYP450-dependent biotransformation of the insecticide fipronil into fipronil sulfone can mediate fipronil-induced thyroid disruption in rats. Toxicol. Sci. 127, 29–41. http://dx.doi.org/10.1093/toxsci/kfs094.

Sheehan, D., Meade, G., Foley, V.M., Dowd, C.A., 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem. J. 360, 1–16. http://dx.doi.org/10.1042/bj3600001.

Slaninova, A., Smutna, M., Modra, H., Svobodova, Z., 2009. A review: oxidative stress in fish induced by pesticides. Neuro Endocrinol. Lett. 30 (Suppl. 1), S2–S12.

Sun, L., Jin, R., Peng, Z., Zhou, Q., Qian, H., Fu, Z., 2014. Effects of trilostane and fipronil on the reproductive axis in an early life stage of the Japanese medaka (*Oryzias latipes*). Ecotoxicology 23, 1044–1054. http://dx.doi.org/10.1007/s10646-014-1248-0.

Tingle, C.C., Rother, J.A., Dewhurst, C.F., Lauer, S., King, W.J., 2003. Fipronil: environmental fate, ecotoxicology, and human health concerns. Rev. Environ. Contam. Toxicol. 176, 1–66. http://dx.doi.org/10.1007/978-1-4899-7283-5_1.

Tomanek, L., 2014. Proteomics to study adaptations in marine organisms to environmental stress. J. Proteom. 105, 92–106. http://dx.doi.org/10.1016/j.jprot.2014.04.009.

Uchendu, C., Ambali, S.F., Ayo, J.O., 2012. The organophosphate, chlorpyrifos, oxidative stress and the role of some antioxidants: a review. Afr. J. Agric. Res. 7, 2720–2728. http://dx.doi.org/10.5897/ajar11.2510.

UNICA, 2015. União da industria da Cana-de-açucar (2012). Unicadata. ⟨http://www.unicadata.com.br⟩.

Woo, S., Yum, S., Kim, D., Park, H., 2009. Transcripts level responses in a marine medaka (*Oryzias javanicus*) exposed to organophosphorus pesticide. Comp. Biochem. Physiol. 149, 427–432. http://dx.doi.org/10.1016/j.cbpc.2008.10.100.

Yan, D., Jiang, X., Xu, S., Wang, L., Bian, Y., Yu, G., 2008. Quantitative structure-toxicity relationship study of lethal concentration to tadpole (Bufo vulgaris formosus) for organophosphorous pesticides. Chemosphere 71, 1809–1815. http://dx.doi.org/10.1016/j.chemosphere.2008.02.033.

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Time- and Oil-Dependent Transcriptomic and Physiological Responses to *Deepwater Horizon* Oil in Mahi-Mahi (*Coryphaena hippurus*) Embryos and Larvae

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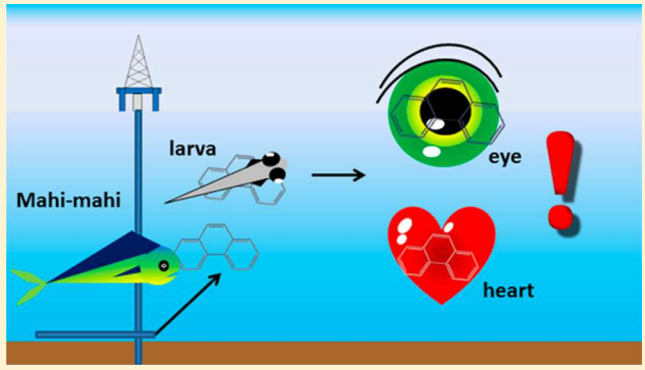
Supporting Information

ABSTRACT: The Deepwater Horizon (DWH) oil spill contaminated the spawning habitats for numerous commercially and ecologically important fishes. Exposure to the water accommodated fraction (WAF) of oil from the spill has been shown to cause cardiac toxicity during early developmental stages across fishes. To better understand the molecular events and explore new pathways responsible for toxicity, RNA sequencing was performed in conjunction with physiological and morphological assessments to analyze the time-course (24, 48, and 96 h post fertilization (hpf)) of transcriptional and developmental responses in embryos/larvae of mahi-mahi exposed to WAF of weathered (slick) and source DWH oils. Slick oil exposure induced more pronounced changes in gene expression over time than source oil exposure. Predominant transcriptomic responses included alteration of EIF2 signaling, steroid biosynthesis, ribosome biogenesis and activation of the cytochrome P450 pathway. At 96 hpf, slick oil exposure resulted in significant perturbations in eye development and peripheral nervous system, suggesting novel targets in addition to the heart may be involved in the developmental toxicity of *DHW* oil. Comparisons of changes of cardiac genes with phenotypic responses were consistent with reduced heart rate and increased pericardial edema in larvae exposed to slick oil but not source oil.



INTRODUCTION

The blow-out of the *Deepwater Horizon* (*DWH*) oil drilling platform initiated the largest oil release at depth in U.S. history, culminating in approximately three million barrels of crude oil released into the northern Gulf of Mexico over several months in the spring and summer of 2010.[1] The timing and location of oil release into the ecosystem from *DWH* coincided with the temporal spawning window for many economically and ecologically important pelagic fish species, such as mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacares*).[2,3] Crude oil-derived polycyclic aromatic hydrocarbons (PAHs) have been shown to adversely impact early life stage fish.[4,5] The composition and structure of individual PAHs in the water column can be significantly altered by natural weathering processes, and it has been shown that weathered surface slick oil is more toxic than source oil on a ∑PAH basis.[6] Weathering typically removes low molecular weight hydrocarbons from oil−water mixtures through evaporation, subsequently producing oil slicks with proportionally higher molecular weight and low solubility PAHs.[7]

The developing fish heart is a sensitive target organ for the toxic effects of crude oil-derived PAHs, particularly those containing three rings such as the phenathrenes, flourenes, and dibenzothiophenes.[5,8] The phenotypes of cardiotoxicity range from bradycardia, arrhythmias, contractility defects, atrium-to-ventricle conduction blockade, and eventually to heart failure.[9,10] In addition, oil exposed fish showed subtle changes

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in heart shape and reduction in swimming performance, indicative of reduced cardiac output.[4,11,12] The primary etiology of defects induced by PAHs has direct effects on cardiac conduction, which have secondary consequences for late stages of kidney development, neural tube structure, and formation of the craniofacial skeleton (eye and jaw).[13,14] Although the mutagenic and adverse whole-organismal effects of crude oil on fish development have been well recognized, the molecular initiating events are less understood. A recent study of select hypothesis-driven genes focusing on the developmental cardiotoxicity of crude oil exposure in mahi-mahi revealed a number of molecular indicators of cardiac stress and injury.[15] High throughput sequencing (HTS) allows unbiased quantification of expression levels of transcripts with a high sensitivity and broad genome coverage, and offers the potential to identify additional molecular indicators that are reflective of the PAH cardiotoxicity phenotype established by traditional microscopic analyses and may reveal other important, yet less overtly apparent, phenotypes and modes of action of oil exposure.

To help identify molecular mechanisms and pathways potentially involved in the developmental toxicity for fish exposed to *DWH* oil, transcriptomic profiles in mahi-mahi embryos exposed to different *DWH* oils (source and artificially weathered oil) were evaluated at different critical windows of development using HTS. Based on differentially expressed transcripts, the most impacted biological processes and pathways were identified at different developmental stages with multiple bioinformatic tools, providing novel insights into the mechanisms of *DWH* oil-induced developmental toxicities. The chemical composition and cardiotoxicity of different *DWH* oil types was also measured with the intent of anchoring the linkages between molecular, functional and morphometric end points during embryonic development.

MATERIALS AND METHODS

Animals. Mahi-mahi broodstock were captured off the coast of Miami, FL using hook and line angling techniques and subsequently transferred to the University of Miami Experimental Hatchery (UMEH) where they were acclimated in 80 m^3 fiberglass maturation tanks (typically 5−7 per tank). Tanks were equipped with recirculating aquaculture systems for water quality and temperature control. All embryos used in experiments were collected using standard UMEH methods.[16] A prophylactic formalin treatment (37 ppm formaldehyde solution for 1 h) was administered to the embryos, followed by a 0.5 h rinse with a minimum of 300% water volume using filtered, UV-sterilized seawater. Fertilization rate and embryo quality was assessed microscopically from a small sample of eggs collected from each spawn. Spawns with low fertilization rate (<85%) or frequent morphological abnormalities (>5%) were not used.

Preparation of Water Accommodated Fractions. Two sources of crude oil from the *DWH* spill that varied with respect to the state of weathering were obtained from British Petroleum under chain of custody for testing purposes: (1) slick oil collected from surface skimming operations (sample ID: OFS-20100719-Juniper-001 A0091G) and (2) oil from the Massachusetts barge (sample ID: SO-20100815-Mass-001 A0075K) which received oil collected from the subsea containment system positioned directly over the well (referred to herein as slick and source oil, respectively). Both types of oil were prepared as high energy water accommodated fractions (HEWAFs) on the day of use by mixing 1 g of oil per liter of 1 μm filtered, UV-sterilized seawater at low speed for 30 s in a Waring CB15 blender (Torrington, CT).[9] The mixture was immediately transferred to a glass separatory funnel, allowed to settle for 1 h and the lower ∼90% drained. The 100% HEWAF (unfiltered) was subsequently diluted in 1 μm filtered, UV-sterilized seawater to obtain test exposures.

Toxicity Testing. Three time course test solutions were subsequently performed using the nominal LC25s determined from the initial bioassays described in Supporting Information (SI) for the purposes of (1) RNA-Seq, (2) phenotypic anchoring of transcriptional responses by imaging analysis of heart rate and pericardial and yolk sac edema and (3) qPCR. The LC25 concentration was chosen as a compromise between attempting to capture initiating events as well as cascade effects while ensuring that a sufficient signal was observed. The first two exposures for RNA-Seq and imaging analysis were performed as described above with the following exceptions: three replicates were used per time point (24, 48, and 96 hpf) with 30 and 25 embryos/larvae per replicate, respectively. Animals hatch between 35 and 40 h. Slick and source oil HEWAF exposures were run concurrently using the same batch of embryos and a shared set of controls for both sets of experiments. The third time course exposure for qPCR was performed using only slick oil HEWAF in the same manner as the previous two exposures with five replicates and 25 embryos per replicate.

Image Analysis. Embryos or larvae were collected from each replicate beaker and imaged at 24, 48, and 96 h for assessment of heart rate and pericardial edema. Embryos or larvae were imaged using either a Fire-i400 or Fire-i530c digital camera (Unibrain, San Ramon, CA) mounted on a Nikon SMZ800 stereomicroscope. Images and videos were collected on a MacBook laptop using iMovie software and calibrated using a stage micrometer.

Water Chemistry Analysis. Samples for PAH analysis were collected in 250 mL amber bottles, stored at 4 °C and shipped overnight on ice to ALS Environmental (Kelso, WA) for analysis by gas chromatography/mass spectrometry−selective ion monitoring (GC/MS-SIM; based on EPA method 8270D). Initial (0 h) PAH samples were collected from bulk dilutions and final (24, 48, or 96 h) samples were composites of approximately equal volumes collected from each of the four corresponding replicates. Only initial samples were collected for control treatments. Reported ∑PAH values represent the sum of 50 select PAH analytes. Temperature, pH, dissolved oxygen (DO) and salinity were also measured (see SI). A summary of all measured water quality parameters and ∑PAH concentrations are provided in SI Tables S1−S3.

RNA Isolation, cDNA Library Construction and Sequencing. Three biological replicates were collected per treatment group. The surviving embryos or larvae from each replicate were pooled and homogenized with a Kontes Pellet Pestle Cordless Motor (Sigma-Aldrich, St. Louis, Missouri) in RNAzol (Molecular Research Center, Cincinnati, Ohio). RNA was isolated and purified with RNeasy Mini Kit (Qiagen, Valencia, CA). The total RNA sample was quantified by NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA degradation and contamination were assessed on 1% agarose gels. RNA integrity was further verified using RNA 6000 Nano Assay chips run in Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). 200 ng of total RNA was used to prepare RNA-Seq libraries using the TruSeq RNA Sample Prep kit following the protocol described

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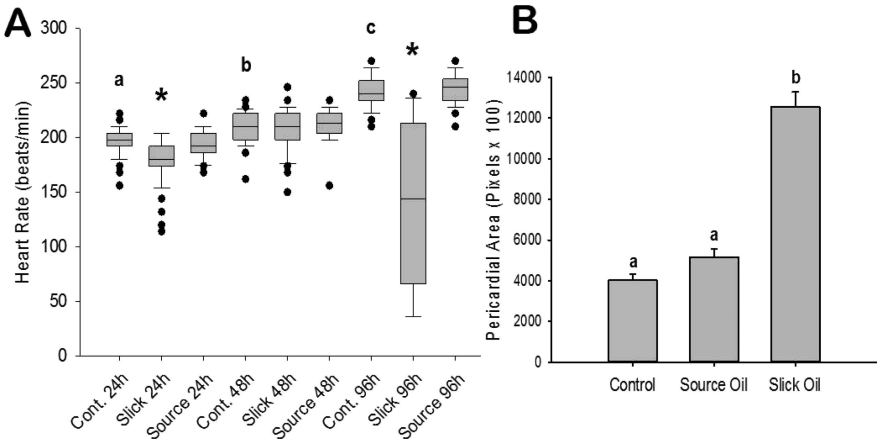

Figure 1. Time course of heart rate assessment in embryonic (24 h) and larval (48–96 h) mahi-mahi exposed to slick or source oil HEWAF (12 and 4.6 μg L^{-1} $\sum$PAHs, respectively) (A). Sample sizes are as follows (from left to right): 60, 57, 60, 42, 53, 48, 47, 25, and 46. Data are presented as box plots indicating the 25th and 75th percentiles; whiskers indicate the 90th and 10th percentiles; filled circles indicate outliers; solid and dashed lines indicate the median and mean, respectively. *Significantly different from time-matched control and source oil treatments by Kruskal–Wallis One Way Analysis of Variance on Ranks followed by Dunn's pairwise multiple comparison procedure. Letters indicate significant differences using the aforementioned statistical method. Pericardial area measured in yolk sac larvae at 48 h exposed to source and slick oil HEWAF (12 and 4.6 μg L^{-1} $\sum$PAHs, respectively) (B). Sample sizes are as follows (from left to right): 42, 52, and 47. Data are presented as mean ± SEM *Different letters* indicate significant differences in pericardial area between control and oil exposed larvae by Kruskal–Wallis One Way Analysis of Variance on Ranks followed by Dunn's pairwise multiple comparison procedure.

by the manufacturer (Illumina, San Diego, CA). Libraries were quantitated with NanoDrop, and four libraries were indexed and sequenced on one lane of an Illumina flow cell (TRUSEQ SBS V3). Single Read 1 × 50 sequencing was performed on an Illumina HiSeq 2500 at the Center for Genomics Medicine, Medical University of South Carolina, Charleston, SC, with each individual sample sequenced to a minimum depth of ~50 million reads (see SI Table S4 for details on sequencing QC and read depths).

Bioinformatic Analysis. Data were subjected to Illumina quality control (QC) procedures (>80% of the data yielded a Phred score of 30). Secondary analysis was carried out on an OnRamp Bioinformatics Genomics Research Platform (OnRamp Bioinformatics, San Diego, CA). OnRamp's advanced Genomics Analysis Engine utilized an automated RNaseq workflow to process the data, including (1) data validation and quality control, (2) read alignment to the *Takifugu rubripes* transcriptome (FUGU4) using BLASTX (against protein sequences): Basic Local Alignment Search Tool,[17] (3) generation of gene-level count data, and (4) differential expression analysis with DEseq2[18] (Genomics Research Platform with RNaseq workflow v1.0.1, including FastQValidator v0.1.1a, Fastqc v0.11.3, DESeq2:1.8.0). The resulting SAM files were sorted and run through the Python package HTSeq to generate count data for gene-level differential expression analyses. Transcript count data from DESeq2 analysis of the samples were sorted according to their adjusted *p*-value or *q*-value, which is the smallest false discovery rate (FDR) at which a transcript is called significant. FDR is the expected fraction of false positive tests among significant tests and was calculated using the Benjamini-Hochberg multiple testing adjustment procedure. The protein FASTA sequences from Ensembl for Fugu were compared using Ensembl's homology to create protein Fasta files that contained a human Entrez gene ID that mapped via *Fugu* to *Mahi-mahi*.

Statistical analysis of pathways and gene ontology (GO) terms was carried out using this sorted transcript list as described by us previously[19] and using Ingenuity Pathway Analysis (IPA, Qiagen, Valencia, CA), Advaita Pathway Guide,[20] and the ToppGene Suite.[21] The rationale behind using IPA, Advaita, and ToppGene is that the degree of annotation available for human is considerably greater than for fish species, and this permits a more sensitive systems level interrogation. Our methodology follows the premise that transcripts are sorted according to their q-value. As we have recently demonstrated releasing the FDR to 0.4 provides a larger gene list for downstream systems analyses. It should be noted that the GO and Pathway performed was itself subjected to FDR testing thereby adding statistical rigor to findings.[40,41]

Primer Design and Quantitative Reverse Transcription Real-Time PCR (qPCR). For targeted genes, National Center for Biotechnology Information (NCBI) nucleotide database was mined for available Perciform sequences (e.g., *Coryphaena hippurus, Notothenia coriiceps, Stegastes partitus, Oreochromis niloticus, Sciaenops ocellatus, Larimichthys crocea, Dicentrarchus labrax, Sparus aurata*, etc.) and available sequences were aligned with DNAMAN 8.0 (http://www.lynnon.com/). Identified regions of conservation were then targeted for qPCR primer design with PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index). Designed primers were target verified (≥80% sequence identity) by NCBI Primer-BLAST. Melt curve analysis and 1% agarose gel electrophoresis were performed to assess the specificity of the qPCR products. SI Table S5 shows the primers used in SYBR quantitative RT-PCR analysis. Differentially expressed genes identified by the above-described method were validated by qPCR using Power SYBR Green QPCR Master Mix (Life Technologies, Carlsbad, CA) on the iCycler-MyIQ (Bio-Rad, Hercules, CA) (see SI).

RESULTS

Chemical Composition of WAF. The PAH profiles exhibited by the slick oil and source oil HEWAFs differed markedly, reflecting the different degrees of weathering among the two oil types (see SI Figure S1, Table S6). The source oil was comprised predominantly of 2-ring PAHs (72%) followed

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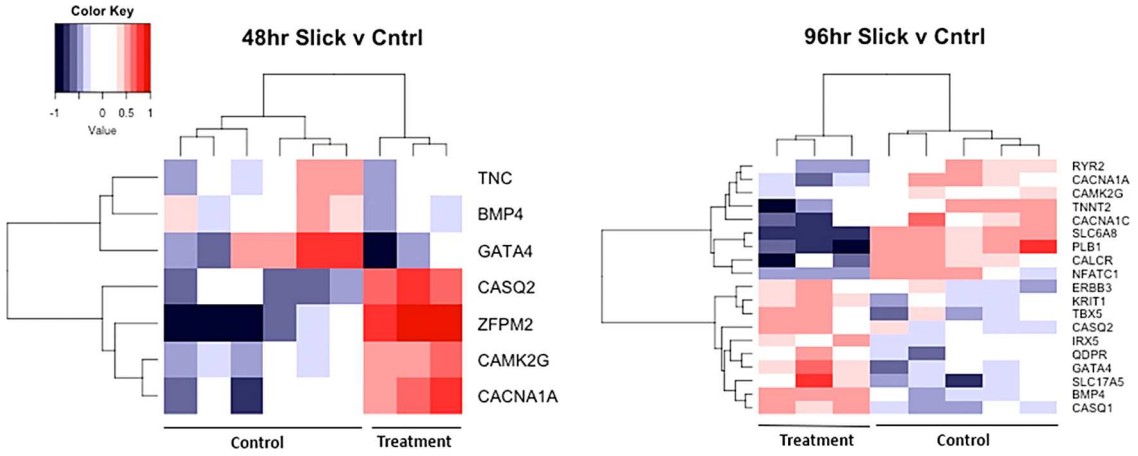

Figure 2. Heat map of gene expression changes in in 48 hpf (left) and 96 hpf (right) embryos in response to slick exposure. Red and blue boxes colors depict relative over- and under-expression in slick oil treated with respect to control. Samples and genes are clustered by similarity. The samples from each treatment group cluster together indicating gene expression differences for the represented transcripts between the two treatments.

by 3-ring PAHs (27%), represented largely by the napthalenes and phenanthrenes/anthracenes, respectively. By contrast, the slick oil was more weathered as evident by the loss of 2-ring PAHs (5%) and enrichment of 3-ring (69%) and 4-ring (25%) PAHs. Both profiles are similar to those obtained from HEWAF preparations using other sources of slick oil and source oil from the *DWH* spill.[6,11,15]

Toxicity Tests. Dose response regressions yielded 96 h % HEWAF LC25 values of 2.12 (0.80−3.09) and 0.09 (0.001−0.24) for the slick and source oils, respectively (values in parentheses indicate upper and lower 95% confidence intervals). Estimated 96 h ∑PAH LC50 values were calculated in two ways: using only the initial concentrations and the geometric mean of the initial and final concentrations. The estimated LC50s (in $\mu g\ L^{-1}$ ∑PAH) using both approaches were 19.5 (10.4−27.6) and 23.5 (12.9−31.25) for the slick oil and 16.5 (1.17−37.5) and 7.33 (0.72−17.5) for the source oil, respectively. The dose response curves for both 96 h bioassays using geometric mean ∑PAH concentrations are shown in SI Figure S2. Time courses of mean percent survival for the RNA-Seq, imaging and qPCR exposures are provided in SI Figure S3.

Physiological Measurements. The slick oil treatment significantly decreased heart rate at 24 and 96 h compared to both time-matched controls and source oil exposed fish (Figure 1A), and the effect was much more pronounced at 96 h. In addition, there was greater variability in heart rates observed among the slick oil treatment at all three time points with the greatest variability again at 96 h. It should be noted that many of the larvae in the 96 h slick oil treatment also exhibited highly variable heart rates (i.e., time interval between heartbeats). Finally, in control treatments heart rate was found to increase with age during this life stage. The slick oil treatment significantly increased pericardial area (see example in SI Figure S4) in larvae at 48 h compared to both controls and source oil exposed fish (Figure 1B).

Quality of Gene Expression Data. The unexposed control samples clustered separately from the slick or source oil treated sample, indicating global transcriptomic differences between the two sets (SI Figure S5).

Transcriptional Responses at 24 hpf after Slick and Source Oil Exposure. In 24 hpf embryos exposed to slick oil, 39 genes were significantly differentially expressed at a False Discovery Rate (FDR) < 0.1, and 95 genes were significantly differentially expressed at an FDR < 0.4 (SI Figure S6A). As for source oil exposure, 90 genes were significantly differentially expressed at FDR < 0.1, and 228 genes were significantly differentially expressed at FDR < 0.4. The significantly enriched Gene Ontology (GO) terms (molecular function, biological process) and Pathways were similar between slick and source oil exposure as assessed by ToppGene (SI Table S7). For the ontology of molecular function, 8 and 39 GO terms were significantly enriched after slick and source oil exposure, respectively. Structural constituents of ribosome and RNA binding were the predominant transcripts altered by oil exposure. Biological processes and pathways changed by oil exposure included transcripts involved in ribosome structure, translation termination, protein targeting to the endoplasmic reticulum, translational elongation, and cytoplasmic ribosomal proteins. IPA predicted an overall decreased activity in the Eukaryotic initiation factor 2 (EIF2) signaling pathway to cell viability in 24 hpf embryos by both slick and source oil exposure (SI Figure S7 A, B).

Transcriptional Responses at 48 hpf after Slick and Source Oil Exposure. Compared to 24 hpf, more genes were significantly altered at 48 hpf after slick and source oil exposure. The enriched GO terms were similar between 24 hpf and 48 hpf after source oil exposure. The most representative GO terms by source oil exposure included RNA binding, and were similar between time points with ribosome structure, translation termination, and protein targeting to the endoplasmic reticulum altered. However, significant differences between source and slick oil exposure started to occur at 48 hpf. In 48 hpf embryos after slick oil exposure, 196 genes were significantly differentially expressed at a FDR < 0.1, and 516 genes were significantly differentially expressed at a FDR < 0.4 (SI Figure S6B). ToppFun analysis using the ToppGene analysis suite revealed development and differentiation as the highest ranked biological functions, including cell development, tissue development, circulatory system development, cardiovascular system development, organ morphogenesis, neurogenesis, and cell morphogenesis (SI Table S8). Several genes (e.g., *casq2*, *cacna1a*) that were significantly differentially expressed after slick oil exposure are involved in cardiac muscle and Ca^{2+} homeostasis (Figure 2). The cardiovascular system

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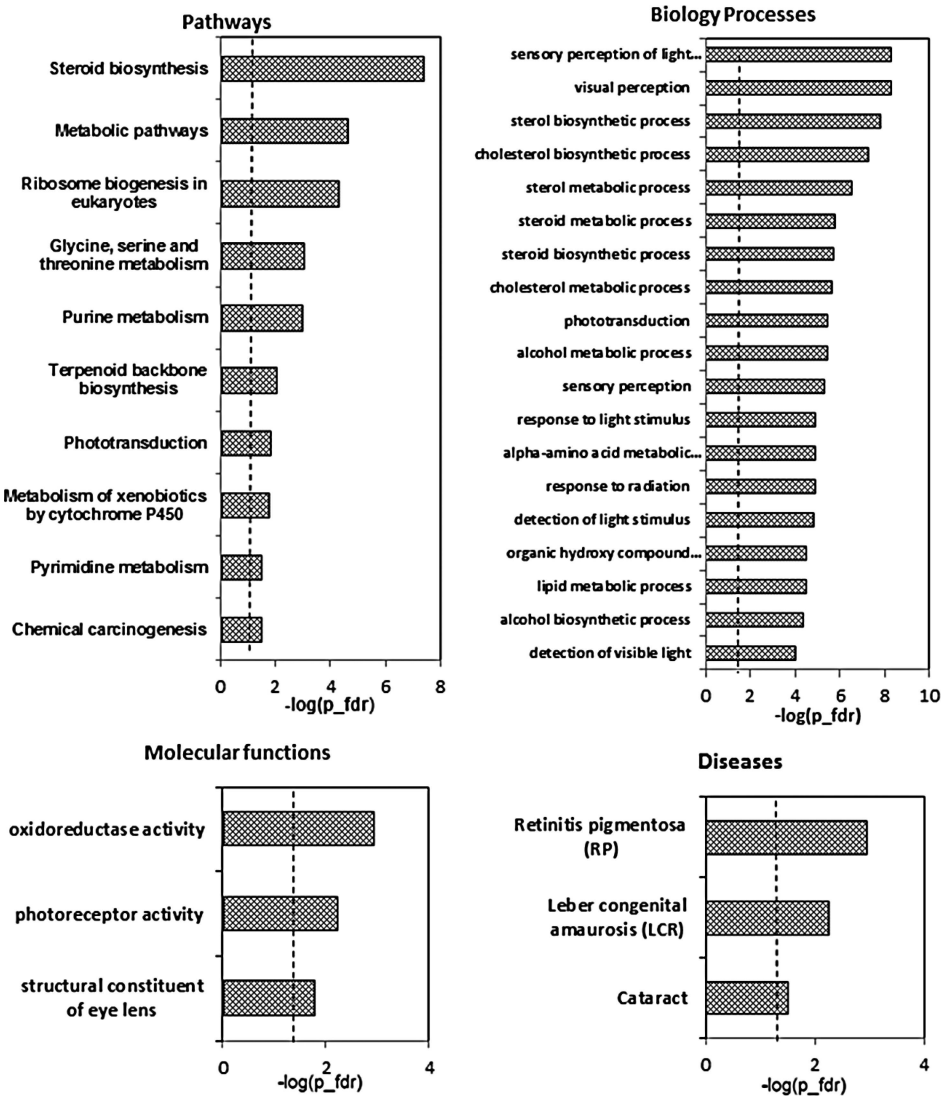

Figure 3. Predicted biological pathways and biological processes using the Advaita Pathway Guide. Mahi-mahi embryos were exposed to slick oil for 96 hpf. The *X*-axis is a negative log of the adjusted *p*-value.

phenotype was the first ranked enriched phenotype in 48 hpf by slick oil (SI Table S9). The significantly enriched canonical pathways included extracellular matrix organization, collagen biosynthesis, focal adhesion, metabolism, integrin signaling pathway, PI3K-Akt signaling pathway, pathways in cancer and metabolism of amino groups, and cardiomyopathy. The representative pathways are shown in SI Table S8. At the molecular level, the most represented GO terms were "binding", including sulfur compound binding, fibronectin binding, heparin binding, glycosaminoglycan binding, growth factor binding, cofactor binding, and collagen binding (SI Table S8). Enzyme binding (72 genes), transition metal ion binding (69 genes) and receptor binding (65 genes) were the top three molecular functions enriched in terms of the number of genes involved.

Transcriptional Responses at 96 hpf after Slick and Source Oil Exposure. The number of significantly differentially expressed genes (FDR < 0.1) greatly increased from 196 at 48 hpf to 1479 genes at 96 hpf after slick oil exposure (SI Figure S6). As for source oil exposure, 128 genes were significantly differentially expressed at FDR < 0.1, and 297 genes were significantly differentially expressed at FDR < 0.4. As with embryos at 24 and 48 hpf, *cyp1a1* was consistently the most strongly upregulated gene (26.5 fold) at 96 hpf after slick oil exposure. Similar to 48 hpf, the most significantly enriched GO molecular functions indicated by ToppGene in 96 hpf after source oil exposure were structural constituents of ribosomes, RNA binding, structural molecule activity, and oxidoreductase activity (SI Table S10). RNA binding was also the most significantly enriched molecular function in 96 hpf after slick oil exposure (>300 genes form input). According to biological function, there were some overlapping GO terms between slick and source oil exposures, such as cellular component disassembly, organic acid metabolic processes, and oxoacid metabolic processes, with many more genes altered after slick oil exposure than source oil exposure (SI Table S10). Extracellular matrix organization was the top enriched pathway in 96 hpf after source oil exposure. Metabolism and biosynthesis was the top enriched pathways identified by ToppFun in 96 hpf after slick oil exposure, including metabolism of amino acids and steroid biosynthesis (specifically cholesterol biosynthetic pathways). Comparing ToppFun and Advaita Pathway Guide analyses, both approaches were consistent with regard to the enriched GO pathways uncovered,

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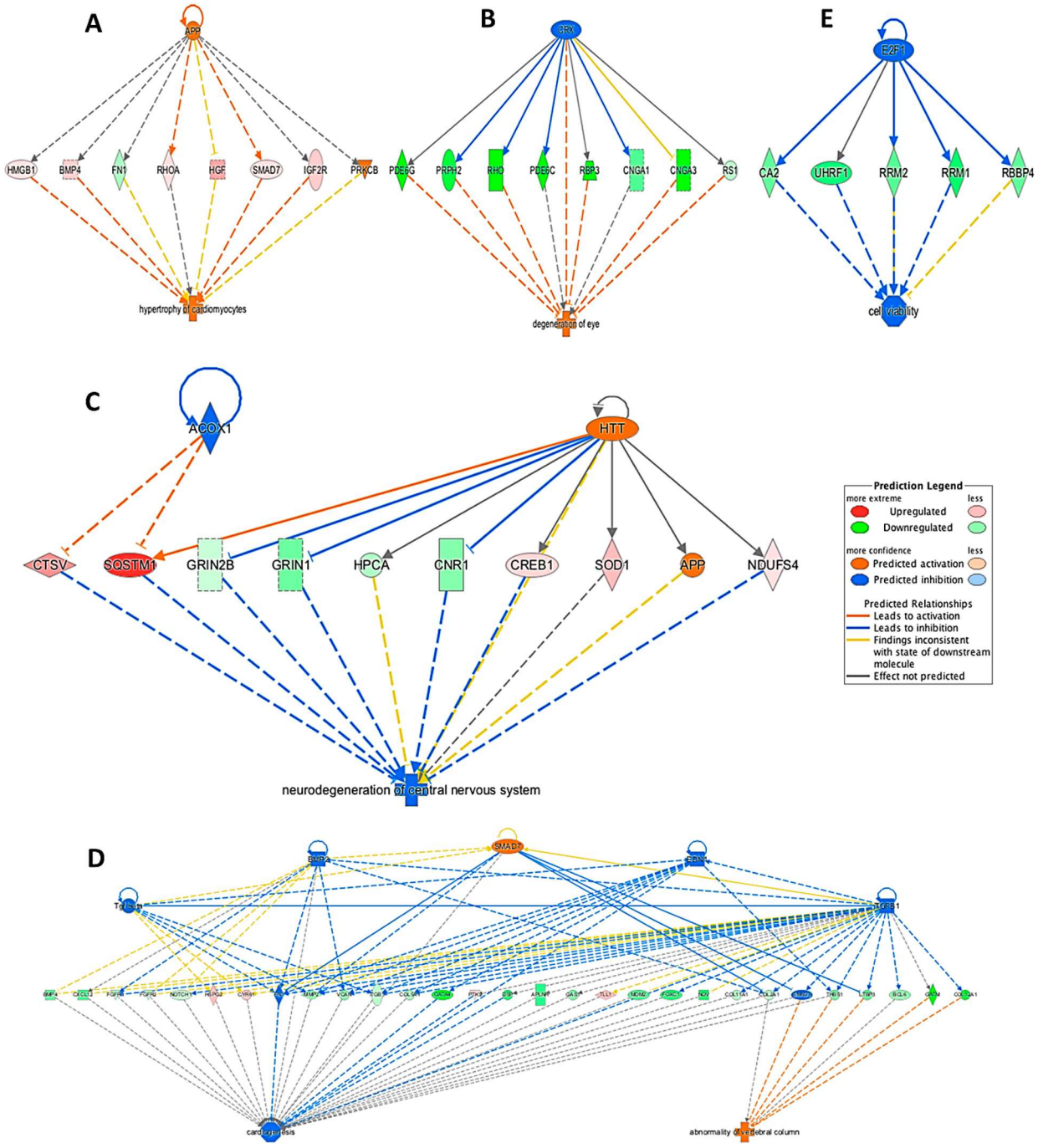

Figure 4. Predicted mechanisms through Ingenuity Pathway Analysis showing how WAF may lead to (A) hypertrophy of cardiomyocytes; (B) degeneration of the eyes; (C) neurodegeneration of central nervous system; (D) cardiogenesis and abnormal vertebral column; (E) decreased cell viability.

with steroid biosynthesis and metabolic pathways being the highest ranked pathways (Figure 3). Advaita also predicted that other biological processes, and molecular functions were altered, including visual perception, sterol biosynthetic processes, lipid metabolic processes, oxidoreductase activity, photoreceptor activity, as well as the disease pathways, retinitis pigmentosa, leber congenital amaurosis and cataract. Notably, 96 hpf after slick oil exposure, the genes involved in steroid biosynthesis were all significantly upregulated (SI Figure S8), while the genes involved in ribosome biosynthesis were all downregulated (SI Figure S9). At 96 hpf after slick oil exposure, ToppFun also identified abnormal cardiovascular system morphology as one of the phenotypes in 96 hpf after slick oil exposure (SI Table S11). Several genes differentially expressed after slick oil exposure were involved in cardiac muscle and Ca^{2+} homeostasis (Figure 2). IPA analysis further predicted the activation of hypertrophy of cardiomyocytes, and inhibition of cardiogenesis by slick oil exposure (Rank #5 Tox Functions predicted by IPA, Figure 4A). In comparison with slick oil, the transcriptomic profile in mahi-mahi embryos and larvae after source exposure were not significantly reflective of effects on cardiac muscle or Ca^{2+} homeostasis. In contrast to 24 hpf, IPA predicted an overall increased activity in EIF2 pathway at 96 hpf by both source and slick oil exposure (SI Figure S7 C, D).

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Given the significant induction of *cyp1a1* in all time points receiving slick oil, IPA was also used to focus on aryl hydrocarbon receptor (AhR) pathways. Notably, 127 AhR-responsive genes were differentially expressed at 96 hpf larvae exposed to slick oil. The interaction between slick oil exposure and AhR largely upregulated the expression of *cyp1a1*, *cyp1b1*, *ugt1a1*, and *ahrr* at 96 hpf.

Validation of Differentially Expressed Genes by qRT-PCR. A subset of genes that were significantly up/down regulated in 96 hpf mahi-mahi larvae after oil exposure by RNaseq were quantified by qRT-PCR. Based on the HTS data, genes involved in the most significantly enriched GO terms or targeted organs by slick oil were selected (SI Figure S11). The genes include *rho* and *rgr* involved in eye development and phototransduction pathways (first ranked Biological Process in Figure 3; *p*-value = 4.8×10^{-9}); *sqle* in steroid biosynthesis (first ranked Pathway in Figure 3; p-value = 4.0×10^{-8}); *cyp1a1 and its upstream ahr, ahrr* in oxidoreductase activity (first ranked Molecular Function in Figure 3; *p*-value = 0.001); and *bmp4*, *gata4*, *cacna1a* involved in abnormal cardiovascular system morphology (Rank #16 Phenotype in SI Table S11; *p*-value $=7.5\times 10^{-4}$). Directions of change by RNaseq and qPCR were consistent with similar magnitudes of fold-change (SI Figure S11).

DISCUSSION

Many studies of fish embryo/larvae have indicated that PAHs associated with oil have caused cardiac phenotypes presenting pericardial edema.[4−10,13,14] While the Aryl hydrocarbon receptor (AhR) plays a role in the cardiotoxic phenotype particularly with tricyclic PAHs, the molecular connections between AhR activation and cardiotoxicity of oil is still unclear, particularly considering similar toxicities from other PAHs and oxygenated derivatives that are poor AhR ligands.[26] More recent studies have evaluated calcium and potassium homeostasis/function and indicate other molecular mechanisms may also be involved in the cardiac phenotype associated with oil toxicity in embryonic/larval stages in fish.[15,24,25] In the current study with mahi-mahi, several genes not associated with AhR were differentially expressed at 48 and 96 hpf after slick oil exposure and are involved in cardiac muscle and Ca^{2+} homeostasis (Figure 2). Proteins coded by *casq1* and *casq2*, calsequestrins, play important roles in maintaining the cellular Ca^{2+} levels and contraction of cardiac muscle. Cardiac ryanodine receptor 2 (*ryr2*) was significantly downregulated 96 hpf after slick oil exposure, also suggesting potential changes in cytosolic Ca^{2+} levels. Significant downregulation of troponin T type 2 (*tnnt2*) which regulates cardiac muscle contraction in response to alterations in intracellular calcium ion concentration[22] was observed in 96 hpf mahi-mahi larvae after slick oil exposure. Changes in this gene have been associated with cardiomyopathies in humans and chicken embryos.[23] *Camk2g* and *cacna1a* were significantly downregulated at 48 hpf but upregulated at 96 hpf (Figure 2). The product of camk2g belongs to the $Ca^{(2+)}$/calmodulin-dependent protein kinase subfamily, which is crucial for plasticity at glutamatergic synapses. Brette et al.[24] showed decreased calcium current (I_{Ca}) and calcium cycling, which disrupted excitation-contraction coupling in cardiomyocytes of bluefin tuna after slick oil exposure. We also found both L type and P/Q type voltage-dependent calcium channel genes were significantly downregulated in 96 hpf after slick oil exposure. It is possible that the decreased calcium channel expression and I_{Ca} levels may affect the cardiotoxicity of slick oil in mahi-mahi larvae. Besides cardiac and Ca^{2+} related pathways, some other organs and pathways were also identified as potential targets for slick oil.

To phenotypically link the affected cardiac associated genes to the higher order cardiac syndrome characteristic of crude oil exposure in fish, embryos and larvae were examined microscopically for a functional effect on heart rate (i.e., bradycardia) as well as pericardial and yolk sac edema. Slick oil exposure caused mild bradycardia at 24 hpf and 96 hpf, but not at 48 hpf, although pericardial edema was clearly increased at this time point. These findings are consistent with previous studies indicating that pericardial edema is the primary response and the most robust indicator of cardiac injury in mahi-mahi.[6,11,15] By contrast, source oil exposure did not induce bradycardia at any time point or cause cardiac edema, consistent with the lack of induced cardiac transcriptional responses to source oil exposure. It should be noted that although ∑PAH concentrations and composition were consistent across experiments for the respective oil types, notably higher mortality than the expected 25% was observed at 96 h for the slick oil imaging and qPCR experiments (57% and 50%, respectively; SI Figure S3). Still, responses at the molecular level were remarkably consistent when comparing genes analyzed by RNA-seq and qPCR (SI Figure S11) supporting a direct link between effects observed among the different experiments. Differences in PAH concentration/composition and acute mortality hinder direct comparisons among the slick and source oil exposures; nevertheless, the lack of functional or transcriptional cardiac effects by the latter likely reflects the lower ∑PAH concentrations and far lower relative abundance of the tricyclic PAHs for which the cardiotoxic phenotype is largely attributable.[8,13]

In addition to genes that indicated cardiac functional impairment, visual perception was the most significantly enriched process uniquely associated with the 96 hpf larvae after slick oil exposure (Figure 3), with 35 out of 70 differentially expressed genes identified in this biological pathway. Genes were associated with the series of events required for an organism to receive a visual stimulus, convert it to a molecular signal, and recognize and characterize the signal. In zebrafish embryos treated with the oxygenated PAH, 1,9-benz-10-anthrone, mRNAs associated with visual perception were also altered.[26] Advaita predicted that phototransduction was significantly suppressed by slick oil at 96 hpf. Consistent with this prediction, the transcript for rhodopsin (rho) was downregulated. *Rho* is an essential G-protein receptor, that when photoexcited, initiates the visual transduction cascade. Transducin (*gnat2*), and GMP-phosphodiesterase (*pde6g*) were also significantly downregulated. These two proteins play critical roles in stimulating the coupling of rhodopsin and cGMP, resulting in the closure of cGMP-gated cation channels subsequently leading to membrane hyperpolarization and release of neurotransmitters.[27] Recovery from light involves the deactivation of the photolyzed rhodopsin by rhodopsin kinase (encoded by *grk7* or *pk*, SI Figure S10); GTP-binding transducin alpha subunit is deactivated through a process that is stimulated by *rgs9* (SI Figure S10). Both *grk7* and *rgs9* were significantly downregulated, which could lead to the perturbation of rhodopsin regeneration and further phototransduction in the 96 hpf larvae by slick oil.

The top associated diseases included retinitis pigmentosa, leber congenital amaurosis and cataract formation (Figure 3). Slick oil may inhibit the activities of photoreceptor-specific

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transcription factor *crx* and lead to downregulation of eye-associated gene expression and degeneration of eyes[28] (Figure 4B). Similarly, Huang et al.[14] used expression microarrays to predict ocular developmental toxicity by Benzo(a)pyrene (BaP), and linked the perturbation of photoreceptor related genes to a decreasing phototactic response behavior. In pufferfish larvae, oil exposure induced significantly smaller and shape-distorted eyes, and subsequent disrupted rhythmic motor movement, and suppressed positive phototaxis of larvae.[29] The authors suggested that the behavioral problems (incorrect swimming) could have originated from the abnormal visual sensation. The observed behavior problem would be significant, as it may cause severe disadvantages during fish life cycles by affecting feeding, escape, and migration responses.

Predictions by IPA also included the neurodegeneration of central nervous system and abnormality of the vertebral column in 96 hpf larvae after slick oil exposure (Figure 4C, D). We found four neurotransmitter transporter genes, *slc6A8* (creatine transporter), *slc6A18* (neutral amino acid transporter), *slc6A4* (serotonin transporter), and *slc6A2* (norepinephrine transporter) that were all significantly downregulated in 96 hpf larvae after slick oil exposure. Prenatal exposure to PAHs has been shown to induce neurological abnormalities such as cognitive impairment, learning difficulties, and loss of short-term memory.[30] Dibenzothiophene and phenanthrene disrupted neural tube structure, and pyrene induced neuronal cell death in zebrafish embryos.[13] BaP decreased brain mass, locomotor activity, dopaminergic neurons and resulted in neurodegeneration in zebrafish.[31] Although the mechanism of neurodegenerative diseases remains unclear, some genes are suggested to be associated with the occurrence of neurodegenerative diseases. A previous study demonstrated that SLC6A3 (solute carrier family 6 (neurotransmitter transporter), member 3) was significantly decreased in Parkinson's disease patients.[32] Gao et al.[31] also reported significantly downregulated SLC6A3 gene expression in zebrafish after BaP exposure.

Four *N*-methyl-D-aspartate receptor (NMDAR) genes (i.e., grin1, grin2a, grin2d and grin2b), and four Ca^{2+}/ calmodulin dependent kinase (CaMK) genes (i.e., *camk2a*, *camk2b*, *camk1g* and *camk2g*) were all significantly downregulated. Glutamate, a major excitatory neurotransmitter, stimulates the NMDAR, located primarily at synapses, causing a transient influx of Ca^{2+} into the postsynaptic neuron. Intracellular Ca^{2+}, along with calmodulin (CaM), can activate CaMKII.[33] As noted above with the cardiac impacts associated with altered Ca^{2+} metabolism, impairment affects many important neuronal processes, particularly in synaptic plasticity and memory.[34] The decrease of CaM and CaMK2 mRNA expression might contribute to altered synaptic plasticity and neuronal survival in exposure to benzo(a)pyrene-exposed rockfish embryos.[35] The downregulation of NMDAR and CaMK suggests neural toxicity may also be an additional target of slick oil through changes in glutamate receptor and Ca^{2+} homeostasis. Since Ca^{2+} appears to also play a significant role in cardiac toxicity, impairment in multiple targets may be responsible for a number of phenotypic responses besides that of cardiac toxicity that may adversely affect development at later life stages.

Another top ranked canonical pathway was the eukaryotic initiation factor 2 (EIF2) in both slick and source oil exposed mahi-mahi larvae at 24 and 96hpf by IPA. An overall decreased activity in the EIF2 signaling pathway at 24 hpf, but an increased activity at 96 hpf by both source and slick oil exposure was predicted. EIF2 plays a key role in global translation initiation and protein synthesis. The regulatory function of EIF2 is mediated via phosphorylation-dephosphorylation of its subunit α. Cellular stress can cause immediate phosphorylation of EIF2 subunit α, which results in inhibition of intracellular translation initiation as an adaptive response.[36] Phosphorylation at Ser 51 abrogates the function of EIF2 α and leads to a shutdown of global mRNA translation and consequently mobilizes stress induced gene expression involved in cell growth, differentiation and apoptosis.[37] EIF2 signaling was identified in the top 3 most significantly affected canonical pathways from the differential exon usage (DEU) genes in 96 hpf zebrafish larvae exposed to BaP.[38] Wang et al.[39] demonstrated that a benzo[a]pyrene metabolite BPDE induced severe cell cycle arrest, apoptosis and decreased cell viability in human amnion epithelial cells, and EIF2 α phosphorylation produced a pro-survival and antiapoptotic effect to alleviate the cellular damage. In the present study, the decreased activity in the EIF2 signaling pathway at 24 hpf followed by increased activity at 96 hpf by both source and slick oil exposure may indicate the 24 hpf stage is more susceptible to embryo lethality since the adaptive response was diminished. The relationship between the temporal responses (i.e., induction at 24 hpf and downregulation at 96 hpf) requires additional study to determine the role of this pathway in acclimation to PAH exposure.

Overall, this is the first study to investigate the time-course transcriptomic responses in marine fish embryos/larvae exposed to two types of *DWH* oil. Slick and source oil exposure induced similar transcriptional responses at early developmental stages (24 hpf), but transcript profiles were different at later developmental stages (48, 96 hpf) in mahi-mahi. IPA indicated slick oil exposure altered the EIF2 signaling which predicted loss of cell viability at early development stage, and induced differentially expressed cardiac-associated genes at 48 and 96 hpf. However, at 96 hpf, slick oil exposure resulted in pronounced perturbations in metabolism, steroid biosynthesis, visual, and cytochrome P450 genes suggesting other targets besides AhR pathways or the heart may be involved in the developmental toxicity of *DWH* oil. Using rapid genomics annotation analyses coupled with advanced informatics tools for a poorly annotated species that was impacted at the site of the *DWH* spill indicates these methods may be used in other species of concern to identify molecular and physiological responses of environmental contamination to reduce uncertainty in assessments of ecological risk and recovery of biota.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02205.

Further information is available that provides PAH measurements (Figure S1; Table S6), water quality measurements (Table S1−S3), an example of larval pericardial edema (Figure S4), a heatmap showing the Euclidean distances between the samples (Figure S5), plots showing relative expression of genes (Figure S6), trends in EIF2 signaling (Figure S7), plots showing perturbations in steroid biosynthesis (Figure S8), ribosome biosynthesis (Figure S9) and phototransduction (Figure S10), primers used in qPCR (Table S5), the top predicted molecular functions, biological processes,

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pathways and phenotype (Table S7−11), the full gene names in Figure 4 (Table S12), methods for determining $LC25_S$, water chemistry, pericardial area, transcript expression, and the standard Illumina QC procedures for mRNA sequencing data (Figure S12) (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) United States of America v. BP Exploration & Production, Inc., et al. 2015. Findings of fact and conclusions of law: Phase Two trial. In re: *Oil spill by the oil rig "Deepwater Horizon" in the Gulf of Mexico*, on April 20, 2010, No. MDL 2179, 2015 WL 225421 (LA. E.D. Jan. 15, 2015). (Doc. 14021). U.S. District Court for the Eastern District of Louisiana.

(2) Gibbs, R. H.; Collette, B. B. On the identification, distribution, and biology of the dolphins, *Coryphaena hippurus* and *C. equiselis*. *Bull. Mar. Sci.* **1959**, *9* (2), 117−152.

(3) Rooker, J. R.; Simms, J. R.; Wells, R. D.; Holt, S. A.; Holt, G. J.; Graves, J. E.; Furey, N. B. Distribution and habitat associations of billfish and swordfish larvae across mesoscale features in the gulf of Mexico. *PLoS One* **2012**, *7* (4), e34180.

(4) Hicken, C. E.; Linbo, T. L.; Baldwin, D. H.; Willis, M. L.; Myers, M. S.; Holland, L.; Larsen, M.; Stekoll, M. S.; Rice, S. D.; Collier, T. K.; Scholz, N. L.; Incardona, J. P. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (17), 7086−7090.

(5) Incardona, J. P.; Collier, T. K.; Scholz, N. L. Oil spills and fish health: Exposing the heart of the matter. *J. Exposure Sci. Environ. Epidemiol.* **2011**, *21* (1), 3−4.

(6) Esbaugh, A. J.; Mager, E. M.; Stieglitz, J. D.; Hoenig, R.; Brown, T. L.; French, B. L.; Linbo, T. L.; Lay, C.; Forth, H.; Scholz, N. L.; Incardona, J. P. The effects of weathering and chemical dispersion on Deepwater Horizon crude oil toxicity to mahi-mahi (*Coryphaena hippurus*) early life stages. *Sci. Total Environ.* **2016**, *543*, 644−651.

(7) Carls, M. G.; Meador, J. P. A perspective on the toxicity of petrogenic PAHs to developing fish embryos related to environmental chemistry. *Hum. Ecol. Risk Assess.* **2009**, *15* (6), 1084−1098.

(8) Bornstein, J. M.; Adams, J.; Hollebone, B.; King, T.; Hodson, P. V.; Brown, R. S. Effects-driven chemical fractionation of heavy fuel oil to isolate compounds toxic to trout embryos. *Environ. Toxicol. Chem.* **2014**, *33* (4), 814−824.

(9) Incardona, J. P.; Swarts, T. L.; Edmunds, R. C.; Linbo, T. L.; Aquilina-Beck, A.; Sloan, C. A.; Gardner, L. D.; Block, B. A.; Scholz, N. L. Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages. *Aquat. Toxicol.* **2013**, *142*, 303−316.

(10) Jung, J. H.; Hicken, C. E.; Boyd, D.; Anulacion, B. F.; Carls, M. G.; Shim, W. J.; Incardona, J. P. Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. *Chemosphere* **2013**, *91* (8), 1146−1155.

(11) Mager, E. M.; Esbaugh, A. J.; Stieglitz, J. D.; Hoenig, R.; Bodinier, C.; Incardona, J. P.; Scholz, N. L.; Benetti, D. D.; Grosell, M. Acute embryonic or juvenile exposure to Deepwater Horizon crude oil impairs the swimming performance of mahi-mahi (*Coryphaena hippurus*). *Environ. Sci. Technol.* **2014**, *48* (12), 7053−7061.

(12) Stieglitz, J. D.; Mager, E. M.; Hoenig, R.; Benetti, D. D.; Grosell, M. Impacts of Deepwater Horizon crude oil exposure on adult mahi-mahi (*Coryphaena hippurus*) swim performance. *Environ. Toxicol. Chem.* **2016**, DOI: 10.1002/etc.3436.

(13) Incardona, J. P.; Collier, T. K.; Scholz, N. L. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **2004**, *196* (2), 191−205.

(14) Huang, L.; Wang, C.; Zhang, Y.; Wu, M.; Zuo, Z. Phenanthrene causes ocular developmental toxicity in zebrafish embryos and the possible mechanisms involved. *J. Hazard. Mater.* **2013**, *261*, 172−180.

(15) Edmunds, R. C.; Gill, J. A.; Baldwin, D. H.; Linbo, T. L.; French, B. L.; Brown, T. L.; Esbaugh, A. J.; Mager, E. M.; Stieglitz, J.; Hoenig, R.; Benetti, D. Corresponding morphological and molecular indicators of crude oil toxicity to the developing hearts of mahi mahi. *Sci. Rep.* **2015**, *5*, 17326.

(16) Stieglitz, J. D.; Benetti, D. D.; Hoenig, R. H.; Sardenberg, B.; Welch, A. W.; Miralao, S. Environmentally conditioned, year-round volitional spawning of cobia (*Rachycentron canadum*) in broodstock maturation systems. *Aquacult. Res.* **2012**, *43* (10), 1557−1566.

(17) Buchfink, B.; Xie, C.; Huson, D. H. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **2015**, *12* (1), 59−60.

(18) Love, M. I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15* (12), 1−21.

(19) Paolini, P.; Pick, D.; Lapira, J.; Sannino, G.; Pasqualini, L.; Ludka, C.; Sprague, L. J.; Zhang, X.; Bartolotta, E. A.; Vazquez-Hidalgo, E.; Barba, D. T. Developmental and extracellular matrix-remodeling processes in rosiglitazone-exposed neonatal rat cardiomyocytes. *Pharmacogenomics* **2014**, *15* (6), 759−774.

(20) Draghici, S.; Khatri, P.; Tarca, A. L.; Amin, K.; Done, A.; Voichita, C.; Georgescu, C.; Romero, R. A systems biology approach for pathway level analysis. *Genome Res.* **2007**, *17* (10), 1537−1545.

(21) Chen, J.; Bardes, E. E.; Aronow, B. J.; Jegga, A. G. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* **2009**, *37* (suppl 2), W305−W311.

(22) Metzger, J. M.; Westfall, M. V. Covalent and noncovalent modification of thin filament action: the essential role of troponin in cardiac muscle regulation. *Circ. Res.* **2004**, *94* (2), 146−158.

(23) Handley-Goldstone, H. M.; Grow, M. W.; Stegeman, J. J. Cardiovascular gene expression profiles of dioxin exposure in zebrafish embryos. *Toxicol. Sci.* **2005**, *85* (1), 683−693.

(24) Brette, F.; Machado, B.; Cros, C.; Incardona, J. P.; Scholz, N. L.; Block, B. A. Crude oil impairs cardiac excitation-contraction coupling in fish. *Science* **2014**, *343* (6172), 772−776.

(25) Alexeyenko, A.; Wassenberg, D. M.; Lobenhofer, E. K.; Yen, J.; Linney, E.; Sonnhammer, E. L. L.; Meyer, J. N. Dynamic zebrafish interactome reveals transcriptional mechanisms of dioxin toxicity. *PLoS One* **2010**, *5* (5), e10465.

(26) Goodale, B. C.; La Du, J.; Tilton, S. C.; Sullivan, C. M.; Bisson, W. H.; Waters, K. M.; Tanguay, R. L. Ligand-specific transcriptional mechanisms underlie aryl hydrocarbon receptor-mediated developmental toxicity of oxygenated PAHs. *Toxicol. Sci.* **2015**, *147*, p.kfv139.

(27) Hofmann, K. P.; Heck, M. Light-induced protein-protein interactions on the rod photoreceptor disc membrane. In Lee AG. *Rhodopsin and G-Protein Linked Receptors, Part A*; JAI Press: Greenwich, CT. 1996; Vol 2 (2 Vol Set), pp 141−198.

(28) Freund, C. L.; Gregory-Evans, C. Y.; Furukawa, T.; Papaioannou, M.; Looser, J.; Ploder, L.; Bellingham, J.; Ng, D.; Herbrick, J. A. S.; Duncan, A.; Scherer, S. W. Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. *Cell* **1997**, *91* (4), 543−53.

DOI: 10.1021/acs.est.6b02205
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(29) Kawaguchi, M.; Sugahara, Y.; Watanabe, T.; Irie, K.; Ishida, M.; Kurokawa, D.; Kitamura, S. I.; Takata, H.; Handoh, I. C.; Nakayama, K.; Murakami, Y. Nervous system disruption and concomitant behavioral abnormality in early hatched pufferfish larvae exposed to heavy oil. *Environ. Sci. Pollut. Res.* **2012**, *19* (7), 2488−2497.
(30) Hood, D. B.; Sheng, L.; Ding, X. X.; Ferguson, M.; McCallister, M.; Rhoades, R.; Maguire, M.; Ramesh, A.; Aschner, M.; Campbell, D.; Levitt, P.; Hood, D. B. Prenatal polycyclic aromatic hydrocarbon exposure leads to behavioral deficits and downregulation of receptor tyrosine kinase, MET. *Toxicol. Sci.* **2010**, *118* (2), 625−634.
(31) Gao, D.; Wu, M.; Wang, C.; Wang, Y.; Zuo, Z. Chronic exposure to low benzo [a] pyrene level causes neurodegenerative disease-like syndromes in zebrafish (*Danio rerio*). *Aquat. Toxicol.* **2015**, *167*, 200−208.
(32) Tissingh, G.; Booij, J.; Bergmans, P.; Winogrodzka, A.; Janssen, A. G.; Van Royen, E. A.; Stoof, J. C.; Wolters, E. C. Iodine-123-N-(omega)-fluoropropyl-2(beta)-carbomethoxy-3 (beta)-(4-iodophenyl) tropae SPECT in healthy controlsand early-stage, drug-naive Parkinson's disease. *J. Nucl. Med.* **1998**, *39* (7), 1143−1148.
(33) Fink, C. C.; Meyer, T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr. Opin. Neurobiol.* **2002**, *12* (3), 293−299.
(34) Kazama, H.; Nose, A.; Morimoto-Tanifuji, T. Synaptic components necessary for retrograde signaling triggered by calcium/calmodulin-dependent protein kinase II during synaptogenesis. *Neuroscience* **2007**, *145* (3), 1007−1015.
(35) He, C.; Wang, C.; Zhou, Y.; Li, J.; Zuo, Z. Embryonic exposure to benzo (a) pyrene influences neural development and function in rockfish (*Sebastiscus marmoratus*). *NeuroToxicology* **2012**, *33* (4), 758−762.
(36) Stolboushkina, E. A.; Garber, M. B. Eukaryotic type translation initiation factor 2: Structure−functional aspects. *Biochemistry (Moscow)* **2011**, *76* (3), 283−294.
(37) Wek, R. C.; Jiang, H. Y.; Anthony, T. G. Coping with stress: eIF2 kinases and translational control. *Biochem. Soc. Trans.* **2006**, *34* (Pt 1), 7−11.
(38) Fang, X.; Corrales, J.; Thornton, C.; Clerk, T.; Scheffler, B. E.; Willett, K. L. Transcriptomic changes in zebrafish embryos and larvae following benzo [a] pyrene exposure. *Toxicol. Sci.* **2015**, *146* (2), 395−411.
(39) Wang, Q.; Jiang, H.; Fan, Y.; Huang, X.; Shen, J.; Qi, H.; Li, Q.; Lu, X.; Shao, J. Phosphorylation of the α-subunit of the eukaryotic initiation factor-2 (eIF2α) alleviates benzo [a] pyrene-7, 8-diol-9, 10-epoxide induced cell cycle arrest and apoptosis in human cells. *Environ. Toxicol. Pharmacol.* **2011**, *31* (1), 18−24.
(40) Irish, J. C.; Mills, J. N.; Turner-Ivey, B.; Wilson, R. C.; Guest, S. T.; Rutkovsky, A.; Dombkowski, A.; Kappler, C. S.; Hardiman, G.; Ethier, S. P. Amplification of WHSC1L1 regulates expression and estrogen-independent activation of ERα in SUM-44 breast cancer cells and is associated with ERα over-expression in breast cancer. *Mol. Oncol.* **2016**, *10* (6), 850−865.
(41) Hardiman, G.; Hazard, S. E.; Savage, S. J.; Wilson, R. C.; Smith, M. T.; Hollis, B. W.; Halbert, C. H.; Gattoni-Celli, S. Systems analysis of the prostate transcriptome in African American men compared to European American men. *Pharmacogenomics*, **2016** DOI: 10.2217/pgs-2016-0025

DOI: 10.1021/acs.est.6b02205
Environ. Sci. Technol. XXXX, XXX, XXX−XXX