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RENAN NUNES COSTA

RESPOSTAS LETAIS E SUBLETAIS DE ANFÍBIOS ANUROS EXPOSTOS À CONTAMINAÇÃO POR PESTICIDAS

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Dedico este trabalho à minha família.

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ÍNDICE

RESUMO

A agricultura mecanizada cobre uma maior área de terra do que qualquer outra atividade humana no mundo. Diretamente associado às extensas terras agrícolas, o uso de pesticidas acontece de maneira indiscriminada e gerenalizada, sendo considerado um dos principais fatores que ameaçam a saúde humana e o meio ambiente. Pesticidas são letais a uma série de organismos aquáticos, como por exemplo, os anfíbios anuros. Estes efeitos letais colaboram diretamente com o declínio de espécies. No entanto, a contaminação pode gerar efeitos subletais que atuam de maneira silenciosa e também contribuem com os declínios populacionais, como por exemplo, alterações morfológicas, comportamentais e ontogenéticas. Geralmente, estas alterações subletais mediadas pela contaminação por pesticidas são negativas e reduzem o fitness das espécies. Neste contexto, o objetivo geral dessa tese foi avaliar as respostas letais e subletais de anfíbios anuros, tanto em fase larval quanto adulta, submetidos à contaminação por pesticidas. Através de uma abordagem cienciométrica, o capítulo 1 trata das principais respostas morfológicas de larvas e metamorfos de anuros em estudos experimentais com contaminação por pesticidas, além das tendências e caminhos dos estudos na área. Nos capítulos 2 e 3, foram realizados experimentos de exposição aguda e crônica a formulações comerciais de herbicidas à base de glifosato para avaliação de respostas letais e subletais em girinos de duas espécies de anuros brasileiros, *Dendropsophus minutus* e *Physalaemus cicada*. Por fim, no capítulo 4 foi realizado um estudo de caso para avaliar se o histórico de aplicação de herbicidas à base de glifosato afeta a estabilidade do desenvolvimento de adultos da espécie *Dendropsophus haddadi*. Considerando que na região Neotropical está a maior diversidade de espécies de anuros e que se trata de uma das regiões com o menor número de espécies consideradas em estudos toxicológicos, os resultados gerados aqui contribuem para a redução dessa lacuna. Além disso, os resultados gerados demonstram que a contaminação por pesticidas leva a diferentes respostas subletais em anuros, que podem contribuir com o declínio populacional e com a perda de espécies. Por fim, o entendimento dos efeitos letais e subletais podem nortear futuras medidas mitigadoras dos impactos de pesticidas sobre a biodiversidade.

PALAVRAS-CHAVE: agroquímicos; assimetria flutuante; contaminação da água doce; estresse ambiental; LC50; morfometria.

ABSTRACT

Mechanized agriculture covers a greater land area than any other human activity in the world. Directly associated to the extensive agriculture lands, the use of pesticides happens in an indiscriminate and generalized way, being considered one of the main factors threatening human health and the environment. Pesticides are lethal to several aquatic organisms, such as amphibian anurans. These lethal effects directly contribute to species declines. However, the contamination can lead to sublethal effects which act silently and also contribute to population declines, such as morphological, behavioral and ontogenetic changes. Often, these sublethal changes mediated by pesticide contamination are negative and reduce the species fitness. In this context, the general objective of this thesis was to evaluate the lethal and sublethal effects of amphibian anurans, both in larval and adult stages, submitted to contamination by pesticides. Through a scientometric approach, chapter 1 deals with the main morphological responses of larvae and metmorphs of anurans in experimental studies with pesticide contamination, beyond the trends and paths of studies in the area. In chapters 2 and 3, acute and chronic exposure experiments were performed using commercial formulations of glyphosate-based herbicides for the evaluation of the lethal and sublethal responses in tadpoles of two Brazilian anurans, *Dendropsophus minutus* and *Physalaemus cicada*. Finally, in chapter 4 we conducted a case study to evaluate if the history of glyphosate-based herbicide application affects the developmental stability in adults of *Dendropsophus haddadi*. Considering that the Neotropical region has the largest anurans diversity and that it is one of the regions with the lowest number of species considered in toxicological studies, the results generated here contribute to fill this gap. Therefore, the results show that pesticide contamination leads to several sublethal responses in anurans, which can contribute to population declines and species loss. Finally, the understanding of lethal and sublethal effects can guide future mitigation measures of the pesticides impacts on biodiversity.

KEY-WORDS: agrochemical; fluctuating asymmetry; freshwater contamination; environmental stress; LC50; morphometry.

INTRODUÇÃO GERAL

Há mais de 50 anos, quando as questões ambientais não eram tão relevantes mundialmente, Rachel Carson fez um dos primeiros alertas sobre os potenciais impactos negativos dos pesticidas sobre o meio ambiente, as espécies e as interações ecológicas em seu livro *Silent Spring* (Carson 1962). Carson destacou o perigoso avanço do interesse econômico pelos pesticidas e os impactos potenciais do capitalismo sobre a natureza. Infelizmente, as previsões de Carson estavam corretas.

As áreas agrícolas cobrem uma maior área de terra do que qualquer outra atividade humana no planeta e essa conversão para o uso agrícola é o principal responsável pelo desmatamento, modificação e contaminação de ambientes terrestres e aquáticos (Foley et al. 2005, Devine e Furlong 2007, Lambin e Meyfroidt 2011). Correlacionado ao rápido avanço das fronteiras agrícolas e ao sistema imposto pelas grandes multinacionais (e.g. Bayer, Syngenta, Monsanto), o uso e comercialização de pesticidas tem crescido significativamente e é considerado um dos principais problemas ambientais e de saúde publica (Wilson e Tisdell 2001, Carneiro et al. 2015, Pedlowski et al. 2012, Bombardi 2017). Essa realidade é bastante pertinente em países como o Brasil, que desde 2008 lidera o ranking mundial de uso e comercialização de agrotóxicos (Carneiro et al. 2015, Bombardi 2017), e que detém uma legislação ambiental com pouco (ou nenhum) embasamento científico, além de órgãos ambientais que deixam a desejar quanto à fiscalização.

De fato, o atual sistema agrícola mediado pela ação de pesticidas e baseado na produção e venda de *commodities* (Bombardi 2017) é economicamente rentável e garante recursos alimentares para a superpopulação humana. Entretanto, o uso em larga escala e a aplicação não seletiva tem contaminado ecossistemas aquáticos e organismos não alvo, contribuindo diretamente para a perda de espécies nativas (Foley et al. 2005, Devine e Furlong 2007, Schiesari e Grillitsch 2011).

Anfíbios anuros apresentam pele com alta permeabilidade e são diretamente afetados por pesticidas, especialmente as espécies com modo de vida bifásico que são dependentes de ambientes aquáticos para deposição dos ovos e desenvolvimento dos girinos (Bishop et al. 1999, Mann et al. 2009). É o grupo de vertebrados com o maior número de espécies em declínio (Stuart et al. 2004, Gallant et al. 2007, Alroy 2015), sendo que a contaminação por pesticidas está entre os principais fatores contribuintes

(Sparling et al. 2001, Blaustein e Kiesecker 2002, Schiesari et al. 2007, Mann et al. 2009, Hayes et al. 2010). Por serem considerados organismos bioindicadores de integridade em ambientes aquáticos, os anfíbios são uma ferramenta interessante para medição do estresse causado por pesticidas (Blaustein e Wake 1995, Kerby et al. 2010), além de serem bons modelos em estudos experimentais sob condições laboratoriais, mesocosmos e/ou campo (Boone e James 2005).

Na natureza, os anfíbios são submetidos a uma série de fatores estressantes que podem ser de origem natural (e.g. pressão de predadores) ou antrópica (e.g. contaminação e degradação do habitat). Quanto à contaminação, as espécies experimentam vários tipos de pesticidas e podem ser submetidos a níveis agudos e crônicos, os quais podem gerar respostas letais e subletais. Por exemplo, as formulações comerciais de glifosato (herbicida mais utilizado no Brasil – Bombardi 2017) variam entre moderado e altamente tóxico para girinos de anuros (Giesy et al. 2000, Relyea 2005, Relyea e Jones 2009); e a exposição aguda e crônica influenciam interações ecológicas e podem levar a alterações nos atributos comportamentais, morfológicos e ontogenéticos (e. g. Relyea 2012, Katzenberger et al. 2014, Costa e Nomura 2016). Ambos os tipos de resposta atuam de forma efetiva sobre o declínio populacional de anfíbios, seja de maneira direta e rápida, como as respostas letais, ou indireta e silenciosa, como as respostas subletais. Além disso, o estresse da contaminação por pesticidas pode atuar de maneira aditivo-sinérgica com fatores estressantes naturais, alterando as respostas espécie-específicas (Katzenberger et al. 2014, Moore et al. 2015). Para tanto, conhecer os efeitos toxicológicos de pesticidas através de respostas letais e alterações nos atributos espécie-específicos pode ser considerado um primeiro passo rumo ao delineamento de medidas mitigadoras que reduzam os impactos destes contaminantes sobre a anurofauna; especialmente no Brasil, que detém a maior riqueza de espécies de anfíbios do planeta e está entre as regiões com um dos menores índices de pesquisadores e espécies consideradas em estudos ecotoxicológicos (Schiesari et al. 2007).

Neste contexto, a presente tese foi baseada na avaliação de respostas letais e sub-letais de anfíbios anuros submetidos à contaminação por pesticidas, com ênfase nas espécies brasileiras e no herbicida glifosato. A tese foi estruturada e organizada em quatro capítulos:

- O **capítulo 1,** intitulado “Pesticide effects on growth and external morphology of larvae and metamorphs (Amphibia, Anura): evidences from experimental studies”, trata de uma avaliação cienciométrica de estudos experimentais que elucidam os caminhos e as tendências na área com foco principal nas respostas morfológicas e no crescimento de larvas e metamórficos de anuros expostos à contaminação por pesticidas. Neste capítulo foram considerados estudos que contemplam espécies de anuros e pesticidas de diferentes regiões do planeta.

- No **capítulo 2,** intitulado “Lethal and sublethal responses of a neotropical tadpole (*Dendropsophus minutus*, Anura: Hylidae) exposed to Roundup Original® and predator cues”, foram realizados dois experimentos em laboratório com o intuito de mensurar respostas letais (mortalidade e LC50) e subletais (comportamento, morfologia geral externa e assimetria flutuante) de girinos submetidos a dois fatores estressantes oferecidos individualmente e em conjunto, sendo um natural (risco de predação) e outro antrópico (contaminação pelo herbicida Roundup Original®).

- No **capítulo 3**, intitulado “Lethal effects of Roundup Original DI® on tadpoles of *Physalaemus cicada* (Anura: Leptodactylidae)”, foram mensuradas as respostas letais do herbicida Roundup Original DI® (i.e. efeito sobre a sobrevivência e definição de uma concentração letal – LC50) sobre girinos. Os resultados foram apresentados através de um Short Communication.

- No **capítulo 4**, intitulado “Fluctuating asymmetry in a small treefrog *Dendropsophus haddadi* (Anura: Hylidae) as a measure of glyphosate contamination history: a case-study from Ecological Reserve of Michelin”, foi avaliado se os níveis de assimetria flutuante (ferramenta utilizada para avaliação de estresse ambiental) em populações de uma espécie de anuro são maiores em habitats com histórico atual do uso de glifosato quando comparados a áreas com histórico de aplicação no passado e áreas sem histórico de aplicação do herbicida.

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CAPÍTULO 1

Pesticide effects on growth and external morphology of larvae and metamorphs (Amphibia, Anura): evidences from experimental studies

Renan Nunes Costa, Fausto Nomura and Mirco Solé

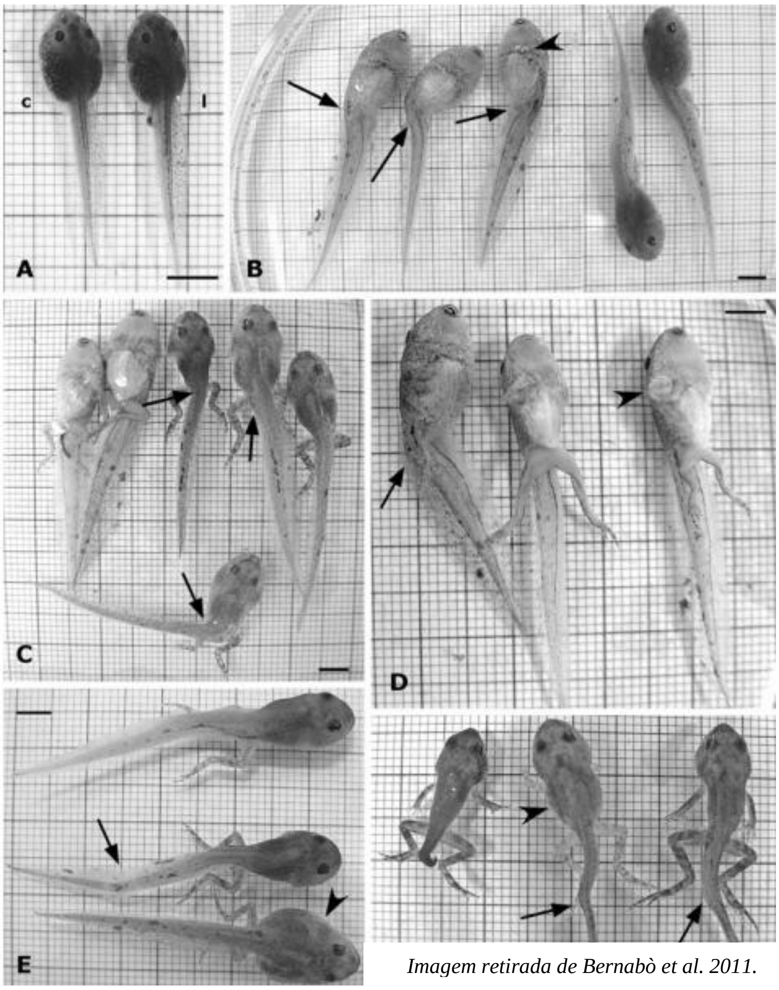

Imagem retirada de Bernabò et al. 2011.

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Pesticide effects on growth and external morphology of larvae and metamorphs (Amphibia, Anura): evidences from experimental studies

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Abstract

The use of pesticides is directly associated with the continuous advance of croplands. Aquatic environments are contaminated by different types of pesticides which affect non-target species, such as amphibian anurans. Besides mortality, contamination by pesticides may influence different species attributes, such as growth and external morphology; mainly in the larvae and metamorph stage which are more dependent of aquatic environments and are the stages with higher hormonal activity associated with metamorphosis. We evaluated evidences from experimental studies that tested the effects on growth and external morphology in larvae and metamorphs of anurans in response to pesticide contamination. We performed a systematic review using a combination of key-words and searched for papers in electronic databases, filtering according to the scope of this study. We found 117 studies, totalizing 356 species-specific cases of contamination. Fifty different species and 43 types of active ingredients were tested, mainly under laboratory conditions. The main effects detected were associated to growth decrease of larvae and metamorphs, followed by morphological changes including malformations and deformities. The most studies were performed in the United States and published in ecotoxicological journals. The knowledge about the sublethal effects of pesticides on amphibians attributes, especially those associated to the individual fitness, as well as the geographical distribution of

these studies, can contribute to design methods and efforts to avoid population size decreases.

Key-Words: agrochemical, freshwater contamination, malformations, deformities, pesticide contamination.

Resumo

O uso de pesticidas está diretamente relacionado ao contínuo avanço dos cultivos agrícolas. Ambientes aquáticos são contaminados por diferentes tipos de pesticidas, o que afeta espécies não alvo, como os anfíbios anuros. Além da mortalidade, a contaminação por pesticidas pode influenciar diferentes atributos das espécies, como o crescimento e a morfologia externa; principalmente nos estágios de larva e metamorfo que são mais dependentes de ambientes aquáticos e são os estágios com a maior atividade hormonal associada à metamorfose. Avaliamos evidências de estudos experimentais que testaram os efeitos sobre o crescimento e a morfologia externa de larvas e metamorfos de anuros em resposta à contaminação por pesticidas. Realizamos uma revisão sistemática usando uma combinação de palavras-chave e buscamos por artigos em bases de dados eletrônicas, filtrando de acordo com o escopo do estudo. Encontramos 117 estudos, totalizando 356 casos espécie-específicos de contaminação. Cinquenta espécies diferentes e 43 tipos de componentes ativos foram testados nestes estudos, principalmente em condições laboratoriais. Os principais efeitos detectados foram associados à redução no crescimento das larvas e metamorfos, seguido pelas mudanças morfológicas que incluem deformidades e más-formações. A maioria dos estudos foi realizada nos Estados Unidos e publicada em revistas de ecotoxicologia. O conhecimento acerca dos efeitos subletais de pesticidas sobre os atributos dos anfíbios, especialmente aqueles associados ao *fitness* individual, assim como a distribuição geográfica desses estudos, pode contribuir para projetar esforços e metodologias para evitar a diminuição do tamanho das populações.

Palavras-Chave: agroquímicos, contaminação da água doce, más-formações, deformidades, contaminação por pesticidas.

Introduction

Pesticides can be classified according to their designation (e.g., herbicide, insecticide, fungicide), toxicity (ranging from highly toxic to non-toxic) and nature (e.g., organic, inorganic), and are highly effective to control agricultural pests, reduce damage to crops and increase food production (Devine and Furlong 2007, Schiesari et al. 2013). However, there is a consensus that several kinds of environmental and social problems are associated to pesticide contamination (Wilson and Tisdell 2001, Pimentel 2009, Pedlowski et al. 2012).

Based on evidences of how damaging pesticides can be for human and wildlife, many countries banned different active ingredients and commercial formulations (e. g. United States, European Union, Sri Lanka, Switzerland). Conversely, countries like Brazil, the world leader in pesticide consumption, followed a different path, allowing the use of a long list of active ingredients and pesticide formulations (Schiesari and Grillitsch 2011, Carneiro et al. 2015, Bombardi 2017). In extensive croplands, high levels of pesticide are indiscriminately applied, leading to habitat perturbation, water systems eutrophication and contamination of ground and surface water, air, soil and biota, directly affecting different groups of non-target organisms and contributing to species loss (Foley et al. 2005, Devine and Furlong 2007, Schiesari and Grillitsch 2011, Schiesari et al. 2013).

Among non-target organisms, amphibians are the most threatened by contamination and habitat loss resulting from agricultural expansion, and are the vertebrate species group with the most population declines in the world (Sparling et al. 2001, Blaustein and Kiesecker 2002, Stuart et al. 2004, Gallant et al. 2007, Mann et al. 2009, Hayes et al. 2010, Alroy 2015). Amphibians are higly susceptible to environment contamination mainly because they have permeable skin and a byphasic lifecycle, being of special concern to species with indirect development with aquatic eggs and larvae (Bishop et al. 1999, Gallant et al. 2007, Schiesari et al. 2007, Mann et al. 2009, Allentoft and O'Brien 2010). This vulnerability of the aquatic stages of amphibians was observed through several negative effects of pesticides on survival (*e.g.*, Relyea 2005, Boone et al. 2007, Relyea and Jones 2009, Hua et al. 2015, Costa and Nomura 2016). Pesticides can act as endocrine disruptors affecting the production, metabolism, and action of natural hormones, which are responsible for growth, behavior and/or developmental regulation in amphibians (Hayes et al. 2006a, Hayes et al. 2010). Thus, pesticide contamination causes sublethal effects on amphibian attributes, especially at the aquatic developmental stages (*i.e.* larvae and metamorphs) and during the metamorphosis process, when hormone regulation is more critical (Hayes et al. 2006a, Mann et al. 2009).

The high phenotypic plasticity of larvae favors ecotoxicological studies in laboratory and outdoor mesocosms (Van Buskirk and Relyea 1998, Steiner and Van Buskirk 2008, Van Buskirk 2009, Fusco and Minelli 2010), allowing the evaluation of sublethal effects of pesticide contamination in a short time. The sublethal effects directly and indirectly affect survival and/or individual fitness, and can be observed by changes in different larval attributes, such as growth (mass and length) and morphology (deformities and variation in body traits) (e.g. Boone and Semlitsch 2002, Arcaute et al. 2012, Relyea 2012, Devi and Gupta 2013, Katzenberger et al. 2014, David and Kartheek 2015). Furthermore, as observed in experimental studies - especially with anuran larvae - a range of biotic and abiotic factors (e.g. temperature, pH, diseases, predator cues, competition) can additively or synergistically interact with pesticides (e.g. Boone and Bridges 2003, Relyea 2006, Jones et al. 2011, Relyea 2012, Rohr et al. 2013, Katzenberger et al. 2014), potentially increasing the lethality and residence time of the chemicals in water bodies (Giesy et al. 2000, Relyea and Hoverman 2006, Relyea 2012). Thus, the amphibian aquatic stages (*i.e.* larvae and metamorphs) are subject to contamination by a range of pesticide types because they are associated with aquatic ecosystems that are located in areas with periodic pesticide application (Boone et al. 2007).

Effects on growth and morphology of larvae and metamorphs are easily observed in experimental or field surveys because these traits are relatively easier to measure compared to behavioral or developmental responses. Also, their measurement has low economic cost compared to physiological and genetical approaches. However, the most common responses associated to growth and morphology of larvae and metamorphs exposed to pesticide contamination are unknown, as well as the geographic distribution and publication tendencies of these studies. Thus, quantitative studies of the scientific production (*i.e.*, scientometrical analysis) can contribute to the understanding of how pesticides affect the growth and morphology of anuran larvae and metamorphs. They can also show patterns, tendencies and how the actual status of the scientific knowledge is developping (Vanti 2002). Herein, we performed a scientometrical review on evidences from experimental studies that investigated pesticide effects on growth and external morphology in anuran larvae and metamorphs. We summarized the most commom effects on growth and morphology, the most common pesticides and species tested, besides the tendencies of the scientific production.

Methods

We searched in the electronic databases Web of Science and Google Scholar using the key words “tadpole”, “larvae” and “metamorph” applying different combinations with eight keywords (pesticide, agrochemical, growth, length, mass, morphology, deformities, and malformation). From the papers found in this search, we also examined the citations and selected the studies related to the scope. We read the abstract and included those articles that apllied an experimental approach to investigate the effect of pesticides in anurans’ larvae and metamorphs between Gosner’s stage 25 and 46 (*i.e.*, corresponding to the stages of larvae and metamorphs; Gosner 1986), carried out in the laboratory, mesocosm or field, restricted to the period between January 2000 and January 2016. We did not select studies that used as subjects embryos, hatchlings and post-metamorphs. We decided to evaluate the effects on larvae and metamorphs because they are the exclusively aquatic developmental stages, and represent the critical moment during the metamorphosis process with hormone-regulated developmental stages (Hayes et al. 2006, Mann et al. 2009). We also excluded all studies that tested the effects of fertilizers or other type of contaminant.

From each included study, we extracted information about (Table S1) the (i) species name, (ii) pesticide tested – alone or mixtures, (iii) pesticide class and commercial formulation (if available), (iv) specific effects on growth and/or morphology, (v) developmental stage tested – larvae and/or metamorph, (vi) type of experimental approach – laboratory, outdoor mesocosm or field experiment, (vii) additive and/or synergetic factors acting with pesticides – if any, (viii) country where the study was conducted, (ix) year of publication and (x) journal of publication. In studies where the authors tested the effects of different types of pesticides (alone or mixed) on different species, we reported each one as a different entry (*i.e.*, the relationship of each species tested with each pesticide type or mixtures – we use throughout the text the term "cases" to define each observation). To understand the main effects of pesticides on growth and morphology, we considered all species-specific cases in relation to five distinct groups: (a) morphological changes – including malformations, deformities and any type of shape changes; (b) length reduction –

including body and tail; (c) length increase – including body and tail; (d) mass reduction and (e) mass increase.

We performed a Principal Component Analisys (PCA) to verify the existence of grouping among the pesticides (Carbaryl, Malathion, Glyphosate, Diazinon, Endosulfan, Chlorpyrifos and Atrazine) and/or an association with morphological responses, for species with at least 30 cases. We performed another PCA with the six most representative species showing at least 20 cases (*Lithobates pipiens, Dryophytes versicolor, Anaxyrus americanus, L. clamitans, L. sylvaticus* and *Xenopus laevis* – see Figure 1) to evaluate if any stress factor would have a relationship with pesticides and their specific growth and morphological responses on larvae and metamorphs. In both cases we disregarded cases that found no effects on growth and/or morphology. Also, we considered only the most representative species, avoiding species with few cases.

Results

We found a total of 117 studies that evaluated the effects on growth and external morphology in anuran larvae and metamorphs exposed to pesticide contamination, totalizing 356 species-specific cases (Table S1). These studies evaluated 50 anuran species, in which *Lithobates pipiens* was the most commonly tested, appearing in 62 cases (Figure 1). We observed a total of 43 different active ingredients, in which carbaryl, malathion and glyphosate were the most common pesticides tested, respectively (Figure 2). The most frequently tested pesticide class was insecticides, followed by herbicides and fungicides (Figure 3).

Effects on growth and external morphology were detected in 68.1% of the cases, while 31.8% reported no detected effects. The main effects detected were associated with the reduction in lenght, suggesting that pesticide contamination affects the growth rate of larvae and metamorphs. The second most frequently effect were morphological changes that include malformations and deformities (Figure 4). We did not observe a grouping among species tested, once a given anuran species could have different morphological responses despite the pesticide type (Figure 5A). Glyphosate seems to be the main agent affecting the growth rate in studies with larvae and metamorphs, either by increasing or reducing the length (Figure 5B – quadrant d); studies with malathion and chlorpyrifos tend to generate morphological malformations and deformities (Figure 5B – quadrant a); atrazine and endosulfan tend to lead to a reduction in mass (Figure 5B

– quadrant b); while carbaryl seems to be linked to increase in mass (Figure 5B - quadrant c).

From the total, 57.4% of the cases tested the larval stage, 8.4% the metamorph stage and 33.8% both stages. Most of the studies were conducted under laboratory conditions (63.2%), followed by outdoor/mesocosm (35%) and outdoor/large-scale experimental ponds (1.6%). Thirty-eight cases tested some stressful factors acting with pesticides to evaluate the additive/synergical effect on growth and morphology (see table S1). The most common factor tested was the presence of predators (12 cases), followed by the presence of competitors (11 cases) (Figure 6). We found that a given anuran species shows different responses independent of the pesticide type and stressors tested (Figure 7A). Considering the relationship among pesticides, other stressors and growth/morphology responses we observed that glyphosate and predators' cues tend to cause a length reduction of larvae and metamorphs (Figure 7B – quadrant b); carbaryl and competitors would lead to a mass increase (Figure 7B – quadrant a); while malathion and atrazine in association to ammonium nitrate and infectious agents could increase the chance of malformations and deformities (Figure 7B – quadrant c).

Studies were performed in 18 different countries, the majority of studies were performed in the United States (58.9%), followed by Canada (8.5%) (Figure 8). We did not observe a trend in the number of publications through the 15 evaluated years, with peaks of publications in the years 2003, 2008 and 2013 (Figure 9). The studies were published in 44 different journals. The majority of studies were published in the journal "Environmental Toxicology and Chemistry" (23.9%), followed by "Archives of Environmental Contamination and Toxicology" (8.5%) and Ecological Applications (7.6%) (Table 1).

Discussion

Effects on growth and external morphology

Different attributes can be used to investigate the sublethal effects of pesticides on larvae and metamorphs, as morphological variation, behavior, physiology and genetics. However, growth changes in mass and body size and external malformations, as hind-limb anomalies, axial and tail deformities, are the most dramatic effects of pesticide exposure (Mann et al. 2009). We found that most experiments reported reduction in length and mass as the main effect of pesticides to larval development. However, larvae and metamorphs can eventually respond to pesticides with a growth increase, with a few studies reporting both effects (increase or reduction in growth) under similar experimental conditions. For example, Figueiredo and Rodrigues (2014) reported the increase and reduction in body size of *Rhinella marina* and *Physalaemus centralis* exposed to five types of herbicides. Groner and Relyea (2011) observed that *Lithobates pipiens* metamorphs exposed to different concentrations of malathion/Malathion Plus® showed either an increase or a reduction in body mass. Also, these contradictory responses on growth and mass can be induced by the same pesticide type [*e.g.*, carbaryl can be associated to mass reduction (e.g., Boone and Bridge 2003, Boone et al. 2007, Groner and Relyea 2011) or mass increase (Boone and Semlitsch 2002, Boone et al. 2004, Boone 2008)]. There are different explanations for these contradictory responses. For example, pesticide contamination can act as an endocrine disruptor; especially on thyroid hormones (TH), which are responsible for regulation of the metamorphosis period (*see review in* Brown and Cai 2007). This mechanism of metamorphosis regulation can also act as an adaptive response to stress, such as pond drying, diseases or contamination (e. g. Denver 1997, Buck et al. 2012, Figueiredo and Rodrigues 2014). Thus, depending on the developmental stage, the pesticides can disrupt the early metamorphosis, resulting in smaller, poorer competitors and/or individuals more susceptible to predation; or disrupt later metamorphosis, resulting in metamorphosis delay, which increases time of exposure to the contamination and increase the susceptibility to adverse environmental effects, like pond drought, diseases and/or other sporadic or stochastic events (e.g. Berrill et al. 1993, Kiesecker 2002, Cauble and Wagner 2005, Hayes et al. 2006a, Sayim and Kaya 2006, Bulen and Distel 2011, Buck et al. 2012).

The growth rates are not only related to metamorphosis time. The contamination by pesticides changes the nutrient dynamic and the primary production, which, in turn, affects the variation in foraging rates, mediates food web disruption and/or trophic cascade effects, and increases competition (Boone and Semlitsch 2001, Boone and Bridges, 2003, Relyea 2006, Whiles et al. 2006, Relyea and Diecks 2008, Hua and Relyea 2014). Thus, growth variations can be associated to different behavioral responses related to food acquisition (*e.g.* increasing or inhibiting of the foraging rate), different levels of species susceptibility to contamination, as well as the species-specific capacity to consume food and convert it to growth (*e.g.*, Bridges 1999, Boone and Semlitsch 2001, Relyea 2004a, Whiles et al. 2006, Denoël et al. 2012). Furthermore, growth changes can be associated to species-specific detoxification mechanisms, which require energy allocation decisions mediated by a trade-off budget (Greulich and Pflugmacher 2004, Venturino and D'Angelo 2005, DuRant et al. 2007). Regardless of the source, these responses on growth of larvae and metamorphs can negatively affect processes observed in the adult stage, such as reduction of overwinter survival and reproductive potential (Smith 1987, Semlitsch et al. 1988, Berven 1990).

Morphological changes, including malformations and deformities, are the second most common response observed and are mainly associated to contamination by malathion and chlorpyrifos (Figure 5B). These morphological changes trigged by chemical substances negatively affect the individual performance and can affect different body parts, as the tail [deeper tails (Relyea 2012, Katzenberger et al. 2014), lateral flexure of the tail from its normal position (Bonfanti et al. 2004, Bernabò et al. 2011, Arcaute et al. 2012), twisting of tail (Greulich and Pflugmacher 2003, David et al. 2012), increase of stiff tails (Krishnamurthy and Smith 2011), bent curved tails (Lajmanovich et al. 2003), necrosis of the tail tip, flexure of the tail tip, fin damage, abnormal growth and blistering on the tail fin (Howe et al. 2004, David and Kartheek 2015)] and fore and hind-limbs [formation of one hind-limb only, formation of three front-limbs (Bridges 2000), limb deformities (Devi and Gupta 2013) and femoral shortening (Fort et al. 2004)]. Furthermore, several kinds of skeletal defects, variations in intestine shape, body deformities and edemas have been reported (e. g. Bridges 2000, Bridges et al. 2004, Bachetta et al. 2008, Mandrillon and Saglio 2009, Bernabò et al. 2011, Krishnamurthy and Smith 2011, David et al. 2012, Relyea 2012, Aiko et al. 2014). As subtler effects, the contamination by pesticides affects the symmetry of morphological traits associated to sensory capabilities (*e.g.*, eyes and nares) (Costa and Nomura 2016), causes deformities in the mouthparts, eyes (Lajmanovich et al. 2003), and spine malformations (*e.g.*, scoliosis, lordosis and kyphosis) (Jayawardena et al. 2010, 2011, Devi and Gupta 2013, Aiko et al. 2014, David and Kartheek 2015). These morphological traits are related to swimming activity, food acquisition, and predator and food detection, and deformities or developmental deviations can result in an increased risk of predation and reduced competitive potential (e. g. Van Buskirk and Relyea 1998, Van Buskirk and McCollum 2000, Relyea 2004c, Van Buskirk 2009, Arendt 2009). Clearly, larvae and metamorphs respond to pesticide stress through variations in body traits, as physical abnormalities. These responses can be better undesrtood by evaluating a fitness-phenotype relationship, especially due to the range of effects observed on behavior and morphological traits (Van Buskirk and McCollum 2000, Arendt 2009, Allentoft and O'Brien 2010, Woodley et al. 2015).

The interactions of pesticides with environmental factors also affect larvae life history (Relyea 2010). These factors act as limiting agents and contribute to the understanding of the additive/synergistic effects on anurans in a contamination scenario (e. g. Kiesecker 2002, Boone et al. 2007, Bancroft et al. 2008, Relyea 2012, Katzenberger et al. 2014). In addition to the negative effects of pesticides alone, we observed that many approaches tested other stressors, but the mainly environmental factor is the effect of predators and competitors (Figure 6). For example, Relyea (2012) observed that larvae of *Lithobates pipiens* presented mass reduction and changes in the tail shape when exposed to the herbicide Roundup Original MAX® in combination with chemical/physical cues of predators. Also, Katzenberger et al. (2014) observed that the contamination by Roundup Power Max®, together with predator presence, reduced the body size and modified the tail morphology of *Dryophytes versicolor* larvae. Investiganting the contamination caused by the insecticide carbaryl/Sevin®, Distel and Boone (2009) observed a mass reduction of *Anaxyrus americanus* metamorphs when density of competitors was high. Buck et al. (2012) observed an increase in growth rates of larvae and metamorphs of *Pseudacris regilla* exposed to carbaryl, and an increase of the pesticide effects when combined with the fungus *Batrachochytrium dendrobatidis* and/or interspecific competition. Growth rate increase was also observed by Cothran et al. (2011) in metamorphs of *Lithobates catesbeianus*, *L. clamitans* and *Dryophytes versicolor* exposed to malathion and the presence/absence of predators. Jones et al. (2011) observed that the contamination by Roundup Original MAX® added to increase of larvae density (*i.e.*, increased competition) leading to an increase of larval growth. Finally, Mills and Semlistch (2004) reported that the species *Lithobates sphenocephalus* exposed to carbaryl/Sevin® respond with an increase or a decrease in mass according to the competitor type and predators' presence/absence (see more species-specific cases in Table S1). Complementary stressors (*e.g.*, predator, diseases, and competitors) can directly contribute to this high response variability, but the amount of variation can be associated to the different species-specific tolerances, variation in experimental conditions, different types of commercial formulation tested, different pesticide action on hormonal regulation and others.

Different stressors increase the negative potential of a specific pesticide due to additive and/or synergistic effects (Sih et al. 2004, Blaustein et al. 2011). Most of the studies we revised had a good accuracy regarding the effects of pesticides, alone or jointly with another stressor, because they were carried out under controlled conditions in the laboratory, but they had a low degree of realism when compared to the experiments carried out in the field (Boone and James 2005). In contrast, field experiments are conducted in a large-scale environment, at the cost of accuracy due to the great number of uncontrolled variables (Boone and James 2005, Mann et al. 2009). Thus, the use of mesocosms can be a better alternative and is the most powerful experimental technique because it allows a greater control of many variables within the experimental system and allows more realism (*see review in* Boone and James 2005). Therefore, these characteristics favor the evaluation of growth and morphological responses in a context closer to natural conditions. Thus, pesticide effects can be strongly mediated by the species-specific susceptibility and different ecological contexts found in a contamination scenario, trying to simulate conditions of freshwater ecosystems.

Distribution and tendencies of publications

Geographical gaps are common in ecological studies (Martin et al. 2012, Trimble and Van-Arde 2012), limiting the knowledge about contamination on species and ecossystens in the world. We observed that most studies that experimentally evaluated the effects of pesticides on larvae and metamorphs were carried out in the United States of America and, consequently, the most tested species are from North American (*Lithobates pipiens*, *Dryophytes versicolor* and *Anaxyrus americanus*). The high number of publications from the United States can be a reflection of the high investiment in science and researcher formation, besides of the financial support obtained from public and private institutions (Mugnaini et al. 2004). However, this hypothesis needs to be tested.

The low number of species from temperate regions compared to tropical regions limits the number of species available to ecotoxicological studies in the former. Considering the recent expanse of areas converted for agriculture and pastures and the number of anuran species in the world (6656 species when this study was conducted - Frost 2016), there is a lack of ecotoxicological studies, mainly about the sublethal effects on growth and morphology. Therefore, regions with the highest numbers of amphibian species (*e.g.*, Neotropical region) have the lowest numbers of species tested regarding the effects of pesticides (Schiesari et al. 2007).

In the 15 years evaluated, we did not observe an increase over time in number of publication that experimentally evaluated the effects of the pesticides on growth and morphology of larvae and metamorphs. Once we focused on studies that measured changes in growth and morphology only, it is possible that studies that measured behavioral, phisiologycal and genetical changes, together with ecotoxicological tests for different contaminants (*e.g.* heavy metals, fertilizers), could explain the variation in the number of studies over time. Also, many studies only evaluated the effects of acute contamination (i.e. lethal effects), not evaluating the sublethal responses of chronic exposure.

The publications were directed to ecotoxicological journals (*e.g.* Environmental Toxicology and Chemistry; Archives of Environmental Contamination and Toxicology), with less publications in ecology, herpetology and/or conservation journals (Table 1). Manuscript publication in specialized ecotoxicological journals can favor the comunication among researchers and the search of comparative studies. Also, publication in specialized journals increases the outreach of new discoveries, potentially increasing the number of citations.

Concluding Remarks

Growth and morphological responses are easily identified and can directly affect fitness and survival of individuals, increasing their susceptibility to stochastic events, the risk of predation and decreasing competitive ability (Brodie Jr. et al. 1991, Steiner and Van Buskirk 2008, Van Buskirk 2009, Relyea 2012). Also, the interactive effects of pesticides with multiple stressors are important to understand the contribution of these factors to population declines (Boone et al. 2007, Baker et al. 2013).

Studies that examined the interaction of pesticides with different stressors, such as predators, competitors, pathogens, climate changes and habitat alterations, can contribute to mitigate ecological impacts and optimize the efforts directed to species conservation (Boone et al. 2007, Baker et al. 2013). It is important that future studies evaluate how pesticides interact with different stressors, especially those resulting from human actions (*i.e.*, global warming, contamination, pollution, habitat changes). It is also necessary to evaluate how the different species traits, especially those related to individual fitness, respond to stressor interactions and how chronic responses contribute to amphibian species loss.

In 2016, 6692 species of anurans were known in the world (Frost 2016) – currently there are 6861 described anuran species (Frost 2017). Considering that only 50 species were identified in this review (0.75% of species), we can conclude that there is a great knowledge gap about the negative effects of pesticides on the growth and morphology of larvae and metamorphs. Furthermore, most studies were conducted with species that occur in countries located in the temperate zones (*e.g.*, United States – where there is money for science). In contrast, few studies have been conducted in tropical zones or countries with high anuran diversity (*e.g.*, Brazil – where there is no money for science). The higher biodiversity in the tropics and the heavy use of pesticides, added to the advance of croplands, turns the study of pesticide impacts and their interaction with different stressors a priority to conservation studies. Also, increasing the number of tested species, especially rare, endemic and/or threatened species, would be important to discriminate between general and specific responses of amphibians to pesticide contamination.

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Figures and Tables

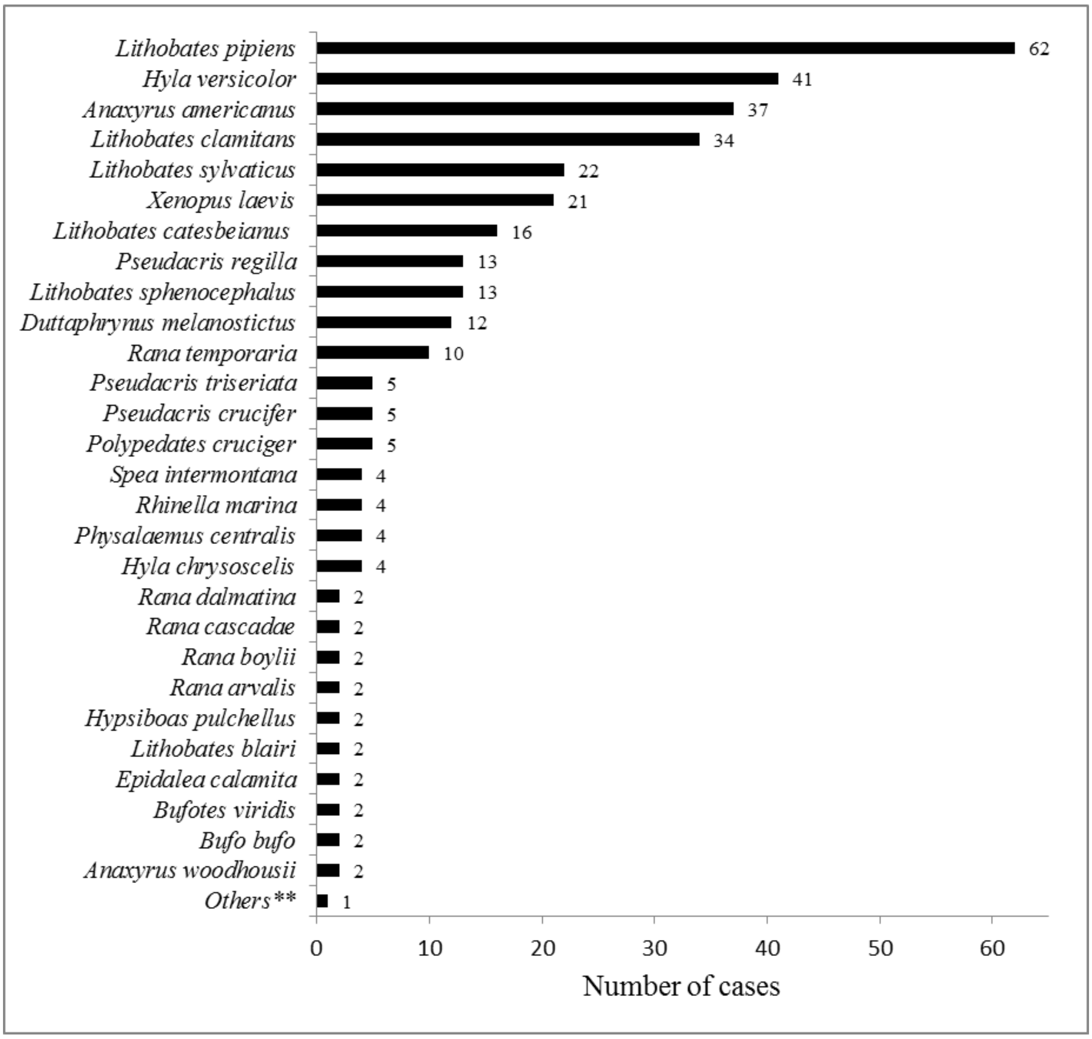

Figure 1. Species considered in the experimental studies and the number of cases found for each species. **Others: species with one case - *Acris crepitans, Bombina variegata, Discoglossus jeanneae, Fejervarya limnocharis, Fejervarya sp.1, Fejervarya sp.2, Fejervarya teraiensis, Gastrophryne olivacea, Hoplobatrachus rugulosus, Hyla arborea, Limnodynastes peronii, Litoria freycineti, Litoria peronii, Osteopilus septentrionalis, Pelobates cultripes, Pelophylax perezi, Pelophylax ridibundus, Physalaemus cuvieri, Rana aurora, Rhinella fernandezae, Scinax nasicus, Spea multiplicata.*

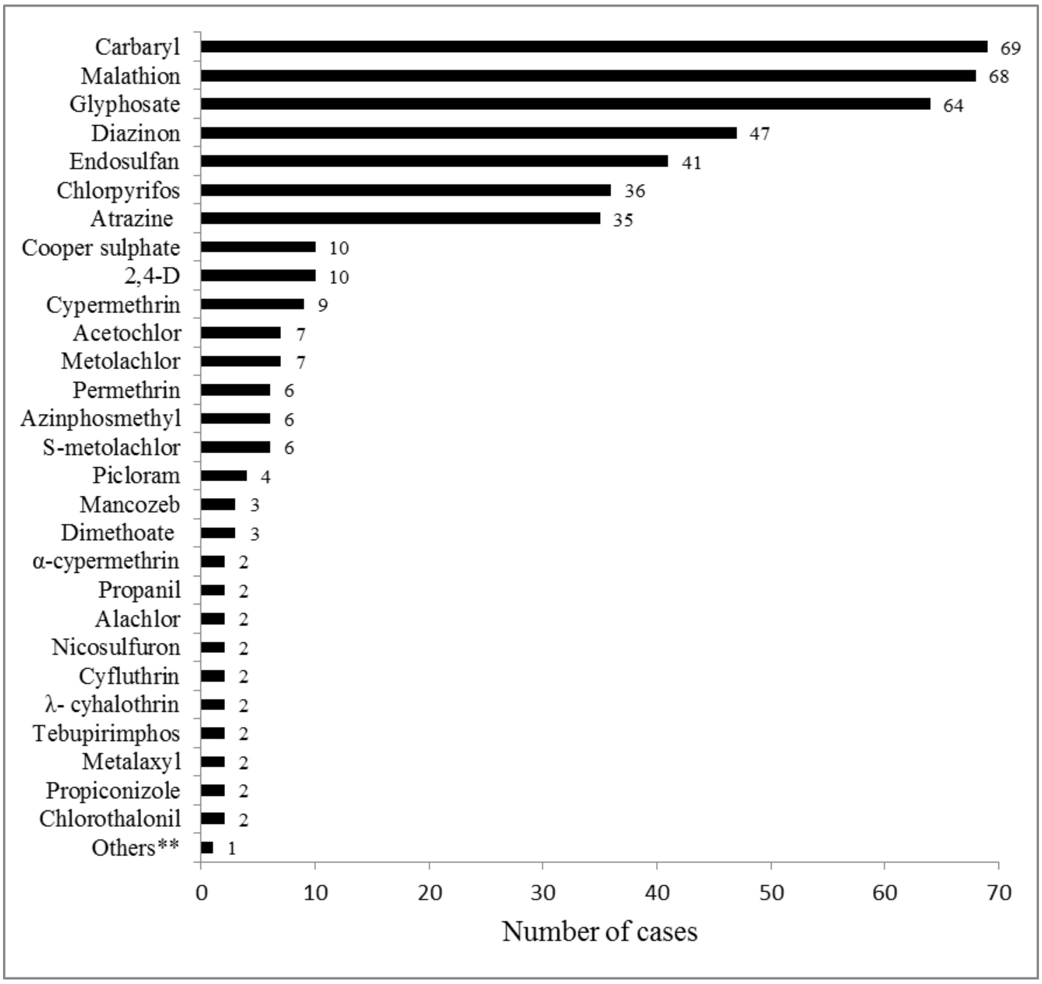

Figure 2. Active ingredients considered in the experimental studies and the number of cases with each active ingredient (including pesticide mixtures). **Others: pesticides with one case – Acephate, Amitrole, Azoxystrobin, Carbendazim, Cyanazine, Edifenphos, Esfenvalerate, Fenpropimorph, MCPA, Methoxychlor, N-butyl isocyanate, Pirimicarb, POEA, Prochloraz, Thiophanate-methyl.

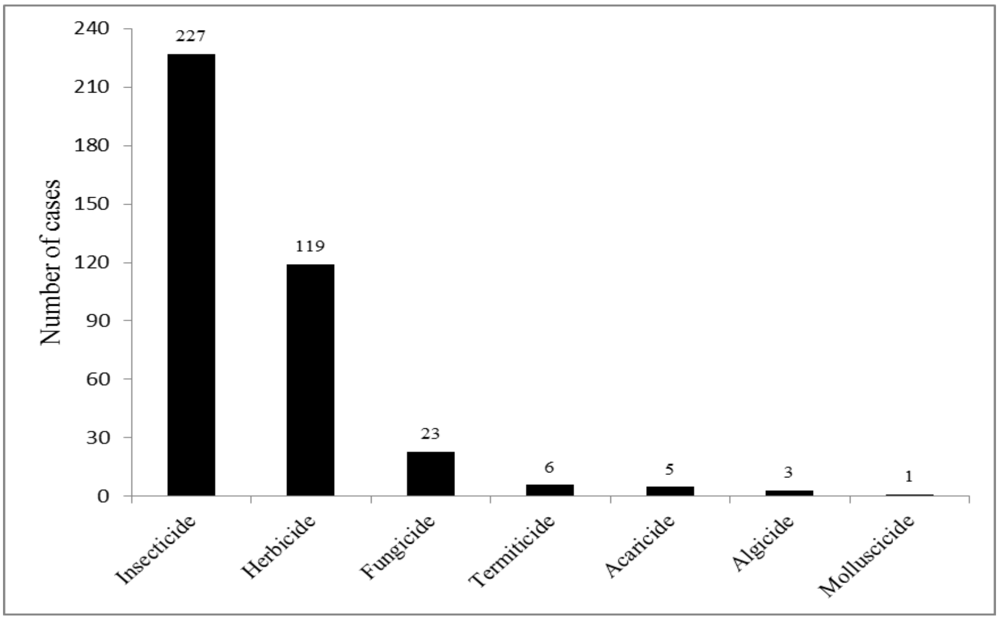

Figure 3. Number os cases that evaluated each pesticide class (including pesticide mixtures).

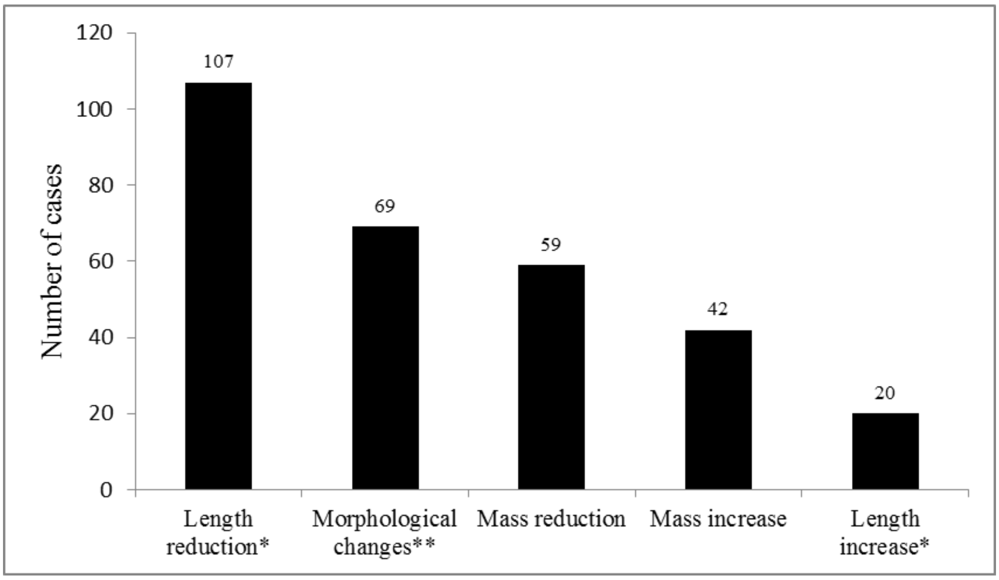

Figure 4. Number of cases that detected the effects on growth and morphology in larvae and metamorphs. *Length reduction and length increase: include body and tail. ** morphological changes – include malformations, deformities and any type of shape changes.

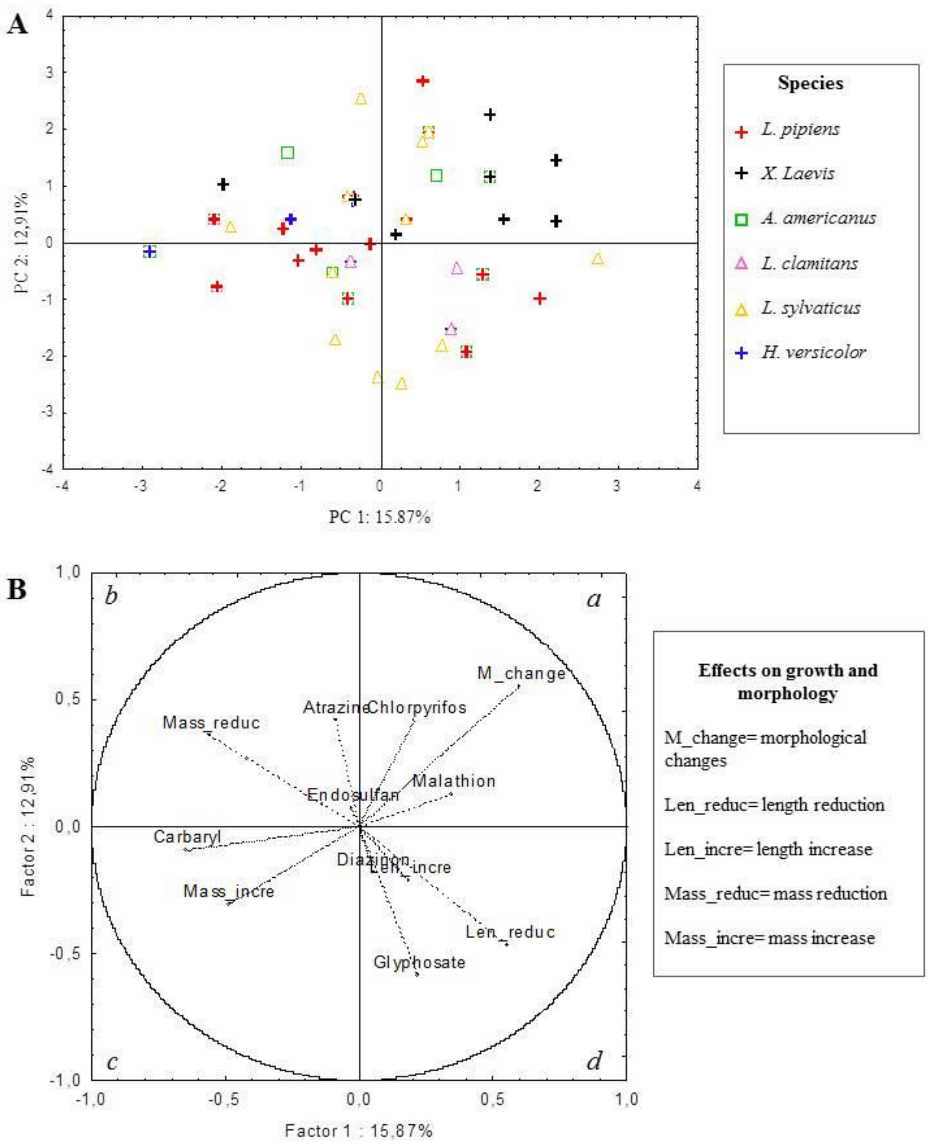

Figure 5. Principal Component Analisys with the most representantive species (A), the most representative pesticides (carbaryl, malathion, glyphosate, diazinon, endosulfan, chlopyrifos and atrazine) and the main effects on growth and morphology (B).

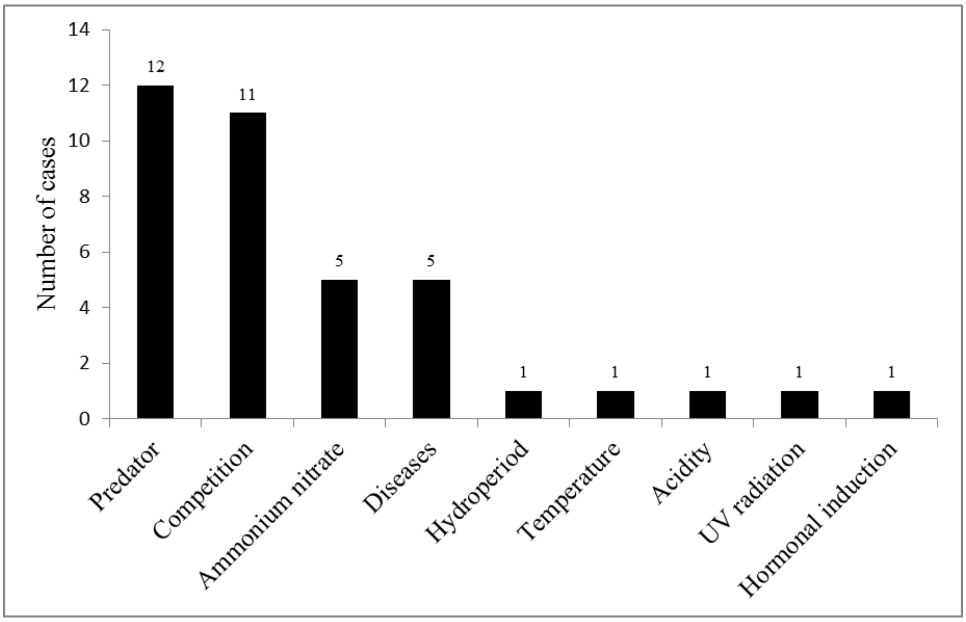

Figure 6. Number of cases that tested other stressors acting with pesticides.

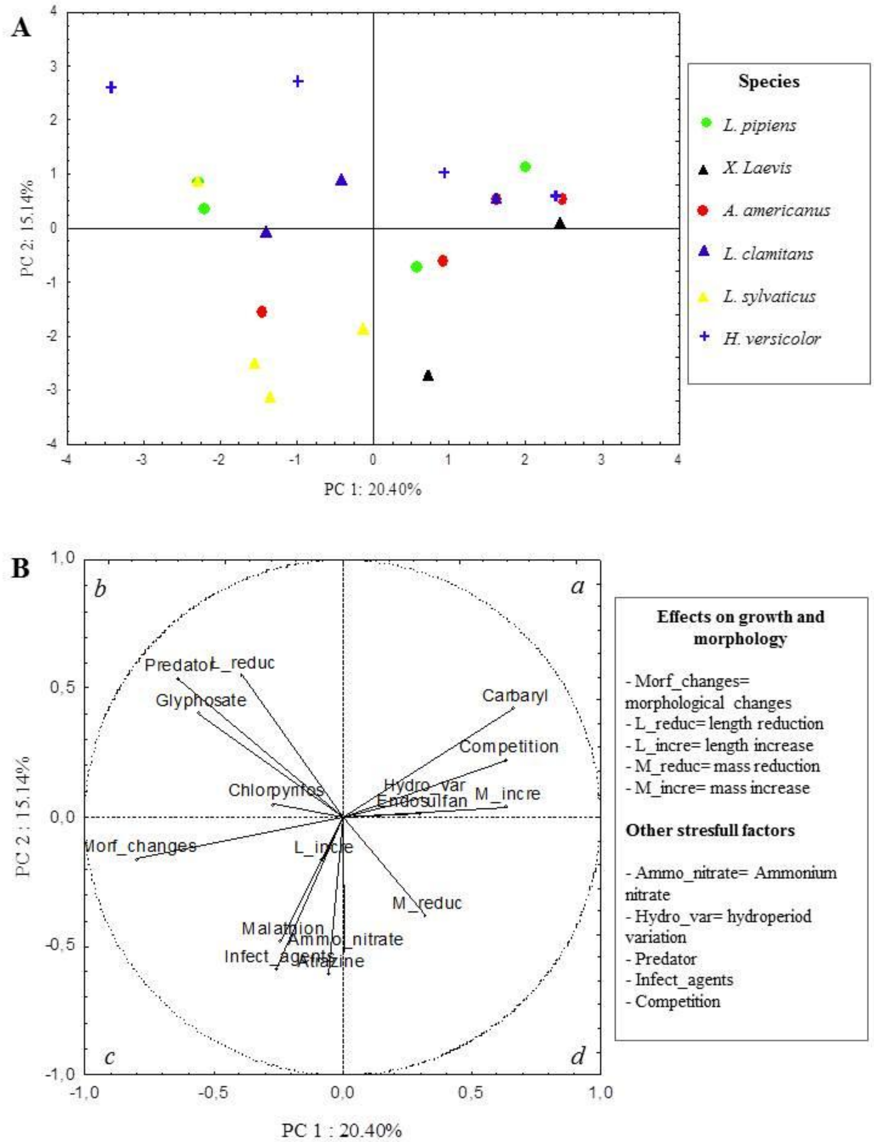

Figure 7. Principal Component Analisys with the most representantive species (A) and pesticides (carbaryl, malathion, glyphosate, endosulfan, chlorpyrifos and atrazine) that tested the effects on growth and morphology acting with other factors (B).

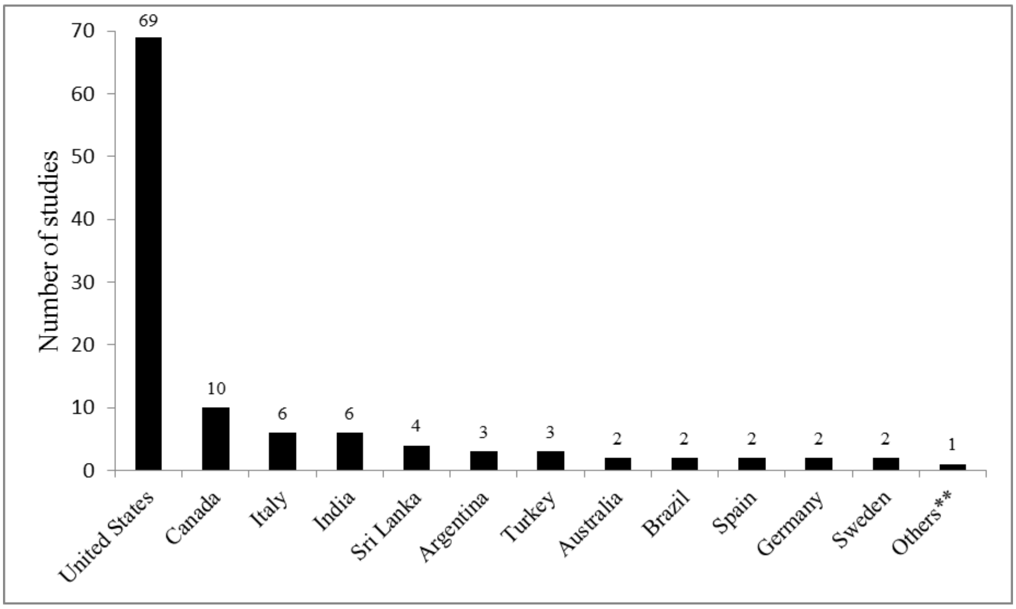

Figure 8. Number of studies conducted in each country. **Others: countries with one study – Albania, Belgium, Denmark, France, Korea, Thailand.

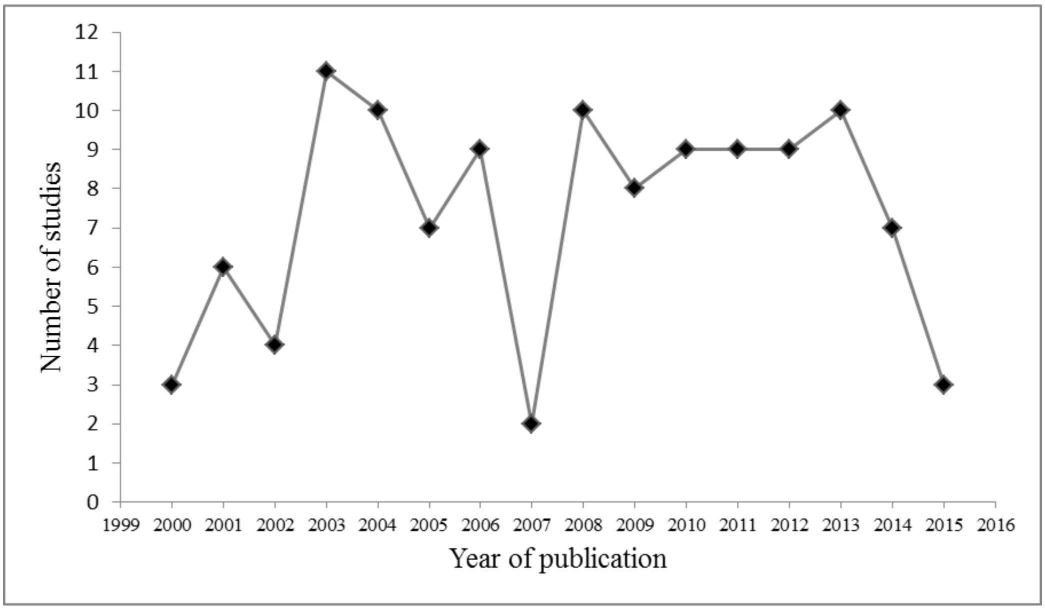

Figure 9. Number of studies published from 2000 to January 2016.

Table 1. Number of studies published in each journal.

Journal of publication	Number of studies
Environmental Toxicology and Chemistry	28
Archives of Environmental Contamination and Toxicology	10
Ecological Applications	9
Aquatic Toxicology	8
Ecotoxicology	6
Bulletin of Environmental Contamination and Toxicology	5
Oecologia	4
Proceedings of the National Academy of Science USA	3
Turkish Jounal of Zoology	3
Environmental Health Perspectives	2
Environmental Pollution	2
Freshwater Biology	2
Functional Ecology	2
Journal of the National Science Foundation of Sri Lanka	2
Plos ONE	2
4th ICE Conference	1
Science of the Total Environment	1
Conservation Biology	1
Biological Conservation	1
BIOS	1
Bioscene	1
Chemosphere	1
Ecological Economics	1
Ecotoxicology and Environmental Safety	1
Environmental Science & Technology	1

Environmental Science and Pollution Research	1
Environmental Toxicology	1
Herpetological Journal	1
Hydrobiologia	1
International Journal of Agriculture and Biology	1
Journal of Applied Ecology	1
Journal of Herpetology	1
Journal of the North American Benthological Society	1
Journal of Toxicology and Environmental Health-Part A	1
Journal of Toxicology, Environment and Health	1
Maejo International Journal of Science and Technology	1
Oikos	1
Pesticide Biochemistry and Physiology	1
Proceedings of the Royal Society B	1
The Journal of Basic and Applied Zoology	1
The Open Zoology Journal	1
Toxicological & Environmental Chemistry	1
Toxicological Sciences	1
Zoo’s Print Journal	1

Supplementary material

Table S1: Species-specific cases of the pesticide effects on growth and external morphology in tadpoles and metamorphs [stage 25 to 46 (Gosner 1960)]. The species names were updated according to Frost (2016). St.= stage; L= Larvae; M= Metamorph; B= Both.

Species	**St.**	**Country**	**Experimental site**	**Active ingredient / formulation (if there)**	**Class**	**Effects on growth and/or external morphology**	**Other stressor factor*** **	**Reference**
Acris crepitans	L	United States	Laboratory	Chlorpyrifos/Dursban TC®	Termiticide	Snout-vent length reduction	No	Widder and Bidwell 2008
Anaxyrus americanus	L	United States	Laboratory	Atrazine	Herbicide	Malformations (wavy tail, lateral tail flexure, facial edema, axial shortening, dorsal tail flexure and blistering)	No	Allran and Kasarov 2001
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone 2008
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Malathion/Malathion®	Insecticide	No effects on growth or morphology	No	Boone 2008
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Permethrin/Cutter's Bug Free Back Yard®	Insecticide	No effects on growth or morphology	No	Boone 2008
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Carbaryl + malathion - mixture/Sevin® + Malathion®	Insecticides	Mass reduction	No	Boone 2008
Anaxyrus americanus	L	United	Outdoor /	Carbaryl/Sevin®	Insecticide	Mass reduction; mass increase	Constant and drying	Boone and James

		States	mesocosm				hydroperiod	2003
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Atrazine/Aatrex®	Herbicide	Mass reduction	No	Boone and James 2003
Anaxyrus americanus	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction	Ammonium nitrate and bullfrogs	Boone et al. 2007
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Bulen and Distel 2011
Anaxyrus americanus	M	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction	High density	Distel and Boone 2009
Anaxyrus americanus	M	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Distel and Boone 2010
Anaxyrus americanus	L	Canada	Laboratory	Glyphosate/Vision®	Herbicide	No effects on growth or morphology	No	Edginton et al. 2004
Anaxyrus americanus	B	Canada	Laboratory	Endosulfan / Thiodan®50WP	Insecticide	Deformities (eye deformities, luxation of the right front limb)	No	Harris et al. 2000
Anaxyrus americanus	B	Canada	Laboratory	Mancozeb/Dithane® DG	Fungicide	Deformities (eyes missing)	No	Harris et al. 2000
Anaxyrus americanus	B	Canada	Laboratory	Azinphos-methyl / Guthion®50WP	Insecticide	No effects on growth or morphology	No	Harris et al. 2000
Anaxyrus americanus	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Anaxyrus americanus	B	United	Outdoor /	Diazinon	Insecticide	No effects on growth or morphology	No	Hua and Relyea

		States	mesocosm					2014
Anaxyrus americanus	B	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Anaxyrus americanus	B	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Anaxyrus americanus	B	United States	Outdoor / mesocosm	Chlorpyrifos + diazinon + malathion + endosulfan – mixture	Insecticides	No effects on growth or morphology	No	Hua and Relyea 2014
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	Mass reduction	No	Jones et al. 2010
Anaxyrus americanus	L	United States	Laboratory	Malathion/Matathion®	Insecticide	Deformities (diamond-shaped body and stiff-tail)	Amonium nitrate	Krishnamurthy and Smith 2010
Anaxyrus americanus	L	United States	Laboratory	Carbaryl/Sevin®	Insecticide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Diazinon	Insecticide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Malathion	Insecticide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Glyphosate/Roundup®	Herbicide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Carbaryl + diazinon - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a

Anaxyrus americanus	L	United States	Laboratory	Carbaryl + malathion - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Carbaryl + glyphosate - mixture/Sevin® + Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Diazinon + malathion – mixture	Insecticides	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Diazinon + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Laboratory	Malathion + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Anaxyrus americanus	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	No effects on growth or morphology	No	Relyea 2012
Anaxyrus americanus	B	United States	Laboratory	Atrazine/Atrazine 4l®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Anaxyrus americanus	B	United States	Laboratory	S-metolachlor/Dual II Magnum®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Anaxyrus americanus	B	United States	Laboratory	Glyphosate/Roundup Original MAX®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Anaxyrus americanus	B	United States	Laboratory	Glyphosate/Roundup WeatherMax®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Anaxyrus woodhousii	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone and Semlitsch 2001

Anaxyrus woodhousii	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	No	Boone and Semlitsch 2002
Anaxyrus woodhousii	B	United States	Outdoor / large-scale experimental ponds	Carbaryl/Sevin®	Insecticide	Mass increase	Low density	Boone et al. 2004
Bombina variegata	L	Germany	Laboratory	Cypermethrin	Insecticide	Physical abnormalities	No	Greulich and Pflugmacher 2004
Bufo bufo	B	Italy	Laboratory	Endosulfan	Insecticide	Mass reduction; malformations (axis, skeletal, tail and mouth malformations, edemas and lateral kink at the base of the tale)	No	Brunelli et al. 2009
Bufo bufo	L	Spain	Laboratory	Cooper sulfate	Fungicide	Total length reduction	No	García-Munõz et al. 2010
Bufotes viridis	L	Albania	Laboratory	Cooper sulfate	Algicide, fungicide and molluscicide	Total length reduction; malformations (spinal cord deformity and edemas)	No	Aiko et al. 2014
Bufotes viridis	L	Turkey	Laboratory	Copper sulfate	Fungicide and algicide	Reduction in body size, body width, and tail length; anomalies (edemas)	No	Gürkan and Hayretdağ 2012
Discoglossus jeanneae	L	Spain	Laboratory	Cooper sulfate	Fungicide	Total length reduction	No	García-Munõz et al. 2010
Duttaphrynus melanostictus	L	India	Laboratory	Malathion	Insecticide	Malformations (scoliosis, lordosis, kyphosis, fin blistering)	No	David and Kartheek 2015
Duttaphrynus melanostictus	L	India	Laboratory	Cypermethrin	Insecticide	Malformations (deformities in coiled intestine, twisting of tail, changes in	No	David et al. 2012

						axial region, loss of conveyance of tail fin and deformities in the head)		
Duttaphrynus melanostictus	B	India	Laboratory	Acephate/Not specified	Insecticide	Growth reduction; malformations (crooked tails, drooped trunk, edema and split at the tail terminal)	No	Ghodageri and Pancharatna 2011
Duttaphrynus melanostictus	B	India	Laboratory	Cypermethrin/Not specified	Insecticide	Length reduction; malformations (drooped trunks, tail distortions and head deformities)	No	Ghodageri and Pancharatna 2011
Duttaphrynus melanostictus	B	Sri Lanka	Laboratory	Chlorpyrifos/Lorsban EC 40® or Pattas®	Insecticide	Snout-vent length increase; mass increase; spine malformations (kyphosis, lordosis, edemas and skin ulcers)	No	Jayawardena et al. 2011
Duttaphrynus melanostictus	B	Sri Lanka	Laboratory	Dimethoate /Dimethoate EC®	Insecticide	Snout-vent length increase; mass increase; spine malformations (kyphosis, lordosis, edemas and skin ulcers)	No	Jayawardena et al. 2011
Duttaphrynus melanostictus	B	Sri Lanka	Laboratory	Glyphosate/Roundup® or Glyphosate®	Herbicide	Snout-vent length increase; mass increase; spine malformations (kyphosis, lordosis, edemas and skin ulcers)	No	Jayawardena et al. 2011
Duttaphrynus melanostictus	B	Sri Lanka	Laboratory	Propanil/3,4 DPA®	Herbicide	Snout-vent length increase; mass increase	No	Jayawardena et al. 2011
Duttaphrynus melanostictus	L	India	Laboratory	Edifenphos/Hinosan EC®	Fungicide	Mass and length reduction	No	Mathew and Andrews 2003

Duttaphrynus melanostictus	L	India	Laboratory	Endosulfan/Endosulfan 3EC®	Insecticide	Mass and length reduction	No	Mathew and Andrews 2003
Duttaphrynus melanostictus	L	Sri Lanka	Outdoor	Diazinon	Insecticide	Body length reduction	No	Sumanadasa et al. 2008a
Duttaphrynus melanostictus	L	Sri Lanka	Laboratory	Diazinon	Insecticide	Size reduction; abnormalities (bent tails, curved tails and slanted bodies)	No	Sumanadasa et al. 2008b
Epidalea calamita	L	Spain	Laboratory	Cooper sulfate	Fungicide	Total length reduction	No	García-Munõz et al. 2009
Epidalea calamita	L	Spain	Laboratory	Cooper sulfate	Fungicide	Total length reduction	No	García-Munõz et al. 2010
Fejervarya limnocharis	L	India	Laboratory	Malathion	Insecticide	Mass reduction; reduction in total lenght, body lenght and tail length	No	Gurushankara et al. 2007
Fejervarya sp.1	B	India	Laboratory	Endosulfan/Hildan 35 EC®	Insecticide and acaricide	Morphological deformities (fore-limb deformities, axial malformation and hind-limbs deformities)	No	Devi and Gupta 2013
Fejervarya sp.2	B	India	Laboratory	Endosulfan/Hildan 35 EC®	Insecticide and acaricide	Morphological deformities (axial malformation)	No	Devi and Gupta 2013
Fejervarya teraiensis	B	India	Laboratory	Endosulfan/Hildan 35 EC®	Insecticide and acaricide	Morphological deformities (fore-limb deformity)	No	Devi and Gupta 2013
Gastrophryne olivacea	L	United States	Laboratory	Chlorpyrifos/Dursban TC®	Termiticide	Mass reduction; snout-vent length reduction	No	Widder and Bidwell 2008
Hoplobatrachus rugulosus	L	Thailand	Laboratory	Atrazine/Not specified	Herbicide	Body length reduction; asymmetrical	No	Trachantong et al.

						limbs		2013
Hyla arborea	L	Turkey	Laboratory	Dimethoate	Insecticide	Total length reduction; tail deformities (bent tail)	No	Sayim and Kaya 2006
Hyla chrysoscelis	M	United States	Laboratory	Carbaryl/Sevin®	Insecticide	Mass increase	No	Gaietto et al. 2014
Hyla chrysoscelis	M	United States	Laboratory	Cooper sulfate	Fungicide	No effects on growth or morphology	No	Gaietto et al. 2014
Hyla chrysoscelis	M	United States	Outdoor / mesocosm	Malathion/Malathion®	Insecticide	Mass increase	No	Mackey and Boone 2009
Hyla chrysoscelis	L	United States	Laboratory	Chlorpyrifos/Dursban TC®	Termiticide	Mass reduction	No	Widder and Bidwell 2008
Hyla versicolor	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	No	Boone and Bridges-Britton 2006
Hyla versicolor	B	United States	Outdoor / mesocosm	Atrazine/Aatrex®	Herbicide	No effects on growth or morphology	No	Boone and Bridges-Britton 2006
Hyla versicolor	B	United States	Outdoor / mesocosm	Carbaryl + Atrazine – mixture	Insecticide and herbicide	No effects on growth or morphology	No	Boone and Bridges-Britton 2006
Hyla versicolor	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction; mass increase	Predator presence; high and low	Boone and Semlitsch 2001

							density	
Hyla versicolor	B	United States	Outdoor / mesocosm	Malathion	Insecticide	Mass increase	No	Cothran et al. 2011
Hyla versicolor	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	Mass increase	No	Hua and Relyea 2014
Hyla versicolor	B	United States	Outdoor / mesocosm	Diazinon	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Hyla versicolor	B	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Hyla versicolor	B	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Hyla versicolor	B	United States	Outdoor / mesocosm	Chlorpyrifos + diazinon + malathion + endosulfan – mixture	Insecticides	Mass increase	No	Hua and Relyea 2014
Hyla versicolor	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	No effects on growth or morphology	No	Jones et al. 2011
Hyla versicolor	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Power Max®	Herbicide	Size reduction; smaller bodies; deeper tails	Predator cues	Katzenberger et al. 2014
Hyla versicolor	B	United States	Laboratory	Atrazine	Herbicide	No effects on growth or morphology	No	LaFiandra et al. 2008
Hyla versicolor	L	United States	Laboratory	Carbaryl/Sevin®	Insecticide	Growth reduction	No	Relyea 2004a

Hyla versicolor	L	United States	Laboratory	Diazinon	Insecticide	Growth reduction	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Malathion	Insecticide	No effects on growth or morphology	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Glyphosate/Roundup®	Herbicide	No effects on growth or morphology	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Carbaryl + diazinon - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Carbaryl + malathion - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Carbaryl + glyphosate - mixture/Sevin® + Roundup®	Insecticide and herbicide	No effects on growth or morphology	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Diazinon + malathion – mixture	Insecticides	Growth reduction	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Diazinon + glyphosate - mixture/Roundup®	Insecticide and herbicide	No effects on growth or morphology	No	Relyea 2004a
Hyla versicolor	L	United States	Laboratory	Malathion + glyphosate - mixture/Roundup®	Insecticide and herbicide	No effects on growth or morphology	No	Relyea 2004a
Hyla versicolor	L	United States	Outdoor / mesocosm	Carbaryl	Insecticide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Relyea 2009

Hyla versicolor	L	United States	Outdoor / mesocosm	Diazinon	Insecticide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Carbaryl + malathion + chlorpyrifos + diazinon + endosulfan – mixture	Insecticides	Mass increase	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Acetochlor	Herbicide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Metolachlor	Herbicide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Glyphosate	Herbicide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	2,4-D	Herbicide	No effects on growth or morphology	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Atrazine	Herbicide	Mass increase	No	Relyea 2009
Hyla versicolor	L	United States	Outdoor / mesocosm	Acetochlor + metolachlor + glyphosate + 2,4-D + atrazine – mixture	Herbicides	No effects on growth or morphology	No	Relyea 2009

Hyla versicolor	L	United States	Outdoor / mesocosm	Carbaryl + malathion + chlorpyrifos + diazinon + endosulfan + acetochlor + metolachlor + glyphosate + 2,4-D + atrazine – mixture	Herbicides and insecticides	Mass increase	No	Relyea 2009
Hyla versicolor	L	United States	Laboratory	Carbaryl	Insecticide	Growth reduction	Predator cues	Relyea and Mills 2001
Hyla versicolor	B	United States	Laboratory	Atrazine/Atrazine 4l®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Hyla versicolor	B	United States	Laboratory	S-metolachlor/Dual II Magnum®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Hyla versicolor	B	United States	Laboratory	Glyphosate/Roundup Original MAX®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Hyla versicolor	B	United States	Laboratory	Glyphosate/Roundup WeatherMax®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Hypsiboas pulchellus	L	Argentina	Laboratory	Cypermethrin	Insecticide	Body length reduction; malformations (axial, eyes, gut, head and face abnormalities)	No	Agostini et al. 2010
Hypsiboas pulchellus	L	Argentina	Laboratory	Cypermethrin/Sherpa®	Insecticide	Body length reduction; malformations (axial, eyes, gut, head and face abnormalities)	No	Agostini et al. 2010
Limnodynastes peronii	L	Australia	Laboratory	Endodulfan/Thiodan®	Insecticide and acaricide	Total length reduction	Egg-rearing temperature (warm and	Broomhall 2004

							cool)	
Lithobates blairi	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone and Semlitsch 2002
Lithobates blairi	L	United States	Laboratory	Glyphosate/Kleeraway®	Herbicide	No effects on growth or morphology	No	Smith 2001
Lithobates catesbeianus	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	No	Boone and Semlitsch 2003
Lithobates catesbeianus	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	Ammonium nitrate and predator	Boone et al. 2007
Lithobates catesbeianus	B	United States	Outdoor / mesocosm	Malathion	Insecticide	Growth increase	Predator	Cothran et al. 2011
Lithobates catesbeianus	L	United States	Laboratory	Malathion	Insecticide	Body length increase	No	Fordham et al. 2001
Lithobates catesbeianus	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	No effects on growth or morphology	No	Jones et al. 2011
Lithobates catesbeianus	L	United States	Laboratory	Carbaryl/Sevin®	Insecticide	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Diazinon	Insecticide	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Malathion	Insecticide	Growth reduction	No	Relyea 2004a

Lithobates catesbeianus	L	United States	Laboratory	Glyphosate/Roundup®	Herbicide	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Carbaryl + diazinon - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Carbaryl + malathion - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Carbaryl + glyphosate - mixture/Sevin® + Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Diazinon + malathion – mixture	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Diazinon + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Laboratory	Malathion + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates catesbeianus	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Growth increase	Acidity increase	Relyea 2006
Lithobates clamitans	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	No	Boone 2008
Lithobates clamitans	L	United States	Outdoor / mesocosm	Malathion/Malathion®	Insecticide	No effects on growth or morphology	No	Boone 2008
Lithobates clamitans	L	United States	Outdoor / mesocosm	Permethrin/Cutter’s Bug Free Back Yard®	Insecticide	Mass increase	No	Boone 2008

Lithobates clamitans	L	United States	Outdoor / mesocosm	Permethrin + malathion - mixture/Cutter’s Bug Free Back Yard® + Malathion®	Insecticides	No effects on growth or morphology	No	Boone 2008
Lithobates clamitans	L	United States	Outdoor / mesocosm	Permethrin + carbaryl - mixture/Cutter’s Bug Free Back Yard® + Sevin®	Insecticides	No effects on growth or morphology	No	Boone 2008
Lithobates clamitans	L	United States	Outdoor / mesocosm	Malathion + carbaryl - mixture/Malathion® + Sevin®	Insecticides	No effects on growth or morphology	No	Boone 2008
Lithobates clamitans	L	United States	Outdoor / mesocosm	Permethrin + malathion + carbaryl - mixture/Cutter's Bug Free Back Yard® + Malathion® + Sevin®	Insecticides	No effects on growth or morphology	No	Boone 2008
Lithobates clamitans	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction	Density increase	Boone and Bridges 2003
Lithobates clamitans	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone and Semlitsch 2001
Lithobates clamitans	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction	No	Boone and Semlitsch 2002
Lithobates clamitans	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction	No	Boone et al. 2001
Lithobates clamitans	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone et al. 2005
Lithobates clamitans	L	United	Laboratory	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone et al. 2013

		States						
Lithobates clamitans	B	United States	Outdoor / mesocosm	Malathion	Insecticide	Growth increase	Predator	Cothran et al. 2011
Lithobates clamitans	L	Canada	Outdoor / large-scale experimental ponds	Glyphosate / VisionMAX®	Herbicide	No effects on growth or morphology	No	Edge et al. 2012
Lithobates clamitans	L	Canada	Laboratory	Glyphosate/Vision®	Herbicide	No effects on growth or morphology	No	Edginton et al. 2004
Lithobates clamitans	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates clamitans	B	United States	Outdoor / mesocosm	Diazinon	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates clamitans	B	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates clamitans	B	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates clamitans	B	United States	Outdoor / mesocosm	Chlorpyrifos + diazinon + malathion + endosulfan – mixture	Insecticides	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates clamitans	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	Mass increase	Tadpole density	Jones et al. 2011
Lithobates clamitans	M	United	Outdoor /	Malathion/Malathion®	Insecticide	Mass increase	No	Mackey and

		States	mesocosm					Boone 2009
Lithobates clamitans	L	United States	Laboratory	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Diazinon	Insecticide	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Malathion	Insecticide	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Glyphosate/Roundup®	Herbicide	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Carbaryl + diazinon - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Carbaryl + malathion - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Carbaryl + glyphosate - mixture/Sevin® + Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Diazinon + malathion – mixture	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Diazinon + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United States	Laboratory	Malathion + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates clamitans	L	United	Outdoor /	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Relyea 2006

		States	mesocosm					
Lithobates pipiens	B	United States	Laboratory	Atrazine	Herbicide	No effects on growth or morphology	No	Allran and Kasarov 2000
Lithobates pipiens	L	United States	Laboratory	Atrazine	Herbicide	Malformations (wavy tail, lateral tail flexure, facial edema, axial shortening, dorsal tail flexure and blistering)	No	Allran and Kasarov 2001
Lithobates pipiens	M	United States	Laboratory	Atrazine + Carbaryl – mixture	Herbicide and insecticide	Mass reduction; increase the number of bony triangles; increase the incidence of skin webbings	UV radiation	Bridges et al. 2004
Lithobates pipiens	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Bulen and Distel 2011
Lithobates pipiens	M	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	Low density	Distel and Boone 2010
Lithobates pipiens	L	Canada	Laboratory	Glyphosate/Vision®	Herbicide	No effects on growth or morphology	No	Edginton et al. 2004
Lithobates pipiens	M	United States	Outdoor / mesocosm	Malathion/Malathion Plus®	Insecticide	Mass reduction; mass increase	No	Groner and Relyea 2011
Lithobates pipiens	M	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass reduction	No	Groner and Relyea 2011
Lithobates pipiens	B	Canada	Laboratory	Endosulfan/Thiodan®50WP	Insecticide	No effects on growth or morphology	No	Harris et al. 2000
Lithobates pipiens	B	Canada	Laboratory	Mancozeb/Dithane®DG	Fungicide	No effects on growth or morphology	No	Harris et al. 2000
Lithobates pipiens	B	Canada	Laboratory	Azinphos-	Insecticide	Deformities (eyes missing)	No	Harris et al. 2000

				methyl/Guthion®50WP				
Lithobates pipiens	M	United States	Laboratory	Atrazine	Herbicide	Snout-vent length reduction; mass reduction	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Alachlor	Herbicide	No effects on growth or morphology	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Nicosulfuron	Herbicide	No effects on growth or morphology	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Cyfluthrin	Insecticide	Snout-vent length reduction	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	λ- cyhalothrin	Insecticide	No effects on growth or morphology	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Tebupirimphos	Insecticide	Snout-vent length reduction; mass reduction	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Metalaxyl	Fungicide	No effects on growth or morphology	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Propiconizole	Fungicide	No effects on growth or morphology	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	S-metolachlor	Herbicide	No effects on growth or morphology	No	Hayes et al. 2006
Lithobates pipiens	M	United States	Laboratory	Atrazine-metolachlor/Bicep II Magnum®	Herbicide	Snout-vent length reduction; mass reduction	No	Hayes et al. 2006
Lithobates pipiens	M	United	Laboratory	Atrazine + s-metolachlor –	Herbicides	Snout-vent length reduction; mass	No	Hayes et al. 2006

		States		mixture		reduction		
Lithobates pipiens	M	United States	Laboratory	Atrazine + alachlor + nicosulfuron + cyfluthrin + λ-cyhalothrin + tebupirimphos + metalaxyl + propiconizole + s-metolachlor – mixture	Insecticides, herbicides and fungicides	Snout-vent length reduction; mass reduction	No	Hayes et al. 2006
Lithobates pipiens	L	Canada	Laboratory	Glyphosate	Herbicide	Snout–vent length reduction	No	Howe et al. 2004
Lithobates pipiens	L	Canada	Laboratory	Polyethoxylated tallowamine surfactant (POEA)/Surfactante substance	Herbicide	Snout–vent length reduction; tail lenght reduction; tail damage (necrosis of the tail tip, flexure of the tail tip, fin damage, abnormal growths on the tail tip and blistering on the tail fin)	No	Howe et al. 2004
Lithobates pipiens	L	Canada	Laboratory	Glyphosate/Roundup Original®	Herbicide	Snout–vent length reduction; tail lenght reduction; tail damage (necrosis of the tail tip, flexure of the tail tip, fin damage, abnormal growths on the tail tip and blistering on the tail fin)	No	Howe et al. 2004
Lithobates pipiens	L	Canada	Laboratory	Glyphosate/Roundup Transorb®	Herbicide	Snout–vent length reduction; tail lenght reduction; tail damage (necrosis of the tail tip, flexure of the tail tip, fin damage, abnormal growths on the tail tip and blistering on the tail fin)	No	Howe et al. 2004
Lithobates pipiens	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014

Lithobates pipiens	B	United States	Outdoor / mesocosm	Diazinon	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates pipiens	B	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates pipiens	B	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates pipiens	B	United States	Outdoor / mesocosm	Chlorpyrifos + diazinon + malathion + endosulfan – mixture	Insecticides	Mass increase	No	Hua and Relyea 2014
Lithobates pipiens	L	United States	Laboratory	Atrazine	Herbicide	No effects on growth or morphology	No	Orton et al. 2006
Lithobates pipiens	L	United States	Laboratory	Carbaryl/Sevin®	Insecticide	Growth reduction	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Diazinon	Insecticide	No effects on growth or morphology	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Malathion	Insecticide	Growth reduction	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Glyphosate/Roundup®	Herbicide	No effects on growth or morphology	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Carbaryl + diazinon - mixture/Sevin®	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates pipiens	L	United	Laboratory	Carbaryl + malathion -	Insecticides	Growth reduction	No	Relyea 2004a

		States		mixture/Sevin®				
Lithobates pipiens	L	United States	Laboratory	Carbaryl + glyphosate - mixture/Sevin® + Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Diazinon + malathion – mixture	Insecticides	Growth reduction	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Diazinon + glyphosate - mixture/Roundup®	Insecticide and herbicide	No effects on growth or morphology	No	Relyea 2004a
Lithobates pipiens	L	United States	Laboratory	Malathion + glyphosate - mixture/Roundup®	Insecticide and herbicide	Growth reduction	No	Relyea 2004a
Lithobates pipiens	L	United States	Outdoor / mesocosm	Carbaryl	Insecticide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Diazinon	Insecticide	Mass reduction	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Endosulfan	Insecticide	Mass increase	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Carbaryl + malathion + chlorpyrifos + diazinon + endosulfan – mixture	Insecticides	No effects on growth or morphology	No	Relyea 2009

Lithobates pipiens	L	United States	Outdoor / mesocosm	Acetochlor	Herbicide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Metolachlor	Herbicide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Glyphosate	Herbicide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	2,4-D	Herbicide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Atrazine	Herbicide	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Acetochlor + metolachlor + glyphosate + 2,4-D + atrazine – mixture	Herbicides	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Carbaryl + malathion + chlorpyrifos + diazinon + endosulfan + acetochlor + metolachlor + glyphosate + 2,4-D + atrazine – mixture	Herbicides and insecticides	No effects on growth or morphology	No	Relyea 2009
Lithobates pipiens	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	Mass reduction; morphological changes (deeper tails, deeper bodies)	Caged predator	Relyea 2012
Lithobates pipiens	M	United States	Outdoor / mesocosm	Malathion/Malathion Plus®	Insecticide	Mass reduction	High tadpole density	Relyea and Diecks 2008
Lithobates pipiens	L	United	Outdoor /	Malathion/Malathion Plus®	Insecticide	Mass reduction	No	Relyea and

		States	mesocosm					Hoverman 2008
Lithobates pipiens	L	United States	Laboratory	Endosulfan/Endosulfan 3EC®	Insecticide	No effects on growth or morphology	No	Shenoy et al. 2009
Lithobates pipiens	L	United States	Laboratory	Mancozeb/Manzate 75DF®	Fungicide	Total length reduction	No	Shenoy et al. 2009
Lithobates pipiens	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	Mass reduction; body shape alterations (wider body, deeper body, deeper tail, longer body, longer forelimbs, longer thighs, longer legs and longer feet)	Predator cues; low food	Woodley et al. 2015
Lithobates sphenocephalus	L	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	No	Boone and James 2003
Lithobates sphenocephalus	L	United States	Outdoor / mesocosm	Atrazine/Aatrex®	Herbicide	Mass reduction	No	Boone and James 2003
Lithobates sphenocephalus	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone and Semlitsch 2002
Lithobates sphenocephalus	B	United States	Outdoor / large-scale experimental ponds	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone et al. 2004
Lithobates sphenocephalus	B	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	No effects on growth or morphology	No	Boone et al. 2007
Lithobates sphenocephalus	B	United States	Laboratory	Carbaryl	Insecticide	Mass reduction; visceral and limb malformations (failure of the ventral surface of the integument, only one hind limb, bends in tail near the trunk	No	Bridges 2000

						and formed of three front limbs)		
Lithobates sphenocephalus	M	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase	No	Bridges and Boone 2003
Lithobates sphenocephalus	B	United States	Laboratory	Thiophanate-methyl	Fungicide	Mass increase, snout-vent length increase	Batrachochytrium dendrobatidis	Hanlon et al. 2012
Lithobates sphenocephalus	L	United States	Laboratory	Glyphosate/Roundup Pro Concentrate®	Herbicide	No effects on growth or morphology	No	Hanlon et al. 2013
Lithobates sphenocephalus	M	United States	Outdoor / mesocosm	Carbaryl/Sevin®	Insecticide	Mass increase; mass reduction	Intra and intraspecific competition; predator presence	Mills and Semlistch 2004
Lithobates sphenocephalus	L	United States	Laboratory	Chlorpyrifos/Dursban TC®	Termiticide	Mass reduction	No	Widder and Bidwell 2006
Lithobates sphenocephalus	L	United States	Outdoor / mesocosm	Chlorpyrifos/Dursban TC®	Termiticide	Mass reduction	No	Widder and Bidwell 2006
Lithobates sphenocephalus	L	United States	Laboratory	Chlorpyrifos/Dursban TC®	Termiticide	Mass reduction	No	Widder and Bidwell 2008
Lithobates sylvaticus	L	United States	Laboratory	Atrazine	Herbicide	Malformations (wavy tail, lateral tail flexure, facial edema, axial shortening, dorsal tail flexure and blistering)	No	Allran and Kasarov 2001
Lithobates sylvaticus	L	United	Outdoor /	Cooper Ethalonamine	Algicide and	Mass reduction	No	Cothran et al.

		States	mesocosm	Complex/Cultrine Plus®	herbicide			2011
Lithobates sylvaticus	M	Canada	Laboratory	Glyhosate/Roundup WeatherMAX®	Herbicide	Mass increase	No	Gahl et al. 2011
Lithobates sylvaticus	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates sylvaticus	B	United States	Outdoor / mesocosm	Diazinon	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates sylvaticus	B	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates sylvaticus	B	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates sylvaticus	B	United States	Outdoor / mesocosm	Chlorpyrifos + diazinon + malathion + endosulfan – mixture	Insecticides	No effects on growth or morphology	No	Hua and Relyea 2014
Lithobates sylvaticus	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	Mass reduction	No	Jones et al. 2010
Lithobates sylvaticus	B	United States	Outdoor / large-scale experimental ponds and laboratory	Atrazine	Herbicide	Mass reduction; limb abnormalities	Trematode infection	Kiesecker 2002
Lithobates sylvaticus	B	United States	Outdoor / large-scale experimental ponds and	Malathion	Insecticide	Mass reduction; limb abnormalities	Trematode infection	Kiesecker 2002

			laboratory					
Lithobates sylvaticus	L	United States	Laboratory	Malathion	Insecticide	Mass reduction; total length alterations (larger and smaller tadpoles); abnormalities (increase of swollen bodies, increase of diamond shape bodies and increase of stiff tails)	No	Krishnamurthy and Smith 2011
Lithobates sylvaticus	L	United States	Laboratory	Malathion	Insecticide	Mass reduction	Ammonium nitrate	Krishnamurthy and Smith 2011
Lithobates sylvaticus	L	Canada	Outdoor / large-scale experimental ponds	Glyphosate/Roundup WeatherMax®	Herbicide	Body length increase	No	Lanctôt et al. 2013
Lithobates sylvaticus	L	Canada	Laboratory	Glyphosate/Roundup WeatherMax®	Herbicide	Mass increase; snout-vent-length reduction	No	Lanctôt et al. 2014
Lithobates sylvaticus	L	Canada	Laboratory	Glyphosate/Vision®	Herbicide	Mass increase; snout-vent-length reduction	No	Lanctôt et al. 2014
Lithobates sylvaticus	B	Canada	Laboratory	Glyphosate/VisionMax®	Herbicide	Mass, tail and snout-vent length increase	No	Navarro-Martín et al. 2014
Lithobates sylvaticus	L	United States	Outdoor / mesocosm	Glyphosate/Roundup Original MAX®	Herbicide	Mass reduction; morphological changes (deeper tail muscles, deeper tails and deeper bodies)	Caged predator	Relyea 2012
Lithobates sylvaticus	M	United States	Outdoor / mesocosm	Malathion/Malathion Plus®	Insecticide	No effects on growth or morphology	No	Relyea and Diecks 2008
Lithobates sylvaticus	L	United	Outdoor /	Malathion/Malathion Plus®	Insecticide	No effects on growth or morphology	No	Relyea and

		States	mesocosm					Hoverman 2008
Lithobates sylvaticus	L	United States	Outdoor / mesocosm	Atrazine	Herbicide	No effects on growth or morphology	No	Rohr and Crumrine 2005
Lithobates sylvaticus	L	United States	Outdoor / mesocosm	Endosulfan	Insecticide	Mass increase; mass reduction	Competition of snails	Rohr and Crumrine 2005
Litoria freycineti	L	Australia	Laboratory	Endosulfan	Insecticide	Total length reduction	No	Broomhall and Shine 2003
Litoria peronii	L	Australia	Laboratory	Endosulfan	Insecticide	Total length reduction	No	Broomhall and Shine 2003
Osteopilus septentrionalis	B	United States	Outdoor / mesocosm	Atrazine	Herbicide	Snout-vent length reduction	Bd fungus	Rohr et al. 2013
Pelobates cultripes	L	Spain	Laboratory	Cooper sulfate	Fungicide	Total length reduction	No	García-Munõz et al. 2010
Pelophylax perezi	L	Spain	Laboratory	Cooper sulfate	Fungicide	Total length reduction	No	García-Munõz et al. 2010
Pelophylax ridibundus	L	Turkey	Laboratory	Malathion	Insecticide	Deformities in head and trunk (tail deformations, abnormal gut coiling and generalized edema)	No	Sayim 2008
Physalaemus centralis	B	Brazil	Laboratory	Glyphosate/Agripec®	Herbicide	Size increase	No	Figueiredo and Rodrigues 2014
Physalaemus centralis	B	Brazil	Laboratory	U46 D-FLUID 2,4-D/Nufarm®	Herbicide	Size increase	No	Figueiredo and Rodrigues 2014

Physalaemus centralis	B	Brazil	Laboratory	Picloram/Padron®	Herbicide	Size reduction	No	Figueiredo and Rodrigues 2014
Physalaemus centralis	B	Brazil	Laboratory	Picloram + 2,4-D - mixture/Tordon®	Herbicide	Size increase	No	Figueiredo and Rodrigues 2014
Physalaemus cuvieri	L	Brazil	Laboratory	Glyphosate/Roundup Original®	Herbicide	Morphological asymmetries (asymmetry in nostril-snout distance and eyes width)	No	Costa and Nomura 2016
Polypedates cruciger	B	Sri Lanka	Laboratory	Chlorpyrifos/Lorsban EC 40® or Pattas®	Insecticide	Snout-vent length reduction; mass reduction; spine malformations (kyphosis, scoliosis, edema and skin ulcers)	No	Jayawardena et al. 2010
Polypedates cruciger	B	Sri Lanka	Laboratory	Dimethoate /Dimethoate EC®	Insecticide	Snout-vent length reduction; mass reduction; spine malformations (kyphosis, scoliosis, edema and skin ulcers)	No	Jayawardena et al. 2010
Polypedates cruciger	B	Sri Lanka	Laboratory	Glyphosate/Roundup® or Glyphosate®	Herbicide	Snout-vent length reduction; mass reduction; spine malformations (kyphosis, scoliosis, edema and skin ulcers)	No	Jayawardena et al. 2010
Polypedates cruciger	B	Sri Lanka	Laboratory	Propanil/3,4 DPA®	Herbicide	Snout-vent length reduction; mass reduction; spine malformations (kyphosis, scoliosis, edema and skin ulcers)	No	Jayawardena et al. 2010
Polypedates cruciger	L	Sri Lanka	Outdoor	Diazinon	Insecticide	Body length reduction	No	Sumanadasa et al.

								2008a
Pseudacris crucifer	B	United States	Outdoor / mesocosm	Chlorpyrifos	Insecticide	Mass increase	No	Hua and Relyea 2014
Pseudacris crucifer	B	United States	Outdoor / mesocosm	Diazinon	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Pseudacris crucifer	B	United States	Outdoor / mesocosm	Malathion	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Pseudacris crucifer	B	United States	Outdoor / mesocosm	Endosulfan	Insecticide	No effects on growth or morphology	No	Hua and Relyea 2014
Pseudacris crucifer	B	United States	Outdoor / mesocosm	Chlorpyrifos + diazinon + malathion + endosulfan – mixture	Insecticides	Mass increase	No	Hua and Relyea 2014
Pseudacris regilla	L	United States	Laboratory	Cypermethrin	Insecticide	No effects on growth or morphology	No	Biga and Blaustein 2013
Pseudacris regilla	B	United States	Outdoor / mesocosm	Carbaryl	Insecticide	Growth increase	Bd fungus; competition with Rana cascadae	Buck et al. 2012
Pseudacris regilla	B	United States	Laboratory	Diazinon	Insecticide	No effects on growth or morphology	No	Kleinhenz et al. 2012
Pseudacris regilla	B	United States	Laboratory	Malathion	Insecticide	No effects on growth or morphology	No	Kleinhenz et al. 2012

Pseudacris regilla	B	United States	Laboratory	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Kleinhenz et al. 2012
Pseudacris regilla	B	United States	Laboratory	Endosulfan	Insecticide and acaricide	No effects on growth or morphology	No	Kleinhenz et al. 2012
Pseudacris regilla	B	United States	Laboratory	Diazinon + malathion + chlorpyrifos + endosulfan – mixture	Insecticide	No effects on growth or morphology	No	Kleinhenz et al. 2012
Pseudacris regilla	B	United States	Laboratory	Chlorpyrifos	Insecticide	No effects on growth or morphology	No	Sparling and Fellers 2009
Pseudacris regilla	B	United States	Laboratory	Endosulfan	Insecticide	Snout-vent length reduction; mass reduction; body abnormalities	No	Sparling and Fellers 2009
Pseudacris regilla	L	Canada	Laboratory	Endosulfan/Thiodan®	Insecticide	Abnormalities (kink tail); lost pigmentation	No	Westman et al. 2010
Pseudacris regilla	L	Canada	Laboratory	Diazinon/Diazinon®	Insecticide	No effects on growth or morphology	No	Westman et al. 2010
Pseudacris regilla	L	Canada	Laboratory	Azinphosmethyl / Guthion®	Insecticide	No effects on growth or morphology	No	Westman et al. 2010
Pseudacris regilla	L	Canada	Laboratory	Endosulfan + diazinon + azinphosmethyl - mixture/Thiodan®+ Diazinon® + Guthion®	Insecticides	No effects on growth or morphology	No	Westman et al. 2010
Pseudacris triseriata	L	United States	Laboratory	Glyphosate/Kleeraway®	Herbicide	No effects on growth or morphology	No	Smith 2001

Pseudacris triseriata	B	United States	Laboratory	Atrazine/Atrazine 4l®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Pseudacris triseriata	B	United States	Laboratory	S-metolachlor/Dual II Magnum®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Pseudacris triseriata	B	United States	Laboratory	Glyphosate/Roundup Original MAX®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Pseudacris triseriata	B	United States	Laboratory	Glyphosate/Roundup WeatherMax®	Herbicide	No effects on growth or morphology	No	Willians and Semlitsch 2010
Rana arvalis	B	Germany	Laboratory	α-cypermethrin	Insecticide	Deformities (tail kinking); Length reduction; mass increase	No	Greulich and Pflugmacher 2003
Rana arvalis	L	Germany	Laboratory	Cypermethrin	Insecticide	Physical abnormalities	No	Greulich and Pflugmacher 2004
Rana aurora	L	United States	Laboratory	Cypermethrin	Insecticide	No effects on growth or morphology	No	Biga and Blaustein 2013
Rana boylii	B	United States	Laboratory	Chlorpyrifos	Insecticide	Snout-vent length and mass reduction	No	Sparling and Fellers 2009
Rana boylii	B	United States	Laboratory	Endosulfan	Insecticide	Mass reduction; body abnormalities	No	Sparling and Fellers 2009
Rana cascadae	L	United States	Laboratory	Cypermethrin	Insecticide	No effects on growth or morphology	No	Biga and Blaustein 2013
Rana cascadae	B	United States	Laboratory	Glyphosate/Roundup®	Herbicide	Mass reduction; abnormalities (bent tails)	No	Cauble and Wagner 2005

Rana dalmatina	B	Italy	Laboratory	Chlorpyrifos	Insecticide	Mass reduction; abnormalities (skeletal defect, abnormal tail lateral flexure, bloated heads and edema)	No	Bernabò et al. 2011
Rana dalmatina	L	Italy	Laboratory	Endosulfan	Insecticide	Mass reduction; snou-vent length reduction; malformations (bloated heads and skeletal malformations)	No	Lavorato et al. 2013
Rana temporaria	M	Denmark	Laboratory	Prochloraz	Fungicide	Mass increase	No	Brande-Lavridsen et al. 2010
Rana temporaria	L	Belgica	Laboratory	Endosulfan	Insecticide	Body mass reduction	No	Denoël et al. 2012
Rana temporaria	B	Sweden	Laboratory	Azoxystrobin	Fungicide	Body length reduction	No	Johansson et al. 2006
Rana temporaria	B	Sweden	Laboratory	Cyanazine	Herbicide	Reduction in body length, tail length and dry weight	No	Johansson et al. 2006
Rana temporaria	B	Sweden	Laboratory	Esfenvalerate	Insecticide	No effects on growth or morphology	No	Johansson et al. 2006
Rana temporaria	B	Sweden	Laboratory	MCPA	Herbicide	No effects on growth or morphology	No	Johansson et al. 2006
Rana temporaria	B	Sweden	Laboratory	Permethrin	Insecticide	Increase in body length, tail length and wet weight	No	Johansson et al. 2006
Rana temporaria	B	Sweden	Laboratory	Pirimicarb	Insecticide	Reduction in tail length and dry weight	No	Johansson et al. 2006
Rana temporaria	L	France	Laboratory	Amitrole	Herbicide	Body and tail length increase; mass	Predator cues	Mandrillon and

						increase; shallower bodies		Saglio 2009
Rana temporaria	B	Sweden	Laboratory	Fenpropimorph	Fungicide	Reduction in mass, tail length, tail fin depth and body length	Predator	Teplitsky et al. 2005
Rhinella fernandezae	L	Argentina	Laboratory	Chlorpyrifos/Lorsban® 48E	Insecticide	Growth reduction; deformities (slight and severe lateral flexure of the tail from its normal position)	No	Arcaute et al. 2012
Rhinella marina	B	Brazil	Laboratory	Glyphosate/Agripec®	Herbicide	No effects on growth or morphology	No	Figueiredo and Rodrigues 2014
Rhinella marina	B	Brazil	Laboratory	U46 D-FLUID 2,4-D/Nufarm®	Herbicide	No effects on growth or morphology	No	Figueiredo and Rodrigues 2014
Rhinella marina	B	Brazil	Laboratory	Picloram/Padron®	Herbicide	Size reduction	No	Figueiredo and Rodrigues 2014
Rhinella marina	B	Brazil	Laboratory	Picloram + 2,4-D - mixture/Tordon®	Herbicide	Size increase	No	Figueiredo and Rodrigues 2014
Scinax nasicus	L	Argentina	Laboratory	Glyphosate/Glyphos®	Herbicide	Cranialfacial and mouth deformities; eye abnormalities; bent curved tails	No	Lajmanovich et al. 2003
Spea intermontana	L	Canada	Laboratory	Endosulfan/Thiodan®	Insecticide	Abnormalities (kink tail)	No	Westman et al. 2010
Spea intermontana	L	Canada	Laboratory	Diazinon/Diazinon®	Insecticide	No effects on growth or morphology	No	Westman et al. 2010
Spea intermontana	L	Canada	Laboratory	Azinphosmethyl / Guthion®	Insecticide	No effects on growth or morphology	No	Westman et al. 2010

Spea intermontana	L	Canada	Laboratory	Endosulfan + diazinon + azinphosmethyl - mixture/Thiodan® + Diazinon® + Guthion®	Insecticides	No effects on growth or morphology	No	Westman et al. 2010
Spea multiplicata	L	United States	Laboratory	Chlorothalonil	Fungicide	Snout-vent-length reduction; tail growth inhibition	No	Yu et al. 2013b
Xenopus laevis	L	Italy	Laboratory	Carbaryl	Insecticide	Malformations (microphthalmia, cardiac or abdominal edema, irregular shape of the intestinal loops, abnomial tail flexure and dorsal flexure)	No	Bachetta et al. 2008
Xenopus laevis	L	Italy	Laboratory	Chlorpyrifos	Insecticide	Malformation (ventral and lateral tail flexure coupled with abnormal gut coiling)	No	Bonfanti et al. 2004
Xenopus laevis	L	Italy	Laboratory	Malathion	Insecticide	Malformation (abnormal tail flexure)	No	Bonfanti et al. 2004
Xenopus laevis	M	United States	Laboratory	Atrazine	Herbicide	No effects on growth or morphology	No	Carr et al. 2003
Xenopus laevis	L	United States	Laboratory	Malathion	Insecticide	Length reduction; axis deformities	No	Chemotti et al. 2006
Xenopus laevis	B	United States	Laboratory	Atrazine	Herbicide	No effects on growth or morphology	No	Coady et al. 2005
Xenopus laevis	L	Italy	Laboratory	Chlorpyrifos	Insecticide	Abnormalities (abnormal tail flexure)	No	Colombo et al. 2005

Xenopus laevis	L	Canada	Laboratory	Acetochlor	Herbicide	Body area reduction (sculpting of the head and reduced tail fin quality)	Induction of thyroid hormones (T3)	Crump et al. 2002
Xenopus laevis	L	Canada	Laboratory	Glyphosate/Vision®	Herbicide	No effects on growth or morphology	No	Edginton et al. 2004
Xenopus laevis	L	United States	Laboratory	Methoxychlor	Insecticide	Malformations (visceral edema, notochord lesions and shortening of the femur)	No	Fort et al. 2004
Xenopus laevis	B	United States	Laboratory	Atrazine	Herbicide	Mass increase; mass decrease	No	Freeman and Rayburn 2005
Xenopus laevis	M	United States	Laboratory	Atrazine	Herbicide	No effects on growth or morphology	No	Hayes et al. 2002
Xenopus laevis	L	United States	Laboratory	Chlorpyrifos	Insecticide	Body length reduction; spinal malformations	No	Richards and Kendall 2003
Xenopus laevis	L	United States	Laboratory	Atrazine	Herbicide	Mass decrease; reduction and increase in snout-vent-length	Amonium nitrate	Sullivan and Spence 2003
Xenopus laevis	L	United States	Laboratory	Malathion/Malathion Plus®	Insecticide	Abnormalities (bent tails at their bases)	No	Webb and Crain 2006
Xenopus laevis	L	Korea	Laboratory	Carbendazim/Benomyl®	Fungicide	Growth inhibition; malformations (optic hernia and dysplasia, narrow head, fin, notochord and tail abnormalities, cephalis edema, optic edema, abdominal edema, gut dysplasia and atrophy)	No	Yoon et al. 2008

Xenopus laevis	L	Korea	Laboratory	N-butyl isocyanate/Benomyl®	Fungicide	Growth inhibition; malformations (blisters, optical hernia and dysplasia, narrow head, fin, notochord and tail abnormalities, optic edema, abdominal edema, gut dysplasia and atrophy)	No	Yoon et al. 2008
Xenopus laevis	L	United States	Laboratory	Malathion	Insecticide	Total length reduction; malformations (edemas, axil and tail deformities)	No	Yu et al. 2013a
Xenopus laevis	L	United States	Laboratory	Endosulfan	Insecticide	Total length reduction; malformations (edemas, axil and tail deformities)	No	Yu et al. 2013a
Xenopus laevis	L	United States	Laboratory	α-cypermethrin	Insecticide	Total length reduction; malformations (edemas, axil and tail deformities)	No	Yu et al. 2013a
Xenopus laevis	L	United States	Laboratory	Chlorothalonil	Fungicide	Snout-vent-length reduction; tail growth inhibition	No	Yu et al. 2013b

*****Other stressful factors**= we considered and added in the table only the factors that really contributed to the effects on growth and external morphology. Factors that were tested, but did not show effects on growth and morphology were not cited in this study.

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CAPÍTULO 2

Lethal and sublethal responses of a neotropical tadpole (*Dendropsophus minutus*, Anura: Hylidae) exposed to Roundup Original® and predator cues

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ABSTRACT

Glyphosate-based herbicides contaminate freshwater ecosystems and are lethal to anuran larvae. In addition to lethal responses, contamination leads to sublethal responses that can change tadpoles' morphology and behavior. Our objectives were to evaluate lethal (survival and $LC50_{96h}$) and sublethal responses (behavior, external morphology and fluctuating asymmetry) of *Dendropsophus minutus* tadpoles submitted to Roundup Original®, the presence of a common predator (Aeshnidae) and the interaction of both factors. We conducted an acute exposure experiment testing increasing concentrations of Roundup (4 days) and a chronic exposure factorial experiment submitting tadpoles to Roundup and predator cues (non-lethal experiment – 17 days). In the acute exposure experiment we observed that survival of tadpoles exposed to Roundup significantly decreased when compared to control. The $LC50_{96h}$ was 2.491 mg a.i./L, classified as moderately toxic. In the chronic exposure experiment we observed that Roundup contamination lead to an increase of swimming activity, and non-contaminated tadpoles maintained a greater distance from predators. The largest changes on external morphology were observed in tadpoles submitted to factorial treatment. Higher deviations on fluctuating asymmetry were observed in tadpoles submitted to Roundup and predator cues together. Anthropogenic stressors increased the negative effects of natural stressors, like predator cues, leading to abnormal morphological and behavioral changes in tadpoles. These changes can lead to fitness reduction, which could reflect in a decrease in population size. Thus, understanding the negative effects of multiples stressors, mainly the anthropogenic ones, can contribute in the elaboration of efficient measures for species conservation.

KEY-WORDS: behavior, fluctuating asymmetry, geometric morphometry, LC50, synergistic effect.

RESUMO

Herbicidas à base de glifosato contaminam ecossistemas de água doce e são letais para larvas de anuros. Além de respostas letais, a contaminação leva a respostas subletais que podem alterar a morfologia e comportamento dos girinos. Estes atributos são naturalmente moldados pelo risco de predação, porém, podem ser alterados pela contaminação por pesticidas. Nosso objetivo foi avaliar respostas letais (sobrevivência e $LC50_{96h}$) e subletais (comportamento, morfologia externa e assimetria flutuante) de girinos de *Dendropsophus minutus* submetidos ao herbicida Roundup Original®, à presença de um predador comum (Aeshnidae) e uma interação de ambos os fatores. Realizamos um experimento de exposição aguda testando concentrações crescentes de Roundup (4 dias) e um experimento fatorial de exposição crônica submetendo os girinos ao Roundup e pistas de predadores (experimento não letal – 17 dias). Na exposição aguda, observamos que a sobrevivência dos girinos expostos ao Roundup reduziu significativamente quando comparada ao controle. O $LC50_{96h}$ foi de 2.491 mg a.i./L, classificado como moderadamente tóxico. Na exposição crônica, observamos que a contaminação por Roundup levou a um aumento na atividade de natação e que girinos não contaminados mantiveram uma maior distância em relação ao predador. As maiores mudanças na morfologia externa foram observadas nos girinos submetidos ao tratamento fatorial. Os maiores desvios na assimetria flutuante foram observados nos girinos submetidos à contaminação por Roundup e às pistas de predadores. Estressores antrópicos aumentam os efeitos negativos de estressores naturais, como as pistas de predadores, levando a anormalidades morfológicas e mudanças no comportamento dos girinos. Essas mudanças podem levar a redução do fitness podendo refletir em uma redução no tamanho populacional. Assim, entender o efeito negativo de múltiplos fatores estressantes, especialmente os antrópicos, pode contribuir na elaboração de medidas eficientes para a conservação das espécies.

PALAVRAS-CHAVE: comportamento, assimetria flutuante, morfometria geométrica**,** LC50, efeito sinérgico.

INTRODUCTION

Commercial formulations of glyphosate (e.g. Roundup®, Vision®) are the most popular herbicides worldwide (Zhang et al. 2011, Battaglin et al. 2014, WHO 2015), being the most applied in Brazilian croplands (Carneiro et al. 2015, Bombardi 2017). Glyphosate-based formulations have surfactant substances (*e.g.* polyethoxylated tallowamine – POEA, aminomethylphosphonic acid – AMPA) which facilitate the penetration in leaves, increasing glyphosate toxicity (Giesy et al. 2000, Mann et al. 2009). High application rates are correlated with the advance of agricultural frontiers (Devine and Furlong 2007, Schiesari and Grillitsch 2011, Bombardi 2017), resulting in several records of freshwater contamination by glyphosate and its surfactant substances (*e.g.* Blanchoud et al. 2007, Peruzzo et al. 2008, Botta et al. 2009, Marques et al. 2009, Battaglin et al. 2009, 2014, Degenhardt et al. 2012).

It's well established that glyphosate-based herbicides are responsible for several lethal and sublethal effects on non-target organisms, such as amphibians (*e.g.* Relyea 2005a, Relyea 2012, Figueiredo and Rodrigues 2014, Katzenberger et al. 2014, Navaro-Martín et al. 2014, Costa and Nomura 2016). Amphibians are highly vulnerable to pesticides and environmental contamination is strongly correlated with population declines (Sparling et al. 2000, Sparling et al. 2001, Davidson et al. 2001, 2002, Blaustein and Kiesecker 2002, Boone et al. 2007, Mann et al. 2009, Hayes et al. 2010). This high susceptibility is consequence of anurans' complex life history and permeable skin, being more harmful to species with indirect development, which have aquatic eggs and tadpoles (Bishop et al. 1999, Gallant et al. 2007, Schiesari et al. 2007, Mann et al. 2009, Allentoft and O'Brien 2010).

Tadpoles have a high phenotypic plasticity and are good tools for short-term ecotoxicological studies because they quickly respond to perturbations from stressor factors (Van Buskirk and Relyea 1998, McDiarmid and Altig 1999, Relyea 2002, Relyea 2004, West-Eberhard 2003, Steiner and Van Buskirk 2008, Van Buskirk 2009, Fusco and Minelli 2010). During the tadpole stage hormone-regulation is more critical, especially at pre-metamorphosis, when the endocrine-disruptor action of pesticides negatively influences production, metabolism and action of natural hormones (Hayes et al. 2006a, Mann et al. 2009, Hayes et al. 2010). Thus, in addition to lethal effects (e.g. Relyea 2005a, Relyea and Jones 2009), tadpoles can show a range of sublethal responses when exposed to glyphosate based-herbicides, significantly changing their behavior, morphology, development and/or physiology (*e.g.* Wojtaszek et al. 2004, Jones et al. 2010, Jones et al. 2011, Relyea 2012, Lajmanovich et al. 2013, Katzenberger et al. 2014, Costa and Nomura 2016).

In natural communities, tadpoles are exposed to a variety of natural and/or anthropogenic stressors (Johnson et al. 2013, Hanlon and Relyea 2013) that can interact with pesticides (Relyea 2012). Predators are one of the most common natural stressors that affect tadpoles, especially due to indirect interactions of tadpoles with the visual and/or chemical clues that indicate predators' presence (*e.g.* Van Buskirk and Relyea 1998, Van Buskirk 2000, Relyea 2001, Relyea 2002a, Jara and Perotti 2009, Jara and Perotti 2010, Relyea 2012, Nomura et al. 2013). Predators and pesticide contamination can act additively or synergistically (Sih et al. 2004, Relyea 2012) leading to different effects on tadpoles, such as the increase of pesticide lethality (e. g. Relyea and Mills 2001, Relyea 2003, Relyea 2005a), changes in interspecific interactions (*e.g.* Boone et al. 2007, Cothran et al. 2011, Hanlon and Relyea 2013) and changes in the antipredator responses, attributes and performance (*e.g.* Relyea and Edwards 2010, Relyea 2012, Katzenberger et al. 2014, Woodley et al. 2015). These negative effects of pesticide contamination can disrupt community interactions, such as top-down or bottom-up trophic cascades (Relyea 2005b, Rohr et al. 2006b, Relyea and Hoverman 2006).

Sublethal responses of multiple stressors acting additive/synergistically can be evaluated through changes on specific attributes of tadpoles, such as the swimming activity (*e.g.* changes in activity time, frequency of atypical behaviors, individual performance and/or refuge use) (Weiss et al 2001, Denoël et al. 2012, Hanlon and Relyea 2013, Katzenberger et al. 2014). Another way is to evaluate changes in general external morphology of tadpoles through alterations in the size and/or shape when exposed to multiple stressors (*e.g.* Relyea 2012, Katzenberger et al. 2014). These behavioral and morphological variations, especially in the tail, can affect the swimming performance of tadpoles (see Van Buskirk and Relyea 1998, Van Buskirk and McCollum 2000b, Katzenberger et al. 2014) and are the main mechanisms that mediate the predator/prey coexistence (Hero et al. 2001). Also, the effects of multiple stressors can be evaluated through deviations in the developmental homeostasis applying

Fluctuating Asymmetry (FA) methods (Palmer and Strobeck 1986, Klingenberg et al. 2002). The FA is based on random deviations between symmetry of bilateral traits and can be used as biomonitoring tool in environment impact studies (Palmer and Strobeck 1986, Sanseverino and Nessimian 2008). The FA is a sensitive biomarker of environmental stress (*i.e.* whether natural or anthropogenic) and can be an indicative of the individual performance and fitness (Clarke 1995, Moller 1997, Bosch and Marquéz 2000, Beasley et al. 2013). The relationship between higher FA levels and anthropogenic stressors has been tested in adult frogs (*e.g.* Gallant and Teather 2001, Lauck 2006, Söderman et al. 2007, Delgado-Acevedo and Restrepo 2008, Eisemberg and Bertoluci 2016, Costa and Nomura 2016, Costa et al. 2017, Zhelev et al. 2015, 2017), but evaluations using tadpoles have recently emerged and appear to be a prominent tool to assess the levels of environmental stress (*e.g.* Eterovick et al. 2015, Eterovick et al. 2016, Costa and Nomura 2016, Costa et al. 2017).

Here, we evaluated the lethal and sublethal responses of *Dendropsophus minutus* (Peters, 1872) tadpoles submitted to contamination by a commercial formula of glyphosate (Roundup Original®), visual/chemical clues of a predator (Aeshnidae) and a combination of both factors (factorial treatment). First, we evaluated the lethal response through survival rates in an acute contamination scenario and estimated the $LC50_{96h}$. Following, we submitted tadpoles to a chronic level of glyphosate contamination and visual/chemical clues of the predator, as well a combination of both factors to evaluate the sublethal responses. We measured sublethal responses through changes in swimming behavior (activity time, air surface breathing, and distance from predator), general external morphology and fluctuating asymmetry of tadpoles. For swimming behavior, we expected a synergistic effect of both stressors in which tadpoles would increase their activity levels, increase their frequency of air surface breathing and keep longer distance from predators when compared to other treatments (*i.e.*, contamination and predator cues only). Furthermore, we expected the same synergistic effect of both stressors on morphological variables with higher changes on general external morphology and higher changes in the fluctuating asymmetry levels when compared to other treatments.

METHODS

Sampling and experimental background

We collected 14 amplexi of *Dendropsophus minutus* from two lentic water bodies (pond 1: 14°07'49.83"S, 47°41'24.65"W; pond 2: 14°08'29.61"S, 47°38'50.06" W – datum SAD69), located in the surroundings of Chapada dos Veadeiros National Park, Alto Paraíso municipality, state of Goiás, Brazil. The ponds are inserted in open areas of Cerrado biome and are located at the edge of a national park, far from croplands and without history of pesticide application. The couples were kept in individual plastic bags with water from the ponds where the adults were collected to oviposition. After oviposition, the adult specimens were released at the original sites. The egg clutches were transported to the Laboratory of Experimental Ecology at the Federal University of Goiás and were combined in a storage tank (glass aquaria - 60cm X 40cm X 40cm) with 8 L of dechlorinated water for acclimatization. After hatching, we sorted tadpoles randomly to the experimental treatments, diluting the potential parental effect, if any, at the treatments. As a predator model, we collected larvae of Aeshnidae in a lentic pond located on a private farm (16°31'55.4"S, 49°16'35.3"W – datum SAD69), at Goiânia municipality, state of Goiás, Brazil. In this habitat, Aeshnidae larvae are very abundant, and we selected larvae of similar body size and morphology.

We performed two experiments under controlled laboratory conditions (air temperature = 28 ± 1°C, and photoperiod = 12 h light/12 h dark). As contaminant, we used the commercial formula of glyphosate (Roundup Original®) with 48% of active ingredient. As experimental units, we used glass aquaria (15 cm X 10 cm X 15 cm) with 1 L of dechlorinated water, without substrate and constant oxygenation with aquarium air compressors. In both experiments, tadpoles were acclimatized in these glass aquaria for 24 h before exposure to treatments. Surviving tadpoles were sacrificed with benzocaine solution (10 mg/l) and preserved in 10% formalin. All specimens were deposited in the Herpetological Collection of the Federal University of Goiás/ZUFG (ZUFG 2502 - 2505). The biological material collected and the experiments were authorized by Sisbio permission nº 48662-1 and by Ethics Committee of Animal Use (CEUA/UFG) nº 032/15.

Acute-toxicity experiment

When tadpoles reached developmental stage 25 (*sensu* Gosner 1960), we randomly assigned 10 individuals to each experimental unit. Based on CONAMA 357 (2005) resolution, we determined the glyphosate concentrations starting from 0.28 mg a.i./L (280 µg/l – glyphosate concentration allowed in class III freshwater types in Brazil). We increased the glyphosate concentrations by uniform increments based on recent studies of toxicity with tadpoles (Relyea 2012, Lajmanovich et al. 2013, Simioni et al. 2013, Costa and Nomura 2016) to simulate an acute exposure. All glyphosate concentrations were calculated using the informed quantity of glyphosate included in the Roundup Original® formulation, as presented in the leaflet of the product.

Following, we prepared treatment-specific solutions with five nominal concentrations of glyphosate (Control = 0 mg a.i./l; T1 = 0.28 mg a.i./l; T2 = 1.5 mg a.i./l; T3 = 3 mg a.i./l; T4 = 6 mg a.i./l). To achieve these treatment-specific solutions of glyphosate, we added 0.583, 3.125, 6.25 and 12.5 µl of Roundup Original®, respectively. To simulate the same hydric stress, we added 12.5 µl of water in the Control. Solutions were applied in a single pulsed dose without the renewal contamination or water replacement. We replicated each treatment seven times, in a total of 35 experimental units with 350 used tadpoles. We randomized the position of experimental units and treatments. Tadpoles were fed *ad libitum* every 2 days with ornamental fish food. The exposure time was 96 h (4 days). We reviewed and removed any dead tadpole every 24 hours, recording the overall survival at the end of the experiment when we also measured the dissolved oxygen, temperature and pH of the water in each experimental unit.

Chronic-toxicity experiment

To optimize the morphological and behavior analysis, we maintained tadpoles in the storage tank for 30 days to grow. After, we randomly collected five tadpoles to be assigned to each experimental unit. We submitted the tadpoles to a factorial treatment with a combination of glyphosate contamination (0 mg a.i./l and 2 mg a.i./l) and chemical/physical cues of predator (absence and presence) [i.e. Control (0 mg a.i./l + predator absence), Tr1 (0 mg a.i./l + predator presence), Tr2 (2 mg a.i./l + predator absence) and Tr3 (2 mg a.i./l + predator presence)]. The lower concentration of glyphosate used is based on $LC50_{96h}$ obtained in acute exposure experiments, ensuring a survival rate and allowing a long exposure time to evaluate the herbicide/predator effects on morphology and behavior. This concentration of glyphosate (i.e. 2 mg a.i./l) was established by adding 4.17 µl of Roundup Original®. We applied the contaminant in a pulsed dose, however, with replacement of the water and renewal of the contamination in experimental units in the middle of the experiment (i.e. on the 8th day). We added the caged predators (non-lethal experiment) in a cylindrical glass aquaria (10 cm X 3 cm) with opening capped with a mosquito net (mesh 3mm), ensuring the visualization of predator and the circulation of water between the experimental unit and the predator cage (visual and chemical cues). In both treatments with predator presence, we added individuals of similar size. For this, we measured the total length of predators and compared them *a priori* with a t-test. We confirmed that there were no differences among predators size between treatments ($t_{(12)}$= 0.024; p= 0.981). We also added empty cages in the experimental units without predator cues (i.e. Control and Tr2) to ensure the same experimental conditions. Cages were added always in the left side of experimental units. During experiment, predators were removed to be fed three times. At the same time we added *ad libitum* ornamental fish food to tadpoles. The exposure time was 17 days (i.e. chronic exposure). We replicated each treatment 7 times, totaling 28 experimental units and 140 used tadpoles. We randomized the position of experimental units and treatments. At the first and last days, we measured the dissolved oxygen, temperature and pH of the water in each experimental unit. At the end, we also measured the total length (TL) and evaluated the developmental stage of each tadpole.

Behavior – To evaluate tadpole behavior, we made video records with three minutes of duration per experimental unit. We performed three recording sessions, totalizing 60 videos and 180 minutes of swimming behavior. Recording sessions were temporally separated – at first period (1st day), at half period (8th day) and at final period (16th day) – to evaluate a potential cumulative effect of treatments on tadpole behavior. In each recording session, we randomly selected five experimental units of each treatment (totalizing 20 videos and 60 minutes per recording session) and positioned a GoPro HD Be a Hero 2 camera laterally at a distance of 15 cm. One minute after placing the camera, we slowly approached to avoid behavioral changes and then started to record. We watched each video five times to evaluate each tadpole, individually. We considered the following swimming behaviors: (i) activity time (seconds) - displacements, feeding and any movement in the tail were considered as activity (e.g. Brunelli et al. 2009, Egea-Serrano et al. 2011, Denoël et al. 2012, Mikó et al. 2017); (ii) air surface breathing (N) – amount of climbs to the surface (Wells 2007, Denoël et al. 2012); and (iii) distance from predator – with a scale attached to aquariums. We measured the distance of each tadpole from the predator cage every 30 seconds, and calculated a mean distance for each tadpole. Using the behavior of each tadpole, we calculated a mean for each behavior type for each experimental unit and used these mean values as response variables.

General external morphology – After the experiment, we randomly collected 30 surviving tadpoles from each treatment for morphological assessment. Tadpoles were sacrificed and positioned against the border of a Petridish using ultrasound gel, and submerged in water. We obtained images in dorsal and lateral view with a Sony a230, 10.2 megapixel camera, equipped with a macro Sigma Zoom 24–70 mm lens ocular, supported on a tripod at a height of 30 cm, to evaluate the shape variation through geometric morphometric techniques. We defined 25 landmarks in lateral view (modified from Van Buskirk 2009 and Katzenberger et al. 2014) and 16 landmarks in dorsal view (modified from Marques and Nomura 2015) (Figure S1) in each tadpole. The landmark configuration provides a coordinate system in a Cartesian plane that represents the shape of organisms, in which the geometric morphometric techniques describe the shape variation without the effects of size, position and rotation (Monteiro and Reis 1999, Zelditch et al. 2004). Thus, homologous landmarks must be observed at all study organisms (Sneath & Sokal 1973). We digitized the landmarks with the tpsUtil (Rohlf 2009) and tpsDig2 (Rohlf 2008) programs.

Fluctuating asymmetry – To evaluate Fluctuating Asymmetry (FA), we used 15 tadpoles from each treatment. We extracted the skin, intestine and eyes of tadpoles and applied a diaphanization protocol (Taylor and Van Dyke 1985). Using an Alltion stereomicroscope with an attached camera BestScope BHC2 – 1080P, we obtained images in ventral view of the hyobranchial skeleton of each tadpole to extract the FA measures. We focused only on the upper structures (i.e. ceratohyal, processus anterior, processus posterior, condylus articularis and basibranchial region – Haas et al. 2003) because they are the most rigid parts of the structure and because it is the region of greater precision in the extraction of true landmarks. The hyobranchial skeleton is a structure with object symmetry (i.e. symmetric itself) but an internal line would be necessary (i.e. midline landmarks) to separate the right side from the left side of the symmetrical plan (see Mardia et al. 2000 and Klingenberg et al. 2002). Thus, we defined 8 paired landmarks and 1 unpaired landmark (Figure S2) to define the midline in the hyobranchial skeleton. This procedure allows the evaluation of the asymmetrical (left-right differences) and symmetrical (individual variation) components of the structure using a single landmark configuration (Klingenberg et al. 2002). However, in this study we chose to display only the asymmetrical component, using the FA as a tool to measure the developmental instability when exposed to contaminants. To account for measurement errors we obtained two images of each tadpole and we digitized each image twice. Landmarks were digitized with the tpsUtil (Rohlf 2009) and tpsDig2 (Rohlf 2008) programs.

Statistical analysis

For acute exposure experiments we performed a Kruskall-Wallis test to compare the survival among treatments of glyphosate contamination, followed by an a posteriori Mann-Whitney test. We used a Probit Regression Analysis to estimate the $LC50_{96h}$ value. We applied one-way ANOVA's to evaluate differences in dissolved oxygen, temperature and pH of water among treatments.

For chronic exposure, we applied repeated measure ANOVA's to compare the dissolved oxygen, temperature and pH of water among treatments and between the first and the last day of experiment. We also applied one-way ANOVA's to compare the total length (TL) and developmental stage of tadpoles among treatments.

For behavioral analyses, we firstly evaluated correlations among response variables applying Pearson's Correlation Coefficient. We found a moderate correlation between (i) activity time and (ii) air surface breathing ($r= 0.626$, $p < 0.001$). Thus, we kept only the variable (i) activity time because it is the most common behavior trait assessed in ecotoxicological studies (Brunelli et al. 2009, Egea-Serrano et al. 2011, Denoël et al. 2012); excluding the variable (ii) air surface breathing from the analysis. We also observed a negligible correlation between (i) activity time and (iii) distance from predator ($r= 0.288$, $p= 0.025$). Due to the low correlation, we decided to keep this variable in the subsequent analysis. Finally, we applied two-way ANOVA's to compare (i) activity time and (iii) distance from predator among treatments (Control, Tr1, Tr2 and Tr3) and periods (initial, middle and end), followed by an a posteriori Tukey test.

To evaluate shape changes in the general external morphology in lateral and dorsal views, we firstly applied a Procrustes superimposition method (Rohlf 1990, Zelditch et al. 2012) using landmark configurations. This method allows visualization of the overall shape variation without effects of size, position, and orientation (Monteiro &

Reis 1999, Zelditch et al. 2004, 2012). Following, we extracted a variance/covariance matrix from Procrustes coordinates to represent the shape variation of tadpoles from each treatment through a Principal Component Analysis (PCA). Using the average shape of all tadpoles, we applied a Canonical Variate Analysis (CVA) to observe if there was an ordination among groups (i.e. treatments). The CVA is more robust to examine shape differences among groups, whereas PCA is more adequate to examine shape differences among individuals within a group (Zelditch et al. 2012). Finally, we calculated a Procrustes distance matrix to evaluate the distance in the shape configurations among pairs of groups, followed by a Permutation test (10.000 randomizations) to assess a statistical significance between treatments (Klingenberg and McIntyre 1998). We performed all shape analyses using the MorphoJ software, version 1.06d (Klingenberg 2011).

To evaluate Fluctuating Asymmetry and measurement errors, we firstly applied a Procrustes ANOVA (Klingenberg & McIntyre 1998, Mardia et al. 2000, Klingenberg et al. 2002) using the landmark configuration of hyobranchial skeleton of all tadpoles. Following, we extracted the variance/covariance matrices for each treatment and applied a Principal Component Analysis to evaluate the changes in the asymmetrical shape of hyobranchial process of tadpoles from each treatment. We applied a Canonical Variate Analysis to observe if the changes on the asymmetrical shape varied among treatments. Finally, we calculate a Procrustes distance matrix followed by a Permutation test (10.000 randomizations) to assess the amount of shape variation among treatments and the statistical significance (Klingenberg and McIntyre 1998). We performed all shape analysis using the MorphoJ software, version 1.06d (Klingenberg 2011).

RESULTS

Acute exposure

Survival and $LC50_{96h}$ - We observed a decrease in survival of tadpoles exposed to Roundup contamination ($H_{(4)}$= 24.579; p< 0.001 – Figure 1). When compared to Control (100% of survivors), the lowest survival rates were observed at the T3= 3 mg a.i./l, T4= 6 mg a.i./l and T2= 1.5 mg a.i./l, respectively (see a post-hoc Mann-Whitney test – Table S1). The estimated $LC50_{96h}$ for tadpoles of *Dendropsophus minutus* is 2.491 mg a.i./L. There were no differences in dissolved oxygen ($F_{(4)}$= 0.964; p= 0.441 -

range = 11–4 ppm), water temperature ($F_{(4)}$= 2; p= 0.080 - range = 22.9–23.7ºC) and pH ($F_{(4)}$= 0.9; p= 0.459 - range = 7.0–7.2) among treatments.

Chronic exposure

For the chronic exposure we observed high survival rates, where only two tadpoles died in Tr1 (i.e. 0 mg a.i./l + predator presence). There were no differences in dissolved oxygen in the water among treatments ($F_{(3)}$= 1.307; p= 0.295); however, there was a difference between the first and the last day of experiment ($F_{(1)}$= 4.422; p= 0.046) in which the dissolved oxygen slightly increased towards the end (mean= 9.67 ppm - range: 11 - 8) when compared to the first day (mean= 8.785 ppm - range: 11 - 5). No differences in water temperature were detected among treatments ($F_{(3)}$= 1.0; p= 0.585) and between the first and the last day of experiment ($F_{(3)}$= 2.0; p= 0.164 – mean: 23.8ºC – range: 24.1 – 23.6). Also, there were no differences in water pH among treatments ($F_{(3)}$= 2.7; p= 0.069); however, there were differences between the first and last day ($F_{(1)}$= 52; p< 0.001), indicating a slightly increase in pH towards the end (mean= 7.142 - range: 7.5 – 6.3) when compared to the first day of experiment (mean= 6.867 - range: 7 – 6.8). There were no differences among treatments in total length ($F_{(3)}$= 0.201; p= 0.895) ($TL_{Control}$= 24.912 mm ± 4.034, range= 35.65 – 18.602; TL_{Tr1}= 25.314 mm ± 3.461, range= 33.492 – 17.832; TL_{Tr2}= 24.597 mm ± 4.199, range= 33.999 – 15.918; TL_{Tr3}= 24.869 mm ± 2.632, range= 30.64 – 20.927) and developmental stage of tadpoles ($F_{(3)}$= 1.012; p= 0.389) ($Dev\_stage_{Control}$= 29.677 ± 3.815, range= 38 – 25; Dev_stage_{Tr1}= 29.133 ± 3.421, range= 35 – 25; Dev_stage_{Tr2}= 28.741 ± 3.245, range= 37 – 25; Dev_stage_{Tr3}= 28.193 ± 3.350, range= 39 – 25).

Behavior – We observed differences in activity time between treatments and periods (F(6)= 2,636; p= 0,027 – Figure 2A). At the 1st day, tadpoles were more active in treatments with contamination, independent of the presence or absence of predators (Tr2 and Tr3, respectively), than in treatments without contamination (Tr1 and Control) (Figure 2A – Table S2). At the 8th day and 16th day, there were no differences in activity time of tadpoles among treatments (Table S2). Compared among periods, tadpoles from Tr2 in the 1st day were more active than all treatments, independent of the period. Also, tadpoles from Tr3 in the 1st day were more active than others, except when compared to Tr2 and Tr3 of the 8th day. We did not observe differences between the 8th day and 16th day of experiment (Figure 2A – Table S2).

There were no differences in the distance from predators among periods (F(6)= 0.697; p= 0.652). However, we observed differences among treatments in the 1st day of experiment (F(3)= 2.803; p= 0.049 – Figure 2B), in which tadpoles maintained greater distance in the treatment with predator cues only (Tr1) when compared to the control (Table S3).

General external morphology - In lateral view, we observed shape differences in general external morphology among treatments, in which higher morphological distance among the mean shapes were observed in tadpoles exposed to Roundup and predator cues (Tr3), Predator cues (Tr1) and Roundup (Tr2), respectively, when compared to the mean shape of control (Table 1A, Figure 3A). The shape changes of tadpoles from control, Tr1 and Tr2 tended to move in the same direction with little changes in tail shape and body shape, especially the deformation associated to body reduction (shallow body) observed in Tr2. However, in the factorial treatment (Tr3) we observed higher deformations in tail shape with an increase in the height of dorsal and ventral fins (deeper tails) and reduction in tail length. Also, body chanhes are directed to the opposite direction of the other treatments (Figure 4).

In dorsal view, we observed shape changes with higher morphological distance in general external morphology of tadpoles exposed to Roundup and predator cues (Tr3) when compared to control tadpoles (Table 1B, Figure 3B). Tadpoles exposed to Tr3 showed a considerable body width reduction (shallow bodies) with a finer appearance (Figure 5). These body changes were observed also in Tr2, however, these were insufficient to express statistical differences from other treatments.

Fluctuating asymmetry – We observed that tadpoles presented Fluctuating Asymmetry in the hyobranchial skeleton due to absence of significant differences between right and left sides and due to random and non-directional FA among individuals (Table 2). Also, the measurement error was smaller than FA ensuring the reliability of the marked landmarks (Table 2). The fluctuating asymmetry levels of the hyobranchial skeleton differed among treatments (Figure 6). When compared to the control, the shape changes representing asymmetry differed statistically for all treatments (Table 3). The highest differences in FA compared to control were observed in Tr2, Tr3 and Tr1, respectively. We observed that shape changes of Control, Tr1 and

Tr2 tended to be displaced to the right side, whereas in the Tr3 the shape changes were displaced to the left side with higher shape deformation (Figure 7).

DISCUSSION

We observed a decrease in the survival of *Dendropsophus minutus* exposed to acute glyphosate contamination. The number of surviving tadpoles was drastically reduced in treatments T2 (31% of survivors), T3 (24% of survivors) and T4 (25% of survivors). Also, the $LC50_{96h}$ for *D. minutus* was 2.491 mg a.i./L, considered as moderately toxic (Giesy et al. 2000, U.S.EPA. 2008) and similar to values revealed by other studies using hylid treefrogs. The negative impact of glyphosate-based herbicides on survival of anuran tadpoles is relatively well documented (e.g. Mann and Bidwell 1999, Giesy et al. 2000, Jones et al. 2010, Relyea 2012, Costa and Nomura 2016), but few studies evaluated survival and LC50 for hylid tadpoles. Relyea (2005a) observed that the survival of *Hyla versicolor* tadpoles was drastically reduced at higher concentrations of Roundup formulation, with a $LC50_{16\text{-days}}$ of 1.35 mg a.i./L. Jones et al. (2011) also observed a survival decrease of *H. versicolor* tadpoles exposed to Roundup Original MAX® with $LC50_{16days}$ values ranging from 1.7 to 2.3 mg a.e./L. Low survival rates were observed for *Pseudacris triseriata* exposed to Roundup formulations Kleeraway® and WeatherMax® (Smith 2001, Williams and Semlitsch 2010). Lajmanovich et al. (2003) also observed a low survival of *Scinax nasicus* tadpoles exposed to GLYFOS® with a $LC50_{96h}$ of 2.64 mg a.i./L. Certainly, hylid tadpoles are vulnerable to glyphosate contamination and this sensitivity differs among species. However, comparisons among species are uncertainly due to methodological differences among studies (Simioni et al. 2013). Thus, the comparative potential could be increased if future studies that assess the glyphosate effects on co-specific tadpoles. Much more information needs to be gathered to assess the real impact of glyphosate use on Hylidae (the second largest anuran family in the world – Frost 2017), especially in the Neotropical region where, despite of a greater anuran diversity suffering high rates of population declines, a gap of ecotoxicological studies exists (Schiesari et al. 2007).

In the chronic exposure experiment, we observed differences among treatments in activity time of *D. minutus* tadpoles at the beginning of the exposure (1st day). An increase of activity time was observed when tadpoles were submitted to contamination only (Tr2) and to combination of contamination and predator cues (Tr3) when compared to control and predator cues only (Tr1). Considering there was no increase of activity time of tadpoles exposed to predator cues only (Tr1 – i.e. absence of anti-predator response associated to activity time) and a similar increase in activity time in Tr2 and Tr3, we found that there are no synergistic effects of both stressors, highlighting the effect of glyphosate contamination alone on activity time. This activity increase in the 1st day can be associated to the stress caused by the first contact with pesticide in which tadpoles may have increased their displacement to avoid it. The effects of glyphosate-based herbicides on tadpole activity are relatively unknown (e.g. Wojtaszek et al., 2004, Katzenberger et al. 2014, Moore et al. 2015, Mikó et al. 2015, 2017). Some authors reported that tadpoles exposed to glyphosate show a decrease in activity with more frequency of hiding behaviors and reduction in basal movements (e.g. Moore et al. 2015, Mikó et al. 2017). However, other authors observed an increase in activity patterns of tadpoles exposed to glyphosate-based herbicides, such as increase in swimming speed and in the number of active tadpoles (e.g. Katzenberger et al. 2014, Mikó et al. 2015). We also observed that activity time in Tr2 and Tr3 decreased over time and did not differ between treatments in other periods (8th day and 16th day). This condition can be associated to pesticide stratification in the water column during the experiments and/or represent a habituation to the contaminated environment (Jones et al. 2010, 2011, Relyea 2012). However, these hypothesis need to be tested.

The physiological mechanism associated to behavioral changes caused by glyphosate-based herbicides is unknown (Mikó et al. 2017). It's know that concentration of methaemoglobin in the blood of tadpoles increases when contaminated by nitrogenous compounds (e.g. fertilizers), affecting oxygen transport (Huey and Beitinger 1980, Hoffmann 2010). This change in blood oxygen parameters can lead to an increase in the activity time associated to displacements, such as vertical movements in the water column to reach surface and breathe air, seeking to reduce the effects associated to hypoxia and/or suffocation (USEPA 1986, Marco and Blaustein 1999, Hoffmann 2010). Glyphosate-based herbicides can act in the same way of nitrogenous compounds, but this hypothesis needs to be evaluated in future studies. Although we did not analyze the variable (ii) air surface breathing, our results can support this observation since we found a significant correlation between the increase of activity time and the increase of air surface breathing behavior. We can highlight that most air surfacing movements were observed in treatments with glyphosate contamination (i.e.

Tr2 and Tr3). Contaminated tadpoles directed their mouth out of the water, often releasing bubbles. This behavior is frequently associated to more active tadpoles and is understood as a complement of aquatic breathing (Wells 2007, Egea-Serrano et al. 2011, Denoel et al. 2012).

We observed that tadpoles submitted to contamination were more active, showed muscle spasms and expressed faster abnormal movements (e.g. erratic displacement without swimming patterns). Two tadpoles (one tadpole in the Tr2 and one tadpole in Tr3) showed abnormal behavior lying on the lateral side in the water column, similar to the atypical behavior described by Fordham et al. (2001) and Denoëel et al. (2012). These behaviors are listed as "atypical" by the literature and are associated to neurotoxic stress (e.g. Fordham et al. 2001, Brunelli et al. 2009, Tu et al. 2010, Denoël et al. 2012), potentially contributing to increase the activity time of tadpoles. However, we did not measure the frequency of atypical behaviors in this study due to the absence of descriptive patterns about the functioning of these atypical behaviors and due to the difficulty in identifying when these behavioral patterns were being performed.

In the 1st day of the experiment, we observed that tadpoles exposed to predator cues remained more distant from predators (Tr1) when compared to other treatments. This greater distance from the predator can be interpreted as an avoidance behavior to reduce predation risk. When submitted to predation risk the most common responses of tadpoles are associated to activity reduction (e.g. immobility, activity time reduction, refuge use) to avoid being detected by the predator (Van Buskirk and Arioli 2002, Relyea 2004, Wells 2007, Jara and Perotti 2009, Jara and Perotti 2010). However, predator-induced behavioral responses are highly plastic and can vary according to predator type and/or specific characteristics of tadpoles (e.g. unpalatability, cryptic coloration) (Relyea 2001, Relyea 2003, Jara and Perotti 2009, Jara and Perotti 2010), as well due to the action of stressor factors. For example, Moore et al. (2015) submitted tadpoles of woodfrogs (*Lithobates sylvatica*) to predator risk and observed that tadpoles failed to express anti-predator responses when submitted to Roundup^{TM}. They suggested that the absence of anti-predator responses is associated to a complete or partial deactivation of the alarm cue system of tadpoles caused by pesticide exposure. As observed by Tierney et al. (2006), an exposure to Roundup^{TM} impaired the olfactory system that can affect recognition of predator cues. Thus, based on our results we also suggest an absence of synergistic effect of both stressors on distance from predator due to the absence of difference between Tr1 and Tr3. It’s possible that glyphosate contamination influences the anti-predator responses of *D. minutus*, leading tadpoles to disregard the predation risk, resulting in them getting closer to the predator in factorial treatments (Tr3).

Considering the general external morphology, tadpoles submitted to factorial treatment (Tr3) showed higher shape changes in lateral and dorsal views when compared to other treatments. Due to the higher changes in morphology of tadpoles from Tr3 when compared to tadpoles from predator cues only (Tr1) and Roundup only (Tr2), we can suggest a synergistic effect of both stressors on morphology. In lateral view, the main shape changes in Tr3 are associated to deeper tail fins and deeper body, mainly in the gut region. These changes are the opposite of the observed in the tadpoles from Control, Tr1 and Tr2, which presented shallow tail fins and shallow bodies. Deeper tails can favor swimming patterns that ensure escaping from the predator and/or act conspicuously to attract the predator attention to less lethal regions than the body, being the most observed adaptive response associated to high predation risk (Caldwell 1982, Brodie Jr. et al. 1991, Relyea 2000, Relyea 2001, Van Buskirk & McCollum 2000, Van Buskirk et al. 2003, 2004, Relyea 2004, Van Buskirk 2009, Relyea 2012, Katzenberger et al. 2014, Woodley et al. 2015). This predator-mediate effect was observed by Katzenberger et al. (2014) that found similar morphological responses (i.e. deeper bodies and deeper tails) in tadpoles exposed to predator cues and tadpoles exposed to a combination of Roundup and predator cues, highlighting the main effect associated to predation risk and not to Roundup contamination effect only. However, Relyea (2012) observed that Roundup led to deeper tails similar to changes induced by predators, suggesting that anti-predator responses can be activated by the herbicide action. Our results corroborate the suggestions made by Relyea (2012) because the anti-predator changes were observed when tadpoles were exposed to factorial treatment only (i.e. predator cues + herbicide). In dorsal view, we can highlight this specific effect of the herbicide on morphology due to the similar shape changes between Tr2 and Tr3, mainly characterized by shallow bodies. Interestingly, shallow body is also commonly a predator-mediated response (e.g. Van Buskirk 2000, Relyea 2001, Relyea 2004, Van Buskirk 2009, Relyea 2012, Woodley et al. 2015). These anti-predation mechanisms (shallow bodies and deeper tails) are associated to reduction of the chances of the predator attacks in vital regions of the prey (i.e. body), directing the predator attention to less vital regions (i.e. tail) (Brodie Jr. et al. 1991). Thus, our results reinforce the herbicide potential to activate anti-predation responses (Relyea 2012).

Compared to control tadpoles, the highest morphological distance among fluctuating asymmetry levels of hyobranchial was observed in Tr2, Tr3 and Tr1, respectively. This result reinforces the contribution of the Roundup contamination to the morphological changes associated to developmental instability of tadpoles (e.g. Costa and Nomura 2016), which is represented by higher FA when compared to non-contaminated tadpoles. Also, the increase in fluctuating asymmetry in the hyobranchial skeleton of tadpoles from Tr3 can be interpreted as a synergistic effect of both stressors. When exposed to both factors, we observed that shape changes in the FA tend to move to the opposite side when compared to tadpoles from Control, Tr1 and Tr2. Furthermore, we showed that tadpoles submitted to predators only (i.e. natural stress) had a considerable FA level when compared to control, reflecting the contribution of natural stressors to developmental instability. Fluctuating asymmetry is widely used as a method to measure the influence of stressful factors on developmental instability in different groups of organisms (Palmer and Strobeck 1986, Sanseverino and Nessimian 2008, Beasley et al. 2013), in which deviations generally are greater when stress increases. Studies using larvae and adult amphibians as a model have found this relationship between asymmetry and anthropogenic stress (e.g. Gallant and Teather 2001, Lauck 2006, Söderman et al. 2007, Delgado-Acevedo and Restrepo 2008, Eisemberg and Bertoluci 2016, Costa and Nomura 2016, Costa et al. 2017, Zhelev et al. 2015, 2017). For example, Costa et al. (2017) observed that the increase of FA levels in tadpoles of *Physalaemus cuvieri* and *Scinax fuscomarginatus* is associated to an increase of agropastoral land use, where higher FA levels are observed in tadpoles from ponds under greater land use. Also, Costa and Nomura (2016) found that tadpoles of *P. cuvieri* showed higher FA levels when exposed to Roundup Original® contamination. Eisemberg and Bertoluci (2016) found higher levels of FA in adults of *P. cuvieri* suffering higher levels of anthropogenic disturbance. Higher FA levels were observed in populations of *Pelophylax ridibundus* from rice paddy ecosystem (Zhelev et al. 2017) as well from populations exposed to domestic sewage pollution and heavy metal pollution (Zhelev et al. 2015). Relationships between FA increase and habitat loss were observed for *Eleutherodactylus coqui* (Delgado-Acevedo and Restrepo 2008), while increase of FA levels was observed in skeletons of *Rana arvalis* from acidified localities

(Söderman et al. 2007). Our results reinforce the contribution of the anthropogenic stressors (i.e. Roundup contamination) on the morphological changes associated to developmental instability of amphibians, mainly for anuran tadpoles.

Often, a symmetrical morphological trait (e.g. sensory and/or locomotor) is associated to performance of individuals that potentially affect their fitness, but this relationship is difficult to measure (Clarke 1995, Møller 1997, Lens et al. 2002). Few studies have experimentally tested the FA/performance/fitness relationship. For example, Bosch and Márquez (2000) found that females of *Alytes obstetricans* with higher tympanum FA had a low accuracy to locate male vocalizations. Møller (1996) observed that higher levels of FA decreased flight performance in *Musca domestica*. Similarly, Swaddle et al. (1996) found that higher FA in primary feathers of *Sturnus vulgaris* affected flight performance. Considering FA in tadpoles, there are no studies that prove the perofrmance/FA relationship. Thus, this relationship is based on inferences, such as the relationship between higher FA in nostrils and eyes and reduction in predator risk recognition (e.g. Costa and Nomura 2016). Probably, higher levels of FA in hyobranchial skeleton leads to lower food intake and/or to respiratory insufficiency with a decrease in oxygen uptake. However, this is an inference which needs to be proven in a future study.

Our results show that glyphosate contamination lead to a survival decrease of *D. minutus* tadpoles and changed sublethal attributes, mainly those predator-mediated. Also, we showed that changes in sublethal attributes can be synergistically affected by multiple external factors (i.e. predators and Roundup contamination). Under natural conditions, changes in species-specific attributes are adaptive responses that reflect the performance and fitness of tadpoles, directly influencing predatory and competitive interactions (Hero et al. 2001, Van Buskirk and McCollum 2000, Van Buskirk and McCollum 2000b, Van Buskirk 2009, Relyea 2012). However, contamination with glyphosate can change these natural responses expressed by tadpoles in freshwater communities, potentially leading to a poorly suited phenotype and to unsuccessful trophic interactions (Relyea 2012). Thus, understanding which species-specific attributes are changed by pesticide contamination, mainly those traits that mediate intra/interspecific interactions, can ultimately contribute to freshwater homeostasis evaluation, as well as to estimate population declines and/or local species loss.

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FIGURES AND TABLES

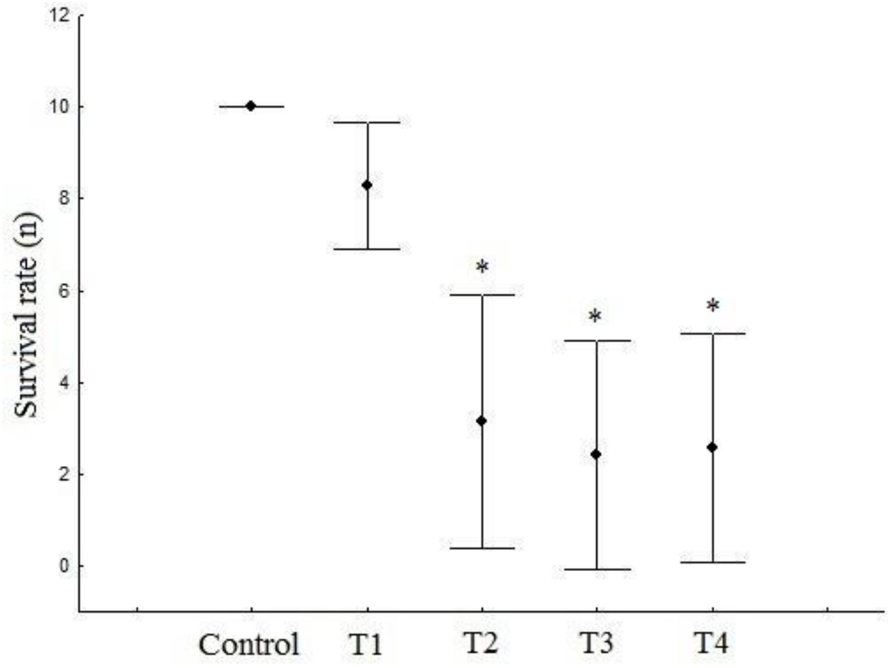


Figure 1: Survival of tadpoles exposed to acute contamination of Roundup Original®. Black points are the means and the bars are the confidence interval (±95%). Significant differences ($p < 0.05$) with respect to control are marked with * (Mann-Whitney post-hoc test).

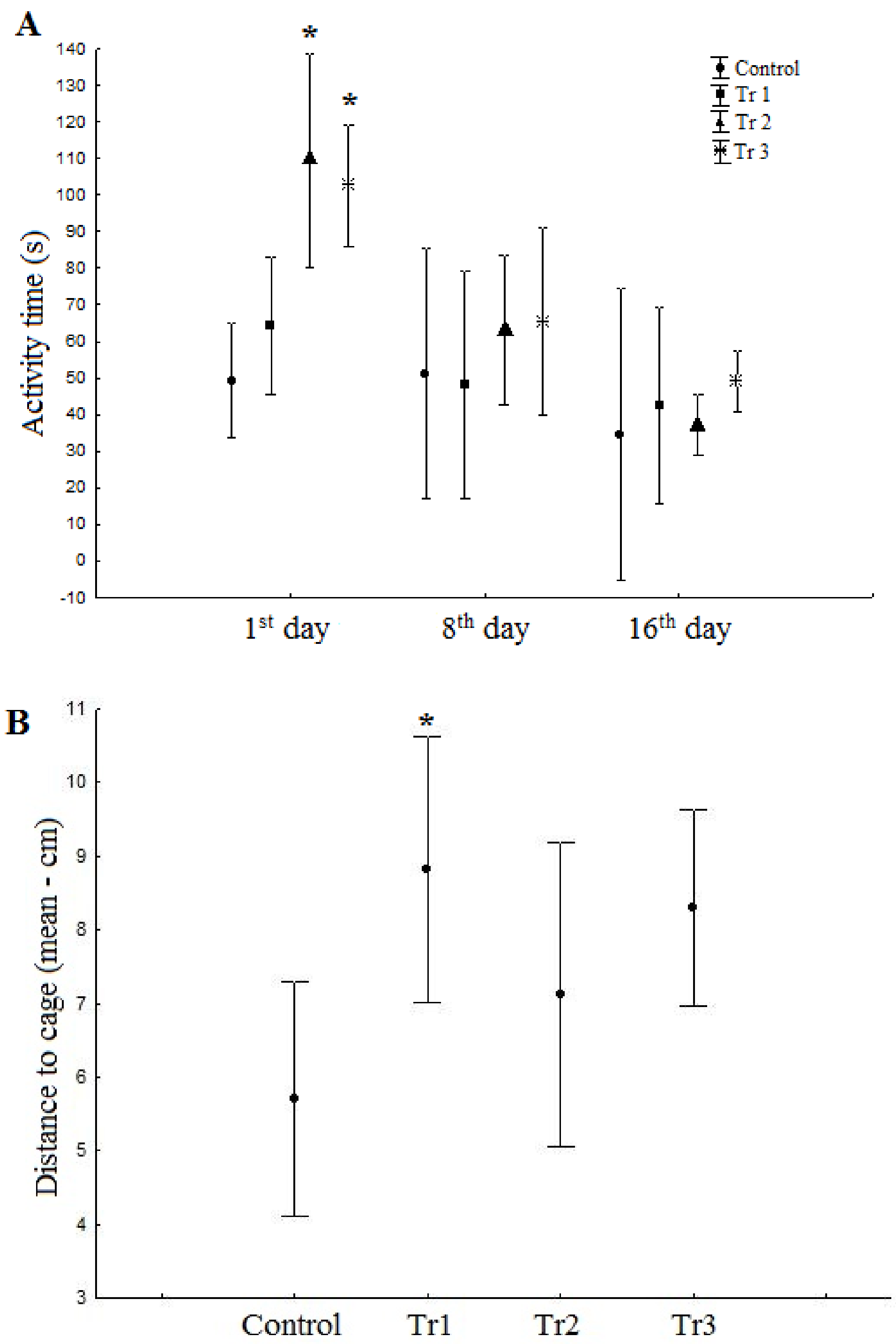


Figure 2: Activity time (A) and distance to cage (B) among treatments. Symbols are the means and the bars are the confidence interval (±95%). Significant differences ($p < 0.05$) with respect to control are marked with * (Tukey post-hoc test).

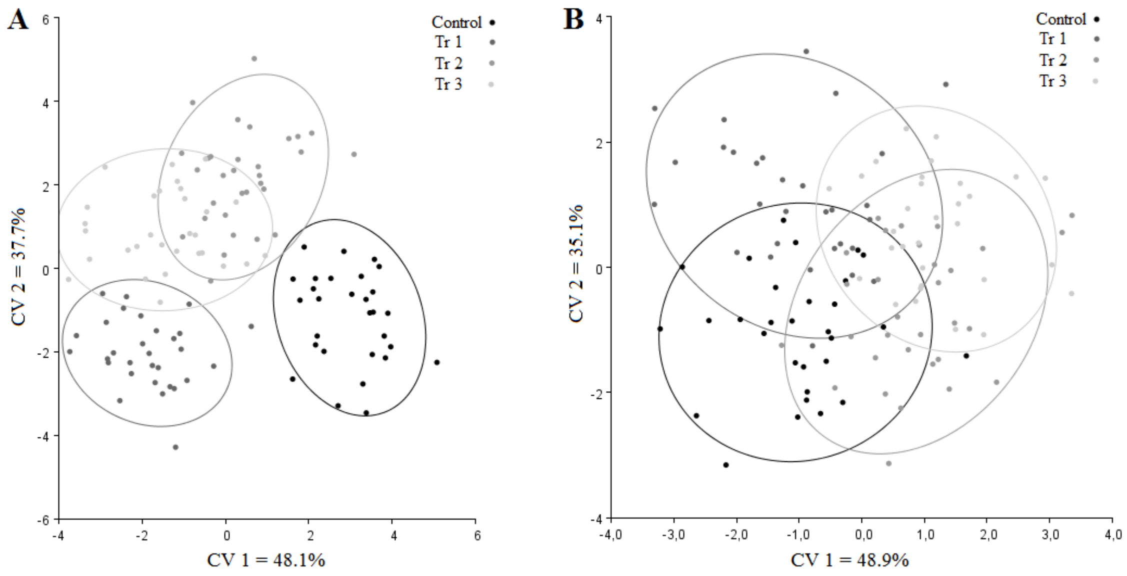


Figure 3: Results of the Canonical Variate Analysis of the general external morphology in (A) lateral view and (B) dorsal view.

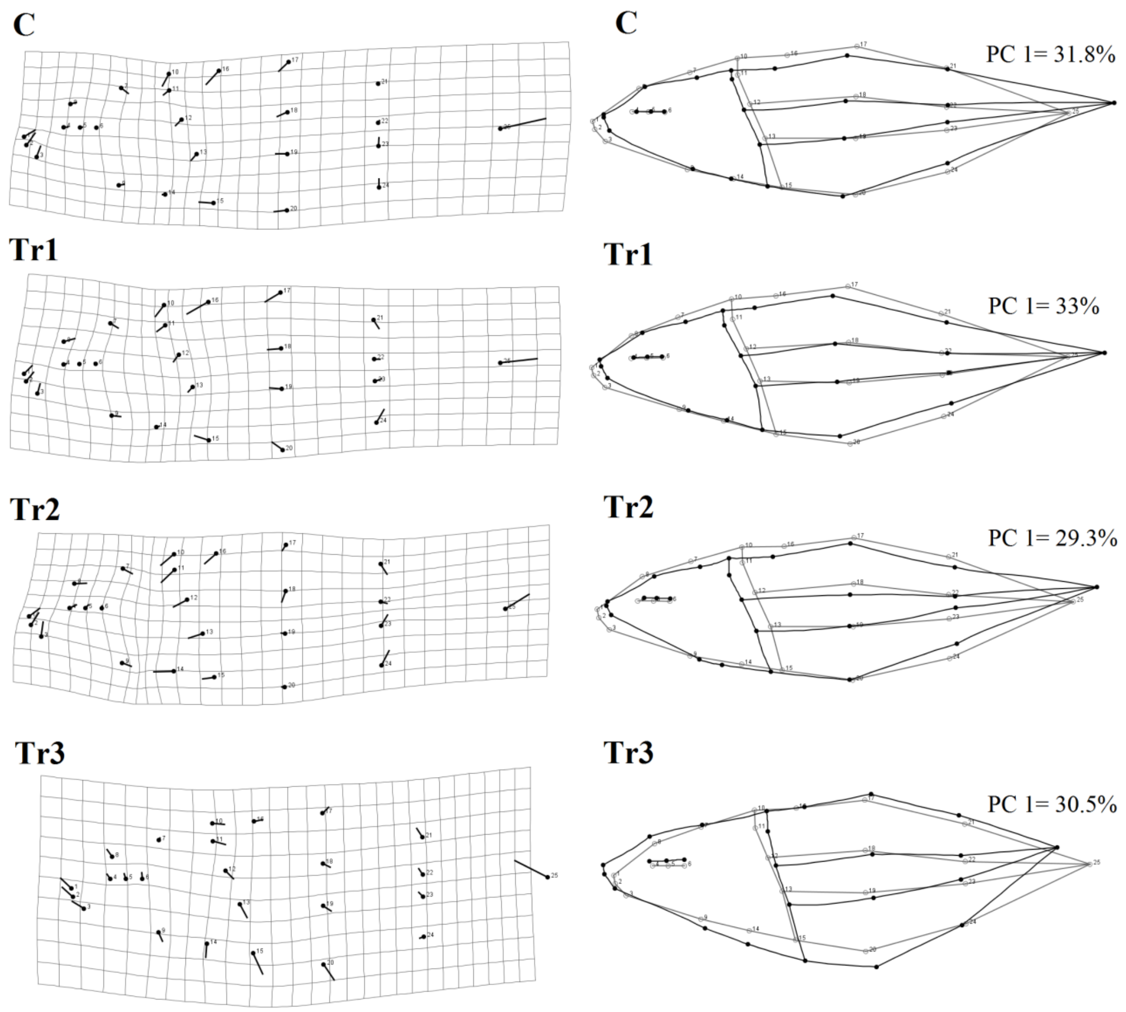


Figure 4: Transformation grid and wireframe outline of the changes on general external morphology of tadpoles in lateral view. In the wireframe graphs, the gray lines represent the shape of Procrustes consensus of the control tadpoles. C= control; Tr1 (exposed to predator cues); Tr2 (exposed to Roundup); Tr3 (exposed to combination of predator cues and Roundup)

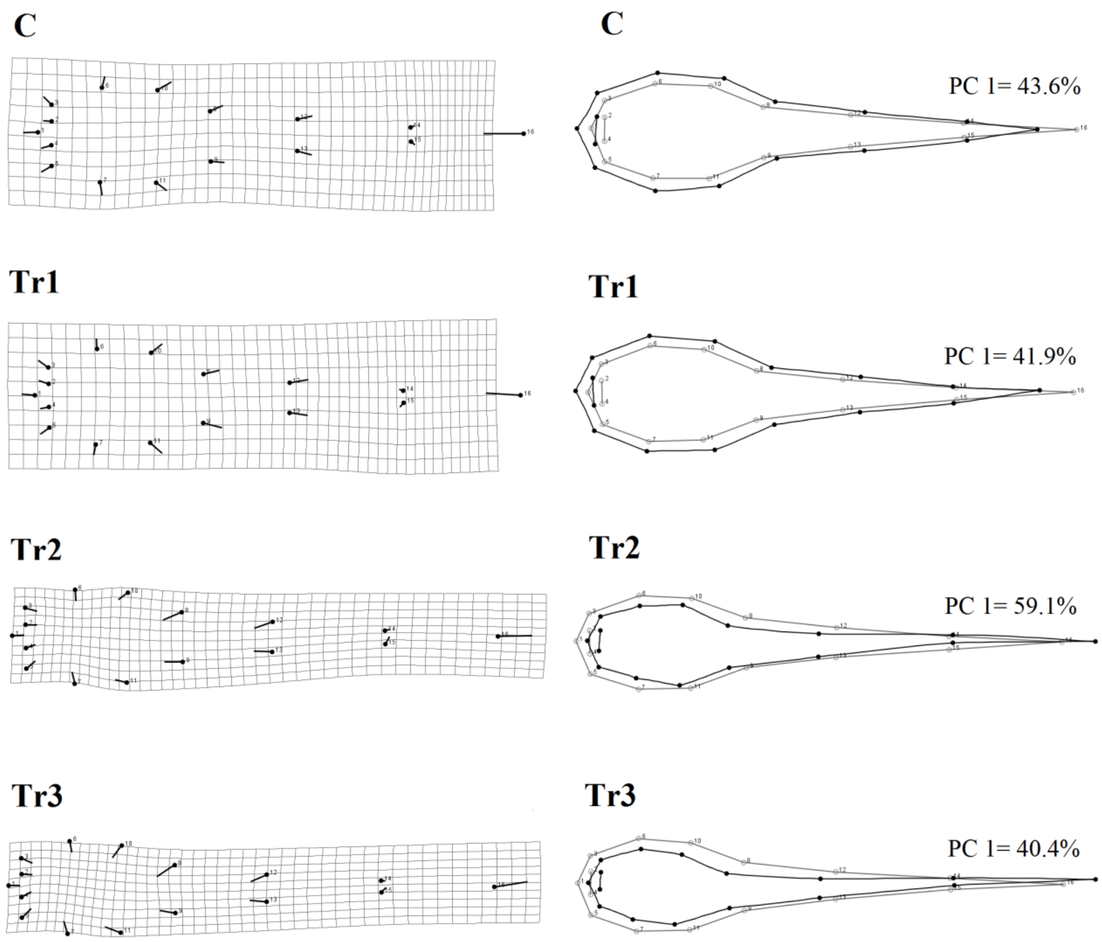


Figure 5: Transformation grid and wireframe outline of the changes in general external morphology of tadpoles in dorsal view. In the wireframe graphs, the gray lines represent the shape of Procrustes consensus of the control tadpoles C= control; Tr1 (exposed to predator cues); Tr2 (exposed to Roundup); Tr3 (exposed to combination of predator cues and Roundup).

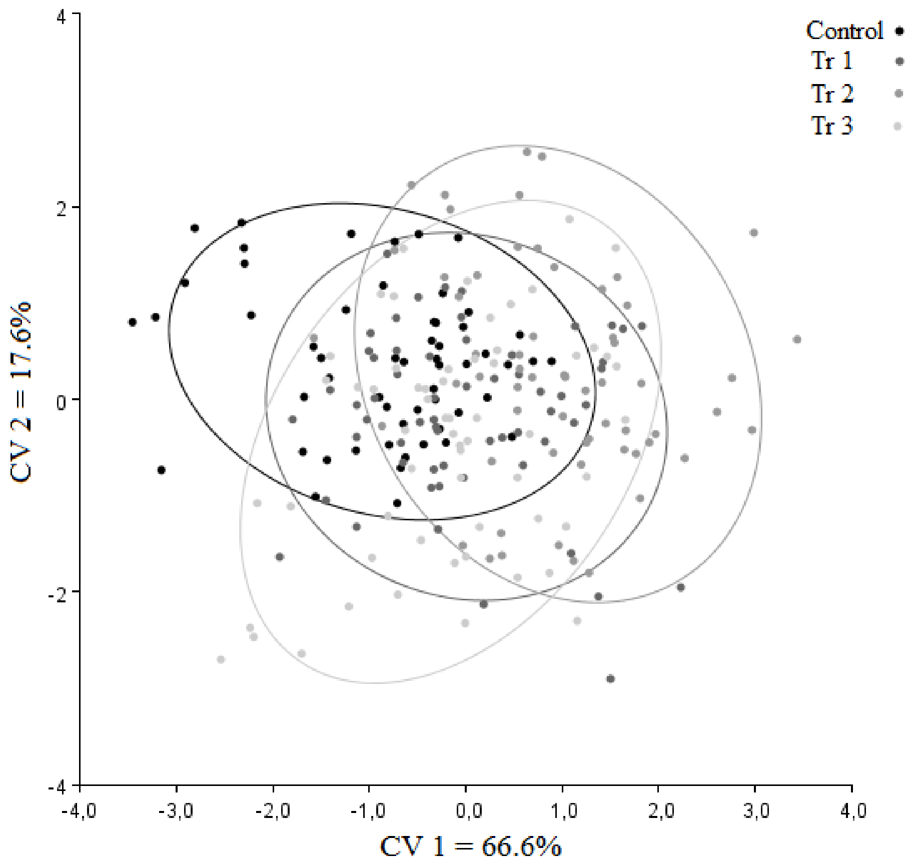


Figure 6: Result of the Canonical Variate Analysis to the fluctuating asymmetry of hyobranquial skeleton.

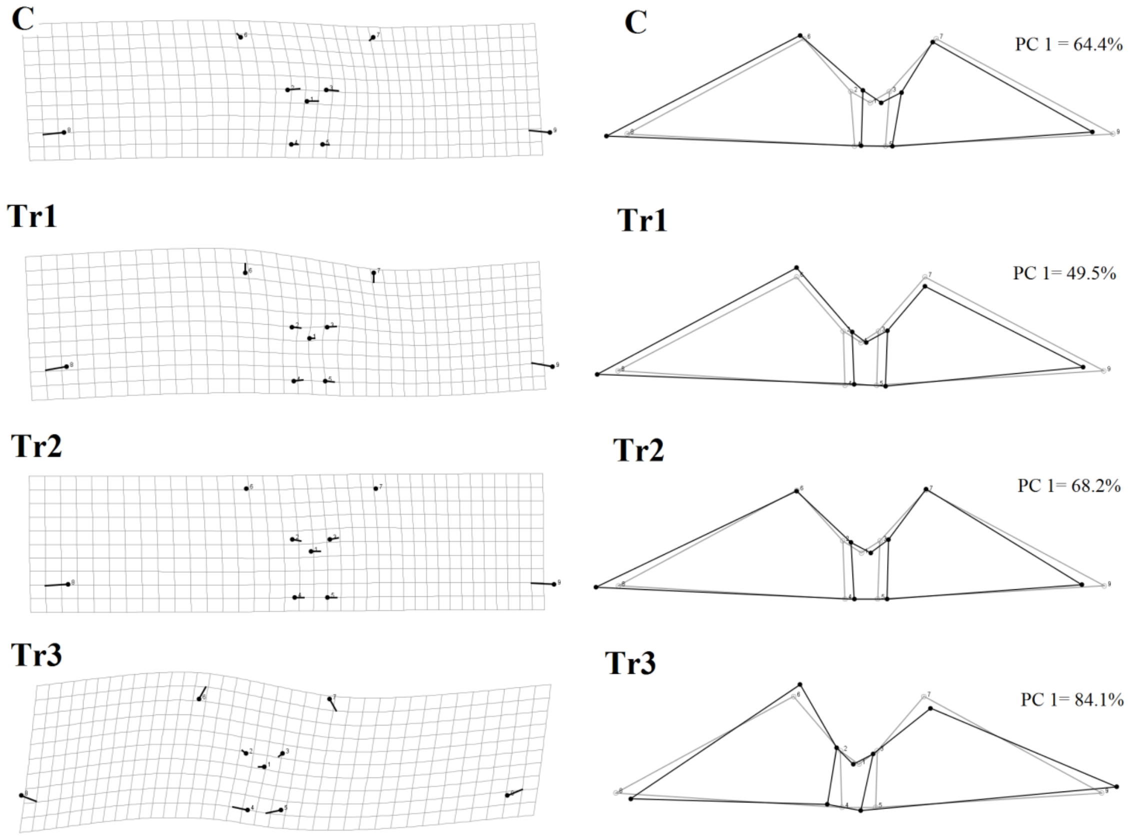


Figure 7: Transformation grid and wireframe outline of the shape changes on the fluctuating asymmetry in the hyobranquial skeleton. In the wireframe graphs, the gray lines represent the shape of Procrustes consensus of the control tadpoles. C= control; Tr1 (exposed to predator cues); Tr2 (exposed to Roundup); Tr3 (exposed to combination of predator cues and Roundup).

Table 1: Procrustes distance among treatments and permutation test results (10000 permutations) for general external morphology in lateral and dorsal view.

A - Lateral view				B - Dorsal view			
	Control	Tr1	Tr2		Control	Tr1	Tr2
Tr1	**0,0293***			Tr1	0,0128		
Tr2	**0,0238****	**0,0332***		Tr2	0,0122	0,0134	
Tr3	**0,0326***	**0,0215****	**0,0237***	Tr3	**0,0191***	**0,0154****	0,0124

Bold= p-values from permutation tests (*p<0.001 and **p<0.05)

Table 2: Results of Procrustes ANOVA to evaluation the fluctuating asymmetry in hyobranquial skeleton.

Effect	MS	Df	F	p
Individual	0.0018553192	98	7.41	**<0.001**
Side	0.0005034600	7	2.01	0.061
Individual*Side	0.0002502470	98	10.48	**<0.001**
Error 1 (image)	0.0000238699	210	0.10	0.999
Error 2 (digit.)	0.0001066914	210	0.44	1.000
Residual	0.0002494606	2940		

Table 3: Procrustes distance among treatments and permutation test results (10000 permutations) for fluctuating asymmetry.

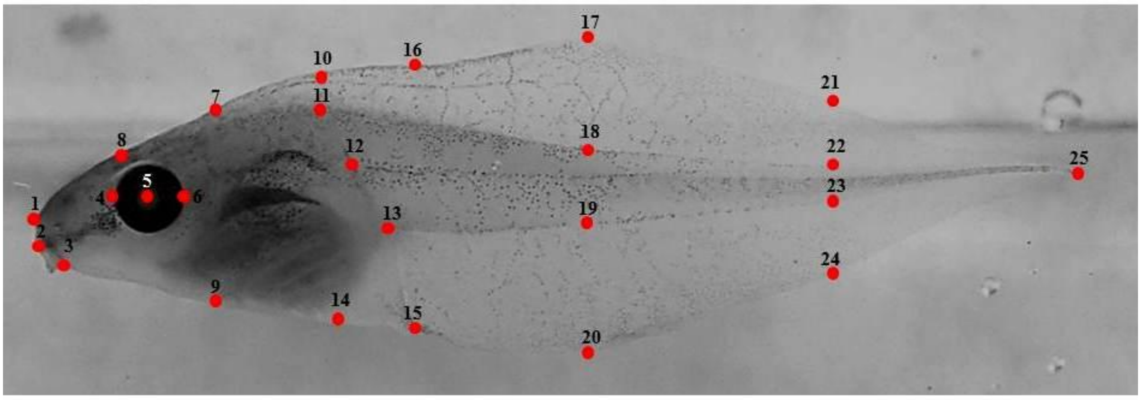
	Control	Tr1	Tr2
Tr1	**0.0098***		
Tr2	**0.0180***	**0.0097***	
Tr3	**0.0111****	0.0060	**0.0104***

Bold= p-values from permutation tests (*p<0.001 and **p<0.05)

SUPPLEMENTARY MATERIAL

Figure S1: Landmark configuration in (A) lateral view and (B) dorsal view in tadpoles of *Dendropsophus minutus*. LM= landmarks.

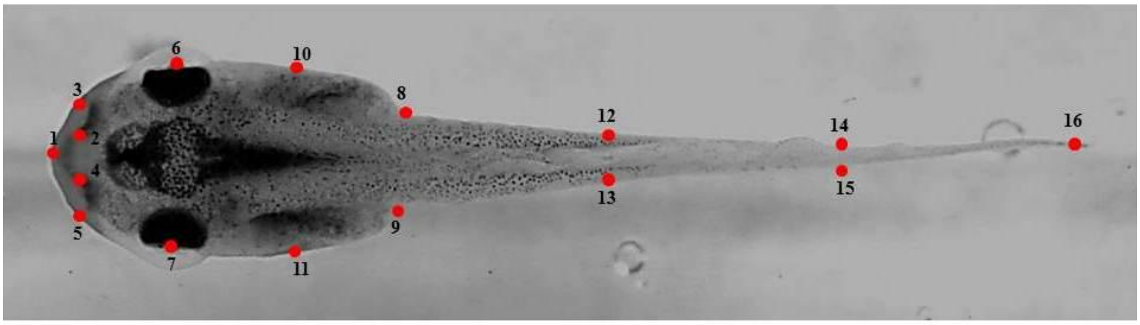
A – Lateral view



LM	Description
1	Anterior-most edge of the snout
2	Anterior point of oral tube when viewed from the side
3	Posterior point of oral tube when viewed from the side
4	Anterior edge of the iris on a horizontal line extending through the center of the eye
5	Center of the pupil
6	Posterior edge of the iris on a horizontal line extending through the center of the eye
7	Point at which the dorsal tail fin joins the top of the body
8	Central point between #1 and #7, representing the snout curvature
9	Ventral edge of the body directly below **#7**
10	Emergency angle of the dorsal fin, parallel to the intersection of the dorsal edge of the tail muscle and the body #11
11	Intersection of the dorsal edge of the tail muscle and the body
12	Intersection of the notochord and the body
13	Intersection of the ventral edge of the tail muscle and the body
14	Intersection of the terminal point of the body and front end of anal tube
15	Posterior end of the anal tube
16	Dorsal edge of the tail fin directly below #15
17	Dorsal edge of the tail fin in the highest point

18	Dorsal edge of the tail muscle directly below #17
19	Ventral edge of the tail muscle directly below #17
20	Ventral edge of the tail fin directly below #17
21	Dorsal edge of the tail located in the middle between #17 and #25
22	Dorsal edge of the tail muscle directly below #21
23	Ventral edge of the tail muscle directly below #21
24	Ventral edge of the tail fin directly below #21
25	Tip of the tail

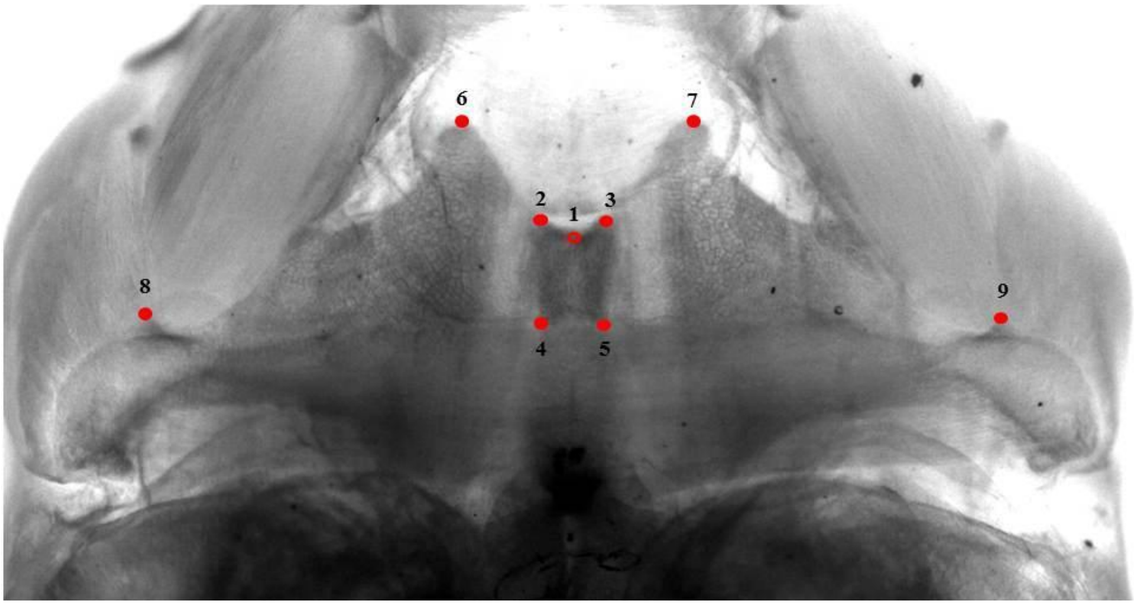
B – Dorsal view



LM	Description
1	Anterior-most edge of the snout
2	Anterior point of the right nostril
3	Edge of the body directly below #2, representing the snout curvature
4	Anterior point of the left nostril
5	Edge of the body directly below #4, representing the snout curvature
6	Center of the right eye
7	Center of the left eye
8	Point at which the tail muscle join to the right side of body
9	Point at which the tail muscle join to the left side of body
10	Central point between #6 and #8, representing the body curvature
11	Central point between #7 and #9, representing the body curvature
12	Muscle tail edge of the right side of the body at 1/3 of distance between #8 e # 16
13	Muscle tail edge of the left side of the body at 1/3 of distance between #9 e # 16

14	Muscle tail edge of the right side of the body at 2/3 of distance between #8 e # 16
15	Muscle tail edge of the left side of the body at 2/3 of distance between #9 e # 16
16	Tip of the tail

Figure S2: Landmarks configuration to fluctuating asymmetry analysis in hyobranquial skeleton of *Dendropsophus minutus* tadpoles. LM 1 = unpaired landmark.



LM	Description
1	Midline point of the hyobranchial skeleton
2	Upper point of the basibranchial – left side
3	Upper point of the basibranchial – right side
4	Bottom point of the basibranchial – left side
5	Bottom point of the basibranchial – right side
6	Maximum curvature point of the processus anterior – left side
7	Maximum curvature point of the processus anterior – right side
8	Maximum curvature point of the condyles articularis – left side
9	Maximum curvature point of the condyles articularis – right side

Post-hoc tables

Table S1: A post-hoc Mann-Whitney test pointed the differences in the survival among treatments.

	Control	0.28 mg	1.5 mg	3 mg	6 mg
R	31.000	25.143	12.286	10.786	10.786
Control					
0.28 mg	1,000000				
1.5 mg	**0,006337**	0,189058			
3 mg	**0,002237**	0,087610	1,000000		
6 mg	**0,002237**	0,087610	1,000000	1,000000	

Table S2: A post-hoc Tukey test pointed the differences in the activity time among treatments.

		1	2	3	4	5	6	7	8	9	10	11	12
		49.360	64.370	109.48	102.63	51.240	48.020	63.050	65.430	34.640	42.550	37.346	49.150
1	First period/ Control												
2	First period/ Tr1	0.987											
3	First period/ Tr2	**0.001**	**0.035**										
4	First period/ Tr3	**0.005**	0.132	0.999									
5	Half period/ Control	1.000	0.995	**0.001**	**0.008**								
6	Half period/ Tr1	1.000	0.976	**0.000**	**0.004**	1.000							

7	Half period/ Tr2	0.994	1.000	**0.026**	0.104	0.998	0.987					
8	Half period/ Tr3	0.979	1.000	**0.043**	0.158	0.992	0.962	1.000				
9	Final period/ Control	0.989	0.456	**0.000**	**0.000**	0.973	0.995	0.525	0.403			
10	Final period/ Tr1	0.999	0.847	**0.000**	**0.001**	0.999	0.999	0.892	0.804	0.999		
11	Final period/ Tr2	0.998	0.599	**0.000**	**0.000**	0.993	0.999	0.669	0.543	1.000	1.000	
12	Final period/ Tr3	1.000	0.986	**0.001**	**0.005**	1.000	1.000	0.993	0.976	0.990	0.999	0.998

Table S3: A post-hoc Tukey test pointed the differences in the distance from the cage among treatments.

	Control	Tr1	Tr2	Tr3
F	5.703	8.830	7.124	8.302
Control				
Tr1	**0,049**			
Tr2	0,622	0,472		
Tr3	0,133	0,969	0,747	

CAPÍTULO 3

Lethal effects of Roundup Orginal DI® on tadpoles of *Physalaemus cicada* **(Anura: Leptodactylidae)**

Renan Nunes Costa and Mirco Solé

Manuscrito a ser submetido para a revista *Environmental Toxicology and Chemistry* (*Short Communication*)

Short Communication

LETHAL EFFECTS OF ROUNDUP ORIGINAL DI® ON TADPOLES OF *Physalaemus cicada* (ANURA: LEPTODACTYLIDAE)

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ABSTRACT

Experimentally, we evaluated the lethal effects of the glyphosate-based herbicide Roundup Original DI® on tadpoles of *Physalaemus cicada.* We found that the contamination significantly decreased tadpole survival. The estimated $LC50_{96h}$ is 5.27 mg a.i./L, considered as moderately toxic. *Physalaemus cicada* has an intermediate pesticide-tolerance when compared with congeneric species. Our results contribute to the increase of knowledge about the glyphosate impact on the *Physalaemus* genus. This genus is widely distributed and potentially can be used as a tool to assess the impact of glyphosate.

KEY-WORDS – survival; LC50; acute exposure experiment; glyphosate; herbicide; congeneric species comparison

INTRODUCTION

Brazilian biomes have been replaced by extensive monocultures to ensure the production and exportation of commodities, such as soybean and sugarcane [1]. To maintain this economic exportation system a large production of quality products is necessary, without the negative effects of agricultural pests. For this, tons of pesticides are intensively applied by farmers and distributed by big companies contributing to environmental contamination and health problems [1, 2, 3, 4].

Glyphosate-based herbicides (e.g. Roundup) are highly applied in Brazilian croplands and the commercialized volume is significantly greater than that of other active ingredients used, being applied in the majority of cropland types [1, 3]. The amount and frequency of application added to the high proximity between water bodies and croplands [5], potentially leads to contamination of freshwater environments [1, 6, 7].

Strongly associated to freshwater environments, amphibians are especially susceptible to contamination because they have permeable skin, and many species colonize temporary and permanent ponds in agricultural landscapes, with aquatic eggs and tadpoles developing under pesticide stress [8]. Experimental studies with tadpoles found that glyphosate formulations negatively affect different species, through measures of survival and based on the lethal concentration (e.g. LC50 – lethal concentration to 50% of a population) [9, 10, 11]. Some Brazilian species were experimentally tested, but there is a significant gap of ecotoxicological studies considering the high anuran diversity in the country [12]. Therefore, few congeneric species have been tested, reducing the comparative potential among studies.

In this short communication, we tested the lethal effects of Roundup Original DI® (i.e. survival and LC50) on tadpoles of *Physalaemus cicada* Bokermann 1966. We choose *P. cicada* because it is a congeneric species of the most representative Brazilian genus with studies about the lethal effects of glyphosate formulations on tadpoles {[*P. cuvieri* [11], *P. centralis* [13] and *P. albonotatus* [14]}, which can increase the robustness of the comparisons. Also, these species are widely distributed throughout Brazil and can be found in habitats with different levels of pesticide application.

MATERIALS AND METHODS

Sample and experimental background

We collected five egg masses of *Physalaemus cicada* from Brumado municipality, state of Bahia, Brazil (14° 3'53.26"S, 41°51'0.89"W). Egg masses were collected in a semi-permanent lentic pond, surrounded by Caatinga vegetation with partially covered canopy. The landscape is fragmented, but the pond is inserted in a relatively well conserved fragment without contact with croplands. Egg masses were transported in plastic bags with water from ponds to the Laboratory of Vertebrate

Zoology at the Universidade Estadual de Santa Cruz - UESC, Ilhéus, Bahia, Brazil. In the lab, egg masses were combined and acclimatized in a glass aquarium with 8L of dechlorinated water, under 25ºC ± 1ºC and photoperiod (12 h light/12 h dark). After hatching, tadpoles were randomized diluting the potential parental effect, if any, at the treatments. We used plastic aquaria (27 cm X 18 cm X 8 cm) as experimental units with 1 L of dechlorinated water, without substrate and constant oxygenation through aquarium air compressors.

Tadpoles were maintained in the lab until they reached the developmental stage 25 [15]. Following, we randomly collected 10 tadpoles to add to each experimental unit. There they were acclimatized for 24 h before treatment exposure. We tested the commercial formulation of glyphosate (Roundup Original DI®) with 44.5% of active ingredient. Based on CONAMA 357 [16] resolution, we calculated the nominal concentrations of glyphosate starting from 0.28 mg a.i./L (280 µg/l – class III freshwater types in Brazil), increasing through uniform increments based on studies with congeneric species [11, 13, 14]. We prepared treatment-specific solutions with five nominal concentrations of glyphosate (Control = 0 mg a.i./l; T1 = 0.28 mg a.i./l; T2 = 1.5 mg a.i./l; T3 = 3 mg a.i./l; T4 = 6 mg a.i./l). To achieve these glyphosate solutions, we added 0.63 (T1), 3.37 (T2), 6.74 (T3) and 13.48 (T4) µl of Roundup Original DI®. We replicated each treatment seven times, totalizing 35 experimental units and 350 tested tadpoles. The position of experimental units and treatments was randomized.

The exposure time was 96 hours (4 days), with revisions every 24 hours to remove any dead tadpole. At the end of the experiments, we calculated overall survival and measured water pH and temperature in each experimental unit. Surviving tadpoles were anesthetized and sacrificed by immersion in benzocaine solution (10 mg/l) and preserved in 10% formalin. Tadpoles were deposited in the Museu de Zoologia da Universidade Estadual de Santa Cruz (MZUESC).

Statistical analysis

We performed a Kruskall-Wallis test to compare tadpole survival among treatments. Following, we performed a posteriori Mann-Whitney test. To estimate the $LC50_{96h}$ value, we used a Probit Regression Analyses. We also applied Kruskall-Wallis tests followed by a Mann-Whitney test to evaluate differences in temperature and pH of water among treatments.

RESULTS

We observed that the contamination by Roundup Original DI® decreased the survival of *Physalaemus cicada* tadpoles ($H_{(4)}$= 17.501; p= 0.001 – Figure 1). When compared to control (91.4% of survivors), the lowest survival was observed in T4 (50%), T3 (52.8%), T1 (61.4%) and T2 (75.7% - non-significant), respectively (Table 1). The estimated $LC50_{96h}$ of *P. cicada* is 5.27 mg a.i./L, considered as moderately toxic.

There were no differences in the temperature of the water among treatments ($H_{(4)}$= 2.991; p= 0.559 – T_{mean}= 21.794°C ± 0.389; range= 22 – 21°C). We observed a slight difference in the pH of the water among treatments ($H_{(4)}$= 18.005; p= 0.001). Differences when compared to control were found in treatment T3 (p< 0.001) and T4 (p= 0.004). However, despite the statistical significance, there was only a small variation in the pH values within treatments ($pH_{control}$ = 4.88 ± 0.095; range= 4.99 – 4.72 / pH_{T1}= 4.86 ± 0.074; range= 5.02 – 4.81 / pH_{T2}= 4.78 ± 0.138; range= 4.98 – 4.6°C / pH_{T3}= 4.51 ± 0.208; range= 4.79 – 4.32 / pH_{T4}= 4.57 ± 0.175; range= 4.89 – 4.42). Considering the increase in water acidity in a pH scale, the variation observed here between control and treatments (i.e. means among 4.88 and 4.57) was very small and we suggest that it was not the factor responsible for the reduction in survival. This observation can be validated if we consider that there was a significant mortality in the treatment T1, in which the pH of the water did not change.

DISCUSSION

The acute contamination by Roundup Original DI® decreased the number of surviving tadpoles of *P. cicada*. This decrease in tadpole survival exposed to glyphosate-based herbicides was also observed for the congeneric species *P. cuvieri* [11], *P. centralis* [13] and *P. albonotatus* [14]. In addition to mortality in higher concentrations, we can highlight the significant mortality in lower concentrations, mainly in the treatment T1 (0.28 mg a.i./L – 38.6% of mortality), which is the level of glyphosate permitted by Brazilian legislation in class III freshwater environments [16]. There are several records of freshwater contamination by glyphosate in Brazil [1, 6, 7], but many cases of acute and chronic contamination can be underestimated and/or are not reported. This mortality can directly contribute to population declines [8, 12, 17, 18, 19, 20, 21] and reflects the fragility of the Brazilian laws for freshwater protection, as well as the absence of scientific support for law creation. Furthermore, the geographical distribution of *Physalaemus* species included in this comparison contemplates the most of Brazil and represents different biomes highly suppressed by croplands where pesticides are used. *Physalaemus cicada* is found in the most of Brazilians Northeast, *P. centralis* and *P. albonotatus* are found in the Southeast and Midwest, while *P. cuvieri* is found in virtually all regions of the country [*see* 22, 23]. This widely distributed species group can be used as a bioindicator tool [24, 25]. An increase in the knowledge about specific widely distributed groups, such as the genus *Physalaemus*, can help to develop mitigation strategies to reduce the impacts of glyphosate-based herbicides in the future.

The estimated $LC50_{96h}$ of *P. cicada* is 5.27 mg a.i./L. This value is similar to the values observed for *P. albonotatus,* with $LC50_{96h}$ of 5.38 mg a.i./L [14]. The $LC50_{96h}$ of *P. cicada* is approximately two times higher than that of *P. cuvieri* ($LC50_{96h}$ of 2.13 mg a.i./l0 [11] and four times smaller than that assessed for *P. centralis* (19.7 mg a.i./L) [13]. Our study is based on the protocol applied by Costa and Nomura [11], which is similar to the protocols applied by Simioni et al. [14] and Figueiredo and Rodrigues [13]. These studies with tadpoles of the genus *Physalaemus* are methodologically similar, but they reveal some differences. For example, we tested the glyphosate formulation Roundup Original DI® with 44.5% of active ingredient, while Costa and Nomura [11] tested Roundup Original® with 48%, Figueiredo and Rodrigues [13] tested Glifosato 480 Agripec® with 48%, and Simioni et al. [14] tested Gliz® 480 SL with 48%. These commercial formulations have different types of "inert ingredients", such as different concentrations of surfactant substances (*e.g.* polyethoxylated tallowamine – POEA, aminomethylphosphonic acid – AMPA), which can increase or not the glyphosate toxicity [8, 26]. However, despite the methodological differences, Bridges and Semlitsch [27] and Simioni et al. [14] suggest that discrepancy among LC50 values can represent the species-specific tolerance. Thus, we can build a glyphosate based-herbicide tolerance scale for *Physalaemus* species tested, in which *P. centralis* is more tolerant, followed by *P. albonotatus*, *P. cicada* and *P. cuvieri*.

There are several ecotoxicological studies with tadpoles, but the power of the comparisons among species can be uncertain and limited. We can list two main causes: (i) less studies with congeneric species and (ii) differences in methodological protocols and conditions. Differences in experimental conditions (e.g. differences in commercial formulations, exposure time, number of replicates, lab conditions, stage/age of organisms and others) combined to phylogenetic distance among species, make comparisons difficult and hamper definitions of which clades or species are most sensitive [8, 10, 14]. Another confounding factor is the stress level of the environment where the egg masses were collected. Some populations can be more pesticide-tolerant than others because they live within and/or closer to agriculture areas that suffer a periodic application of agrochemicals [28, 29, 30, 31].

We conclude that the glyphosate-based herbicide Roundup Original DI® decreases the survival of *Physalaemus cicada* tadpoles, being considered as moderately toxic. Comparing with congeneric species, *P. cicada* has an intermediate pesticide-tolerance, similar to *P. albonotatus*. This congeneric comparison is relatively robust because the experimental conditions of these studies were very similar, and can represent a first step for the development of mitigating measures in the future. However, comparisons should be made carefully, mainly when experiments have large methodological differences.

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FIGURES AND TABLES

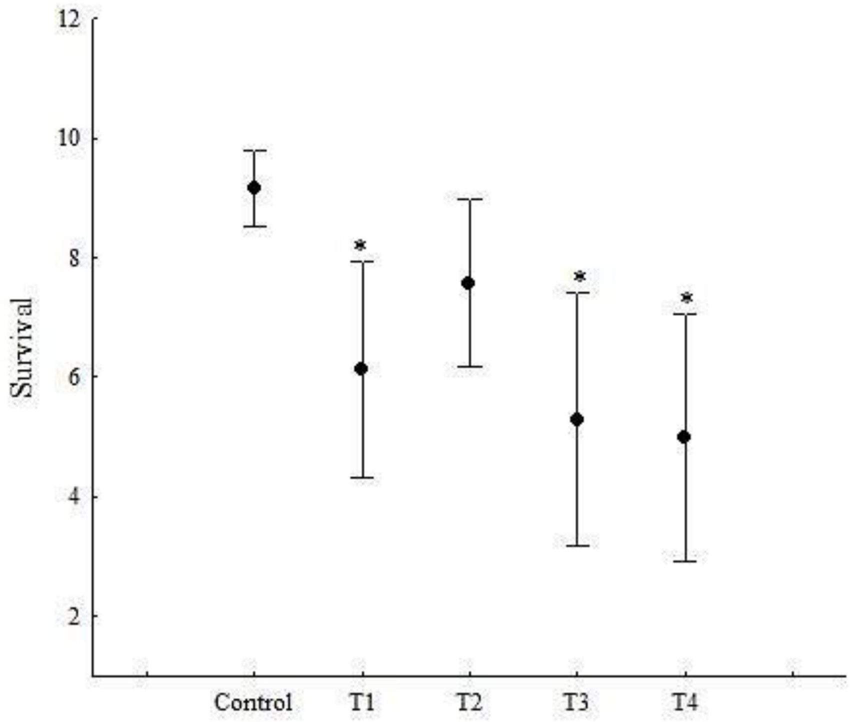

Figure 1: Survival of *Physalaemus cicada* tadpoles submitted to different concentrations of Roundup Original DI®. Points represent the means, and bars represent the confidence interval (95%). Statistical differences from the control are marked with (*), based on Mann-Whitney test.

Table 1: Results of a post-hoc Mann-Whitney test. H= results of the pairwise comparisons. Bold values represent significant differences.

	Control	T1	T2	T3	T4
H	30.500	14.857	21.071	12.429	11.143
Control					
T1	**0.042**				
T2	0.857	1.000			
T3	**0.009**	1.000	1.000		
T4	**0.004**	1.000	0.698	1.000	

CAPÍTULO 4

Fluctuating asymmetry in a small treefrog (*Dendropsophus haddadi:* Anura: Hylidae) as a measure of the history of glyphosate application: a case study from Plantações Michelin da Bahia

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Fluctuating asymmetry in a small treefrog (*Dendropsophus haddadi:* Anura: Hylidae) as a measure of the history of glyphosate application: a case study from Plantações Michelin da Bahia

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ABSTRACT

Glyphosate-based herbicides are periodically applied in long-cycle rubber plantations to open trails among the trees. Often, these areas have a history of glyphosate contamination, potentially leading to contamination of freshwater environments and non-target organisms. Amphibians are susceptible to chronic levels of glyphosate and can be used as a measuring tool to assess the stress through sublethal responses, such as deviations in the symmetry of bilateral traits (i.e. fluctuating asymmetry – FA). We investigated the effects of the history of glyphosate application in rubber plantations of the Plantações Michelin da Bahia (PMB) on the FA levels of *Dendropsophus haddadi*. For this, we compared populations from ponds among (i) areas without application history of glyphosate – conserved areas; (ii) areas with application in the past – regenerated rubber plantations without application for at least 10 years; and (iii) areas under current application – active rubber and rubber/cacao plantations. We also compared FA levels among each sampled site. We collected adult, calling males in lentic ponds and measured six morphological traits on the right and left sides to calculate FA indexes. We excluded three from the six morphological traits due to measurement errors and directional asymmetry. We did not observe differences in FA levels among areas with different history of glyphosate application. Also, we did not observe differences in FA levels among ponds. Our results show that the developmental stability of *D. haddadi* populations inserted in rubber plantations of PMB is not affected by the history of glyphosate application and/or local condition. Not necessarily all organisms tested here experienced contamination besauce the application history of glyphosate is an indirectly inferred factor. However, some environmental stressors, such as history of pesticide application, may not lead to changes in developmental stability due to populational tolerance. Some populations can be more pesticide-tolerant, which can result from natural selection in proximity to the agricultural areas and/or long term exposure to contaminants. Pesticide tolerance may vary depending on resistance of each species and ability to survive under anthropogenic stress.

KEY-WORDS: developmental instability, rubber plantations, environmental stress, non-target organisms.

RESUMO

Herbicidas à base de glifosato são aplicados periodicamente em plantações de borracha de ciclo longo para abrir as trilhas entre as árvores. Geralmente, essas áreas tem um histórico de contaminação por glifosato, potencialmente levando à contaminação dos ambientes de água doce e organismos não alvo. Anfíbios são suscetíveis a níveis crônicos de glifosato e podem ser usados como uma ferramenta mensurável de estresse através de respostas subletais, como os desvios na simetria de traços bilaterais (i.e. Assimetria Flutuante – AF). Investigamos se existe efeito do histórico de aplicação de glifosato em plantações de borracha na Plantações Michelin da Bahia (PMB) sobre os níveis de AF de *Dendropsophus haddadi*. Comparamos populações de poças entre (i) áreas sem histórico de aplicação – áreas conservadas; (ii) áreas com aplicação no passado – plantações de borracha em regeneração sem aplicação há pelo menos 10 anos; e (iii) áreas sob aplicação atual – plantações de borracha e plantações de borracha com cacau. Nós também comparamos os níveis de AF entre cada poça amostrada. Coletamos machos adultos em corpos d'água lênticos e medimos seis traços morfológicos no lado direito e esquerdo para calcular os índices de AF. Excluímos três dos seis traços morfológicos devido a erros de medição e assimetria direcional. Não observamos diferenças nos níveis de AF entre as áreas com diferentes históricos de aplicação de glifosato. Além disso, não observamos diferenças nos níveis de AF entre poças. Nossos resultados mostram que a estabilidade do desenvolvimento das populações de *D. haddadi* inseridas nas plantações de borracha da PMB não é afetada pelo histórico de aplicação de glifosato e/ou pela condição local. Não necessariamente todos os organismos testados aqui experimentaram a contaminação

porque o histórico de aplicação de glifosato é um fator inferido indiretamente. No entanto, alguns estressores ambientais, como o histórico do uso de pesticidas, podem não levar a alterações na estabilidade do desenvolvimento devido à tolerância populacional. Algumas populações podem ser mais tolerantes aos pesticidas, o que pode resultar de seleção natural na proximidade de áreas agrícolas e/ou pela exposição ao contaminante em longo prazo. Esta capacidade de tolerância a pesticidas pode variar de acordo com a resistência de cada espécie em sobreviver sob estresse antrópico.

PALAVRAS-CHAVE: instabilidade do desenvolvimento, plantações de borracha, estresse ambiental, organismos não alvo.

INTRODUCTION

Brazil is the world leader in pesticide consumption since 2008 (Carneiro et al. 2015, Bombardi 2017). This condition is correlated with the advances of the agriculture frontiers that replace natural vegetation by extensive monocultures (Devine and Furlong 2007, Schiesari and Grillitsch 2011, Bombardi 2017). Often, these croplands are located close to freshwater ecosystems (Baker et al. 2013), significantly increasing the risk of contamination through different processes, such as lixiviation, runoff, intentional or inaccurate application and overspray in aerial application (e.g. Goldsborough and Beck 1989, Giesy et al. 2000, Peruzzo et al. 2008, Queiroz et al. 2011, Degenhardt et al. 2012, Bombardi 2017). As a consequence, several cases of freshwater contamination (e.g. superficial and underground water, rainwater, potable water) are reported in different federative states (e.g. Silva et al. 2003, Silva et al. 2009, Armas et al. 2007, Mattos et al. 2002, Dores et al. 2006, Marques et al. 2009, Freire et al. 2012, Gomes and Barizon 2014, Bombardi 2017).

Among the pesticides, glyphosate-based herbicides (e. g. Roundup®) are the most applied and commercialized agrochemicals in Brazil and worldwide (Zhang et al. 2011, Battaglin et al. 2014, Bombardi 2017). This herbicide is highly effective and can be repeatedly applied to weed control in the same growing season in short-cycle crops (e.g. soybean, sugarcane and corn) (Battaglin et al. 2014), as well as to clean trails among trees in long-cycle cultivations, such as mature and developing rubber plantations (Ismail et al. 2002, Moraes et al. 2008, Flesher 2014, 2015, Krashevska et al. 2015). Glyphosate formulations are water soluble and when drained to freshwater environments can be found in surface water and/or associated to aquatic sediments, aquatic vegetation and soil of rivers, streams, ponds and wetlands (e.g. Battaglin et al. 2005, Gillion et al. 2007, Smalling et al. 2012, Degenhardt et al. 2012, Moreira et al. 2012, Battaglin et al. 2014).

Displacement and persistence time of glyphosate formulations vary according to the environmental conditions (Siimes et al. 2006, Queiroz et al. 2011) and can be observed in freshwater environments from two to 91 days after application (Giesy et al. 2000, Grunewald et al. 2001, Silva et al. 2003, National Pesticide Information Center 2008, Vera et al. 2010, Bergstrom et al. 2011). Levels of freshwater contamination fluctuate spatially and temporally and can be associated to application rates (e.g. amount and frequency), abiotic conditions (e.g. pH, soil type, landform), stratification potential and/or rainfall regime, that can dilute or not the contaminant (Sudo et al. 2004, Boone et al. 2007, Ma et al. 2008, Mann et al. 2009, Pedlowski et al. 2012, Lenhardt et al. 2014). This range of confounding factors makes it difficult to measure and compare the glyphosate levels in situ, hampering the comprehension of the impact on non-target organisms submitted to chronic contamination for a long time. Thus, evaluating changes in attributes of species with bioindicator characteristics is a good tool to measure the effects of anthropogenic stressors (Noss 1990, Niemelä 2000, Rainio and Niemelä 2003, Heink and Kowarik 2010).

Amphibians are a thermometer of the global crisis of biodiversity and are considered good bioindicators of water quality, reflecting the biotic and abiotic integrity of habitats and favoring the evaluation of pesticide stress in aquatic ecosystems (Blaustein and Wake 1995, Kerby et al. 2010, Alroy 2015). This condition is associated with their biological and ecological traits, such as high sensitivity to environmental changes, permeable skin and dependence upon aquatic environments, especially due to the biphasic lifestyle (McDiarmid and Altig 1999, Gallant et al. 2007, Allentoft and O'Brien 2010, Aiko et al. 2014). Lethal effects of glyphosate formulations on amphibians have been documented experimentally, mainly for larval stages (e.g. Mann and Bidwell 1999, Relyea 2005, Jones et al. 2010, 2011, Relyea 2012, Costa and Nomura 2016). However, some amphibian species are more pesticide-tolerant and survive under periodic application, using small ponds located within or adjacent to agricultural areas (Bridges 1997, Gallant and Teather 2001, Hua et al. 2013a, Lenhardt et al. 2014, Hua et al. 2015, Miko et al. 2017). For example, Lenhardt et al. (2014)

showed an overlap between amphibian occurrence and pesticide applications in agriculture areas.

Fluctuating asymmetry (FA) is a cheap and easy tool to assess the impact of stressors on organisms, being used in biomonitoring and environmental impact studies (Johnson et al. 1993, Sanseverino & Nessimian 2008, Beasley et al. 2013). Fluctuating Asymmetry is based on small, subtle, random and non-directional deviations in the symmetry of bilateral morphology traits, in which the differences between right and left sides are normally distributed (about a mean of zero) and do not differ significantly from zero (Palmer and Strobeck 2003, Palmer 2004, Graham et al. 2010). Fluctuating asymmetry differs from directional asymmetry (DA), in which the greater development of a character is directed to one side with a mean significance different from zero. Fluctuating asymmetry also differs from antisymmetry (AS), in which a greater development of a character can occur to either side, with a bimodal distribution about a mean of zero (Palmer and Strobeck 2003, Palmer 2004, Sanseverino and Nessimian 2008). Levels of FA naturally reflect the organism’s genotype, but higher FA levels may reflect developmental instability of organisms submitted to stressors (Palmer and Strobeck 1986, Sanseverimo and Nessimian 2008). Usually, higher deviations are associated to performance and fitness reduction of individuals (Bosch and Marquéz 2000) that lead to population changes in the long term (Markow 1995). Studies with adults and larvae of amphibians have found relationships among increase of FA levels and stressors, such as logging (Lauck 2006), agropastoral land use (Gallant and Teather 2001, Costa et al. 2017, Zhelev et al. 2017), habitat loss and disturbance (Söderman et al. 2007, Eisemberg and Bertoluci 2016), domestic sewage pollution, heavy metal pollution (Zhelev et al. 2015), tannery effluents (Montalvão et al. 2017) and pesticide contamination (Costa and Nomura 2016).

Our objective was to evaluate if there is an effect in FA levels of a common small treefrog [*Dendropsophus haddadi* (Bastos and Pombal 1996)] populations that occur in the Plantações Michelin da Bahia in areas with different histories of glyphosate application [(i) without application, (ii) application in the past and (iii) current application]. We expected the populations from habitats under a current scenario of glyphosate application to show higher FA levels than populations from areas without history of application and populations from areas with application in the past (i.e.

regenerated areas). We also aimed to evaluate if there are differences in FA levels among each studied site.

METHODS

Study Area

We conducted fieldwork in the private property Plantações Michelin da Bahia (PMB) located in Ituberá and Igrapiúna municipalities (13º50’S, 39º10’W), state of Bahia, Brazil. The PMB is inserted in the Atlantic Forest biome and is characterized by a mosaic of secondary forest patches under different successional stages, regenerated rubber plantations, mixed tree crops, rubber monocultures and rubber/cacao plantations (*see* Flesher 2015). The most conserved areas of forest are located in the Reserva Ecológica da Michelin (REM) that is composed by fragments of secondary forest with different histories of hunting and logging. Some of the largest forest fragments are Pancagê (550 ha) that is contiguous with a 13,000 ha forest located outside the reserve, followed by Vila 5 and Pancada Grande (625 ha) and Luíz Inácio (140 ha). Currently the reserve is protected and monitored to reduce the action of hunters and loggers. In these fragments there is no history of application of glyphosate-based herbicides, as well as of other pesticides, because these areas were never replaced by croplands (Flesher and Laufer 2013, Flesher 2014, *personal information*).

In the REM, the largest fragments of secondary forest are connected by abandoned rubber plantations in different successional stages. These areas were intentionally abandoned by the company and reforested with native species between the lines of rubber trees (Flesher and Laufer 2013, Flesher 2014, Flesher 2015). In the past, these areas were active rubber plantations treated with glyphosate-based pesticides to maintain the trails among the trees clean to allow rubber extraction (*personal information*).

In rubber monocultures of the PMB the glyphosate-based herbicides (mainly the Roundup formulations) are applied once or twice per year to maintain the trails clean (Flesher 2014, Flesher 2015, *personal information*). Also, the PMB is composed by rubber/cacao plantations where the use of pesticides is widespread (Flesher 2015). In rubber/cacao plantations other pesticides (e.g. fungicides, insecticides) are applied by farmers associated to PMB. However, all ponds considered in our study were inserted in areas of a known history of glyphosate application. Information about the landscape structure and history of glyphosate-based herbicide applications were obtained from the scientific literature (Flesher and Laufer 2013, Flesher 2014, 2015) and interviews with the REM team [manager of the REM for 8 years (KMF) and the administrator of the REM for 30 years (ASS)].

Data Collection

Dendropsophus haddadi is a small sized tree frog that inhabits Atlantic Forest areas from Espírito Santo to Pernambuco states (Frost 2017). It is classified as being of Least Concern by the IUCN red list (IUCN 2017). This species was chosen because it is commonly found throughout the study area and populations can be found in a gradient from conserved to anthropogenic matrices (i.e. with presence and absence of rubber plantations).

In seven days we sampled a total of 10 permanent/semi-permanent lentic ponds with at least 1 km distance between them (except for P1, P2 and P3 with approximately 500 m between them – Table 1). We selected these ponds according to the following categories of the history of glyphosate application: (i) without application – ponds located within the most conserved forest in the REM (Pacangê). In this category, we included ponds relatively closer (i.e. P1, P2 and P3), within the same forest fragment to reduce the effects associated to different histories of logging. (ii) Application in the past – ponds located within areas of regenerated rubber plantations with unmanaged rubber (at least for the last four years), with history of glyphosate application in the past (at least 10 years ago – *personal information*). (iii) Current application – ponds located within areas of active rubber monocultures and rubber/cacao plantations with current application of glyphosate-based herbicide (once or twice per year) (Table 1).

At night (18:00 – 00:00) we collected adult males of *D. haddadi* through acoustic and visual search. In each pond we collected between five to 11 individuals (Table 1) that were transported to the lab within plastic bags. We collected a total of 82 individuals (13708-1 - ICMBio) representing 30 individuals from preserved areas, 15 from regenerated areas and 37 from active crop areas. Specimens were anesthetized and euthanized with a saturated benzocaine solution, fixed in formalin solution (10%) and preserved in 70% alcohol. All voucher specimens were deposited in the Museu de

Zoologia da Universidade Estadual de Santa Cruz – MZUESC (vouchers among 18542 – 18724).

Each individual was positioned near to a ruler (scale) and photographed in dorsal view. We used a standardized photographic system with a digital Samsung ST-66 HD camera positioned at 15 cm of height. Following, we measured the right and left sides of the six bilateral morphological traits from the photographs to calculate the FA indexes (Figure 1, Table 2). Each morphological trait was measured twice by the same sampler (RNC) to reduce the measurement errors. Repetitions were taken blind (without the knowledge what treatment each individual came from) and done on separate days (Palmer and Strobeck 2003). Individuals were similarly positioned to maximize the trait measures and to minimize errors associated to fixation. However, some measures were excluded due to fixation problems. Thus, we excluded four measures for snout-eye distance, 12 for hand digit length, one for femur length, five for foot length and 12 for foot digit length. We also measured the snout-vent length (SVL) of individuals. To take the measures we used the ImageJ 1.46r software.

Statistical analysis

For the FA analysis we followed the protocol proposed by Palmer and Strobeck (1986, 2003). We firstly searched for outliers and removed them according to critical values suggested by Grubbs (1969). We applied Grubbs' test to search for outliers from original measurements of right and left sides in both repetitions. We detected and excluded one data for snout-eye distance, one for femur length and two for tibia-fibula length. Also, we calculated a mean value with repeated measures for right and left sides and extracted the differences between sides [i.e. FA= (R – L)] to search for outliers with Grubbs test. Here, we detected and excluded one data for hand-digit length, one for tibia-fibula length and two for foot digit length. Thus, after data exclusion due to fixation problems and outliers we conducted the subsequent analyses with 77 measures for snout-eye distance (SED), 69 for hand-digit length (HDL), 80 for femur length (FEL), 79 for tibia-fibula length (TFL), 75 for foot length (FOL) and 70 for foot digit length (FDL).

After exclusion of outliers, we used the original repeated measures to apply a two-way mixed model ANOVA's for each morphological trait (response variables), with sides (i.e. right and left) as a fixed factor and individuals as a random factor. This procedure was applied to measure the contribution of the measurement errors based on the mean square of error and mean square of the side by individual interaction, as well as the inference of the symmetry type (Palmer and Strobeck 1986, 2003). We also applied a one-sample t-test to verify whether the means differed significantly from zero, evaluating the existence of directional asymmetry (DA) of each morphological trait. With a Kolmogorov–Smirnov test (K–S) we evaluated the normality of the differences between right and left sides. We also visually inspected the distribution of differences between right and left sides to evaluate the existence of antisymmetry (AS). Pearson's correlations were applied to verify the effects of each trait size on FA levels, as well as the effects of snout-vent-length on FA levels (Palmer and Strobeck 1986, 2003). Due to the absence of correlations (Table 4), no size-dependence corrections were required. Thus, subsequent analyses were realized with the absolute values of fluctuating asymmetry for each trait (i.e. index FA1= $|D – E|$) (Palmer 1994).

After extracting the FA1 index, we calculated the mean FA for each trait of individuals from each pond. We performed one-way ANOVA's to compare the mean FA to each morphological variable among the categories of the history of glyphosate application. Also, we performed one-way ANOVA's to evaluate if the mean FA differed between ponds.

RESULTS

We observed that measurement error was higher than FA for trait TFL because the mean square of error was higher than the mean square of the side by individual interaction (Table 3). Traits FEL and FOL showed directional asymmetry because there was significant difference among sides (Table 3) and the means were significantly different from zero (Table 4). Directional asymmetry in the FEL is directed to the left side and in the FOL to the right side. Thus, we excluded these traits from our hypothesis test (i.e. TFL, FEL and FOL). We observed that the other traits had FA indexes with normal distribution about a mean of zero without patterns of antisymmetry; and that the FA indexes were not correlated with each trait size and/or snout-vent-length (Table 4).

We did not observe differences in the FA levels of the morphological traits of *D. haddadi* among areas (i) without application, (ii) with application in the past and (iii) current application (Table 5A). Also, we did not observe differences in the FA levels among ponds (Table 5B).

DISCUSSION

Measurement errors can be responsible for the detection of non-expected patterns (e.g. directional asymmetry and antisymmetry) and significantly contribute to noise in FA studies, leading to misleading conclusions (Simmons et al. 1999, Palmer and Strobeck 2003, Palmer 2004). The most parsimonious suggestion in these cases is the exclusion of the traits (Palmer and Strobeck 1992, Palmer 2004). We excluded three traits due to the higher measurement error (TFL) and due to the presence of directional asymmetry (FEL and FOL). In FA studies with amphibians, evidences of DA were found in the femur (Eisemberg and Bertoluci 2016, Eterovick et al. 2016), tibia-fibula (Eterovick et al. 2016), radio-ulna and eyespot area (Gallant and Teather 2001) for adults, and eye-nostril distance for tadpoles (Eterovick et al. 2015). This directional asymmetry can be associated to preferential use of the same side in specific behaviors, such as the habit to jump to the same side (Malashichev 2002) and/or use the right forelimb during handling (Bisazza et al. 1996). Despite the noise associated to measurement errors and due to unexpected patterns, the statistical robustness ensures that FA is a good tool to measure environmental stress (Palmer 2004, Palmer and Strobeck 2003).

The history of glyphosate contamination applied in rubber/cacao plantations of PMB did not affect FA levels of *Dendropsophus haddadi*. Some studies have reported this absence of cause-effect relationship in anurans, in which increases of environmental stress did not reflect in increase of FA levels (e.g. Eterovick et al. 2015, Delgado-Acevedo and Restrepo 2007). *Dendropsophus haddadi* is a common and abundant species that survives in ponds altered by different anthropogenic factors (IUCN 2017, Frost 2017). These characteristics can be associated to species-specific tolerance (Simioni et al. 2013, Hua et al. 2014), ensuring the occurrence in habitats under chronic levels of pesticide contamination. Pesticide-tolerance of amphibians can be increased according to spatially proximity with agriculture areas (Cothran et al. 2013, Hua et al. 2014, Hua et al. 2015). For example, Hua et al. (2015) conducted a study with *Lithobates sylvaticus* and showed that populations living closer to agriculture were more pesticide-tolerant than populations living far from agricultural areas. Therefore, some amphibians cross croplands and are commonly found in ponds inserted in areas under intensive pesticide applications (Gallant and Teather 2001, Fryday and Thompson 2012, Lenhardt et al. 2014). It’s possible that *D. haddadi* populations living in the active plantations of PMB have a considerable pesticide-tolerance. Thus, chronic levels of glyphosate contamination did not affect developmental stability and did not increase FA levels in exposed populations.

Takahasi (2007) found that *Hyla versicolor* and *Hyla chrysoscelis* can identify ponds contaminated with chronic levels of Roundup® avoiding these environments and not laying their eggs. This behavior can reduce the contact of adults and offspring with contaminants, not affecting their development. However, this behavior needs to be evaluated for more species, mainly those that occur in ponds inserted in agricultural landscapes. Also, in agricultural landscapes reproductive and non-reproductive habitats often are located closer or surrounded by croplands (Baker et al. 2013, Lenhardt et al. 2014). During a reproductive season, amphibians can cross kilometers between these habitats, being contaminated during the dispersion process (Becker et al. 2007, Berger et al. 2012, 2013, Lenhardt et al. 2014). Thus, it is possible that some temporary ponds within disturbed areas are occupied by colonizers from uncontaminated or non-stressed areas, as well as ponds within preserved areas may be occupied by some colonizers from contaminated areas (i.e. an effect of the surrounding landscape).

We used the history of glyphosate application as an indirect meassure of environmental contamination and not necessarily all individuals analized here experienced the contamination. Lenhardt et al. (2014) observed a temporal coincidence among breeding migrations of amphibians and pesticide application. This overlap increases the risk of individual contamination. However, multiple reproductive events throughout the year and/or multiple events in the same reproductive season (Wells 2007) can reduce temporal overlap between amphibians and pesticides. Also, pesticide concentration in environments can vary according to anthropogenic and/or abiotic conditions (e. g. Sudo et al. 2004, Boone et al. 2007, Ma et al. 2008, Mann et al. 2009, Pedlowski et al. 2012, Lenhardt et al. 2014). For example, the amount and frequency of glyphosate applications in the PMB can vary according to the planting age, being more frequent in younger rubber plantations or when rubber is planted with cacao (Flesher 2014, Flesher 2015). These fluctuations can lead to different levels of environment contamination, as well as to non-contamination of some habitats.

Independently of the presence or absence of glyphosate in environments, each studied pond has a considerable level of anthropogenic stress due to changes derived from substitution of natural vegetation by rubber plantation and/or rubber-cacao plantations. It's known that habitat disturbance can lead to an increase of FA levels in amphibians (e. g. Lauck 2006, Eisemberg and Bertoluci 2016, Montalvão et al. 2017, Costa et al. 2017). However, we found that there were no differences in the FA levels among ponds. Probably, the level of stress associated to rubber/rubber-cacao plantation is not so severe and does not affect the developmental stability of *D. haddadi*.

In conclusion, our results show that the history of glyphosate applications in PMB did not affect the developmental stability of *D. haddadi*, as well as the local environment stress (i.e. in each pond). Despite the indirect estimative of environmental contamination, these results can be associated to species-specific tolerance through the developmental homeostasis of populations tested. However, for robustness increase in future studies we suggest more control of confounding factors as well as the use of organisms directly impacted by the stressors during their development, such as tadpoles developing in contaminated ponds. In addition, a laboratorial study would be interesting to understand if the tested organisms can respond through changes in FA levels and potentially increase the inferences in field studies.

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FIGURES AND TABLES

Table 1: Coordinates of the sampled ponds with the number of individuals per site (N). Cat.: categories of glyphosate application history. Matrix: description of the matrix surrounding the pond.

Pond	Lat (S)	Long (W)	Cat.	Matrix	N
P1	13°49'58.3"	39°15'02.1"	(i)	Forest (Pacangê)	10
P2	13°49'51.5"	39°15'02.3"	(i)	Forest (Pacangê)	11
P3	13°50'01.7"	39°14'47.1"	(i)	Forest (Pacangê)	9
P4	13°46'53.7"	39°09'21.9"	(iii)	Rubber	6
P5	13°49'06.4"	39°09'59.0"	(iii)	Rubber	10
P6	13°50'04.9"	39°13'37.0"	(ii)	Regenerating rubber	6
P7	13°50'31.9"	39°13'27.1"	(ii)	Regenerating rubber	9
P8	13°50'19.9"	39°12'00.8"	(iii)	Rubber, cacao and banana	8
P9	13°49'22.4"	39°11'51.5"	(iii)	Rubber and cocoa	8
P10	13°48'52.6"	39°09'11.4"	(iii)	Rubber	5
Total	-	-	-	-	82

Table 2: Criteria for measurement acquisition. (*) Measurements were always taken using the greatest length of the structure.

Morphological variable	Abbreviation	Description
Snout-eye distance	SED	Distance from the tip of the snout to upper edge of eyes.
Hand digit length	HDL	Distance from the junction of the finger one and arm to the tip of the finger.
Femur length	FEL	Distance from the cloaca to the tip of the thigh.*
Tibia-fibula length	TFL	Distance from the highest point of the tibia to the lowest point.*
Foot length	FOL	Distance from the tip of the foot to the junction of the toe one and foot.*
Foot digit length	FDL	Distance from the junction of the toe one and foot to the tip of the toe.

Table 3: Results of two-way mixed model ANOVA's for all morphological variables. (*) $p < 0.05$; (**) $p < 0.001$.

	Side			Individual			Side X individual			Error	
Traits	M.S.	F	d.f.	M.S.	F	d.f.	M.S.	F	d.f.	M.S.	d.f.
SED	<0.001	0.001	1	**0.272****	13.449	76	**0.020****	24.513	76	<0.001	154
HDL	0.024	0.34	1	**0.519****	7.44	68	**0.070****	9.61	68	0.007	138
FEL	**0.44****	8.35	1	**0.49****	9.22	79	**0.05****	3.45	79	0.02	160
TFL	0.02	1.82	1	**0.69****	53.87	78	0.01	0.69	78	0.02	158
FOL	**0.141***	4.64	1	**0.343****	11.30	74	**0.030****	5.07	74	0.006	150
FDL	0.01	0.08	1	**0.70****	8.44	69	**0.08****	5.22	69	0.02	140

Table 4: Results of tests for directional asymmetry, normality and size-dependence correlations. K – S = Kolmogorov-Smirnov.

		T-test (one sample)			K-S		Pearson – trait size		Pearson – SVL	
Trait	N	T	d.f.	P	D	P	R	p	R	P
SED	77	-0.034	76	0.972	0.064	>0.20	-0.082	0.475	-0.041	0.720
HDL	69	-0.584	68	0.560	0.066	>0.20	-0.205	0.090	-0.125	0.304
FEL	80	-2.889	79	**0.004**	0.062	>0.20	-0.026	0.813	-0.151	0.180
TFL	79	1.348	78	0.181	0.063	>0.20	-0.035	0.760	-0.072	0.527
FOL	75	2.154	74	**0.034**	0.087	>0.20	-0.174	0.135	0.093	0.554
FDL	70	-0.286	69	0.775	0.113	>0.20	0.148	0.220	0.059	0.624

Table 5: Comparison of the FA index of *Dendropsophus haddadi* among areas without glyphosate application, with application in the past and current application (A); and comparison among ponds (B).

A				
Trait	**d.f.**	**M.S.**	**F**	**p**
SED	2	0.020	2.713	0.07
HDL	2	0.016	0.681	0.509
FDL	2	0.057	1.512	0.227
B				
Trait	**d.f.**	**M.S.**	**F**	**p**
SED	9	0.010	1.432	0.192
HDL	9	0.030	1.370	0.222
FDL	9	0.033	0.850	0.573

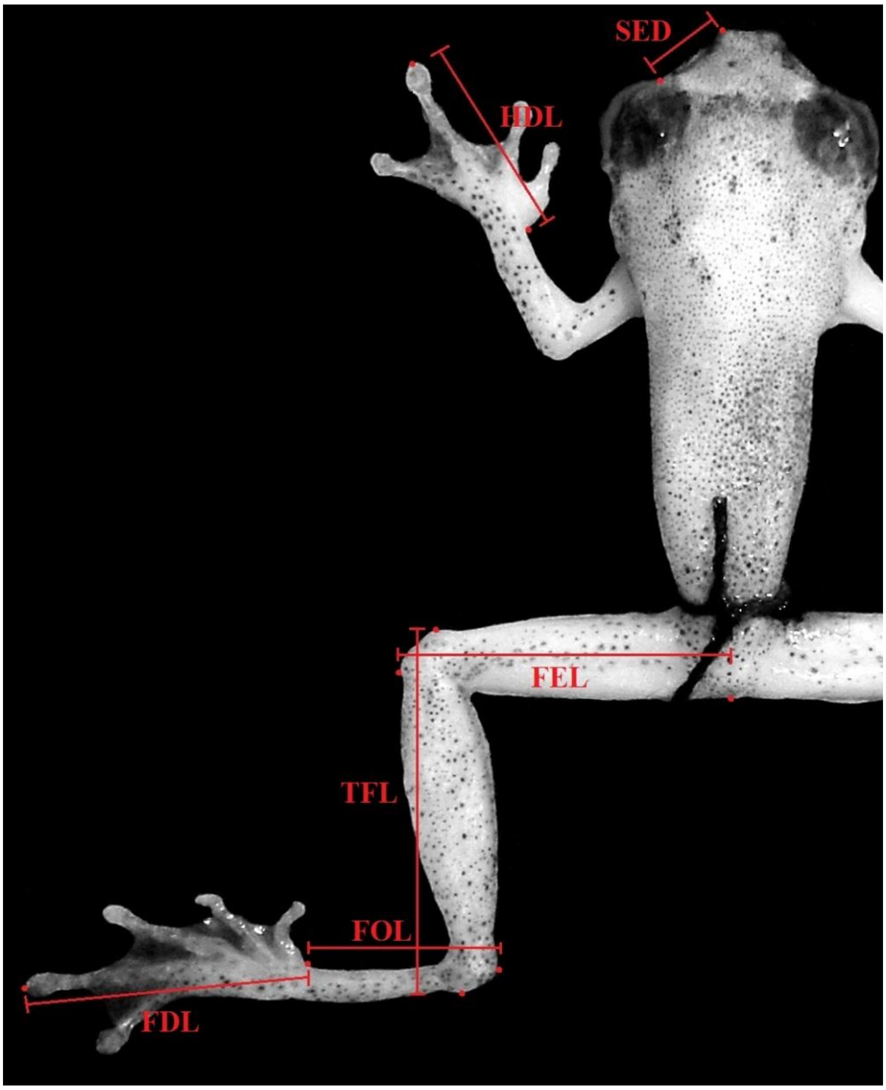

Figure 1: Measured morphological structures in the right and left sides to achieve the FA index. See abreviations in the Table 2.

CONCLUSÃO GERAL

As formulações de Roundup testadas levaram a respostas letais em anfíbios anuros, como a redução na sobrevivência de girinos de *Dendropsophus minutus* (Capítulo 2) e *Physalaemus cicada* (Capítulo 3), e são consideradas moderadamente tóxicas para ambas as espécies. Com base nesses resultados, é possível concluir que os herbicidas a base de glifosato afetam negativamente os anfíbios anuros, podendo colaborar diretamente com os declínios populacionais e a perda de espécies. Além disso, novos resultados foram gerados para o gênero com maior representatividade quanto ao número de estudos ecotoxicológicos no Brasil (i.e. *Physalaemus*) (Capítulo 3). Essa ampliação do conhecimento sobre espécies congenéricas pode aumentar a robustez durante comparações.

Conclui-se também que os pesticidas são responsáveis por uma série de respostas subletais nos anuros e que os estudos ecotoxicológicos que envolvem efeitos sobre o crescimento e a morfologia abordam poucas espécies quando comparado à alta diversidade no planeta. Além disso, estes estudos não estão distribuídos no espaço geográfico de forma homogênea (Capítulo 1). Por exemplo, dentre as respostas subletais, foi observado que girinos de *D. minutus* submetidos ao Roundup Original® apresentaram alterações comportamentais e morfológicas, como alterações na forma geral do corpo e maiores níveis de assimetria flutuante. Essas alterações podem ser mediadas pela presença de estressores naturais, como os predadores, levando ou não a efeitos sinérgicos (Capítulo 2). De maneira geral, as respostas subletais atuam de maneira silenciosa e gradual, o que pode refletir sobre a redução do fitness das espécies.

Com os resultados do capítulo 4, é possível concluir que alguns estressores ambientais, como o histórico do uso de pesticidas, podem não levar a alterações na estabilidade do desenvolvimento, sem alterar os índices de assimetria flutuante. Algumas espécies podem ser mais tolerantes ao estresse ambiental do que outras, ou algumas populações podem ser mais tolerantes aos pesticidas por estarem associadas a áreas agrícolas e/ou expostas a um contaminante por um longo tempo.