UNIVERSIDADE ESTADUAL PAULISTA
"JULIO DE MESQUITA FILHO"
INSTITUTO DE PESQUISA EM BIOENERGIA

PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO EM BIOENERGIA

ECOLOGICAL AND ECOTOXICOLOGICAL IMPACTS OF SUGARCANE CULTURES ON AMPHIBIAN TADPOLE COMMUNITIES

DAVID SÁNCHEZ DOMENE

Tese apresentada ao Instituto de Pesquisa em Bioenergia de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientador(a):

Eduardo Alves de Almeida

Rio Claro – SP

Abril 2019

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David Sánchez Domene

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Comissão examinadora

Prof. Dr. Eduardo Alves de Almeida
FURB – Universidade Regional de Blumenau, Santa Catarina, Brasil

Dra. Juliane Silberschmidt Freitas
Pós-Doutoranda USP – Universidade de São Paulo, São Paulo, Brasil

Prof. Dr. Luís Olímpio Menta Giasson
FURB – Universidade Regional de Blumenau, Santa Catarina, Brasil

Prof. Dr. Camilo Dias Seabra Pereira
UNIFESP – Universidade Federal de São Paulo, São Paulo, Brasil

Prof. Dra. Maria Stela Maioli
UNESP – Universidade Estadual Paulista, São Paulo, Brasil

Conceito: *Aprovado*

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RESUMO

O Estado de São Paulo é responsável pela produção da metade da cana-de-açúcar no Brasil, e segue crescendo, sendo esse cultivo um dos principais responsáveis pelo desmatamento no interior do estado. A atual expansão da cana-de-açúcar está ocorrendo principalmente sob áreas de cultivos anuais e pastagens de pecuária extensiva. Embora possa parecer positivo, essa conversão de pastagens supõe um grande impacto sobre as poças de água construídas como bebedouros para o gado, as quais abrangem altas proporções de biodiversidade a sua vez. Nesse cenário, os anfíbios são especialmente vulneráveis, uma vez que a grande maioria das espécies de anuros, registrados no interior do estado, se reproduz nestas poças que estão sendo transformadas ou mesmo desaparecendo. Além disso, o cultivo intensivo de cana-de-açúcar exige o uso de agrotóxicos que devido as fortes precipitações sazonais provocam escoamento superficial a estes leitos aquáticos e sua subsequente contaminação por misturas complexas de pesticidas. Nesta dissertação de doutorado são apresentados os resultados de um estudo sobre os impactos ecológicos e ecotoxicológicos em girinos de diferentes espécies de anuros que habitam poças temporárias anexas aos cultivos de cana-de-açúcar. O estudo foi realizado na área agrícola circundante da cidade de São José do Rio Preto, uma das principais áreas de produção de cana-de-açúcar no noroeste paulista. A vegetação nativa dessa região tem sido continuamente desmatada desde o século XIX, e nas últimas décadas tem experimentado uma intensa expansão do cultivo de cana-de-açúcar, substituindo principalmente pastagens, o que torna essa região um local idôneo para pesquisa da anurofauna, a qual tem sido extensivamente estudada desde meados dos anos 60. Para compreender os impactos ecológicos e ecotoxicológicos nos girinos que habitam as poças temporais anexas aos cultivos de cana-de-açúcar, esta dissertação está formada por quatro capítulos nos quais são avaliados i) os impactos do cultivo da cana-de-açúcar sobre a estrutura das comunidades de anuros associadas a poças temporárias por meio do estudo dos efeitos das características locais das poças, da paisagem entorno a elas e da contaminação por pesticidas, ii) as malformações oculares encontradas em girinos coletados durante as amostragens de campo, iii) a confiabilidade do teste de desempenho de natação de escape como ferramenta para a detecção de comprometimento da mobilidade em organismos aquáticos expostos a pesticidas, e iv) os efeitos nas comunidades de água doce das misturas de pesticidas em um experimento microcosmos. Os resultados desses estudos vão desde a confirmação da importância das poças para a conservação de anfíbios, até a detecção de contaminação generalizada na região de São José do Rio Preto, bem como a apresentação dos primeiros casos de malformações anfíbias em paisagens agrícolas no Brasil, e a primeira linha de base de malformações para anfíbios na América do Sul.

Palavras-chave: Anfíbios, Cana-de-açúcar, Pesticidas, Mudança do uso do solo

ABSTRACT

São Paulo state cultivates half of the sugarcane in Brazil, being this culture among the main responsible for deforestation in the inland of the state. Current expansion of sugarcane is taking place mostly over annual croplands and extensive cattle ranching pastures. Although it may seem positive, this pasture conversion supposes a major impact on cattle ponds which support high proportions of biodiversity. In this scenario, amphibian populations are especially vulnerable since the vast majority of the anurans registered in the inland of the state breed in cattle ponds which are been transformed or even disappearing. In addition, intensive sugarcane culture demands the usage of pesticides which can enter ponds when heavy precipitation events cause surface run-off. Consequently, complex mixtures of pesticides occur in ponds near to cultures. In this doctoral dissertation are presented the results of a study of the ecological and ecotoxicological impacts on tadpoles of anurans species inhabiting temporary breeding ponds annex to sugarcane cultures. The study was conducted in the surrounding sugarcane-dominated agricultural area of the city of São José do Rio Preto, which stands out as an appropriate place for the research purpose, since it is part of São José do Rio Preto macro region, in the northwestern of São Paulo state, one of the major sugarcane production areas in Brazil, its native vegetation has been intensely deforested since the 19th century, in the last decades has experienced one of the greatest sugarcane expansions, mostly replacing cattle pastures, and its anurofauna has been extensively studied since mid-1960s. The dissertation includes four chapters in which are assessed i) the impacts of sugarcane cultivation on the anuran communities structure associated to temporary breeding ponds by assessing the effects of ponds characteristics, landscape and pesticides contamination, ii) eye malformation found in tadpoles collected during field samplings, iii) the reliability of the escape swimming performance test as a tool for the detection of motility impairment in aquatic organisms exposed to a pesticides, and iv) the effects on freshwater communities of pesticides mixtures in an outdoor microcosms experiment. The findings from these studies range from the confirmation of the importance of cattle ponds for amphibian conservation to the detection of widespread contamination in São José de Rio Preto region, as well as the introduction of the first report of amphibian malformations in agricultural landscapes in Brazil, as well as first malformation baseline for amphibians in South America.

Keywords: Amphibians, Sugarcane, Pesticides, Land-use-change

INDEX

Chapter II

Chapter III

Chapter IV

GENERAL INTRODUCTION

In the early 70s, the report "Limits to Growth" alarmed about the global ecological constraints caused by the economic and population growth (Meadows et al. 2002), however much of the forecasts exposed by this report, as declines in food production, population level and energy availability, were overcome as a result of the research and technical development (RTD) of agricultural technologies, and its adoption by developing countries (Glaeser, 2011; Pingali and Raney, 2005). This RTD, named as "Green Revolution", was based principally in the genetic improvement of crops, the mechanization of farming, the expansion of irrigation infrastructures, and the generalized use of synthetic fertilizers and pesticides, which doubled and tripled crop yields (Glaeser, 2011; Jordan, 2013). In fact, food production per capita was higher in the year 2000 than in 1970, despite having doubled population, and even though the arable land per person declined by 40 percent, from 0.43 ha in 1961/63 to 0.26 ha in 1997/99 (Meadows et al. 2002; FAO, 2003). Thus, Green Revolution driven intensification saved an estimated 18 to 27 million hectares of natural land from being brought into agricultural production (Stevenson *et al.* 2013). Nevertheless, it is scientific consensus that agriculture is the activity that has most modified the earth surface (Vitousek, 1997; World Bank 2001; Tilman *et al*. 2001; Foley, 2005). In 2016, agriculture used near to one-third of total land area, with India, United States of America, Russian Federation, China and Brazil alone representing 40 percent (FAO, 2018).

In Brazil, which holds the greatest diversity of amphibian species in the world (AmphibiaWeb, 2019), land use transformation is the main threat to amphibians (Eterovick *et al.* 2005; Silvano and Segalla, 2005). Amphibians endangerment is a global phenomenon, in fact the International Union for Conservation of Nature (IUCN) reports that approximately 40% of the world's amphibian species are endangered, making them the most vulnerable group among vertebrates (IUCN, 2019). Different studies have shown that amphibian population

declines are the reflect of complex interactions of impacts such as exotic species introduction (Fisher and Shaffer, 1996; Kiesecker *et al.* 2001), UV-radiation increase (Blaustein *et al.* 1994, 1997; Anzalone *et al.* 1998), climatic changes (Pounds, 2001), the emergence infectious diseases (Berger *et al.* 1998, 1999), the habitat loss and land-use changes (Stuart *et al.* 2004; Cushmann, 2006), and the environmental contamination by anthropogenic pollutants (Blaustein *et al.* 2003).

In the last two decades, large areas in Brazil have been transformed by agribusiness because of the National Alcohol Program - Proálcool (Friberg, 2009, Lapola, 2010), which promoted sugarcane expansion to replace petroleum by sugar-derived ethanol in 1975. Further reinforcing this strategy, as a consequence of the Paris climate agreement within the United Nations Framework Convention on Climate Change (2015), the Brazilian government announced the "RenovaBio" program to boost participation of renewable fuels in its energy mix, which seeks an increase in ethanol production from 28 billion liters per year in 2015 to around 50 billion liters by 2030 (MME, 2017). Although in the last years has experienced a decline, Brazil remains the largest producer of sugar, and will continue to be the main producer by 2027, producing 34% of the world's sugarcane (OECDE-FAO, 2018).

The state of São Paulo cultivates half of the sugarcane in Brasil (UNICA, 2018). Consequently, sugarcane is among the main responsible for deforestation in the inland of the state (Kronka, 2005), however, current deforestation rate by sugarcane expansion is a minor problem since it is taking place mostly over annual croplands and extensive cattle ranching pastures (Aguiar *et al.* 2009; Egeskog *et al.* 2014). Although it may seem positive, this pasture conversion is supposing a major impact on cattle ponds (Snodgrass et al. 2000; Beja and Alcazar 2003), which support higher proportions of biodiversity of aquatic organisms compared to larger freshwater systems (Santi *et al.* 2010; Biggs *et al.* 2014). In this scenario, amphibians populations are especially vulnerable since the vast majority of the anurans

registered in the inland of the state breed in cattle ponds (Da Silva et al. 2012), which are disappearing dramatically (Rodrigues *et al.* 2008; Joly *et al.* 2010), while the remaining ponds suffer the simplification of their vegetation (Da Silva *et al.* 2012), and pesticides contamination (Schiesari and Grillitsch, 2011). Intensive sugarcane culture demands the usage of pesticides such as herbicides, fungicides and insecticides, in order to prevent yield losses by pest infestations (Oerke and Dehne, 2004; Velasco *et al.* 2012). Heavy precipitation events, typical during the breeding season of amphibians in most inner São Paulo state, cause surface run-off which are the major route for pesticides entry into agricultural water bodies, (Leu *et al.* 2004; Taghavi *et al.* 2010; Stehle and Schulz, 2015). Consequently, complex mixtures of pesticides occur in water bodies near to cultures (Leu *et al.* 2004; Armas *et al.* 2005; Stehle and Schulz,2015; Sánchez-Domene *et al.* 2018). Thus, whereas vegetation simplification reduces the number of microhabitats available to meet anuran species-specific requirements (Da Silva *et al.* 2012), pesticides contamination cause alterations in reproduction and development, malformations, biochemical disfunctions, immunosuppression, and mortality (*e.g.* Relyea 2005, 2008; Rohr *et al.* 2009; Mann et al. 2009; Shuman-Goodier and Propper, 2016; Freitas *et al.* 2017).

This doctoral dissertation presents a study of the ecological and ecotoxicological impacts on tadpoles of anurans species inhabiting temporary breeding ponds annex to sugarcane cultures. The study was done in the surrounding sugarcane-dominated agricultural area of the city of São José do Rio Preto, which stands out as an appropriate place for the research purpose, since it is part of São José do Rio Preto macro region, in the northwestern of São Paulo state, one of the major sugarcane production areas in Brazil, with around 894.736,80 hectares cultivated in 2018; involving 96 cities and a population of 1.5 million, approximately (IEA, 2019; SEADE, 2019). The native vegetation of this region has been intensely deforested since the 19th century, being replaced by pastures, croplands, and urban areas (Kronka *et al.* 1993).

Currently just a 4% of the native semi-deciduous Atlantic forests and Cerrado formations remains (Nalon *et al.* 2008), making this region the most deforested and fragmented in the state (Rodrigues *et al.* 2008). In the last decades, the region has experienced one of the greatest sugarcane expansions, mostly replacing cattle pastures (Aguiar *et al.* 2009). This, together with the extensive knowledge of its anurofauna, studied since mid-1960s (Provete *et al.* 2011), makes this region an appropriate place to study the impact of sugarcane expansion over the anurans associated to temporary breeding ponds.

The dissertation includes four chapters:

1. **Effects of habitat characteristics and landscape on amphibian communities inhabiting a sugarcane dominated agroecosystem,** in which was studied the impact of sugarcane cultivation on the anuran communities structure associated to temporary breeding ponds by assessing the effects of local (ponds characteristics), landscape (land uses surrounding ponds), and contamination (pesticides in water) factors.
2. **Eye malformation baseline in *Scinax fuscovarius* larvae populations that inhabit agroecosystem ponds in southern Brazil**, in which were studied rare eye malformations found in tadpoles collected during field samplings.
3. **First vs. Best response analysis in escape swimming performance tests for ecotoxicology in tadpoles (*Boana lundii*)**, in which the reliability of the escape swimming performance test as a tool for the detection of motility impairment in aquatic organisms exposed to a pesticide was assessed.
4. **Effects of pesticides mixtures occurring at tropical agroecosystems small water bodies on representative freshwater communities: a microcosm approach**, in which the effects on freshwater communities (phytoplankton-periphyton-tadpoles) of a field-based mixture of pesticides, at two concentrations, were assessed in an outdoor microcosms experiment.

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Chapter I

Effects of pesticides, habitat characteristics and landscape on amphibian communities inhabiting a sugarcane dominated agroecosystem

Effects of habitat characteristics and landscape on amphibian communities inhabiting a sugarcane dominated agroecosystem

David Sánchez-Domene[1], Alba Navarro-Lozano[2], Raphael D. Acayaba[3], Diogo B. Provete[4], Cassiana C. Montagner[5], Denise de C. Rossa-Feres[2], Fernando R. da Silva[6], Germán M. López-Iborra[7], Eduardo A. Almeida[8].

[1] UNESP - São Paulo State University, Bioenergy Research Institute, Rua 10, 2527, 13500-230, Rio Claro, SP, Brazil.
[2] UNESP - São Paulo State University, Laboratory of Theorical Ecology, Department of Zoology and Botany, Rua Cristóvão Colombo, 2265, 15054-000, São José do Rio Preto, SP, Brazil.
[3] School of Tecnology, University of Campinas, Rua Paschoal Marmo, 1888, 13484-332, Limeira, SP, Brazil.
[4]Institute of Biosciences, Federal University of Mato Grosso do Sul, Campo Grande, 79002-970, Mato Grosso do Sul, Brazil.
[5] Environmental Chemistry Laboratory, University of Campinas / Institute of Chemistry, Cidade Universitária "Zeferino Vaz", Caixa Postal 6154, 13484-332, Campinas, SP, Brazil.
[6] Theorical Ecology Laboratory: Integrating Time, Biology e Space (LET.IT.BE), Department of Environmental Science, Federal University of São Carlos, Rodovia Washington Luis, km 235, 13565-905, Sorocaba, SP, Brazil
[7] Department of Ecology/IMEM Ramon Margalef, Universidad de Alicante, Campus de San Vicente del Raspeig, Apdo. correos 99, 03080 Alicante, Spain.
[8] Department of Natural Sciences, Regional University of Blumenau, Rua Antônio da Veiga, 140 - Itoupava Seca, 89030-903 Blumenau, SC, Brazil

ABSTRACT

Global demand for crops will continue increasing over the next few decades to cover both food and biofuel needs. This entails a parallel demand of arable land that is being satisfied with deforestation and agriculture intensification. Consequently, the last decades have witnessed a worldwide loss of biodiversity at an unprecedent scale, being Amphibia the most affected class within vertebrates. The Regional Administration of São José do Rio Preto, in the northwest of the São Paulo State (Brazil), is a traditional cattle ranching region that has experienced one of the greatest sugarcane expansions, mostly replacing cattle pastures. In this paper we study the impact of sugarcane expansion over the structure of anuran communities, incidence and abundance, associated to temporary breeding ponds. To that end, we assessed the effects of local (ponds characteristics), landscape (land uses surrounding ponds) and pesticides contamination over anuran communities that inhabited nineteen temporary ponds with different surrounding main land uses, sugarcane and pasture, during the breeding season 2015 – 2016. Our results show that species incidence and abundance of amphibian tadpoles in the studied tropical agroecosystem were differently governed. While landscape (distance to forest fragments) was the only factor driving species richness and incidence of species at ponds (≈ 15%), local pond variables (mostly related to pond vegetation) and to a lesser extent pesticides contamination explained species abundance (≈ 49%). Our findings highlight the importance of maintaining man-made cattle ponds and native forest fragments endangered by Brazilian monoculture expansion to achieve a successful amphibian conservation. However, its simple maintenance is not enough to ensure their conservation, priority must be given to ponds with abundant stratified vegetation, near to forest fragment, with short and permeable connecting space, and far from pesticides sources.

Keywords: *agroecosystem, amphibian communities, landscape, contamination.*

INTRODUCTION

Global demand for crops will continue increasing over the next few decades to cover both food and biofuel needs (Godfray *et al.* 2010; Lotze-Campen *et al.* 2010). Consequently, arable land is suffering a parallel demand that is being satisfied with deforestation and agriculture intensification. Deforestation, which mostly consist in the conversion of natural vegetation into agricultural uses (Lambin *et al.* 2003), is one of the most important drivers of biodiversity loss (*e.g.* Newbold *et al.* 2011 and 2016, Gibson *et al.* 2015). Current technology-driven agricultural intensification is reducing past rates of deforestation (Byerlee *et al.* 2014). However, intensification implies both the creation of large monoculture fields and the application of large amounts of agrochemicals (pesticides and fertilizers) that maximize crops yield but also represent a threat for biodiversity (Donald *et al.* 2001; Tscharntke *et al.* 2005; Schiesari and Grillitsch, 2010; Vanbergen *et al.* 2013; Schiesari *et al.* 2015). Together, deforestation and the intensification of agriculture convert complex natural landscapes into simplified matrices with small remnants of native vegetation, which leads to the reduction of resources and conditions that allow a set of species with different niches to occur, resulting in low biodiversity areas (Laurance, 1999; Tews *et al.* 2004; Tscharntke *et al.* 2005). As a consequence, the last decades have witnessed a worldwide loss of biodiversity at an unprecedent scale, being Amphibia the most affected class within vertebrates (Matson *et al.* 1997; Tilman *et al.* 2001; Catenazzi, 2015).

In the last two decades, large areas in Brazil have been transformed by agribusiness because of the National Alcohol Programme - Proálcool (Friberg, 2009, Lapola, 2010), which promotes sugarcane expansion to replace petroleum by alcohol derived from sugar since 1975. Brazil accounts for 40% of the global production of sugarcane and São Paulo

state is responsible for over half of it (UNICA, 2018), being this crop the main responsible for deforestation in São Paulo state (Kronka, 2005), where cultivated area reaches 6.16 Mha. However, current deforestation rate by sugarcane expansion is a minor problem since it is taking place mostly over annual croplands and extensive cattle ranching pastures (Aguiar *et al.* 2009; Egeskog *et al.* 2014).

The native vegetation of northwestern São Paulo State has been intensely deforested since the 19th century, and has been replaced by pastures, croplands, and urban areas (Kronka *et al.* 1993). Currently just a 4% of the native semi-deciduous Atlantic forests and Cerrado formations remains (Nalon *et al.* 2008), making this region the most deforested and fragmented in the state (Rodrigues *et al.* 2008). The Regional Administration of São José do Rio Preto, in the northwest of São Paulo state, is traditional cattle ranching region that has experienced in the last decades one of the greatest sugarcane expansions. Currently, after intensive and fast deforestation, sugarcane expansion is taking place mostly over annual croplands and extensive cattle ranching pastures. This rapid land use change occurred is threatening amphibians as it produces conditions different from those that most species have evolved to exploit (Siqueira, 2015). Among main habitat changes resulting from sugarcane expansion stands out: i) the loss of temporary ponds, which despite being built as water reservoirs for cattle, have become important breeding sites for amphibian species living in agroecosystems (Da Silva *et al.* 2012a), ii) the increase of environmental harshness due to the management techniques used, which almost every year requires the cutting of stalks, or its burning, producing instant and drastic changes in the landscape, and iii) the exposition to agrochemicals, while pastures employ no pesticides, sugarcane is among the top consumers of these chemicals in Brazil (D'Anunciação *et al.* 2014, Pignati, 2017). Together, these habitat changes impose greater metabolic costs and mortality rates to amphibians (Chesson and

Huntly 1997; Shea and Chesson 2002; Preest *et al.* 2003), which must cope with increased heat and desiccation risk, agrochemicals contamination, greater exposure to predators (Denoel *et al.* 2005), and increased exposure to UV-B radiation (Bancrof *et al.* 2008).

The fast land-use change, together with the extensive knowledge of its anurofauna, studied since mid-1960s (Provete et al. 2011), makes the northwest region of São Paulo state an appropriate place to study the impact of sugarcane expansion over the anuran communities inhabiting these recently formed agroecosystems. At this sugarcane-dominated agroecosystems, amphibians populations are especially vulnerable since the vast majority of the anurans registered breed in cattle ponds (Da Silva *et al.* 2011), which are disappearing dramatically (Rodrigues et al. 2008; Joly *et al.* 2010), while the remaining ponds suffer the simplification of their vegetation (Da Silva *et al.* 2012a), and pesticides contamination (Schiesari and Grillitsch, 2011). Thus, to understand the consequences of this habitat changes on amphibian communities, and the relative importance of the local condition of the ponds, and their surrounding matrix, we studied amphibian populations breeding at ponds annex to sugarcane and those breeding at pasture ponds.

MATERIALS AND METHODS

Sampling design

We sampled tadpoles in 19 temporary ponds (Fig.1) by dipnetting for 40 minutes every four weeks during the rainy season (Nov 2015 – Mar 2016), totaling four surveys per pool. This sampling period covered almost the whole breeding period of most anuran species that occur in the region (Provete *et al*. 2011). Sampled ponds were selected based on their distance to sugarcane fields, resulting in 10 ponds at distances less than or equal to 50 m from sugarcane fields and 9 at greater distances (Tab.S1) Once collected, larvae

were anesthetized in benzocaine, fixed in a 1:1 solution of ethanol (70%) and formalin (15%), and deposited in the tadpole collection DZSJRP at Universidade Estadual Paulista, São José do Rio Preto. Animals were collected under license n.49969-1, authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio).

Landscape and local environmental variables

Variables measured were those that have previously shown to affect amphibian communities in tropical open area ponds (Da Silva *et al.* 2011; Da Silva *et al.* 2012ab; D'Anunciação *et al.* 2013; Schiesari and Corrêa, 2016). We used Google Earth Pro (v7.1.8.3036; see thesis annex B) to calculate the following landscape metrics: i) proportion of area of the surrounding land use types land within 1 km radius buffers around each pond, and ii) shortest distance from pond to agriculture, forest fragments, linear structures, and urban. Land uses were classified as: agriculture (mostly sugarcane, but also soybean, orange trees, and others), forestry (eucalyptus and rubber tree), forests fragments (remnants of native vegetation), linear structures (roads, rail-ways and motor-ways), pastures (extensive non-pesticide managed cattle pastures), urban (cities and rural residences), and waterbodies (mostly fish-farms); and ii) distance from each pond~~s~~ to the nearest agriculture, forest fragment, linear structure and urban patch. Local variables measured were i) maximum pond depth, ii) maximum surface area, estimated according to the geometric shape (ellipse, circle, triangle or square), iii) proportion of vegetation cover on the margins and within the ponds at three different height categories ("creeping" <10 cm, "erect" 10 to 50 cm and "high" >50 cm; visually estimated), and iv) tadpoles density (maximum density among the four surveys; total ind/m^2) (Tab.S1).

Pesticide contamination

We assessed the concentrations of the 14 most used pesticides for sugarcane management in Brazil (IBAMA, 2012). We took water samples (1 L) from each pond in decontaminated amber bottles, and preconcentrated in solid phase 6cc Oasis HLB Extraction Cartridge (500 mg) LP (Waters, Milford, MA). We determine concentrations using a 1200 series Liquid Chromatograph System, equipped with a binary pump, and coupled to a 6410 triple quadrupole Mass Spectrometer with an electrospray ionization source; all from Agilent Technologies Santa Clara, CA, USA. (see Sánchez-Domene *et al.* 2018 for further details).

Statistical analysis

Prior to analyses, proportion variables and density of tadpoles, were arcsin-transformed, and distance variables were log-transformed (Anderson *et al.* 2006; Zuur *et al.* 2010). As *Leptodactylus labyrinthicus* was represented by only one individual (pond SO_B), this species was not included in the analyses. The, predictor variables were standardized to zero mean and unit variance in the R package vegan (Oksanen *et al.* 2015). Finally, for statistical analysis we grouped pesticides into chemical classes, and we used incidence of pesticides classes instead of their concentration, since the breeding season of amphibians in the region matches with torrential rains and high temperatures that cause high water volume fluctuations, and consequently, in the concentration of pesticides. Variables were tested for multicollinearity, only retaining those with a variation inflation factor ≤ 3 (Zuur *et al.* 2010).

Predictors of amphibian species richness in ponds

We built a set of models with Poisson Generalized Linear Model (Zuur *et al.* 2009) which were submitted to a model selection procedure, in order to find the best model to

explain the influence of local and landscape variables on tadpole species richness at ponds. Diagnostic plots showed no overdispersion. We built different models by combining the variables measured to represented distinct hypotheses (Table 1). Then, we ranked models using Akaike's information criterion, corrected for small sample sizes (AICc), and if necessary, we selected those with $\Delta AICc < 2$ (Burnham & Anderson, 2002) to conduct model averaging. We also assessed model support using Akaike weights, which indicates the weight of evidence towards a given model among those considered.

Influence of local and landscape variables on tadpole species composition

We used partial redundancy analyses, coupled with variation partitioning, to disentangle species response to environmental variables (landscape, local, and pesticides contamination) using the R package vegan (Oksanen *et al.* 2015). We assessed both incidence and abundance matrices, this last after Hellinger transformation (Legendre & Legendre, 2012).

Prior to the analysis, we used forward selection to remove environmental variables with little explanatory power using the double-stopping criteria (Blanchet et al. 2008) in the R package adespatial (Dray *et al.* 2018). To control for spatial autocorrelation, we built a neighboring network linking ponds according to potential dispersal routes for individual adult anurans. This neighbor network considered landscape structure and penalized roads, highways, railways and other natural barriers to dispersal (McIntire and Fajardo, 2009; Fig.S1). This analysis was implemented in the R package spdep using the edit.NB function (Bivand *et al.* 2013). From this neighbor network we calculated a spatial weighting matrix as a function of the inverse of distance given by the neighbor network (Bauman *et al.* 2018). Afterwards, we calculated Moran Eigenvector Maps (MEM) from this spatial weighting matrix in order to describe the spatial arrangement of ponds in the

landscape at multiple spatial scales according to the neighbor network (Dray *et al.* 2012, Legendre and Legendre 2012). Then, we included all MEMs that described significant positive autocorrelation structures in the analysis. From this preliminary analysis, we found that the spatial arrangement of ponds only explains a negligible proportion of species composition. Similarly, explanatory environmental variables were little spatially autocorrelated (Tab.S2) Thus, space was not included in further analyses.

RESULTS

Pesticides contamination

We found 10 pesticides (see thesis annex A) in 11 of the 18 studied ponds (Tab.2 and S.3), being 6 herbicides (2-Hydroxy-Atrazine, Hexazinone, Tebuthiuron, Ametrine, Atrazine and Diuron), 2 insecticides (Imidacloprid and Malathion), and 2 fungicides (Carbendazime and Tebuconazole). As mentioned above, for statistical analyses we grouped them into chemical classes: Azole (Tebuconazole), Benzidimazole (Carbendazime), Neonicotinoid (Imidacloprid), Organophosphates (Malathion), Triazine (2-Hydroxy-Atrazine, Ametrine, Atrazine), Triazinone (Hexazinone), and Urea (Diuron, Tebuthiuron). Pesticides analysis of the SO_C pond could not be performed; thus, it was not included in the pRDA analyses.

Predictors of amphibian species richness in ponds

We recorded tadpoles of 10 amphibian species belonging to three families (Table 3). The most abundant species, and the only ones present in all the studied ponds, were *Scinax fuscovarius* (31,651 individuals) and *Physalaemus nattereri* (13,766 individuals). Mean species richness per pond was 5.16 (±1.60 SD; range 3–9). Only two models had ΔAICc < 2: "Null" and "Distance", and none was overwhelmingly supported by its Akaike

weight (Table 4). After screening the "Distance" model, we detected that Forest distance (For_d) was highly related with species richness. Therefore, we constructed a model for this single variable and repeated the model selection procedure (Tab.4). This analysis clearly ranked this new model as best in both ΔAICc and Akaike weight, being the distant ponds to forest fragments the poorest in species richness (Figure 2). In addition, since collinearity problems between density of tadpoles at ponds (Tad) and $Agri_d$ were detected, we perform an ad hoc linear regression which showed the existence of more tadpoles per-unit area in ponds furthest away from agriculture (Fig.2)

Tadpole species composition

Variation in species incidence and abundance were differently explained by environmental variables (Figure 3). The incidence of *Trachycephalus typhonius*, *Leptodactylus podicipinus*, *Dendropsophus nanus*, *Dermatonotus muelleri,* and *Dendropsophus minutus* were partially explained (Adj. R-square ≈ 15%) only by the pond distance to the nearest forest fragments (Tab.5). However, variance in species abundance were explained by contamination (pesticides presence) and pond local characteristics (Adj. R-square ≈ 49%). Contamination (Triazines) accounted for 10%, while pond's local characteristics (pond area, proportion of high vegetation at ponds margins, and proportion of erect vegetation in the interior) accounted for 48%, however the shared component Contamination-Local explained 9%, remaining variation explanation as: Contaminatin = 1%, Local = 39% and Contamination-Local = 9% (Tab.5 and Fig.3). *P.nattereri* and *L.fuscus* were more abundant in ponds with greater proportion of high vegetation at margins, while *S.fuscovarius* and *D.muelleri* were in ponds with high proportion of erect vegetation inside. At the same time, abundances of *D.muelleri*, together with *T.typhonius,* were higher in larger ponds.

DISCUSSION

Our results show that incidence and abundance of amphibian tadpoles respond differently to environmental variables. While distance to forest fragments was the only factor driving the incidence of species at ponds, local pond variables, mostly related to vegetation, were the only ones explaining their variation in abundance. Incidence and abundance have been stressed one over other in different papers, several authors support the study of abundance over richness because consider that this parameter is not as easy to affect by species specific characteristics as incidence is (*e.g.* Ernst and Rödel, 2008; Matthews *et al.* 2014). However, in the light of the differences found in factors driving amphibian incidence and abundance in our study, we believe that the joint study of both community descriptors is much more informative than the study of just one of them, as has been already point out by other researchers (Da Silva *et al.* 2011a; Schneider-Maunoury *et al.* 2016)

The fact that ponds closer to forest fragments were the richest in species might be explained by a positive edge-effect, which entails higher number of species in the transitional area between natural and human-modified habitats than in those habitats alone (Ries *et al.* 2014). However, we cannot assume that the higher richness is consequence of the sum of forest interior species with those from open area, since all species recorded are generalist, typical from open areas and tolerant to anthropogenic modifications but also found at forests (Provete *et al.* 2011; IUCN, 2018). Another study carried out close to our study area, which built artificial pools at different distances from forest fragments (Silva *et al.* 2012b), found greater species richness in pools near to forest fragments, concluding that the relation between distance to forest fragment and pools richness was a proxy to the main factor controlling the ability of amphibian species to establish and sustain breeding populations, heat and water stress resistance (Da Silva *et*

al. 2012b). Amphibian species richness has been explained by ponds distance to nearest forest fragments in several other studies conducted in this same region (Da Silva *et al.* 2011; Da Silva *et al.* 2012ab; Prado and Rossa-Feres, 2014), evidencing a spatial pattern, but ponds local variables were also very important. A possible explanation to why we did not detect the effect of pond local variables on species richness is that the spatial configuration of the set of ponds studied, distance to forest fragments and land uses surrounding them, were masking the explanatory power of local characteristics. Sugarcane plantations are inhospitable for adult amphibians due to management practices (D'Anunciação *et al.* 2014), which may favor species with traits features adapted to desiccation (Schneider-Maunoury *et al.* 2016). Thus, our study would have reflected this fact by only highlighting distance to forest fragment as the main driven factor for species richness. This fits with the concept of habitat split, defined as anthropogenic disconnection between habitats used by the different life history stages of a species (Becker *et al.* 2008), which has been pointed out to be more important than habitat loss and fragmentation for the incidence of amphibian species at ponds (Becker *et al.* 2008; Lion *et al.* 2014). In addition, the fact that ponds near to agriculture presented lower density of tadpoles than those ponds farthest, further reinforce the hypothesis that habitat split is affecting the studied region since the arrival of individuals to that ponds could be particularly challenging for some species.

Tadpole abundance was mainly driven by the presence of high vegetation at ponds margins. Ponds with greater proportion of high vegetation at margins (mainly sugarcane reeds) and low proportion of erect vegetation inside, had high abundances of *P.nattereri* and *L.fuscus*, species whose males call to attract females on the ground or floating on the water (Santos and Rossa-Feres, 2007). This vegetation configuration, related to ponds annex to sugarcane plantations, grouped two kind of ponds among the studied: i) those of

constructed at margins of plantations as retention ponds, where the low proportion of erect vegetation Bseems to be caused by its great depth, and ii) naturally occurring puddles at plantations margins resulted from the accumulation of the recurrent torrential rains at the time of the study, where the low proportion of erect vegetation inside them was probably due to its recent formation.

Our results emphasize the importance of maintaining ponds near forest fragments in agroecosystems in seasonally dry tropical regions, since they are needed for the conservation of amphibian communities. According to Rittenhouse and Semlitsch (2007), most of adult amphibian, and recently metamorphosed individuals, use forest habitats near breeding ponds (30 – 200 meters) during non-reproductive seasons. However, is noteworthy that the sole maintenance forests fragments and ponds appears not to be enough for conservation, but also their spatial arrangement (Becker et al., 2007; Lion et al. 2014). Thus, the best solution would be to have ponds with diverse vegetation structure near to each other connected by permeable matrix (D'Anunciação *et al.* 2013), and near to forest fragments.

Although we found traces of pesticides in 11 of the 18 ponds, revealing a widespread contamination in the studied sugarcane-dominated agroecosystem, its concentration seems not to be enough to affect amphibian communities inhabiting the region. Indeed, although the presence of herbicides of the Triazine chemical class (Ametrine, Atrazine and 2-Hydroxy-Atrazine) explain 10% of the total variation of species abundance, only 1% of the variation was explained by only their presence, being the remaining 9% explanation shared with ponds local variables. The detected contamination was not restricted to ponds subjected to direct runoff, but also occurred at ponds distant to sugarcane, which could be a sign of contamination by pesticides drift because of spraying or aerial fumigation. Regarding the apparent low impact of pesticides on the studied

amphibian communities, it could be explained because of the low effects on tadpoles of the concentrations found, or because of the development of tolerance to pesticides, which has been described in amphibian populations living near to agriculture (Hua *et al.* 2015ab). Two different mechanisms have been proposed as responsible for this tolerance, phenotypic plasticity (Hua *et al.* 2015b, Jones *et al.* 2018) and detoxification pathway (Oziolor *et al.* 2017). Both mechanisms need several pesticides low concentrations exposures to induce tolerance, which seem to be occurring at assessed ponds. Nevertheless, it is worth remembering that low concentrations found in the ponds seem be result of the diluting effect of the torrential rains events occurring in the region during amphibian breeding season, therefore increasements in the concentrations of pesticides due to the high evaporation occurring between rain events, may lead higher concentrations, which negatively affect the growth and development of tadpoles and increase susceptibility to diseases and behavioral alterations (Maan *et al.* 2009; Egea-Serrano *et al.* 2012).

CONCLUSIONS

Our findings highlight the importance of maintaining man-made cattle ponds and native forest fragments to achieve a successful amphibian conservation. However, its simple maintenance is not enough to ensure their conservation, priority must be given to ponds with abundant stratified vegetation, near forest fragment, with short and permeable connecting matrix, and far from pesticides contamination sources. In addition, in spite of no impacts of pesticides on amphibian community were detected, although we cannot rule out their existence, widespread contamination was. Therefore, we recommend monitoring amphibian populations inhabiting this increasingly scarce breeding ponds in

order to detect dangerous concentration pesticide pulses whose effects could endanger tadpoles and juveniles inhabiting them, thereby risking population recruitment.

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FIGURES

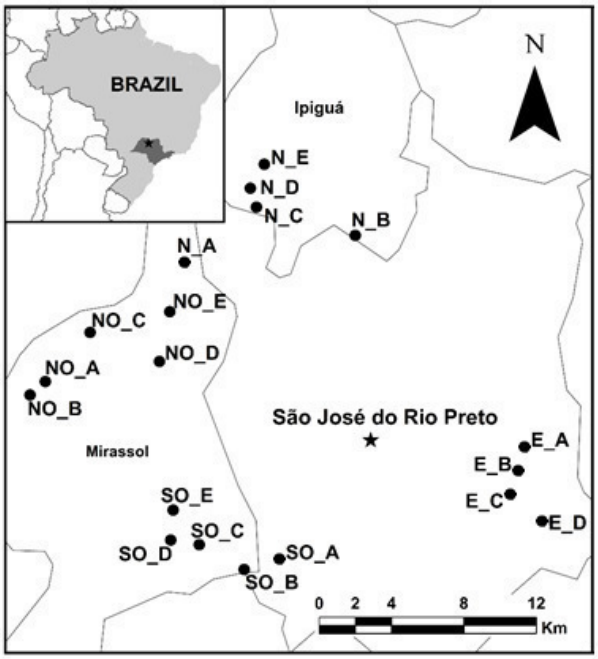

Figure 1. Temporary ponds from agroecosystems with different degrees of sugarcane intensification assessed during the rainy season 2015/2016 in São José do Rio Preto, São Paulo State (Brazil). E_(A,B,C,D) for East ponds, SO_(A,B,C,D,E) for Southwest ponds, NO_(A,B,C,D,E) for Northwest ponds and N_(A,B,C,D,E) for North ponds.

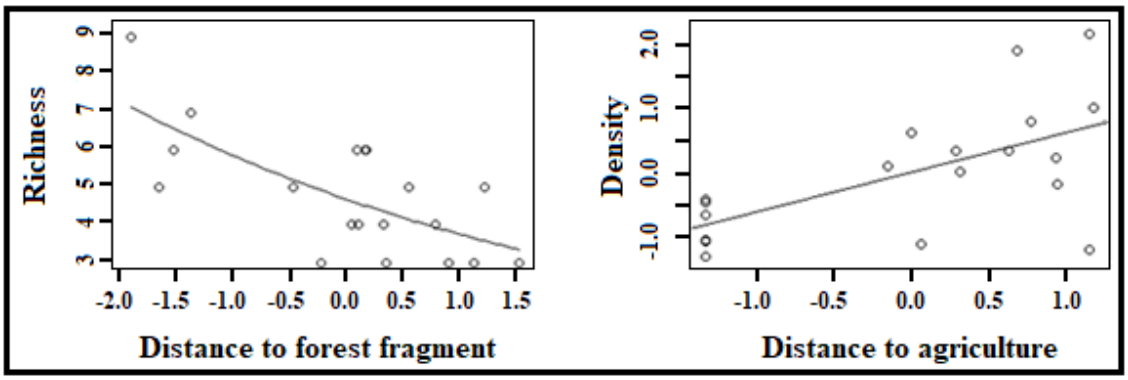

Figure 2. Relationship between tadpole species richness and ponds' distances to nearest forest fragments (on the left) and between tadpole density at ponds and distance to agriculture fields (on the right), in 19 temporary ponds from sugarcane dominated agroecosystem assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil). Distances to nearest forest fragment, distance to agriculture and tadpole density were log-transformed and standardized to zero mean and unit variance for statistical analyses purposes, on the graphs negative values means lower distances or density.

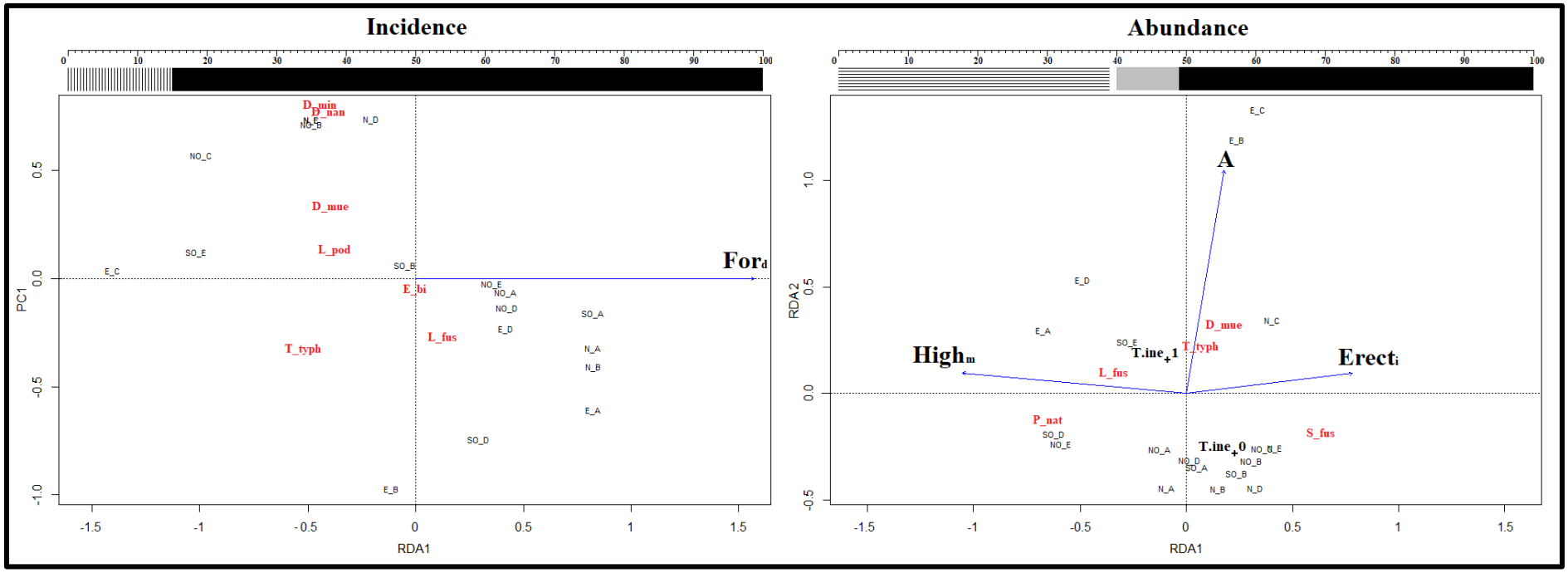

Figure 3. Partial redundancy analyses (pRDA) ordination triplots showing main variables explaining species incidence and abundance in 19 temporary ponds from sugarcane dominated agroecosystem assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil). Sampled ponds (in capital letters), amphibian species (in small size bold letters), quantitative explaining variables (in large size bold letters), qualitative explaining variables (in medium size bold letters, crosses representing centroids of levels). The scores of species close to the origin are not labelled; for abbreviations meanings refer to the text. The bars on the top of the plots represent the result of variation partitioning; landscape (vertical lines), local (horizontal lines), local-contamination (white), contamination shared (grey), and unexplained variation (black).

TABLES

Table 1. Candidate models used in GLM analysis of species richness

Model	Variables	Explanation
Landscape		
Distance	$For_d + Agri_d$	Distance to forest fragment (For_d; adult shelter and/or foraging areas) and to agriculture ($Agri_d$; pesticides contamination more likely to occur), as major determinants for tadpole species richness at ponds.
Proportion	$For_p + Agri_p$	Proportion of forest fragments (For_p; adult shelter and/or foraging areas) and of agriculture ($Agri_p$; adult inhospitable land-uses), as major determinants for tadpole species richness at ponds.
Distance-Proportion	$Agri_d + For_d + Agri_p + For_p$	Combination of landscape variables.
Local	Tad + Erect	Tadpole density (Tad; individuals per square meter of pond) and proportion of erect vegetation at interior of ponds (Erect; related to the structural complexity in the breeding sites and species-specific requirements.), as major determinants for tadpole species richness at ponds.
Local + Landscape		Combinations of landscape and local variables.
Local-Distance	$Tad + Erect + Agri_d + For_d$	
Local-Proportion	$Tad + Erect + Agri_d + For_p$	
Global	$For_d + For_p + Agri_p + Tad + Erect$	$Agri_d$ not included because of collinearity problems (VIF > 3).

Table 2. Concentration (ng L^{-1}) of the chemical classes of pesticides found in water samples from 18 temporary ponds from agroecosystems with different amounts of sugarcane assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil). For concentrations of each pesticide see supplementary material Table S3.

Pond	Urea	Benzidimazole	Triazinone	Triazine	Neonicotinoid	Azole	Organophosphorus
E_A	**448**	<DL	**236**	**179**	<DL	<QL	<QL
E_B	**705**	<QL	**1211**	**325**	<DL	<QL	<QL
E_C	**29.5**	<DL	<QL	<QL	<DL	<QL	<QL
E_D	**35.3**	<DL	<QL	**34.2**	<DL	<QL	<QL
NO_A	<DL	<DL	<DL	<DL	<DL	<DL	<DL
NO_B	<DL	<DL	<DL	<DL	<DL	<DL	<DL
NO_C	<DL	<DL	<DL	<DL	<DL	<DL	<DL
NO_D	<DL	<DL	<DL	<DL	<DL	<DL	<DL
NO_E	<DL	<DL	<DL	**2.94**	**3.33**	<DL	<DL
N_A	<DL	<DL	<DL	<DL	**3.86**	<DL	<DL
N_B	<DL	<DL	<DL	<DL	<DL	<DL	<DL
N_C	<DL	<DL	<DL	<DL	<DL	<DL	<DL
N_D	**7.96**	<DL	**3.95**	**12.07**	**3.89**	<DL	<DL
N_E	<DL	<DL	<DL	<DL	<DL	<DL	<DL
SO_A	**22.6**	**23.2**	<QL	**24.2**	<DL	<QL	<QL
SO_B	**22.2**	<DL	<QL	<QL	<DL	<QL	<QL
SO_D	<QL	<DL	<DL	<QL	<DL	<QL	<QL
SO_E	<QL	<DL	<DL	<QL	<DL	<DL	<QL

<DL = Below the Detection Limit (for statistical analysis it is assumed absence of the compound)
<QL= Below the Quantification Limit (for statistical analysis it is assumed presence of the compound)

Table 3. Total number of tadpoles sampled in 19 temporary ponds from agroecosystems with different amounts of sugarcane assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil).

Pond	*Dendropsophus nanus*	*Dendropsophus minutus*	*Dermatonotus muelleri*	*Elachisocleis bicolor*	*Leptodactylus fuscus*	*Leptodactylus labyrinthicus*	*Leptodactylus podicipinus*	*Physalaemus nattereri*	*Scinax fuscuvarius*	*Trachycephalus typhonius*
E_A	0	0	0	0	239	0	0	187	25	0
E_B	1	0	1010	0	69	0	0	86	1202	247
E_C	7	21	1210	17	6	0	275	23	1695	995
E_D	1	0	92	10	443	0	1	498	175	0
NO_A	0	0	52	2	53	0	0	1746	2242	0
NO_B	12	27	11	0	4	0	0	129	1746	0
NO_C	39	7	54	0	0	0	107	301	5948	0
NO_D	0	0	27	0	21	0	0	838	1956	0
NO_E	0	2	0	0	32	0	0	1915	244	0
N_A	0	0	0	0	52	0	0	1380	2244	0
N_B	0	0	0	0	16	0	0	375	2011	0
N_C	41	5	699	0	5	0	0	77	3064	0
N_D	25	17	0	0	0	0	0	51	776	0
N_E	83	60	6	0	8	0	0	17	1649	0
SO_A	0	0	0	0	68	0	0	180	803	0
SO_B	0	59	3	0	30	1	0	308	3000	0
SO_C	1	1	0	0	124	0	0	195	1091	0
SO_D	0	0	0	0	24	0	0	2921	255	2
SO_E	5	3	98	0	136	0	0	2539	1525	503

Table 4. Model selection results for predicting the amphibian species richness in ponds from agroecosystems with different amounts of sugarcane assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil). On the left first analysis performed, on the right same analisys including the model "Distance to forest fragment".

Model	AICc	df	ΔAICc	AICw
Null	76.076	1	0.0	0.467
Distance	76.692	3	0.6	0.343
Local	79.572	3	3.5	0.081
Proportion	79.657	3	3.6	0.078
Distance-Proportion	83.258	5	7.2	0.013
Local-Distance	83.426	5	7.3	0.012
Local-Proportion	85.782	5	9.7	0.004
Global	87.358	6	11.3	0.002

Model	AICc	df	ΔAICc	AICw
Distance to forest fragment	74.087	2	0.0	0.558
Null	76.076	1	2.0	0.206
Distance	76.692	3	2.6	0.152
Local	79.572	3	5.5	0.036
Proportion	79.657	3	5.6	0.034
Distance-Proportion	83.258	5	9.2	0.006
Local-Distance	83.426	5	9.3	0.005
Local-Proportion	85.782	5	11.7	0.002
Global	87.358	6	13.3	<0.001

AICc (Akaike information criteria corrected for small sample sizes), df (degrees of freedom), ΔAICc (delta AICc; AICc differences) and AICw (AIC weights).

Table 5. Percentage of explained variation (adjusted R square) results of partial redundancy analyses for amphibian species incidence and abundance of whole models and each of the significant variables composing the final reduced models. Reduced model obtained by applying forward selection using the double-stopping criteria (significant p-value and cumulative adjusted R square value).

	Pond variables	Whole Model		Reduced Model	
		explained variation	p-value	explained variation	p-value
Abundance	Sourrounding Landscape	8.88	n.s	//	//
	Local	49.61	< 0.001	48.12%	< 0.001
				High vegetation at margins 36.23%	< 0.001
				Area 6.06%	< 0.05
				Erect vegetation at interior 5.83%	< 0.05
	Contamination	20.98	< 0.05	10.03%	< 0.05
				Triazine 10.03%	< 0.05
Incidence	Sourrounding Landscape	24.51	< 0.05	15.86%	< 0.001
				Distance to riparian forest 15.86%	< 0.001
	Local	13.21	n.s	//	//
	Contamination	0	n.s	//	//

Supplementary Material

Figure S1. Neighboring network (Delaunay triangulation) linking ponds according to potential dispersal routes for individual adult anurans. In red, highways with a great density of traffic considered as dispersal barrier to anurans.

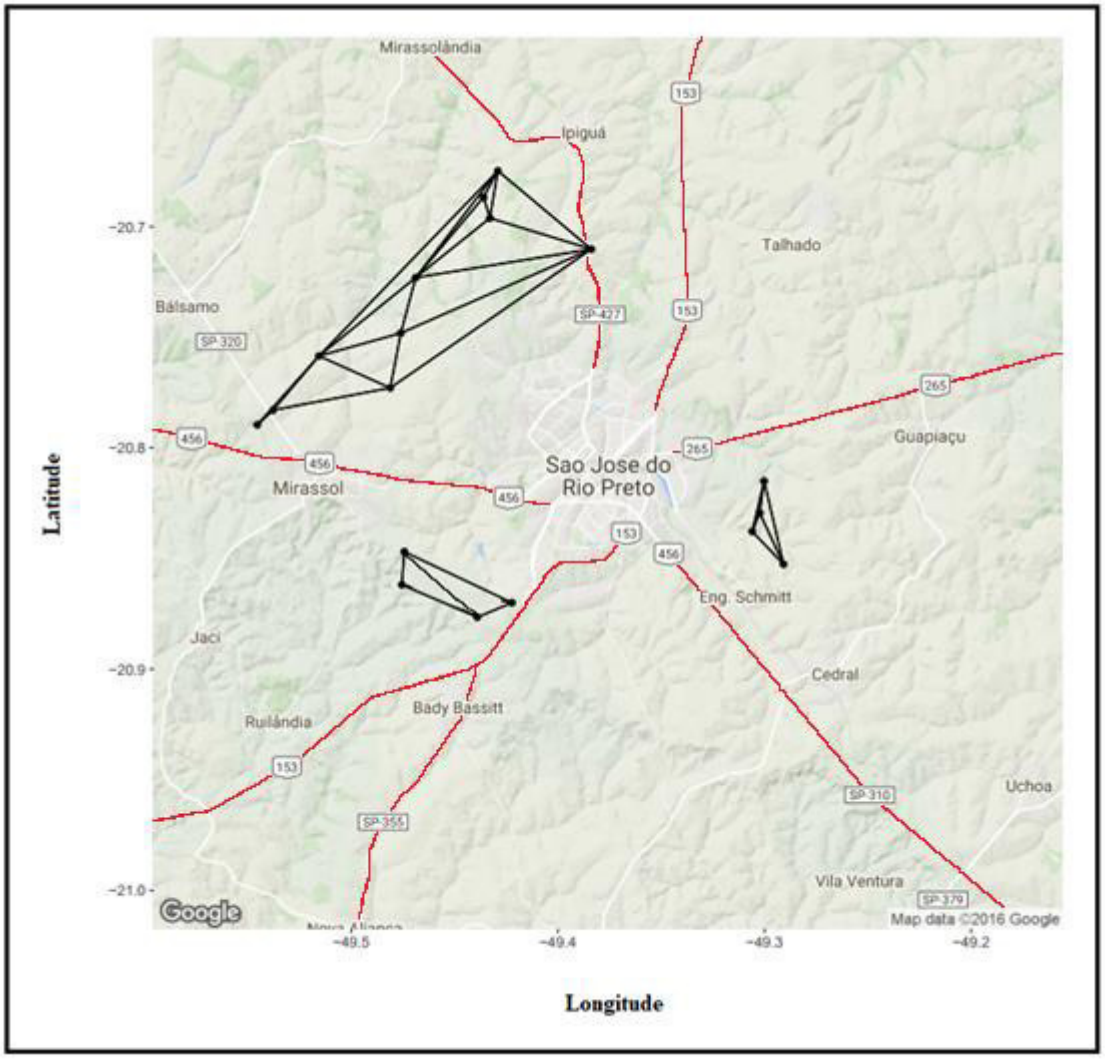

Table S1. Landscape and local variables of 19 temporary ponds from agroecosystems with different amounts of sugarcane assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil).

Ponds	Ponds landscape proportion variables							Ponds landscape distance variables (m)				Ponds local variables								
												Depth	Area (m^2)	Proportion of vegetation at Margin			Proportion of vegetation at Interior			Tadpoles density
	Agriculture	Forestry	Forest fragments	Linear structures	Pasture	Urban	Waterbodies	Agriculture	Forest fragments	Linear structures	Urban	(cm)		Creep	Erect	High	Creep	Erect	High	(ind/m^2)
E_A	0.379	0.005	0.058	0.004	0.550	0.003	0.000	1	134	69	65	100	288.8	0	0.05	0.2	0	0.05	0.15	0.744
E_B	0.414	0.000	0.112	0.003	0.410	0.052	0.009	1	30	886	768	100	923.54	0.3	0.5	0	0	0.25	0	1.691
E_C	0.241	0.000	0.228	0.013	0.469	0.037	0.012	162	23	25	135	100	352.81	0.15	0.85	0	0.15	0.45	0	8.373
E_D	0.213	0.026	0.069	0.013	0.414	0.262	0.003	1	301	15	28	86	286.37	0	0.8	0.05	0	0.3	0.05	1.589
NO_A	0.443	0.023	0.030	0.048	0.402	0.055	0.000	243	607	23	68	100	317.07	0.85	0	0	0.2	0	0	4.822
NO_B	0.402	0.020	0.038	0.052	0.429	0.057	0.002	17	187	92	84	100	185.13	0	1	0	0	0.4	0	5.899
NO_C	0.687	0.000	0.085	0.000	0.228	0.000	0.000	50	34	1387	1478	72	465.82	0	0.95	0	0	0.5	0	6.730
NO_D	0.276	0.006	0.084	0.000	0.624	0.008	0.003	402	387	1050	400	65	158.6	0.35	0	0	0.15	0	0	13.367
NO_E	0.657	0.009	0.077	0.005	0.179	0.070	0.002	1	178	1	307	72	166.16	0	0.35	0.2	0	0.1	0.1	3.816
N_A	0.200	0.079	0.201	0.006	0.503	0.010	0.000	430	437	22	96	51	128.61	0	1	0	0	0.4	0	9.198
N_B	0.075	0.032	0.122	0.018	0.472	0.277	0.004	240	305	17	165	79	149.49	0.7	0.3	0	0	0.4	0	6.375
N_C	0.442	0.054	0.054	0.009	0.427	0.014	0.000	113	202	24	390	68	260.88	1	0	0	0	0.5	0	6.746
N_D	0.417	0.150	0.041	0.013	0.353	0.026	0.000	29	238	10	660	51	317.77	0	1	0	0	0.6	0	1.441
N_E	0.666	0.052	0.065	0.007	0.204	0.006	0.000	1	203	16	489	73	220.49	0	0.15	0	0	0.4	0	3.991
SO_A	0.053	0.005	0.026	0.022	0.677	0.218	0.000	401	845	23	29	77	362.87	0.8	0	0	0	0.15	0	1.130
SO_B	0.374	0.025	0.152	0.012	0.383	0.052	0.002	25	104	15	230	100	131.87	0.4	0.6	0	0	0.5	0	7.788
SO_C	0.611	0.002	0.070	0.016	0.292	0.007	0.003	1	554	1	454	31	190.16	0	0.3	0.5	0	0.6	0.1	3.087
SO_D	0.018	0.010	0.351	0.007	0.591	0.018	0.005	53	190	43	50	64	389.63	0.6	0.2	0.1	0	0.1	0	5.528
SO_E	0.282	0.099	0.186	0.012	0.418	0.002	0.001	130	40	115	860	75	172.14	0	0.7	0	0	0.1	0	12.397

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Table S2. Ponds spatial autocorrelation (Moran's I).

Moran's I			
Eigenvector	**Eigenvalue**	**stat**	**p-value**
1	3.083	1.037	0.01
2	2.428	0.817	0.01
3	2.220	0.747	0.01
4	0.903	0.304	0.01
5	0.018	0.006	0.38
6	0.012	0.004	0.22

Table S3. Concentration of the pesticides found in water samples from 18 temporary ponds from agroecosystems with different amounts of sugarcane assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil).

	Concentration of pesticides (ng/L)									
Pond	**Tebuconazole**	**Malation**	**Tebuthiuron**	**Carbendazim**	**Hexazinone**	**Ametryne**	**Diuron**	**Hydroxy_atrazine**	**Atrazine**	**Imidacloprid**
E_A	<LQ	<LQ	**163**	<LD	**236**	**179**	**285**	<LD	<LD	<LD
E_B	<LQ	<LQ	**271**	<LQ	**1211**	**325**	**434**	<LD	<LD	<LD
E_C	<LQ	<LQ	<LQ	<LD	<LQ	<LQ	**29.5**	<LD	<LD	<LD
E_D	<LQ	<LQ	**35.3**	<LD	<LQ	**34.2**	<LQ	<LD	<LD	<LD
NO_A	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
NO_B	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
NO_C	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
NO_D	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
NO_E	<LD	<LD	<LD	<LD	<LD	<LD	<LD	**2.94**	<LD	**3.33**
N_A	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	**3.86**
N_B	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
N_C	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
N_D	<LD	<LD	**4.92**	<LD	**3.95**	**4.74**	**3.04**	**3.8**	**3.53**	**3.89**
N_E	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
SO_A	<LQ	<LQ	**22.6**	**23.2**	<LQ	**24.2**	<LQ	<LD	<LD	<LD
SO_B	<LQ	<LQ	**22.2**	<LD	<LQ	<LQ	<LQ	<LD	<LD	<LD
SO_D	<LQ	<LQ	<LQ	<LD	<LD	<LQ	<LQ	<LD	<LD	<LD
SO_E	<LD	<LQ	<LQ	<LD	<LD	<LQ	<LQ	<LD	<LD	<LD

DL = Below the Detection Limit (for statistical analysis it is assumed absence of the compound)

<QL= Below the Quantification Limit (for statistical analysis it is assumed presence of the compound)

Chapter II

Eye malformation baseline in Scinax fuscovarius larvae populations that inhabit agroecosystem ponds in southern Brazil

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Eye malformation baseline in *Scinax fuscovarius* larvae populations that inhabit agroecosystem ponds in southern Brazil

David Sánchez-Domene[1*], Alba Navarro-Lozano[2], Raphael Acayaba[3], Katiuce Picheli[2], Cassiana Montagner[4], Denise de Cerqueira Rossa-Feres[2], Fernando Rodrigues da Silva[5], Eduardo Alves de Almeida[6]

[1] Instituto de Pesquisa em Bioenergia, Universidade Estadual Paulista, Rio Claro, SP 13500-230, Brazil.
[2] Laboratório de Ecologia Teórica, Departmento de Zoologia e Botânica, Universidade Estadual Paulista, São José do Rio Preto, SP 15054-000, Brazil.
[3] Faculdade de Tecnologia, Universidade de Campinas, Limeira, SP 13484-332, Brazil.
[4] Laboratório de Química Ambiental, Departamento de Química Analítica, Instituto de Química, Universidade de Campinas, Campinas, SP 13484-332, Brazil.
[5] Laboratório de Ecologia Teórica: Integrando Tempo, Biologia e Espaço (LET.IT.BE), Departmento de Ciências Ambientais, Universidade Federal de São Carlos, Sorocaba, SP 18052-780, Brazil.
[6] Departamento de Ciências Naturais, Fundação Universidade Regional de Blumenau, Itoupava Seca, SC 89030-903, Brazil.

ABSTRACT

Events of mass malformations in amphibian populations that have exceeded historical records have been reported over the past thirty years. Many of these events have been linked to human activities that occurred near amphibian breeding habitats. The rise in biofuels has promoted, and continues to promote, the growth of sugarcane plantations in Brazil, with the northwest region of São Paulo State having experienced the largest sugarcane expansion over the past few decades. In this region, we sampled temporary ponds located in agroecosystems dominated to different degrees by sugarcane. We found several larvae of *Scinax fuscovarius* with eye malformations (anophthalmia, aphakia, microphthalmia and sub-development). In this study, we assessed whether the distance from the ponds to the nearest sugarcane crop, the proportion of sugarcane surrounding the ponds, the presence of pesticides in the ponds, or the proportion of land uses with potential teratogens that surround the ponds were related to the frequencies of amphibian eye malformations. We found pesticides present in 11 of the 18 ponds, but none of the predictor variables was associated with the frequencies of amphibian eye malformations. Thus, our results suggest that the observed frequencies of amphibian eye malformations could be a consequence of natural mutation rates, and these data could be used as a malformation baseline for the region. This malformation baseline is the first reported for amphibians in South America and may be useful in future surveys on amphibian populations in tropical agroecosystems.

Keywords: *pesticides*, *amphibians*, abnormalities, *sugarcane*, *teratogens*.

INTRODUCTION

Malformations are permanent structural defects resulting from morphogenesis errors in organisms (Meteyer et al. 2000; Lannoo, 2008). In amphibians, a large range of malformations have been linked to exposure to different teratogens emitted into the environment by human activities, such as electromagnetic radiation, chemicals and metals, as well as natural agents, such as viruses and parasites (Ouellet, 2000; Lannoo, 2008). Although mass malformations in amphibians have coincided with global population declines (Alroy, 2015; Whitfield et al. 2016), the link between malformations and population declines is not strongly supported (Houlahan et al. 2000; Ouellet, 2000; Ankley et al. 2004; Lunde and Johnson, 2012). Indeed, it is not even clear whether the link between proximity to human activities and the high occurrences of malformations in amphibian populations are due to teratogen exposure or to the intensive surveillance of freshwater systems over the past decades (Ouellet, 2000). Therefore, until further studies clarify the causes and implications of malformations, amphibian populations inhabiting areas susceptible to exposure to potential teratogens must be monitored (Burkhart et al. 2000).

Most studies that assess the occurrences of amphibian malformations in wild populations have been conducted in developed countries (Ouellet, 2000; Lannoo, 2008), which have a longer history of environmental contamination than that of most developing countries (Guerra and Aráoz, 2016). The lack of studies assessing malformations and their causes in amphibian biodiversity hotspots is worrisome. For example, in Brazil, which has the greatest diversity of amphibian species in the world with 1080 species (Jenkins et al. 2013; Segalla et al. 2016), only three reports of malformations in wild populations have been described (e.g. Dias and de Carvalho-e-Silva, 2012; Tolledo and Toledo, 2015;

Ramalho et al. 2017), and the causative agents of these malformations have not yet been studied.

To address this knowledge gap, we assessed the relationship between human activities in agriculturally dominated landscapes and the occurrences of eye malformations in several populations of *Scinax fuscovarius* larvae inhabiting temporary ponds in the northwest of São Paulo State, Brazil. This region is among the most deforested and fragmented in São Paulo State, and it has fewer conservation areas due to the commercial demands over the past three decades that contributed to the loss of the original vegetation, which was destroyed by agricultural activities (Rodrigues et al. 2008). In addition, the region has recently experienced one of the largest expansions of sugarcane plantations in Brazil, mainly replacing extensive non-pesticide managed cattle pastures (Rodrigues et al. 2008; Joly, 2010).

Schiesari & Grillitsch (2011) compiled a list of all the pesticides registered for use on sugarcane in Brazil and found 225 formulations, with 117 considered "highly dangerous" or "very dangerous" to the environment. Considering that small temporary ponds located in agricultural areas are usually flooded during intense periods of rain that results in several agrochemicals to leach from the soil into the ponds (Bridges et al. 2004), we hypothesized that sugarcane management is related to the exposure of amphibian larvae to pesticides (potential teratogens). Therefore, we predict that variables associated with sugarcane cultivation and/or human activities surrounding the ponds are related to eye malformation occurrences. Essentially, we aimed to answer the following question: Are the occurrence of eye malformations in *Scinax fuscovarius* larvae related to sugarcane plantations or other human activities?

METHODS

Study of organism and area

We studied *Scinax fuscovarius* (A. Lutz, 1925) larvae with eye malformations that were found among individuals sampled in the region of São José do Rio Preto, São Paulo, Brazil (Fig. 1). This treefrog (Hylidae) is widely distributed in open habitats of South America, being very common in agricultural and urban areas where it breeds in both temporary and permanent ponds during the rainy season (Aquino et al. 2010; AmphibiaWeb, 2017). These characteristics, together with the large numbers of eggs per spawn (Rodrigues et al. 2005), make *S. fuscovarius* an suitable indicator species for aquatic habitats embedded in agroecosystems. Larvae were collected during the rainy season (Nov 2015 – Mar 2016) in 19 temporary ponds that had different amounts of sugarcane. We selected these temporary ponds based on their distance to sugarcane fields (Table 1), resulting in ten ponds at distances less than or equal to 50 meters from sugarcane fields and nine at greater distances from the fields. Once collected, the larvae were anesthetized in benzocaine, fixed in a 1:1 solution of ethanol (70%) and formalin (15%), and deposited in the tadpole collection at Universidade Estadual Paulista, São José do Rio Preto (DZSJRP).

Determination of the malformations

Four classes of eye malformations were detected among the larvae (Fig. 2): (i) anophthalmia (absence of eye), (ii) aphakia (absence of eye lens), (iii) microphthalmia (one or both eyes abnormally small) and (iv) sub-development (small amorphous and not emerged eye). Since some abnormalities present in amphibians can result from erroneous

regenerations of traumatic injuries (Ouellet, 2000; Ballengee and Sessions, 2009; Lannoo, 2009), we examined the larvae under a stereoscopic microscope (Leica MZ75) discarding those that presented tissue tearing or scars in the ocular region. This methodology is likely to discard some malformed larvae, but it avoids the inclusion of false-positive individuals in subsequent analyses.

Determination of the pesticides in the ponds

For each pond, we determine the concentrations of the fourteen most used pesticides on sugarcane in Brazil (IBAMA, 2012). We collected water samples (one liter per pond) in decontaminated amber bottles and preconcentrated in solid phase 6cc Oasis HLB Extraction Cartridge (500 mg) LP (Waters, Milford, MA). The presence of pesticides was determined using a 1200 series liquid chromatograph system, equipped with a binary pump, and coupled to a 6410 triple quadrupole mass spectrometer with an electrospray ionization source (LC-ESI-MS/MS). The chromatographic separation was performed in a thermostatted column compartment (TCC G1316A) at 20 ^{o}C, using a reversed-phase Zorbax SB-C18 column (2.1 x 30 mm, particle size of 3.5 µm) from Agilent Technologies. Ammonium formate (5 mmol L^{-1}) (A) and methanol (B) were used as mobile phase. At a flow rate of 0.3 mL min^{-1}, the gradient elution was programmed by increasing the relative organic solvent concentration from 30% to 60% in 3 minutes and followed by an increase to 67% in 10 minutes. After readjusting the initial conditions, the system was re-equilibrated for 5 minutes. The injection volume was 10 µL. After the chromatographic separation, the tandem MS conditions were optimized for the positive ionization mode, using the ESI source. The following parameters were adjusted to maximize ionization: a drying gas flow rate of 10 L min^{-1}, drying gas temperature of 350 ^{o}C, nebulizer gas pressure at 50 psi, and capillary voltage of 3000 V. Nitrogen was used

as a collision gas. Based on Montagner et al. (2014), multiple reaction monitoring (MRM) transitions were employed for confirmation and quantification of the target compounds. The detection limits of the method varied between 0.1 to 2.0 ng L^{-1}.

Causative agents of eye malformations

We carried out a systematic literature review to identify potential agents that have been proven to be causes of amphibian eye malformations. For the literature review, we used databases from ScienceDirect, Google Scholar and Scopus, covering all publications through December 2016. We used a combination of key words: "malformation AND tadpoles", "malformation AND amphibian", "abnormalities AND tadpole", "abnormalities AND amphibian", "amphibian AND eye", and "amphibian AND ocular". We also used the references of selected articles to search for additional studies.

Local and landscape variables

The amphibian breeding season in the region of study occurs during torrential rains and high temperatures, which causes water volume fluctuations in ponds and, consequently, in the concentration of pesticides. Thus, we used the maximum water depth of ponds (MD) as a local variable related to the concentration of pesticides. The depth was measured in the field using a 2-meter-long millimeter cable. We used Google Earth Pro (v7.1.8.3036) to calculate the following landscape variables (land uses and distances) related to sugarcane crops and human activities in the region (Table 1): i) the distance from the pond to the nearest sugarcane field (DNS; calculated considering the shortest distance between the edges of the polygons from the pond to the nearest sugarcane crop), ii) the surrounding sugarcane fields (SS; calculated based on the proportion of sugarcane

crops within the circular buffers with a radius of 1 km centered on each pond), and iii) surrounding areas with teratogens (SAT; calculated based on the proportion of areas with the potential presence of teratogens within circular buffers with a radius of 1 km centered on each pond). To determine SAT, we created binary maps derived from land-use maps (also created for this study) of the circular buffers with a radius of 1 km centered on each pond (supplementary figure 1). The binary maps reclassified the categories of the land use maps into two categories: i) "Land uses with potential presence of teratogens", which includes those associated with human activities that use or emit potential teratogens into the environment such as agriculture (sugarcane, soybean and orange trees, among others), forestry (eucalyptus and rubber tree), waterbodies (fish-farm), urban areas (cities and rural residences) and linear structures (roads, rail-ways and motor-ways); and ii) "Land uses virtually free of teratogens", which includes riparian forests (remnants of native flora) and pastures (extensive non-pesticide managed cattle pastures).

Statistical Analyses

Prior to the analyses, the proportional explanatory variables (SS and SAT) were arcsin transformed, and the DNS was log transformed. Then, the entire set of explanatory variables were standardized and tested for multicollinearity. According to Quinn and Keough (2002), high VIF values (>10) indicate high multicollinearity. Because we found VIF values smaller than four among the explanatory variables all further analyses were performed without considering multicollinearity.

To assess if the frequencies of eye malformations (percentage of malformed individuals of the total larvae sampled in each pond, regardless the type of eye malformations they presented) were related to contamination or local or landscape variables, we generated a linear mixed effects (LME) model using the R-package `nlme´

(Pinheiro et al. 2017). To determine the optimal model, we started with a model in which the fixed effect contained the five explanatory variables previously listed, and the pond identities were considered as random effect. Then, we generated sub-model sets from the global model using the dredge function implemented in the MuMIn package (Barton 2017). We used Akaike's information criterion corrected for small sample sizes (AICc) (Burnham and Anderson 2002) and Akaike weights to evaluate model selection uncertainty.

In addition to the TCP values assessed with the LME, we decided to analyze the presence/absence of pesticides separately using Fisher's exact test to identify their association with the occurrence of eye malformations. Thus, we constructed 11 contingency tables (2 x 2) with a set of binary variables: malformation (which had a value 0 or 1, depending on whether or not the ponds presented malformed larvae), pesticides (which assumed the value 0 or 1, depending on whether or not the ponds presented the pesticide assessed), and contamination (which had a value 0 or 1, depending on whether or not the ponds presented at least one of the analyzed pesticides). The pesticide analysis of the SO_C pond could not be performed, so it was excluded from the statistical analyses. All analyses were performed using R Statistical Software (v.3.4.3, The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Contamination of ponds and causative agents of eye malformations

We found traces of pesticides in 11 of the 18 studied ponds (Table 1 and 2): i) six herbicides (2-Hydroxy-Atrazine, Hexazinone, Tebuthiuron, Ametryn, Atrazine and Diuron), ii) two insecticides (Imidacloprid and Malathion), and iii) two fungicides

(Carbendazime and Tebuconazole). Although our literature review revealed 44 agents (including a mixture of pesticides) that have been demonstrated to cause amphibian eye malformations (supplementary table 1), none of the pesticides detected in our study have been linked with eye malformations before.

Malformations

We collected 31,651 *Scinax fuscovarius* larvae of which 29 individuals (0.09%) presented eye malformations. Larvae with eye malformations were found in 12 of the 19 sampled ponds. Frequencies of eye malformations within the ponds with malformed larvae varied between 0.02% and 0.41% (Table 3). Among malformed larvae, 13 presented anophthalmia, seven microphthalmia (three of them bilateral), three aphakia and six sub-development (Fig. 2; Table 3). We did not observe malformations that affected other larvae structures, such as the limbs, tail or jaws.

Assessment of the frequencies of malformations

None of the models supported the data better than the NULL-model (supplementary table 2).

Pesticides and Malformations relationship

We found significant associations between occurrence of eye malformations and pesticide contamination of the ponds (Table 4). However, the associations were not as we would expect. For example, while only 36.4% (4 / 11) of the contaminated ponds (ponds with the presence of at least one of the analyzed pesticides) presented tadpoles with eye

malformations, all uncontaminated ponds (7 / 7) had malformed individuals (Fisher's exact test; n = 18, two-tailed $P < 0.05$).

DISCUSSION

We found that *Scinax fuscovarius* larvae populations presented a 0.09% prevalence of eye malformations. This frequency appears to be relatively low, as events of malformations in wild populations that do not exceed an estimated background malformation frequency of 2–5% are considered of no concern (Ouellet, 2000; Blaustein and Johnson, 2003). However, it should be noted that malformations included in our study are restricted to larval eyes, while most studies have presented baseline total malformation frequencies for post-metamorphic amphibians (Piha et al. 2006; Lunde and Johnson, 2012). Our results are the first that describe amphibian malformations in agricultural landscapes in Brazil. In addition, this study confirms our suspicions of widespread pesticide contamination in sugarcane-dominated agroecosystem ponds in northwest São Paulo State, which places the amphibian populations inhabiting these important, and increasingly scarce, breeding habitats at risk (Santos et al. 2007; Da Silva et al. 2011). Thus, we recommend monitoring amphibian populations in these habitats.

The SAT-model suggested that some unknown agent present in the surrounding areas of the ponds could be affecting tadpole populations when they were assessed; however, if this is true, we have been unable to determine the identity of this unknown agent. Identifying causative agents responsible for mass malformation events in amphibian populations is not always possible. In fact, it is most common to find reports of mass malformation events that state that multiple factors are responsible, acting at local or regional scales and usually related to human activities (e.g., Taylor et al. 2005; Agostini

et al. 2013; Kang et al. 2016). Perhaps, this study experienced the same situation as those studies. The difficulties in identifying the agents responsible for the eye malformations in the field are evident in view of the large number of agents (44 compiled in our literature review) associated with eye malformations in amphibians, not to mention the agents that despite being eye developmental disruptors have not yet been described as such.

Nevertheless, the NULL-model was indisputably the best model. Two likely causes could explain it, either our methodological design was not adequate for the identification of the causative agent of the malformations or the frequencies of eye malformations identified were effect of natural mutation rates. Supporting the natural mutation rates explanation, a thorough literature review uniquely revealed two papers that clearly offer the frequencies of eye malformations in larvae populations (Johnson et al. 2001 and 2002). In these papers, the frequencies reported for eye malformations ranged from 0% to 0.74%, even though they were part of mass malformations events (total malformations ranged from 10.72% to 37.86%, mostly affecting limbs) linked with trematode (*Ribeiroia*) infections (the involvement of pesticides and metals as causative agents were discarded). As *Ribeiroia* infections have been discarded as the cause of eye malformations (Lanoo, 2008), it is reasonable to believe that the frequencies of eye malformations found by Johnson et al. (2001 and 2002) and those found in our study area, might be due to natural mutation rates (see similar frequencies in Levey et al. 2003 and Williams et al. 2008).

Our findings suggest that there is no evidence that sugarcane cultivation practices are responsible for the eye malformations described. Indeed, we argue that malformations may be the result of natural mutations, and therefore, these frequencies could be used as eye malformation baseline levels for populations of *S. fuscovarius* larvae. It should be noted that, unlike most other malformation frequency baselines for amphibians, our

frequency baseline is based on larvae populations, which unlike with those based on adults, offers natal pond information, causes less impacts on population viability, and allows accurate estimates of the frequencies of malformations, since malformed adults only represent a minor fraction of the malformed larvae (Lunde et al. 2012; Lunde and Johnson, 2012). Therefore, this malformation baseline, which is the first reported for amphibians in South America, will be useful in future studies on amphibian populations that inhabit water bodies in tropical agroecosystems.

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FIGURES

Figure 1. Temporary ponds from agroecosystems with different amounts of sugarcane assessed during the 2015/2016 rainy season in São José do Rio Preto, São Paulo State (Brazil). Black dots for ponds where malformed tadpoles were found, grey dots for ponds where malformed tadpoles were not found. E_(A,B,C,D) for east ponds, SO_(A,B,C,D,E) for southwest ponds, NO_(A,B,C,D,E) for northwest ponds and N_(A,B,C,D,E) for north ponds.

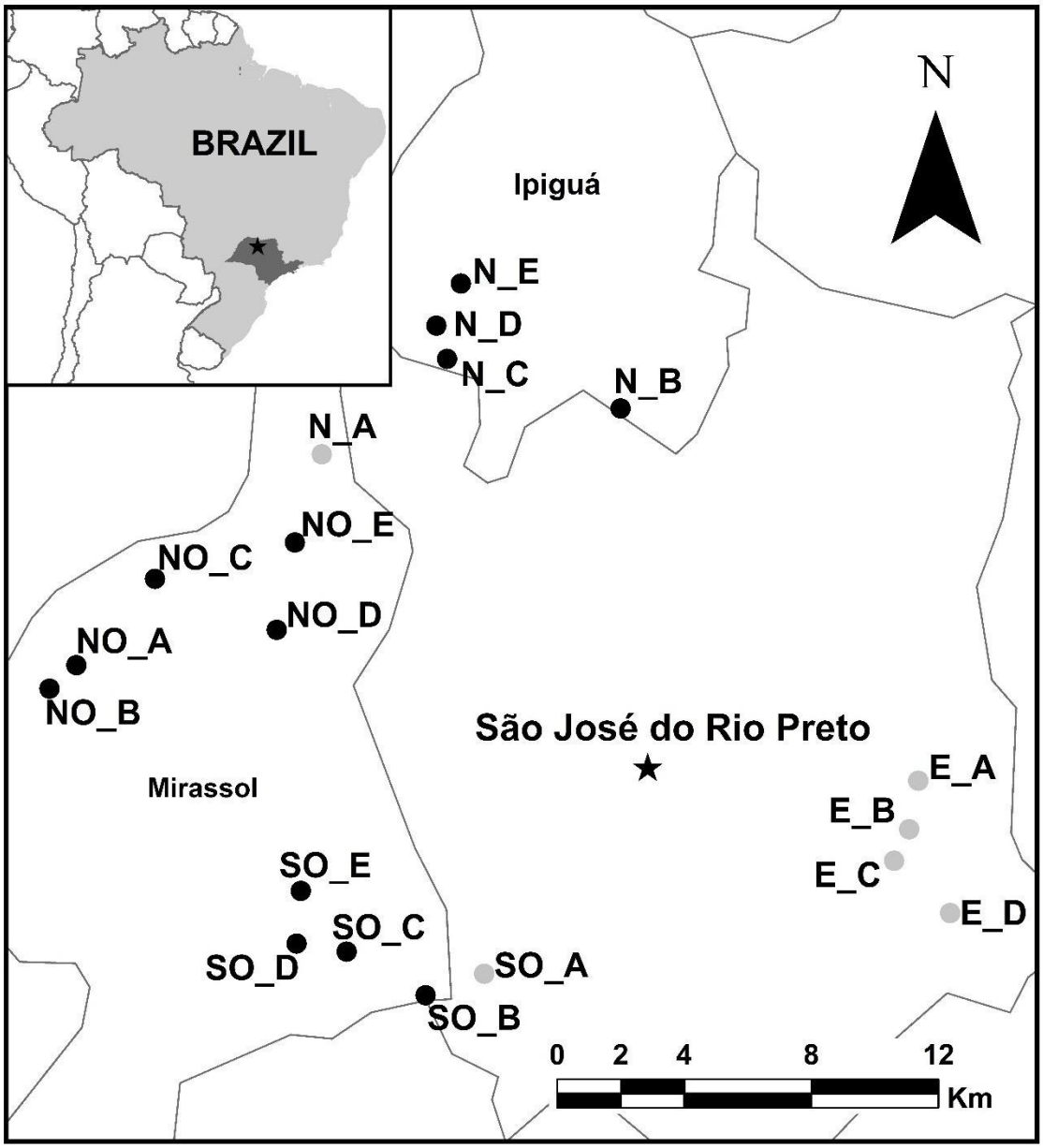
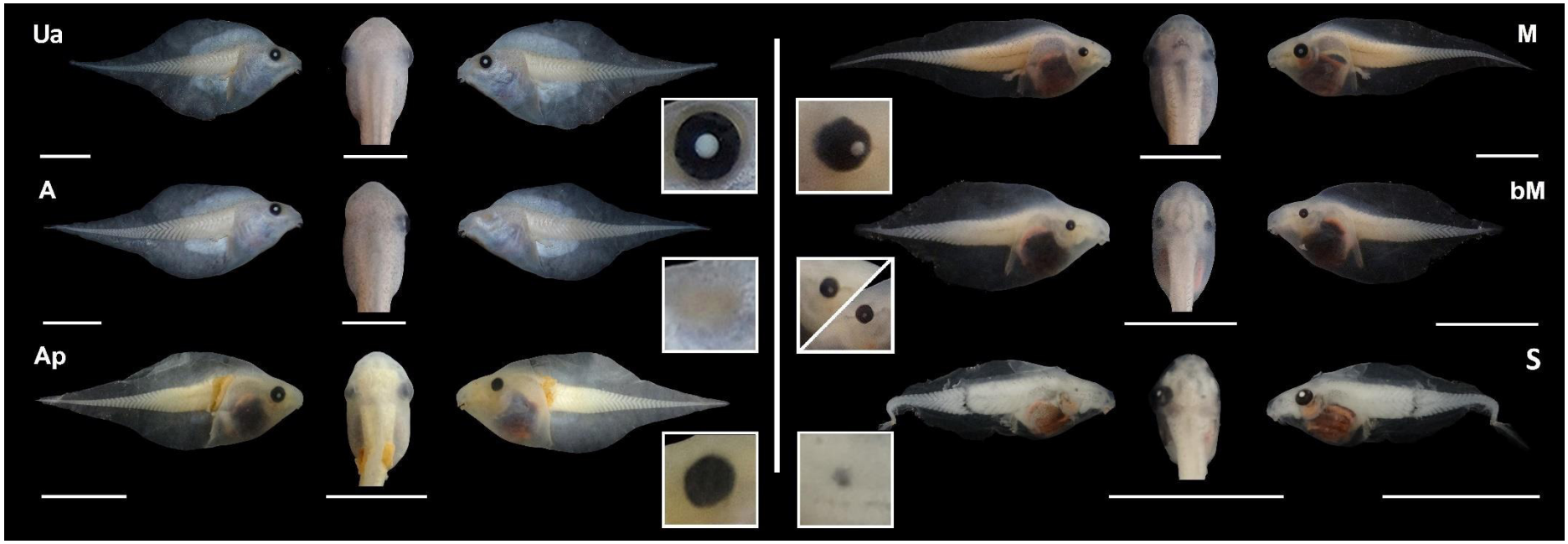


Figure 2. Eye malformations detected in *Scinax fuscovarius* larvae collected during the 2015/2016 rainy season in temporary ponds from agroecosystems with different amounts of sugarcane in São José do Rio Preto, São Paulo (Brazil). Ua = unaffected, A = anophthalmia (absence of eye), Ap = aphakia (absence eye lens), M = microphthalmia (eye abnormally small), bM = bilateral microphtalmia and S = sub-development (small amorphous and not emerged eye). Scale bars = 1 cm.



TABLES

Table 1. Coordinates (datum = WGS84), local and spatial variables of 19 temporary ponds from agroecosystems with different amounts of sugarcane studied during the 2015 / 2016 rainy season in São José do Rio Preto, São Paulo (Brazil). Depth = maximum water depth in pond. Distance = shorter distance between the pond and the nearest sugarcane field. Sugarcane and potential presence of teratogens = proportion of these land uses within the circular buffer area of a 1km radius surrounding each pond. Concentration of pesticides = total concentration of pesticides in ponds.

Ponds	Longitude	Latitude	Depth (cm)	Distance (m)	Sugarcane	Potential presence of Teratogens	Concentration of Pesticides (ng L^{1})
E-A	-49.300177	-20.815041	>100	1	0.33	0.39	863.00
E-B	-49.302334	-20.829412	>100	1	0.41	0.48	2241.00
E-C	-49.305974	-20.837824	>100	162	0.23	0.30	29.50
E-D	-49.290806	-20.852632	86	1	0.19	0.52	69.50
NO-A	-49.537884	-20.782967	>100	243	0.26	0.57	0.00
NO-B	-49.545502	-20.789611	>100	17	0.35	0.53	0.00
NO-C	-49.515660	-20.758477	72	50	0.69	0.69	0.00
NO-D	-49.481147	-20.772899	65	402	0.28	0.29	0.00
NO-E	-49.476052	-20.748140	72	1	0.64	0.74	6.27
N-A	-49.468836	-20.723020	51	430	0.18	0.30	3.86
N-B	-49.383798	-20.710139	79	899	0.02	0.41	0.00
N-C	-49.432938	-20.696070	68	193	0.13	0.52	0.00
N-D	-49.435978	-20.686656	51	35	0.23	0.61	27.87
N-E	-49.429059	-20.674697	73	1	0.48	0.73	0.00
SO-A	-49.422243	-20.870024	77	659	0.04	0.30	70.00
SO-B	-49.439017	-20.876562	>100	25	0.35	0.47	22.20
SO-C	-49.461409	-20.864171	31	1	0.61	0.64	No data
SO-D	-49.475542	-20.861890	64	846	0.01	0.06	0.00
SO-E	-49.474356	-20.846970	75	130	0.28	0.40	0.00

Table 2. Presence/absence of pesticides in water samples from 18 temporary ponds from agroecosystems with different amounts of sugarcane collected during the 2015/2016 rainy season in São José do Rio Preto, São Paulo (Brazil). D = detected; – = not detected.

Pesticide	Pond																	
	E-A	E-B	E-C	E-D	NO-A	NO-B	NO-C	NO-D	NO-E	N-A	N-B	N-C	N-D	N-E	SO-A	SO-B	SO-D	SO-E
2-Hydroxy-Atrazine	–	–	–	–	–	–	–	–	D	–	–	–	–	–	–	–	–	–
Ametryne	D	D	D	D	–	–	–	–	–	–	–	–	D	–	D	D	D	D
Atrazine	–	–	–	–	–	–	–	–	–	–	–	–	D	–	–	–	–	–
Carbendazime	–	D	–	–	–	–	–	–	–	–	–	–	–	–	D	–	–	–
Diuron	D	D	D	D	–	–	–	–	–	–	–	–	D	–	D	D	D	D
Hexazinone	D	D	D	D	–	–	–	–	–	–	–	–	D	–	D	D	–	–
Imidacloprid	–	–	–	–	–	–	–	–	D	D	–	–	D	–	–	–	–	–
Malathion	D	D	D	D	–	–	–	–	–	–	–	–	–	–	D	D	D	D
Tebuconazole	D	D	D	D	–	–	–	–	–	–	–	–	–	–	D	D	D	–
Tebuthiuron	D	D	D	D	–	–	–	–	–	–	–	–	D	–	D	D	D	D
Azoxystrobin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Carbofuran	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Clomazone	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Simazine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 3. Occurrences /frequencies (%) of eye malformations detected in larvae populations of *Scinax fuscovarius* inhabiting temporary ponds from agroecosystems with different amounts of sugarcane. N = number of tadpoles sampled in the pond. Anophthalmia (absence of eye), Aphakia (absence eye lens), Microphthalmia (eye abnormally small), Sub-development (small amorphous and not emerged eye). The number of bilateral malformation occurrences is in parentheses.

Ponds	Eye malformation				Total eye malformations	N
	Anophthalmia	Microphthalmia	Aphakia	Sub-development		
E-A	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	25
E-B	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	1202
E-C	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	1695
E-D	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	175
NO-A	0 / 0.00%	1 / 0.04%	0 / 0.00%	1 / 0.04%	2 / 0.09%	2242
NO-B	1 / 0.06%	0 / 0.00%	0 / 0.00%	0 / 0.00%	1 / 0.06%	1746
NO-C	0 / 0.00%	0 / 0.00%	0 / 0.00%	1 / 0.02%	1 / 0.02%	5948
NO-D	0 / 0.00%	4 (3) / 0.20%	1 / 0.05%	0 / 0.00%	5 / 0.26%	1956
NO-E	1 / 0.41%	0 / 0.00%	0 / 0.00%	0 / 0.00%	1 / 0.41%	244
N-A	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	2244
N-B	1 / 0.05%	1 / 0.05%	0 / 0.00%	1 / 0.05%	3 / 0.15%	2011
N-C	0 / 0.00%	1 / 0.03%	1 / 0.03%	1 / 0.03%	3 / 0.10%	3064
N-D	3 / 0.39%	0 / 0.00%	0 / 0.00%	0 / 0.00%	3 / 0.39%	776
N-E	4 / 0.24%	0 / 0.00%	0 / 0.00%	0 / 0.00%	4 / 0.24%	1649
SO-A	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	803
SO-B	2 / 0.07%	0 / 0.00%	1 / 0.03%	0 / 0.00%	3 / 0.10%	3000
SO-C	0 / 0.00%	0 / 0.00%	0 / 0.00%	2 / 0.18%	2 / 0.18%	1091
SO-D	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	0 / 0.00%	255
SO-E	1 / 0.07%	0 / 0.00%	0 / 0.00%	0 / 0.00%	1 / 0.07%	1525
REGION	13 / 0.04%	7 / 0.02%	3 / 0.01%	6 / 0.02%	29 / 0.09%	31,651

Table 4. Fisher's exact tests (two-tailed) for associations between ponds with water contaminated with pesticides and occurrence of eye malformations in larvae of *Scinax fuscovarius*. N= number of ponds; M = number of ponds where we recorded at least one individual with eye malformation; C = number of contaminated ponds; U = number of uncontaminated ponds.

Pesticide	Fisher's exact test			
	N	M / C	M / U	*P*-value
2-Hydroxy-Atrazine	18	2 / 2	9 / 16	0.497
Imidacloprid	18	2 / 3	9 / 15	0.999
Carbendazime	18	0 / 2	11 / 16	0.137
Hexazinone	18	2 / 7	9 / 11	< 0.05
Tebuthiuron	18	3 / 9	8 / 9	< 0.05
Ametrine	18	3 / 9	8 / 9	< 0.05
Atrazine	18	1 / 1	7 / 17	0.444
Diuron	18	3 / 9	8 / 9	< 0.05
Malation	18	2 / 8	9 / 10	< 0.05
Tebuconazole	18	1 / 7	10 / 11	< 0.01
Contamination[†]	18	4 / 11	7 / 7	< 0.05

[†]Contamination reflects presence of at least one of the analyzed pesticides in the pond.

Supplementary material

Table S1. Teratogens known to cause eye malformations. To elaborate this table was performed a search covering all publications until December 2016, using the following search terms: malformation OR abnormalities AND tadpole OR amphibian AND eye OR ocular, were applied in the scientific search engines ScienceDirect, Google Schoolars and Scopus. Bibliographies of selected articles were also used to search for additional studies. Studies included in the review table met, (1) when several studies pointed to a same teratogen, just the one/s which include malformations descriptions were included, and (2) when a teratogen was pointed by an unique study it was including even without malformations description. A (Anophtalmia), Ap (Aphakia), As (Asymetries in eye formation), Ca (Cataracts), Co (chorodial or iris Coloboma), C.d (Corneal dystrophy), Cy (Cyclopia), Cys (corneal Cyst), E (Edema), H (Hypopigmentation,including depigmentation), He (Hernia), HY (Hyperpigmentation), M (Microphtalmia), M.l (Misplaced lens), M.g (Misshapen globe) and N-d (Non-described).

Teratogens	Eye malformations	Species / developmental phase	Reference
CHEMICALS			
Atrazine, Chlorpyrifos and Monosodium Methanearsonate (Asses in mixture)	A	Hyla chrysoscelis / tadpoles	Britson and Threlkeld, 1999
Polychlorinated biphenyls	Co, H, M and M.g	Xenopus laevis / tadpoles	Gutleb et al. 1999
Sodium fluoride	M	Xenopus laevis / embryos	Goh and Neff, 2003
Trinitrotoluene	M and M.g	Xenopus laevis / embryos	Saka, 2004
Dichlorodiphenyltrichloroethane	M and M.g	Xenopus laevis / embryos	Saka, 2004
4-amino-2,6-dinitrotoluene	M and M.g	Xenopus laevis / embryos	Saka, 2004
2-amino-4,6-dinitrotoluene	M and M.g	Xenopus laevis / embryos	Saka, 2004
Bisphenol A	Ap, M and H	Xenopus laevis / embryos	Kazunobu et al. 2009 San-Segundo et al. 2013
Cypermethrin	N.d	Hypsiboas pulchellus / tadpoles	Agostini et al. 2010

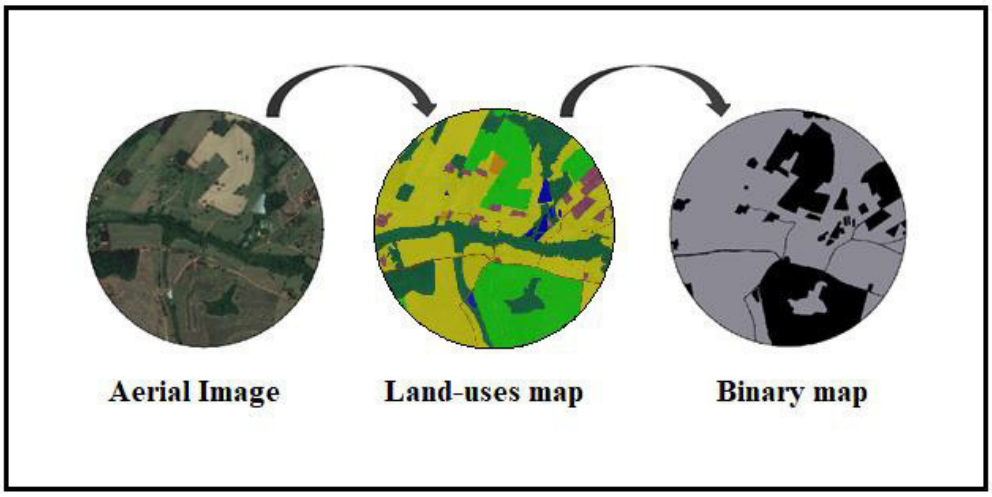
Glyphosate	A and M	Discoglossus pictus / embryos Xenopus laevis / embryos	Paganelli et al. 2010 Wagner et al. 2017 (on-line in 2016)
Tributyltin	Ap, Ca, E, H and M	Xenopus laevis / embryos Xenopus tropicalis / tadpoles	Liu et al. 2012 Hu et al. 2015
Methylparaben	N.d	Xenopus laevis / embryos	San-Segundo et al. 2013
Chlorpyrifos	M	Xenopus laevis / embryos	San-Segundo et al. 2013
Antagonist of retinoid X receptor (UVI3003)	H and M.g	Xenopus tropicalis / embryos	Zhu et al. 2014a
Alitretinoin	A, H and M	Xenopus tropicalis / embryos	Zhu et al. 2014b
Triphenyltin	Ca, E and M	Xenopus laevis / embryos	Hu et al. 2015
Pyraclostrobin	Ca, E and M	Xenopus laevis / embryos	Hu et al. 2015
Perfluorohexanoic	N.d	Xenopus laevis / embryos	Kim et al. 2015
Perfluoroheptanoic	N.d	Xenopus laevis / embryos	Kim et al. 2015
Tributyl phosphate	N.d	Xenopus tropicalis / embryos	Zhang et al. 2016
Tricresyl phosphate	N.d	Xenopus tropicalis / embryos	Zhang et al. 2016
Tris (2-chloroisopropyl) phosphate	N.d	Xenopus tropicalis / embryos	Zhang et al. 2016
Tris (1,3-dichloro-2-propyl) phosphate	N.d	Xenopus tropicalis / embryos	Zhang et al. 2016
Pyraclostrobin	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Picoxystrobin	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Azoxystrobin	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Fludioxonil	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Folpet	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a

Bixafen	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Tebuconazole	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Myclobutanil	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Isopyrazam	Ca and M	Xenopus tropicalis / embryos	Li et al. 2016a
Dimethyl phthalate	As, B.s, Cy and M	Silurana tropicalis / tadpoles	Mathieu-Denoncourt et al. 2016
Monomethyl phthalate	As, B.s, Cy and M	Silurana tropicalis / tadpoles	Mathieu-Denoncourt et al. 2016
Dicyclohexyl phthalate	As, B.s, Cy and M	Silurana tropicalis / tadpoles	Mathieu-Denoncourt et al. 2016
Butachlor	N.d	Xenopus laevis / embryos	Li et al. 2016b
METALS			
Cadmium	Co, H, M, M.g and M.l	Xenopus laevis /embryos	Sunderman et al. 1991
Cobalt	Co, H, M, M.g and M.l	Xenopus laevis / tadpoles	Plowman et al. 1991
Nickel	Ca, Co, Cys, H, He and M	Xenopus laevis / embryos	Hopfer et al. 1991 Hauptman et al. 1993
Chromium	N.d	Triturus vulgaris meridionalis / embryos	Calevro et al. 1998
Lead	Cys and He	Pelophylax nigromaculata / tadpoles	Huang et al. 2014
Selenium	B.s, Co, E, M and M.l	Xenopus laevis / embryos	Massé et al. 2015
RADIATIONS			
UV radiation (UV-A, UV-B and visible)	An, C.d, Hy and M	Bufo boreas boreas / tadpoles Rana pipens / tadpoles	Worrest and Kimeldorf, 1976 Ankely et al. 2002
GSM-like radiofrequency	C, Co and M	Xenopus laevis / embryos	Boga et al. 2016

Table S2. Dredge of the Linear Mixed Effects (LME) models predicting the relationship between frequencies of eye malformations in *Scinax fuscovarius* tadpoles and different local, contamination and landscape descriptors. Int = Intercept; DNS = shortest distance between ponds and nearest sugarcane crop; MD = maximum water depth of ponds; SAT = proportion of areas with potential presence of teratogens within circular buffers with a radius of 1 km centered on each pond; SS = calculated based on the proportion of sugarcane crops within circular buffers with a radius of 1 km centered on each pond; TCP = total concentration of pesticides in ponds; df = degrees of freedom; logLik = Log-Likelihood; AICc = corrected Akaike's Information Criteira; ΔAICc = delta AICc; wAICc = weights of AICc; NULL = model without a predictor variable (considering only intercept). Models ranked by AICc.

Model	Int	DNS	MD	SAT	SS	TCP	df	logLik	AICc	ΔAICc	wAICc
1	0.1039						3	8.612	-9.5	0	0.742
5	0.1039			0.06726			4	8.445	-5.8	3.7	0.117
3	0.1039		-0.05567				4	7.636	-4.2	5.31	0.052
9	0.1039				0.03994		4	6.864	-2.7	6.86	0.024
17	0.1039					-0.03532	4	6.695	-2.3	7.2	0.02
7	0.1039		-0.06221	0.07285			5	8.371	-1.7	7.77	0.015
2	0.1039	-0.02469					4	6.395	-1.7	7.8	0.015
21	0.1039			0.06708		-0.03496	5	6.554	1.9	11.4	0.002
11	0.1039		-0.06523		0.05195		5	6.546	1.9	11.42	0.002
4	0.1039	-0.05008	-0.07307				5	6.351	2.3	11.81	0.002
13	0.1039			0.08271	-0.02097		5	6.341	2.3	11.83	0.002
6	0.1039	0.02424		0.08177			5	6.273	2.5	11.96	0.002
18	0.1039	-0.05293				-0.06016	5	5.374	4.3	13.76	0.001
25	0.1039				0.04782	-0.0439	5	5.281	4.4	13.95	0.001
19	0.1039		-0.04944			-0.01563	5	5.27	4.5	13.97	0.001
10	0.1039	0.001747			0.04106		5	4.624	5.8	15.26	0
15	0.1039		-0.0616	0.07655	-0.0051		6	6.043	7.6	17.06	0
8	0.1039	-0.00489	-0.06366	0.07005			6	5.932	7.8	17.28	0
23	0.1039		-0.05735	0.07235		-0.0121	6	5.806	8	17.53	0
20	0.1039	-0.0657	-0.06199			-0.04148	6	4.633	10.4	19.88	0
12	0.1039	-0.0262	-0.07144		0.03623		6	4.454	10.7	20.24	0
29	0.1039			0.07261	-0.0075	-0.03358	6	4.375	10.9	20.4	0
22	0.1039	-0.00229		0.0657		-0.03604	6	4.369	10.9	20.41	0
27	0.1039		-0.05672		0.05442	-0.02251	6	4.264	11.1	20.62	0
14	0.1039	0.02048		0.08827	-0.01188		6	4.181	11.3	20.78	0
26	0.1039	-0.0318			0.02944	-0.05553	6	3.364	12.9	22.42	0
16	0.1039	-0.00727	-0.06343	0.07439	-0.00785		7	3.709	17.8	27.29	0
24	0.1039	-0.01729	-0.05954	0.06216		-0.0194	7	3.628	17.9	27.45	0
31	0.1039		-0.05727	0.07342	-0.00146	-0.01186	7	3.525	18.1	27.66	0
28	0.1039	-0.04477	-0.06189		0.02914	-0.03693	7	2.578	20	29.55	0
30	0.1039	-0.00474		0.07072	-0.00881	-0.03558	7	2.268	20.7	30.17	0
32	0.1039	-0.01905	-0.05941	0.06585	-0.00646	-0.0191	8	1.427	29.1	38.66	0

Figure S1. Schematic overview of the maps' construction of the circular buffer areas of 1 km radius that surround each of the ponds. Land-use maps with multiple categories and, derived from them, Binary maps. The binary maps reclassified the categories reflected in the land-use maps into two categories: Land-uses with potential presence of teratogens (in black), which includes those associated with human activities that use or emit potential teratogens into the environment such as agriculture (sugarcane (light green) and other cultures (orange)), forestry (blue-green), waterbodies (blue), urban areas (purple) and linear structures (brown); and Land-uses virtually free of teratogens (in grey), which includes riparian forests (dark green) and pastures (yellow). The creation of the maps was done using Google Earth Pro v7.1.8.3036.



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Chapter III

First vs. Best response analysis in escape swimming performance tests for ecotoxicology in tadpoles (Boana lundii)

First *vs.* Best response analysis in escape swimming performance tests for ecotoxicology in tadpoles (*Boana lundii)*

David Sánchez-Domene[1*], Alba Navarro Lozano[2], Alexandre Vidotto Barboza Lima[3], Eduardo Alves de Almeida[4].

[1] Instituto de Pesquisa em Bioenergia, Universidade Estadual Paulista Júlio de Mesquita Filho, Rua 10, 2527, Bairro: Santana, 13500-230, Rio Claro (São Paulo), 13500-230, Brazil.
[2] Departamento de Zoologia e Botânica, Universidade Estadual Paulista Júlio de Mesquita Filho, Rua Cristovão Colombo, 2265, 15054-000, São José do Rio Preto (São Paulo), Brazil.
[3]Departamento de Biologia, Universidade Estadual Paulista Júlio de Mesquita Filho, Rua Cristovão Colombo, 2265, 15054-000, São José do Rio Preto (São Paulo), 15054-000, Brazil.
[4]Departamento de Ciências Naturais, Fundação Universidade Regional de Blumenau, Av. Antonio da Veiga 140, Itoupava Seca, 89030-903, Blumenau (Santa Catarina), Brazil.

ABSTRACT

Within behavioral aquatic toxicology, escape swimming performance test is employed to assess how the exposure to a stressor affects the escape response of aquatic organisms. Although it is a well-established test, researches using it are applying, mainly, two different approaches for the selection of the escape response to be analyzed, analyzing the first (response to the first predation simulated attempt to which the animal is subjected) or the best response (response with better performance among several attempts). Despite the use of these different approaches, conclusions derived from them are usually coincident, focusing on the ecological implications that the impairment of the escape swimming performance could have on the survival of the evaluated species. The detection of these divergent approaches (first *vs.* best), encourage us to develop an experiment to find out if both approaches offer similar or different results. For this, tadpoles of *Boana lundii* where subjected to a 48h toxicity test in a randomized block design with two environmentally relevant concentrations of Chlorpyrifos (0.6 μg L^{-1} and 1 μg L^{-1}) and two temperatures (28°C and 34°C), after which first and best escape swimming performance, and acetylcholinesterase activity where analyzed. While no reduction in acetylcholinesterase activity was detected, usual biomarker of organophosphorus insecticides as Chlorpyrifos, escape's distance and burst speed were reduced by the higher Chlorpyrifos concentration used. This reduction was shown only by the best response analysis, whereas first response analysis did not detect any affection over escape components. The decrease in escape performance was attributed to bioenergetic impairment caused by non-cholinergic effects of Chlorpyrifos. We manage to prove that the two main analytical approaches (first and best) used in escape swimming performance test can offer different results. In addition, our results suggest that the use of acetylcholinesterase as biomarker in tadpoles may be underestimating the effects of environmentally relevant concentrations of Chlorpyrifos, since escape's performance was affected by non-cholinergic effects.

Keywords: escape swimming performance, amphibians, organophosphates; non-cholinergic effects; acetylcholinesterase.

INTRODUCTION

Intensive agriculture cause the entry of large amounts of chemicals into surface freshwater systems, causing negative effects on non-target organisms that inhabit them (Pimentel, 1995; Relyea and Hoverman, 2006; Nogueira *et al.* 2012; Giesy *et al.* 2014). Indeed, pesticides contamination has been pointed as one of the most relevant threats for freshwater organisms (MEA, 2005).

Behavioral studies for aquatic toxicology allow the quantification of the effects caused by the exposure to different stressor agents such as pesticides, drugs or metals. Although these studies have been used successfully for decades, the emergence of new low-cost technological tools have increased their use, making scientific inquiry more accessible (Melvin and Wilson, 2013). These experiments currently stand out for their simplicity and quick execution, which make of them an extremely useful tool for ecotoxicology assays (Gerhardt, 2007; Melvin and Wilson, 2013). In addition, these experiments offer whole organism responses which can be extrapolated to ecological scenarios for assessing the effects of environmental contamination on individuals, populations and/or communities (Gerhardt, 2007; Melvin and Wilson, 2013).

Among behavioral studies for aquatic toxicology, escape swimming performance test is commonly used, since it allows to assess how the exposure to stressor agents affects individuals ability to avoid predation, and consequently, to survive (Bridges, 1997; Walker et al. 2005). This test consist, in essence, in the simulation of predation attempts on experimental specimens, usually with a tactile or an electrical stimulus, which evoke escape responses that are analyzed to assess whether the exposure to a stressor affect any of the most important components for the success of the escape response such as average speed, burst speed, distance and time (Van Buskirk and McCollum, 2000; Eidietis, 2005).

Although escape swimming performance test is widely used, it is easy to detect in the literature that researchers who are using it are adopting, mainly, two approaches for the selection of the escape response to be analyzed, with some analyzing the first escape response (response to the first predation attempt) and some others the best escape response (response with better performance among several attempts). However, notwithstanding the use of these different approaches (hereafter first and best), conclusions derived from these studies are usually focused on the ecological implications that the impairment of the escape swimming performance could have on the survival of the evaluated species.

Thus, the detection of the use of these two different approaches encouraged us to assess if they offer similar or different results for a same experiment. To that end, we assessed the escape swimming performance of tadpoles of the species *Boana lundii,* using both first and best approaches. Before the escape swimming performance test, tadpoles were exposed during 48h to two environmentally relevant concentrations of Chlorpyrifos (CPF), one of the most used organophosphate (OP) insecticides worldwide (Eaton et al. 2008). Numerous studies described the impairment of swimming performance as a result of the exposure to OPs (*e.g.* Widder and Bidwell, 2008; Pereira et al. 2012). This impairment has been associated with the irreversible inhibition that OPs cause in acetylcholinesterase (AChE), which results in a synaptic hyper-stimulation due to the acetylcholine (ACh) accumulation in the post-synaptic regions of nervous tissue, leading to physiologic and behavioral disorders (Szabó *et al.* 1992; Peakall, 1996; Behra *et al.* 2002; Eaton *et al.* 2008; Pereira *et al.* 2012; Tilton et al. 2012). In addition to the CPF exposure, tadpoles were submitted to two different temperature treatments to assess synergic effects of water temperature with CPF. Temperature can interfere in the amphibian's responses to some environmental contaminants, being this effect of

temperature on pesticides action on organisms one of the current challenges in environmental toxicology (Freitas *et al.*, 2016, 2017ab; Hooper *et al.*, 2013).

In this paper we study the effect of temperature and CPF on tadpoles swimming escape response and explore if first and best analytical approaches produce different results for a same experiment.

MATERIALS AND METHODS

Test organism

Tadpoles assessed were obtained from a spawn of the species *B.lundii* collected in a floodplain forest pond from Caetetus ecological station (São Paulo, Brazil). The spawn was maintained in controlled conditions in aerated aquariums with dechlorinated water (pH 7.5-8.0; 28 ± 1°C) until tadpoles hatched. Then, tadpoles were fed *ad libitum* with commercial tropical fish food until reached Gosner stages 25-27 (Gosner, 1960). The pond were the spawn was collected was selected to avoid historical contamination since tolerance to pesticides has been described for amphibians inhabiting ponds with recurrent pollution (Cothran *et al.* 2013), what might underestimate toxicant effects in low doses.

Experimental design

Tadpoles were exposed for 48 h to two nominal CPF concentrations (0.6 µg L^{-1} and 1.0 µg L^{-1}) at two different temperatures (28°C and 34°C) resulting in six treatments (C-28, C-34, 0.6-28, 0.6-34, 1.0-28 and 1.0-34; C for control) of four replicas with three tadpoles each. Exposure were performed as static systems (without water renewal from the acclimation phase) in aerated three liters glass aquariums capacity, filled with one liter of dechlorinated water (pH 7.5-8.0), and under natural photoperiod. The CPF stock

solution used for the treatments was prepared in acetone, not exceeding in any case 16.7 μL L^{-1} of acetone per aquarium. These volumes of acetone are considered atoxic for amphibians, according with Bridges (1997), Richards and Kendall (2003) and Robles-Mendoza et al. (2009), so solvent control was not considered. Experimental temperatures were gradually reached during 24 h (acclimation phase) in controlled water baths to avoid thermal shock. From that moment, tadpoles were under experimental conditions and not fed until the final of the experiment to avoid prandial effects, which ensures the equitability in the physiological state of animals. Experimental conditions (temperature, exposure time and CPF concentrations) were selected to perform an environmentally relevant experiment. Thus, temperatures assessed were based on those found in northwest Brazil open area ponds during summer, when reproduction of most part of neotropical amphibians occurs (Freitas et al. 2016); exposure time on the fact that most exposures to peak concentrations of CPF in agroecosystems are less than 48h, followed by periods of fewer or no exposure (Robles-Mendoza *et al*. 2011; Williams *et al.* 2014), and concentrations of CPF on reports of CPF concentrations found in Brazilian agricultural water bodies (Albuquerque *et al.* 2016).

Escape swimming performance

To perform the escape swimming performance test, tadpoles were placed individually in a narrow glass swimming-channel (50 × 2 × 5 cm) with 2 cm depth of water from their respective aquariums. Channel dimensions and water depth were adapted to tadpoles' sizes, covering them completely but limiting their responses to a two-dimensional movement (Egea-Serrano and Tejedo, 2014). After one minute of acclimation, a predation attempt was simulated touching tadpole's tail with a glass rod (see thesis annex D). A total of three responses, with one minute of recovery between them, were evoked. If a

stimuli did not evoke a response (escape) the individual was classified as non-reactive. Responses were assessed using the analytical approaches first and best (response to the first predation attempt, and the longest distance response among the three attempts).

Escape responses were videotaped (SONY® HDR-XR160; 25 frames per second) and analyzed using the visual tracking software Kinovea (Kinovea© v.0.8.24); being the snout of the tadpoles selected as tracking point. Three swimming escape components were measured: Time (T; seconds elapsed since the first tail movement to its total stop), Burst speed (BS; escape response rate between the first tail movement frame and the subsequent frame, in mm/sec), and Distance (D; linear length of the escape response, in mm). After the third predation attempt, tadpoles were gently euthanized by submersion in Benzocaine solution (0.95 mg L^{-1}). Then, despite the minor differences among tadpoles' sizes each tadpole was photographed laterally to measure tail length (TL) and maximum tail muscle depth (TMD) using ImageJ (Abràmoff *et al.* 2004). Once photographed, each tadpole was splitted into tail and body (Fig.1) and immediately frozen at -80°C for further analysis of cholinesterase activity.

AChE activity

Tail and body tissues were homogenized in a proportion 1:4 (w/v) of Tris-HCl buffer 0.1 mol L^{-1}, pH 8.0 and centrifuged for 30 min at 9168 g at 4°C. The supernatant was then collected and frozen at -80°C. AChE activity was measured according to the method described by Ellman *et al.* (1961), modified for microplate reader. To this analysis 1 µL of body or 10 µL of tail sample were added to the reaction medium containing potassium phosphate buffer 0.1 mol L^{-1}, pH 8.0; acetylthiocoline iodide 0.5 mmol L^{-1} and DTNB 1 mmol L^{-1}. The protein quantitation was performed according to Bradford (1976). The

specific AChE activity of each replica was characterized in duplicate using a pool of their three tadpoles and is given in U mg^{-1} of protein from the sample.

Data analyses

Prior to the analyses described below, we performed linear regressions between control best performance escape components (T, BS and D) and the tail's metrics (TL and TMD) to determine the need to apply some of this metrics as covariate. These regressions were restricted to control best performance responses since they reflect the full performance of tadpoles and highlight, if any, the covariate that most contribute to the escape (Van Buskirk and McCollum 2000). Two-way ANOVAs were used to assess the effects of CPF concentration and temperature, as well as their interaction, on the first and best escape performance and on the activities of AChE in tail and body tissues. In turn, the differences on reactivity to the predation attempts between the treatments and the analytical approaches (first and best) were assessed using two-sided Fisher's exact test. Normality and homoscedasticity of the variables' residuals were visually confirmed to meet parametric statistical test assumptions; distance and time were log-transformed to meet parametrical requirements. Significant interactions (p<0.05) were further analyzed with unequal N HSD post-hoc tests. All analyses were performed using R Statistical Software (v.3.4.0, The R Foundation for Statistical Computing, Vienna, Austria) and graphed in Excel® 2013 (Microsoft Inc.).

RESULTS

There were no deaths during the experiment; however, two of the 72 tadpoles were censured from the escape test (one in the L28 treatment due to our mishandling and other

in the C34 treatment due to damages in its tail). First response analysis was composed by 53 escapes and best response by 66). First response had 17 non-reactive tadpoles while 4 tadpoles were non-reactive in any of the simulated predator attempts. 16 responses were shared between first and best response analysis (cases where first response performance was also the best).

Covariate selection

Best escape components (T, BS and D) of the best response escape from tadpoles of at control treatment did not correlated with their tail metrics (Fig.2). Therefore, we do not use tail metrics as covariates in the analyses.

Escape swimming performance

First response swimming escape components were not affected by temperature, CPF concentration, nor its interaction (Tab.3). However, the analysis of best response showed that increasing CPF concentration decreased escape's distance and marginally burst speed, while time was not affected (Tab.3, Fig. 3A, 3B and 3C); post hoc analysis for distance swum was not significant. Temperature and the interaction of temperature and CPF concentration did not affect escape components of best response.

Reactivity to stimuli

Differences in the react to stimuli were not attributable to CPF concentrations neither for first, nor best nor within concentration treatments between first and best response (Fig. 3). Given the non-affection of CPF in the reactivity, differences between total first and best reacts (all individuals reacts without taking into account concentration treatments)

were analyzed, showing a significant increase (p = 0.0037) in the reactivity percentage rate for best response (94.29%, 66/70) compared to first response (75.71%, 53/70). The non-significant differences of the analyses within concentration treatments between first and best response, despite their similarity with total firs vs best percentage rates (73.91 *vs.* 91.30 for control, 78.26 *vs.* 95.65 for low concentration, and 75 *vs.* 95.83 for high), could be a result of the conservatism of Fisher's test for low sample size (Andrés and Tejedor 1995).

AChE activity

Tadpoles' tail and body AChE activity were not affected by the treatments (Fig. 3D).

DISCUSSION

Body AChE activity values found in *B.lundii* varied in the range between 1.5 and 1.9 U mg^{-1}, which is in accordance to activities reported in other tadpoles species, like *Hyla chrysoscelis*, *Lithobates sphenocephalus*, *Acris crepitans*, *Gastrophryne olivacea* and *Scinax fuscovarius* (Widder and Bidwell 2008; Leite *et al.* 2010). The non-affection of CPF concentrations assessed on AChE activity could be result of the protective role that carboxylesterase (CbE) has over AChE. This enzyme, with greater sensitivity to OP than AChE, acts as an alternative target, being consider an early factor for OP detoxification (Barata *et al.* 2004; Ferrari *et al.* 2011). Thus, concentrations of CPF used in the experiment could be insufficient to overcome CbE barrier during the first 48h of exposure.

Despite non-affection on AChE activity, the best response analysis with higher CPF concentration decreased escape's distance and burst speed. Environmentally relevant

concentrations of OP had been associated with increases in reactive oxygen species (ROS) generation in amphibian tadpoles (Ferrari *et al.* 2011; Liendro *et al.* 2015). The increase of ROS in the organism cause dysfunctions on mitochondrial energy metabolism and alterations in the antioxidant system and the calcium homeostasis (Kaur *et al.* 2007; Gupta and Milatovic, 2011; Karami-Mohajeri and Abdollahi 2013; Sotomayor *et al.* 2015). These non-cholinergic effects of OP are responsible for bioenergetics impairment in intoxicated organism, due to the extra energy consumption for biotransformation processes (detoxification), and to the diminished ATP production at mitochondrion (Kaur *et al.* 2007; Gupta and Milatovic 2011; Karami-Mohajeri and Abdollahi, 2013). Thus, we attribute the decrease in distance swum to non-cholinergic effects of OP, which could increment fatigue in tadpoles subjected to a high-energy consumption (as is the case of our best escape response, composed by several escape attempts with short-time recovery). This hypothesis is also supported by the lack of effects on first escape responses, where presumably, tadpoles from all treatments had enough energy to perform similar escapes, but as the number of escapes responses increase (we evoked three escapes per tadpole), the fatigue started to appear on tadpoles, producing minor escape's distances. The fact that escape's time did not differ between treatments, shows that responses of animals exposed to higher CPF concentration were less efficient than those observed in animals from the control group and the group exposed to lower concentration. Unfortunately, our experiment was designed just to analyze AChE activity and there was not enough tissue left for the analysis of oxidative stress markers, making our explanation just a hypothesis.

If we attribute differences in escape response to an energy deficit, and we hypothesize that in best response fatigue is accentuated, it is not clear why all escape components analyzed were better in best than in first response. Sensitization process could explain this contradiction, it is a non-associative phylogenetically preserved learning process in

which repeated administrations of potentially threatening stimuli results in a progressive amplification of the response to the same or weaker stimuli presented later. It allows to the organism a better response to a previously experienced threat, increasing survival probabilities (Antelman, 1988; Pijanowska *et al.* 2006; Crook *et al.* 2014). Thus, despite of best responses had better performance than first, attributable to sensitization, best responses showed fatigue symptoms while first response did not.

In brief, environmentally relevant concentration of CPF (1.0 μ L^{-1}) induced shorter escape responses and initial high-velocity escape reaction (burst speed) when best response approach was applied. According to our hypothesis, as the number of a predation attempts increase, escape responses diminish their performance due to the fatigue accentuation, making tadpoles more susceptible to predation. This, could be dangerous in high predation pressure scenarios, such as ponds associated to agriculture where many neotropical amphibians develop in presence of pesticides (Schiesari and Corrêa, 2016).

Paragraphs above evidence differences in the results, for a same toxicological test, depending on the analytical approach (first or best). While first response analysis ruled out negative effects of CPF concentration in tadpoles' escape performance, best response determined negative effects. Therefore, we firmly believe that a debate for the consensus of the analytical approach to be use in escape swimming performance tests for ecotoxicological studies should be open.

Why this lack of consensus? A reason that may cause the choice of best response analysis by ecotoxicologists is the fact that this approach assures a greater N for statistical analyses (or at least the same) than the analyses of first response. Our results showed 53 swimming escapes for first response and 66 for best response. Differences in reactivity in our research were not caused by CPF concentrations, but to response analyzed (first or best), what suggest that sensitization process may also be acting, increasing reactivity rate

to each new stimuli which tadpole is submitted (*e.g.* Teplitsky et al. 2005). Therefore, despite in our experiment reactivity to stimuli was not significantly affected by the toxicant, the single use of best response analysis approach might result in an over estimation of the reactivity rates when toxicants affects negatively the reactivity (*e.g.* García-Muñoz *et al.* 2009). This could lead to invalid conclusions, since in nature the reactivity, or not, to a predator strike is the difference between survival or death (Figiel Jr. and Semlitsch, 1991).

The table 1 shows that while researchers using escape swimming performance test for ontogeny, predation or tail shape studies, choose mainly best analysis approach (with 7 of the 11 papers collected in the table), ecotoxicological studies are more dissonant (with 3 papers using best response, 3 first response, 2 analyzing all the responses evoked and 1 using the average of the evoked responses). Van Buskirk and McCollum (2000) described best response as the one that reflect tadpoles' full capacity, therefore, in our opinion, best response approach should be restricted to studies where the knowledge of the maximum performance is the aim, as hydrodynamic or ontogeny studies. In addition, we believe that best response is an artifact response that does not reflect ecological conditions, fundamental purpose of ecotoxicological studies. Consequently, we suggest the abandonment of best response analysis for ecotoxicological studies.

CONCLUSSIONS

Despite our experimental design failed in the selection of the biomarker analyzed (AChE) to assess the effects of environmentally relevant concentrations of CPF on escape response of *B.lundii*, we propose a consistent explanation for the effects detected in the escape performance. The study demonstrates that the two main approaches (first and best)

used in the analyses of escape swimming performance tests can offer different results, leading to different conclusions when assessing the impact of toxicants over organisms. In addition, our results suggest that the use of AChE as biomarker in tadpoles may be underestimating the effects of environmentally relevant concentrations of CPF, since escape's performance may be affected by non-cholinergic effects.

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FIGURES

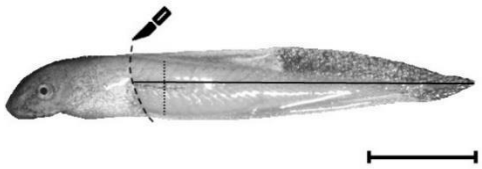

Figure 1. Lateral image of *B.lundii* tadpole. Dashed line indicates where the tadpole was splitted into two parts (body and tail), solid line and dotted line illustrating the locations of measurements of tail length (TL) and maximum tail muscle depth (TMD) respectively. Scale bar, 1 cm.

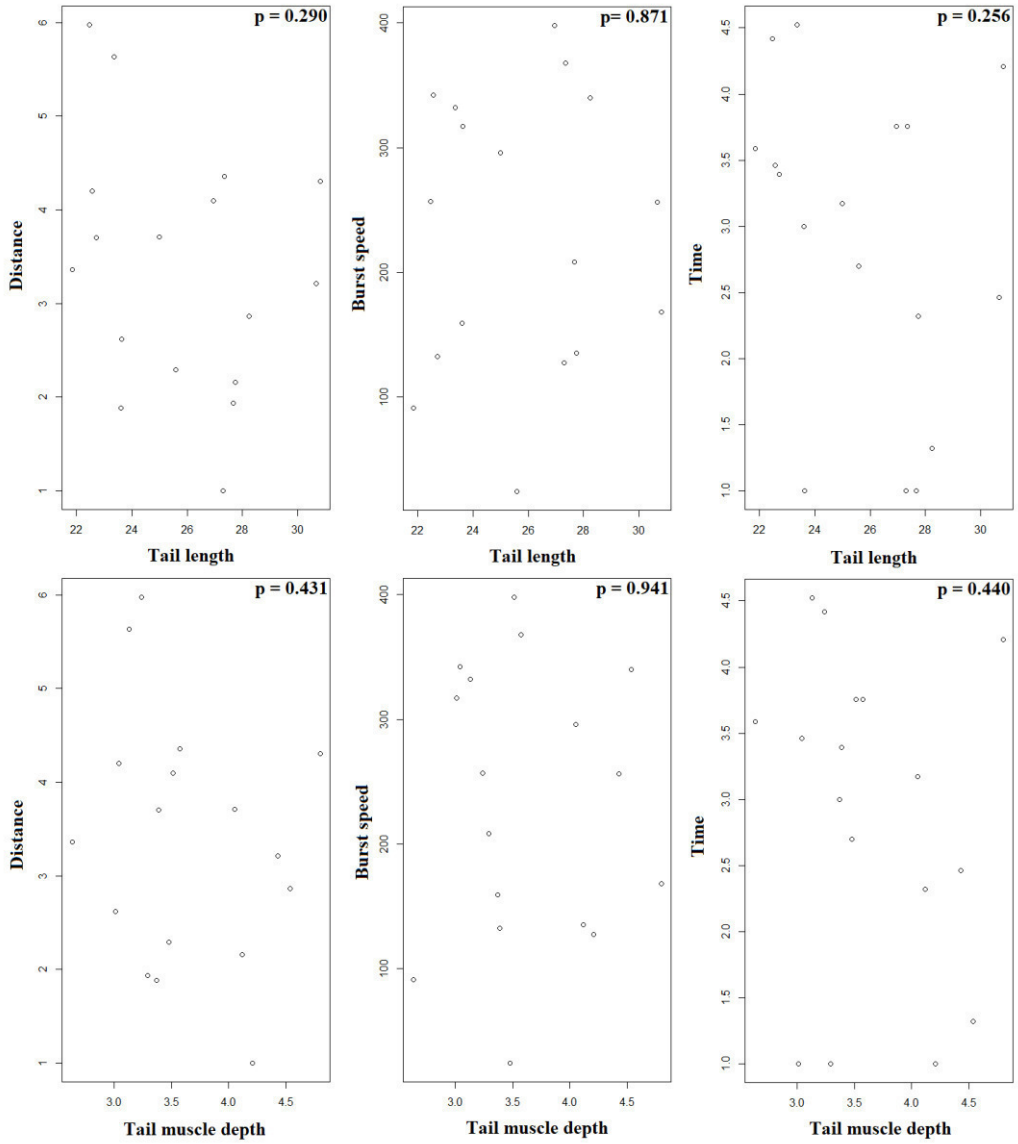

Figure 2. Correlations between best escape response components and

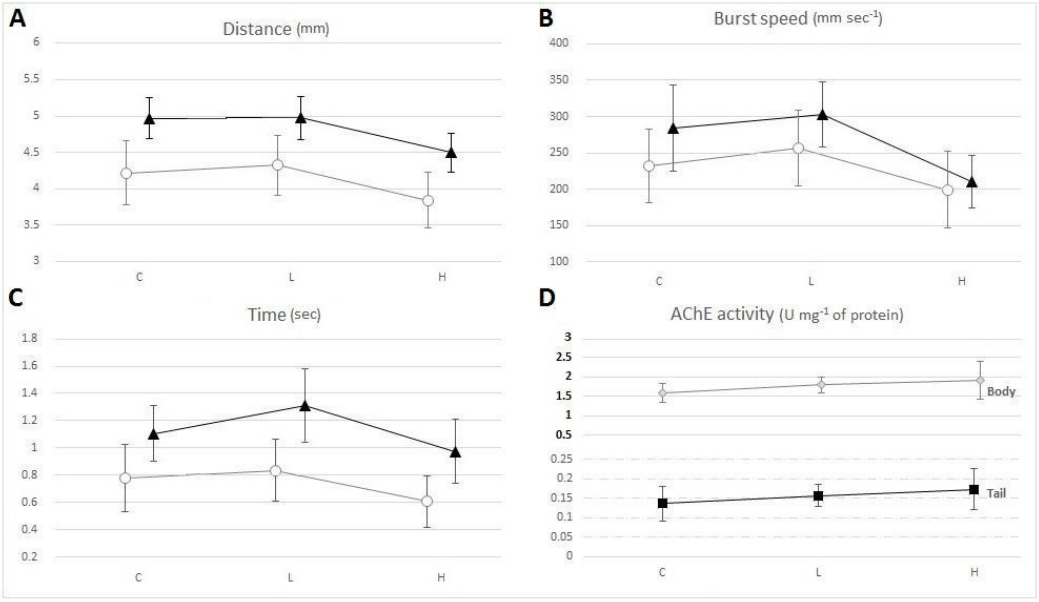

Figure 3. Effects of Chlorpyrifos concentration on escape swimming performance components: distance (A), burst speed (B) and time (C). First response values in white circles and best response values in black triangles. Values represents mean ± 95% confidence interval. D) Enzymatic activity of acetylcholinesterase in body (grey circles) and tail (black squares) of *B.lundii* tadpoles for different Chlorpyrifos concentrations. Each value representing mean value ± 95% confidence interval of four pools of three animals, each done in duplicate. Note the different scales in the horizontal axis. C, L and H for control, low concentration and high concentration, respectively. Graphics restricted to concentration treatments. Refer to text for statistical differences.

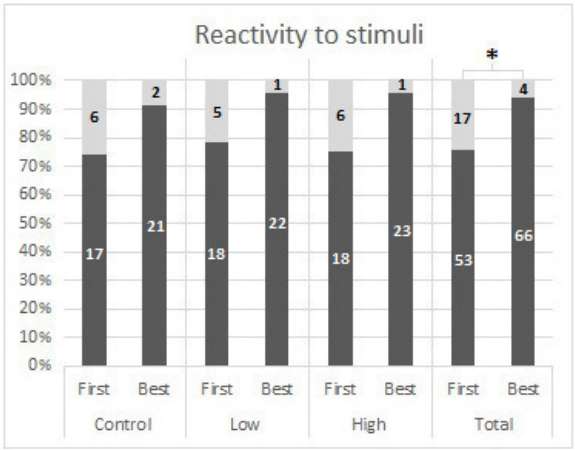

Figure 4. Percentage of reactivity (reactive in dark grey and non-reactive in light grey) of first and best responses, for Control, Low and High Chlorpyrifos concentration treatments, and for total (all individuals without taking into account concentration treatments). Numbers inside bars indicates number of tadpoles of each group. Asterisk indicates significant difference in reactivity rates using Fisher's exact test.

Tables

Table 1. Summary of studies using scape swimming performance test with tadpoles.

E.R.	A.R.	R.T.	Morphological measurements	Arena	Study area	Reference
7	All	24 hours	*Not used*	*N.I.* x 1 x 5 (concenctric channel)	Ecotoxicology	Bridges, 1997
5	Average	1 minute	Total length	33.5 x 20 x 4.5	Ecotoxicology	Britson and Threlkeld, 1998
1	First	No rest	Body length	100 x 5 pipe	Ecotoxicology	Savage et al., 2002
5	All	24 hours	Total length	40 x 30 x 10	Ecotoxicology	García-Muñoz et al., 2009
4	Best	No rest	Body length	30 x 2 x 1	Ecotoxicology	Widder and Bidwell, 2008
3	Best	30 seconds	Body length	102 x 4 x 5	Ecotoxicology	Mitchkash et al. 2013
1	First	No rest	*Not used* (all tadpoles with similar size)	90 x 45 x 5	Ecotoxicology	Wood and Welch, 2014
1	First	No rest	*Not used*	40 x 1 x 2	Ecotoxicology	Egea-Serrano and Tejedo, 2014
3	Best	*N.I.*	PCA of digital tail shape stimations	*N.I.*	Ecotoxicology	Levis et al. 2016
2	First	No rest	Body length and tail length	*N.I.* x 2.54 x 3 (concentric channel)	Tail shape	Figiel and Semlitsch, 1991
3	Best	No rest	Tail length, tail depth (max and at half-length)	28 x 28 x 1.8	Tail shape	Van Buskirk and McCollum, 2000
5	Best	30 seconds	Body length and tail length	140 x 102 x 6	Ontogeny	Brown and Taylor, 1995
10	Best	*N.I.*	Body length	30 x 30 x 5	Ontogeny	Wilson and Franklin, 1999
3	Best	30 minute	Tail length, tail depth (max), tail muscle depth (max), body depth and length	4 x 200 x 4	Ontogeny	Teplitsky et al., 2005
1	First	No rest	Tail length (three size classes)	100 x 2 x 2	Predation	Richards and Bull, 1990
5	Average	*N.I.*	Total length	100 x 50 x 6	Predation	Chovanec, 1992
4	Best	No rest	Body length	100 x 8.5 x 4	Predation	Watkins, 1996
3	Best	*N.I.*	PCA of total length, tail length, tail muscle depth (max), tail depth (max), tail and body area	34 x 25.6 x 2.5	Predation	Richardson, 2002
1	First	No rest	Digital tail shape stimation	100 x 40 x 10	Predation	Dayton, 2005
5	Best	*N.I.*	Total length and tail area	Water depth twice tadpole's depth (Plate)	Predation	Eidietis, 2005

E.R. (evoked responses); A.R. (analyzed responses); R.T. (rest time between responses)
Arena (length x width x water depth; in cm). *N.I.* (not informed; information not presented in the paper)

Table 2. Summary statistics for two-way ANOVAs performed to evaluate effects of Chlorpyrifos concentration, temperature treatments and its interaction, on tadpoles' escape components in First response and Best response analysis.

			Burst Speed			Distance			Time		
		df	MS	F	p	MS	F	p	MS	F	p
FIRST	Temp	1	89	0.006	0.937	2.351	1.419	0.240	0.377	0.301	0.586
	Conc	2	14869	1.069	0.352	1.680	1.013	0.371	0.990	0.789	0.460
	Temp * Conc	2	658	0.047	0.954	0.137	0.083	0.921	0.076	0.061	0.941
	Error	47	13913			1.657			1.254		
BEST	Temp	1	27765	2.094	0.153	0.108	0.106	0.746	0.006	0.011	0.917
	Conc	2	54358	4.099	0.021*	3.378	3.330	0.042*	1.300	2.156	0.125
	Temp * Conc	2	4917	0.371	0.692	0.176	0.173	0.841	0.054	0.089	0.915
	Error	60	13262			1.014			0.603		

Significant differences ($p<0.05$) marked with asterisk.
df = Degrees of Freedom; MS = Mean Squares; F = F-ratio; p = p-value

Chapter IV

Effects of pesticides mixtures occurring at tropical agroecosystems small water bodies on representative freshwater communities: a microcosm approach

Effects of pesticides mixtures occurring at tropical agroecosystems small water bodies on representative freshwater communities: a microcosm approach

David Sánchez-Domene[1]*, Alba Navarro-Lozano[2], Larissa Gonçalves Santos[3], Raphael Acayaba[4], Cassiana Montagner[5], Alexandre Vidotto Barboza Lima[6], Danilo Grünig Humberto da Silva[7], Eduardo Alves de Almeida[8]

[1] Instituto de Pesquisa em Bioenergia, Universidade Estadual Paulista, Rio Claro, SP13500-230, Brazil.
[2] Laboratório de Ecologia Teórica, Departmento de Zoologia e Botânica, Universidade Estadual Paulista, São José do Rio Preto, SP 15054-000, Brazil.
[3] Departamento de Zoologia e Botânica – IBILCE, Universidade Estadual Paulista – UNESP, Rua Cristóvão Colombo, 2265, Jardim Nazareth, CEP 15054-000 São José do Rio Preto, SP, Brasil
[4] Faculdade de Tecnologia, Universidade de Campinas, Limeira, SP 13484-332, Brazil.
[5] Laboratório de Química Ambiental, Departamento de Química Analítica, Instituto de Química, Universidade de Campinas, Campinas, SP 13484-332, Brazil.
[6] Departamento de Biologia, Universidade Estadual Paulista Júlio de Mesquita Filho, Rua Cristovão Colombo, 2265, 15054-000, São José do Rio Preto (São Paulo), 15054-000, Brazil.
[7] Departamento de Química e Ciências Ambientais, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista (UNESP), São José do Rio Preto, Brazil.
[8] Departamento de Ciências Naturais, Fundação Universidade Regional de Blumenau, Itoupava Seca, SC 89030-903, Brazil.

ABSTRACT

Agriculture has become one of the most important drivers of freshwater ecosystems degradation in the world. Modern agriculture management requires the use of a wide range of pesticides which can accumulate in complex mixtures in water bodies near to cultures. Most studies that investigate the effects of the exposition to pesticides on organisms do it in isolation, despite of mixtures of pesticides cause unpredictable impacts than pesticides alone. In fact, mixtures of pesticides may act in additive, synergic or antagonistic ways when entering the environment simultaneously. Recent findings of pesticides mixtures contaminating waterbodies of the agricultural area surrounding the city of São José do Rio Preto (Brazil), led us to study their effects on the experimental freshwater communities inhabiting those ponds. To that end we exposed a representative freshwater community (phytoplankton-periphyton-tadpoles) to a field-based mixture of pesticides (Imidacloprid, Ametrine, Hexazinone and Tebuthiuron) in two different concentrations (Low: 4,12, 4 and 8, and High: 8, 325, 1210 and 705; ng L-1) in outdoor microcosms. While after 5 days of exposition periphyton did not result affected by the pesticides mixtures, we detected significant differences in the abundance of the phytoplankton classes Bacillariophyceae, Chlorophyceae, Cryptophyceae and Trebouxiophyceae, and the body length of tadpoles. However, the overall results indicate that concentrations assessed do not appreciably affect the freshwater community assessed. Although no dramatical effects on the assessed freshwater community were detected, the effects observed, caused by minimal pesticides concentrations, are worrisome. In addition, the ground water contamination detected adds to the allegations of widespread pesticides contamination in Brazil.

Keywords: *pesticides mixture*, *freshwater communities*, microcosm, *sugarcane*, contamination.

INTRODUCTION

Today is not unusual to find agrochemicals contaminating small water bodies annex to agricultural lands (Stehle and Schulz, 2015, Chau *et al.* 2018, Ippolito and Fait, 2019). Modern agriculture management requires the co-use of pesticides such as fungicides, insecticides and herbicides, which result in the input of complex mixtures to rain and irrigation runoff-fed water bodies (Green, 2016; Leu *et al*. 2004; Stehle and Schulz,2015). The extensive use of these chemicals has allowed the increase in crop yields but have also affected non-target species inhabiting agroecosystems, especially those that are water dependents (Olivier *et al.* 2012). In fact, agriculture has become one of the most important drivers of freshwater ecosystem degradation in the world (Smukler *et al.* 2012; Arts and Hanson, 2019). Several ecotoxicological studies have shown that water bodies contaminated with pesticides, mostly by agricultural runoff (Wauchope, 1978; Van der Werf,1996 Aparicio *et al.,* 2015), can cause alterations in reproduction and development, malformations, biochemical disfunctions, immunosuppression, and even mortality on living organisms inhabiting them (*e.g.* Relyea 2005; Rohr *et al.* 2009; Mann *et al.* 2009; Köhler and Rita, 2013; Shuman-Goodier and Propper, 2016). Many studies have also revealed the occurrence of complex pesticides mixtures on water bodies annex to agriculture (*e.g.* Gilliom *et al.* 2007, Agostini *et al.* 2013, Sánchez-Domene *et al.* 2018), nevertheless few studies have been carried out on the effects of those mixtures on freshwater communities inhabiting small water bodies, which are important components of freshwater ecosystems as they support higher proportions of biodiversity compared to larger freshwater systems (Biggs *et al.* 2014; Lorenz *et al.* 2016).

Recently, we found several pesticides contaminating waterbodies of the agricultural area surrounding the city of São José do Rio Preto (Sánchez Domene *et al.* 2018). This traditional cattle ranching region, at the northwest of the State of São Paulo, has experienced one of the greatest sugarcane expansions in Brazil (Aguiar *et al.* 2009), which is one of the most pesticide

demanding crop in the country (Pignati, 2017). These findings made us to concern about the effects of those agrochemicals to freshwater communities inhabiting waterbodies close to sugarcane cultures. To better understand that, we planned a microcosms study where we assessed the effects a pesticides mixture, based on those that are most frequently found at waterbodies close to sugarcane cultivation in São José do Rio Preto, at two concentrations, on a relatively simple freshwater community (phytoplankton-periphyton-tadpoles). The mixture was composed by Imidacloprid, a neonicotinoid insecticide with systemic action against piercing-sucking pests (Tomizawa and Casida, 2005), and the herbicides listed as not approved in the EU Pesticides database: Ametrine and Hexazinone, organic compounds of the triazine family which inhibit the photosystem II (Kubilius and Bushway, 1998; Jones and Kerswell, 2003), and Tebuthiuron, an organic compound from the urea family which also inhibits photosynthesis (Hatzios and Penner, 1980). The joint action of agrochemicals can act in additive, synergic or antagonistic ways when affecting communities simultaneously (Piggott et al., 2015). To try to understand how agrochemicals mixtures affect the phytoplankton, periphyton and tadpoles, it became almost inevitable to limit factors affecting the studied communities. The use of microcosms, which act as ecosystems proxies held in partially isolated containers, are useful tools to understand the way freshwater communities are affected by such stressors. In addition, microcosms enable the replication and study of simplified ecosystems in a robust statistical experiment (Beyers and Odum, 1993).

In this paper, we study the effects of two different concentrations of a pesticides mixture, based on data collected in the field, on experimental freshwater communities (phytoplankton-periphyton-tadpoles) maintained in outdoor microcosms. We hypothesized that microcosms contaminated with the selected pesticides will suffer both the decrease in the abundance of primary producers (phytoplankton and periphyton), because of the effects of the herbicides Ametrine, Hexazinone and Tebuthiuron present in the pesticides mixture, and the impairment

of tadpoles because of the synergic effects of food scarcity (periphyton decrease) and the biochemical changes induced by the pesticides.

MATERIALS AND METHODS

Microcosms construction

We constructed twelve outdoor microcosms, four replicas per treatment, arranged according to a completely randomized experimental design, at the Sao Paulo State University (UNESP) facilities in São Jose do Rio Preto, Sao Paulo, Brazil. Construction started on 01-Feb-2017 (day -35, see Fig.1). Each microcosm consisted in a 40 L polypropylene basin, filled with 30 L of non-chlorinated well ground water, to which were added 400 mL of freshwater from the pond where experimental tadpoles came from (as periphyton and plankton inoculum, and at time as tadpole feed) and 60 g of dried mango tree leaf litter (as initial organic nutrient source, and as tadpoles' habitat structurer). Once constructed, microcosms were covered with shade-cloth lids complying with the Brazilian government recommendations to reduce the mosquito (*Aedes aegypti*) vector of Zika virus. At time, this lid avoided direct incidence of sun and insect colonization.

Response of periphyton to pesticides mixtures

On day -34, once the leaf litter was at the bottom of the microcosms, we added a 15 x 7 cm ceramic tile (non-finished surface up) as substrate for periphyton biomass sampling. The biomass of periphyton developed in each of the pesticides mixture treatments was calculated as the difference in weight between the clean tile and the tile colonized by periphyton, both oven-dried at 80ºC for 24h. We used an analytical balance with readability of 0.1 mg.

Pesticides mixtures

We prepared two different concentrations of a mixture of pesticides based on those found at sugarcane-dominated agroecosystem forming the agricultural area surrounding São José do Rio Preto (Sánchez-Domene et al. 2019a). The mixture was formed by the insecticide Imidacloprid (Imi) and the herbicides Ametrine (Ame), Hexazinone (Hex) and Tebuthiuron (Teb), all of them among the 14 most used pesticides for sugarcane management in Brazil (IBAMA, 2012). Two different concentrations of this mixture, based on those concentrations found at the agricultural area of São José do Rio Preto (Sánchez-Domene et al. 2019a), were assessed, i) "Low" (Imi = 4, Ame = 12, Hex = 4, and Teb = 8; ng L^{-1}), and ii) "High" (Imi = 8, Ame = 325, Hex = 1210, and Teb = 705; ng L^{-1}). Once the pesticides mixtures were added (day 0), after refilling the microcosms up to 30 L to compensate the evaporation occurred from day -35, we assessed the concentrations of pesticides on days 0, 2 and 5 (pesticides samples; see Fig.1) by collecting 500 mL of water from each microcosm and concentrating these water samples through a solid phase 6cc Oasis HLB Extraction Cartridge (500 mg) LP (Waters, Milford, MA). Concentrations in the final eluate were determined using a 1200 series Liquid Chromatograph System, equipped with a binary pump, and coupled to a 6410 triple quadrupole Mass Spectrometer with an electrospray ionization source; all from Agilent Technologies Santa Clara, CA, USA (see Sánchez-Domene *et al.* 2018 for further details).

Response of phytoplankton community to pesticides mixtures

We assessed the effects of the exposure to the two treatments of the pesticides mixture on phytoplankton community abundance and on the concentration of chlorophyll a (Chl-a) on the days 0, 2 and 5 (phytoplankton samples; see Fig.1). We only determined abundances of the most representative phytoplankton classes: Bacillariophyceae, Chlorophyceae,

Chrysophyceae, Cryptophyceae, Coscinodiscophyceae, Cyanophyceae, Dinophyceae, Euglenophyceae, Synurophyceae, Trebouxiophyceae, Xanthophyceae and Zygnematophyceae. To that end we analyzed microcosms water samples using inverted microscope (Leica DM IL LED, 400× of magnification) after 24h of sedimentation in 10 mL sedimentation chamber (Uthermöhl, 1958). We obtained these samples on days 0, 2 and 5 by filtering 400 mL of microcosm water (25 µm mesh size) and conserved them until their analysis with lugol's iodine solution. To obtain statistically robust result, we counted in each sample at least 100 individuals/colonies of the most abundant class in not less than 30 counting diameter transects (Edler and Elbrächter, 2010).

Concentration of Chl-a (µg L^{-1}) was determined following Golterman *et al.* (1978). To this end, 300 mL of water from each microcosm were filtered using Milipore® AP20 filters which were immediately wrapped in aluminum foil and frozen until chlorophyll extraction. For the extraction, the filters were macerated with acetone (90%). The resultant extracts were transferred to test tubes wrapped in aluminum foil and maintained under refrigeration for 12 h; after that period of time tubes were centrifuged (10 min at 30,000 rpm). The absorbances (663 and 750 nm) of the supernatant were measured in duplicate using the ultraviolet spectrophotometer UV-151 (BEL Engineering®) in 1 cm quartz cuvette. Concentrations of Chl-a were calculated following the formula:

$$Chl_a = U \times \left(\frac{10^6}{k}\right) \times \left(\frac{Vol_e}{Vol_f}\right)$$

U: difference between 663 nm and 750 nm absorbance values K: extinction coefficient = 89
Vol_e: volume of chlorophyll extract (mL) Vol_f: volume of microcosm water filtered (mL)
Chl_a: concentración of chlorophyll a (µg mL^{-1})

Response of tadpoles to pesticides mixture

We obtained experimental tadpoles from a spawn of the species *Physalaemus nattereri* collected in a pond (pond SO_E throughout this thesis; see Fig.S1) from the agricultural area of São José do Rio Preto. The spawn was maintained in controlled conditions in aerated aquariums with dechlorinated water (pH 7.5-8.0; 28 ± 1°C) until tadpoles hatched. Then, tadpoles were fed ad libitum with commercial tropical fish food until reached Gosner stages 25-27 (Gosner, 1960). A total of 108 of those tadpoles were used for the microcosm experiment, nine per replica. We assessed the effects of the pesticides mixtures on tadpoles' growth by comparing their mass and length on days -1 (the afternoon before its introduction to microcosms) and 5 (immediately after tadpoles' catching). On day -1 we used an analytical balance with readability of 0.1 mg to weigh each tadpole; we gently remove excess of water by placing them for a moment on filter paper. Immediately, we photographed them laterally to further measure their body length (see Fig.2) using the software ImageJ (Schneider *et al.* 2012). We used body length rather than total length because tails could be easily injured (Annibale *et al.* 2019). On day 5, once weighed and photographed following the procedure described above, tadpoles were submitted to activity and escape swimming performance assessments (see below). Immediately afterwards we gently euthanized individuals by submersion in Benzocaine solution (0.95 mg L^{-1}) and stored them individually at -80°C for subsequent enzymes evaluations.

To assess the effects of pesticides mixture on the activity of tadpoles, we follow the methodology described in Bridges (1997). Briefly, we selected randomly two tadpoles per microcosm and placed them in small individual aquariums (with water from their respective microcosms). The aquariums were filmed from above during 80 min (SONY® HDR-XR160). The first 30 min of video was considered as acclimation time; the rest of the video was analyzed by observing each tadpole for 5 sec every 4 min to determine activeness or inactiveness.

Activeness was defined as any tadpole movement. A total of 20 observations per aquarium were performed and the proportion of 5 sec observations periods with tadpoles' activeness were registered for each aquarium. In addition, we assessed the affection on tadpoles escape swimming performance by placing them individually in a narrow glass swimming-channel (50 × 2 × 5 cm) with 2 cm depth of water from their respective microcosms (two tadpoles, without tail damages, per microcosm). Channel dimensions and water depth were adapted to tadpoles' sizes, covering them completely but limiting their responses to a two-dimensional movement (Egea-Serrano and Tejedo, 2014). After one minute of acclimation, we simulated a predation attempt by touching tadpole's tail with a glass rod. We only simulate one predation attempt per individual since first response analysis emulates natural predatory pressure (Sánchez-Domene at al. 2019b). If the predation attempt did not evoke escape, the individual was classified as non-reactive. We recorded escape responses (25 frames per second) and analyzed the videos using the visual tracking software Kinovea (Kinovea© v.0.8.24); snouts of the tadpoles were used as tracking points. We measure two swimming escape components, time (seconds elapsed since the first tail movement to its total stop), and distance (linear length of the escape response, in mm).

We performed three different sample preparations to assess tadpole biochemical changes caused by exposure to the pesticides mixtures. For glutathione-S-transferase (GST) measurements, we homogenized two tadpoles of each microcosms (n=24), entirely and individually in a ratio 1:4 (w/v), in ice-cold homogenization buffer (Tris-HCl 20 mM, EDTA 1 mM, DTT 1 mM, sucrose 0.5 M, KCL 0.15 M and PMSF 1 mM; pH 7.4). We centrifuged the homogenate obtained at 9000 g for 30 min at 4°C, then we collected the supernatant and centrifuged it again at 50,000 g for 60 min at 4°C. We analyzed GST activity in the resulting supernatant. For acetylcholinesterase (AChE) and carboxylesterase (CbE) measurements, we again homogenized two tadpoles of each microcosms in ice-cold solution (Tris HCl 0,1 mol L^{-}

[1]; pH 8.0; ratio 1:4) and centrifuged the homogenate at 10,000 g for 30 min at 4°C. We analyzed AChE and CbE activities in the resulting supernatant fraction. Finally, to assessed lipid peroxidation, we homogenized two tadpoles of each microcosms in ice-cold homogenization buffer (PBS 0.05 M and Na2-EDTA 0.003 M; pH 7.4; ratio 1:4) and centrifuged the homogenate at 20,000 g for 10 min at 4°C. We gently aspirated the resulting supernatant to measure malondialdehyde (MDA) concentration.

We measured the activity of enzymes on a Victor TM X3 microplate reader (Perkin Elmer). To measure GST activity, we followed the method described by Keen *et al.* (1976). This method monitors the formation of the conjugate of CDNB with GSH catalyzed by GST in the sample (PBS 0.2 M, CDNB 1mM and GSH 1mM; pH 6.5) at 340 nm. To measure AChE and CbE activities, we followed the methodology of Ellman *et al.* (1961). This method measures the formation of a thiol derivative produced by the enzyme action on the substrate, which reacts with DTNB producing a yellow compound. We used ACh as substrate for AChE analysis and phenylthioacetate for CbE; these reactions were monitored at 412 nm at 25°C. Specific activities were expressed as U per mg of protein^{-1}. We quantified proteins of the samples following the Bradford assay (1976) with Coomassie brilliant blue G-250 at 595 nm. We compared the results with the analytical curve prepared with BSA as the standard. Finally, following Esterbauer and Zollner (1989), with few modifications, we evaluated the levels of MDA using the adduct formed with thiobarbituric acid (TBA). According to Domijan *et al.* (2015), we used HPLC-FD (Shimadzu Corporation, Kyoto, Japan) to detect MDA-TBA adduct. We calculated MDA levels (*p*mol per mg of tissue^{-1}) based on a calibration curve, prepared according to same procedure described above for the samples, using authentic standard.

Statistical analysis

We used Kruskall-Wallis test to assess for differences among the studied responses to the different concentrations of pesticides mixtures; Anova analisys could not be applied since data did not fit the assumptions of normality and homoscedasticity, even after data transformations. When significant differences were detected, we use Dunn's test, with Bonferroni adjustment (Rice, 1989), for post-hoc comparisons. Using the lateral photographs of the tadpoles, and the software ImageJ, we measure tail length (TL) of tadpoles submitted to scape swimming performance test. In case of substantial differences among individuals, the correlation between TL and the time and length of the scape responses will be study to determine the need of its use as covariates in the statistical analysis of scape swimming performance (Sánchez-Domene *et al.* 2019b). All analyses were performed using R Statistical Software (R Development Core Team, 2008).

Results

Pesticides mixtures

The analyses of the water samples of the microcosms, of the days 0, 2 and 5, revealed several issues important for the experiment. First of all, we did not achieve the exact experimental concentrations stablished for the experiment (see Tab.1), nevertheless the concentrations achieved were environmentally relevant for open area ponds assessed in the agricultural area surrounding São José do Rio Preto (Sánchez-Domene et al. 2019a). On the other hand, we found quantifiable concentrations of Imidacloprid and 2-hydroxyatrazine in all water samples from the day 0 (see Tab.1), including the 4 replicas of the control treatment. The only possible explanation to this, is the contamination of the well ground water used in the experiment. Reinforcing this hypothesis, traces of other chemical compounds such as Caffeine, Diuron,

Azoxystrobin and Carbendazim, not added to water by us, were found in the water samples (see thesis annex C). In addition, it should be highlighted that despite of the mesocosm experiment was planned to have different concentrations of Imidacloprid at Low and High concentration treatments, the concentration presented in the well water employed for the experiment made that all microcosms, including control replicas, presented similar concentration of this pesticide, therefore differences among treatments are only due to herbicides' concentration differences among treatments.

Effects of pesticides mixtures on periphyton

The biomass of periphyton developed in the surface of the tiles disposed on the microcosms for this purpose did not revealed significant differences among treatments (Kruskal-Wallis test $p = 0.74$; see Fig.S2)

Effects of pesticides mixtures on phytoplankton community

The Kruskal-Wallis tests performed to phytoplankton data revealed significant differences within the classes Bacillariophyceae, Chlorophyceae, Cryptophyceae and Trebouxiophyceae, however, post-hoc analyses were non-significant in all cases (see Fig.3). The exposure to the two concentrations of the herbicides mixture did not affect in a same way those classes, while Bacillariophyceae and Chlorophyceae showed a reduction in their abundances from day 0 to 2 at microcosms treated with High concentration, with a minimal recovery on day 5, at Control and Low treatments they increased their abundances from day 0 to 5. In the case of Cryptophyceae, cells almost disappeared from microcosms after the exposition to Low and High treatments. Finally, the class Trebouxiophyceae follow the same abundance pattern in all treatments, including Control, being the significant differences detected by the analysis

attributable to the increment in abundances at day 5. With respect to the analysis of the Chl-a concentration, the results revealed a significant decrease in the concentration of Chl-a after the addition of the Low concentration pesticides mixture; on this occasion the Dunn's test revealed significant differences from day 0 to day 5 (see Fig.4).

Effects of pesticides mixtures on tadpoles

The variables selected to assess the growth of the tadpoles during the 5 days of exposure to the pesticides mixtures revealed different results. While weight of tadpoles did not change significatively among treatments (see Fig.5), the length (body length) of tadpoles increase in control and low concentration treatment, while remain the same at high concentration treatment. Regarding motility assays performed, neither activity assay (Kruskal-Wallis test $p = 0.92$; see Fig.S3) nor escape swimming performance test (Kruskal-Wallis test for Distance $p = 0.95$, for Time $p = 0.89$; see Fig.S3) presented significant alterations. The study performed to assess the need to include tail length of tadpoles as a covariate for the analysis of the swimming escape performance did not show any significant correlation between the tail length of the tadpoles and the swimming escape performance variables assessed (see Fig.S4). In addition, no significant differences among tail length of the tadpoles assessed from each microcosm were detected (see Fig.S4). Therefore, we decided not to include tail length of tadpoles as a covariate. In the case of the biochemical variables assessed on tadpoles, none changed significantly (Kruskal-Wallis test for GST $p = 0.08$, for AChE $p = 0.10$, for CbE $p = 0.86$, for MDA $p = 0.09$; see Fig.S5).

DISCUSION

The overall results of our microcosms experiment indicate that concentrations employed do not affect, in a significant way, the freshwater community assessed. However, we would like to highlight that these results are not determinant to discard affections of pesticides on communities inhabiting waterbodies from sugarcane-dominated agroecosystem of the northwest of São Paulo state, since i) we weren't able to match the higher concentrations of the pesticides found in the agroecosystem surrounding São José do Rio Preto, ii) those higher concentrations found at field, could not be considered as the higher existing since analyzed water samples were collected during the amphibian breeding season of the region, which occurs during torrential rains and high temperatures causing high water volume fluctuations in ponds, and consequently, in the concentration of pesticides affecting organisms (Sánchez-Domene et al. 2018), and iii) exposure time at the experiment was restricted to 5 days, while in nature the exposures could cover the full life of organisms.

The detected increasement in the abundance of Bacillariophyceae and Chlorophyceae phytoplankton classes from day 0 to day 5 at Control microcosms was a pattern not expected. One possible explanation for this unexpected behavior is the handling, sampling procedures, to which microcosms were submitted from day 0 to day 5 to obtain experimental data. This handling could upset the existing balance at microcosms, such as the light penetration range (Huisman *et al.* 1999) and the CO_2/O_2 concentration (Engel *et al.* 2007). A balance upset can benefit the development of some phytoplankton classes in detrimental of others (Cermeño *et al.* 2011). Assuming this was what happen, the higher increasement in the abundance of Bacillariophyceae and Chlorophyceae from day 0 to day 5 at Control microcosms, compared with those registered at Low treatment, may reveal a detrimental effect of herbicides on these phytoplankton classes. Further reinforcing this conjecture, the concentration of chlorophyll-a at microcosms also seem to reveal the effect of herbicides mixtures to phytoplankton

community at both Low and High treatment microcosms. However, again Control microcosms, showed a decrease in concentration from day 0 to day 2; we think this could be due to both the phytoplankton sampling method we employed, in which filtered water was returned to microcosms, and the upset of the microcosms balance.

Tadpoles exposed to herbicides mixtures did not show any detrimental effect neither on the biochemical indicators nor motility or activity. However, although their mass did not differ among treatments, body length did. While control and low treatments showed a significant body length growth during the 5 days of exposure to the mixture of herbicides, tadpoles on microcosms treated with the high concentration mixture did not increment their body length. Rossa-Feres *et al.* (2004) showed that tadpoles of *P. nattereri* mostly feed on periphyton, therefore, since periphyton did not result affected by herbicides treatments, this smaller body growth rate on high concentration treatment cannot be associated to deficient nourishment, in fact the mass of the tadpoles did not differ among treatments. None of the variables assessed on tadpoles clarifies the reason behind this effect on body length. After a deep literature review, we did not find any study on amphibians within the range of herbicides' concentrations we applied to microcosms. It should be noted that although named as "High" throughout this article, the concentration attributed to High concentration microcosms, which sum around 1.25 $\mu g\ L^{-1}$, is low compared to most lab and microcosms research published. In example, Berrill *et al*. (1994) reported no affections on tadpoles from the species *Rana pipiens* exposed to 100 mg L^{-1} of hexazinone for 8 days, and Saka *et al.* (2018) reported decrease in total body length and body mass in tadpoles from the species *Silurana tropicalis,* but exposed to 200 $\mu g\ L^{-1}$ of ametrine for 26 days, finding no effects in the exposition to 20 $\mu g\ L^{-1}$. Therefore, we cannot explain the cessation in body growth, nevertheless it is very interesting to have detected this effect at such low concentrations of pesticides. One possible explanation to this effect at such

low concentration could be a synergic effect of the mixture of herbicides acting together (Relyea, 2008).

Although it was not the objective of this study, we were surprised to discover that well ground water from the facilities of UNESP (São José do Rio Preto) were contaminated by at least 9 pesticides (see annex C). The joint detection of caffeine in the samples of ground water used at microcosms suggest contamination by human wastewater discharge (Linden *et al.* 2015). This alarming finding add up to the widespread pesticide contamination detected at ponds from the sugarcane-dominated agroecosystem surrounding São José do Rio Preto (Sánchez-Domene *et al.* 2018).

CONCLUSIONS

Although no dramatical effects on the assessed freshwater community were detected, the effects observed on phytoplankton and tadpoles are of interest given that were caused by minimal pesticides concentrations. In addition, the ground water contamination detected is an alarming fact which adds to the allegations of widespread pesticides contamination in Brazil.

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FIGURES

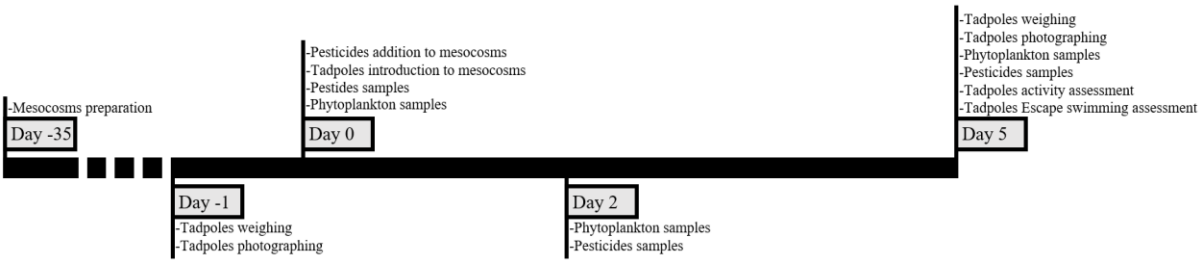


Figure 1. Detailed experimental timeline.

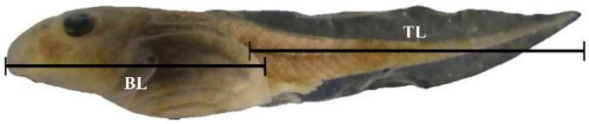


Figure 2. Lateral image of *Physalaemus nattereri* tadpole. BL and TL indicate the measuring lines followed for body length and tail length measurements.

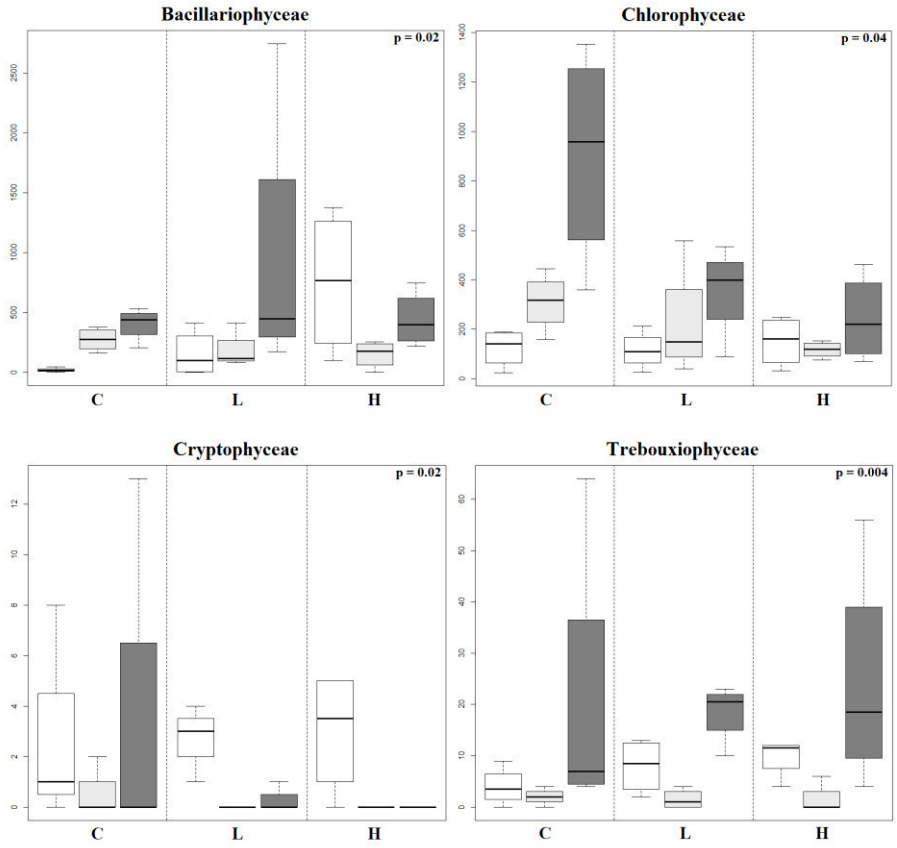


Figure 3. Abundance (individuals counted in a sedimentation chamber of 10 mL) of the Bacillariophyceae, Clorophyceae, Cryptophyceae and Trebouxiophyceae phytoplankton classes before (day 0, white boxes) and after (day 2, light grey boxes, and day 5, dark grey boxes) their exposition to two different concentrations of a pesticides mixture for five days at outdoor microcosms. C (control microcosms), L (microcosms with low concentration of pesticides mixture) and H (microcosms with high concentration of pesticides mixture). Kruskal-Wallis results (p-value) in the upper-right corner of the graphs; Dunn's test, with Bonferroni adjustment, did not show significant differences.

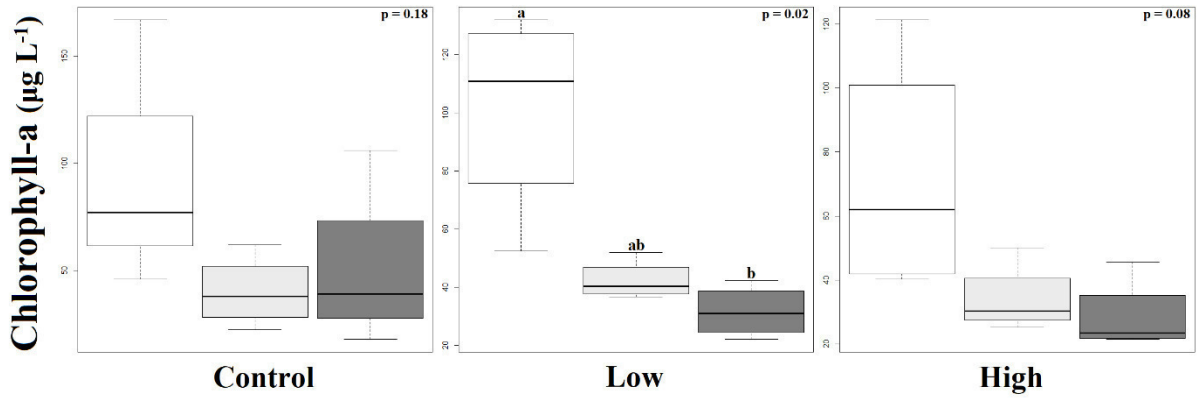


Figure 4. Concentration of Chlorophyll-a at microcosms before (day 0, white boxes) and after (day 2 and day 5, light grey and dark grey boxes respectively) the addition of two different concentrations of a pesticides mixture at outdoor conditions. Kruskal-Wallis results (p-value) in the upper-right corner of the graphs; bold lowercase letters for significant differences detected by Dunn's test with Bonferroni adjustment.

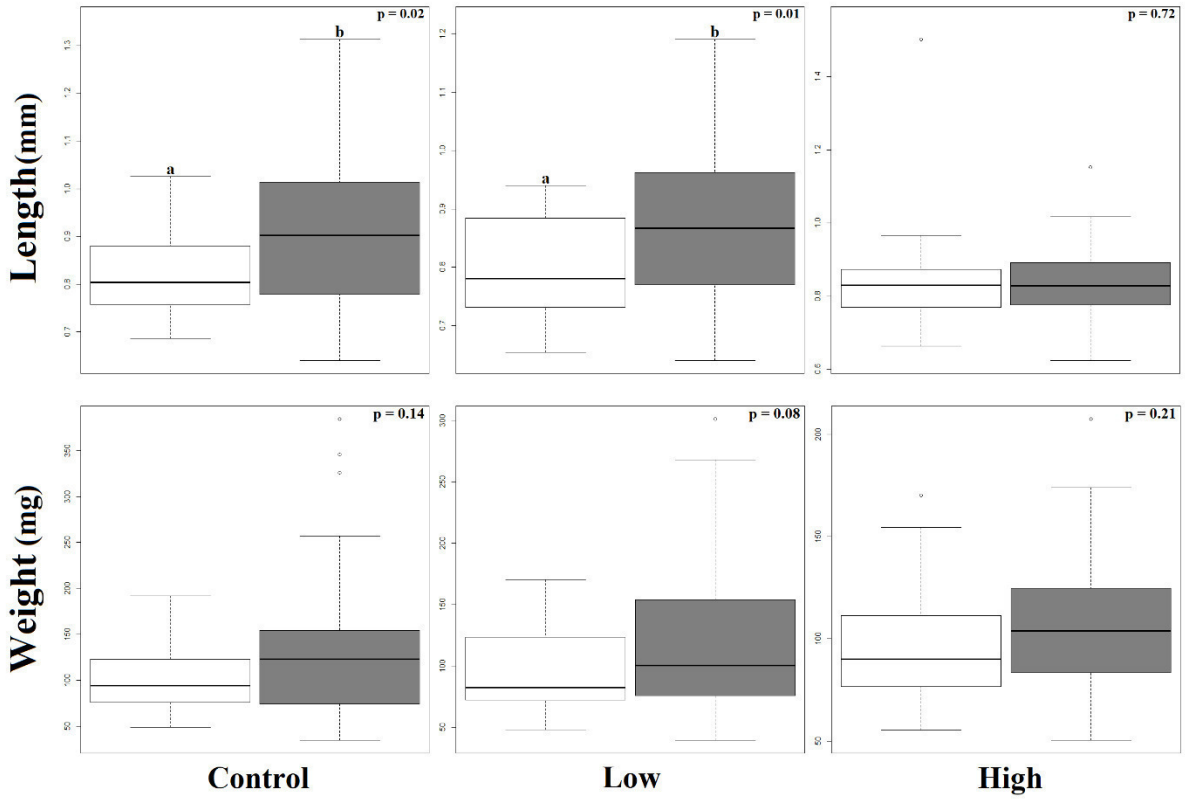


Figure 5. Length (body length) and weight (total tadpole's mass) of tadpoles before and after their exposition to two different concentrations of a pesticides mixture for five days at outdoor microcosms. Day -1 (white box), Day 5 (gray box). Kruskal-Wallis results (p-value) in the upper-right corner of the graphs; bold lowercase letters for significant differences detected by Dunn's test with Bonferroni adjustment.

TABLES

Table 1. Target concentrations (italics) and real concentrations (bold) on the day 0 of the experiment.

Concentration of the pesticides in the experimental mixtures (ng L^{-1})					
Treatment	**IMI**	**AME**	**HEX**	**TEB**	**2-HA**
Control	- / **9.7**	*0* / **<LQ**	*0* / **<LQ**	*0* / **<LQ**	- / **7.9**
Low	*4* / **10.3**	*12* / **<LQ**	*4* / **5.6**	*8* / **6.9**	- / **10.2**
High	*8* / **10.8**	*325* / **97.7**	*1210* / **900.2**	*705* / **232.7**	- / **11.1**

QL = Quantification limit

SUPPLEMENTARY MATERIAL

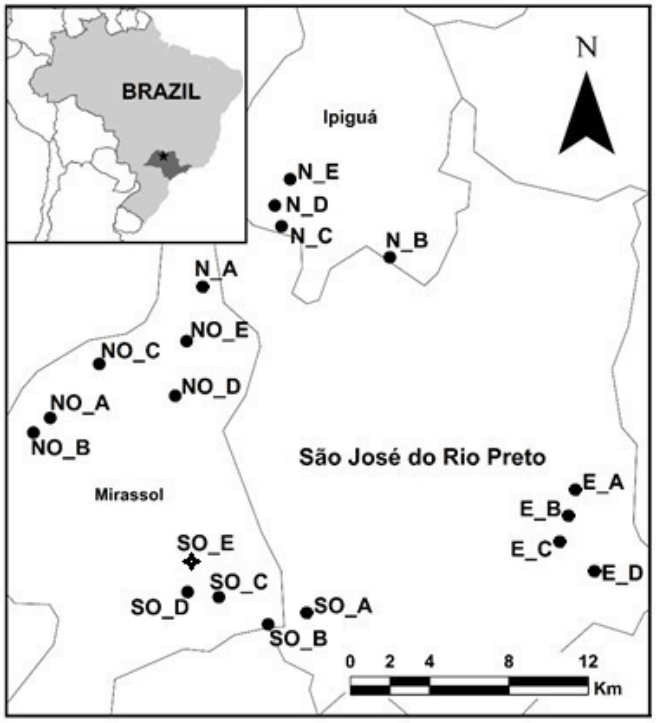


Figure S1. Temporary ponds from agroecosystems with different degrees of sugarcane intensification assessed during the rainy season 2015/2016 in São José do Rio Preto, São Paulo State (Brazil). E_(A,B,C,D) for East ponds, **SO**_(A,B,C,D,**E**) for Southwest ponds, NO_(A,B,C,D,E) for Northwest ponds and N_(A,B,C,D,E) for North ponds.

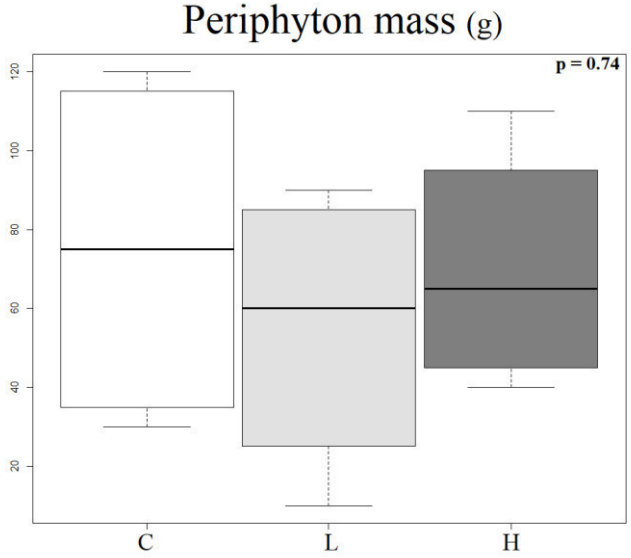


Figure S2. Biomass of periphyton (g) developed during 34 days in outdoor microcosms. The last five days of the experiment, the microcosms were submitted to two different treatments: L (low concentration of pesticides mixtures; light grey box) and H (high concentration of pesticides mixtures; dark grey box), control replicas (white box) were also assessed. Kruskal-Wallis result (p-value) in the upper-right corner of the graph.

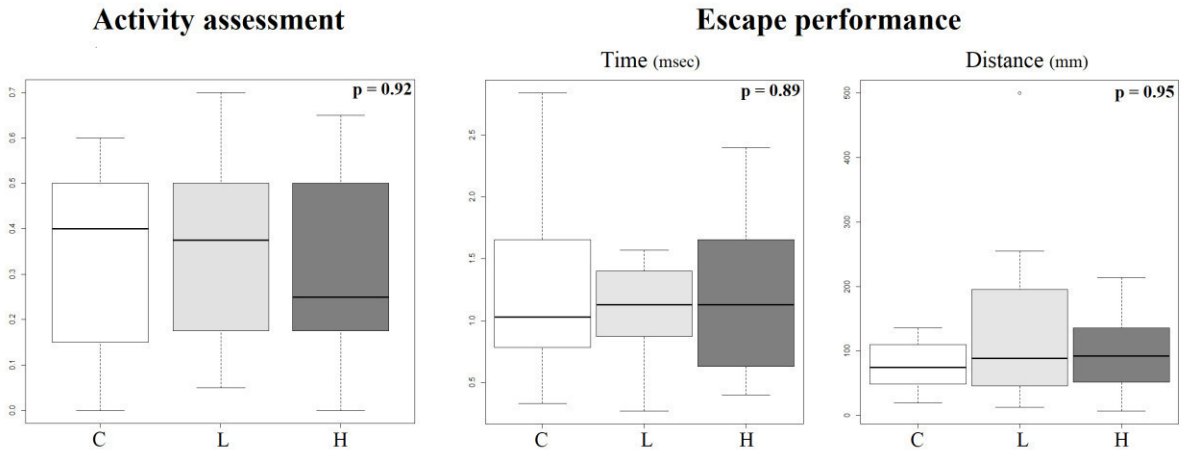


Figure S3. Motility assays (activity and escape swimming performance) performed on tadpoles after their exposition to two different concentrations of a pesticides mixture for five days at outdoor microcosms. C (white box), L (light grey box) and H (dark grey box) for Control, Low and High concentration treatments of pesticides mixtures respectively. Kruskal-Wallis result (p-value) in the upper-right corner of the graph.

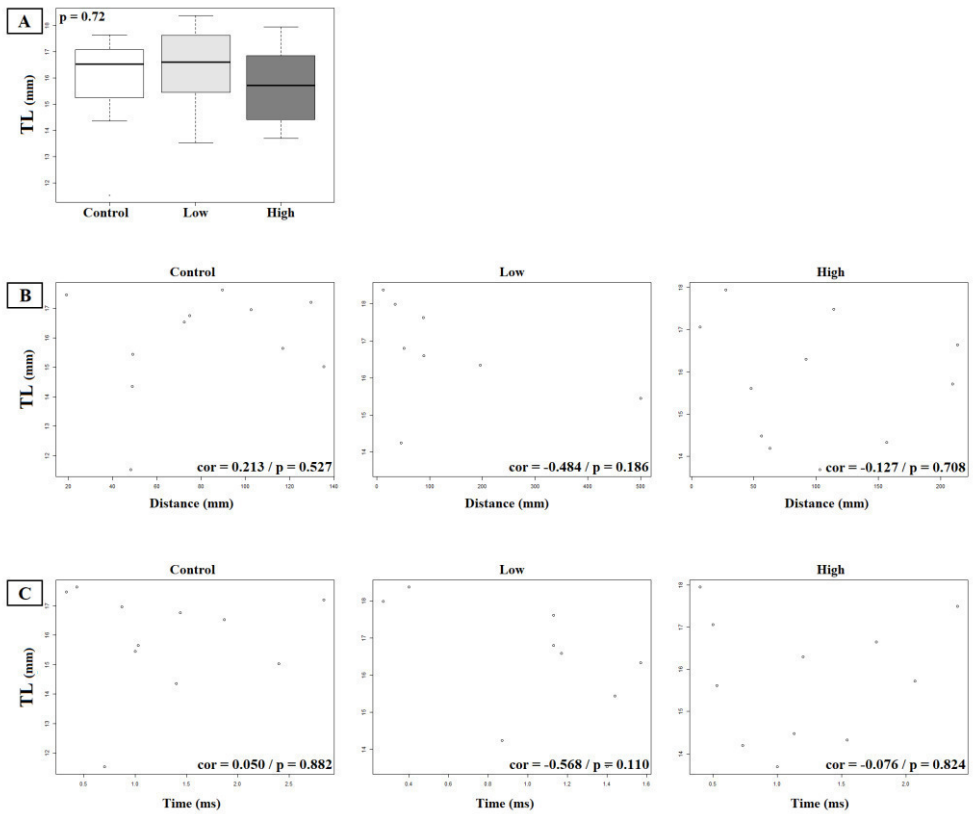


Figure S4. Tail length of tadpoles submitted to escape swimming performance assessment (**A**); Kruskal-Wallis result (p-value) in the upper-left corner of the graph. Linear regression analyses, by treatment, of escape swimming variables (time and distance), versus tail length (**B** and **C**); cor = Pearson correlation coefficients, p = regression p-value.

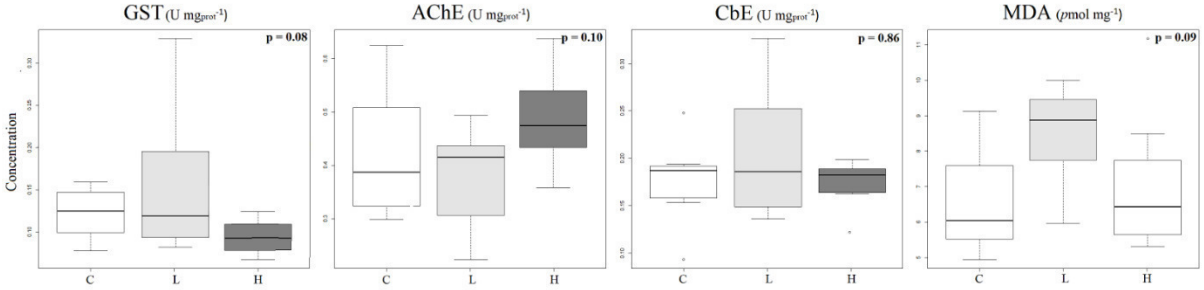


Figure S5. Concentrations of glutathione-S-tranferase (GST), acetylcholinesterase (AChE), carboxylesterase (CbE) and malondialdehyde (MDA) on tadpoles after their exposition to two different concentrations of a pesticides mixtures for five days at outdoor microcosms. C (white box), L (light grey box) and H (dark grey box) for Control, Low-concentration and High-concentration treatments of pesticides mixtures respectively. Kruskal-Wallis result (p-value) in the upper-right corner of the graph.

Water temperature (ºC)

Day		-1				0				1				2				3				4			
Replica		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control	Morning	24.74	25.06	26.01	25.32	26.12	26.59	26.63	27.54	24.84	25.20	25.13	25.53	25.69	25.88	25.94	26.09	24.23	24.76	25.86	25.03	26.35	26.42	26.65	27.12
	Afternoon	35.65	37.31	37.35	36.85	34.31	34.81	35.10	34.97	26.12	26.59	26.63	27.54	36.46	38.02	38.25	38.63	34.86	37.02	36.65	37.03	34.24	34.56	34.95	35.12
Low	Morning	24.89	25.19	25.52	25.04	26.29	26.57	27.13	26.78	24.95	25.32	25.75	25.45	25.78	26.04	26.39	26.15	24.18	24.89	25.26	25.34	26.32	26.48	26.89	26.65
	Afternoon	36.15	37.35	37.48	37.90	34.86	35.17	35.88	36.88	26.29	26.57	27.13	26.78	37.37	38.29	38.66	38.75	36.85	38.02	37.33	37.54	35.12	35.23	36.17	36.94
High	Morning	25.01	25.00	25.04	25.17	26.44	26.72	26.74	27.48	25.14	25.39	25.45	25.83	26.10	26.16	26.51	26.53	24.86	25.18	25.68	24.76	26.32	27.03	26.69	27.24
	Afternoon	36.69	36.84	37.43	37.09	35.20	35.26	35.27	36.22	26.44	26.72	26.74	27.48	37.73	38.44	38.36	38.87	37.02	36.54	37.15	36.86	35.37	35.16	34.87	36.02

Water conductivity (ms/cm3)

Day		-1				0				1				2				3				4			
Replica		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control	Morning	0.231	0.233	0.229	0.235	0.232	0.233	0.231	0.245	0.232	0.232	0.234	0.235	0.235	0.232	0.237	0.237	0.231	0.232	0.233	0.239	0.234	0.230	0.236	0.235
	Afternoon	0.229	0.243	0.227	0.233	0.229	0.227	0.237	0.228	0.232	0.233	0.231	0.245	0.234	0.232	0.236	0.242	0.231	0.228	0.239	0.230	0.233	0.230	0.237	0.240
Low	Morning	0.237	0.235	0.242	0.235	0.239	0.239	0.236	0.241	0.233	0.229	0.239	0.235	0.237	0.232	0.244	0.236	0.241	0.238	0.235	0.239	0.240	0.232	0.243	0.235
	Afternoon	0.233	0.227	0.240	0.227	0.234	0.228	0.137	0.230	0.239	0.239	0.236	0.241	0.237	0.232	0.244	0.233	0.233	0.230	0.140	0.232	0.238	0.230	0.246	0.234
High	Morning	0.236	0.236	0.238	0.241	0.237	0.237	0.220	0.241	0.236	0.235	0.235	0.238	0.235	0.240	0.237	0.241	0.237	0.236	0.226	0.239	0.236	0.239	0.238	0.239
	Afternoon	0.235	0.235	0.233	0.238	0.234	0.233	0.231	0.236	0.237	0.237	0.220	0.241	0.238	0.241	0.234	0.244	0.233	0.235	0.233	0.235	0.238	0.238	0.236	0.240

Dissolved Oxygen in water (mg/L)

Day		-1				0				1				2				3				4			
Replica		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control	Morning	3.17	3.57	4.53	3.98	2.07	1.73	2.24	2.26	2.05	1.71	1.85	1.73	2.39	1.82	1.98	1.82	2.04	1.78	2.19	2.23	2.43	1.88	2.02	1.77
	Afternoon	8.10	5.53	9.96	10.22	7.09	8.17	5.12	6.93	2.07	1.73	2.24	2.26	8.44	8.39	8.82	6.32	7.12	8.23	5.08	7.02	8.41	8.42	8.79	6.28
Low	Morning	2.60	3.85	2.95	2.03	1.31	1.45	2.22	2.17	1.44	2.48	1.93	2.04	1.66	2.18	2.11	1.73	1.34	1.48	2.25	2.21	1.68	2.09	2.15	1.82
	Afternoon	8.89	9.96	10.76	9.81	6.48	7.13	7.30	8.53	1.31	1.45	2.22	2.17	8.14	7.21	7.31	8.52	6.53	7.09	7.27	8.63	8.04	7.25	7.27	9.03
High	Morning	2.89	3.31	3.72	2.83	1.49	2.19	2.20	2.33	1.63	1.18	2.23	1.84	2.20	2.03	2.34	1.89	1.43	2.14	2.18	2.44	2.21	1.97	2.33	1.85
	Afternoon	8.38	7.46	9.48	10.05	7.04	7.29	7.48	7.40	1.49	2.19	2.20	2.33	7.33	7.43	8.32	6.91	7.08	7.24	7.52	7.36	7.27	7.45	8.28	7.03

Water pH

Day		-1				0				1				2				3				4			
Replica		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control	Morning	7.05	7.04	6.57	7.04	7.26	7.25	7.40	7.35	7.55	7.18	7.22	7.20	7.50	7.30	7.32	7.24	6.98	7.01	6.66	6.96	7.38	7.28	7.33	7.19
	Afternoon	8.01	7.33	8.25	8.03	7.47	7.94	7.53	7.82	7.26	7.25	7.40	7.35	8.04	8.22	9.13	7.99	8.03	7.44	8.12	7.95	7.98	8.18	9.09	8.01
Low	Morning	7.01	7.04	6.87	6.82	7.22	7.25	7.29	7.57	7.14	7.21	7.22	7.25	7.27	7.29	7.31	7.32	7.01	7.02	6.97	7.01	7.33	7.23	7.33	7.28
	Afternoon	8.02	8.25	8.13	8.27	7.60	7.74	7.97	7.93	7.22	7.25	7.29	7.57	8.15	8.10	8.01	8.32	8.11	8.15	8.09	8.20	8.14	8.12	7.99	8.28
High	Morning	6.98	7.01	7.01	6.68	7.57	7.29	7.44	7.40	7.20	7.27	7.31	7.28	7.28	7.32	7.40	7.32	6.98	7.01	6.97	6.75	7.31	7.29	7.38	7.29
	Afternoon	7.89	7.99	8.19	8.14	7.79	7.82	7.84	7.83	7.17	7.29	7.44	7.40	7.96	7.90	8.12	7.92	7.95	7.89	8.03	8.15	8.03	8.00	8.15	7.94

Figure S6. Water characteristics of the microcosms during the experimental days (from day -1 to day 4). Measured with multiparameter Horiba-U10®.

General conclusions

The main conclusions obtained after the work carried out during this research are listed below.

GENERAL CONCLUSIONS

1. Widespread pesticides contamination in ponds annex to sugarcane-dominated agroecosystems in northwest São Paulo have been detected. Traces of 10 different compounds were identified.

2. Species incidence and abundance of amphibian tadpoles in ponds from sugarcane-dominated agroecosystems are differently governed. While distance to forest fragments was the only factor driving the incidence, and also richness, of species, local pond variables, mostly those related to vegetation, were the only ones explaining their abundances.

3. The importance of maintaining ponds and native forest fragments, both endangered by sugarcane monoculture expansion, for successful amphibian conservation plans has been supported. In addition, the priority of preserving ponds with abundant stratified vegetation, with short and permeable connecting space with forest fragments, and located where runoff contamination is unlikely, has been argued.

4. First report of amphibian malformations in agricultural landscapes in Brazil, as well as first malformation baseline for amphibians in South America have been presented. This will be useful for future ecotoxicological surveys on amphibian populations inhabiting tropical agroecosystems.

5. We manage to prove that the two main analytical approaches used in escape swimming performance tests, first and best response analysis, can offer different results, thus leading to different conclusions when assessing the impact of toxicants over organisms.

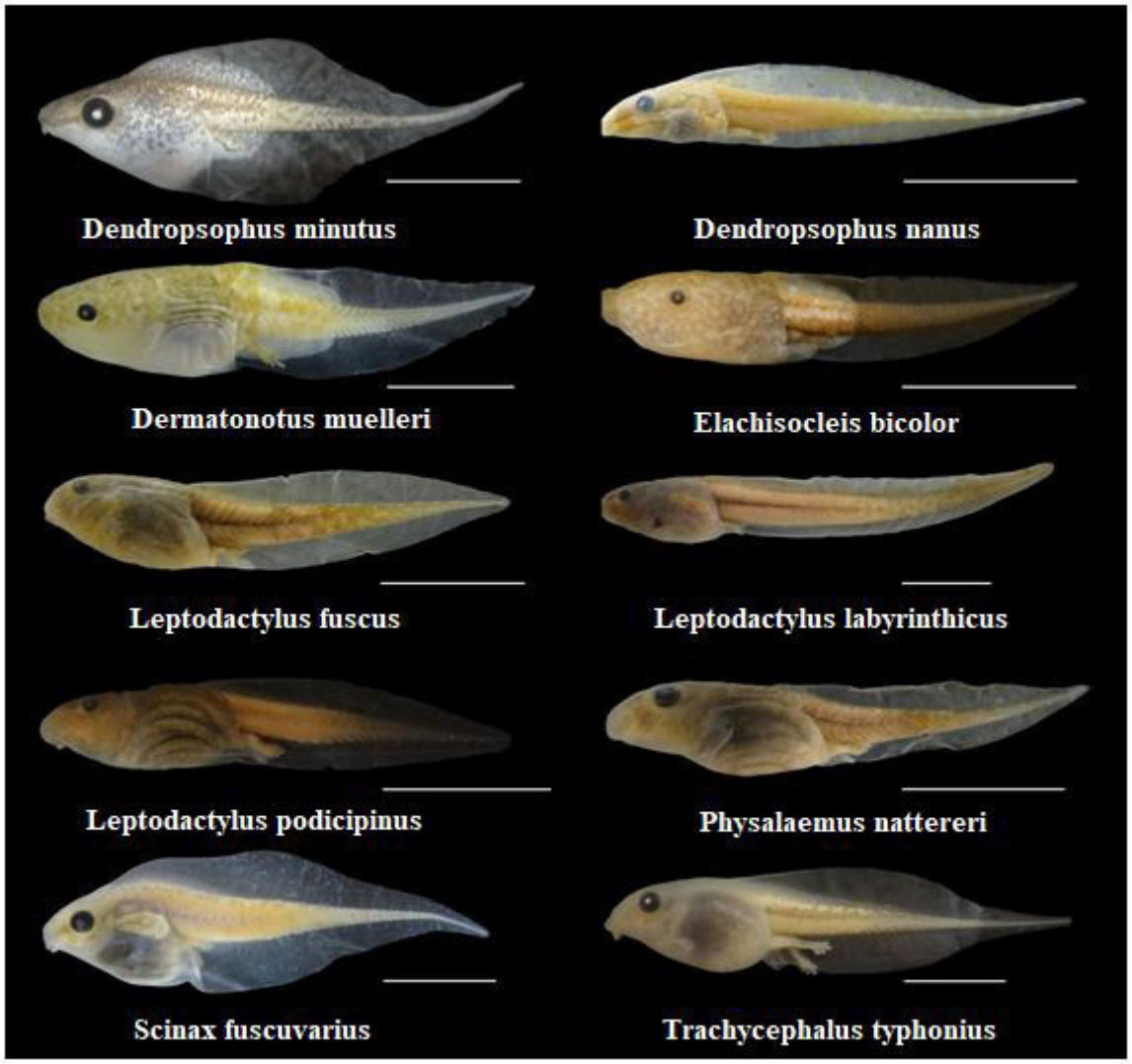
6. The sole use of acetylcholinesterase as biomarker of exposure to organophosphate insecticides in tadpoles may be underestimating the effects of environmentally relevant concentrations given that escape's performance is also affected by non-cholinergic effects.

7. Although pesticides concentrations found in ponds from the sugarcane-dominated agroecosystems surrounding São José do Rio Preto did not show any dramatical effect neither on periphyton, phytoplankton, nor amphibians, the widespread contamination detected represents a potential danger for freshwater communities inhabiting agricultural ponds. Therefore, monitoring ponds annex to sugarcane plantations is recommended in order to favor early detections of dangerous concentrations of pesticides which could endanger freshwater communities inhabiting them.

8. Well ground water from the facilities of UNESP-Ibilce (São José do Rio Preto) were contaminated by at least 9 pesticides. This is added to the widespread pesticide contamination detected at ponds from the sugarcane-dominated agroecosystem surrounding São José do Rio Preto. These findings join to the allegations of widespread pesticides contamination in Brazil.

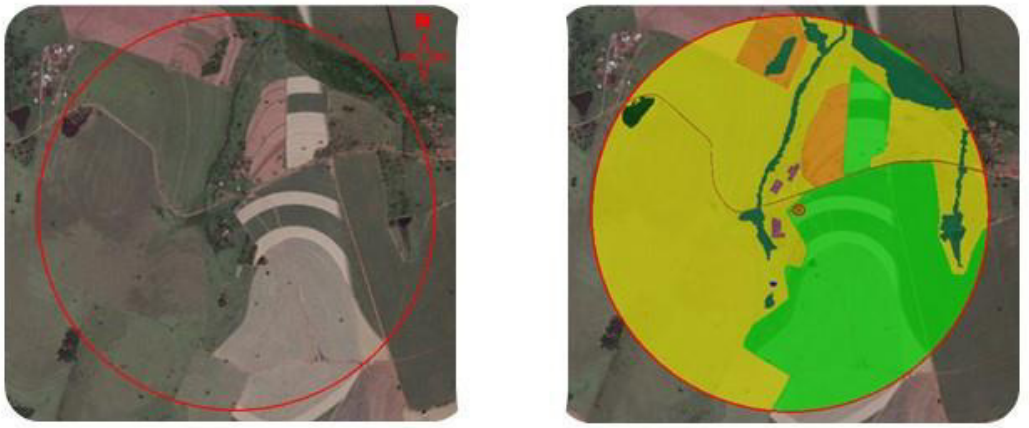
THESIS ANNEX MATERIAL

A. Tadpoles from the species sampled in the ponds from the sugarcane-dominated agroecosystem surrounding São José do Rio Preto.



B. Characteristics of the ponds from the sugarcane-dominated agroecosystem surrounding São José doo Rio Preto.

E-A



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 32.56	Paved Road: 0.00
Other cultures: 5.37	Railway: 0.00
Pasture: 54.99	Water body: 0.02
Urban: 0.35	Forest fragment: 5.82
County Road: 0.44	Forestry: 0.45

Distance to

Sugarcane: 1	Roads: 69
Urban: 65	Forest fragment: 134

Environmental variables

Area: 288.80	Depth: >100
Creep margin: 0	Creep inside: 0
Erect margin: 5	Erect inside: 5
High margin: 20	High inside: 15

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 187
E. bicolor: 0	*S. fuscovarius*: 25
L. fuscus: 239	*T. typhonius*: 0

Pesticides

Tebuconazole: <LQ	Ametrine: 179
Malation: <LQ	Diuron: 285
Tebuthiuron: 163	Atrazine-2-hydroxy: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: 236	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

E-B

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 40.90	Paved Road: 0.00
Other cultures: 0.51	Railway: 0.00
Pasture: 40.98	Water body: 0.90
Urban: 5.19	Forest Fragment: 11.24
Road: 0.28	Forestry: 0.00

Distance to

Sugarcane: 1	Roads: 886
Urban: 768	Forest fragment: 30

Environmental variables

Area: 923.54	Depth: >100
Creep margin: 30	Creep inside: 0
Erect margin: 50	Erect inside: 25
High margin: 0	High inside: 0

Tadpoles

D. nanus: 1	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 1010	*P. nattereri*: 86
E. bicolor: 0	*S. fuscovarius*: 1202
L. fuscus: 69	*T. typhonius*: 247

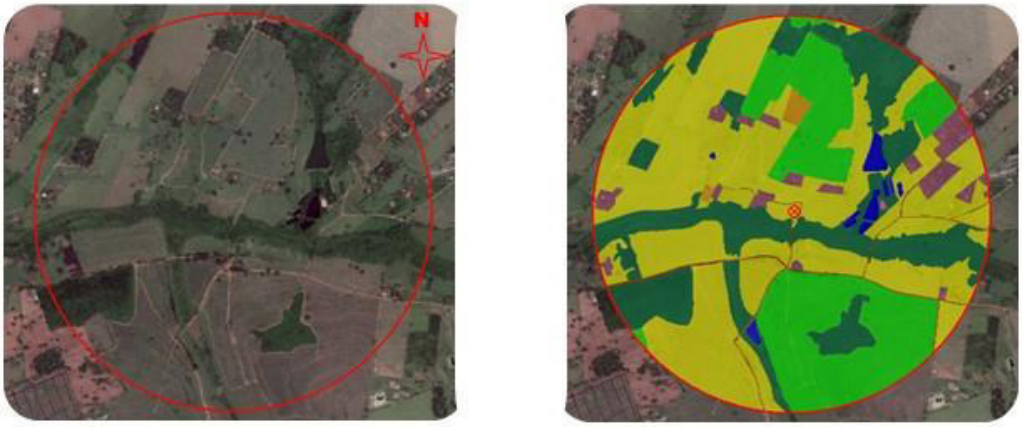
Pesticides

Tebuconazole: <LQ	Ametrine: 325
Malation: <LQ	Diuron: 434
Tebuthiuron: 271	2-hydroxyatrazine: <LD
Carbendazim: <LQ	Atrazine: <LD
Hexazinone: 1211	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

E-C



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 23.44	Paved Road:0.00
Other cultures: 0.64	Railway: 0.00
Pasture: 46.90	Water body: 1.25
Urban: 3.69	Forest Fragment: 22.83
Road: 1.25	Forestry: 0.00

Distance to

Sugarcane: 162	Roads: 25
Urban: 135	Forest fragment: 23

Environmental variables

Area: 352.81	Depth: >100
Creep margin: 15	Creep inside: 15
Erect margin: 85	Erect inside: 45
High margin: 0	High inside: 0

Tadpoles

D. nanus: 7	*L. labyrinthicus*: 0
D. minutus: 21	*L. podicipinus*: 275
D. muelleri: *1210*	*P. nattereri*: 23
E. bicolor: 17	*S. fuscovarius*: 1695
L. fuscus: 6	*T. typhonius*: 995

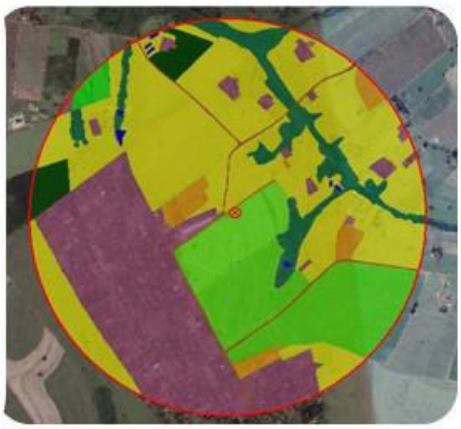
Pesticides

Tebuconazole: <LQ	Ametrine: <LQ
Malation: <LQ	Diuron: 29.5
Tebuthiuron: <LQ	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LQ	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

E-D



Sugarcane culture
Other cultures
Pasture
Urban
Road
Water body
Forest fragment
Forestry
Studied pond

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 18.50	Paved Road: 0.00
Other cultures: 2.83	Railway: 0.00
Pasture: 41.44	Water body: 0.27
Urban: 26.18	Forest Fragment: 6.92
Road: 1.27	Forestry: 2.59

Distance to

Sugarcane: 1	Roads: 15
Urban: 28	Forest fragment: 301

Environmental variables

Area: 286.37	Depth: 86
Creep margin: 0	Creep inside: 0
Erect margin: 80	Erect inside: 30
High margin: 5	High inside: 5

Tadpoles

D. nanus: 1	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 1
D. muelleri: 92	*P. nattereri*: 498
E. bicolor: 10	*S. fuscovarius*: 175
L. fuscus: 443	*T. typhonius*: 0

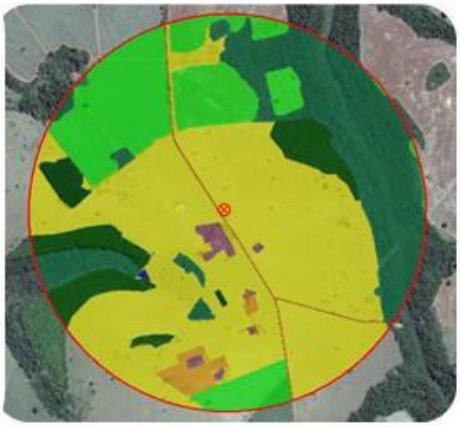
Pesticides

Tebuconazole: <LQ	Ametrine: 34.2
Malation: <LQ	Diuron: <LQ
Tebuthiuron: 35.3	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LQ	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

N-A



Sugarcane culture
Other cultures
Pasture
Urban
Road
Water body
Forest fragment
Forestry
Studied pond

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 18.38	Paved Road: 0.00
Other cultures: 1.58	Railway: 0.00
Pasture: 50.30	Water body: 0.04
Urban: 1.03	Forest Fragment: 20.08
Road: 0.64	Forestry: 7.95

Distance to

Sugarcane: 430	Roads: 22
Urban: 96	Forest fragment: 437

Environmental variables

Area: 128.61	Depth: 51
Creep margin: 0	Creep inside: 0
Erect margin: 100	Erect inside: 4
High margin: 0	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 1380
E. bicolor: 0	*S. fuscovarius*: 2244
L. fuscus: 52	*T. typhonius*: 0

Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: 3.86

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

N-B

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 2.06	Paved Road: 1.40
Other cultures: 5.46	Railway: 0.00
Pasture: 47.18	Water body: 0.39
Urban: 27.74	Forest Fragment: 12.16
Road: 0.42	Forestry: 3.17

Distance to

Sugarcane: 899	Roads: 17
Urban: 165	Forest fragment: 246

Environmental variables

Area: 149.49	Depth: 79
Creep margin: 70	Creep inside: 0
Erect margin: 30	Erect inside: 40
High margin: 0	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 375
E. bicolor: 0	*S. fuscovarius*: 2011
L. fuscus: 16	*T. typhonius*: 0

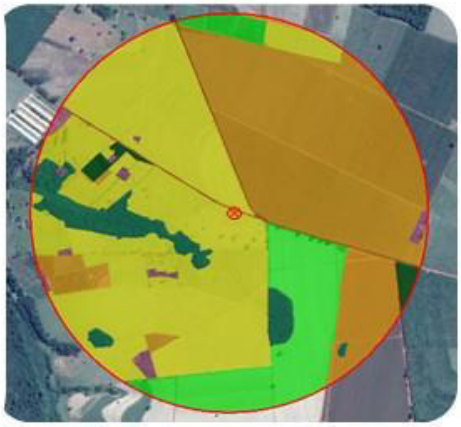
Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 1	Microphtahlmia: 1
Aphakia: 0	Underdevelopment: 1

N-C



Sugarcane culture
Other cultures
Pasture
Urban
Road
Water body
Forest fragment
Forestry
Studied pond

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 13.01	Paved Road: 0.00
Other cultures: 31.17	Railway: 0.00
Pasture: 42.74	Water body: 0.00
Urban: 1.35	Forest Fragment: 5.36
Road: 0.93	Forestry: 5.43

Distance to

Sugarcane: 193	Roads: 24
Urban: 390	Forest fragment: 202

Environmental variables

Area: 260.88	Depth: 68
Creep margin: 100	Creep inside: 0
Erect margin: 0	Erect inside: 50
High margin: 0	High inside: 0

Tadpoles

D. nanus: 41	*L. labyrinthicus*: 0
D. minutus: 5	*L. podicipinus*: 0
D. muelleri: 699	*P. nattereri*: 77
E. bicolor: 0	*S. fuscovarius*: 3064
L. fuscus: 5	*T. typhonius*: 0

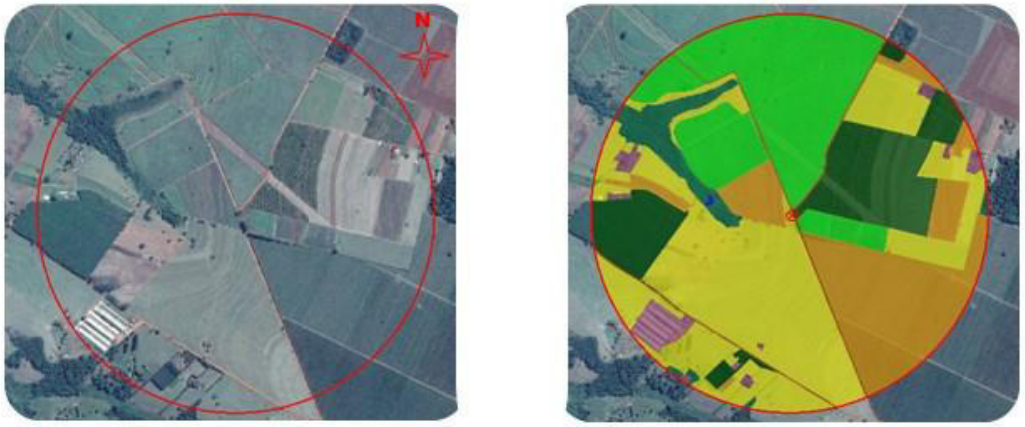
Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 1
Aphakia: 1	Underdevelopment: 1

N-D



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 23.07	Paved Road: 0.00
Other cultures: 18.63	Railway: 0.00
Pasture: 35.27	Water body: 0.05
Urban: 2.61	Forest Fragment: 4.07
Road: 1.31	Forestry: 15.00

Distance to

Sugarcane: 35	Roads: 10
Urban: 660	Forest fragment: 238

Environmental variables

Area: 317.77	Depth: 51
Creep margin: 0	Creep inside: 0
Erect margin: 100	Erect inside: 60
High margin: 0	High inside: 0

Tadpoles

D. nanus: 25	*L. labyrinthicus*: 0
D. minutus: 17	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 51
E. bicolor: 0	*S. fuscovarius*: 776
L. fuscus: 0	*T. typhonius*: 0

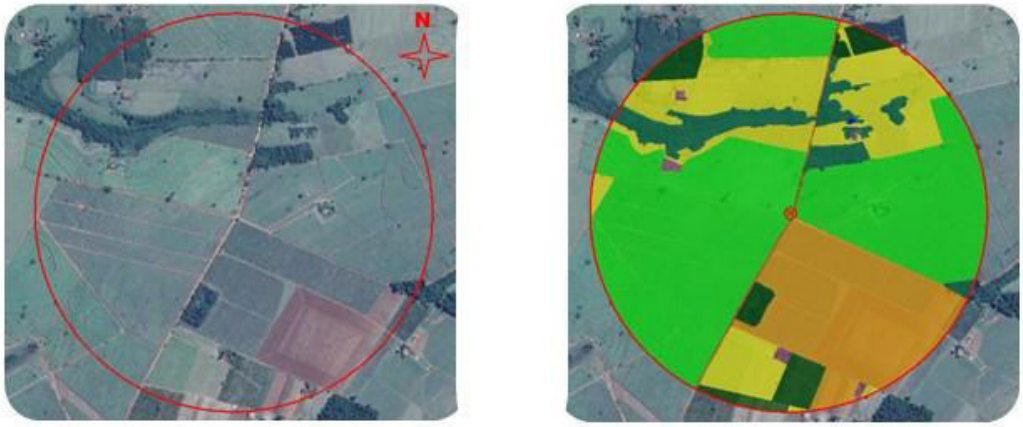
Pesticides

Tebuconazole: <LD	Ametrine: 4.74
Malation: <LD	Diuron: 3.04
Tebuthiuron: 4.92	2-hydroxyatrazine: 3.80
Carbendazim: <LD	Atrazine: 3.53
Hexazinone: 3.95	Imidacloprid: 3.89

Eye malforations

Anophthalmia: 3	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

N-E



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 48.04	Paved Road: 0.00
Other cultures: 18.59	Railway: 0.00
Pasture: 20.40	Water body: 0.02
Urban: 0.57	Forest Fragment: 6.50
Road: 0.66	Forestry: 5.21

Distance to

Sugarcane: 1	Roads: 16
Urban: 489	Forest fragment: 203

Environmental variables

Area: 220.49	Depth: 73
Creep margin: 0	Creep inside: 0
Erect margin: 15	Erect inside: 40
High margin: 0	High inside: 0

Tadpoles

D. nanus: 83	*L. labyrinthicus*: 0
D. minutus: 60	*L. podicipinus*: 0
D. muelleri: 6	*P. nattereri*: 17
E. bicolor: 0	*S. fuscovarius*: 1649
L. fuscus: 8	*T. typhonius*: 0

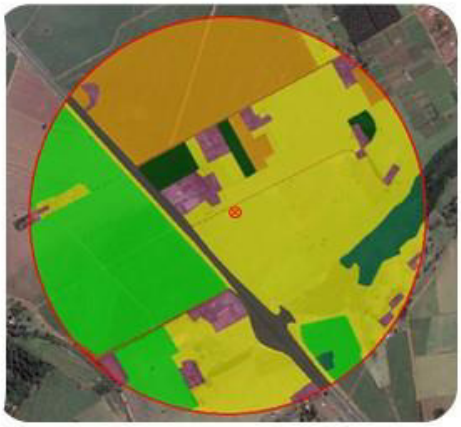
Pesticides

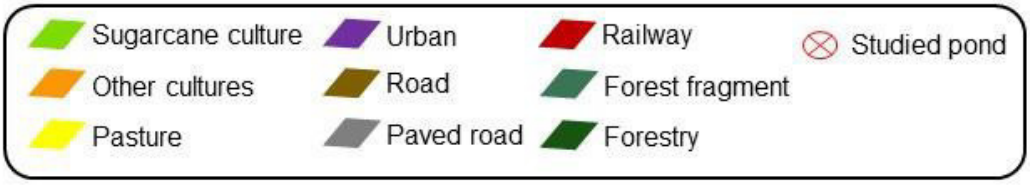
Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 4	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

NO-A





Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 25.95	Paved Road: 3.91
Other cultures: 18.34	Railway: 0.05
Pasture: 40.16	Water body: 0.00
Urban: 5.51	Forest Fragment: 3.01
Road: 0.82	Forestry: 2.26

Distance to

Sugarcane: 243	Roads: 23
Urban: 68	Forest fragment: 607

Environmental variables

Area: 317.07	Depth: >100
Creep margin: 85	Creep inside: 20
Erect margin: 0	Erect inside: 0
High margin: 0	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 52	*P. nattereri*: 1746
E. bicolor: 2	*S. fuscovarius*: 2242
L. fuscus: 53	*T. typhonius*: 0

Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 1
Aphakia: 0	Underdevelopment: 1

NO-B

Sugarcane culture | Urban | Water body | Forest fragment
Other cultures | Road | Railway | Studied pond
Pasture | Paved road | Forestry

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 34.78	Paved Road: 2.13
Other cultures: 5.41	Railway: 1.77
Pasture: 42.89	Water body: 0.25
Urban: 5.67	Forest Fragment: 3.82
Road: 1.29	Forestry: 2.00

Distance to

Sugarcane: 17	Roads: 92
Urban: 84	Forest fragment: 187

Environmental variables

Area: 185.13	Depth: >100
Creep margin: 0	Creep inside: 0
Erect margin: 100	Erect inside: 40
High margin: 0	High inside: 0

Tadpoles

D. nanus: 12	*L. labyrinthicus*: 0
D. minutus: 27	*L. podicipinus*: 0
D. muelleri: 11	*P. nattereri*: 129
E. bicolor: 0	*S. fuscovarius*: 1746
L. fuscus: 4	*T. typhonius*: 0

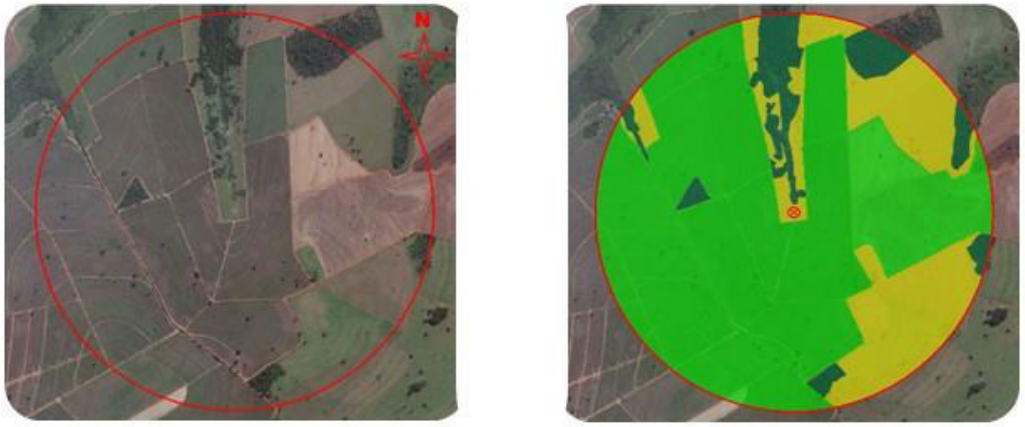
Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 1	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

NO-C



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 68.73	Paved Road: 0.00
Other cultures: 0.00	Railway: 0.00
Pasture: 22.77	Water body: 0.00
Urban: 0.00	Forest Fragment: 8.50
Road: 0.00	Forestry: 0.00

Distance to

Sugarcane: 50	Roads: 1387
Urban: 1478	Forest fragment: 34

Environmental variables

Area: 465.82	Depth: 72
Creep margin: 0	Creep inside: 0
Erect margin: 95	Erect inside: 50
High margin: 0	High inside: 0

Tadpoles

D. nanus: 39	*L. labyrinthicus*: 0
D. minutus: 7	*L. podicipinus*: 107
D. muelleri: 54	*P. nattereri*: 301
E. bicolor: 0	*S. fuscovarius*: 5948
L. fuscus: 0	*T. typhonius*: 0

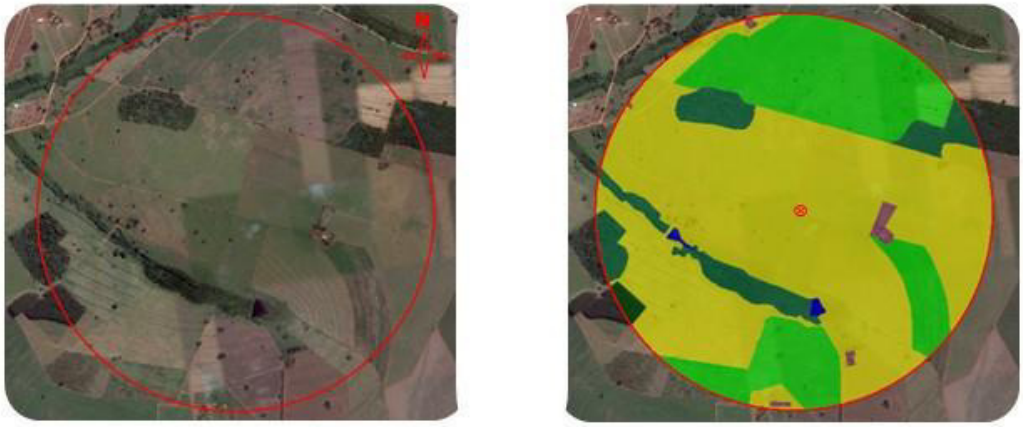
Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 1	Underdevelopment: 1

NO-D



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 27.62	Paved Road: 0.00
Other cultures: 0.00	Railway: 0.00
Pasture: 62.39	Water body: 0.30
Urban: 0.77	Forest Fragment: 8.35
Road: 0.00	Forestry: 0.57

Distance to

Sugarcane: 402	Roads: 1050
Urban: 400	Forest fragment: 387

Environmental variables

Area: 158.60	Depth: 65
Creep margin: 35	Creep inside: 15
Erect margin: 0	Erect inside: 0
High margin: 0	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 27	*P. nattereri*: 838
E. bicolor: 0	*S. fuscovarius*: 1956
L. fuscus: 21	*T. typhonius*: 0

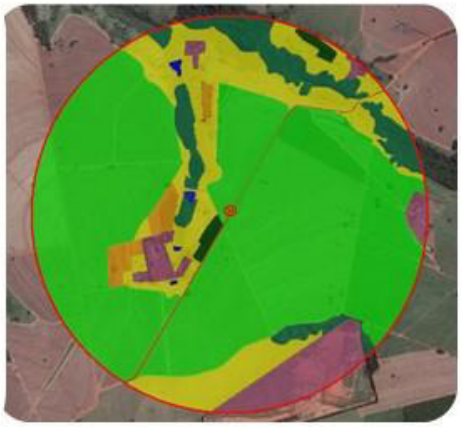
Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 4
Aphakia: 1	Underdevelopment: 0

NO-E



Sugarcane culture
Other cultures
Pasture
Urban
Road
Water body
Forest fragment
Forestry
Studied pond

Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 63.79	Paved Road: 0.00
Other cultures: 1.90	Railway: 0.00
Pasture: 17.95	Water body: 0.22
Urban: 6.99	Forest Fragment: 7.75
Road: 0.53	Forestry: 0.87

Distance to

Sugarcane: 1	Roads: 1
Urban: 307	Forest fragment: 178

Environmental variables

Area: 166.16	Depth: 72
Creep margin: 0	Creep inside: 0
Erect margin: 35	Erect inside: 10
High margin: 20	High inside: 10

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 2	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 1915
E. bicolor: 0	*S. fuscovarius*: 244
L. fuscus: 32	*T. typhonius*: 0

Pesticides

Tebuconazole: <LD	Ametrine: <LD
Malation: <LD	Diuron: <LD
Tebuthiuron: <LD	2-hydroxyatrazine: 2.94
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: 3.33

Eye malforations

Anophthalmia: 1	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

SO-A

Data of the image: 10/28/2015
Coordinates: Pasture

Land use

Sugarcane: 4.21	Paved Road: 1.46
Other cultures: 1.05	Railway: 0.00
Pasture: 67.69	Water body: 0.00
Urban: 21.75	Forest Fragment: 2.60
Road: 0.74	Forestry: 0.50

Distance to

Sugarcane: 659	Roads: 23
Urban: 29	Forest fragment: 845

Environmental variables

Area: 362.87	Depth: 77
Creep margin: 80	Creep inside: 0
Erect margin: 0	Erect inside: 15
High margin: 0	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 180
E. bicolor: 0	*S. fuscovarius*: 803
L. fuscus: 68	*T. typhonius*: 0

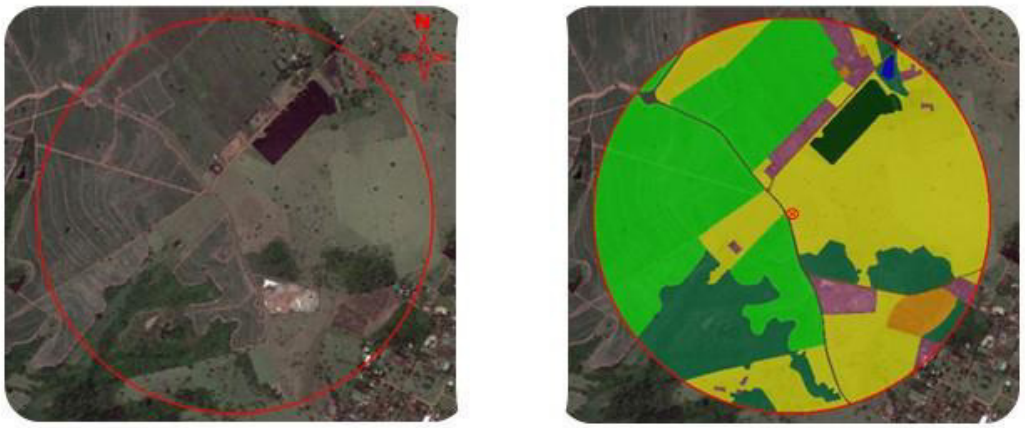
Pesticides

Tebuconazole: <LD	Ametrine: 24.2
Malation: <LQ	Diuron: <LQ
Tebuthiuron: 22.6	2-hydroxyatrazine: <LD
Carbendazim: 23.2	Atrazine: <LD
Hexazinone: <LQ	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

SO-B



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 35.47	Paved Road: 0.74
Other cultures: 1.95	Railway: 0.00
Pasture: 38.31	Water body: 0.19
Urban: 5.16	Forest Fragment: 15.18
Road: 0.48	Forestry: 2.52

Distance to

Sugarcane: 25	Roads: 15
Urban: 230	Forest fragment: 104

Environmental variables

Area: 131.87	Depth: >100
Creep margin: 40	Creep inside: 0
Erect margin: 60	Erect inside: 50
High margin: 0	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 1
D. minutus: 59	*L. podicipinus*: 0
D. muelleri: 3	*P. nattereri*: 308
E. bicolor: 0	*S. fuscovarius*: 3000
L. fuscus: 30	*T. typhonius*: 0

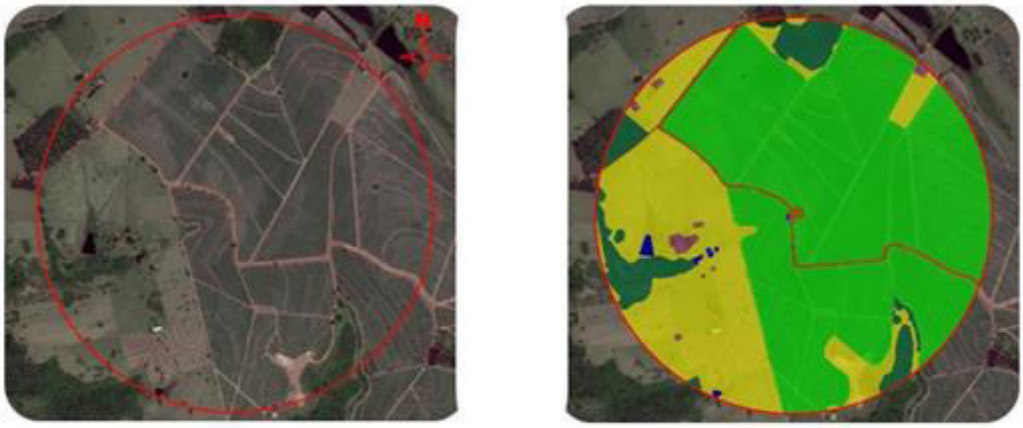
Pesticides

Tebuconazole: <LQ	Ametrine: <LQ
Malation: <LQ	Diuron: <LQ
Tebuthiuron: 22.2	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LQ	Imidacloprid: <LD

Eye malforations

Anophthalmia: 2	Microphtahlmia: 0
Aphakia: 1	Underdevelopment: 0

SO-C



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 61.05	Paved Road: 0.00
Other cultures: 0.00	Railway: 0.00
Pasture: 29.16	Water body: 0.34
Urban: 0.68	Forest Fragment: 6.97
Road: 1.61	Forestry: 0.19

Distance to

Sugarcane: 1	Roads: 1
Urban: 454	Forest fragment: 554

Environmental variables

Area:	Depth:
Creep margin:	Creep inside:
Erect margin:	Erect inside:
High margin:	High inside:

Tadpoles

D. nanus: 1	*L. labyrinthicus*: 0
D. minutus: 1	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 195
E. bicolor: 0	*S. fuscovarius*: 1091
L. fuscus: 124	*T. typhonius*: 0

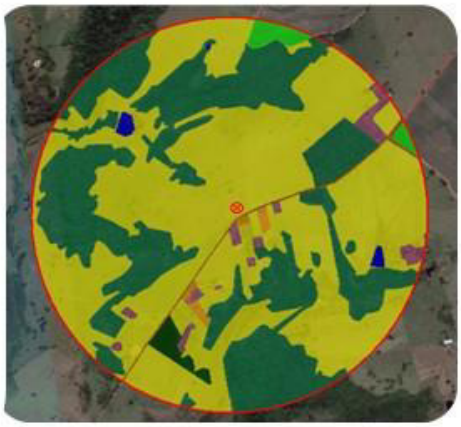
Pesticides

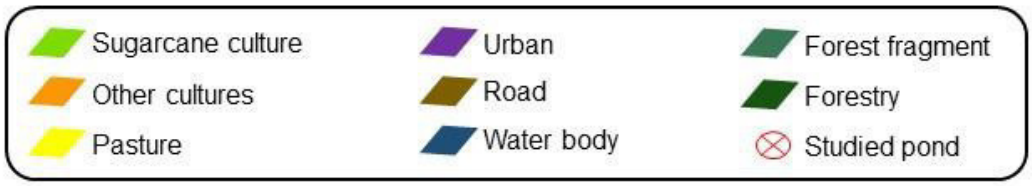
Tebuconazole:	Ametrine:
Malation:	Diuron:
Tebuthiuron:	2-hydroxyatrazine:
Carbendazim:	Atrazine:
Hexazinone:	Imidacloprid:

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 2

SO-D





Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 1.27	Paved Road: 0.00
Other cultures: 0.58	Railway: 0.00
Pasture: 59.09	Water body: 0.45
Urban: 1.83	Forest Fragment: 35.12
Road: 0.66	Forestry: 0.99

Distance to

Sugarcane: 846	Roads: 43
Urban: 50	Forest fragment: 190

Environmental variables

Area: 389.63	Depth: 64
Creep margin: 60	Creep inside: 0
Erect margin: 20	Erect inside: 10
High margin: 10	High inside: 0

Tadpoles

D. nanus: 0	*L. labyrinthicus*: 0
D. minutus: 0	*L. podicipinus*: 0
D. muelleri: 0	*P. nattereri*: 2921
E. bicolor: 0	*S. fuscovarius*: 255
L. fuscus: 24	*T. typhonius*: 2

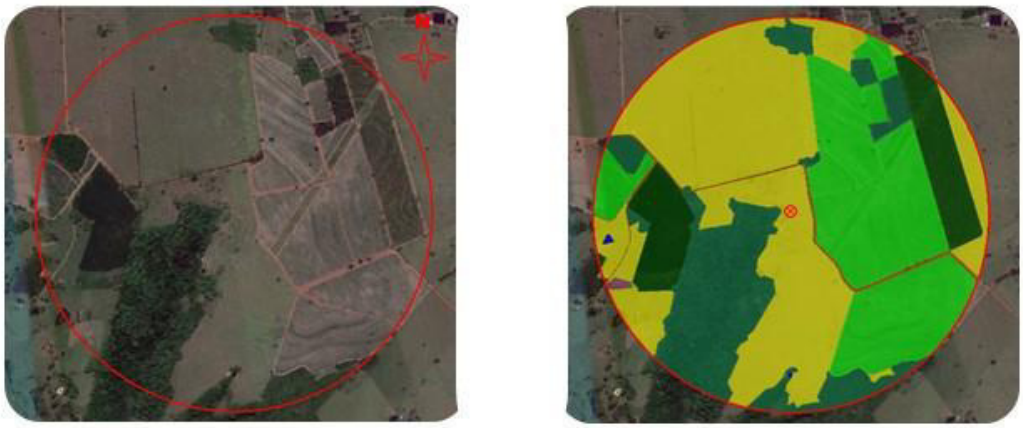
Pesticides

Tebuconazole: <LQ	Ametrine: <LQ
Malation: <LQ	Diuron: <LQ
Tebuthiuron: <LQ	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 0	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

SO-E



Data of the image: 10/28/2015

Coordinates:

Land use

Sugarcane: 28.25	Paved Road: 0.00
Other cultures: 0.00	Railway: 0.00
Pasture: 41.79	Water body: 0.07
Urban: 0.18	Forest Fragment: 18.63
Road: 1.18	Forestry: 9.91

Distance to

Sugarcane: 130	Roads: 115
Urban: 860	Forest fragment: 30

Environmental variables

Area: 172.14	Depth: 75
Creep margin: 0	Creep inside: 0
Erect margin: 70	Erect inside: 10
High margin: 0	High inside: 0

Tadpoles

D. nanus: 5	*L. labyrinthicus*: 0
D. minutus: 3	*L. podicipinus*: 0
D. muelleri: 98	*P. nattereri*: 2539
E. bicolor: 0	*S. fuscovarius*: 1525
L. fuscus: 136	*T. typhonius*: 503

Pesticides

Tebuconazole: <LD	Ametrine: <LQ
Malation: <LQ	Diuron: <LQ
Tebuthiuron: <LQ	2-hydroxyatrazine: <LD
Carbendazim: <LD	Atrazine: <LD
Hexazinone: <LD	Imidacloprid: <LD

Eye malforations

Anophthalmia: 1	Microphtahlmia: 0
Aphakia: 0	Underdevelopment: 0

C. Analysis report of the microcosms water samples.

Instituto de Química
Universidade Estadual de Campinas
Profa. Cassiana C. Montagner Raimundo
Laboratório de Química Ambiental – LQA
montagner@iqm.unicamp.br

Campinas, 03 de abril de 2017.

Resultado das análises das amostras de março/2017

Interessado: Eduardo Alves de Almeida
Endereço: Química e Ciências Ambientais, IBILCE – UNESP, Av Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, SP, Brasil

Data de recebimento das amostras: 16/03/2017
Quantidade de Amostra: 28
Matriz: Água
Coleta: As coletas foram feitas pelo interessado e os cartuchos contendo os analitos foram recebidos no LQA nas datas acima citadas.

PREPARO DE AMOSTRAS

A eluição dos compostos foi feita a 8 mL min^{-1} em um *manifold* à vácuo de 12 portas (PrepSep - Fisher Scientific) por meio da adição de 4 mL de metanol e 4 mL de acetonitrila em cada cartucho de extração. Cada eluato foi recolhido em tubo de ensaio com tampa de PTFE (Teflon® - politetrafluoretileno). Cada tubo de ensaio foi completamente seco, utilizando fluxo constante de N_2. Ao tubo seco foi adicionado uma solução de H_2O:MeOH 70:30 (v/v), com volume específico para cada amostra, permitindo ao final do processo um fator de concentração de 1000 vezes. O tubo de ensaio foi agitado vigorosamente, utilizando um Vortex. O volume foi transferido quantitativamente para um filtro de seringa (nylon 0,22 µm) e por fim para um *vial* de 2,0 mL de capacidade munido de tampa com septo (Agilent).

QUANTIFICAÇÃO DOS ANALITOS

A quantificação dos compostos alvo foi realizada por cromatografia líquida acoplada à espectrometria de massas em tandem (LC-MS/MS). Foi utilizado um cromatógrafo Agilent modelo 1200, equipado com bomba binária, injetor automático e compartimento de coluna termostatizado. A separação cromatográfica foi realizada com uma coluna Zorbax SB-C18 (2,1x30 mm, tamanho de partícula de 3,5 µm) a 25°C. A fase móvel foi constituída de água ultrapura (A) e metanol (B), previamente filtrados em membranas com 0,2 µm de porosidade, contendo 5 mM de formiato de amônio, aditivo esse que favorece à formação de íons. A composição do gradiente, em função da concentração do solvente B, foi a seguinte: início com 30% e aumento para 60% em 3 minutos e por fim subir para 67% em 10 minutos. Entre cada corrida cromatográfica o sistema foi mantido à 30% de B por 5 minutos para recondicionamento da coluna. A identificação e a quantificação dos compostos foram realizadas por espectrometria de massas em um equipamento Agilent com triplo quadrupolo (modelo 6410B). Os compostos foram ionizados em uma fonte de *electrospray* no modo positivo, e foram monitorados pelo modo MRM (*MultipleReactionMonitoring*), de acordo com os parâmetros descritos na Tabela 1. As curvas analíticas foram construídas de acordo com a área obtida para cada composto em função de sua massa na coluna.

Tabela 1 – Transições precursor-produto e as respectivas energias de colisão (EC) selecionadas para a quantificação dos 13 íons empregando o modo MRM do espectrômetro de massas.

Composto	*Polaridade*	*Fragmentor (V)*	*Precursor*	*Quantificação*		*Confirmação 1*		*Confirmação 2*	
			(m/z)	*m/z*	*EC (V)*	*m/z*	*EC (V)*	*m/z*	*EC (V)*
Ametrina	+	100	228,2	186	15	158	20	138	20
Azoxistrobina	+	100	404,2	372	5	344,1	20	-	-
2 hidroxiatrazina	+	100	198,2	156,2	15	114,1	20	86,1	20
Carbendazin	+	100	192,1	160	20	132	30	105	35
Carbofurano	+	100	222	165	10	123	20	55	16
Clomazona	+	100	240,1	125	1	-	-	-	-
Diuron	+	100	233 / 235	72,1	20	46	16	72,1	20
Hexazinona	+	100	253,2	171	8	71,1	31	85,1	30
Imidacloprido	+	100	256	175	15	209	10	-	-
Malation	+	100	331	99	15	285	1	-	-
Simazina	+	100	202	124	15	132	15	104	25
Tebuconazole	+	100	308,2	70	20	70	10	125	30
Tebutiuron	+	110	229,1	172	10	116	30	57,2	34

*m/z ± 0,1

Resultados

Os limites de detecção e quantificação, para cada composto, estão destacados na Tabela 2. Os resultados obtidos para as amostras analisadas encontram-se na Tabela 3 a 5.

Tabela 2 – Limites de detecção e quantificação e o coeficiente de determinação (r^2) para cada composto analisado

Composto	Limite de detecção (ng L^{-1})	Limite de quantificação (ng L^{-1})	r^2
Imidacloprido	2,1	6,1	0,998
Carbendazim	0,9	2,8	0,999
Simazina	2,7	8,3	0,997
Carbofurano	1,6	4,9	0,999
Hexazinona	1,4	4,1	0,999
Tebutiuron	2,0	6,0	0,998
Diuron	1,1	3,4	0,999
Clomazona	2,5	7,5	0,998
Ametrina	1,7	5,2	0,999
Malation	1,8	5,3	0,999
Tebuconazole	1,6	4,8	0,999
Azoxistrobina	1,9	5,7	0,998
2 hidroxiatrazina	2,2	6,7	0,998

Tabela 3 – Concentrações dos compostos alvo determinados nas amostras em nanograma do composto por litro de amostra (ng L^{-1}).

Compostos	Amostras								
	C1 dia1	C2 dia1	C3 dia 1	C4 dia 1	H1 dia 1	H1 dia 2	H1 dia 3	H2 dia 1	H2 dia 2
Imidacloprido	10,9	9,6	8,8	9,4	11,5	12,3	12,0	9,4	8,9
Carbendazim	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
2-Hydroxy Atrazine	7,2	10,2	7,1	7,4	11,9	15,8	24,7	8,8	10,9
Hexazinona	<LD	<LQ	<LD	<LD	886	894	821	938	886
Tebutiuron	<LQ	<LQ	<LQ	<LQ	220	236	221	252	231
Diuron	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Clomazona	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Ametrina	<LQ	<LD	<LD	<LD	96	58	24	104	56
Azoxistrobina	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Tebuconazole	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Simazina	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Malation	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Carbofurano	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD

<LD: compostos abaixo do limite de detecção

<LQ: compostos abaixo do limite de quantificação
Em vermelho: fora da faixa linear

Tabela 4 – Concentrações dos compostos alvo determinados nas amostras em nanograma do composto por litro de amostra (ng L^{-1}).

Compostos	Amostras								
	H2 dia 3	H3 dia 1	H3 dia 2	H3 dia 3	H4 dia 1	H4 dia 2	H4 dia 3	L1 dia 1	L1 dia 2
Cafeina	19,0	19,1	18,1	27,4	40	64	44	25	37
Imidacloprido	8,9	10,8	10,7	11,3	11,5	10,9	9,9	10,5	11,2
Carbendazim	<LQ	<LQ	<LQ	<LQ	3,0	2,9	<LQ	<LQ	<LQ
2-Hydroxy Atrazine	16,1	12,0	17,6	32,3	11,6	25	40	10,0	11,2
Hexazinona	886	873	812	818	904	856	801	5,5	5,5
Tebutiuron	232	219	200	208	240	218	204	6,6	6,1
Diuron	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Clomazona	<LD	<LQ	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Ametrina	28,1	96	38,2	13,5	95	<LQ	<LQ	<LQ	<LQ
Azoxistrobina	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Tebuconazole	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Simazina	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Malation	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Carbofurano	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD

<LD: compostos abaixo do limite de detecção
<LQ: compostos abaixo do limite de quantificação
Em vermelho: fora da faixa linear

Tabela 5 – Concentrações dos compostos alvo determinados nas amostras em nanograma do composto por litro de amostra (ng L^{-1}).

Compostos	Amostras									
	L1 dia 3	L2 dia 1	L2 dia 2	L2 dia 3	L3 dia 1	L3 dia 2	L3 dia 3	L4 dia 1	L4 dia 2	L4 dia 3
Imidacloprido	10,2	10,7	10,3	11,1	8,8	8,9	9,4	11,4	12,1	12,0
Carbendazim	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	2,8	2,8	3,2
2-Hydroxy Atrazine	11,2	8,4	8,8	8,6	9,5	10,7	10,7	13,0	14,5	16,1
Hexazinona	5,0	6,3	5,5	5,7	5,1	5,0	4,8	5,6	5,8	6,3
Tebutiuron	<LQ	7,2	7,1	7,5	6,0	<LQ	6,1	7,7	8,6	8,6
Diuron	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Clomazona	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LQ	<LD	<LQ
Ametrina	<LQ	<LQ	<LQ	<LD	<LQ	<LD	<LD	<LQ	<LQ	<LD
Azoxistrobina	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Tebuconazole	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Simazina	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Malation	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Carbofurano	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD

<LD: compostos abaixo do limite de detecção
<LQ: compostos abaixo do limite de quantificação

D. QR code. Scan to see a demonstration video of the escape swimming performance test performed for thesis chapters 3 and 4.