UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Rinneu Elias Borges

Avaliação anatomofuncional e biomarcadores genotóxicos de girinos coletados em áreas agrícolas e em reserva biológica, na região sudoeste do estado de Goiás

Biomarcadores Genotóxicos em Anuros

São José do Rio Preto
2019

Rinneu Elias Borges

Avaliação anatomofuncional e biomarcadores genotóxicos de girinos coletados em áreas agrícolas e em reserva biológica, na região sudoeste do estado de Goiás

Biomarcadores Genotóxicos em Anuros

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Orientador: Prof. Dr. Classius de Oliveira

São José do Rio Preto
2019

B732a

Borges, Rinneu Elias

Avaliação anatomofuncional e biomarcadores genotóxicos de girinos coletados em áreas agrícolas e em reserva biológica, na região sudoeste do estado de Goiás : biomarcadores genotóxicos em anuros / Rinneu Elias Borges. -- São José do Rio Preto, 2019

80 p. : tabs., fotos, mapas

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto

Orientador: Classius de Oliveira

1. Anfíbios. 2. Anura. 3. Genotoxicidade. 4. Malfomações. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

Rinneu Elias Borges

Avaliação anatomofuncional e biomarcadores genotóxicos de girinos coletados em áreas agrícolas e em reserva biológica, na região sudoeste do estado de Goiás

Biomarcadores Genotóxicos em Anuros

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Comissão Examinadora

Prof. Dr. Classius de Oliveira
UNESP – Câmpus de São José do Rio Preto
Orientador

Prof. Dr. Rodrigo Zieri
IFSP - Câmpus Barretos

Profª. Drª. Monica Jones Costa
UFScar – Câmpus Sorocaba

Profª. Drª. Rafaela Maria Moresco
Unioeste – Câmpus Curitiba

Prof. Dr. Vitor Hugo Mendonça do Prado
UEG – Câmpus Anápolis

São José do Rio Preto
18 de abril de 2019

Dedicatória

Agradeço a meus pais, Ivair Camilo Borges e Izabel Secco Borges por incentivar os estudos e mostrar o melhor caminho. A minha esposa Lia Raquel, e aos meus filhos Rafael Santos Secco Borges e Gabriel Santos Secco Borges.

.

Agradecimentos

Agradeço a minha esposa, Lia Raquel, pelo apoio e orientações durante toda a caminhada durante o meu doutorado.

Aos meus filhos Rafael Santos Secco Borges e Gabriel Santos Secco Borges pelo carinho de sempre renovar minhas forças, passando boas energias.

Ao meu irmão Ricardo Luiz Borges;

Meu grande tio Ivo, *in memórian*, esse sempre meu parceiro, muita saudades, gostaria muito que ele estivesse aqui para ver que conseguimos.

Ao meu orientador Prof. Classius, por ter confiado e me dado a oportunidade de poder realizar esse sonho.

Meus colegas de serviço de campo e laboratório: Marcelino, Rhayane, Reyla, Richelle, Wadson, Itamar, Marco Antonio, Tainã, Quemuel, Eloisa, Bruno.

Aos Pesquisadores Marcelino Benvindo e Rhayane Alves pela ajuda de campo e nos manuscritos.

Aos pesquisadores e amigos Rodrigo Zieri e Lilian Belussi pelas orientações e ajuda com os trabalhos.

Minha ex-orientadora Denise Rossa-Feres, por ter mostrado como é apaixonante trabalhar com girinos.

Aos amigos Celso, Rodrigo Braguiroli e Ricardo Goulart pelo companheirismo, cachaças e conselhos.

Aos colegas professores e funcionários da Universidade de Rio Verde.

A todos os meus familiares.

RESUMO

A crescente demanda para produções agrícolas e o consequente uso de agrotóxicos nas lavouras, vem se tornando foco das atenções sobre o uso e as conseqüências destes contaminantes químicos ambientais. Utilizando-se de anuros coletados em ambientes agrícolas e em uma Unidade de Conservação, o Parque Nacional das Emas (PNE), ambos região Sudoeste do Estado de Goiás, investigou-se a influência de contaminantes de origem agrícola nos aspectos anatomorfológicos em larvas de anuros bem como as possíveis alterações genótoxicas destes compostos. Para isto, foram coletadas larvas de anuros de 21 espécies de área agrícola e nove da Unidade de Conservação, em diferentes ambientes. Foram realizadas análises de malformações no padrão morfológico (27 espécies), bem como análises de alterações eritrocitárias em três espécies. Os resultados demonstraram alta porcentagem de anormalidades em anuros provenientes de agroecossistemas, quando comparados aos de ambiente natural. Entretanto, as análises de alterações eritrocitárias nucleares nas larvas não mostram significância para a formação de micronúcleo em girinos entre os ambientes estudados. No entanto foi registrada uma maior frequência de células reniformes em girinos de ambiente antropizado. Portanto, nosso estudo aponta que girinos associados a ambientes agrícolas são mais suscetíveis a algum tipo de alteração morfológica, o que reforça a importância da preservação de áreas originais e ainda um olhar mais critico quanto ao uso de pesticidas em lavouras.

Palavras-chave: Anfíbios. Anura. Genotoxicidade. Malfomações.

ABSTRACT

The growing demand for agricultural production and the consequent use of agrochemicals in crops has become a focus of attention on the use and consequences of these environmental chemical contaminants. Using anurans collected in agricultural environments and in a Conservation Unit, the Emas National Park (PNE), both Southwest region of the State of Goiás, investigated the influence of contaminants of agricultural origin on the anatomorphological aspects in anuran larvae as well as the possible genotoxic changes of these compounds. For this, anuran larvae were collected from 21 species of agricultural area and nine from the Conservation Unit, in different environments. Analyzes of malformations in the morphological pattern (27 species) were carried out, as well as analyzes of erythrocyte alterations in three species. The results showed a high percentage of anurans abnormalities from agroecosystems when compared to those from the natural environment. However, the analyzes of nuclear erythrocyte alterations in the larvae do not show significance for the formation of micronucleus in tadpoles between the studied environments. However, a higher frequency of reniform cells was recorded in anthropic environment tadpoles. Therefore, our study indicates that tadpoles associated with agricultural environments are more susceptible to some type of morphological alteration, which reinforces the importance of the preservation of original areas and a more critical view regarding the use of pesticides in crops.

Keywords: Amphibians. Anura. Genotoxicity. Malformations.

LISTA DE ILUSTRAÇÕES

Figura 1	Áreas de amostragem dos girinos	16
Figura 2	Análise morfológica e triagem	17
Figura 3	Procedimento para avaliação eritrocitária	18
Figura 1 [Cap1]	Análise da água	33
Figura 2 [Cap1]	Anormalidades corporais em girinos	37
Figura 3 [Cap1]	Anormalidades orais em girinos	39
Figura 4 [Cap1]	Anormalidades orais em girinos	41
Figura 5 [Cap1]	Anormalidades orais em girinos	43
Figura 6 [Cap1]	Anormalidades corporais em girinos	44
Figura 1 [Cap2]	Alterações eritrocitárias	61
Figura 2 [Cap2]	Alterações eritrocitárias	61
Figura 1 [Cap3]	Vista dorsal de *Physalaemus albonotatus*	76
Figura 2 [Cap3]	Vista ventral de *Physalaemus albonotatus*	76
Figura 3 [Cap3]	Distribuição de *Physalaemus albonotatus*	78

LISTA DE TABELAS

Tabela 1 [Cap1]	Tipos de alterações morfológicas em girinos	34
Tabela 1 [Cap2]	Frequências de alterações eritrocitárias	60
Tabela 1 [Cap3]	Medidas morfométricas de *Physalaemus albonotatus*	77

SUMÁRIO

1. **INTRODUÇÃO GERAL** 12

2. **HIPOTESE** 15

3. **OBJETIVOS** 15

4. **METODOLOGIA** 16

4.1 Locais de amostragem dos girinos 16

4.2 Coleta dos exemplares 17

4.3 Avaliações das anormalidades 17

4.4 O teste do micronúcleo e outras alterações eritrocitárias nucleares (ANs) 18

4.5 Amostras de água 19

4.6 Procedimentos éticos, legais e acondionamento do material 19

4.7 Análises estatísticas 19

5. **RESULTADOS GERAIS** 20

6. **CONSIDERAÇÕES FINAIS** 20

7. **REFERÊNCIAS** 20

8. **APÊNDICE A** - **CAPÍTULO I** -Manuscript publication to Environmental Science and Pollution Research 27

9. **APÊNDICE B - CAPÍTULO II** - Manuscript accept to Archives of Environmental Contamination and Toxicology 54

10. **APÊNDICE C - CAPÍTULO III** - Manuscript submetted for Herpetology Notes 75

INTRODUÇÃO GERAL

As técnicas que utilizam bioindicadores para avaliação de impactos advindos de agrotóxicos podem ser divididas em duas linhas principais, sendo a primeira responsável pelo monitoramento de alterações em populações, comunidades e ecossistemas, e a segunda responsável pela avaliação de alterações individuais na taxa de crescimento, comportamento, malformações, reprodução e alterações bioquímicas e fisiológicas em nível celular e molecular (ROSENBERG 1993; TAVERA-MENDOZA et al. 2002; CARR et al. 2003; LAM, P. K. S.; GRAY, J. S 2003; ROHR e PALMER 2005).

Um biomarcador de desequilíbrio em um ecossistema é o registro de ocorrências de malformações nos girinos, o que também pode representar um dos fatores que contribuem para o declínio dos anuros (LANNOO 2008), embora as causas exatas dessas malformações ainda não são totalmente compreendidas (TAYLOR et al. 2005). Malformações podem ser provocadas por quaisquer fatores bióticos e abióticos, incluindo a infestação por trematódeos (JOHNSON et al. 1999, 2004), predação (BALLENGÉE e SESSIONS 2009; BOWERMAN et al. 2010), radiação UVB (BACON et al. 2013) e exposição a pesticidas (AGOSTINI et al. 2013; BACON et al. 2013, BORGES et al., 2019).

Muitos tipos de malformações podem ser encontrados em larvas de anfíbios, embora as anormalidades da boca estejam entre as mais importantes (BURGER e SNODGRASS 2000; DRAKE et al. 2007; BACON et al. 2013), dado seus efeitos potenciais sobre a alimentação comportamento e, consequentemente, no crescimento larval (Rowe et al. 1996; Venesky et al. 2009, 2010, PEREZ-IGLESIAS et. al., 2019). Crescimento reduzido e conseqüentemente o menor tamanho corporal pode expor indivíduos a um maior risco de predação durante a transição entre os ambientes aquáticos e terrestres, a fase de vida de maior vulnerabilidade dos anuros (TOLEDO 2005). Anormalidades causadas por mutações, erros de desenvolvimento e lesões físicas são comuns, mas normalmente são encontrados em menos de 5% de uma população (BLAUSTEIN e JOHNSON 2003; LUNDE e JOHNSON 2012).

No entanto, estudos recentes registraram maiores taxas de anomalia, de 15 a 90%, relacionadas a um número de diferentes fatores (Blaustein e Johnson, 2003), incluindo pesticidas (BORGES et al., 2018 EGEA-SERRANO et al. 2012; AGOSTINI et al. 2013; BACON et al. 2013).

Avaliando pelo aspecto genético, determinar os efeitos de contaminantes na estrutura genética das populações pode fornecer informações sobre as implicações dos xenobióticos ao nível populacional (THEODORAKIS 2001; SANTOS et al. 2017). Portanto, o estudo dos efeitos de contaminantes antropogênicos na estrutura e função do DNA deve ser um componente essencial da ecotoxicologia, biomonitoramento e avaliação de riscos ecológicos (THEODORAKIS 2001). As células do sangue refletem todas as mudanças físicas e químicas em organismos que são expostos a diferentes grupos de inseticidas e pesticidas (AMBALI et al. 2011).

Entre os sistemas de testes citogenéticos, a avaliação do micronúcleo (MN), seja em sangue ou células esfoliadas, é comumente usada para avaliar aberrações cromossômicas estruturais e numéricas induzidas por agentes clastogênicos e/ou aneugênicos (THOMAS et al. 2009; GONZÁLES et al. 2017). O teste MN é considerado simples, sensível e apresenta um resultado rápido dos efeitos xenobióticos em uma determinada área (POLLO et al., 2015; CARRAFA et al., 2013).

Alguns critérios são considerados para a formação de MN em eritrócitos sanguíneos, tais como o diâmetro do MN, que deve variar de 1/16 a 1/3 do núcleo principal, a refringência e cor do micronúcleo, que devem ser as mesmas do núcleo principal (CARRAFA et al., 2013). A origem desses MN ocorre durante a divisão mitótica, onde fragmentos ou ate mesmo cromossomos inteiros aparecem desassociados do núcleo principal (MAJER et al., 2001; ANGELIERI et al., 2014; SARPAL et al., 2016). Esta formação pode surgir quando alguma substancia induz a ruptura cromossômica (clastogênica) ou quando agentes afetam o aparelho do fuso mitótico (aneugênica) (BELIEN et al., 1995, MAJER et al., 2001; SARPAL et al., 2016).

Assim o teste do MN tornou-se um importante bioindicador de poluição, e, desse modo, tem sido uma ferramenta para a previsão do potencial de efeitos em longo prazo de xenobióticos em espécies selvagens (GONZÁLEZ et al. 2017). Além disso, é um teste de custo relativamente baixo e de rápida execução (SCHAUMBURG et al. 2012, BORGES et al., 2019).

O Brasil vem assumindo o lugar de maior consumidor de agrotóxicos no mundo e o processo produtivo agrícola brasileiro está cada vez mais dependente dos agrotóxicos e fertilizantes químicos (CARNEIRO et al. 2012; AUGUSTO et al. 2012; RIGOTTO et al. 2012).

Em 2008, o Brasil ultrapassou os Estados Unidos e assumiu o posto de maior mercado mundial de agrotóxicos. O consumo médio de agrotóxicos vem aumentando em relação à área plantada, passando de 10,5 litros por hectare (2002) para 12,0 l/ha (2011), de forma que áreas de grande concentração da monocultura coincidem com as áreas de maior consumo de agrotóxicos (RIGOTTO et al. 2012). Nos últimos dez anos, segundo CARNEIRO et al. (2012) o mercado mundial de agrotóxicos cresceu 93%, o mercado brasileiro cresceu 190%.

A utilização desses produtos em sistemas abertos, como o meio ambiente, impossibilita qualquer medida efetiva de controle, pois não há como enclausurar essas fontes de contaminação e proteger os compartimentos ambientais (água, solo, ar), além da flora e fauna associada a estes ecossistemas. Os resíduos de fertilizantes e agrotóxicos representam os principais contaminantes de origem agrícola, sendo que através da água da chuva e da irrigação ou percolação no solo podem atingir os corpos d'água diretamente ou indiretamente, chegando aos lençóis freáticos (ARIAS et al. 2007).

Contaminantes ambientais são considerados uma grande ameaça para a vida selvagem (GUILLETTE et el. 1995), e vários organismos fornecem informações úteis sobre a qualidade química e física da água a qual estão expostos durante toda a vida (HAYWOOD et al. 2004). Os anfíbios, devido às características morfofisiológicas (respiração pulmonar, cutânea e branquial, fase larva aquática e adulto terrestre) que lhes são peculiares, são sugeridos como bioindicadores da qualidade ambiental (BLAUSTEIN e WAKE 1990; Phillips 1990; GRIFFITHS e BEEBEE 1992; HALLIDAY 2000) e desta forma têm se tornado foco de muitos estudos. Segundo dados mais recentes da Sociedade Brasileira de Herpetologia SBH (2016), o Brasil apresenta 1080 espécies de anfíbios descritos, e o cerrado um dos locais que abriga boa parte desta biodiversidade, com 209 espécies, das quais 108 são endêmicas (VALDUJO et al. 2012).

Alguns trabalhos relatam que contaminantes ambientais, como defensivos agrícolas, podem atuar como desreguladores endócrinos dem muitas espécies de animias. (GUILLETE et al. 1995; MCCOY et al. 2008; HAYES et al. 2003). A exposição aos desreguladores endócrinos químicos podem alterar a sinalização hormonal normal durante o desenvolvimento embrionário e modificar a anatomia, comportamento e função do sistema reprodutivo de muitos vertebrados, incluindo peixes (JOBLING et al. 2006), anfíbios (Hayes et al. 2003), répteis (GUILLETTE et al. 1995; GUILLETTE e Iguchi 2003), aves (FRY e TOONE 1981) e mamíferos (SONNE et al. 2006).

2. HIPÓTESES

a) Água coleta em area preservada esteja livre de agrotóxicos, ou dentro do limente permitido na legislação;
b) Encontrar diferença quanto ao aspecto de malformações e citogenéticas, nas duas regiões amostradas;
c) Reforçar a importância da conservação de áreas para a manutenção das espécies;

3. OBJETIVOS

O presente trabalho teve como objetivo geral avaliar aspectos anatomorfológicos e genotóxicos de girinos coletados *in locu* de duas áreas distintas quanto ao diferente uso da terra (Matriz agrícola e Unidade de Conservação). Especificamente objetivou-se:

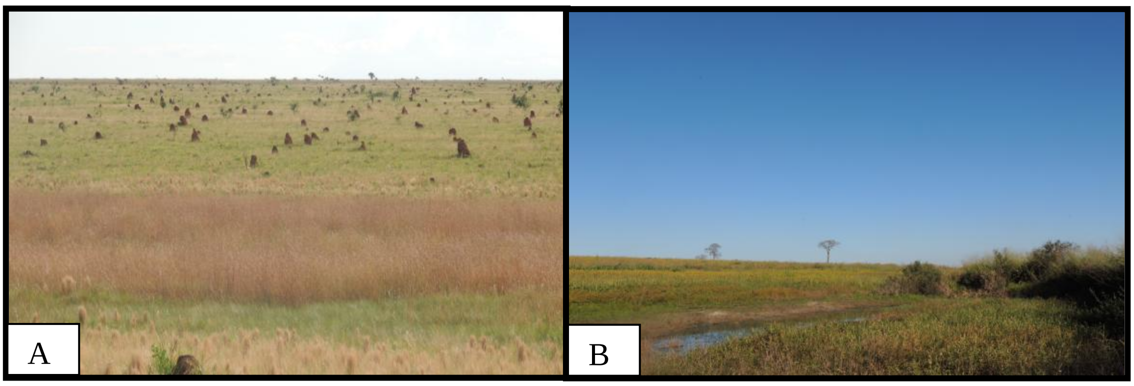
a) Identificação dos espécimes coletados nas duas diferentes áreas;
b) Avaliar e comparar os padrões anatomorfológicos dos girinos inseridos em áreas agrícolas e de girinos de Unidades de Conservação.
c) Avaliar a frequência de micronúcleos e outras alterações eritrocitárias nucleares de algumas espécies estudadas;

4. METODOLOGIA

4.1 Locais de amostragem dos girinos

Os ambientes de coleta dos dados biológicos foram conduzidos em duas distintas áreas que representassem diferentes uso da terra (Figura 1). Assim, foram coletados girinos no: I- Parque Nacional das Emas (PNE, Unidade de Conservação Federal) e II – Ambiente agrícola localizado no Município de Rio Verde, Goiás, Brasil. O Parque Nacional das Emas (18° 2′ 37,64″ S, 52° 56′ 30,11″ W) é uma unidade de conservação federal localizada na fronteira sudoeste do estado brasileiro Goiás, com área total de 132.133 ha, localizada nos municípios de Mineiros, em Goiás, e Costa Rica, no estado vizinho de Mato Grosso do Sul. No município de Rio Verde (17° 47′ 21.87″ S, 50° 52′ 53.77″ O), a área agrícola é constituída por uma matriz dominada por plantações de milho e soja, intercaladas com alguns fragmentos de floresta. Este município recentemente foi classificado entre os dez primeiros no país que mais consomem pesticidas (PIGNATI et al. 2017).

Figura 1: Áreas de amostragem dos girinos. A: Ambiente integro, Unidade de Conservação, Parque Nacional das Emas; B: Área agrícola no Município de Rio Verde, Goiás.



Fonte: Resgistrado pelo autor, dezembro de 2014.

4.2. Coleta dos exemplares

As larvas de anuros foram coletadas durante a estação reprodutiva dos indivíduos adultos entre novembro de 2013 a janeiro de 2017 nos referidos ambientes. A coleta foi realizada por meio de puçás, que consiste em um cabo de madeira acoplado em uma peneira (3 mm^2), o qual foi passado em lagoas permanentes e temporárias, e brejos por aproximadamente 1 h em cada ambiente. Os girinos foram anestesiados em solução de benzocaína (5 g / L), e posteriormente depositados em formalina a 10% (Heyer et al. 1994) (Figura 2). No laboratório, os girinos foram triados por morfotipos semelhantes, observados ao microscópio estereoscópico e procedida a identificação dos espécimes baseada em chaves de identificação especializadas, de acordo com Brandão (2002), Rossa-Feres e Nomura (2006) e Pezzuti (2011).

Figura 2: Análise morfológica e triagem. A: Identificação dos espécimes com auxilio de microscópio estereoscópico e avaliação do padrão morfológico; B: Acondicionamento dos exemplares.

Fonte: Resgistrado pelo autor, setembro de 2017.

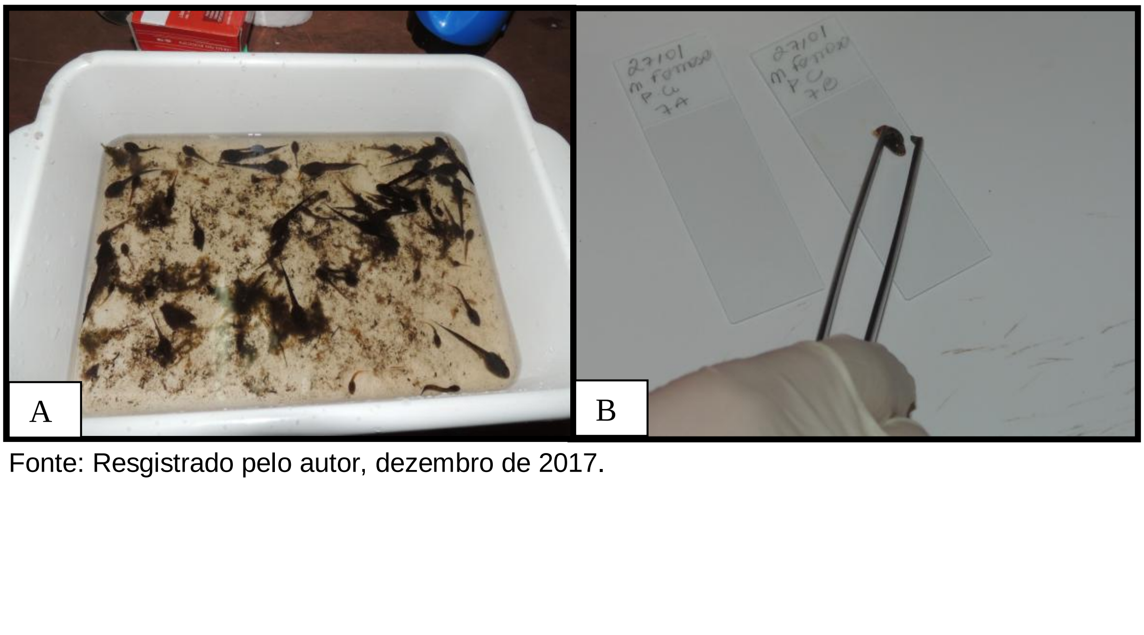
4.3. Avaliação das anormalidades

Para a avaliação das anormalidades, o estabelecimento da condição morfológica normal, foi considerado. Desta forma a partir da comparação com o padrão normal foi possível identificar as variações nos organismos. Para isso foram considerados os trabalhos de Bantle et al. (1991), com adaptações, Krishnamurthy e Smith (2011), Peltzer et al. (2013), Babini et al. (2015) e Perez-Iglesias (2015). Foram analisados, e comparados com o padrao normal para cada espécie, os seguintes parâmetros: olho, narina, aparato bucal, padrão da cauda e intestino e formato do corpo.

4.4 O teste do Micronúcleo (MN) e outras Alterações Eritrocitárias Nucleares (AENs)

Para a realização do teste do MN e demais alterações eritrocitárias nucleares, foram avaliados três espécies (*Dendropsophus minutus, Physalaemus cuvieri* and *Scinaxfus fuscovarius)* de girinos comuns ocorrentes em ambos os ambientes (agrícola e Unidade de Conservação). Após a identificação dos espécimes, o teste do MN foi aplicado aos eritrócitos sanguíneos circulantes obtidos, de acordo com Pérez-Iglesias et al. (2015). Os girinos foram anestesiados pela primeira vez por imersão em água gelada e amostras de sangue foram então obtidas através de uma secção cranial feita atrás do opérculo (Figura 3). Dois esfregaços sanguíneos foram obtidos em lâminas limpas para cada animal, que foram então secas ao ar, fixadas em metanol frio (4° C) por 20 minutos, e então coradas em Giemsa a 5% durante 12 minutos. Uma amostra de 1000 células foi analisada para cada girino para determinar a freqüência de MN e AENs, usando um microscópio com câmera acoplada e uma ampliação de 100x. Para a identificação das alterações eritrocitárias (MN e AENs), foram considerados os seguintes critérios (Pérez-Iglesias et al. 2015): (i) o diâmetro é menor que 1/16 a 1/3 do núcleo principal, (ii) o material é não-reflexivo, com o mesma ou menor intensidade de coloração em comparação com o núcleo principal, e (iii) a estrutura não está ligada ou se sobrepondo ao núcleo principal.

Figura 3: Procedimento para avaliação eritrocitária. A: Triagem dos girinos em campo; B: Confecção do esfregaço sanguíneo.



Fonte: Resgistrado pelo autor, dezembro de 2017.

4.5 Amostras de água

Uma amostra da água foi coletada para cada regiões de estudo, Parque Nacional das Emas e no município de Rio Verde. Foram coletadas subamostras nos pontos de amostragem e no mesmo período de coleta dos girinos e, em seguida armazenada em uma única amostra por área, representado cada região. As amostras foram preservadas a uma temperatura de 4°C e enviado para o laboratório para análises específicas. Ensaios físico-químicos foram realizados por cromatografia gás-líquido com espectrometria de massa para verificar a presença de carbamato, organocloreto, e pesticidas organofosforados.

4.6 Procedimentos Éticos, Legais e Acondicionamento do material

Todos os procedimentos foram encaminhados à apreciação pela Comissão de Ética da Universidade de Rio Verde (CEUA; Universidade de Rio Verde, nº 0316), com a devida licença (SISBIO n.41919-1) emitida pelo Instituto Chico Mendes de Conservação da Biodiversidade. O material biológico está depositado na coleção herpetologica do Laboratório de Biologia Animal do Instituto Federal Goiano, *campus* Rio Verde.

4.7 Análises Estatísticas

As alterações morfológicas em girinos nas duas áreas de estudo foram analisadas pelo qui-quadrado em uma tabela de contingência de 2 × 2, com nível de significância P <0,05. Este teste foi executado no Programa R.

Uma análise fatorial de variância (ANOVA), com o teste post-hoc de Tukey, foi utilizada para avaliar o conjunto de anomalias nucleares (AENs). Neste caso, os dados foram transformados na raiz para satisfazer a hipótese de homogeneidade de variância. A significância de p <0,05 foi considerada para todas as análises.

5. RESULTADOS GERAIS

Como resultados deste estudo, apresentamos dois capítulos diretamente relacionado a proposta do trabalho. No entanto apresentamos também uma nota científica, oriundo das excursões ao campo, permitiu o registro de uma espécie (*Physalaemus albonotatus*) para uma nova área de ocorrência, ampliando assim a distribuição desta espécie para a Unidade de Conservação. Assim segue em anexo a esta tese os capítulos:

6. CONSIDERAÇÕES FINAIS

Estratégias de conservação são necessárias para minimizar os danos morfológicos observados em larvas de anuros em áreas agrícolas e ressaltamos ainda a importância da manutenção das unidades de conservação.

Fornecemos também dados *in situ* sobre as alterações morfológicas bem como nucleares para espécies nativas do Cerrado Brasileiro. Salientamos ainda que *Physalaemus cuvieri* e *Scinax fuscovarius* apresentaram alterações eritrocitárias e são potenciais candidatos a espécies sentinelas para ambientes perturbados.

7. REFERÊNCIAS

AGOSTINI, M.G.; KACOLIRIS F.; DEMETRIO, P.; NATALE, G.S.; BONETTO, C.;RONCO, A.E. (2013) Abnormalities in amphibian populations inhabiting agroecosystems in northeastern Buenos Aires Province, Argentina. Dis Aquat Org 104:163–171.

AMBALI S, F.; AYO J.O.; ESIEVO KAN, Ojo S. A. 2011. Hemotoxicity induced by chronic chlorpyrifos exposure in Wistar rats: Mitigating effect of vitamin C. *Veterinary Medicine International*. 2011: 945439.

ANGELIERI, F.; FRANCHI, L.; CEVIDANES, L.H.S.; SCANAVINI, M.A.; MCNAMARA-Jr J.A. (2014) Long-term treatment effects of the FR-2 appliance: a prospective evaluation 7 years post-treatment. Eur J Orthod 36: 192–199.

ARIAS, A.R.L.; BUSS, D.F.; ALBURQUERQUE ,C.; INÁCIO, A.F.; FREIRE; M.M.; EGLER, M.; MUGNAI, R.; BAPTISTA, D.F. 2007. Utilização de bioindicadores na avaliação de impacto e no monitoramento da contaminação de rios e córregos por agrotóxicos. *Ciênc. Saúd. Col.* 12: 61 - 72.

AUGUSTO, L.G.S.; CARNEIRO, F.F.; PIGNATI, W.; RIGOTTO, R.M.; FRIEDRICH, K.; FARIA N.M.X.; BÚRIGO, A.C.; FREITAS, V.M.T.; GUIDUCCI Filho, E. 2012. Dossiê ABRASCO - Um alerta sobre os impactos dos agrotóxicos na saúde. ABRASCO, Rio de Janeiro, junho de 2012. 2ª Parte. 135p.

BABINI, M.S.; BIONDA, C.L.; SALAS, N.E.; MARTINO, A.L. (2015) Health status of tadpoles and metamorphs of Rhinella arenarum (Anura, Bufonidae) that inhabit agroecosystems and its implications for land use. Ecotoxicol Environ Saf 118:118–125.

BACON, J.P.; FORT, C.E.; TODHUNTER, B.; MATHIS, M.; FORT, D.J. (2013) Effects of multiple chemical, physical, and biological stressors on the incidence and types of abnormalities observed in Bermuda's cane toads (Rhinella marina). J Exp Zool 320B:218–237.

BALLENGÉE, B.; SESSIONS, S.K. (2009) Explanation for missing limbs in deformed amphibians. J Exp Zool 312:1–10.

BANTLE, J.A.; DUMONT, J.N.; FINCH, R.A.; LINDER, G. (1991) Atlas of abnormalities: a guide for the performance of FETAX. Oklahoma State University Press, Oklahoma.

BELIEN, J.A.M.; COPPER, M.P.; BRAAKUIS B.J.M.; SNOW, G.B.; BAAK, J.P.A. (1995) Standardization of counting micronuclei - definition of a protocol to measure genotoxic damage in human exfoliated cells. Carcinogenesis 16:2395–2400.

BLAUSTEIN, A.R.; JOHNSON, P.T. (2003) The complexity of deformed amphibians. Front Ecol Environ 1:87–94.

BLAUSTEIN, A.R.; WAKE, D.B. 1990. Declining amphibian populations: a global phenomena? *Trends in Ecology and Evolution.* 5: 203–204.

BORGES, R.E.; SANTOS L. R. S.; ASSIS R.A.; BENVINDO-SOUZA M.; FRANCO-BELUSSI L.; OLIVEIRA C. (2019) Monitoring the morphological integrity of neotropical anurans. Environmental Science and Pollution Research, 26:2623–2634

BOWERMAN, J.; JOHNSON, P.T.J.; BOWERMAN, T. (2010) Sublethal predators and their injured prey: linking aquatic predators and severe limb abnormalities in amphibians. Ecology 91:242–251.

BRANDÃO, R.A. (2002) A new species of Phyllomedusa Wagler, 1830 (Anura: Hylidae) from Central Brazil. J Herpetol 36:571–578.

BURGER, J.; SNODGRASS, J.W. (2000) Oral deformities in several species of frogs from the Savannah River site, USA. Environ Toxicol Chem 19:2519–2524.

CARNEIRO, F.F.; PIGNATI, W.; RIGOTTO, R.M.; AUGUSTO, L.G.S,.;RIZOLLO, A.; MULLER, N.M.; ALEXANDRE, V.P.; FRIEDRICH, K.; MELLO, M.S.C. 2012. Dossiê ABRASCO - Um alerta sobre os impactos dos agrotóxicos na saúde. ABRASCO, Rio de Janeiro, abril de 2012. 1ª Parte. 98p.

CARAFFA, E. et al. Determinación de la frecuencia de micronúcleos en eritrocitos de Bufo arenarum que habitan ambientes urbanizados. Acta Toxicológica Argentina, v. 21, n. 2, p. 78-84, 2013**.**

CARR, J.; GENTLES, A.; SMITH, E.; GOLEMAN, W.; URQUIDI, L.; THUETT, K.; KENDALL R.J.; GIESY, J.P.; GROSS, T.S.; SOLOMON KR, VAN DER KRAAK G. 2003. Response of larval *Xenopus laevis* to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environ. Toxicol. Chem.* 22: 396 – 405.

DRAKE, D.L.; ALTIG, R.; GRACE, J.B.; WALLS, S.C. (2007) Occurrence of oral deformities in larval anurans. Copeia 2:449–458.

EGEA-SERRANO, A.; RELYEA, R.A.; TEJEDO, M.; TORRALVA, M. (2012) Understanding of the impact of chemicals on amphibians: a metaanalytic review. Ecol Evol 2:1382–1397.

FRY, D.M.; TOONE C.K.. 1981. DDT-induced feminization of gull embryos. *Science.* 213:922-924 Sonne C, Leifsson PS, Dietz R, Born EW, Letcher RJ, Hyldstrup L. 2006. Xenoendocrine pollutants may reduce size of sexual organs in East Greenland polar bears (*Ursus maritimus*). *Environ Sci Technol.* 40(18):5668–5674.

GONZALES, E,C,L.; LARRIERA, P.A.; POLETTA, G.L. 2017. Micronuclei and other nuclear abnormalities on Caiman latirostris (Broad-snouted caiman) hatchlings after embryonic exposure to different pesticide formulations. *Ecotoxicology and Environmental Safety*. 136: 84-91.

GRIFFITHS, R.; BEEBEE, T. 1992. Decline and fall of the amphibians. *New Scientist*. 134 (1827): 25–29.

GUILLETTE, L.J. Jr.; GROSS, T.S.; GROSS, D.; ROONEY, A.A.; PERCIVAL H.F. 1995. Gonadal steroidogenesis in vitro from juvenile alligators obtained from contaminated and
control lakes. *Environ Health Perspect* 103(4):31-36.

GUILLETTE, L.J. Jr.; IGUCHI, T. 2003. Contaminant-induced endocrine and eproductive alterations in reptiles. *Pure Appl Chem.* 75(11–12):2275–2286.

HALLIDAY, T. 2000. Dofrogsmake good canaries? *BioScience.*47: 143–146.

HAYES, T.; HASTON, K.; TSUI, M.; HOANG, A.; HAEFFELE, C.; VONK, A. 2003. Atrazine-Induced hermaphroditism at 0.1 ppb in american leopard frogs (*Rana pipiens*): laboratory and field evidence. *Environ. Health Perspect.* 111:568-575.

HAYWOOD, L.K.; ALEXANDER, G.J.; BYRNE, M.J.; CUKROWSKA, E. 2004. *Xenopus laevis* embryos and tadpoles as models for testing for pollution by zinc, copper, lead and cádmium. *African Zoology.* 39(2): 163–174.

HEYER, W.R.; DONNELLY, M.A.; MCDIAMIRD, R.W.; HAYEK, L.C.; FOSTER, M. (1994) Measuaring and monitoring biological diversity standard methods for amphibians. Smithsonian Institutions Press, Washington.

JOBLING, S.; WILLIAMS, R.; JOHNSON, A.; TAYLOR, A.; GROSS-SOROKIN, M.; NOLAN, M. 2006. Predicted exposures to steroid estrogens in UK rivers correlate with widespread
sexual disruption in wild fish populations. *Environ Health Perspect.* 114:32–39.

JOHNSON, P.T.J.; LUNDE, K.B.; RITCHIE, E.G.; LAUNER, A.E. (1999) The effect of trematode infection on amphibian limb development and survivorship. Science 284:802–804.

Johnson PTJ, Sutherland DR, Kinsella JM, Lunde KB (2004) Review of the trematode genus Ribeiroia (Psilostomidae): ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. Adv Parasitol 57:191–253.

Krishnamurthy SV, Smith GR (2011) Combined effects of malathion and nitrate on early growth, abnormalities, and mortality of wood frog (Rana sylvatica) tadpoles. Ecotoxicology 20:1361–1367.

LAM, P. K. S.; GRAY, J. S. The use of biomarkers in environmental monitoring programmes. Marine Pollution Bulletin. v. 46, n. 1, p. 182-186, 2003.

LANNOO, M, (2008) The collapse of aquatic ecosystems: malformed frogs. University of California press, Berkeley.

LUNDE, K.B.; JOHNSON, PTJ (2012) A practical guide for the study of malformed amphibians and their causes. J Herpetol 46:429–441.

MAJER, B.J.; LAKY, B.; SNASMULLER, S.; KAISSIE. F. (2001) Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage and in chemoprevention trials. Mutat Res 489:147–172.

MCCOY KA, BORTNICK LJ, CAMPBELL CM, HAMLIN HJ, GUILLETTE JR LJ, ST MARY CM. 2008. Agriculture alters gonadal form and function in the toad *Bufo marinus*. *Environ. Health Perspect.* 116(11):1526-1532.

PELTZER, P.M.; LAJMANOVICH, R.C.; ATTADEMO, A.M.; JUNGES, C.M.; CABAGNA- ZENKLUSEN, M.C.; REPETTI, M.R.; SIGRIST. M.E; BELDÓMENICO. H. (2013) Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (Trachycephalustyphonius) tadpoles. Ecotoxicol Environ Saf 98: 142–151.

PÉREZ-IGLESIAS, J.M. (2015) Biomarcadores para avaliação dos efeitos da atrazina em girinos de Rhinella schneideri (Bufonidae) e Physalaemus nattereri (Leiuperidae): utilização de parâmetros tradicionais e pigmentação visceral. Dissertation, Universidade Estadual Paulista.

PEZZUTI, T.L. (2011) Girinos do quadrilátero ferrífero, sudeste do Brasil: Ecomorfologia e chave de identificação interativa. Dissertation, Universidade Federal de Minas Gerais.

PIGNATIAW, LIMA FANS, LARA, S.S.; CORREAMLM, BARBOSA, J.R.; LEÃO, L.H.C.; PIGNATTI, M.G. (2017) Spatial distribution of pesticide use in Brazil: a strategy for health surveillance. Ciência & Saúde Coletiva 22:3281– 3293.

PHILLIPS, K. 1990. Where have all the frogs and toads gone? *BioScience.* 40: 422–424.

POLLO, F.V. et al. Common toad Rhinella arenarum (Hensel, 1867) and its importance in assessing environmental health: test of micronuclei and nuclear abnormalities in erythrocytes. Environmental Monitoring and Assessment. V. 187, p. 581, 2015.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

RIGOTTO, R.M.; PORTO, M.F, FOLGADO, C,; FARIA, N.M.; AUGUSTO, L.G.; BEDOR, C.; BURIGO, A.; CARNEIRO, F.F.; CASTRO, F.P.; FERNANDES, G.B.; FERREIRA, M.J.M.; FRIEDRICH, K. MARINHO, A.M.C.P.; MONTEIRO, D.; PIGNATI, A.W.; PINHEIRO, T.M.M.; RIZZOLO, A. SILVA, N. TYGEL, A. 2012. Dossiê Abrasco - Agrotóxicos, conhecimento científico e popular: construindo a ecologia de saberes. Porto Alegre, novembro de 2012. 3ª Parte. 244p.

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

ROSENBERG, D.M.; R.E.S.H. VH. 1993. Freshwater Biomonitoring and benthic macroinvertebrates, Chapman & Hall, NewYork.

ROSSA-FERES, D.C.; NOMURA, F. (2006) Morphological characterization and taxonomic key for tadpoles (Amphibia: Anura) from northwestern region of São Paulo state, Brazil. Biota Neotropica 6:1–26.

ROWE, C.L.; KINNEY, O.M.; FIORI, A.P.; CONGDON, J.D. (1996) Oral deformities in tadpoles (Rana catesbeiana) associated with coal ash deposition: effects on grazing ability and growth. Freshw Biol 36:723–730.

SARPAL, R.S., TANEJA, N., SHERGILL, N.K., RAVINDRA, S.V., 2016. Biomonitoring of Buccal Mucosa Cells in Chronic Smokers and Nonsmokers. World Journal of Dentistry, 7:189- 194.

SBH. 2016. Sociedade Brasileira de Herpetologia. Disponível em: http://www.sbherpetologia.org.br. Acessado em fevereiro de 2019.

SANTOS, C.S.A.; BRANDÃO, R.; MONTEIRO, M.S.; BASTOS, A.C.; SOARES AMVM, LOUREIRO, S. Assesment of DNA damage in Ardea cinerea and Ciconia ciconia: A 5-year study in Portuguese birds retrieved for rehabilitation. *Ecotoxicology and Environmental Safety*. 136: 104-110; 2017***.***

SCHAUMBURG, L.G.; POLETTA, SIROSKI, P.A.; MUDRY, M.D. 2012. Baseline values of Micronuclei and Comet Assay in the lizard Tupinambis merianae (Teiidae, Squamata). *Ecotoxicology and Environmental Safety*. 84: 99-103.

SONNE, C.; LEIFSSON, P.S.; DIETZ, R.; BORN, E.W.; LETCHER, R.J.; HYLDSTRUP, L. 2006. Xenoendocrine pollutants may reduce size of sexual organs in East Greenland polar bears (*Ursus maritimus*). *Environ Sci Technol.* 40(18):5668–5674.

TAVERA-MENDOZA, L.; RUBY, S. BROUSSEAU, P.; FOURNIER, M.; CYR, D.; MARCOGLIESE, D. 2002. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environ. Toxicol. Chem*. 21: 527 - 531.

TAYLOR, B.; SKELLY, D.; DEMARCHIS, L.K.; SLADE, M.D.; GALUSHA, D.; RABINOWITZ, P.M. (2005) Proximity to pollution sources and risk of amphibians limb malformation. Environ Health Perspect 113:1497–1501.

THEODORAKIS, C.W. 2001. Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. *Ecotoxicology*. 10: 245-256.

THOMAS, P.; HOLLAND, N.; BOLOGNESI, C.; KIRSCH-VOLDERS, M. BONASSI, S. ZEIGER, E. KNASMUELLER, S. FENECH, M. 2009. Buccal micronucleus cytome assay. *Nature Protocols*. 4: 825–837.

TOLEDO, L.F. (2005) Predation of juvenile and adult anurans by invertebrates: current knowledge and perspectives. Herpetol Rev 36:395– 400.

VALDUJO, P.H., SILVANO, D.L., COLLI, G. & MARTINS, M. 2012. Anuran species composition and distribution patterns in Brazilian Cerrado, a Neotropical Hotspot. South Am. J. Herpetol. 7(2):63-78. http://dx.doi. org/10.2994/057.007.0209

VENESKY, M.D.; PARRIS, M.J.; STORFER, A. (2009) Impacts of Batrachochytrium dendrobatidis infection on tadpole foraging performance. EcoHealth 6:565–575.

VENESKY, M.D.; WASSERSUG, R.J.; PARRIS, M.J. (2010) How does a change in labial tooth row number affect feeding kinematics and foraging performance of a ranid tadpole (Lithobates sphenocephalus)? Biol Bull 218:160–168. https://doi.org/10.1086/BBLv218n2p160.

8. APÊNDICE A - CAPÍTULO 1: Manuscript publication to Environmental Science and Pollution Research.

Monitoring the morphological integrity of neotropical anurans

Rinneu Elias Borges[1] & Lia Raquel de Souza Santos[2] & Rhayane Alves Assis[2] & Marcelino Benvindo-Souza[2] & Lilian Franco-Belussi[3,4] & Classius de Oliveira[3]

Received: 24 May 2018 /Accepted: 15 November 2018 /Published online: 24 November 2018 Springer-Verlag GmbH Germany, part of Springer Nature 2018
DOI 10.1007/s11356-018-3779-z

Abstract

Amphibians are considered to be excellent bioindicators to their morphophysiological characteristics and life cycle. In this context, the present study investigated the morphological integrity of anuran larvae collected in preserved environments in the Emas National Park, in the municipality of Mineiros (Goiás state, Brazil), and in environments representative of the agricultural matrix of the Rio Verde region, also in Goiás, where there is a long history of the use of agricultural pesticides. Samples of water from temporary ponds, permanent dams, and veredas were analyzed for the presence of pesticides and, especially atrazine (5350 μg/L), found at significantly higher concentrations in the agricultural matrix. We observed a high percentage (approximately 10%) of morphological malformations including alterations of the fins in *Boana albopunctatus* and *Scinax fuscovarius*; alteration in oral structures in *B. albopunctatus*, *Dermatonotus muelleri, Physalaemus centralis*, *Physalaemus cuvieri*, and *Leptodactylus fuscus* mainly in the tadpoles collected in the agricultural environment in comparison with those from the protected area (3.5%; $P < 0.0001$, $\chi 2$ 31.75). However, changes in the eyes, mouth, intestines, and nostrils, as well as amelia were observed only in the agricultural environment. The vast majority of the observed malformations were associated with the tail and oral disc, which suggests that these anatomical parameters may be used as sensitive morphological biomarkers. Given these findings, we reinforce that areas of agricultural land may have a deleterious effect on the morphological integrity of the tadpoles and consequently, on their development, and that these features may be used as indicators of environmental quality and health.

Keywords Amphibians. Ecotoxicology. Malformations. Bioindicators. Pesticides. Agriculture

Introduction

The current worldwide decline in amphibian populations has been attributed to a number of different factors (Blaustein et al. 2011). One indicator of disequilibrium in an ecosystem is the occurrence of malformations in tadpoles, which may also represent one of the factors contributing to the decline in anuran populations (Lannoo 2008), although the exact causes of these malformations are still not fully understood (Taylor et al. 2005). Malformations may be provoked by any number of biotic and abiotic factors, including infestation by trematodes (Johnson et al. 1999, 2004), predation (Ballengée and Sessions 2009; Bowerman et al. 2010), UVB radiation (Bacon et al. 2013), and exposure to pesticides (Bridges 2000; Egea-Serrano et al. 2012; Agostini et al. 2013; Bacon et al. 2013). Some authors have also discussed the potential role of chemical endocrine disrupters or even natural processes as a cause of abnormalities (McCoy et al. 2008).

Many types of malformation can be found in amphibian larvae, although abnormalities off the mouth are among the most important (Burger and Snodgrass 2000; Drake et al. 2007; Bacon et al. 2013), given their potential effects on feeding behavior, and consequently on larval growth (Rowe et al. 1996; Venesky et al. 2009, 2010). Reduced growth and a consequently smaller body size may expose individuals to a higher risk of predation during the transition between aquatic and terrestrial environments, which is the most vulnerable anuran life phase (Toledo 2005). Abnormalities caused by mutations, errors of development, and physical injury are common, but they are typically found in less than 5% of a population (Blaustein and Johnson 2003; Lunde and Johnson 2012). However, recent studies have recorded much higher rates of anomaly, of 15–90%, related to a number of different factors (Blaustein and Johnson 2003), including agricultural pesticides (Bridges 2000; Egea-Serrano et al. 2012; Agostini et al. 2013; Bacon et al. 2013).

Overall, approximately 38% of the Earth’s land area is dedicated to agricultural practices (Foley et al. 2011), resulting in unprecedented environmental impacts, which encourages monitoring of one of the most pesticidally consumed areas in Brazil, in this case the state of Goiás, municipality of Rio Verde (Pignati et al. 2017). The state is part of the Brazilian Cerrado, where the biome is reported for intense agricultural activities (Schiesari and Grillitsch 2011), however, there are still few in situ reports on morphological integrity analysis of anurans in the face of environmental disturbance risks. In contrast, many studies show genotoxic effects of pesticides in anurans (Gonçalves et al. 2015, 2017). In general, this need for toxicological studies extends to the entire Neotropical region, even though it presents a greater diversity of anurans and points to higher rates of population decline (Schiesari et al. 2007). A systematic evaluation of the morphological integrity of anurans of agricultural area is important in predicting mortality from environmental changes. In addition, external morphological characteristics besides easy evaluation are low cost.

Materials and methods

Study area and data collection

Anatomical alterations in tadpole larvae were investigated in two distinct areas between November 2013 and January 2017: I- Emas National Park and II - the agricultural matrix of the municipality of Rio Verde, Goiás, Brasil. Emas National Park (18° 2′ 37.64″ S, 52° 56′ 30.11″ W) is a federal conservation unit located on the southwestern border of the Brazilian state of Goiás, with a total area of 132,133 ha, located in the municipalities of Mineiros, in Goiás, and Costa Rica, in the neighboring state of Mato Grosso do Sul. The vegetation of this national park encompasses a number of different Cerrado savanna formations, including grassland savanna, shrubby savanna, veredas (palm swamps), and gallery forests. The tadpoles were collected in a total of 11 sampling points, distributed among sidewalks, temporary pools, and permanent and nascent ponds. All sampling areas were selected in order to avoid the anthropogenic interference that exists at the Park boundary, with sampling points with a mean distance of 15 km in the Conservation Unit. By contrast, the agricultural matrix of Rio Verde (17° 47′ 21.87″ S, 50° 52′ 53.77″ O) is dominated by plantations of maize and soybean, interspersed with a few fragments of forest. This municipality has recently been ranked among the top ten in the country that most consume pesticides (Pignati et al. 2017). In this environment, the specimens were collected in six water bodies inside the crop, including temporary pools, permanent and semi-permanent ponds, without the action of any other effluent.

The anuran larvae were collected (SISBIO collecting license no. 41919-1 e Committee on Animal Use at the University of Rio Verde, Goias, Brazil (Comissão de Ética no Uso de Animais da Universidade de Rio Verde, CEUA/ Universidade de Rio Verde, no. 0316) from temporary and permanent ponds and marshes using a wire mesh (3 mm^2) hand-net, which was used to sample the margins and the interior of each environment during approximately 1 h. The tadpoles were anesthetized in a benzocaine solution (5 g/L), fixed in 10% formalin (Heyer et al. 1994), and in the laboratory, they were observed under stereoscopic microscope for species identification, based on Brandão (2002), Rossa-Feres and Nomura (2006) and Pezzuti (2011).

Identification of abnormalities

All the tadpoles collected in both areas (agricultural matrix and national park) were included in this analysis to guarantee the broadest possible perspective of the natural conditions found in each study area. Thus, the analyzes evaluated the morphology of 825 tadpoles between development stages 26 and 40 (Gosner 1960), belonging to 21 species collected from the agricultural matrix in Rio Verde, and 1047 tadpoles of nine species obtained from the conservation unit (Emas National Park). The abnormalities encountered during this evaluation were assigned to the categories proposed by Bantle et al. (1991), with adaptations, Krishnamurthy and Smith (2011), Peltzer et al. (2013), Babini et al. (2015), and Perez-Iglesias (2015). Following analysis, the animals were photographed under a stereoscopic microscope to record the specific characteristics observed. Voucher specimens were deposited in the Animal Biology Laboratory of the Rio Verde campus of Goiano Federal Institute (IF Goiano).

Analysis of water samples

Water samples were collected from both study areas, the Emas National Park and the agricultural matrix of the municipality of Rio Verde. Subsamples were collected from each sampling point and then stored in a single container, representing each region. The samples were preserved at a temperature of 4 °C and sent to the laboratory for analysis. Physical-chemical assays were run by gas-liquid chromatography with mass spectrometry to verify the presence of carbamate, organochloride, and organophosphate pesticides. The assays tested for the following substances: 2,4-D + 2,4,5-T; alachlor; aldicarb + aldicarb-sulfone + aldicarb-sulfoxide; aldrin; dieldrin; aldrin + dieldrin; atrazine; carbendazim + benomyl; carbofuran; chlordane (cis + trans); chlorpyrifos + chlorpyrifos-oxon; DDT (4,4-DDT + 4,4- DDE + 4,4-DDD); diuron, endosulfan (alfa + beta + sulfate); endrin; glyphosate + AMPA; lindane; mancozeb; metolachlor; molinate; methyl parathion; pendimethalin; permethrin; profenofos; simazine; tebuconazole; terbufos, and trifluralin, with parameters being based on Brazilian Health Ministry ordinance 2914 of December 12th, 2011.

Statistical analysis

The relative frequencies of the presence of morphological alterations observed in the tadpoles collected from the two study areas were analyzed by chi-square in a 2 × 2 contingency table, with a $P < 0.05$ significance level. This test was run in the R program.

Results

Water parameters

The herbicide atrazine was the only chemical compound recorded in high concentrations in the agricultural matrix. The concentration of atrazine recorded in this area was 5349.94 µg/L, thousands of times higher than the legal Maximum Permissible Value (MPV) of 2 µg/L based on Brazilian Health Ministry ordinance 2914 of December 12th, 2011 (Table supplementary (S1)). A number of the other chemical compounds tested were also found in this sample, but always at concentrations below the MPV established in Brazilian legislation. In the protected area, by contrast, all the chemical compounds recorded were at concentrations below their respective MPVs, and in all cases, the concentrations recorded in the national park were lower than those recorded in the agricultural matrix (Fig. 1 and Table S1).

Abnormalities recorded in the anuran larvae

A number of different, uncommon alterations of the external morphology and anatomical configuration were found in the tadpole samples analyzed. In the agricultural sample, a total of 825 tadpoles were analyzed, of which, 82 (9.93%) presented morphological abnormalities ($\chi 2 = 31.7$; $P < 0.0001$). Six different types of abnormality were found in nine of the 21 anuran species collected in the agricultural matrix (Table 1).

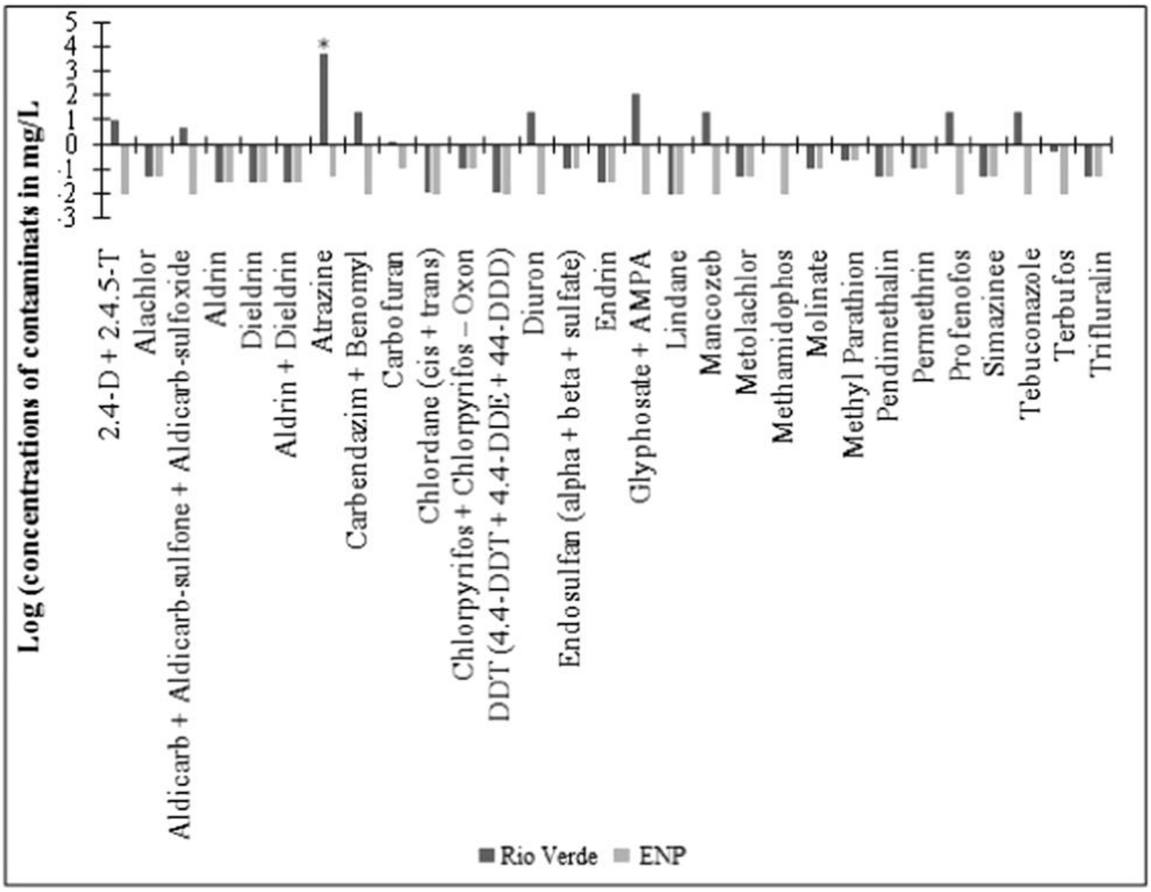


Fig. 1 Concentrations (log10 transformed) of contaminants recorded in the water samples collected in the agricultural matrix of the municipality of Rio Verde (dark bars) and the protected area, in Emas National Park (ENP) (light bars). *Concentration above the Maximum Permissible Value (MPV)

Abnormalities of the tail were the most common alteration, being found in 6.18% of the tadpoles analyzed from the agricultural matrix. These abnormalities, including irregular and double fins, were especially common in five species, *Dermanotus mulleri, Leptodactylus fuscus, Physalaemus centralis, Physalaemus nattereri, and Scinax fuscovarius* (Fig. 2a, b). A range of alterations was also found in the oral morphology of the tadpoles from the agricultural matrix, including interrupted, overlapping, folded, and absent denticles, as well as alterations of the papillae and dermal flaps (Fig. 3a, b). These abnormalities were recorded in 3.27% of the tadpoles and were most common in *P. cuvieri and L. fuscus* (Figs. 4 and 5). A smaller proportion (1.69%) of the tadpoles had other malformations, including changes in the intestinal morphology, the absence or displacement of the eyeball, nasal asymmetry, and amelia, all of which were only observed in the tadpoles from the agricultural matrix (Fig. 6 and Table 1).

Table 1 Quantity and types of morphological alterations observed the tadpole specimens collected in the agricultural matrix of the municipality of Rio Verde (RV) and in the protected area, in Emas National Park (ENP)

Environments	Species	Types of morphological alterations															
		N		Tail		Oral disc		Intestine		Ocular		Nostril		Amelia		Total	
		ENP	RV	ENP	RV	ENP	RV	ENP	RV	ENP	RV	ENP	RV	ENP	RV	ENP	RV
	D. minutus	32	36	2	–	–	–	–	–	–	–	–	–	–	–	2	0
SCE	*P. cuvieri*	165	305	–	–	4	11	–	2	–	1	–	1	–	–	4	14
	S. fuscovarius	157	65	–	26	–	–	–	–	–	–	–	–	–	–	0	26
	B. albopunctatus	594	12	10	–	17	–	–	–	–	–	–	–	–	–	27	0
	D. mulleri		47		6		1	–	–	–	–	–	–	–	–		7
	L. fuscus		92		2		9	–	4	–	4	–	–	–	1		15
Rio Verde	*P. centralis*		8		3		5	–	–	–	1	–	–	–	–		5
	P. nattereri		51		14		–	–	–	–	–	–	–	–	–		14
	Pseudopaludicola sp.		5		–		1	–	–	–	–	–	–	–	–		1
	*Other species analyzed	99	204														0
Total		1047	825	12	51	21	27	0	6	0	6	0	1	0	1	33	82

SCE species common to both environments (Emas National Park and Rio Verde) that presented some type of malformation N total number of individuals analyzed *Other species analyzed from the agricultural matrix and Emas National Park which did not present any morphological alteration

Endoparasites were also observed in four tadpole species (*P. nattereri, P. cuvieri, P. fuscomaculatus, and L. fuscus*) from this environment.

In the protected area, by contrast, where 1047 specimens were collected, only two types with morphological alteration were observed in four (*Dendropsophus minutus, Boana albopunctatus, P. cuvieri, and S. fuscovarius*) of the nine species sampled (Table 1). These alterations (recorded in 3.15% of the specimens) were found only in the tail (bifurcated) and oral disc (Figs. 2C and 3C).

Six of the tadpole species were recorded at both sites, although malformations were recorded in only four of these species. In these four species, morphological alterations were observed in 9.8% of the specimens collected in the agricultural matrix, and in only 3.48% of those from the protected area. Overall, in all species in both environments, malformations of the tail and oral disc were by far the most common alterations, being found in approximately 90% of the cases. This indicates that these parameters are the most important for morphological analyses (Table 1).

Discussion

The findings of the present study indicate that the tadpoles collected in areas of agricultural land had significantly higher rates of morphological alterations (approximately 10% of specimens) in comparison with the protected area, in which only 3.5% of the specimens were affected. The presence of anomalies is common in natural anuran populations, although the normal rate of abnormalities resulting from mutations, developmental errors and lesions, is typically less than 5% (Blaustein and Johnson 2003; Lunde and Johnson 2012). The malformation rate recorded in the protected area was thus consistent with that expected for a natural population. In the agricultural matrix of Rio Verde, however, the much higher rate of 9.93% indicates that other, unnatural processes are affecting the tadpole population.

A number of studies have shown that exposure to pesticides provokes both morphological (Lajmanovich et al. 2003; Carezzano and Cabrera 2011; Babini et al. 2016; Perez-Iglesias et al. 2016) and cytogenetic alterations in anurans, indicated by the formation of micronucleated cells (Rudek and Rozek 1992; Candioti et al. 2010; Pérez-Iglesias et al. 2014).

In addition to the damage to the DNA of these animals, survival rates also tend to be lower, given that morphological malformations, in particular, those associated with the oral disc (Carezzano and Cabrera 2011; Babini et al. 2016) affect the growth and development of the anuran larvae. As around 89% of threatened anuran species are affected by farming practices (Young et al. 2004), pesticides are a major concern for natural ecosystems and even human health, given their impacts (Pérez-Iglesias et al. 2015) on the anuran fauna.

In addition to the impacts of pesticides, identified in a wide range of studies (Bridges, 2000; Blaustein and Johnson 2003; Ankley and Rodney 2004; Robles-Mendoza et al. 2009; Egea- Serrano et al. 2012; Agostini et al. 2013; Bacon et al. 2013), other factors may also cause anomalies in amphibians, including predation (Ballengée and Sessions 2009; Bowerman et al. 2010), UVB radiation (Blaustein and Johnson 2003; Ankley and Rodney 2004; Bacon et al. 2013; Franco-Belussi et al. 2016), and infection by parasites (Johnson et al. 1999, 2004). In recent work, effluents of mores were responsible for developmental impacts and morphological characters of tadpoles of *P. cuvieri* (Montalvão et al. 2018). In the present study, endoparasites were recorded in five anuran species, only in specimens obtained from agricultural environments. Laboratory findings have shown that pesticides may reduce resistance to parasites (Kiesecker 2002; Budischak et al. 2008; Rohr and McCoy 2010), given their toxicity to immune cells which may thus reduce the efficiency of the mechanisms that protect these hosts from infection (Voccia et al. 1999; Martin et al. 2010). In particular, Mann et al. (2009) showed that the immune system of amphibians is suppressed by the presence of pesticides, provoking a number of different responses, such as an increase in the proportion of encysted cercaria (Rohr et al. 2008; Kiesecker 2002) and pulmonary infection rates (Christin et al. 2003; Gendron et al. 2003).

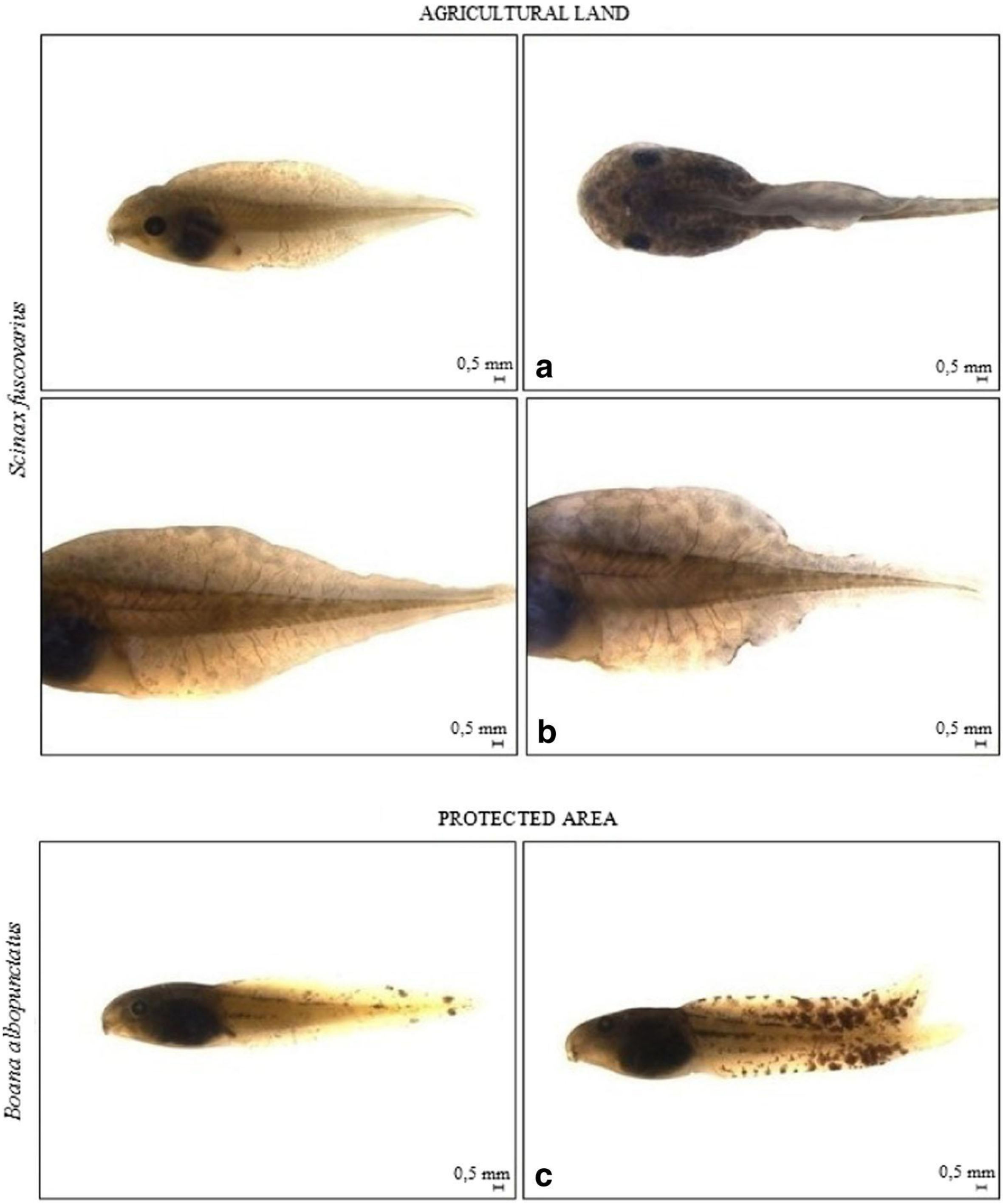


Fig. 2 Photo-documentation of the tail of the tadpole specimens collected in the agricultural matrix of the municipality of Rio Verde and in the protected area in the Emas National Park in Goiás (Brazil). Left-hand column: morphological configuration considered to be normal for the species; right-hand column: morphological alterations observed. a: Double caudal membrane (dorsal view); b: Ridges in the superior and inferior margins of the caudal fin; c: Tail with bifurcated extremity

The analyses of water quality also indicated extremely high concentrations of the herbicide atrazine, and this herbicide is genotoxic and mutagenic for a Neotropical anuran *Dendropsophus minutus* (Gonçalves et al. 2017). In addition to relatively high concentrations of other pesticides, albeit within the legal limits, but possibly responsible for synergic effects of their active ingredients (Cedergreen 2014), leading to an increase in toxicity due to the cumulative effects of stressors (Peltzer et al. 2013). In this case, the combined effects of these compounds are likely to interfere with the biological cycle of the tadpoles that inhabit the agricultural matrix, making them more vulnerable to parasites than the animals found in more pristine environments. Based on data from WHO (World Health Organization) and the Environmental Protection Agency (EPA), the International Union of Pure and Applied Chemistry (Iupac 2018) classifies atrazine as a mildly toxic and dangerous herbicide. This herbicide is the seventh most used in Brazil, with 13,911.37 tons of the active ingredient being sold annually (Ibama 2014). In a recent report Perez- Iglesias et al. (2016), they verified that different levels of atrazine can cause retraction and until the partial or total loss of the denticles of *Rhinella schneideri and P. nattereri*, besides retarding the growth and development of the anuran larvae.

In the present study, the analyses showed that morphological alterations occurred in six anatomical structures. Deformations of the oral disc, the second most common type of alteration observed in the specimens from the agricultural matrix, may be highly deleterious to the nutritional condition of the animal. This type of abnormality may affect foraging behavior and reduce the nutritional efficiency of the animal (Rowe et al. 1996), and the absence of or defects in the denticles of tadpoles may make them less able to anchor themselves to the substrate when feeding (Venesky et al. 2010). In fact, in addition to malformed or absent denticles, some of the tadpoles collected in the present study had little or no intestinal content, which may reflect an inability to feed adequately as a consequence of the damage to their oral structures. Babini et al. (2015, 2016) also recorded malformations in the oral morphology of anuran larvae collected in agricultural systems, in addition to an increase in the frequency of micronuclei and nuclear anomalies, which they attributed to the presence of chemical contaminants in the environment.

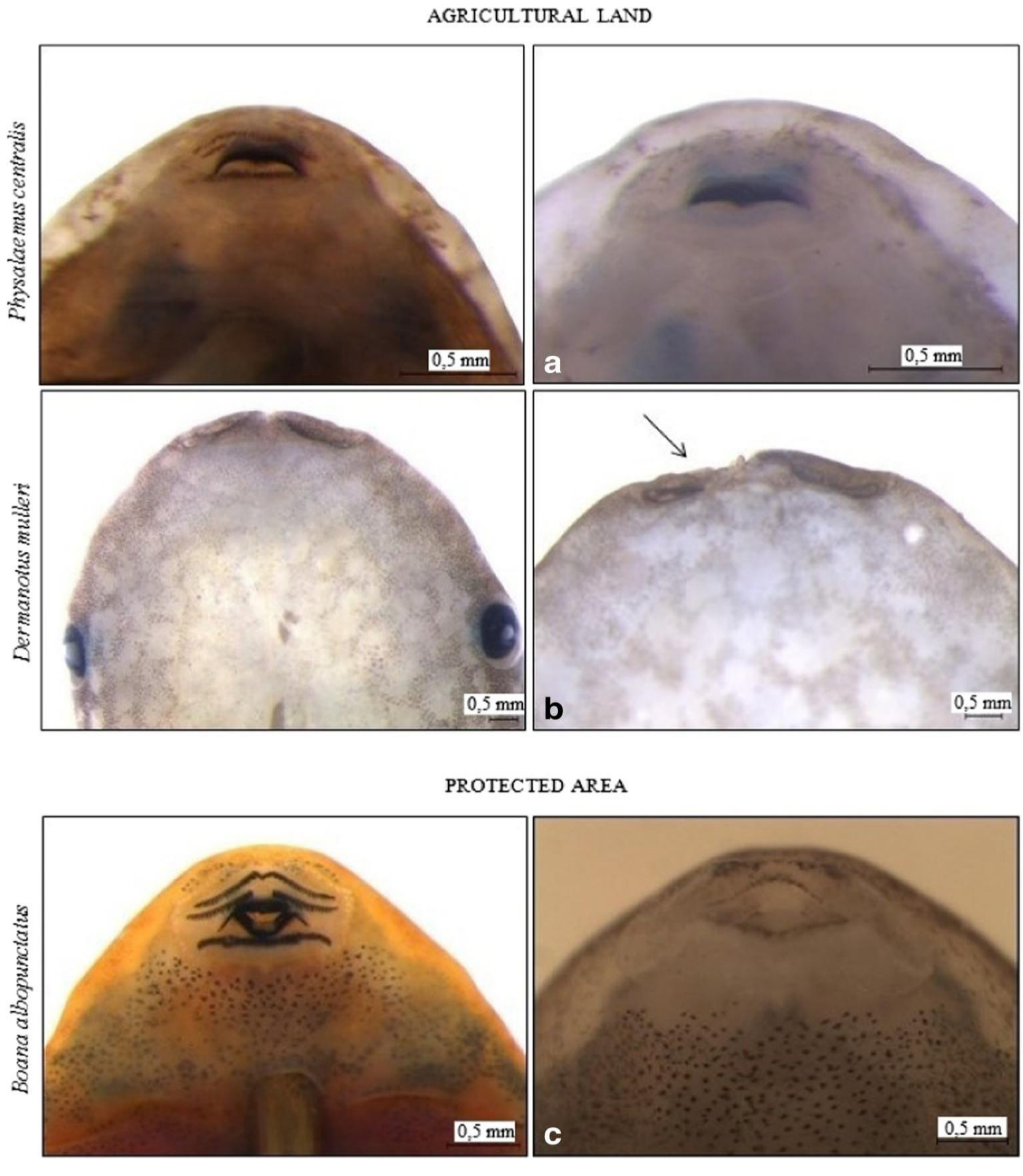


Fig. 3 Photo-documentation (ventral view) of the oral morphology of hysalaemus cuvieri tadpoles collected in the agricultural matrix of the municipality of Rio Verde, Goiás (Brazil). Left-hand column: normal configuration of the species; right-hand column: morphological alterations observed. A Complete absence of denticles. B Absence of dermal flaps. C Absence of denticles

Deformations of the tail were also relatively common in the present study, ranging from the insertion of its root to the presence of ridges and bifurcations associated with the fin membranes. Dawson (1932) and Lynn (1950) observed that the duplication of the whole tail or parts of this organ is generally related to the hyper-generation of the organ following mechanical damage caused by predators or chemical compounds, such as copper (Chen et al. 2007). However, Brandão et al. (2011) observed malformations of the tail and reduced body size in tadpoles exposed to copper, which is commonly used in industrial processes and is easily introduced into the environment in pesticides, domestic effluents, and the consumption of fossil fuels. Even so, a number of other studies have linked malformations of the tail to the exposure of the larvae to pesticides (Baier et al. 2016), and substantial deformations of the tail (around 50–90%) to the presence of agricultural chemicals, in particular, glyphosate, in the environment. As the tail of tadpoles contains important reserves of energy and calcium for metamorphosis (Vitt and Caldwell 2013), it would seem likely that alterations in this structure will result in smaller individuals in the postmetamorphic stages.

In the present study, alterations of the eyes (absence and/or sinking/dislocation of the eyeball), the absence of members, and intestinal irregularities (such as a dislocated spiral, and the formation of an intestinal loop prior to the cloaca) were also observed, albeit at much lower rates and exclusively in the specimens from the agricultural environment. These types of abnormality have been observed in studies that tested atrazine as a contaminating agent in anuran larvae and juveniles (Britson and Threlkeld 1998; Allran and William 2001; Hayes et al. 2002; Carr et al. 2003; Coady et al. 2005; Hayes et al. 2006; Svartz et al. 2012). Deformities have also been reported in tadpoles exposed to Endosulfan, a widely-used organochloride pesticide (Brunelli et al. 2009). Given this, the alterations observed in the present study may also have been related to the action of a certain pesticide, especially as high concentrations of certain chemical compounds may cause malformations in anuran larvae, as well as having sub-lethal effects on swimming behavior and growth, and an increase in the frequency of micronuclei (Perez-Iglesias 2015; Perez-Iglesias et al. 2016).

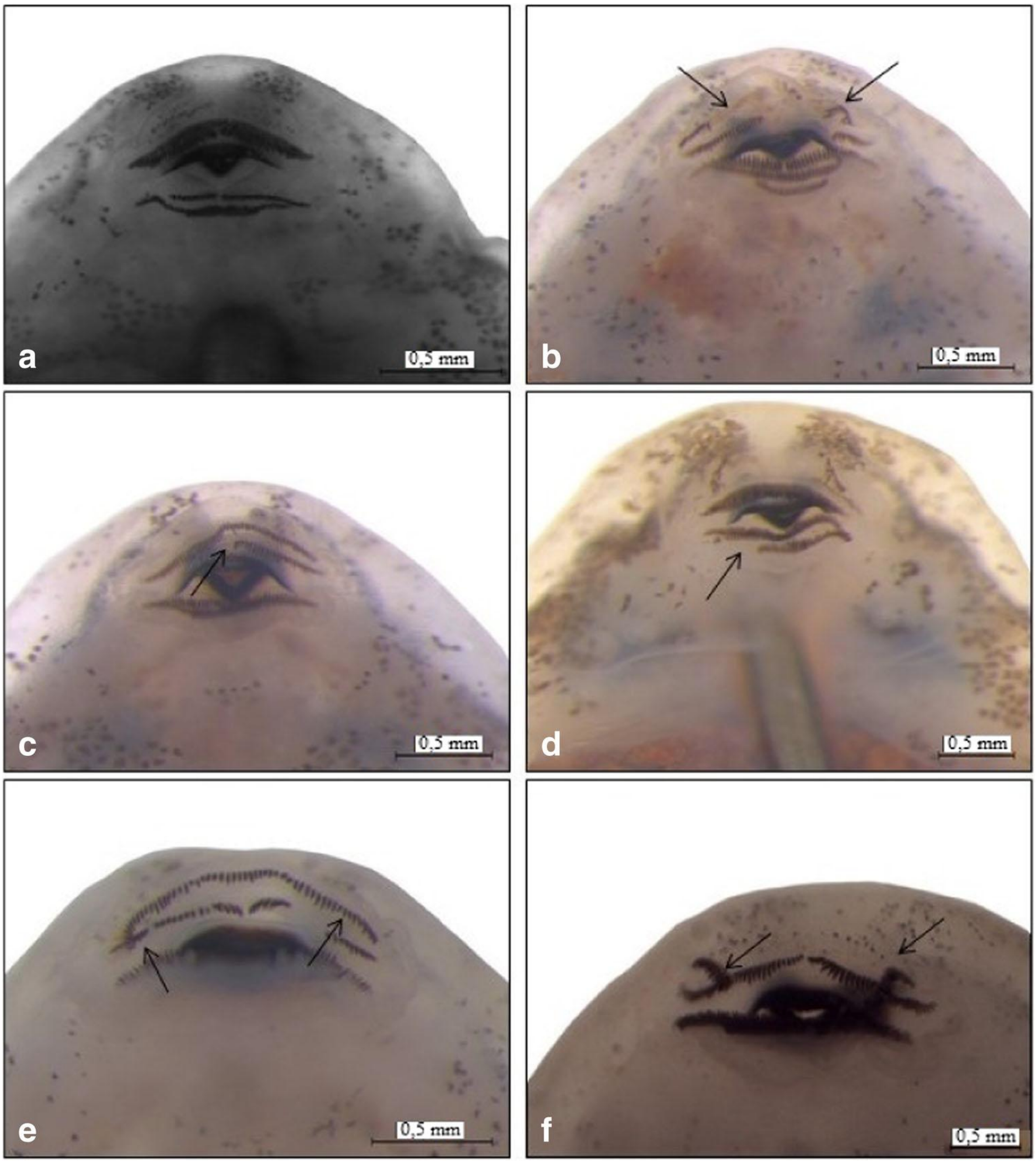


Fig. 4 Photo-documentation (ventral view) of the oral morphology of Physalaemus cuvieri tadpoles collected in the agricultural matrix of the municipality of Rio Verde, Goiás (Brazil). a. Oral disc considered normal for the species. b. Folding and absence denticle row. C Absence of the anterior row of denticles. d Absence of posterior row of denticles. e, f Overlapping and absent denticles. The arrows indicate the observed alterations

Lajmanovich et al. (2003) conducted experiments using glyphosate and observed a range of malformations in the anuran larvae, such as alterations of the eyes, crooked tails, and in particular, deformities of the denticles. As reported by Rowe et al. (2002), McDaniel et al. (2004) and Pérez-Iglesias (2015), the presence of agricultural toxins and heavy metals in aquatic systems may have lethal and sub-lethal effects on amphibians. Overall, then, the presence of pesticides in agricultural systems, and in particular, in freshwater environments, is preoccupying, given that these cumulative, toxic compounds impact the quality of the aquatic environments that anurans depend on for their survival (Schmutzer et al. 2008; Babini et al. 2016). Despite the implications of the morphological damage diagnosed in the study specimens, it is far from easy task to interpret the real ecological impacts of these alterations (Köhler and Triebskorn 2013). In this case, anatomical studies will be important to provide a more ample perspective, focusing on the population or community level, especially considering that the presence of contaminants in the environment, combined with the morphological damage observed in the present study may ultimately lead to the local extinction of certain species, which may decrease the functional diversity of the community (Mouillot et al. 2008). This may be a result of morpho-anatomical defects, such as impaired swimming ability due to damage to the caudal membrane or reduced nutritional status related to defects in the denticles, resulting in a loss of the biological function of the organism in the local habitat and community. As data from natural environments are still scarce, it is important to note that, irrespective of the type of malformation observed, the probable causes are still uncertain, given that this study was conducted in loco, where many other abiotic and biotic factors may play a role in the determination of the observed defects. Even so, a clear difference was found in the composition of the water samples collected in the two environments, which indicates the occurrence of intense contamination by pesticides the agricultural matrix, whether unintentional or through disregard for environmental regulations, which is likely to represent a considerable risk to the local anuran fauna (Peltzer et al. 2013; Wagner et al. 2014; Cedergreen 2014).

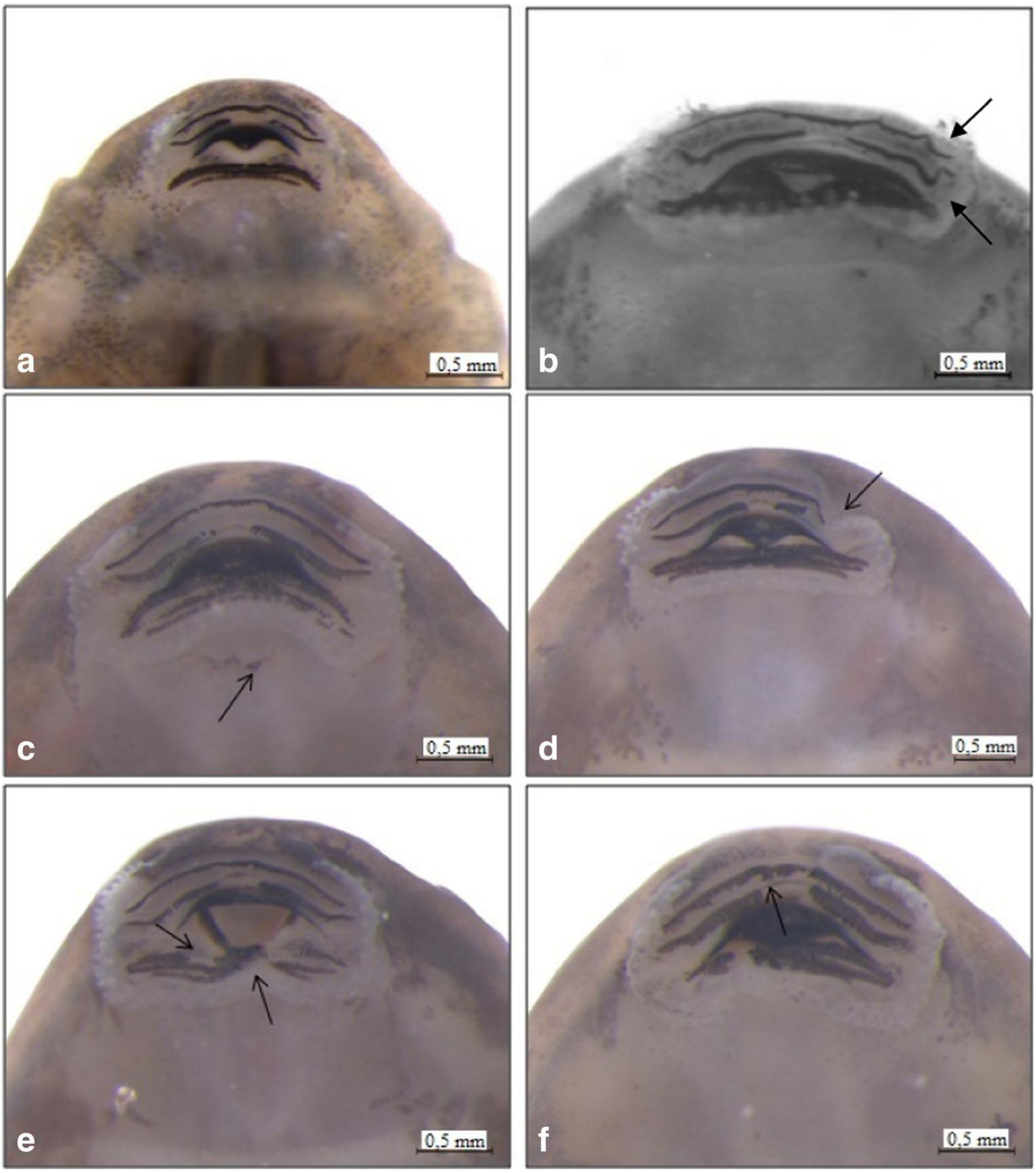


Fig. 5 Photo-documentation (ventral view) of the oral morphology of *Leptodactylus fuscus* tadpoles collected in the agricultural matrix of the municipality of Rio Verde, Goiás (Brazil). a Normal oral disc. b Folds in the anterior denticle row. c Abnormal keratinization. d Fold in the row o papillae. e Gaps and ridges in the three posterior rows of denticles. f Denticles of different sizes. The arrows indicate the observed alterations

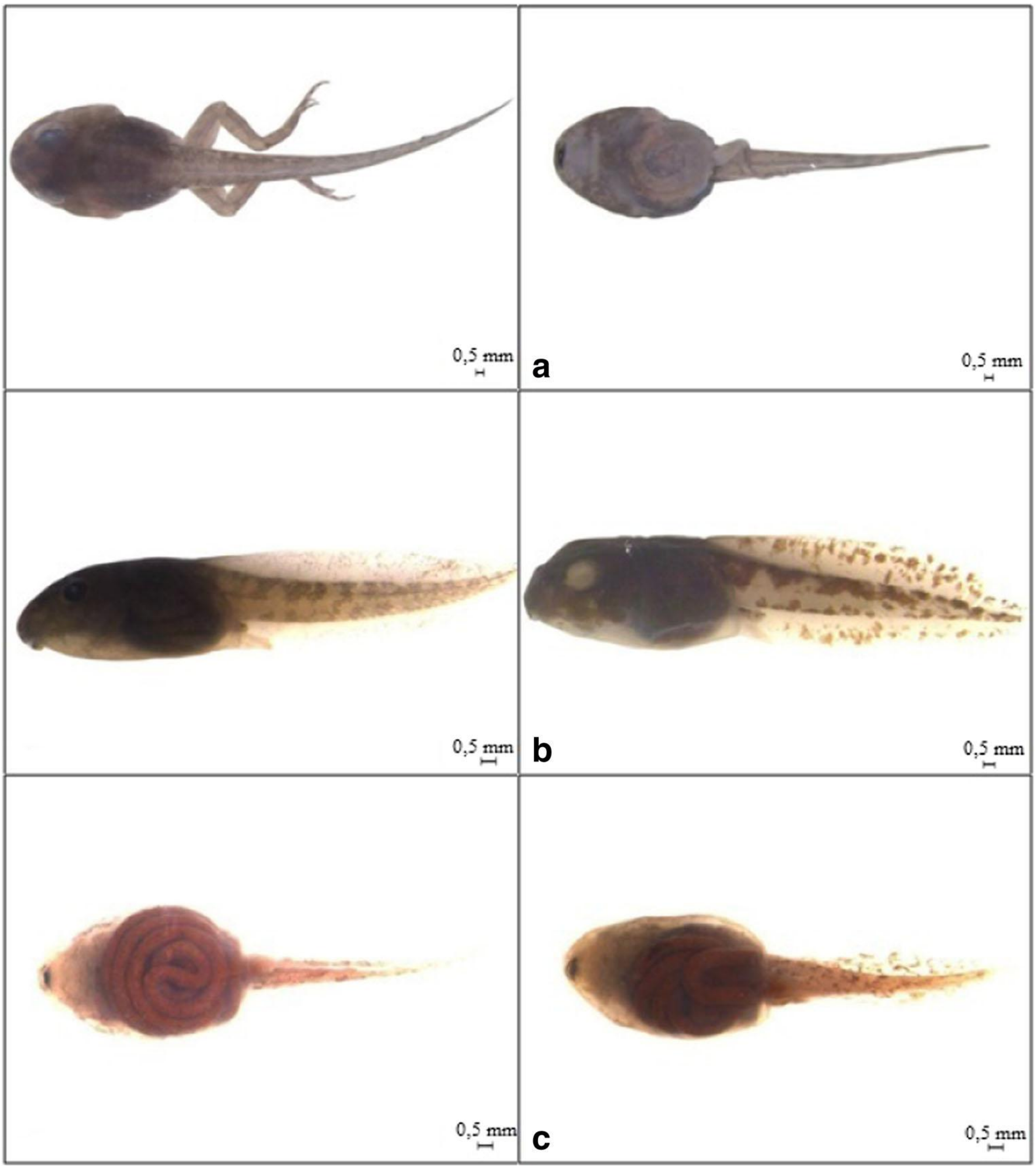


Fig. 6 Photo-documentation of tadpoles collected on agricultural land in the municipality of Rio Verde, Goiás (Brazil). Left-hand column: normal configuration of the species; right-hand column: morphological alterations observed. A: Ventral view of Amelia in L. fuscus; B: Lateral view of L. fuscus, showing the absence of an eye; C: Ventral view of intestinal loop in P. cuvieri

The rate of malformation recorded in the agricultural environment was almost twice the threshold considered normal (5%), reinforcing the need for the monitoring of this community (Wagner et al. 2014). In this study, we analyze nine Neotropical species (*B. albopunctatus, D. minutus, L. fuscus, P. cuvieri, D.*

mulleri, P. centralis, P. nattereri, S. fuscovarius, and Pseudopaludicola sp.) that focus on morphological abnormalities and resistance to pesticides in situ, but some this species (e.g., *P. cuvieri, L. fuscus, S. fuscovarius*, and *B. albopunctatus*) have a relatively ample geographic distribution, and are also relatively tolerant of anthropogenic impacts, which may account for their typical association with areas of farmland. Then, these species represent good study models, given that they are typically present in areas in which pesticide use is constant and present a large occurrence.

Conclusion

The rates of morphological alterations observed in the present study reinforce the importance of the maintenance and preservation of conservation units, given the low frequency of malformations recorded in these protected areas. On the other hand, it is observed in this study that anurans inhabit agricultural areas are more susceptible to damage caused by the action of environmental contaminants of agricultural origin. In this way important mitigating measures are taken to minimize these damages, such as management plans for species that inhabit these environments, special attention for the insertion of nonpriority groups in conservation strategies, since many studies demonstrate the absence of anurans in these groups, including also information on their susceptibility to these agents in commercial pesticide formulations. It will nevertheless be important to identify the proximate cause of the induction of these anomalies in order to better understand the health and degradation of the environment, as well as its associated fauna. As the majority of the alterations were found in the caudal fin and oral disc, these morpho-anatomical structures may be useful as biomarkers for the monitoring of the morphological integrity of anuran larvae.

Acknowledgments

We are grateful to the Brazilian National Council for Scientific and Technological Development (CNPq) for supporting this study through grant number 477044/2013-1, and the São Paulo State Research Foundation (FAPESP) for supporting laboratory procedures (process no. 2013/02067-5). LFB was also supported by a CAPESPNPD postdoc fellowship during the final preparation of this manuscript. We also thank Rio Verde University (UniRV) for providing a researcher stipend, and the Chico Mendes Institute for Biodiversity Conservation (IBAMA/ICMBio) and the administration of Emas National Park for authorizing and supporting the fieldwork.

References

Agostini MG, Kacoliris F, Demetrio P, Natale GS, Bonetto C, Ronco AE (2013) Abnormalities in amphibian populations inhabiting agroecosystems in northeastern Buenos Aires Province, Argentina.

Dis Aquat Org 104:163–171 Allran JW, William HK (2001) Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environ Toxicol Chem 20:769– 775

Ankley GT, Rodney DJ (2004) Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J 45:469–483

Babini MS, Bionda CL, Salas NE, Martino AL (2016) Adverse effect of agroecosystem pond water on biological endpoints of common toad (Rhinella arenarum) tadpoles. Environ Monit Assess 188:2–14

Babini MS, Bionda CL, Salas NE, Martino AL (2015) Health status of tadpoles and metamorphs of Rhinella arenarum (Anura, Bufonidae) that inhabit agroecosystems and its implications for land use. Ecotoxicol Environ Saf 118:118–125

Bacon JP, Fort CE, Todhunter B, Mathis M, Fort DJ (2013) Effects of multiple chemical, physical, and biological stressors on the incidence and types of abnormalities observed in Bermuda's cane toads (Rhinella marina). J Exp Zool 320B:218–237

Baier F, JedingerM, Gruber E, Zaller JG (2016) Temperature-dependence of glyphosate-based herbicide’s effects on egg and tadpole growth of common toads. Front Environ Sci 4:51

Ballengée B, Sessions SK (2009) Explanation for missing limbs in deformed amphibians. J Exp Zool 312:1–10

Bantle JA, Dumont JN, Finch RA, Linder G (1991) Atlas of abnormalities: a guide for the performance of FETAX. Oklahoma State University Press, Oklahoma

Blaustein AR, Han BA, Relyea RA, Johnson PTJ et al (2011) The complexity of amphibian population declines: understanding the role of co-factors in driving amphibian losses. Ann N YAcad Sci 123:108– 119

Blaustein AR, Johnson PT (2003) The complexity of deformed amphibians. Front Ecol Environ 1:87–94

Bowerman J, Johnson PTJ, Bowerman T (2010) Sublethal predators and their injured prey: linking aquatic predators and severe limb abnormalities in amphibians. Ecology 91:242–251

Brandão FP, Marques S, Rodrigues S, Santos B, Travasso R, Venâncio C, Pereira R, Ortiz-Santaliestra M, Soares A, Gonçalves F, Lopes I (2011) Influência da temperatura na toxicidade de cobre em girinos de rã verde Pelophylax perezi. Captar 3:66–77

Brandão RA (2002) A new species of Phyllomedusa Wagler, 1830 (Anura: Hylidae) from Central Brazil. J Herpetol 36:571–578 Bridges CM (2000) Long-term effects of pesticide exposure at various life stages of the southern leopard frog (Rana sphenocephala). Arch Environ Contam Toxicol 39:91–96

Britson CA, Threlkeld ST (1998) Abundance, metamorphosis, developmental, and behavioral abnormalities in Hyla chrysoscelis tadpoles following exposure to three agrichemicals and methyl mercury in outdoor mesocosms. Bull Environ Contam Toxicol 61:154–161
Brunelli E, Bernabó I, Berg C, Lundstedt-Enkel K (2009) Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behaviour in Bufo bufo tadpoles. Aquat Toxicol 91:135– 142

Budischak SA, Belden LK, Hopkins WA (2008) Effects of malathion on embryonic development and latent susceptibility to trematode parasites in ranid tadpoles. Environ Toxicol Chem 27:2496–2500

Burger J, Snodgrass JW (2000) Oral deformities in several species of frogs from the Savannah River site, USA. Environ Toxicol Chem 19:2519–2524

Candioti JV, Natale GS, Soloneski S, Ronco AE, Larramendy RL (2010) Sublethal and lethal effects on Rhinella Arenarum (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida®. Chemosphere 78:249–255

Carezzano FJ, CabreraMR (2011) Variación en la fórmula dentaria larval de Physalaemus biligonigerus (Amphibia, Leptodactylidae) de humedalesen agroecosistemas del sur de Córdoba, Argentina. Boletín de la Sociedad Zoológica del Uruguay 20:28–33

Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van Der Krak G (2003) Response of larval

Xenopus laevis to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environ Toxicol Chem 22:396–405

Cedergreen N (2014) Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9: 96580

Chen TH, Jackson G, William K (2007) Adverse effects of chronic copper exposure in larval northern leopard frogs (Rana pipiens). Environ Toxicol Chem 26:1470–1475

ChristinMS, GendronAD, Brousseau P, Me'nard L, Marcogliese DJ, Cyr D, Ruby S, Fournier M (2003) Effects of agricultural pesticides on the immune system of Rana pipiens and on its resistance to parasitic infection. Environ Toxicol Chem 22:1127–1133

Coady KK, Murphy MB, Villeneuve DL, Hecker M, Jones PD, Carr JA, Solomon KR, Smith EE, Van Der Kraak G, Kendall RJ, Giesy JP (2005) Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in Xenopus laevis. Ecotoxicol Environ Saf 62:160–173

Dawson AB (1932) Aventral accessory tail in Triturus viridescens and its duplication experimentally. Anat Rec 52:139–149

Drake DL, Altig R, Grace JB,Walls SC (2007) Occurrence of oral deformities in larval anurans. Copeia 2:449–458

Egea-Serrano A, Relyea RA, Tejedo M, Torralva M (2012) Understanding of the impact of chemicals on amphibians: a metaanalytic review. Ecol Evol 2:1382–1397

Foley JA, Ramankutty N, BraumanKA, Cassidy ES, Gerber JS, Johnston M, Balzer C (2011) Solutions for a cultivated planet. Nature 478: 337–342

Franco-Belussi L, Sko HN, Oliveira C (2016) Internal pigment cells respond to external UV radiation in frogs. J Exp Biol 219:1378– 1383

Gendron AD, Marcogliese DJ, Barbeau S, Christin MS, Brousseau P, Ruby S, Cyr D, Fournier M (2003) Exposure of leopard frogs to a pesticide mixture affects life history characteristics of the lungworm Rhabdias ranae. Oecologia 135:469–476

Goncalves MW, Gambale PG, Godoy FR, Alves AA, Rezende PHD, Maciel NM, Nomura F, Bastos RP, Marco P (2017) The agricultural impact of pesticides on Physalaemus cuvieri tadpoles (Amphibia: Anura) ascertained by comet assay. Zoologia 4:e19865

Goncalves MW, Vieira TB, Maciel NM, Carvalho WF, Lima LSF, Gambale PG, Cruz AD, Nomura F, Bastos RP, Silva DM (2015) Detecting genomic damages in the frog Dendropsophus minutus: preserved versus perturbed areas. Environ Sci Pollut Res 22:3947– 3954. https://doi.org/10.1007/s11356-014-3682-1

Gonçalves MW, de Campos CBM, Batista VG, da Cruz AD, de Marco Junior P, Bastos RP, e Silva, D. D. M. (2017) Genotoxic and mutagenic effects of Atrazine Atanor 50 SC on Dendropsophus minutus Peters, 1872 (Anura: Hylidae) developmental larval stages. Chemosphere 182:730–737

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

Hayes TB, Collins A, Mendonza M, Noriega N, Stuart AA, Vonk A (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc Natl Acad Sci 99:5476–5480

Hayes TB, Stuart AA, Mendonza CA, Noriega N, Vonk A, Johnston G, Liu R, Kpodzo D (2006) Characterization of atrazine-induced gonadal malformations in African clawed frogs (Xenopus laevis) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17β-estradiol): support for the demasculinization/feminization hypothesis. Environ Health Perspect 114:134–141
Heyer WR, Donnelly MA, Mcdiamird RW, Hayek LC, Foster M (1994) Measuaring and monitoring biological diversity standard methods for amphibians. Smithsonian Institutions Press, Washington Ibama, Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis (2014) Relatórios de comercialização de agrotóxicos 2014. http://www.ibama.gov.br/areas-tematicas-qa/relatoriosdecomercializacao- de-agrotoxicos/pagina-3. Accessed 15 september 2017

Iupac, International Union Of Pure and Applied Chemistry (2018) Global availability of information on agrochemicals. Atrazine. https://sitem.herts.ac.uk/aeru/iupac/Reports/43.htm. Accessed 15 september 2017

Johnson PTJ, Lunde KB, Ritchie EG, Launer AE (1999) The effect of trematode infection on amphibian limb development and survivorship. Science 284:802–804

Johnson PTJ, Sutherland DR, Kinsella JM, Lunde KB (2004) Review of the trematode genus Ribeiroia (Psilostomidae): ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. Adv Parasitol 57:191–253

Kiesecker JM (2002) Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proc Natl Acad Sci 99:9900–9904

Köhler HR, Triebskorn R (2013) Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? Science 341:759–765

Krishnamurthy SV, Smith GR (2011) Combined effects of malathion and nitrate on early growth, abnormalities, and mortality of wood frog (Rana sylvatica) tadpoles. Ecotoxicology 20:1361–1367

Lajmanovich RC, Sandoval MT, Peltzer PM (2003) Induction of mortality and malformation in Scinax nasicus tadpoles exposed by glyphosate formulations. Bull Environ Contam Toxicol 70:612–618

Lannoo M (2008) The collapse of aquatic ecosystems: malformed frogs. University of California press, Berkeley

Lunde KB, Johnson PTJ (2012) A practical guide for the study of malformed amphibians and their causes. J Herpetol 46:429–441

Lynn WG (1950) A case of duplication of the tail in Plethodon. Herpetologica 6:81–84

Mann RM, Ross VH, Catherine BC, Scott PW (2009) Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ Pollut 157:2903–2927

Martin LB, Hopkins WA, Mydlarz LD, Rohr JR (2010) The effects of anthropogenic global changes on immune functions and disease resistance. Ann N YAcad Sci 1195:129–148

Mccoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette JR, Lj ST, Mary CM(2008) Agriculture alters gonadal formand function in the toad Bufomarinus. Environ Healt Perspect 116:1526–1532

McDaniel TV, Martin PA, Ross N, Brown S, Lesage S, Pauli BD (2004) Effects of chlorinated solvents on four species of North American amphibians. Arch Environ Contam Toxicol 47:101–109

Montalvão MF, da Silva Castro AL, de Lima Rodrigues AS, Oliveira Mendes B,Malafaia G (2018) Impacts of tannery effluent on development and morphological characters in a neotropical tadpole. Sci Total Environ 610-611:1595–1606

Mouillot D, Culioli JM, Pelletier D, Tomasini JA (2008) Do we protect biological originality in protected areas? A new index and an application to the Bonifacio Strait Natural Reserve. Biol Conserv 141: 1569–1580

Peltzer PM, Lajmanovich RC, Attademo AM, Junges CM, Cabagna- Zenklusen MC, Repetti MR, Sigrist ME, Beldómenico H (2013) Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (Trachycephalustyphonius) tadpoles. Ecotoxicol Environ Saf 98: 142–151

Pérez-Iglesias JM (2015) Biomarcadores para avaliação dos efeitos da atrazina em girinos de Rhinella schneideri (Bufonidae) e Physalaemus nattereri (Leiuperidae): utilização de parâmetros tradicionais e pigmentação visceral. Dissertation, Universidade Estadual Paulista

Pérez-Iglesias JM, Arcaute CR, Nikoloff N, Dury L, Soloneski S, Natale GS, Larramendy ML (2014) The genotoxic effects of the imidacloprid-based insecticide formulation GlacoxanImida on Montevideo tree frog Hypsiboas pulchellus tadpoles (Anura, Hylidae). Ecotoxicol Environ Saf 104:120–126

Pérez-Iglesias JM, Franco-Belussi L, Moreno L, Tripole S, Oliveira C, Natale GS (2016) Effect of glyphosate on hepatic tissue evaluating melanomacrophages and erythrocytes responses in neotropical anuran Leptodactylus latinasus. Environ Sci Pollut Res 23:9852–9861
Pezzuti TL (2011) Girinos do quadrilátero ferrífero, sudeste do Brasil: Ecomorfologia e chave de identificação interativa. Dissertation, Universidade Federal de Minas Gerais

PignatiAW, Lima FANS, Lara SS, CorreaMLM, Barbosa JR, Leão LHC, Pignatti MG (2017) Spatial distribution of pesticide use in Brazil: a strategy for health surveillance. Ciência & Saúde Coletiva 22:3281–3293

Robles-Mendoza C, García-Basilio C, Cram-Heydrich S, Hernández- Quiroz M, Vanegas-Pérez C (2009) Organophosphorus pesticides effect on early stages of the axolotl Ambystoma mexicanum (Amphibia: Caudata). Chemosphere 74:703–710

Rossa-Feres DC, Nomura F (2006) Morphological characterization and taxonomic key for tadpoles (Amphibia: Anura) from northwestern region of São Paulo state, Brazil. Biota Neotropica 6:1–26

Rohr JR, McCoy KA (2010) A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ Health Perspect 18:20–32

Rohr JR, Raffel TR, Sessions SK, Hudson PJ (2008) Understanding the net effects of pesticides on amphibian trematode infections. Ecol Appl 18:1743–1753

Rowe CL, Kinney OM, Fiori AP, Congdon JD (1996) Oral deformities in tadpoles (Rana catesbeiana) associated with coal ash deposition: effects on grazing ability and growth. Freshw Biol 36:723–730

Rowe CL, Hopkins WA, Congdon JD (2002) Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: a review. Environ Monit Assess 80:207–276

Rudek Z, Rozek M (1992) Induction of micronuclei in tadpoles of Rana temporaria and Xenopuslaevis by the pyrethroid Fastac 10 EC. Mutat Res 298:25–29

Schiesari L, Grillitsch B (2011) Pesticides meet megadiversity in the expansion of biofuel crops. Front Ecol Environ 9:215–221

Schiesari L, Grillitsch B, Grillitsch H (2007) Biogeographic biases in research and their consequences for linking amphibian declines to pollution. Conserv Biol 21:465–471

Schmutzer A, Chandler Gray MJ, Burton EC, Miller DL (2008) Impacts of cattle on amphibian larvae and the aquatic environment. Freshw Biol 53:2613–2625
Svartz GV, Jorge H, Cristina SPC (2012) Sublethal effects of atrazine on embryo-larval development of Rhinella arenarum (Anura: Bufonidae). Ecotoxicology 21:1251–1259

Taylor B, Skelly D, Demarchis LK, Slade MD, Galusha D, Rabinowitz PM (2005) Proximity to pollution sources and risk of amphibians limb malformation. Environ Health Perspect 113:1497–1501

Toledo LF (2005) Predation of juvenile and adult anurans by invertebrates: current knowledge and perspectives. Herpetol Rev 36:395– 400

Venesky MD, Parris MJ, Storfer A (2009) Impacts of Batrachochytrium dendrobatidis infection on tadpole foraging performance. EcoHealth 6:565–575
Venesky MD, Wassersug RJ, Parris MJ (2010) How does a change in labial tooth row number affect feeding kinematics and foraging performance of a ranid tadpole (Lithobates sphenocephalus)? Biol Bull 218:160–168. https://doi.org/10.1086/BBLv218n2p160

Voccia I, Blakley B, Brousseau P, Fournier M (1999) Immunotoxicity of pesticides: a review. Toxicol Ind Health 15:119–132

Wagner D, Brühl M, Veith PP, Lötters S (2014) Evaluating the risk of pesticide exposure for amphibian species listed in Annex II of the European Union Habitats Directive. Biol Conserv 176:64–70

Young B, Stuart SN, Chanson JS, Cox NA, Boucher TM (2004) Disappearing jewels: The status of New World amphibians. Nature Serve, Arlington

9. APÊNDICE B - CAPÍTULO 2: Manuscript accept to Archives of Environmental Contamination and Toxicology.

Genotoxic evaluation in tadpoles associated with agricultural in the central cerrado, Brazil

Rinneu Elias Borges[1]. Lia Raquel de Souza Santos[2]. Marcelino Benvindo-Souza[2], Richelle Sousa Modesto[1], Rhayane Alves Assis[2] Classius Oliveira[3]

Abstract Many agricultural practices cause environmental degradation that affects the cellular integrity of anurans. In the present study, we provided in situ data of *Dendropsophus minutus, Physalaemus cuvieri* and *Scinaxfus fuscovarius* collected in soybean/corn and conservation units in the Brazilian Cerrado. The in situ data showed no significant variation in the micronucleus frequency between the sites, only the reniform cells had a higher rate for the agricultural environment. A combined analysis of all nuclear erythrocyte abnormalities (ENAs = reniform nuclei, apoptotic cell, binucleated and anucleated cells), was recorded higher frequencies in farmland. Overall, *Scinax fuscovarius* was considered the best potential bioindicator for soybean/corn plantations. Finally, we recommend expanding the micronucleus test for in situ studies in order to expand our understanding of the sensitivity of native anuran species and provide a more systematic assessment of the adverse effects of environmental pollutants on wildlife.

Keywords Agriculture, Amphibians, Nuclear abnormalities, Biomarker, Conservation

Introduction

The micronucleus test has now been used to monitor the erythrocytes of anurans for the presence of xenobiotics throughout the world for more than thirty years (Krauter et al. 1987), emphasizing the potential of this procedure as a biomarker, which is still widely used in the present day (Cruz-Esquivel et al. 2017; Natale et al. 2018). Micronuclei are fragments of chromosomes that have been excluded from the principal nucleus in tissue cells under division (Bosch et al. 2011). An increase in the frequency of micronuclei is considered to be an early response to chromosomal damage (Garaj-Vrhovacet al. 2008). Given this, micronucleus test is one of the most effective cytogenetic assays for the detection of genotoxic damage induced by clastogenic and aneugenic agents (Udroiu et al. 2015), and the use of these biomarkers can provide a useful early warning system for the presence of pollutants before any irreversible damage occurs to the ecosystem (Depledge and Fossi 1994; Josende et al. 2015). Xenobiotic agents may cause carcinogenic effects (Anderson et al. 1994), teratogenic, embryotoxicity, as well as a number of health disorders referred to as genotoxic disease syndrome (Kurelec 1993). The genotoxic agents, besides damaging the DNA (Gonçalves et al. 2015), may lead to a number of sublethal effects such as liver damage, morphological abnormality, and affect the growth of anurans (Pérez-Iglesias et al. 2019). When genomic damage arises and is not repaired, it may be corrected in the subsequent cell cycle, at which point mutations capable of affecting the animals' survival may occur (Gonçalves et al. 2017a).

In this context, amphibians are widely used as bioindicators of environmental disturbance (Cruz-Esquivel et al. 2017), given that a large number of studies have shown that these vertebrates are highly sensitive to pesticides, due to their biological and ecological characteristics, including their metabolism, aquatic life cycle, sensitive skin, and unprotected eggs (Hayes et al. 2006, 2010; Brühl et al. 2011; Bradford et al. 2011; Nikoloff et al. 2014; Arcaute et al. 2014; Cruz-Esquivel et al. 2017). Evidence on the negative effects of exposure to pesticides in anurans has been growing progressively for more than thirty years (Berger 1989). These environmental pollutants are now considered to be predictors of the risk of decline of amphibian populations throughout the world (Mann et al. 2009; Jing et al. 2017), indicating that this group of vertebrates is one of the most vulnerable to the effects of agricultural contaminants (Sparlinget al. 2010).

Anurans play an important role in both natural and agricultural ecosystems, given that, in addition to their value as bioindicators, these animals are important natural predators of many agricultural pests (Arcaute et al. 2014; Cruz-Esquivel et al. 2017). Species that reproduce in bodies of water are probably the most affected by toxic waste, given that they are exposed directly to pollutants, with serious, often fatal consequences, which can cause populations to decline (Hayes et al. 2010; Babini et al. 2016). The unprecedented expansion of farmland in South American countries, such Brazil (Dias et al. 2016), reinforces the need for more systematic data on the effects of contamination on the region's anuran fauna. In the present study, we investigated the tadpoles of three anuran species *in situ* in temporary pools found within soybean plantations, and compared the results with data on the same three species from a protected area, in order to evaluate the variation in the levels of genotoxic damage.

One of these species was *Dendropsophus minutus* (Anura: Hylidae), which has an ample distribution in South America (Gehara et al. 2014). Recently, Gonçalves et al. (2017b) found evidence that the herbicide atrazine has genotoxic and mutagenic effects in this species. The second species is *Scinax fuscovarius*, which is found in open habitats, such as pastures, in southern, southeastern, and midwestern Brazil, as well as eastern Bolivia and Paraguay, and northern Argentina and Uruguay. The third species, *Physalaemus cuvieri*, is found throughout most of southern, central, and eastern Brazil, in Missions and Entre Rios, in Argentina, and in eastern Paraguay (Mijares et al. 2010). This species is highly adaptable, and occurs in many different types of habitat, including pastures and savanna wetlands (Mijares et al. 2010). Gonçalves et al. (2017a) considered this species to be a good model for the detection of the genotoxic effects of the agricultural inseticides used on plantations, and as it is a common and abundant species, it would be ideal for environmental biomonitoring. This is the first *in situ* micronucleus study of both *Scinax fuscovarius* and *Physalaemus cuvieri.*

Brazil is a key region, since it is among the regions of the world with greater diversity of anurans, however, the expansion of agriculture is a constant threat. Given this, to expand the database on the genotoxic parameters of anuran populations *in situ*, we analyzed wild-caught specimens of the tadpoles of three species, *Dendropsophus minutus, Physalaemus cuvieri* and *Scinax fuscovarius* using the micronucleus test.

Material and Methods

Sampling design of the in situ study

The tadpoles were collected in two different areas in 2014 and 2016, with the aid of a dip net in temporary and semi-permanent water pools and packed in plastic containers until sorting. Two areas were selected in order to contemplate the different land use histories. One of these areas was Emas National Park, which was considered to be the control, in this environment the animals captured in side the conservation unit were in puddles, swamps and lagoons distant about 26 km from the edge of the park. The second site was an area of farm land in the municipality of Rio Verde, representing an environment with a greater genotoxic susceptibility, the tadpoles were collected, concomitantly to the harvest period (soybean) in a level curve and temporary pools located in the interior of plantations. Both these areas are located within the Cerrado biome of central Brazil, in the south of the state of Goiás. The Emas National Park (18°2'37.64" S, 52°56'30.11" W) covers an area of 132,000 hectares, most of which is located within the municipality of Mineiros, in Goiás, as well as the adjacent municipality of Costa Rica, in the state of Mato Grosso do Sul. This national park is considered to be the most important protected area within the Cerrado biome, due to its considerable diversity of fauna and flora (IBDF 1981; Redford and Fonseca 1986). More than two-thirds (68.1%) of the park is covered in open savanna, while a quarter (25.1%) is dense savanna, and the rest of the area encompasses riparian forests and marsh lands (Ramos-Neto and Pivello 2000). By contrast, the landscape of Rio Verde (17°47’53.24” S, 50°54’6.61” W) is dominated by plantations of soybean and corn (Pignati et al. 2017), with only a few scattered patches of forest. Rio Verde was chosen for the present study because this municipality is one of the principal consumers of pesticides in Brazil (Pignati et al. 2017). Another aspect that justifies the importance of this study is the scarcity of *in situ* investigations throughout South America for the test of micronuclei in anurans (Supplementary).

Tadpoles (Gosner 1960, phases 34–38, pre-metamorphic stages whose species characteristics are already defined) of three anuran species (*Physalaemus cuvieri*, *Scinax fuscovarius*, and *Dendropsophus minutus*) were with stereo microscope and identification keys (Rossa-Feres and Nomura 2006) and analyzed in the present study. Ten specimens of each species were collected frome ach study area, with a total sample of 60 specimens, 20 for eachspecies (license SISBIO no. 41919-1; CEUA no. 0316).

Micronuclei and other nuclear abnormalities of the erythrocyte

The micronucleus test (MN) was applied to erythrocytes obtained following the processing and identification of the specimens, based on the protocol of Pérez-Iglesias et al. (2015). The tadpoles were first anesthetized by immersion in freezing water and blood samples were then obtained through an incision made behind the operculum. Two smears of blood were obtained on clean slides for each animal, which were then air-dried, fixed with cold (4°C) 100% methanol for 20 minutes, and then stained in 5% Giemsa during 12 minutes. A sample of 1000 cells was analyzed in each tadpole to determine the frequency of micronuclei (MN) and other erythrocyte nuclear abnormalities

(ENAs), using a magnification of 100x, as recommended by Pollo et al. (2015). The MNs were identified using the following criteria (Pérez-Iglesias et al. 2015): (i) the diameter is smaller than 1/16 to 1/3 of the principal nucleus, (ii) the material is non-reflective, with the same or a lower intensity of staining in comparison with the principal nucleus, and (iii) the structure is not linked to or overlapping the principal nucleus. In addition to the frequency of MNs, we quantified five other types of ENAs: Nuclear Buds (NB), reniform nuclei (RE), apoptotic cell (AP), binucleated (BI) and anucleated (AN) cells (Cruz-Esquivel et al. 2017). The frequency of ENAs is presented per type of abnormality, and for the total set of abnormalities (NB+RE+AP+BI+AN), which is the standard approach in this type of analysis (Pollo et al. 2015).

Water analysis

Water samples were collected and analyzed in a specific laboratory for the evaluation of organochlorine compounds, organophosphorates and carbamates. Quantitative and qualitative results of the samples indicated high Atrazine indices (5000μg/L) in samples of agricultural environment compared to the samples from the Conservation Unit (Borges et al., 2019).

Statistical analyses

We presented the *in situ* data as means±standard deviation. We evaluated the variation in the frequency of micronuclei (MN) and other nuclear abnormalities (NB+RE+BI+AP+AN) between study areas and among species using the nonparametric Kruskal-Wallis analysis of variance, followed by Dunn's test. A factorial Analysis of Variance (ANOVA), with Tukey's*post-hoc* test was used to evaluate the set of erythrocytenuclear abnormalities (ENAs). In this case, the data were root-transformed to satisfy the assumption of homogeneity of variance. A significance of $p < 0.05$ was considered for all analyses.

Results

In this study, no genotoxic damage was found related to the formation of micronucleated-cells for the three species evaluated between the study environments (*H* = 4.8391, *P* = 0.43) (Table 1). However, reniform cells were significantly more frequent (*H* = 13.6809, *P* = 0.01) in the tadpoles from the farm land, in comparison with the control (Table 1). The data on the micronuclei and other erythrocyte nuclear abnormalities (NB, RE, BI, AP, and AN) in the three anuran species are shown in Table 1 and Figure 1. No significant variation was found among the three species (*D. minutus, P. cuvieri* and *S. fuscovarius*) in the frequency micronuclei, either at Rio Verde (*H* = 3.463, *P* = 0.20) or in Emas National Park (*H* = 0.5446, *P* = 0.76).

Table 1 Mean±standard deviation of the frequency of micronuclei and other erythrocyte nuclear abnormalities (ENAs) observed per 1000 cells in tadpoles, comparing anuran species and environments

Species/Area	MN/1000 cells	Others erythrocyte nuclear abnormalities/1000 cells				
		NB	**RE**	**BI**	**AP**	**AN**
PNE						
D. minutus	0.20±0.42	0.00±0.00	0.70±0.67	0.20±0.63	0.00±0.00	0.80±2.53
P. cuvieri	0.20±0.63	0.10±0.32	0.20±0.42	0.10±0.32	0.00±0.00	0.40±1.26
S. fuscovarius	000±0.00	0.00±0.00	0.60±1.07	0.10±0.32	0.00±0.00	0.00±0.00
Rio Verde						
D. minutus	0.40±0.52	0.20±0.42	1.60±2,55	0.50±0.71	0.10±0.32	0.60±1.90
P. cuvieri	0.60±0.97	0.40±0.70	3.50±5.23*	0.00±0.00	0.30±0.67	0.70±1.89
S. fuscovarius	0.00±0.00	0.30±0.67	2.90±2.56	1.00±1.05	0.00±0.00	0.20±0.42

PNE = Emas National Park; Rio Verde = farmland in the municipality of Rio Verde, Goiás. MN = Micronucleus, NB = nuclear buds, RE = nuclei reniform, BI = binucleated cell, AP = Apoptotic cell, AN = anucleated cell. *Significant difference (p <0.05) comparing PNE species to reniform cells

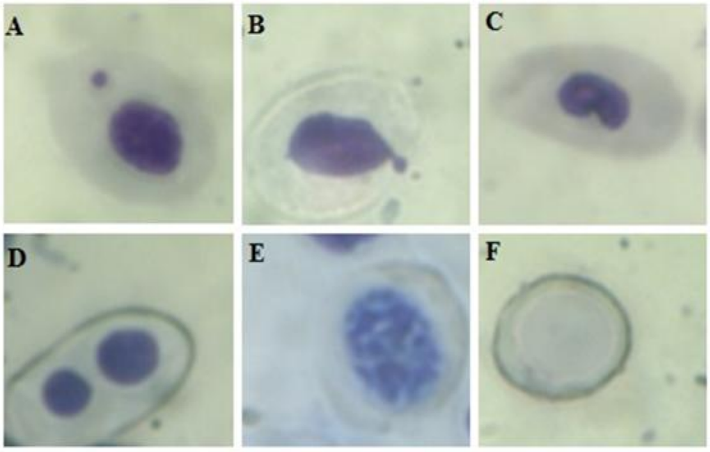


Fig. 1 Erythrocytes of tadpoles, magnified 1000x: (A) micronucleus, (B) nuclear bud; (C) reniform nucleus; (D) binucleated cell; (E) Apoptotic cell, and (F) anucleated cell

In the combined analysis of all erythrocyte nuclear abnormalities (ENAs), a significant difference was found between environments ($F_{1,54}$= 16.340, P < 0.001), with higher frequencies being recorded in the farmland (Fig. 2 A). Overall, then, in the comparison between the two study areas, only *S. fuscovarius* presented a significant difference between environments in the set of erythrocyte abnormalities found in the tadpoles (Fig. 2 B).

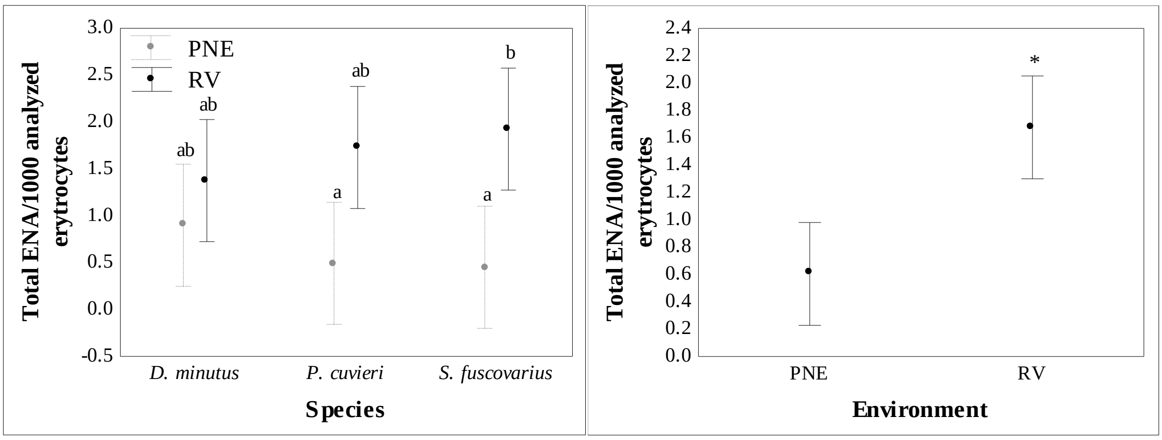


Fig. 2 Erythrocyte nuclear abnormalities (ENAs) in tadpoles sampled in a protected area (Emas National Park = PNE) and farmland (Rio Verde = RV). The root-transformed data are presented as the mean (circles) and standard deviations (vertical bars). A: the asterisk indicates a significant difference in the

ENAs recorded in RV in comparison with the control (PNE). B: different letters indicate significant differences, whereas the values marked with the same letters are statistically similar to one another, based on the factorial ANOVA.

Discussion

Although Brazil is rich in anurans diversity, the use of hematological test, such as the micronucleus assay, has been applied in these animals for a little more than a decade (Ferreira et al. 2003), to track environmental toxicity. Thus, in order to expand the knowledge of the anthropic impact on tadpoles for an on-site approach. In the present study here we find no significant variation between species of anurans within each environment (Emas National Park and agricultural land), either by micronuclei or other nuclear erythrocyte abnormalities, that is, nuclear buds, apoptotic cells and anucleated cells. In contrast, there was a higher rate of reniform nucleus cells in *P. cuvieri* than in the control area. Lajmanovich et al. (2014) and Arcaute et al. (2014) observed that nuclear abnormalities may be a consequence of exposure to environmental contaminants with cytotoxic, genotoxic, or mutagenic activity, which is consistent with our findings. Binucleated cells and nuclear buds can be considered to be indicators of genetic damage (Cavas and Ergene-Gözükara 2005; GökalpMuranli and Güner 2011), and even though no significant differences were found in the present study, the findings were similar to those on the micronuclei. A number of previous *in situ* studies have detected an increase in the frequency of micronuclei in agricultural environments, in comparison with control areas (Gonçalves et al. 2015; Pollo et al. 2016; Cruz-Esquivel et al. 2017).

Experimental *ex situ* studies have also shown the impact of pesticides, through the induction of micronuclei and other nuclear abnormalities (Arcaute et al. 2014; Jing et al. 2017; Gonçalves et al. 2017b; Natale et al. 2018). In general, *in situ* studies are popular tools in aquatic toxicology, and their application for the determination of specific toxicological effects of the environment is highly recommended (Castro et al. 2003; Antunes et al. 2008).

We emphasize that even though there is no statistical significance for the frequency of MN for the studied species, *Dendropsophus minutus* and *Physalaemus cuvieri* from the agricultural environment always presented mean MN frequencies higher than those recorded in Conservation Units. However, we suggest that these organisms, especially *S.fuscovarius*, can be considered sentinels for environmental disturbance, since in the ENAs analysis, this species responded to the different land use. As far as we know, this is the first micronucleus test study on *S. fuscovarius* and *P. cuvieri* under natural conditions for the detection of genotoxic effects, although comet assays are available for *P. cuvieri* tadpoles from pools in soybean plantations, where the specimens presented significantly more DNA damage, in comparison with the control (Gonçalves et al. 2017a). Gonçalves et al. (2015) also found that *D. minutes* specimens from disturbed areas had large quantities of DNA damage in blood cells, in comparison with animals from areas with no agricultural activities. In a more recent study, these same authors (Gonçalves et al. 2017b) concluded that atrazine is clastogenic, with dose-dependent genotoxic and mutagenic effects in *D. minutus*, depending on the larval development phase. Studies of this kind are important for the identification of sentinel species that are more sensitive to environmental pressures, and require more data. Brodeur et al. (2009) and Zaya et al. (2011) concluded that atrazine may provoke a range of effects, ranging from morphological alterations, such as the inversion of the sex of the adults, to shifts in behavior, as seen in the reduced swimming capacity of the tadpoles.

The integrated analysis of all nuclear abnormalities nevertheless revealed effects of environmental disturbance. Agricultural environments favor an increase in nuclear abnormalities, as confirmed in *in situ* studies (Babini et al. 2016), as well as in areas impacted by mining (Pollo et al. 2016; Pollo et al. 2017). In an *ex situ* study, Lajmanovich et al. (2014) observed that *R. arenarum* exposed to herbicide presented an increase in the frequency of damaged erythrocytes.

Our findings are also consistent with the results of controlled *ex situ* studies of agricultural contaminants (Lajmanovich et al. 2014; Montalvão and Malafaia 2017). In these cases, the frequency of ENAs was consistently higher than that of micronuclei, as also shown by Pollo et al. (2016). These findings indicate a greater genetic instability in *S. fuscovarius* from farmland, in comparison with preserved areas. Pesticides were shown to provoke a biochemical response in *S. fuscovarius* tadpoles, with an increase in susceptibility to environmental contaminants (Margarido et al. 2013). All thees findings point clearly to an increase in genotoxic susceptibility in disturbed areas, in comparison with more preserved environments.

Overall, then, the present study provides important insights into the *in situ* vulnerability of anurans of the Brazilian Cerrado to genotoxic effects in agricultural environments, for which data are still scant. Further research is clearly needed on these species, in particular for the different stages of development. In Gonçalves et al. (2017b), the pre-metamorphic stage of *D. minutus* presented a significantly larger quantity of DNA damage and micronuclei, although, depending on the dose, the most extensive DNA damage was found in stages 42–43GS. The sampling of adult specimens would also provide important insights, as would the more systematic evaluation of disturbance, through the analysis of water quality, for example. The morphological analysis of tissue may also provide an ampler perspective on the health of both the amphibians and the environment. Based on these biomarkers, combined with the MN test, it should be possible to reduce the potential for false positives, and provide a more reliable and systematic analysis of the phenomenon. Reinforcing these conclusions, Barni et al. (2007) pointed out that these organisms react to environmental contaminants through alterations in certain specific structural and functional parameters (biomarkers) at different hierarchical levels, that is, in the molecules, cells, tissue, and organs.

Conclusion

This study showed the application of the micronucleus test in anurans in Brazil. We provides new in situ data from soybean plantations in the Brazilian Cerrado savanna, which demonstrated that *P. cuvieri* (increase in reniform nuclei) and *S. fuscovarius* (increase in all erythrocyte nuclear anomalies) are potential candidates for sentinel species in disturbed environments. Overall, the micronucleus test applied to the analysis of tadpoles is a rapid and effective method for the detection of genotoxic damage, which would be recommendable for in situ studies of the response of anurans to environmental disturbance.

Acknowledgments We are grateful to the Brazilian National Council for Scientific and Technological Development (CNPq), for supporting this study through personal research grant number 477044/2013-1, and the São Paulo State Research Foundation (FAPESP) for supporting the analyses (process no. 2013/02067-5). We would also like to thank the Chico Mendes Institute for Biodiversity Conservation (ICMBio/SISBIO), and the Emas National Park for authorizing fieldwork.

Referências

Amaral DF, Montalvao MF, Mendes BD, de Souza JM, Chagas TQ, Rodrigues ASD, Malafaia G (2018a) Insights about the toxic effects of tannery effluent on *Lithobates catesbeianus* tadpoles. Science of The Total Environment 621:791-801

Amaral DF, Montalvao MF, Mendes BD, Castro ALD, Malafaia G (2018b) Behavioral and mutagenic biomarkers in tadpoles exposed to different abamectin concentrations. Environmental Science and Pollution Research 25:12932-12946

Agarwal S and Sohal RS (1994) DNA oxidative damage and life expectancy in houseflies. Proc. Nat. Acad. Sci. USA 91:12332-5

Anderson S, Sadinski W, Shugart L, Brussard P, Depledge M, Ford T, Hose J, Stegeman J, Suk W, Wirgin I, Wogan G (1994) Genetic and Molecular Ecotoxicology: A Research Framework. Environmental Health Perspectives 102:3-8

Antunes SC, Castro BB, Nunes B, Pereira R, Gonçalves F (2008) In situ bioassay with *Eisenia andrei* to assess soil toxicity in an abandoned uranium mine. Ecotoxicology and Environmental Safety, 71:620-631

Arcaute CR, Pérez-Inglesis JM, Nikoloff N, Natale G.S, Soloneski S, Larramendy ML (2014) Genotoxicity evaluation of the insecticide imidacloprid on circulating blood cells of Montevideo tree frog *Hypsiboaspulchellus* tadpoles (Anura, Hylidae) by comet and micronucleus bioassays. Ecological Indicators 45:632-639

Babini MS, Bionda CL, Salas NE, Martino AL (2015) Health status of tadpoles and metamorphs of *Rhinella arenarum* (Anura, Bufonidae) that inhabit agroecosystems and its implications for land use. Ecotoxicology and Environmental Safety, 118:118-125

Babini MS, Bionda CL, Salas NL, Martino AL (2016) Adverse effect of agroecosystem pond water on biological endpoints of common toad (*Rhinella arenarum*) tadpoles. Environmental Monitoring and Assessment188:459

Babini MS, Bionda CD, Salinas ZA, Salas NE, Martino AL (2018) Reproductive endpoints of *Rhinella arenarum* (Anura, Bufonidae): Populations that persist in agroecosystems and their use for the environmental health assessment. Ecotoxicology and environmental Safety 154:294-301

Barni S, Boncompagni E, Grosso A, Bertone V, Freitas I, Fasola M, Fenoglio C (2007) Evaluation of *Rana snkesculenta* blood cell response to chemical stressors in the environment during the larval and adult phases. Aquatic Toxicology 81:45-54

Berger L (1989) Disappearance of amphibian larvae in the agricultural landscape. Ecology International Bulletin 17: 65-73

Bolognesi C Perrone E, Roggieri P, Pampanin DM, Sciutto A (2006) Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquatic Toxicology 78S:93-98

Bolognesi C, Knasmueller S, Nersesyan A, Thomas P, Fenech M (2013) The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay – An update and expanded photogallery. Mutation Research/Reviews in Mutation Research 753:100-113

Borges RE, Santos LRS, Assis RA, Benvindo-Souza M, Franco-Belussi L, Oliveira C (2019) Monitoring the morphological integrity of neotropical anurans. Environmental Science and Pollution Research 26:2623-2634.

Bosch B, Mañas F, Nora G, Aiassa D (2011) Micronucleus test in post metamorphic Odontophrynuscordobae and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. Journal of Toxicology and Environmental Health Sciences 3:155-163

Bradford DF, Knapp RA, Sparling DW, Nash MS, Stanley KA, Tallent-Halsell NG, McConnell LL, Simonich SM (2011) Pesticide distributions and population declines of California, USA, alpine frogs, *Rana muscosa* and *Rana sierra*. Environmental Toxicology and Chemistry30:682-691

Brodeur JC, Svartz G., Perez-Coll CS, Marino DJG, Herkovits J (2009) Comparative susceptibility to atrazine of three developmental stages of *Rhinella arenarum* and influence on metamorphosis: Non-monotonous acceleration of the time to climax and delayed tail resorption. AquaticToxicology 91:161-170

Brühl CA, Pieper S, Weber B (2011) Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. Environmental Toxicology and Chemistry30:2465-2472

Castro BB, Guilhermino L, Ribeiro R (2003) In situ bioassay chambers and procedures for assessment of sediment toxicity with *Chironomus riparius*. Environ Pollution 125:325-335

Cavas T, Ergene-GözükaraS, (2005) Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. Aquatic Toxicology 74:264-271

Cavas T, Garanko NN, Arkhipchuk VV (2005) Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. Food and Chemical Toxicology 43:569–574

Cavalcante DG.SM, Martinez CBR, Sofia SH (2010) Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*, Mutation Research - Genetic Toxicology and Environmental Mutagenesis695:41-46

Crane M, Burton G.A, Culp JM, Greenberg MS, Munkittrick KR, Ribeiro R, Salazar MH, St-Jean SD (2007) Review of Aquatic In Situ Approaches for Stressor and Effect Diagnosis. Integrated Environmental Assessment and Management 3:234-245

Cruz-Esquivel A, Viloria-Rivas J, Marrugo-Negrete J (2017) Genetic damage in *Rhinella marina* populations in habitats affected by agriculture in the middle region of the SinA River, Colombia. Environmental Science and Pollution Research 24:27392-27401

Depledge MH, Fossi MC (1994) The role of biomarkers in environmental assessment. Ecotoxicology 3:161-172

Dias LCP, Pimenta FM, Santos AB, Costa MH, Ladle RJ (2016) Patterns of land use, extensification, and intensification of Brazilian agriculture. Global Change Biology 22:2887-2903

Ernst F, Alonso B, Colazzo M, Pareja L, Cesio V, Pereira A, Marquez A, Errico E, Segura AM, Heinzen H, Perez-Parada A (2018) Occurrence of pesticide residues in fish from south American rainfed agroecosystems. Science of the Total Environment 631-632:169-179

Fenech M (2000) The in vitro micronucleus technique. Mutation Research - Genetic Toxicology and Environmental Mutagenesis 455:81-95

Ferreira CM, Bueno-Guimaraes MJ, Ranzani-Paiva HM, Soares SRC, Rivero DH, Saldiva PH (2003) Hematological markers of copper toxicity in *Rana catesbeiana* tadpoles (Bullfrog). Revista brasileira de toxicologia16:83-88

Franco-Belussi L, Skold HN, de Oliveira C (2016) Internal pigment cells respond to external UV radiation in frogs. Journal of Experimental Biology 219:1378-1383

Garaj-Vrhovac V, Gajski G, Ravlić S (2008) Efficacy of HUMN criteria for scoring the micronucleus assay in human lymphocytes exposed to a low concentration of p,p'-DDT. Brazilian Journal of Medical and Biological Research41:473- 476

Gehara M, Crawford AJ, OrricoVGD, Rodríguez A, et al. (2014). High Levels of Diversity Uncovered in a Widespread Nominal Taxon: Continental Phylogeography of the Neotropical Tree Frog Dendropsophusminutus. Plos One9:e111829

Gonçalves MW, Vieira TB, Maciel NM, Carvalho WF, Lima LSF, Gambale PG, da Cruz AD,Nomura F, Bastos RP, Silva DM (2015) Detecting genomic damages in the frog *Dendropsophus minutus*: preserved versus perturbed areas. Environmental Science and Pollution Research 22:3947-3954

Gonçalves MW, Gambale PG, Godoy FR, Alves AA, Rezende PHD, Maciel NM, Nomura F, Bastos RP, de Marco P (2017a) The agricultural impact of pesticides on *Physalaemus cuvieri* tadpoles (Amphibia: Anura) ascertained by comet assay. Zoologia4:e19865

Gonçalves MW, de Campos CBM, Batista VG, da Cruz AD, de Marco P, Bastos RP, Silva DDE (2017b) Genotoxic and mutagenic effects of Atrazine Atanor 50 SC on *Dendropsophus minutus* Peters, 1872 (Anura: Hylidae) developmental larval stages. Chemosphere 182:730-737

Gosner KL (1960) A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification. Herpetologica16:183-190

GökalpMuranli FD, Güner U (2011) Induction of micronuclei and nuclear abnormalities in erythrocytes of mosquito fish (*Gambusia affinis*) following exposure to the pyrethroid insecticide lambda-cyhalothrin. Mutation Research 726:104-108

Glomski CA, Tamburlin J, Hard R et al. (1997) The phylogenetic odyssey of the erythrocyte. IV. The amphibians. Histology and Histopathology 12:147-70

Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J (2006) Pesticide mixtures, endocrine disruption, and Amphibian declines: are we underestimating the impact? Environmental Health Perspectives114:40-50

Hayes TB, Falso P, Gallipeau S, Stice M (2010) The cause of global amphibian declines: a developmental endocrinologist's perspective. Journal of Experimental Biology213:921-933

Harabawy AS, Mosleh YL (2014) The role of vitamins A, C, E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of Nile tilapia, *Oreochromis niloticus*. Ecotoxicology and Environmental Safety 104:28-35

IBDF (Instituto Brasileiro de Desenvolvimento Florestal) (1981) Plano de manejo-Parque Nacional das Emas PNE. I.B.D.F./F.B.C.N., Brasília, 90 pp.

Jing X, Yao GJ, Liu DH, Liu C, Wang F, Wang P, Zhou ZQ (2017) Exposure of frogs and tadpoles to chiral herbicide fenoxaprop-ethyl. Chemosphere 186:832-838

Josende ME, Tozetti MA, Alalan TM, Filho MV, Ximenez SS, Silva Júnior FMR, Martins ES (2015) Genotoxic evaluation in two amphibian species from Brazilian subtropical wetlands. Ecological Indicators 49:83-87

Kiechle FL, Zhang X (2002) Apoptosis: biochemical aspects and clinical implications.Clinica ChimicaActa326:27-45

Kurelec B (1993) The genetic disease syndrome. Marine Environmental Research 35:341-348

Krauter PW, Anderson SL, Harrison FL (1987) Radiation-induced micronuclei in peripheral erythrocytes of Ranacatesbeiana: an aquatic animal model for in vivo genotoxicity studies. Environmental and Molecular Mutagenesis 10:285-96

Lajmanovich RC, Cabagna M, Peltzer PM, Stringhini G.A, Attademo AM (2005) Micronucleus induction in erythrocytes of the Hylapulchella tadpoles (Amphibia: Hylidae) exposed to insecticide endosulfan. Mutation Research 587:67-72

Lajmanovich RC, Cabagna-Zenklusen MC, AttademoAM,Junges CM, PeltzerPM,BassoA,Lorenzatti E (2014) Induction of micronuclei and nuclear abnormalities in tadpoles of the common toad (*Rhinella arenarum*) treated with the herbicides Liberty® and glufosinate-ammonium. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 769:7-12

Lima PD, Vasconcellos MC, Montenegro RC, Bahia MO, Costa ET, Antunes LM, et al. (2011) Genotoxic effects of aluminum, iron and manganese in human cells andexperimental systems: a review of the literature. Human & Experimental Toxicology, 30:1435-1444

Londero JEL, dos Santos CP, Segatto ALA, Schuch AP (2017) Impacts of UVB radiation on food consumption of forest specialist tadpoles. Ecotoxicology and Environmental Safety 143:12-18

Machado da Rocha C (2011) The micronucleus test in erythrocytes of amphibian larvae as tool for xenobiotic exposure risk assessment: a brief review and an example using *Lithobates catesbeianus* exposed to copper sulphate.Middle East Journal of Scientific Research8:23-29

Mann RM, Hyne RV, Choung CB, Wilson SP (2009) Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ Pollution 157:2903-2927

Mahboob S, Al-Balwai HFA, Al-Misned F, Ahmad Z (2014) Investigation on the genotoxicity of mercuric chloride to freshwater *Clarias gariepinus*. Pakistan Veterinary Journal 34:100-103

Margarido TCS, Felicio AA, Rossa-Feres DD, de Almeida EA (2013) Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. Marine Environmental Research, 91:61-67

Martínez-Valenzuela C, Waliszewski SM, Amador-Muñoz O, Meza E, Calderón-Segura ME, Zenteno E, Huichapan-Martínez J, Caba M, Félix-Gastélum R, Longoria-Espinoza R. (2017) Aerial pesticide application causes DNA damage in pilots from Sinaloa, Mexico. Environmental Science and Pollution Research, 24: 2412-2420

Mesak C, Mendes BD, Ferreira RD, Malafaia G. (2018) Mutagenic assessment of *Lithobatescatesbeianus* tadpoles exposed to the 2, 4-D herbicide in asimulated realistic scenario. Environmental Science and Pollution Research 25:15235-15244

Mijares A, Rodrigues MT, Baldo D (2010) *Physalaemus cuvieri.* The IUCN Red List of Threatened Species 2010:e.T57250A11609155

Montalvao MF and Malafaia G (2017) Effects of abamectin on bullfrog tadpoles: insights on cytotoxicity. Environmental Science and Pollution Research 24:23411-23416

Natale GS, Vera-Candioti J, de Arcaute CR, Soloneski S, Larramendy ML, Ronco AE (2018)Lethal and sublethal effects of the pirimicarb-based formulation Aficida (R) on *Boana pulchella* (Dumeril and Bibron, 1841) tadpoles (Anura, Hylidae). Ecotoxicology and Environmental Safety 147:471-479

Nikoloff N, Natale G.S, Marino D, Soloneski S, Larramendy ML (2014) Flurochloridone-based herbicides induced genotoxicity effects on *Rhinella arenarum* tadpoles (Anura: Bufonidae). Ecotoxicology and Environmental Safety 100:275-281

Nori JN, Lescano P, Illoldi-Rangel N, Frutos MR, Cabrera G.C, Leynaud (2013) The conflict between agricultural expansion and priority conservation areas: making the right decisions before it is too late. Biological Conservation 159:507-513

Ossana NA, Castane PM, Poletta GL, Mudry MD, Salibian A (2010) Toxicity of Waterborne Copper in premetamorphic tadpoles of *Lithobates cat esbeianus* (shaw, 1802). Bulletin of Environmental Contamination and Toxicology 84:712-715

Pérez-Iglesias JM, Arcaute CR, Nikoloff N, Dury L, Soloneski S, Natale G.S, Larramendy ML (2014) Ecotoxicology and Environmental Safety 104:120-126

Pérez-Iglesias JM, Soloneski S, Nikoloff N, Natale GS.Larramendy ML (2015) Toxic and genotoxic effects of the imazethapyr-based herbicide formulation Pivot H® on montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae). Ecotoxicology and Environmental Safety 119:15-24

Pérez-Iglesias JM, Natale GS, Soloneski S, Larramendy ML (2018) Are the damaging effects induced by the imazethapyr formulation Pivot (R) H in *Boana pulchella* (Anura) reversible upon ceasing exposure? Ecotoxicology and Environmental Safety 148:1-10

Pérez-Iglesias, JM, Franco-Belussi, L, Natale, GS, Oliveira, C (2019) Biomarkers at different levels of organisation after atrazine formulation (SIPTRAN 500SC®) exposure in *Rhinella schineideri* (Anura: Bufonidae) Neotropical tadpoles. Environmental Pollution 244:733-746

Pignati AW, Lima FANS, Lara SS, Correa MLM, Barbosa JR, Leão LHC, Pignatti MG, (2017) Spatial distribution of pesticide use in Brazil: a strategy for Health Surveillance. Ciência&SaúdeColetiva22:10

Pollo FE, Bionda CL, Salinas ZA, Salas NE, Martino AL (2015) Common toad *Rhinella arenarum* (Hensel, 1867) and its importance in assessing environmental health: test of micronuclei and nuclear abnormalities in erythrocytes. Environmental Monitoring and Assessment 187:1-9

Pollo FE, Grenat PR, Otero MA, Salas NE, Martino AL (2016) Assessment in situ of genotoxicity in tadpoles and adults of frog *Hypsiboas cordobae* (Barrio 1965) inhabiting aquatic ecosystems associated to fluorite mine. Ecotoxicology and Environmental Safety 133: 466-474

Pollo FE, Grenat PR, Salinas ZA, Otero MA, Salas NE, Martino AL (2017) Evaluation in situ of genotoxicity and stress in South American common toad

Rhinella arenarumin environments related to fluorite mine. Environmental Science and Pollution Research 24:18179-181872

Qin YJ, Li X, Xiang Y, Wu D, Bai LW, Li ZZ, Lang Y (2017) Toxic effects of glyphosate on diploid and triploid fin cell lines from *Misgurnus anguillicaudatus*. Chemosphere 180: 356-364.

Ramos-Neto MB, Pivello VR (2000) Lightning Fires in a Brazilian Savanna National Park: Rethinking Management Strategies. Environmental Management26:675-684

Ray MR, Basu C, Mukherjee S, Roychowdhury S, Lahiri T (2005) Micronucleus frequencies and nuclear anomalies in exfoliated buccal epithelial cells of firefighters. International Journal of Human Genetics 5:45-58

Redford KH, Fonseca GAB (1986) The Role of Gallery Forests in th Zoogeography of the Cerrado's Non-volant Mammalian Fauna. Biotropica 18:126-135

Rossa-Feres DC, Nomura F (2006) Caracterização e chave de identificação taxonômica para girinos (Amphibia: Anura) da região noroeste do estado de São Paulo, Brasil. Biota Neotropica 6(1)

Rodilla V (1993) Origin and evolution of binucleated cells and binucleated cells with micronuclei in cisplatin-treated CHO cultures. Mutation Research 300:281-91

Santana JM, Dos Reis A, Teixeira PC, Ferreira FC, Ferreira CM (2015) Median lethal concentration of formaldehyde and its genotoxic potential in bul lfrog tadpoles(*Lithobates catesbeianus*). Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes 50:896-900

Schuch AP, dos Santos MB, Lipinski VM, Peres LV, dos Santos CP, Cechin SZ, Schuch NJ, Pinheiro DK, Loreto ELDS(2015) Identification of influential events concerning the Antarctic ozone hole over so uthernBrazil and thebiological effects induced by UVB and UVA radiation in an endemic treefrog species. Ecotoxicology andEnvironmental Safety 118:190-198

Silva CI, PolaquiniCR,Ferreira H, Pavan F (2017) Evaluation of cytotoxic, apoptotic, mutagenic, and chemopreventive activities of semi-synthetic esters of gallic acid. Food and Chemical Toxicology 105:300-307

Sparling DW, Linder G, Bishop CA, Krest S (2010) Ecotoxicology of amphibians and reptiles. Boca Raton, USA. CRC Press, Taylor & Francis

Theodorakis CW (2001) Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicology 10:245-256

Udroiu I, Sgura A, Vignoli L, Bologna MA, D’Amen M, Salvi D, Ruzza A, Antoccia A, Tanzarella C (2015) Micronucleus Test onTrituruscarnifex as a Tool for Environmental Biomonitoring. Environmental and MolecularMutagenesis 56:412-417

Veronez ACD, Salla RV, Baroni VD, Barcarolli IF, Bianchini A, Martinez CBD, Chippari-Gomes AR (2016) Genetic and biochemical effects induced by iron ore, Fe and Mn exposure in tadpoles of the bullfrog Lithobatescatesbeianus.AquaticToxicology 174:101-108

Yanéz M, Altamiranda M, Oyataga L (2009) Diversidad de La Herpetofauna de Tobar Donoso, Prov. Carchi, Ecuador. Technical Report

Zaya RM, Amini Z, Whitaker AS, Kohler SL, Ide CF (2011.) Atrazine exposure affects growth, body condition and liver health in Xenopuslaevis tadpoles. Aquatic Toxicology104:243-253

10. APÊNDICE C - CAPÍTULO 3: Manuscript submetted for Herpetology Notes

First occurrence of *Physalaemus albonotatus* (Steindachner, 1864) (Anura, Leiuperidae) for the state of Goiás, Brazil.

Rinneu Elias Borges[1], Eliane Andreia dos Santos Oliveira[2], Lia Raquel de Souza Santos[2,*] Marcelino Benvindo de Souza[2], Alessandro Ribeiro Morais[2], Natan Medeiros Maciel[3], Classius deOliveira[4]

Currently, the *Physalaemus cuvieri* species group comprises the following species: *P. albonotatus* (Steindachner, 1864); *P. centralis* Bokermann, 1962; *P. cicada* Bokermann, 1966; *P. cuqui* Lobo, 1993; *P. cuvieri* Fitzinger, 1826; *P. ephippifer* (Steindachner, 1864), *P. erikae* Cruz and Pimenta, 2004; *P. fischeri* (Boulenger, 1890), *P. atim* Brasileiro e Haddad, 2015 and *P. kroyeri* (Reinhardt and Lütken, 1862) (Nascimento et al., 2005; Lourenço et al., 2015; Brasileiro e Haddad, 2015). *Physalaemus albonotatus* occurs in Argentina, Bolivia, Brazil, and Paraguay (Frost 2018). In Brazil, specifically, this species occurs only in the Mato Grosso and Mato Grosso do Sul states (Frost 2018).

On January 26, 2017, during fieldwork in the Emas National Park municipality of Mineiros (18º15’47.78”S, 52º53’23.90”W; Datum = WGS84; 783 m a.s.l.), state of Goiás, Brazil, we found two males of *P. albonotatus* in calling activity. We observed these individuals in a temporary pond (e.g., dammed water), next to Formoso river (~ 215 meters), characterized by having scarce vegetation in its interior, but dense vegetation in its margin. We manually collected these individuals, euthanized with 5% benzocaine, fixed in 10% formaldehyde and preserved in 70% ethanol and housed at the Herpetological Collection of the Instituto Federal Goiano – Campus Rio Verde (IFGoianoRV 405 and 420).

We identified the specimen based on morphological visual characters, such as dark gray coloration (Fig. 1) with discrete dark horizontal stripes on the legs and a whitish ventral color (Fig. 2).

Figure 1. Dorsal view of an adult specimen of *Physalaemus albonotatus* collected in the Emas National Park.

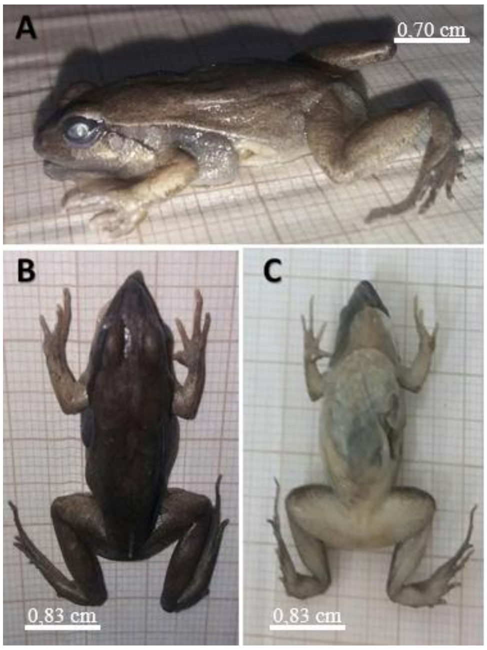


Figure 2. Side view (A), dorsal (B) and ventral (C) of the adult specimen of *Physalaemus albonotatus* collected in the Emas National Park.

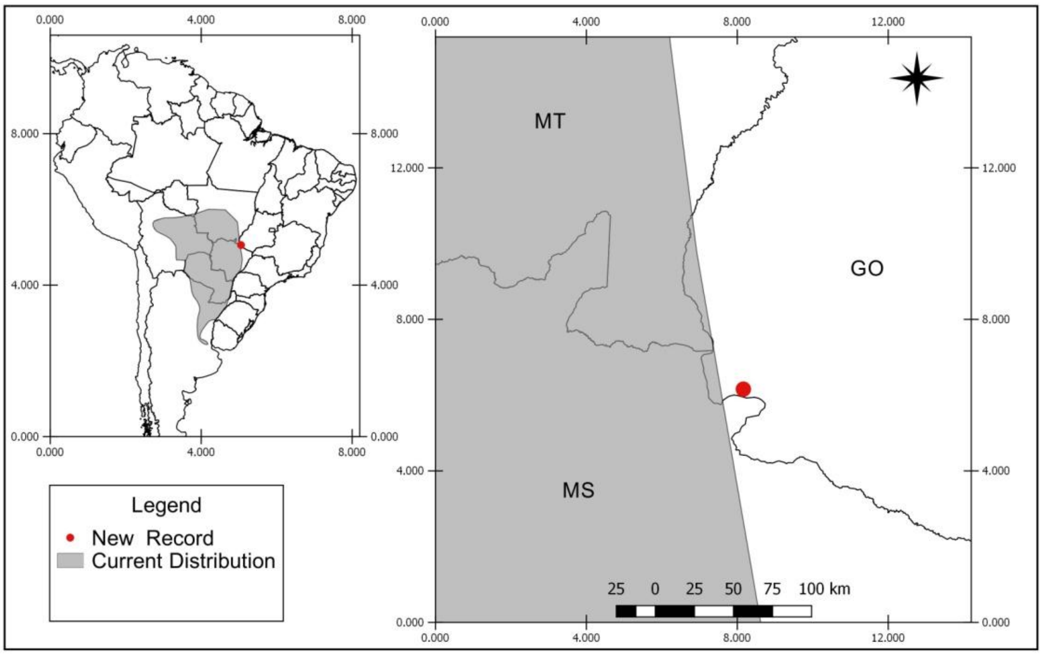
Also we measured the individuals with a caliper (nearest 0.05 mm) using the following morphometric data: total length (LT), head width (WH), interocular distance (DI), eye-nostril distance (DEN), distance inter-nostril (DIN), length of thigh (LTH), length of tibia (LTB), head length (LH); a snout length index (ILS) was calculated by DEN/ LH according to Brasileiro and Haddad (2015) (Tab. 1).

Table 1. Morphometric traits of males of *Physalaemus albonotatus* (in mm; *n* = 2). The abbreviations of the characteristic measurements are defined in Material and Methods.

P. albonotatus	LT	WH	DI	DEN	DIN	LTH	LTB	LH	ILS
Specimen 1	29,12	10,09	5,18	2,33	2,02	10,97	11,30	10,79	0,22
Specimen 2	29,16	9,57	5,25	2,36	2,04	10,94	11,39	11,05	0,21

Our report represents the first occurrence of the *Physalaemus albonotatus* in Goiás (Figure 3). In their study with the anuran community from Emas National Park, Kopp et al. (2010) did not register this species. Additionally, Morais et al. (2011) conducted intense sampling of anurans in the southwest region from Goiás state; however, no specimens of *Physalaemus albonotatus* were found. Morais et al. (2012) reported the occurrence of *P.* aff. *albonotatus* at Silvânia National Forest, Goiás state. Additional sampling and analyzes (e.g., acoustical and/or morphological) are necessary for the confirmed the identification of this population. This population could also be *P. atim* due to the resemblance with *P. albonotatus*, both morphological and acoustic. The straight-line distance between the area studied by Morais et al. (2012) and the area in which the specimen of *Physalaemus albonotatus* was found is 488km. However, distance between type-locality of *P. atim* (Campo Largo, Goiás state) the area studied by Morais et al. (2012) are approximately 70 Km. The discovery of *Physalaemus albonotatus* in the State of Goiás reinforces the need for further surveys to understand patterns of occurrence and distribution of anurans species to the southwest region of the state, especially due to the strong anthropogenic pressures that threaten the fauna of the Cerrado amphibians Brazilian.

Figure 3. Current distribution of *Physalaemus albonotatus* and location of the new record (18º15'47.78 ''S; 52º53'23.90''W) of the species.



Acknowledgments. To the Foundation for Research Support of the State of Goiás (FAPEG) for the post-graduate scholarship for EASO. To the administration of the Emas National Park for the support during the collection of data. To PELD Emas: PELD EMAS site 13 (PELD/MCTI/CNPq/FAPEG) for financial support. To CNPq for the fellowship to NMM. Wadson Rodrigues Rezende for the help in the field collections. We also thank the Instituto Chico Mendes de Conservação da Biodiversidade (IBAMA/ICMBio) for authorizing the fieldwork (license number 53799-1), and Roniel Freitas for making the species distribution map.

REFERENCES

Aquino, L., S. Reichle, D. Silvano and J. Langone. 2004. *Physalaemus albonotatus*. The IUCN Red List of Threatened Species 2004: e.T57239A11607238. Acessed at http://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T57239A11607238.en, 18 February 2015.

Brasileiro,C.A.; Haddad, C.F.B. A new species of Physalaemus from central Brazil (Anura: Leptodactylidae). Herpetologica, 71 (4), 2015, 280-288.

Falico, D.A., J.A. Lopez, C.A. Antoniazzi and A.H. Beltzer. 2012. Variación interpoblacional y ontogenética en la dieta de la rana llorona *Physalaemus albonotatus* (Anura: Leiuperidae). Revista Mexicana de Biodiversidad 83:1187-1193. doi: http://dx.doi.org/10.7550/rmb.28122.

Forti, L.R. and L.C. Encarnação. 2012. Water-wave production in the Neotropical frogs *Physalaemus albonotatus* and *Pseudopaludicola mystacalis*: a seismic signal? Salamandra 48(3):181-184.

Kehr, A., E.A. Schaefer and M.I. Duré. 2004. The tadpole of *Physalaemus albonotatus* (Anura: Leiuperidae). Journal of Herpetology 38(1):145-148. doi: http://dx.doi.org/10.1670/135-02N.

Kopp, K., L. Signorelli and R.P. Bastos. 2010. Distribuição temporal e diversidade de modos reprodutivos de anfíbios anuros no Parque Nacional das Emas e entorno, Estado de Goiás, Brasil. Iheringia, Série Zoologia 100(3):192-200.

Morais, A.R., R.P. Bastos, R. Vieira and L. Signorelli. 2012. Herpetofauna da Floresta Nacional de Silvânia, um remanescente de Cerrado no Brasil Central. Neotropical Biology & Conservation 7(2):114-121.

Morais, A.R., L. Signorelli, P.G. Gambale, K. Kopp, F. Nomura, L.D. Guimarães, W. Vaz-Silva, J. Ramos and R.F. Bastos. 2011. Anfíbios anuros associados a corpos d'água do sudoeste do estado de Goiás, Brasil. Biota Neotropical 11(3):355-363.

Oliveira, F.F.R., L.B. Nascimento, P.C. Eterovick, and I. Sazima. 2013. Description of the Tadpole and Redescription of the Advertisement Call of Physalaemus evangelistai (Anura, Leiuperidae), with Notes on Its Natural History. Journal of Herpetology, 47(4):539-543. doi: http://dx.doi.org/10.1670/12-116.

Ruggeri, J., L.R. Weber. 2012. A survey of the internal oral features and external morphology of Physalaemus larvae (Anura, Leptodactylidae). Zootaxa 3200: 1–26.

Vittorazzi, S.D., Y.R.S.D. Quinderé, S.M. Recco-Pimentel, C. Tomatis, D. Baldo, J.R.F. Lima, J.M. Ferro, J.D. Lima and L.B. Lourenço. 2014. Compartive cytogenetics of *Physalaemus albifrons* and *Physalaemus cuvieri* species groups (Anura, Leptodactylidae). Comparative Cytogenetics 8(2): 102-123.