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Ação de desreguladores endócrinos em *Rana catesbeiana* (Anura) em estádio larval e juvenil: efeitos genotóxicos, morfológicos e respostas imunológicas.

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DOUTORADO

PÓS GRADUAÇÃO
EM BIOLOGIA ANIMAL

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

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Financiadora: CAPES

Orientador: Prof. Dr. Classius de Oliveira

Co-Orientador: Dr. Javier Goldberg

São José do Rio Preto
2019

G821a

Gregorio, Lara Salgueiro de

Ação de desreguladores endócrinos em Rana catesbeiana (Anura) em estádio larval e juvenil: efeitos genotóxicos, morfológicos e respostas imunológicas / Lara Salgueiro de Gregorio. -- São José do Rio Preto, 2019

107 p. : tabs., fotos

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

Orientador: Classius de Oliveira

Coorientador: Javier Goldberg

1. Toxicologia Genética. 2. Melanina. 3. Reprodução. 4. Leucócitos. 5. Poluentes. 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).

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São José do Rio Preto
01 de Março de 2019

Dedico aos meus pais, Rosiani e Sérgio, por sempre acreditarem em mim e possibilitarem a minha trajetória até aqui.

Agradecimentos

Primeiramente agradeço aos meus pais, Rosiani e Sérgio, por sempre apoiarem as minhas decisões e fazerem de tudo pelo meu crescimento pessoal e profissional, pela educação que me proporcionaram e a dedicação que sempre tiveram.

Agradeço também aos meus avós, Diva e José, pois sem eles nada disso seria possível. Eles são a base da minha família e sempre me deram amor e apoio em todas as situações.

Ao meu orientador e amigo, Dr. Classius de Oliveira, pela orientação, conselhos e ideias que trilharam meus caminhos durante a execução do projeto. Agradeço também por ter sempre acreditado e confiado em mim durante todos esses anos trabalhando em conjunto.

Ao meu co-orientador, Javier Goldberg, pela parceria estabelecida e pelos conhecimentos que pude adquirir, além da confiança depositada em mim e no nosso grupo de pesquisa do Laboratório de Anatomia.

Ao auxílio financeiro concedido na forma de bolsa de doutorado, visto que o presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Aos professores e funcionários do Ibilce/Unesp, que contribuíram para minha formação acadêmica e por serem exemplos a serem seguidos (ou não serem) no âmbito profissional.

A todos os colegas do Laboratório de Anatomia, por me auxiliarem em várias etapas do desenvolvimento do projeto e pelo divertimento nas horas de tensão.

Ao meu marido, Victor Marcondes Sacchi, por ser um dos responsáveis pela minha paixão pela Biologia, por estar sempre ao meu lado, por todo amor e respeito nos momentos difíceis e todo apoio durante minha formação acadêmica.

Às minhas queridas amigas de graduação, Ana Flávia, Ana Beatriz e Ayla, por me acompanharem desde o início do curso, ainda que agora um pouco mais difícil de nos ver, mas que ainda assim compartilham das alegrias dos meus melhores momentos e me dão força e conselhos nas horas mais difíceis.

A todos os meus amigos que não são do Ibilce, que apesar de não estarem presentes no meio acadêmico, também sempre me incentivaram e estiveram presentes garantindo muitas risadas nos momentos de descontração.

RESUMO

Os anfíbios são bons indicadores de qualidade ambiental devido a várias características que os tornam suscetíveis a alterações do ambiente. A contaminação aquática, principalmente por desreguladores endócrinos, tem sido apontada como uma causa para o declínio dos anfíbios. O 4-nonilfenol (NP) é um detergente com efeito estrogênico, que já demonstrou efeitos deletérios em alguns grupos de vertebrados. Já o Acetato de Ciproterona (CPA) é um medicamento com propriedade antiandrogênica e, apesar da maioria dos estudos serem com roedores, tem-se observado alto potencial tóxico deste composto. Neste estudo o objetivo foi avaliar os efeitos genotóxicos do NP e do CPA, e sua ação na pigmentação hepática, um biomarcador morfológico de efeito, importante para confirmar a ação dos compostos, além da morfologia gonadal e da contagem leucocitária de girinos e jovens de *Rana catesbeiana*, em três diferentes concentrações ambientalmente relevantes, após exposição por 28 dias. Nossos resultados mostraram que o NP e o CPA são genotóxicos para os girinos, sendo o CPA de toxicidade muito maior em todas as concentrações, enquanto nos jovens apenas o CPA gerou resposta, nas duas maiores dosagens. Na pigmentação hepática houve aumento apenas nos jovens expostos à concentração média de NP e mínima de CPA. A gônada dos girinos ainda estava indiferenciada, portanto não pode ser avaliada, enquanto na dos jovens não houve alteração na razão sexual nem condições intersexuais. Já o perfil leucocitário variou em ambas as fases. O NP reduziu os linfócitos e aumentou os neutrófilos na dosagem mínima para os girinos, enquanto o CPA aumentou os eosinófilos nas duas maiores concentrações. Nos jovens os neutrófilos reduziram em todos os tratamentos, os trombócitos aumentaram em todos os grupos, exceto na maior dose de CPA, e houve também aumento de basófilos em todos os grupos do CPA. Estes resultados em conjunto nos mostram que ambos os compostos apresentam toxicidade para *R. catesbeiana*, principalmente citotoxicidade, visto que as principais alterações observadas foram genotóxicas e nas células imunológicas relacionadas à resposta inata. Além disso, houve uma pequena alteração na pigmentação melânica dos jovens, que também está relacionada à defesa. A gônada não apresentou variações, o que pode estar relacionado à alta resistência da espécie. Assim, concluímos que ambos os compostos são tóxicos, mesmo em concentrações muito baixas e em uma espécie resistente como *R. catesbeiana*, ainda que alguns parâmetros morfológicos não tenham sido alterados. Observamos ainda que a fase de vida pode interferir no grau de resposta aos xenobióticos.

Palavras–chave: EDCs; Genotoxicidade; Melanina; Reprodução; Leucócitos.

ABSTRACT

Amphibians are good indicators of environmental quality due to several characteristics that make them susceptible to environmental changes. Aquatic contamination, especially by endocrine disrupters, has been indicated as a cause for amphibian decline. 4-nonylphenol (NP) is a detergent with estrogenic effect, which has already demonstrated deleterious effects in some groups of vertebrates. Cyproterone Acetate (CPA), in turn, has antiandrogenic properties, it is used in medicines, and although most of the studies are with rodents, it has been observed a high toxic potential of this compound. In this study, our aim was to evaluate the genotoxic effects of NP and CPA, and its action on hepatic pigmentation, a morphological biomarker, important to confirm the effects of the compounds, as well as gonadal morphology and leukocyte count of tadpoles and juveniles of *Rana catesbeiana*, in three different environmentally relevant concentrations, after 28 days exposure. Our results showed that NP and CPA are both genotoxic for tadpoles, but CPA showed much higher toxicity at all concentrations, while in juveniles only CPA increased nuclear abnormalities at the two highest dosages. In hepatic pigmentation there was an increase only in the juveniles exposed to the medium concentration of NP and minimum dosage of CPA. The tadpole gonad was still undifferentiated, so it can not be evaluated, while in the juvenile there was no change in the sex ratio and any intersexual conditions. The leukocyte profile varied in both stages with the treatments. NP reduced lymphocytes and increased neutrophils at minimum dosage for tadpoles, while CPA increased eosinophils at the two highest concentrations. In the juveniles, neutrophils were reduced in all treatments, thrombocytes increased in all groups, except for the highest dose of CPA, and there was also an increase in basophils in all CPA groups. These results, taken together, demonstrate that both compounds are toxic to *R. catesbeiana*, showing mainly cytotoxicity, since the main alterations observed were genotoxic and in the innate immunological cells. In addition, there was a slight change in melanin pigmentation in juveniles, which is also related to the defense of the organism. The gonad did not vary with treatment, which may be related to the high resistance of the species. Thus, we conclude that both compounds are toxic, even at very low concentrations and in a resistant species such as *R. catesbeiana*, although some morphological parameters have not been altered. We also observed that the life stage of the animals may interfere with the response to xenobiotics.

Keywords: EDCs; Genotoxicity; Melanin; Reproduction; Leukocytes.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

SUMÁRIO

1. INTRODUÇÃO GERAL

1.1. Declínio Populacional dos Anfíbios

Dados da IUCN (International Union for Conservation of Nature) (2018) mostram que 31,1% das espécies de anfíbios estão na lista vermelha de ameaça a extinção, enquadrando-se em categorias como criticamente ameaçadas (8,2%), ameaçadas (13,4%) ou vulneráveis (9,5%). O declínio dos anfíbios está ocorrendo mundialmente em taxas alarmantes, possivelmente com números subestimados (STUART et al., 2004), sendo estes animais relativamente mais afetados que os outros vertebrados, como aves e mamíferos (IUCN, 2018). Alguns autores consideram, inclusive, que estes altos índices de extinções (de anfíbios e outros grupos animais em conjunto), possam ser a sexta extinção em massa da Terra (WAKE; VREDENBURG, 2008).

Há vários fatores causando o declínio da população de anfíbios como, por exemplo, a introdução de espécies exóticas, o aumento de doenças infecciosas como a quitridiomicose e as diversas alterações ambientais tais como aquecimento global, poluição, destruição e a alteração dos habitats (WAKE; VREDENBURG, 2008; HAYES et al., 2010). As alterações ambientais, tal como o aquecimento global, podem interferir diretamente na estrutura do habitat por estimular a proliferação de patógenos (POUNDS et al., 2006). É inegável que o ser humano tem participação considerável em quase todas essas mudanças que vem ocorrendo mundialmente na natureza, e os anfíbios, devido às suas características intrínsecas, são muito suscetíveis a essas variações, tornando-se vulneráveis a extinção (WAKE; VREDENBURG, 2008).

Há várias particularidades que tornam os anfíbios bons indicadores de qualidade ambiental, tais como: o ciclo de vida em dois estádios distintos, dependentes da água e do ambiente úmido, o que os expõe a diferentes desafios ao longo da sua vida; a permeabilidade dos ovos, brânquias e pele, que os torna suscetíveis a poluentes ambientais; além de serem animais ectotérmicos, sendo suscetíveis a variações de temperatura (DUNSON; WYMAN; CORBETT, 1992; BURLIBAŞA; GAVRILĂ, 2011; WAKE; VREDENBURG, 2008; CATENAZZI, 2015). Dentre as principais diferenças entre os estádios larval e adulto dos anfíbios podemos citar a dieta, que via de regra é herbívora para as larvas e carnívora para os adultos, as vias respiratórias, branquial no girino e pulmonar/cutânea no adulto, além do habitat que para os girinos é aquático e para os adultos normalmente é terrestre/semi-terrestre (WAKE; VREDENBURG, 2008). Essas distinções fazem com que cada fase do animal seja

exposta a contaminantes e alterações ambientais de maneiras diferentes, podendo responder também diferentemente ao longo de sua vida a estes estressores.

Segundo Hayes et al. (2010), as cinco alterações ambientais globais que são as causas finais do declínio dos anfíbios são: as mudanças atmosféricas (como temperatura, chuvas e radiação UV), perda de habitat, patógenos, introdução de espécies exóticas e poluentes ambientais. Vários contaminantes podem, ainda, alterar as propriedades cutâneas dos anfíbios e/ou enfraquecer seu sistema imune, favorecendo infecções (CATENAZZI, 2015). Sendo assim, a preocupação com a contaminação aquática tem aumentado significativamente nos últimos anos, principalemente tendo em vista alguns compostos como fármacos, desreguladores endócrinos e poluentes orgânicos, que podem atuar em concentrações extremamente baixas (BILA; DEZOTTI, 2007).

1.2. Compostos Desreguladores Endócrinos (EDCs)

Os compostos desreguladores endócrinos (EDCs) constituem uma classe de substâncias que podem interferir no sistema endócrino dos organismos (WELSHONS et al., 2003; BILA; DEZOTTI, 2007). Até hoje se discute a definição mais correta para esse conjunto de compostos, no entanto, de maneira geral, todos os autores concordam que são substâncias que tem a capacidade de interferir no sistema endócrino por alguma via, seja ela direta ou indireta, gerando respostas locais ou sistêmicas. Estes contaminantes ocorrem no ambiente normalmente em concentrações em nível de µg/L e ng/L e podem ser substâncias naturais (exemplo: estrogênios e fitoestrogênios) ou sintéticas (exemplos: medicamentos, pesticidas, bisfenol A, etc) (BILA; DEZZOTI, 2007).

Há alguns anos os autores buscam explicar como os EDCs podem atuar em concentrações tão baixas. Segundo Welshons et al. (2003), o sistema endócrino está ajustado para responder a doses muito baixas dos hormônios naturais do organismo. Os receptores hormonais têm alta afinidade por seus ligantes, de modo que possuem capacidade de desencadear respostas específicas e eficientes em baixas concentrações (VANDENBERG et al., 2012). Sendo assim, já que os EDCs estão diretamente relacionados com o sistema endócrino, os compostos capazes de interagir com os receptores hormonais poderiam gerar respostas também em concentrações baixas, da mesma maneira como os hormônios (WELSHONS et al., 2003). Além disso, não necessariamente há relação entre a concentração e a quantidade de receptores ligados e/ou a concentração e uma resposta mais significativa (WELSHONS et al., 2003; VANDENBERG et al., 2012). Esta relação vai depender das

propriedades do composto e dos receptores, de modo que as respostas em uma determinada dosagem não necessariamente poderão predizer as respostas em outras dosagens (WELSHONS et al., 2003; VANDENBERG et al., 2012).

1.2.1. 4-Nonilfenol

O 4-nonilfenol (NP) é um composto moderadamente ativo, mimético do hormônio 17-β-estradiol e, portanto, considerado um EDC (VAZQUEZ-DUHALT et al., 2005). Ele é utilizado principalmente na fabricação de detergentes (cerca de 80% da demanda), podendo também ser utilizado como intermediário na produção dos nonilfenóis na sua forma etoxilada (VAZQUEZ-DUHALT et al., 2005). Estes compostos são amplamente utilizados, de modo que acabam contaminando principalmente o meio aquático, apesar de também ser encontrado nos ambientes terrestres (YING; WILLIAMS; KOOKANA, 2002).

Apesar de muitos produtos utilizarem a forma etoxilada do NP, no meio ambiente há micro-organismos capazes de transformar estes compostos novamente à forma de 4-Nonilfenol, que é ainda mais tóxica (VAZQUEZ-DUHALT et al., 2005). Sua concentração encontrada nos rios de diversos países varia de níveis não detectáveis até a faixa de µg/L, sendo que ambientes com até 1 µg/L são considerados pouco contaminados; de 1 a 10 µg/L são moderadamente contaminados; e acima de 10 µg/L são muito poluídos (VAZQUEZ-DUHALT et al., 2005). A ocorrência de compostos nonilfenólicos foi detectada, inclusive, em água potável, em concentrações até a faixa de 6.7 µg/L, mas podendo chegar a 43.3 µg/L em casos mais extremos (BERRYMAN et al., 2004).

Há vários estudos com NP em girinos, mostrando que o composto pode influenciar em diversos aspectos. Foram observadas alterações na proporção entre machos e fêmeas (KLOAS; LUTZ; EINSPANIER, 1999), aumento da taxa de indivíduos intersexo (MACKENZIE et al., 2003), redução do comprimento dos indivíduos, redução da área de dispersão dos melanóforos cutâneos, o que consequentemente reduziu a pigmentação (PARK; KANG; GYE, 2010), malformações morfológicas como curvatura do corpo e alterações histopatológicas no cordão espinhal, notocorda, fígado e nos olhos (SAYED et al., 2012). Estudos com outras espécies mostram ainda que o NP pode apresentar efeitos genotóxicos, como visto em eritrócitos de peixes (TELES et al., 2004; AL-SHARIF, 2012) e linfócitos humanos (HARRÉUS et al., 2002), além de comprometer o sistema imune de ratos, interferindo, por exemplo, na produção de citocinas e na regulação de células NK (XIA et al., 2013).

1.2.2. Acetato de Ciproterona

O Acetato de Ciproterona (CPA) é um composto que apresenta efeito antiandrogênico, progestágeno e antigonadotrópico (NEUMANN; TÖPERT, 1986) e, portanto, também pode ser classificado como um EDC. Está presente em alguns contraceptivos e fármacos normalmente indicados para o tratamento do câncer de próstata, acne em estágios graves, hirsutismo e reposição hormonal (NCBI, 2018).

Os compostos antiandrogênicos, como o CPA e a Flutamida, atuam competindo pelos receptores de andrógenos (PRATT et al., 1994). A Flutamida, ao ser administrada sozinha, impede que a testosterona se ligue aos receptores de andrógenos e, portanto, bloqueia o mecanismo de feedback negativo de agir na pituitária, aumentando os níveis tanto de testosterona, como de LH e FSH no plasma sanguíneo (VIGUIER-MARTINEZ et al., 1983; PRATT et al., 1994). O CPA, apesar de competir pelos receptores de andrógenos, também é capaz de inibir a síntese de testosterona (efeito antigonadotrópico) (NEUMANN; TÖPERT, 1986). Além disso, alguns estudos mostram que, quando administrado em dosagens mais altas, o CPA também pode atuar como agonista, mimetizando os efeitos dos andrógenos (KEMPPAINEN et al., 1992). Dessa forma, o CPA pode atuar de maneiras diferentes dependendo de dose administrada, reforçando a ausência de um comportamento padrão esperado, já observada para alguns EDCs.

Ainda não há estudos avaliando os níveis de CPA no meio ambiente. No entanto, na Inglaterra e País de Gales foram feitos cálculos preditivos para avaliar suas possíveis concentrações nos rios, com base no consumo e taxa de excreção do composto (GREEN et al., 2015). A maioria das predições não ultrapassou 10 ng/L, no entanto, em alguns pontos foram calculadas concentrações bem maiores (GREEN et al., 2015). No Brasil, não há dados ou predições sobre o CPA nos corpos d'água, no entanto, sabe-se que alguns anticoncepcionais como Diane e Selene, que contém o composto, são amplamente utilizados no país.

Os efeitos que o CPA pode induzir nos organismos podem comprometer diversos aspectos, mas para anfíbios há poucos estudos. Em anuros há trabalhos avaliando aspectos reprodutivos, mostrando que o CPA causa redução da pigmentação dos ovários em diferenciação, bem como da quantidade de indivíduos fêmeas (HAYES et al., 2006). O composto pode ainda interromper a maturação dos espermatozoides e causar desintegração do tecido intersticial, afetando principalmente as células de Leydig (HAIDER, 1980), além de interferir na diferenciação sexual de girinos (HSÜ; HSÜ; LIANG, 1979). Em peixes o CPA

também altera os parâmetros reprodutivos, comprometendo espermatozoides e o desenvolvimento do ovário (KIPARISSIS et al., 2003), e em répteis, afeta o comportamento sexual (TOKARZ, 1987). Para mamíferos há vários estudos que evidenciam, por exemplo, efeitos genotóxicos, propriedades hepatotóxicas e o favorecimento de tumores no fígado pela exposição ao CPA (NEUMANN et al., 1992; RABE et al., 1996; KASPER, 2001; SIDDIQUE; AFZAL, 2005).

1.3. Genotoxicidade

As alterações genotóxicas são aquelas que ocorrem na estrutura ou conteúdo do DNA ou dos cromossomos devido à exposição a compostos tóxicos (AL-SABTI; METCALFE, 1995). Dentre as técnicas utilizadas podemos citar a contagem de micronúcleos, que são cromossomos, em partes ou inteiros, que se perdem durante a anáfase celular e, assim, não são incluídas no núcleo principal, formando um ou mais núcleos secundários de tamanho reduzido (AL-SABTI; METCALFE, 1995). Além disso, outras anormalidades nucleares têm sido consideradas nas análises genotóxicas, tais como alterações do formato nuclear (riniforme, lobado, com reentrâncias ou vesículas, dentre outros), células binucleadas, anucleadas ou multinucleadas, apoptóticas, com picnose nuclear (estágio precedente da apoptose) ou com "buds", que são estruturas semelhantes aos micronúcleos, porém que mantém ligação com o núcleo principal (FENECH et al, 2003; LAJMANOVICH et al, 2013; 2014; JOSENDE et al., 2015).

As análises genotóxicas têm sido amplamente utilizadas como biomarcadores (ADAMS et al., 2001), os quais podem ser definidos como parâmetros funcionais ou estruturais de um organismo que podem ser alterados por um composto tóxico (DEPLEDGE; FOSSI, 1994; VAN GESTEL; VAN BRUMMELEN, 1996; BARNI et al., 2007). O uso destas análises favorecem estudos ecotoxicológicos por serem um meio quantitativo de se avaliar efeitos de contaminantes ambientais (ADAMS et al., 2001), além de serem técnicas simples e de fácil acessibilidade. Além disso, os anfíbios são ótimos modelos biológicos para estudos de genotoxicidade (BURLIBAȘA; GAVRILĂ, 2011), no entanto a maior parte destes estudos está relacionada a defensivos agrícolas.

Estudos com NP e CPA evidenciando potencial genotóxico abrangem, em sua maioria, outros grupos de vertebrados. Para NP há diversos estudos com peixes (TELES et al., 2004; MEKKAWY; MAHMOUD; SAYED, 2011; AL-SHARIF, 2012; SHARMA; CHADHA, 2017), enquanto para o CPA a maioria dos trabalhos são com mamíferos (NEUMANN et al.,

1992; RABE et al., 1996; KASPER, 2001; SIDDIQUE; AFZAL, 2005). Ambos os compostos são EDCs, podendo agir em concentrações muito baixas, além de ocorrer contaminando os corpos d'água. Sendo assim, é importante que sejam avaliados seus efeitos em organismos como os anfíbios, que são muito suscetíveis a alterações ambientais e estão sofrendo declínio populacional em escala mundial.

1.4. Morfofisiologia Hepática

O fígado é considerado o principal órgão responsável pelo metabolismo, de modo que está em contato direto com poluentes ambientais (SALEH, 1982; BRAUNBECK; STORCH; BRESCH, 1990). É responsável por funções como o acúmulo de substâncias de reserva (BUCKE; WATERMANN; FEIST, 1984) e processos de detoxificação, bem como a biotransformação de agentes nocivos (FENOGLIO et al., 2005). Nos animais exotérmicos, o fígado pode ser afetado por diversos parâmetros biológicos e ambientais, estando diretamente relacionado com a fisiologia animal (BRUSLÈ; ANADON, 1996).

Nos anfíbios, os órgãos hematopoiéticos apresentam células pigmentares com atividade fagocítica denominadas melanomacrófagos (AGIUS, 1980). Estas células possuem formato arredondado (FRANCO-BELUSSI; CASTRUCCI; OLIVEIRA, 2013) e podem ocorrer de forma aglomerada, formando os centros de melanomacrófagos (AGIUS, 1980). A principal função destas células está relacionada com a fagocitose de material celular oriundo do catabolismo (ELLIS; MUNROE; ROBERTS, 1976), indicando papéis de detoxificação ou reciclagem de substâncias endógenas e exógenas (HERRÁEZ; ZAPATA, 1986). Atuam também na proteção contra bactérias e esporos parasitas (ROBERTS, 1975), gerando respostas imunes e bactericidas (FRANCO-BELUSSI; CASTRUCCI; OLIVEIRA, 2013), além de estarem relacionados com a estocagem do ferro após a eritrofagocitose (AGIUS; ROBERTS, 2003).

No citoplasma dos melanomacrófagos existem diferentes tipos de grânulos contendo substâncias químicas diversificadas, sendo o principal pigmento a melanina (AGIUS; AGBEDE, 1984; HERRÁEZ; ZAPATA, 1991). A melanina é um polímero complexo sintetizado endogenamente (CÉSARINI, 1996; FRANCO-BELUSSI; CASTRUCCI; OLIVEIRA, 2013) e está relacionada à neutralização de radicais livres, cátions e outros compostos tóxicos provenientes da degradação do material fagocitado (ZUASTI et al., 1989; AGIUS; ROBERTS, 2003). Além disso, alguns precursores da melanina agem como bactericidas (CHRISTIANSEN et al., 1996). Vários estudos têm demonstrado que os

pigmentos hepáticos respondem à exposição a diversos contaminantes aquáticos, sendo considerados bons biomarcadores morfológicos de efeito para estudos ecotoxicológicos (DE OLVEIRA et al., 2017).

O NP mostrou-se como intereferente na dispersão dos melanoforos cutâneos de girinos *Bombina orientalis* (PARK; KANG; GYE, 2010) e o CPA afetou a pigmentação dos ovários de *Xenopus laevis* (HAYES et al., 2006). No fígado há poucos estudos avaliando o efeito destes compostos, porém sabe-se que o NP causa redução do número de melanomacrófagos em *Bufo regularis* (SAYED et al., 2012). A exposição de girinos *Rhinella schneideri* a formulações contendo atrazina, um herbicida com propriedade estrogênica, assim como o NP, causa aumento da quantidade de melanomacrófagos no fígado já nas primeiras 48h de exposição (PÉREZ-IGLESIAS et al., forthcoming 2019). Não se sabe o efeito do CPA na melanina hepática dos anuros, porém outros compostos antiandrogênicos como o medicamento Flutamida induziu aumento da pigmentação em adultos *Rhinella schneideri*. Dessa maneira, pode-se observar que a melanina é responsiva a diversos compostos, podendo variar a resposta de acordo com as propriedades do composto.

1.5. Desenvolvimento e Diferenciação Gonadal

De maneira geral, o desenvolvimento gonadal em anuros se inicia nos girinos entre as fases 24 a 26 de Gosner (1960), quando ocorre a migração das células germinativas primordiais (PGCs) para a região da crista genital (OGIELSKA; KOTUSZ, 2004). A diferenciação tanto masculina quanto feminina foi descrita em 10 estágios, de modo que nos três primeiros estágios a gônada encontra-se indiferenciada (OGIELSKA; KOTUSZ, 2004; HACZKIEWICZ; OGIELSKA, 2013). A partir do estágio IV, no ovário é possível observar a formação de uma cavidade central (lúmen) na região medular da crista genital, originado pela degeneração das células somáticas ali presentes (OGIELSKA; KOTUSZ, 2004). Já na diferenciação testicular essa degeneração não ocorre, mantendo a medula consistente e sólida, na forma de metâmeros (HACZKIEWICZ; OGIELSKA, 2013). Além disso, no córtex feminino há oogônias primárias que proliferam e se diferenciam em secundárias, sendo circundadas por células foliculares; enquanto no masculino há espermatogônias primárias, que também proliferam e se diferenciam em secundárias, circundadas por precursoras das células de Sertoli (OGIELSKA; KOTUSZ, 2004; HACZKIEWICZ; OGIELSKA, 2013).

Existem três padrões diferentes de diferenciação gonadal. No (1) Diferenciado, a gônada diferencia diretamente em ovário ou testículo; no (2) Indiferenciado, também há

desenvolvimento direto em ovário ou a gônada permanece um tempo maior indiferenciada para, então, transformar-se em testículo; no (3) Semi-diferenciado, os indivíduos tem gônadas diferenciadas primeiramente em ovários (ainda que geneticamente macho) e, só posteriormente, formam-se os testículos, degenerando-se as estruturas femininas (GRAMAPUROHIT; SHANBHAG; SAIDAPUR, 2000). Para *Rana catesbeiana* já foram descritos os padrões diferenciado e semi-diferenciado (HSÜ; LIANG, 1970), e atualmente a literatura ainda é bastante controversa quanto ao desenvolvimento e a diferenciação gonadal desta espécie.

De maneira geral, a diferenciação gonadal ocorre entre os estágios 28 e 35 de Gosner (1960), podendo em alguns casos ocorrer no estágio 25 (GRAMAPUROHIT; SHANBHAG; SAIDAPUR, 2000; RIZZI et al., 2015) ou até depois da metamorfose (FABREZI et al., 2012). No entanto, já se sabe que o desenvolvimento somático, categorizado por Gosner, não necessariamente está alinhado ao desenvolvimento gonadal (OGIELSKA; KOTUSZ, 2004). Estudos de Chang e Hsu (1987) relacionando idade, estágio larval e o desenvolvimento ovariano de *R. catesbeiana* evidenciaram que indivíduos no mesmo estágio, porém mais velhos, apresentavam maior nível de desenvolvimento gonadal, mostrando que a idade tem maior importância do que o estágio neste aspecto.

A influência de hormônios sexuais no desenvolvimento e diferenciação gonadal também é discutida. A espécie *Rana curtipes* apresenta padrão semi-diferenciado de diferenciação gonadal; quando exposta à testosterona e 17-β-estradiol apenas no período crítico da formação do ovário ou do testículo, a proporção final de machos e fêmeas originados não se altera (SAIDAPUR; GRAMAPUROHIT; SHANBHAG, 2001). No entanto, quando a exposição ocorre durante todo o desenvolvimento larval essa proporção muda de acordo com o hormônio recebido (SAIDAPUR; GRAMAPUROHIT; SHANBHAG, 2001). Outras espécies que foram expostas ao longo de todo o período larval a estes hormônios também apresentaram alterações nas proporções de machos e fêmeas ou alterações morfológicas nas gônadas resultantes, como alterações no seu desenvolvimento ou situações de intersexo (PETRINI; ZACCANTI, 1998; HAYES; MENENDEZ, 1999; MACKENZIE et al., 2003; MALI; GRAMAPUROHIT, 2016). Dessa maneira, os hormônios sexuais se mostram fundamentais para o desenvolvimento gonadal, porém não necessariamente para a sua diferenciação, indicando haver variações de acordo com a sensibilidade da espécie em questão (MALI; GRAMAPUROHIT, 2016).

Tendo em vista esta importância dos hormônios sexuais no desenvolvimento e/ou diferenciação gonadal destes animais, é esperado que a exposição aos desreguladores endócrinos também cause alterações. Há vários trabalhos demonstrando estes efeitos principalmente com defensivos agrícolas, porém pouco se sabe sobre os efeitos do NP e CPA, sobretudo nos girinos. Estudos com NP mostraram que há aumento da taxa de indivíduos com fenótipo feminino ou com características intermediárias, categorizadas como intersexo (KLOAS; LUTZ; EINSPANIER, 1999; MACKENZIE et al., 2003). Já com o CPA foram observadas alterações morfológicas como redução da pigmentação ovariana (HAYES et al., 2006) e, quando expostos a altas dosagens, foram vistos efeitos contrários ao esperado, como a masculinização dos ovários, sendo que o composto é antiandrogênico (HSÜ; HSÜ; LIANG, 1979). Já em anuros machos adultos o CPA afeta a maturação dos espermatozoides e causa degeneração no tecido intersticial, inclusive das células de Leydig (HAIDER, 1980).

1.6. Leucócitos e Sistema Imune

Há vários princípios hematológicos básicos que se aplicam a todos os vertebrados (ALLENDER; FRY, 2008). O sangue é constituído de plasma, o componente líquido, e das células sanguíneas, que são os eritrócitos e os leucócitos (JUNQUEIRA; CARNEIRO, 2004). A função primária dos eritrócitos nos anfíbios também é o transporte de oxigênio através da hemoglobina (ALLENDER; FRY, 2008). Os leucócitos são divididos em diferentes tipos celulares, cada um com diferentes funções relacionadas ao sistema imune (JUNQUEIRA; CARNEIRO, 2004). Dentre eles há linfócitos e monócitos, categorizados como agranulócitos, além de eosinófilos, basófilos e neutrófilos (ou heterofilos) que são chamados de granulócitos (ARIKAN; ÇIÇEK, 2014). A morfologia e função dos leucócitos nos anfíbios são semelhantes à das outras espécies (ALLENDER; FRY, 2008). No sangue dos anfíbios podem ainda ser encontrados trombócitos, que são células de função equivalente às plaquetas dos mamíferos, porém com morfologia distinta (ALLENDER; FRY, 2008).

Os linfócitos são o tipo de leucócito predominante no sangue periférico dos anfíbios e, em conjunto com os monócitos, podem corresponder a até 80% da contagem leucocitária (ALLENDER; FRY, 2008; ARIKAN; ÇIÇEK, 2014). Os linfócitos podem ser diferenciados em grandes e pequenos de acordo com sua morfologia (ARIKAN; ÇIÇEK, 2014). Ambos são arredondados, mas os pequenos, além de menores, apresentam núcleo mais cromofílico e ocupando praticamente toda a célula, enquanto os grandes são obviamente maiores, mas núcleo menor, mais deslocado para a periferia e com citoplasma mais evidente (ARIKAN;

ÇIÇEK, 2014). Os monócitos são bastante semelhantes aos linfócitos grandes, porém normalmente são ainda maiores e apresentam o núcleo com formato riniforme e o citoplasma corado com menor intensidade e normalmente possui vacuolizações (JUNQUEIRA; CARNEIRO, 2004; ARIKAN; ÇIÇEK, 2014). Os trombócitos são semelhantes aos linfócitos, porém com formato alongado, algumas vezes até fusiforme (ARIKAN; ÇIÇEK, 2014).

Os granulócitos normalmente possuem o núcleo com formato irregular e sempre apresentam grânulos específicos, envoltos por uma membrana (JUNQUEIRA; CARNEIRO, 2004). Os neutrófilos comumente tem núcleo lobado e seus grânulos são eosinofílicos e com formato mais alongado, no entanto em alguns casos é difícil de percebê-los em microscopia de luz (ARIKAN; ÇIÇEK, 2014). Podem ser encontrados também neutrófilos com núcleo sem divisão em lobos e grânulos intensamente corados, os quais são chamados de heterófilos (ARIKAN; ÇIÇEK, 2014). Os eosinófilos costumam ter núcleo bilobulado, os grânulos tem intensa eosinofilia, com formato arredondado, os quais normalmente recobrem o núcleo (JUNQUEIRA; CARNEIRO, 2004; ARIKAN; ÇIÇEK, 2014). Já os basófilos apresentam grânulos menores, arredondados, com intensa basofilia, cuja coloração costuma impedir a visualização do núcleo (ARIKAN; ÇIÇEK, 2014).

O sistema imune dos vertebrados é dividido em inato e adaptativo (ROBERT; OHTA, 2009). A resposta inata é primária, a qual age pela ativação de células como neutrófilos e macrófagos, que eliminam patógenos por fagocitose ou citotoxicidade (ROBERT; OHTA, 2009). A ativação da resposta imune inata leva, por sua vez, à iniciação da resposta adaptativa, a qual é caracterizada por linfócitos T e B e sua expressão de antígenos específicos para determinados agressores (ROBERT; OHTA, 2009).

A maior parte dos leucócitos está envolvida com o sistema imune inato, com exceção dos linfócitos que são parte da resposta adaptativa e, além disso, os monócitos tem função dupla como células apresentadoras de antígeno (APC) (ROBERT; OHTA, 2009). Os neutrófilos, quando recrutados em um tecido, tem função fagocitária (JUNQUEIRA; CARNEIRO, 2004). Os eosinófilos também possuem ação fagocitária, mas estão mais relacionados a casos de hipersensibilidade, sendo atraídos para áreas de inflamação (JUNQUEIRA; CARNEIRO, 2004). Já os basófilos contém histamina nos seus grânulos, que é um fator quimiotático para eosinófilos e neutrófilos (JUNQUEIRA; CARNEIRO, 2004). Os linfócitos agem na resposta adaptativa, de modo que são ativados por antígenos específicos e respondem produzindo anticorpos contra os mesmos (JUNQUEIRA; CARNEIRO, 2004).

Atualmente, sabe-se que alguns EDCs como Bisfenol A, Atrazina e o NP possuem efeitos imunotóxicos, reduzindo a resistência dos animais a doenças (YIN et al., 2007; SHELLEY et al., 2012). O CPA também pode atuar no sistema imunológico, reduzindo a quantidade de linfócitos (ABOUDKHIL et al., 1991). A exposição a um conjunto de pesticidas, dentre eles a atrazina, também afetou a proliferação de linfócitos T em *Rana pipiens* (CHRISTIN et al., 2004). As infecções por patógenos têm sido apontadas como uma das causas do declínio dos anfíbios (HAYES et al., 2010) e, portanto, a exposição a estes contaminantes pode facilitar a entrada de micro-organismos e contribuir para este processo.

2. OBJETIVOS

Este trabalho teve como objetivos gerais avaliar os efeitos dos desreguladores endócrinos nonilfenol (NP) e acetato de ciproterona (CPA) em aspectos genotóxicos, morfológicos e imunológicos de girinos e jovens de *R. catesbeiana*. Os objetivos específicos foram:

a) Avaliar se os compostos afetam a frequência de anormalidades nucleares nos eritrócitos em ambos os estádios.

b) Avaliar se a pigmentação melânica hepática responde ao tratamento com os contaminantes em ambos os estádios.

c) Verificar o grau de diferenciação e desenvolvimento gonadal dos girinos nos estádios iniciais e, se já houver diferenciação, como os compostos podem afetá-la.

d) Avaliar se os desreguladores hormonais afetam a razão sexual dos indivíduos e/ou o desenvolvimento das gônadas.

d) Avaliar o perfil leucocitário dos indivíduos em ambos os estádios e quais os efeitos dos tratamentos nesta linhagem de células.

3. DELINEAMENTO EXPERIMENTAL

A fim de viabilizar a execução dos experimentos, os mesmos foram conduzidos em três etapas (Figura 1). A cada dois dias era necessário trocar grande volume de água para os girinos, de modo que seria inviável realizar os experimentos com os dois compostos ao mesmo tempo. Dessa maneira, foi feito primeiramente um experimento de exposição dos girinos ao NP e, posteriormente um segundo experimento com exposição ao CPA. Já para os jovens, pelo fato da quantidade de água ser mais reduzida, foram então realizados, ao mesmo tempo, os experimentos de exposição ao NP e ao CPA.

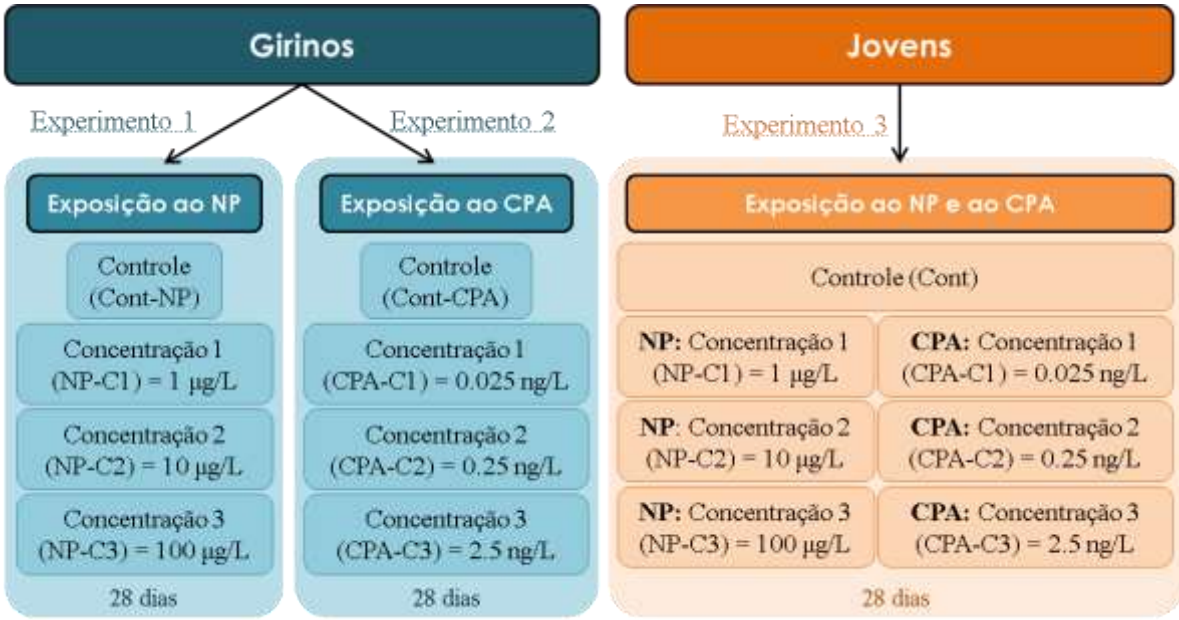

Figura 1: Delineamento experimental realizado neste estudo. Foram realizados três experimentos, dois com girinos e um com jovens. Os girinos foram submetidos inicialmente a um experimento de exposição ao NP em três concentrações distintas, além do grupo controle. Posteriormente, foram realizados os mesmos procedimentos, mas com exposição ao CPA em três concentrações distintas e o grupo controle. O terceiro experimento foi realizado com indivíduos jovens, que também foram expostos às mesmas concentrações de NP e CPA que os girinos, além do grupo controle. Todos os experimentos tiveram duração de 28 dias.

Nos dois experimentos com girinos foram utilizados um total de 72 animais (fornecidos pelo CAUNESP, Jaboticabal – SP), sendo 9 indivíduos para cada grupo experimental (dois controles e seis tratados), organizados em três réplicas. Dessa maneira, cada grupo continha três recipientes plásticos (réplicas), contendo três animais em cada um, preenchidos com três litros de água declorada (1 L/animal) (Figura 2). Nos grupos tratados os compostos eram diluídos inicialmente em um litro de água e, então, através de cálculos ($C_1.V_1=C_2.V_2$), foram distribuídos os devidos volumes de água contaminada para cada grupo experimental. Já nos experimentos com os indivíduos jovens (também fornecidos pelo CAUNESP), foi adicionada mais uma réplica por grupo experimental, totalizando 84 animais (12 animais/grupo; um controle e seis tratados). Assim, neste experimento cada grupo foi organizado em quatro recipientes plásticos (réplicas), cada um contendo três animais e 200

mL de água declorada. Nos grupos tratados, a contaminação da água foi feita da mesma maneira, diluindo primeiramente em um litro de água.

Figura 2: Fotografia do experimento 2 (exposição dos girinos ao CPA) mostrando a organização dos grupos Controle, CPA-C1, CPA-C2 e CPA-C3 em recipientes plásticos, cada um com três réplicas (1, 2 e 3) contendo três animais e três litros de água.

Em todos os experimentos as trocas de água eram realizadas a cada 48 horas, com os compostos sempre diluídos no mesmo dia. A meia-vida do NP em ambiente aquático estático é de aproximadamente 17 dias, sendo considerado um composto de alta persistência nesses locais (NCBI, 2019). Já sua meia-vida em sistemas biológicos pode chegar a 99 horas no fígado de peixes (COLDHAM et al., 1998), uma vez que este composto pode ser metabolizado. Para o CPA não há dados sobre a meia-vida ambiental, porém sabe-se que em humanos sua metabolização ocorre em cerca de 1,9 dias (NCBI, 2018). Sendo assim, o período para troca da água de 48 horas foi utilizado a fim de garantir que as concentrações desejadas dos compostos na água fossem mantidas, e também para evitar interferência dos produtos de excreção dos animais. Após as trocas era fornecido o alimento (rações formuladas pelo CAUNESP especificamente para a espécie).

Ao final dos experimentos os animais foram eutanasiados em benzocaína (5 g/L), o sangue foi coletado (nos jovens coletou-se antes da eutanásia, com os animais anestesiados), a dissecação foi feita e o os órgãos coletados seguiram para o processamento histológico. Todos os procedimentos e as análises feitas foram aprovados pelo Comitê de Ética no Uso de Animais (CEUA) do Instituto de Biociências, Letras e Ciências Exatas (Ibilce – Unesp) e serão detalhados nos capítulos a seguir.

4. CAPÍTULO 1: Manuscript submitted to *Environmental Pollution*.

Genotoxic effects of Nonylphenol and Cyproterone Acetate in *Rana catesbeiana* (Anura) tadpoles and juveniles

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Abstract

Ecotoxicological studies commonly use genotoxic analyses as early biomarkers to investigate the potential effects of environmental contaminants in biological models. Several pollutants are capable of inducing DNA damage and, therefore, counting micronuclei and other nuclear abnormalities are efficient tools to evaluate the genotoxic potential of the compounds. Some pollutants such as 4-nonylphenol (NP), a detergent used mainly in industries, and Cyproterone Acetate (CPA), an antiandrogenic medicine, have already shown genotoxic effects on some vertebrates, however, the effects of these compounds on anurans are not yet known. Since these animals are declining worldwide due to water contamination and other factors, and they are bioindicators of environmental quality, it is important to know how these pollutants affect anurans in order to discuss appropriate solutions to avoid the extinction of the species. Therefore, this study investigates the genotoxic effects of NP and CPA at three different concentrations on *Rana catesbeiana* by counting the nuclear abnormalities observed in tadpoles and juveniles after exposure to the compounds. The tested dosages were 1, 10 and 100 μg/L for NP, and 0.025, 0.25 and 2.5 ng/L for CPA in experiments that lasted 28 days. The experimental conditions were the same except for the water volume since tadpoles and juveniles exhibit different habits at different developmental stages. The tadpoles were more susceptible than juveniles to both compounds, showing increased nuclear abnormalities in the highest NP concentration and all CPA exposures. The juveniles, on the other hand, responded only to the two highest CPA concentrations. We concluded that CPA, even at very low concentrations, is extremely harmful to both anuran developmental stages, but especially to tadpoles. Although only the 100 μg/L NP dosage induced a genotoxic response in tadpoles, it still is a significant response since this is considered an environmental relevant dosage. Besides, the tadpoles were more sensitive to pollutants than the juveniles.

Capsule: This study emphasizes the importance of knowing the response of genotoxic effects of endocrine disrupting chemicals, such as NP and CPA, on anurans at different developmental stages when exposed to environmentally relevant doses.

Key words: Micronuclei; Nuclear Abnormalities; EDCs; Biomarkers; Bioindicator

4.1. Introduction

Using early or precocious biomarkers in ecotoxicological studies is very important to investigate the action of environmental contaminants in biological models using measurable parameters (ADAMS et al., 2001). These biomarkers can be defined as structural or functional parameters of organisms, in different levels, which are altered by environmental toxicants (DEPLEDGE; FOSSI, 1994; VAN GESTEL; VAN BRUMMELEN, 1996; BARNI et al., 2007). Some of the most widely used biomarkers are genotoxic effects, evaluations of genetic diversity, immunological responses and reproductive impairment aspects (ADAMS et al., 2001).

A commonly used genotoxic technique is the micronuclei count, which are defined by lost chromosomes (or fragments) that form other nucleus (one or more) at a lower proportion (AL-SABTI; METCALFE, 1995). However, several other nuclear abnormalities have been considered such as alterations of the nucleus shape, nuclear buds, anucleated, binucleated or multinucleated cells, apoptotic cells and nuclear pyknosis, which precedes apoptosis (FENECH et al., 2003; LAJMANOVICH et al., 2013; 2014; JOSENDE et al., 2015). Recently, ecotoxicological studies have been using genotoxic analysis increasingly since several pollutants are capable of inducing nuclear abnormalities among species.

The amphibians are considered as bioindicators of the health status of an ecosystem and good biological models for environmental studies (DUNSON; WYMAN; CORBETT, 1992). They are very susceptible to environmental contaminants due to some characteristics such as complex life-cycle, the permeability of eggs, gills, and skin, rapid growth and ectothermia (DUNSON; WYMAN; CORBETT, 1992; BURLIBAŞA; GAVRILĂ, 2011). Furthermore, the amphibians are experiencing a worldwide population decline caused by water contamination, among other factors (BLAUSTEIN et al., 2011). Thus, it is important to know the biological effects of these compounds and use sensitive tools to understand how they are affecting the species.

It is well known that various contaminants increase the frequency of nuclear abnormalities in anurans, such as several agrochemicals (LAJMANOVICH et al., 2013; 2014; NIKOLOFF et al., 2013; PÉREZ-IGLESIAS et al., 2016), heavy metals (MOUCHET et al., 2006), and other pollutants like benzo-α-pyrene (FANALI et al., 2018). The 4-Nonylphenol (NP) is an alkylphenol produced during petroleum refinement, used mainly in detergent production and found in significant concentrations in the air, soil, water and sediments (VAZQUEZ-DUHALT et al., 2005). It has estrogenic effects, being considered an endocrine-

disrupting chemical (EDC) (VAZQUEZ-DUHALT et al., 2005). Although NP genotoxic effect on fish is well established (TELES et al., 2004; MEKKAWY; MAHMOUD; SAYED, 2011; AL-SHARIF, 2012; SHARMA; CHADHA, 2017), there is only one study demonstrating the genotoxic potential for amphibians, and it was used high concentrations (HUANG et al., 2009). The Cyproterone Acetate (CPA), present especially in drugs against prostate cancer and contraceptives, is also classified as an EDC due to the antiandrogenic, progestogen and antigonadotropic effects (NEUMANN; TÖPERT, 1986; NCBI, 2018). This compound is also a water contaminant with significant genotoxic effects on mammals (NEUMANN et al., 1992; RABE et al., 1996; KASPER, 2001; SIDDIQUE; AFZAL, 2005), but studies on aquatic vertebrates, such as fish and amphibians, are still lacking.

Thus, this study aims at evaluating whether different NP and CPA concentrations could induce nuclear abnormalities, such as micronucleus, buds and binucleated, anucleated and/or apoptotic cells, in the erythrocytes of *Rana catesbeiana* in different developmental stages in a long-term exposure. Because the EDC compounds probably can act in very low doses, we tested low concentrations, expressed as μg/L and ng/L. We hypothesize that both compounds cause the nuclear abnormalities to increase, with more expressive responses in tadpoles, since earlier developmental stages are probably more susceptible to the contaminants.

4.2. Materials and Metods

Animals

A total of 72 *Rana catesbeiana* tadpoles in Gosner stage 25 (1960) were obtained from the Centro de Aquicultura of Unesp (CAUNESP), in Jaboticabal, SP. Two experiments with 36 tadpoles of each were conducted, but we randomly chose 20 from each trial for the genotoxic analyses. CAUNESP also provided 84 *R. catesbeiana* juveniles, 10 days after metamorphosis with 7.92 g mean weight. The experiments were approved by the Ethics Committee on the Use of Animas (CEUA) from Instituto de Biociências, Letras e Ciências Exatas (Ibilce – Unesp), protocol number 128/2015.

Tadpoles Experiments

All animals were acclimated for seven days before the experiments. The experiments with the tadpoles were separated in two. In one experiment, the tadpoles were exposed to NP (4-Nonylphenol, Ref: 46405, Sigma-Aldrich) while in the other, they were exposed to CPA

(Cyproterone Acetate, Ref: C989100, TRC Canada) under exactly the same experimental conditions, which are described below.

The 72 tadpoles were separated into eight groups: two control group (Cont-NP and Cont-CPA, one for each experiment) and six treatment groups exposed to three dosages of NP and three dosages of CPA. The tested NP concentrations were 1, 10 and 100 μg/L (NP-C1, NP-C2, and NP-C3, respectively), chosen based on Vazquez-Duhalt et al. (2005), since surface waters containing such NP concentrations are classified as low, medium and high pollution, respectively. The CPA concentrations were first tested based on Green et al. (2015), but pilot tests showed lethal responses even at low doses (10 and 100 ng/L), so we determined as feasible the concentrations of 0.025, 0.25 and 2.5 ng/L (CPA-C1, CPA-C2, and CPA-C3, respectively).

The tadpoles were maintained in 5 L plastic containers with three animals in each containing 3 L of dechlorinated water (1 L/animal), with three replicates per experimental group. If any animal died during the experiment, the water proportion of 1 L/animal was maintained to ensure the same contaminant proportion and population density. The experiment lasted 28 days, the water was changed every 48h and food was supplied after water renewal (protein-based formula for *R. catesbeiana*, made by CAUNESP). The used mineral water (Hidroleve ®) was tested for physicochemical and bacteriological properties to ensure no external contamination. During the experiments, the animals were kept at room temperature (mean: 26ºC) and photoperiod of approximately 14/10h light/dark.

After the experimental period, 5 animals were randomly chosen from each experimental group (*N*=5) and euthanized in benzocaine (5 g/L). Subsequently, because the animals were too small (approximately 1.5 cm) to collect blood via puncture, their heads were cut off with a razor and the blood was left to drip onto the glass slides. The blood smears were prepared immediately to prevent clotting.

Juveniles Experiments

The experimental design for the juveniles was the same as described for the tadpoles, they were also maintained in 5L plastic containers with three animals in each, but one more replicate was added to each experimental group, totalizing 84 animals (*N*=12 per group). Because the juvenile habit is semi-terrestrial, we reduced the water volume to 200 mL, so the animals could be in contact with the water, but could be still supported on their four legs at

the bottom surface of the containers. All other parameters such as compound concentrations, exposure time, water change and food supply were kept the same for both stages.

After the experimental period, the juveniles were anesthetized with xylocaine ® (lidocaine) in the inner thigh region, which was then cleaned with cotton (to remove excess) and the blood was collected from the femoral vein with heparinized syringes and needles. The smears were prepared immediately after the blood collection and the animals were euthanized in benzocaine (5 g/L) soon after.

Analysis of Nuclear Abnormalities

After drying, the blood slides were fixed in 4ºC methanol for 20 minutes and stained with Giemsa at 7.5% for 15 minutes. Under a light microscope (Leica DM 4000 B), a total of 1000 erythrocytes per animal (tadpoles and juveniles) was counted and examined to find nuclear abnormalities, following the parameters and methodology applied by Lajmanovich et al. (2013, 2014). Because the objective of this study was not to evaluate the causes or particularities of each abnormality, all deformities per animal were summed up to evaluate the genotoxic effects of the compounds in general.

Statistical Analysis

The ratio of nuclear abnormality counts was modeled using a Generalized Linear Model (GLM) with binomial distribution and *log* link function including the treatment (categorical predictor with 7 levels) and the stage of the animal (categorical predictor with two levels) and their interaction. We tested model assumptions using diagnostic plots in the R (Team Core, 2017) package *sjplot* (LÜDECKE, 2016). Residuals had homogeneous variance and normal distribution.

Because the experiments with NP and CPA on tadpoles were divided into two stages, we have two control groups, one for each experiment (Cont-NP and Cont-CPA). However, since these two control groups were not statistically different ($p = 0.3722$), we calculate a mean value and considered a single control group (Cont) for comparisons in the statistical analysis.

4.3. Results

The nuclear abnormalities (Figure 1) varied with the tested compounds, concentrations and animal developmental stage. The responses to CPA were more aggressive while the

response to NP was observed for the highest concentrations only. Additionally, the tadpoles were more vulnerable to the exposition.

Effects of NP and CPA on Tadpoles

The response to the NP treatment was more aggressive only in the highest concentration (NP-C3 group), showing significantly increased nuclear abnormalities compared to control ($p < 0.01$). The other NP treatments did not show genotoxic effects (Cont – NP-C1: $p = 0.9912$; Cont – NP-C2: $p = 0.9183$). On the other hand, the CPA treatment showed increased genotoxicity in all concentrations tested ($p < 0.001$ for all comparisons). The results for tadpoles are shown in Figure 2.

An interesting fact observed in the CPA groups, especially in the highest concentration, is the number of apoptotic cells found. Although these cells were not present in the control groups, they were found in regular numbers in NP-C3 but in aberrant/abnormal numbers in the CPA treated groups (Table 1/Figure 3).

Effects of NP and CPA on Juveniles

Compared to control group (Cont), the NP treatment did not affect the count of nuclear abnormalities in any concentration tested in this study (Cont–NP-C1: $p = 0.8097$; Cont–NP-C2: $p = 0.4828$; Cont–NP-C3: $p = 0.2297$). On the juveniles the CPA treatments also caused more expressive responses, but genotoxicity did not increase significantly in CPA-C1 ($p = 0.9878$). The significant differences were found only in CPA-C2 and CPA-C3 ($p < 0.01$ for both comparisons). The results for juveniles are shown in Figure 4.

Contrary to what happened to tadpoles, apoptotic cells were not observed in juveniles, neither in the control nor in the treated groups (Table 2/Figure 5).

Tadpoles vs Juveniles

The results obtained for tadpoles and juveniles show that the older animals were more resistant to the studied compounds. Also, the tadpoles had increased nuclear abnormalities in the NP-C3 treatment and in all CPA concentrations, compared to control. Comparing tadpoles and juveniles at these same concentrations (NP-C3 and all CPA dosages), there is also statistical difference between the life stages ($p < 0.001$ in all comparisons). Even at CPA-C2 and CPA-C3 that both tadpoles and juveniles had increased nuclear abnormalities, the

increase in the tadpoles was statistically higher than in the juveniles. These results are shown in Figure 6.

4.4. Discussion

Our results show that both compounds tested were genotoxic to the different developmental stages of anuran life cycle. However, tadpoles in the initial developmental stages were more responsive to the studied EDCs in general (NP and CPA), whereas juveniles responded only to CPA, which also induced more aggressive responses than NP.

In the literature, genotoxic studies on exposition to NP show significant responses to the studied treatments for fish (TELES et al., 2004; MEKKAWY; MAHMOUD; SAYED, 2011; AL-SHARIF, 2012; SHARMA; CHADHA, 2017) and for the anuran *Rana nigromaculata* (HUANG et al., 2009). Our results corroborate the genotoxic potential of NP since we also found nuclear abnormalities in tadpoles at the highest concentration. However, all these authors tested higher concentrations than we did, except for the similar doses used by Al-Sharif (2012). It should be emphasized that even the highest NP dose tested in our study, can be found in the environment. In the United States, concentrations of 94 μg/L NP have already been detected in water bodies (DACHS; VAN RY; EISENREICH, 1999) while concentrations up to 343 and 644 μg/L NP have also been reported in Spain (SOLÉ et al., 2000). In both tadpoles and juveniles the NP presented a dose-response effect, so it is possible that higher concentrations would have induced more expressive responses. However, because EDCs probably have complex action mechanisms (WELSHONS et al., 2003), further studies testing a wider range of concentrations should be performed to understand better and, in more detail, how NP acts on these organisms.

The responses of both tadpoles and juveniles were more expressive to the CPA treatments, however, the genotoxicity of this compound to aquatic vertebrates is still poorly understood. Because CPA is used in medicines (NCBI, 2018), mammals are the main biological model for these studies, which frequently evaluate the potential collateral effects on humans. Despite this, several studies have already shown genotoxic effects (NEUMANN et al., 1992; RABE et al., 1996; KASPER, 2001; SIDDIQUE; AFZAL, 2005), so our study corroborate these findings. The nuclear abnormalities increased significantly for all three concentrations tested (except for the lowest dose in juveniles), showing that CPA is a potent toxicant given that we tested very low concentrations (ng/L) in this study. Data for

environmental concentrations are lacking in the literature. However, Green et al. (2015) made predictive calculations based on CPA consumption in England and Wales and its excretion rate, concluding that the concentrations should vary from 34 to 74 ng/L in four untreated effluents, but not higher than 10 ng/L in most rivers. In our study, much lower CPA concentrations caused severe genotoxic effects to the anurans while our pilot tests with 10 and 100 ng/L killed all the animals. Thus, we observed that CPA is very toxic to *R. catesbeiana*, especially tadpoles, and more attention should be given to environmental contamination by this compound, as well as the effects on organisms that could be exposed to it.

Besides the high increase in general nuclear abnormalities that we observed for tadpoles exposed to CPA, we noted a specific high frequency of apoptotic cells. These cells are initially called by pykinotic cells, identified by its intense chromatin condensation, strongly stained in the nucleus (FENECH et al., 2002; ELMORE, 2007). As the apoptosis process progresses, the nucleus starts to fragment (FENECH et al., 2002), since apoptosis is a programmed cell death mechanism, which is activated automatically when drastic DNA alterations occur (ELMORE, 2007). This high number of apoptotic cells in CPA treated groups indicates that this compound is probably inducing such severe DNA damage to the erythrocytes leading them to death by apoptosis. The high frequency observed in micronuclei counts is also indicative of the extreme DNA damage caused by CPA.

The exposure to both NP and CPA showed milder effects on juveniles compared to tadpoles, since juveniles were not affected by exposure to NP and affected by CPA exposure only in the highest concentrations (CPA-C2 and CPA-C3). Studies comparing tadpoles and adults in non-polluted and polluted areas showed higher micronuclei frequency in tadpoles, even in preserved areas (BARNI et al., 2007). The larvae live fully immersed in water and depend on the gills for respiration, which favors greater contact between the individual and the environment, making the tadpoles more susceptible to contaminants (WRIGHT; WRIGHT, 1996; BARNI et al., 2007). Also, the detoxification processes in larvae are less effective than in adults or juveniles, since the liver, which is the main detoxification organ, is under development during larval stages (BARNI et al., 2007).

4.5. Conclusion

In conclusion, we observed that both NP and CPA increased nuclear abnormalities in tadpoles. The NP had significant responses only at 100 μg/L, while the response to CPA was harmful in all doses. For the juveniles, only the treatment with CPA in the highest

concentrations (0.25 and 2.5 ng/L) caused genotoxic effects. The tadpoles are more susceptible to these pollutants than the juveniles. It is important to emphasize that the results obtained with CPA were significant in doses considered very low. Although NP has induced responses only in higher concentrations, the dosage is environmentally relevant and should also be taken into account. Since the amphibian populations are already in decline, we strongly encourage performing more studies with these and other EDCs to evaluate the adverse effects that could be triggered by these compounds.

4.6. Acknowledgments

We would like to thank the entire Anatomy Lab team for their technical support during the experiments.

4.7. Funding Sources

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) – Financing Code 001 and LSG doctoral fellowship, a research grant to CO provided by São Paulo Research Foundation, FAPESP (#2013/02067-5, #2015/12006-9 and #2018/01078-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (#305081/2015-2). LFB received post-doctoral fellowship from FAPESP (Process 2014/00946-4). Language revision was also financed by CAPES (Financing Code 001).

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4.9. Figures and Tables

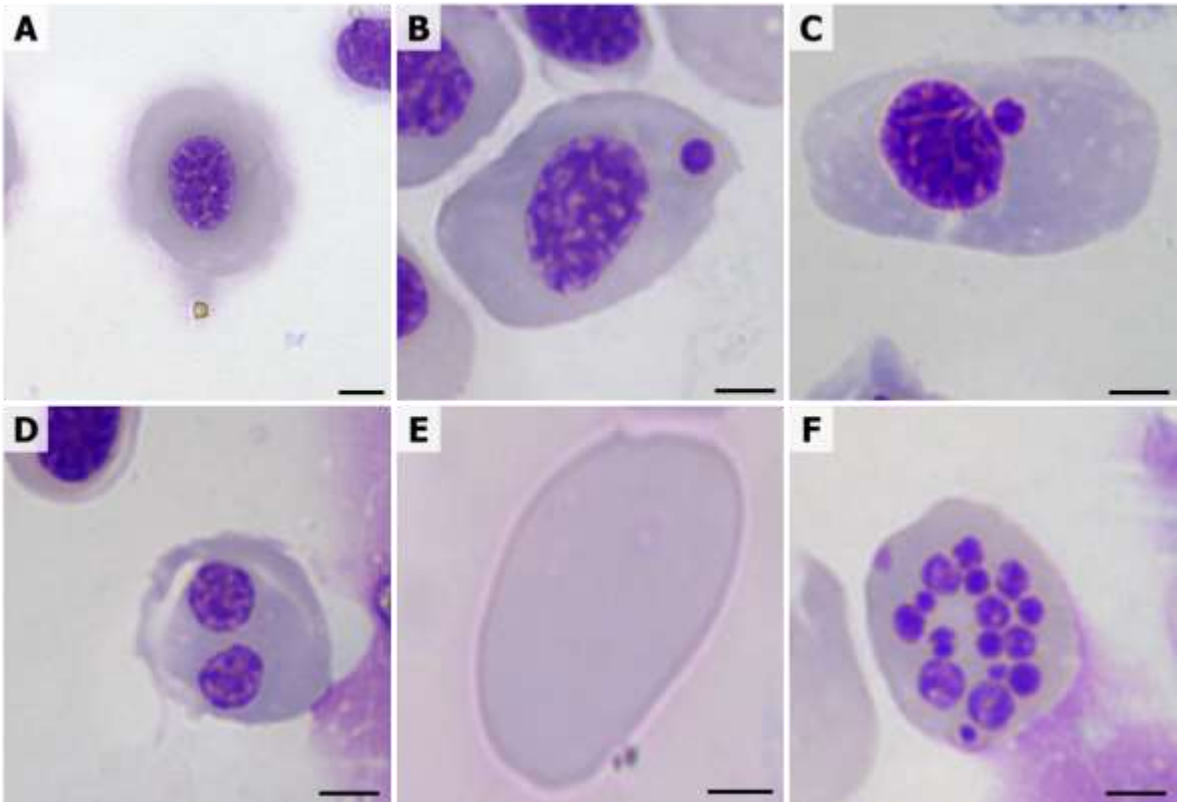

Figure 3 [1]. Normal erythrocyte (**A**) and the nuclear abnormalities found in this study for tadpoles: micronucleus (**B**), nuclear bud (**C**), binucleated (**D**), anucleated (**E**) and apoptotic (**F**) cells. The juveniles erythrocytes had similar morphology, and no apoptotic cell at this stage. Staining: Giemsa. Scale bars: 5 μm.

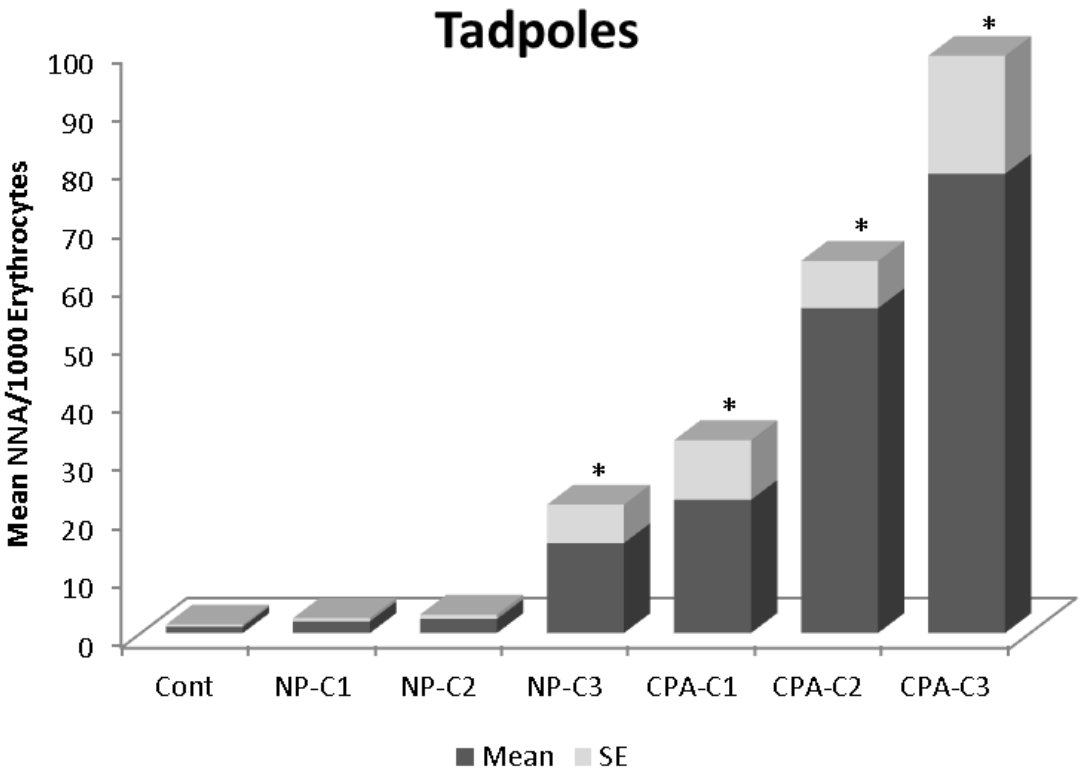

Figure 4 [2]: Mean number of nuclear abnormalities (NNA) found in 1000 erythrocytes from each tadpole, comparing the NP and CPA exposed groups to control group (Cont). The asterisk (*) means statistically significant difference. Mean ± Standard Error (SE).

Table 1: Number of nuclear abnormalities counted in a total of 5000 erythrocytes from 5 tadpoles: micronucleus (MN), buds, binucleated (BN), anucleated (AN) and apoptotic (AP) cells.

	Total	**MN**	**Bud**	**BN**	**AN**	**AP**
Cont-NP	5000	6	0	1	0	0
Cont-CPA	5000	2	1	1	0	0
NP-C1	5000	7	0	3	0	0
NP-C2	5000	10	0	2	0	0
NP-C3	5000	32	17	7	0	21
CPA-C1	5000	27	6	14	0	67
CPA-C2	5000	136	13	17	3	109
CPA-C3	5000	82	16	20	5	270

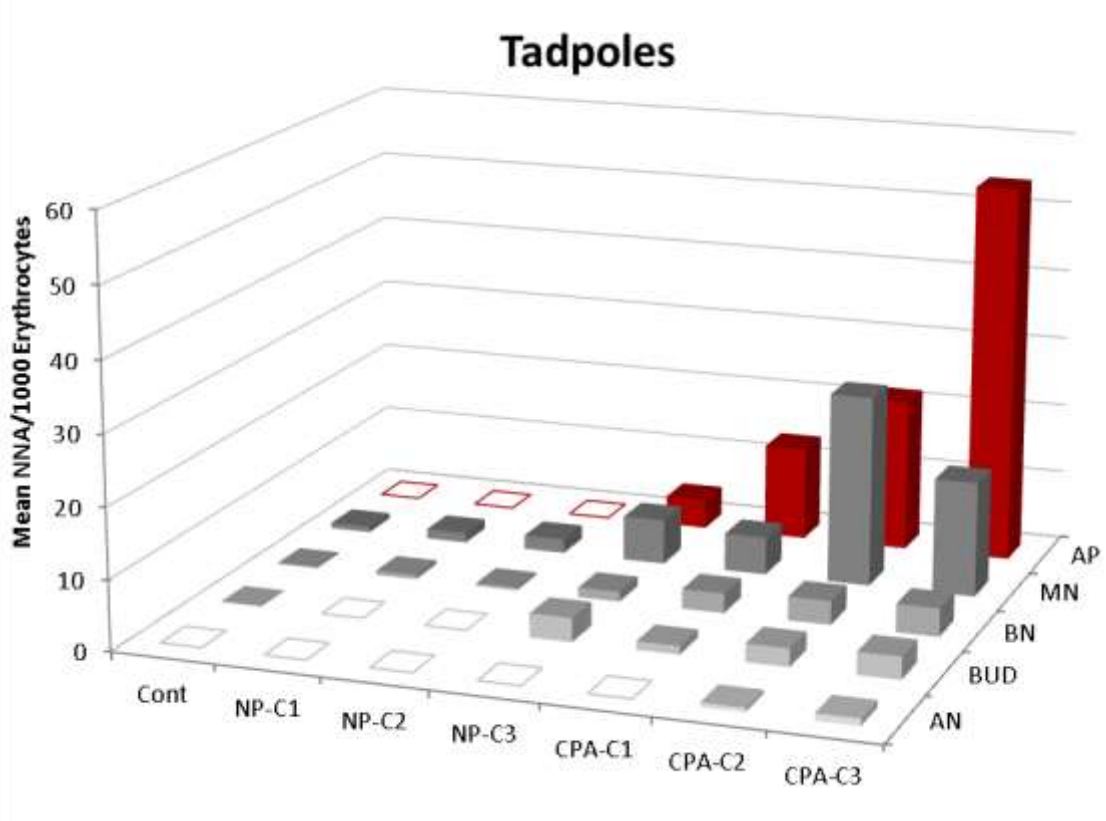

Figure 5 [3]: Mean number of nuclear abnormalities (NNA) counted per 1000 erythrocytes of tadpoles from control (Cont) and the three treated groups of each compound (NP and CPA): anucleated cells (AN), buds, binucleated (BN), micronucleus (MN) and, highlighted in red, apoptotic (AP) cells. The empty columns (contoured) represent absence of that abnormality.

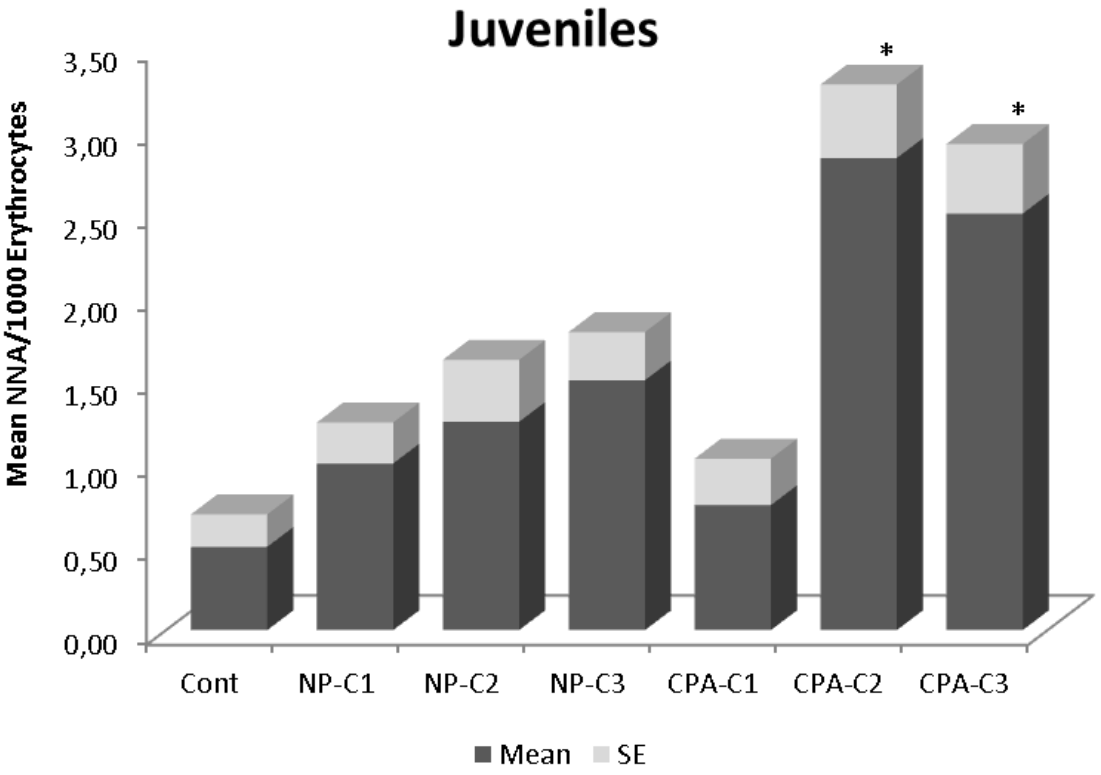

Figure 6 [4]: Mean number of nuclear abnormalities (NNA) found in 1000 erythrocytes from each juvenile, comparing the NP and CPA exposed groups to the control (Cont). The asterisk (*) means statistically significant difference. Mean ± Standard Error (SE).

Table 2: Total nuclear abnormalities found in 12000 erythrocytes from 12 juveniles: micronucleus (MN), buds, binucleated (BN), anucleated (AN) and apoptotic (AP) cells.

	Total	**MN**	**Bud**	**BN**	**AN**	**AP**
Cont	12000	3	0	2	1	0
NP-C1	12000	5	1	3	3	0
NP-C2	12000	12	0	0	3	0
NP-C3	12000	12	1	2	3	0
CPA-C1	12000	7	0	0	2	0
CPA-C2	12000	31	1	1	1	0
CPA-C3	12000	24	2	1	3	0

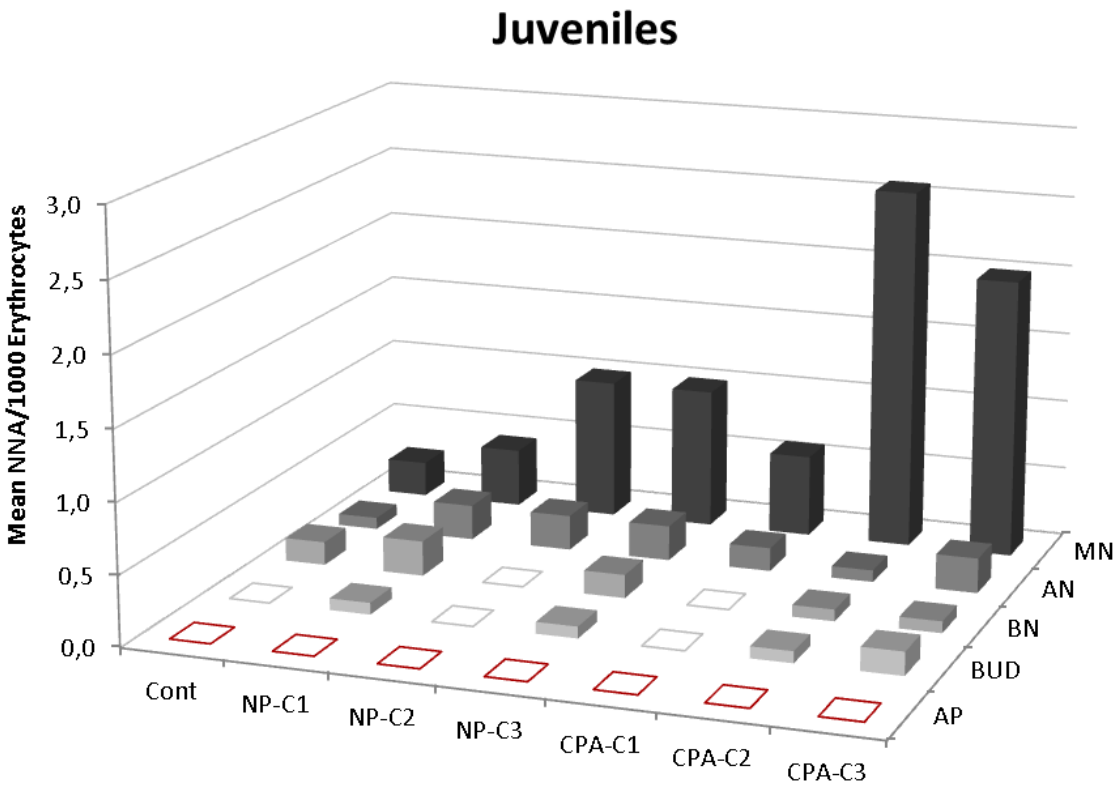

Figure 7 [5]: Mean number of nuclear abnormalities (NNA) counted per 1000 erythrocytes of juveniles from the control (Cont) and the three treated groups for each NP and CPA: apoptotic cells (AP), which in this stage was not found, buds, binucleated (BN), anucleated (AN) and micronucleus (MN). The empty columns (contoured) represent absence of that abnormality.

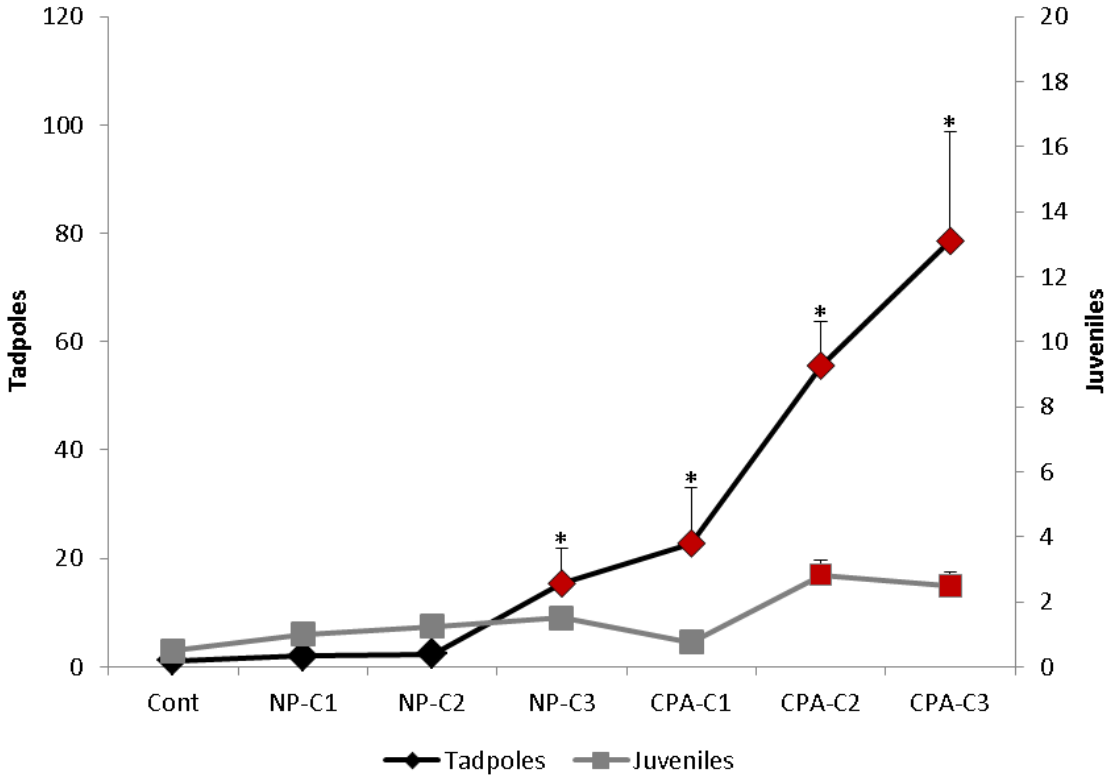

Figure 8 [6]. Mean number of nuclear abnormalities/1000 erythrocytes for each tadpole and juvenile, for control (Cont) and the three treated groups of each NP and CPA. The red markers highlight the significant difference between the treatment and the control group for tadpole (black line) or juvenile (grey line) and the asterisk (*) indicates statistical difference between the stages.

5. CAPÍTULO 2: Manuscript in preparation to be submitted to *Environmental Science and Pollution Research*.

Morphological effects of Nonylphenol and Cyproterone Acetate in the liver and gonads of *Rana catesbeiana* (Anura)

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Abstract

Recently, the concern about amphibians' population decline is increasing. The environmental pollution is one ultimate contributing factor for this phenomenon and the contamination with endocrine disrupting chemicals (EDCs) has particularly worried the scientific community because of its ability to cause adverse effects at very low doses. Pollutants like 4-nonylphenol (NP), an estrogenic detergent, and Cyproterone Acetate (CPA), an antiandrogenic medicine, are EDCs that occur contaminating water bodies. We hypothesized that the exposure to NP and CPA could exhibit effects in the liver, since it is the main organ for detoxification, and in the gonads, due to the hormonal disrupting action. The effects of these chemicals, however, are poorly known, especially in anurans. Thus, we investigated the effects of three different concentrations of NP and CPA in liver melanin pigmentation and gonads morphology of tadpoles and juveniles *Rana catesbeiana*. The NP concentration was 1, 10 and 100 μg/L, and CPA was 0.025, 0.25 and 2.5 ng/L, with experiments lasting 28 days. Both stages were submitted to the same experimental conditions. The tadpoles did not vary the liver pigmentation and the gonads were not differentiated yet. However, the animals had impairment in growth rate, so in all treatments the tadpoles had less body mass and length. The juveniles showed increased melanin pigmentation at 10 μg/L (NP) and 0.025 ng/L (CPA). The sex ratio and the morphology of the gonads at this stage were not altered by the treatments, as well as their growth. The melanin has defensive, cytoprotective roles, so the increase in its area is related to the protection of the organisms against toxic effects. The tadpoles are still under development, their immune system probably does not respond properly as the juveniles, which may have more efficient response mechanisms. As regarding the gonads, some studies discuss that the effects of sexual hormones on the differentiation may vary according to species. Thus, *R. catesbeiana* should be resistant to NP and CPA at the concentrations used in this study, since any signal of intersexualization was observed. Although the compounds showed slight effects in hepatic pigmentation, we should emphasize that the dosages are very low and the species is resistant. Also, EDCs should not present dose-response effects, so the effects are not necessarily predictive. Therefore, we still need to be aware of the environmental levels of NP and CPA and its effects on other species and at different dosages.

Key words: EDCs; Pigmentation; Melanin; Sex ratio; Morphology.

5.1. Introduction

According to International Union for Conservation of Nature (IUCN, 2018), 31,1% of amphibians species are in the red list, threatened with extinction. These animals have several characteristics that make them susceptible to water contamination, such as egg, gill and skin permeability, two-stage life cycle, with different particularities but both dependent on water and ectothermia (DUNSON; WYMAN; CORBETT, 1992; BURLIBAŞA; GAVRILĂ, 2011; WAKE; VREDENBURG, 2008). Thus, among other factors, the environmental pollution has been considered an important contributor to amphibians' population decline worldwide (WAKE; VREDENBURG, 2008; HAYES et al., 2010).

Endocrine disrupting chemicals (EDCs) are natural or synthetic substances, capable of interfering direct or indirectly in the endocrine system, found in the environment at levels of µg/L e ng/L (WELSHONS et al., 2003; BILA; DEZOTTI, 2007). The 4-Nonylphenol (NP) is an example of EDC, showing estrogenic properties, used mainly in detergent production (VAZQUEZ-DUHALT et al., 2005). The Cyproterone Acetate, in turn, is an antiandrogenic, antigonadotropic and progestogen compound used in medicines, been another example of EDC (NEUMANN; TÖPERT, 1986). This class of contaminants is capable to induce responses in very low doses, since they interact with endocrine system that is adjusted to respond to hormones, which also occurs in even lower concentrations (WELSHONS et al., 2003). Therefore, the concern about environmental pollution by EDCs and the effects in amphibians' organisms is legitimate and it is important to be investigated.

Ectothermic animals such as fishes, amphibians and reptiles have peculiar pigmented phagocytic cells in hematopoietic organs like liver and spleen, named melanomacrophages (AGIUS, 1980; SCALIA et al, 1988; WOLKE, 1992; CHRISTIANSEN; GRZYBOWSKI; KODAMA, 1996). These cells are derived from the neural crest, round-shaped, and are capable to synthetize melanin endogenously (SCALIA et al, 1988; CÉSARINI, 1996; FRANCO-BELUSSI; CASTRUCCI; OLIVEIRA, 2013). The melanin pigment has bactericidal properties (CHRISTIANSEN et al., 1996) and is also able to neutralize toxicant agents derived from degraded phagocyte material (ZUASTI et al., 1989; AGIUS; ROBERTS, 2003), showing important cytoprotective roles (McGRAW, 2005).

Recently, the hepatic melanin is being used as biomarker for ecotoxicological studies, since it is responsive to several aquatic contaminants (DE OLVEIRA et al., 2017). It is known that some EDCs are capable to alter melanomacrophages and/or melanin proportion in hepatic tissues of amphibians and fishes, such as drugs like Flutamide (GREGORIO et al., 2016) and

testosterone cypionate (ZIERI et al., 2015), pesticides like Atrazine (PÉREZ-IGLESIAS et al., forthcoming 2019) and other products used in commercial formulations like 4-nonylphenol (SAYED et al., 2012; SAYED; ABD-ELKAREEM; ABOU-KHALIL, forthcoming 2019) and triclosan (CHAI et al., 2017).

In addition to hepatic responses, EDCs commonly also interfere in reproductive aspects, due to its close relation to endocrine system. The real role of sex hormones in anurans' gonadal development and differentiation is still discussed in the literature. In a general way, exposure to testosterone or estradiol during the whole larval development leads to impairments in gonadal development, morphology and, in some cases, alters the sex ratio (PETRINI; ZACCANTI, 1998; HAYES; MENENDEZ, 1999; SAIDAPUR; GRAMAPUROHIT; SHANBHAG, 2001; MACKENZIE et al., 2003; MALI; GRAMAPUROHIT, 2016). However, it has been suggested that the presence of these hormones should be essential for the development of the gonads, but the effects on differentiation may depend on the sensibility of the species (MALI; GRAMAPUROHIT, 2016).

In view of the endocrine disrupting properties of NP and CPA, we hypothesized that the chronic exposure of tadpoles and post-metamorphic juveniles *Rana catesbeiana* to these compounds could: 1. Alter the melanin-pigmented area in the liver, probably increasing the pigmentation due to melanin cytoprotective roles; 2. Affect the morphology of the gonads, probably with a sex ratio tending to female-biased in NP treatment and male-biased in CPA treatment; and 3. Induce more expressive responses in juveniles, since they probably have more efficient immunological system, due to their more advanced developmental stage, and the melanomacrophages are part of the innate immune system.

5.2. Metodology

Animals

We used 72 tadpoles of *R. catesbeiana* at stage 25 (Gosner, 1960), approximately 1 week after hatching and less than 1 cm long, and 84 juveniles with 10 days after metamorphosis and mean mass of 7.92 g, obtained in from Centro de Aquicultura of Unesp – Jaboticabal (CAUNESP). The experiments with tadpoles were separated in two parts while the experiments with juveniles were performed in one single time (experimental design explained below). The experimentations were approved by Ethics Committee on the Use of

Animas (CEUA) from Instituto de Biociências, Letras e Ciências Exatas (Ibilce – Unesp), protocol number 128/2015.

Experimental Design – Tadpoles

Since the water volume used for tadpoles was too large, the experiments were separated in two parts as a matter of logistics. First the animals were exposed to NP (4-Nonylphenol, Ref: 46405, Sigma-Aldrich) and then to CPA (Cyproterone Acetate, Ref: C989100, TRC Canada), ensuring that all the experimental conditions were exactly the same for both experiments, varying only the compounds. The acclimation occurred seven days before the experiments.

The 72 animals were separated in eight groups: one control group for each compound (Cont-NP or Cont-CPA) and three different treated groups for NP or CPA, totalizing six treatment groups. The concentrations of NP were 1 μg/L (NP-C1 group), 10 μg/L (NP-C2 group) and 100 μg/L (NP-C3 group), chosen based on environmental relevant doses (VAZQUEZ-DUHALT et al., 2005). The CPA dosages were based on environmental studies made by Green et al (2015) and pilot tests, so it was determined 0.025 ng/L (CPA-C1 group), 0.25 ng/L (CPA-C2 group) and 2.5 ng/L (CPA-C3 group).

Each experimental group contained three replicates, each one composed of three animals (*N*=9 per group), maintained in plastic containers filled with 3 L of dechlorinated water (1 L water/animal). During the experiments some animals died, so we maintained the proportion of 1 L water/animal to avoid interferences. The tadpoles were submitted to this experimentation during 28 days, each 48h the water was changed and, after this procedure, the food was supplied (with *R. catesbeiana* specific formulation by CAUNESP). The portions were equally distributed by replicate to avoid differences in the final mass due to different amounts of food. The room conditions of temperature and photoperiod were measured at a mean of 26ºC and 14/10h light/dark.

The euthanasia of the animals was made in benzocaine (5 g/L), they were weighted in precision analytical balance (± 0.001 g) and measured (cm) and then the animals were fixed in Metacarn (60% methanol, 30% chloroform and 10% acetic acid) for three hours. After fixation we dissected the animals and collected the liver and the gonads associated with the kidney, which were not weighted, since the scale did not recognize their low mass. The organs were included in historesin (Leica).

Experimental Design – Juveniles

The juveniles were also exposed to NP and CPA, but we added one more replicate per group. Thus, we had four replicates, each one with three animals (*N*=12 per group), organized in one control group (Cont) and three treated groups for each compound, NP and CPA, at the same concentrations used for tadpoles. Except for the volume of water, all the other conditions described for tadpoles were applied for juveniles. Since their life habit is semi-terrestrial, the water volume used here was of 200 mL, so the animals could be in contact with the surface and the water at the same time. The food supply was made the same way as the tadpoles, after the water change and in equal amounts per replicate.

After 28 days of exposure, the juveniles were euthanized in benzocaine (5 g/L), they were weighted in precision analytical balance (± 0.001 g) and followed to dissection to collect the liver and the gonads associated with the kidney. The organs were also weighted and then were fixed in Metacarn (60% methanol, 30% chloroform and 10% acetic acid) for three hours and were included in historesin (Leica).

Morphological and Morphometric Analyses

For the tadpoles we obtained the final body mass (g) in precision analytical balance (± 0.001 g) and the total length (cm) of the tadpoles were compared between the groups to evaluate if the compounds affected their growth rate. We also compared the intial and final mass of the juveniles, as well as the hepatosomatic and the gonadosomatic indexes.

The liver of both life stages and the gonads was sectioned 2 μm thick with a microtome (Leica RM 2255), stained with Hematoxilin-Eosin (H/E) and was analyzed under a light microscope (Leica DM4000 B) associated with an image capture system (Leica DMC 4500). In the liver the H/E staining was used in histological sections to quantify the melanin area in the tissue. These measurements were made based on different staining patterns (SANTOS et al., 2014), in 25 random histological fields per animal to avoid areas with pigment historegionalization, using the software Image Pro Plus (Media Cybernetics Inc v.6.0). The gonads in both life stages were evaluated whether the animals were still undifferentiated, males, females or with intermediate characteristics to both sexes, based on their histological characteristics.

Statistical Analyses

Since the experiments with tadpoles were done in two parts, with two control groups (Cont-NP and Cont-CPA), we calculate the average values between them for each analysis to facilitate the interpretation and the comparisons. All the data were first tested for normality and homogeneity, and then transformed with log when needed to meet the parametric principles. These parametric data, such as body masses, length and visceral-somatic indexes were submitted to one-way Anova variance test, followed by Tukey test. To model the melanin-pigmented area we used a Generalized Linear Model (GLM) with beta distribution and *log* link function with the treatment (categorical predictor with 7 levels) and the stage of the animal (categorical predictor with two levels) and their interaction. We tested model assumptions using diagnostic plots in the R (Team Core, 2017) package *sjplot* (LÜDECKE, 2016). Residuals had homogeneity of variance and normal distribution. To compare the sex ratio between the experimental groups we used G test. This test was implemented using the code provided by Peter Hurd (available at http://www.psych.ualberta.ca/~phurd/cruft/g.test.r).

5.3. Results

At the beginning of the experiments the tadpoles were at Gosner stage 25 and, after 28 days all the animals were still at the same stage. However, we observed alterations in their growth rate, in parameters such as weight and length. The liver pigmentation was not affected and all the gonads were undifferentiated.

The juveniles, on the other hand, were not impacted in body and organs weights, but the liver pigmentation did alter with some treatments. The sex ratio was not affected by the compounds.

5.3.1. Effects on Growth Rate

The exposure of the tadpoles to all concentrations of both compounds, NP and CPA, severely impacted on their growth rate, interfering on final body mass and length of the animals (Figure 9 [1]). Compared to control, the body mass of NP- and CPA-treated groups was lower ($F = 17.03$; $p < 0.01$ for all groups, except NP-C2 that $p = 0.01$). The same pattern was observed for the length of the animals, being smaller in all treated groups ($F = 20.8$; $p < 0.01$ for all comparisons).

For the juveniles, at the beginning of the experiments the animals were weighted to avoid discrepancies and there were no differences between the groups ($F = 0.5$; $p = 0.806$). At the end of the experiments, the animals were weighted again and the body mass of the animals

were not different from control as well (Figure 10 [2]), so the treatments did not interfere in mass gain of the juveniles ($F = 0.228$; $p = 0.966$). Also, there were no differences for hepatosomatic ($F = 0.511$; $p = 0.798$) and gonadosomatic indexes ($F = 1.088$; $p = 0.377$) between the groups (Figure 11 [3]).

5.3.2. Effects on Hepatic Melanin

The liver of the tadpoles were poorly pigmented even in control group, especially when compared to juveniles' liver (Figure 12 [4]). The treatment with NP and CPA did not affect the tadpoles' hepatic melanin area ($p = 1$, for all comparisons). The juveniles, in turn, had an increase in hepatic pigmentation in NP-C2 and CPA-C1 groups ($p < 0.01$ for both groups, compared to control) (Figure 13 [5]).

5.3.3. Effects on the Gonads

After experimental period, the gonads of all tadpoles were in very early developmental stages (gonadal primordium), so it was impossible to make any comparative analysis (Figure 14 [6]). For the juveniles we analyzed the histology of the gonads (Figure 15 [7]) and categorized individuals into male or females. All the groups showed a female-biased sex ratio and the treatments with NP and CPA did not interfered in this final proportion (Table 3 [1]). Also, any sign of masculinization of ovaries or feminization of testis was noted. We observed only one case in NP-C2 group of a male with testicular oocytes, but we did not categorized as intersex or NP-caused effect because it may be a result from semi-differentiated pattern of gonadal differentiation (see discussion).

5.4. Discussion

In this study we observed some general somatic effects of NP and CPA on tadpoles, reflected as lower growth rate of treated animals, while more specific responses such as liver pigmentation were not affected. On the other hand, the juveniles showed the opposite type of response: the general aspects such as body mass and visceral somatic indexes did not alter with treatment, but liver pigmentation increased in some groups. The sex ratio, in turn, was not influenced by the exposures.

The three tested concentrations of NP and CPA caused a decrease in tadpoles' growth rate, both in body mass and in larval length. Studies evaluating the action of estrogenic compounds like NP, bisphenol A and estradiol on *Xenopus laevis* embryonic growth showed

similar results, considering it a malformation and a teratogenic effect (SONE et al., 2004). Exposure of *Rana chensinensis* embryos and tadpoles to Fluoride also decreased body mass and length, which was explained due to lower osmorregulatory abilities and less developed detoxification mechanisms in animals at earlier stages (CHAI et al., 2016). Also, in nature, it has been proved that smaller bodies is associated with increased vulnerability to mortality and decreased fitness (CABRERA-GUZMÁN, 2013).

The liver is involved in detoxification processes and biotransformation of toxic agents (FENOGLIO et al., 2005). The melanomacrophages present in this organ play an important role in these processes, recycling endogenous and exogenous substances (ROBERTS, 1975) and phagocytizing catabolic material (ELLIS; MUNROE; ROBERTS, 1976). In our study we observed that the tadpoles' liver is much less pigmented than juveniles' probably because they are in initial developmental stage. This is an indication that the detoxification mechanism at this early stage would be less effective, as suggested by Chai al (2016), which could, therefore, lead to the lower growth rate observed after exposure to NP and CPA.

There was also an increase in melanin pigmented area of juveniles' liver in NP-C2 and CPA-C1 groups, which is another indicative of better capacity to respond to contaminants exposure that did not occur in tadpoles. The melanin is related to neutralization of components from phagocytosed material (ZUASTI et al., 1989; AGIUS; ROBERTS, 2003), with cytoprotective roles (McGRAW, 2005) and also has antioxidant properties (McGRAW, 2005). It is responsive to several pollutants, which make it a good biomarker for ecotoxicological studies (DE OLVEIRA et al., 2017). The NP already showed to induce oxidative stress in fish (XU et al., 2013; LEE et al., 2018) and rats (AYDOGAN et al., 2008; LI et al., 2017), which could stimulate melanin production. The CPA is understudied, especially in amphibians, but it is known that it causes oxidative DNA damage in rats (DING et al., 2014) with a possible role of reactive oxygen species (ROS) (SIDDIQUE; AFZAL, 2005). Thus, the increase in melanin could be probably a result from oxidative stress caused by the exposure to the contaminants.

A dose-response effect was not observed in our study. However, as regard to EDCs, the quantity of bound receptors is not necessarily related to the concentration of the compound and/or the magnificence of its response; it depends on the properties of the toxicant (WELSHONS et al., 2003; VANDENBERG et al., 2012). The most effective dosage for NP observed in the liver pigmentation was 10 μg/L, while for CPA it was the lowest concentration, 0.025 ng/L. These results show that even low concentrations of NP and CPA

can be cytotoxic to anurans, which cannot predict the effects in higher dosages, and more attention should be taken to environmental pollution by these compounds.

The gonadal development and differentiation in *R. catesbeiana* is still very discussed in the literature. Our results showed that the tadpoles at Gosner stage 25 had very small, completely undifferentiated gonads. On the other hand, there are studies with *R. catesbeiana* tadpoles at the same stage with gonads already differentiated, showing ovaries with diplotene oocytes and testis with secondary spemartogonia (RIZZI et al., 2015). The difference between our tadpoles and Rizzi's is the size; we started our experiments with tadpoles about 1 cm long, while they used animals of approximately 8 cm. It is a big difference in body length, which indicates that their animals are probably older than ours, having more time to grow. Studies already compared tadpoles at the same stage, but with different ages, and the results showed that older animals have more developed ovaries than the younger (CHANG; HSU, 1987). Also, species such as *Boana riojana* already showed that the testicular development is stage-dependent, but the ovarian development is time-dependent (GOLDBERG et al., 2019). Thus, as regarding the gonadal development, it is not necessarily associated to somatic development (as staged by Gosner), being the life time more relevant in some cases (CHANG; HSU, 1987; OGIELSKA; KOTUSZ, 2004).

The pattern of gonadal differentiation of *R. catesbeiana* is also another point of discussion in the literature. There are three patterns: differentiated, undifferentiated and semi-differentiated (GRAMAPUROHIT; SHANBHAG; SAIDAPUR, 2000). In the differentiated type the ovary or testis develops directly. In undifferentiated type the ovary develops directly or the gonad remains undifferentiated for a longer period and then forms a testis. The semi-differentiated type occurs when first the gonad forms an ovary, independent on the genetic sex, and then, if the individual is a male, it transforms into a testis. The study evaluating gonadal development and/or differentiation of *R. catesbeiana* has been done for several years (SWINGLE, 1925; 1926; PUCKETT, 1940; HSÜ; LIANG, 1970; IWASAWA; TAKASAWA, 1974). The first studies (SWINGLE, 1925; 1926; PUCKETT, 1940) considered two races from differentiated and undifferentiated patterns. However, the undifferentiated description of them nowadays is better included as semi-differentiated type. The differentiated and semi-differentiated patterns were also observed in Hsü and Liang (1970) studies, and Iwasawa and Takasawa (1974) studied a semi-differentiated race.

We did not analyze the whole development of the animals, so we could not have conclusive data about the pattern of differentiation. However, based on unpublished data that

showed that pre-metamorphic tadpoles have female-biased sex ratio, and given that in most part of the literature this specie shows semi-differentiated pattern, we believe that the race used in this study should have this type of differentiation as well. Other evidence is the individual found in NP-C2 group with testicular oocytes. Since there was no statistical difference in sex ratio or other morphological abnormalities caused by NP, our hypothesis is that this occurrence of female cells in male gonads should be an occasional abnormal retention from the transition from ovary to testis. These oocytes will probably degenerate with time, turning into atretic cells and then being reabsorbed (which were already observed).

The exposure to NP and CPA did not interfere in sex ratio or in normal gonadal morphology of juveniles at any concentration tested. Although the gonad is already differentiated, adult anurans with mature gonads exposed to pollutants can show morphological alterations such as increased intersexuality and/or hermaphroditism (REEDER et al, 2005; McCOY et al; 2008; HAYES et al., 2011; MORESCO; MARGARIDO; DE OLIVEIRA, 2014) and other impairments like reduced spermatozoa (GREGORIO et al., 2016). However, the susceptibility to toxicants may vary depending on the species' sensibility (BRIDGES; SEMLITSCH, 2000). These results suggest that in *R. catesbeiana* sexual differentiation is relatively resistant to the compounds, probably due to its late differentiation. However, other species could be more susceptible to NP and CPA exposures, leading to reproduction impairment.

5.5. Conclusion

In this study we concluded that recently hatched *R. catesbeiana* tadpoles respond with somatic generalized effects after long-term exposition to NP and CPA, showing reduced growth rate (body mass and length) in all treatments. The juveniles, in turn, showed more specific responses in the liver, increasing melanin area as a cytoprotective action against contamination by NP (at 10 μg/L) and CPA (at 0.025 ng/L). The hepatic function of detoxification may be one factor contributing to protection of the gonads, which showed no alterations in juveniles after the treatments. However, it is important to note that the NP and CPA dosages used here were very low and still were capable to induce responses in a resistant species such as *R. catesbeiana*. We highly encourage further studies to be done with these contaminants, especially on more sensitive species, to assess the real potential of these EDCs as a threat to amphibians.

5.6. Acknowledgments

We would like to thank the entire Anatomy Lab team for their technical support during the experiments.

5.7. Funding Sources

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) – Financing Code 001 and LSG doctoral fellowship, a research grant to CO provided by São Paulo Research Foundation, FAPESP (#2013/02067-5, #2015/12006-9 and #2018/01078-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (#305081/2015-2). LFB received post-doctoral fellowship from FAPESP (Process 2014/00946-4).

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5.9. Figures and Tables

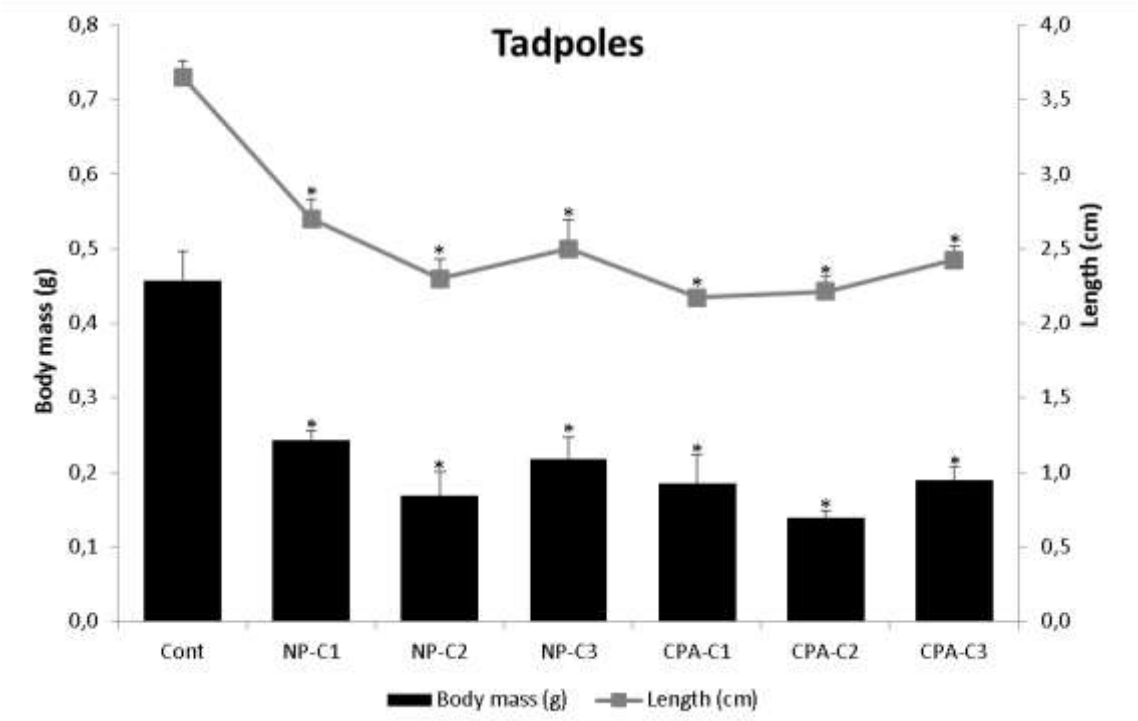

Figure 9 [1]: Mean body mass (g) and length (cm) of the tadpoles after 28 days of exposure for control (Cont) and NP- and CPA-treated groups. The asterisk (*) means statistical difference to control group. Mean ± SE.

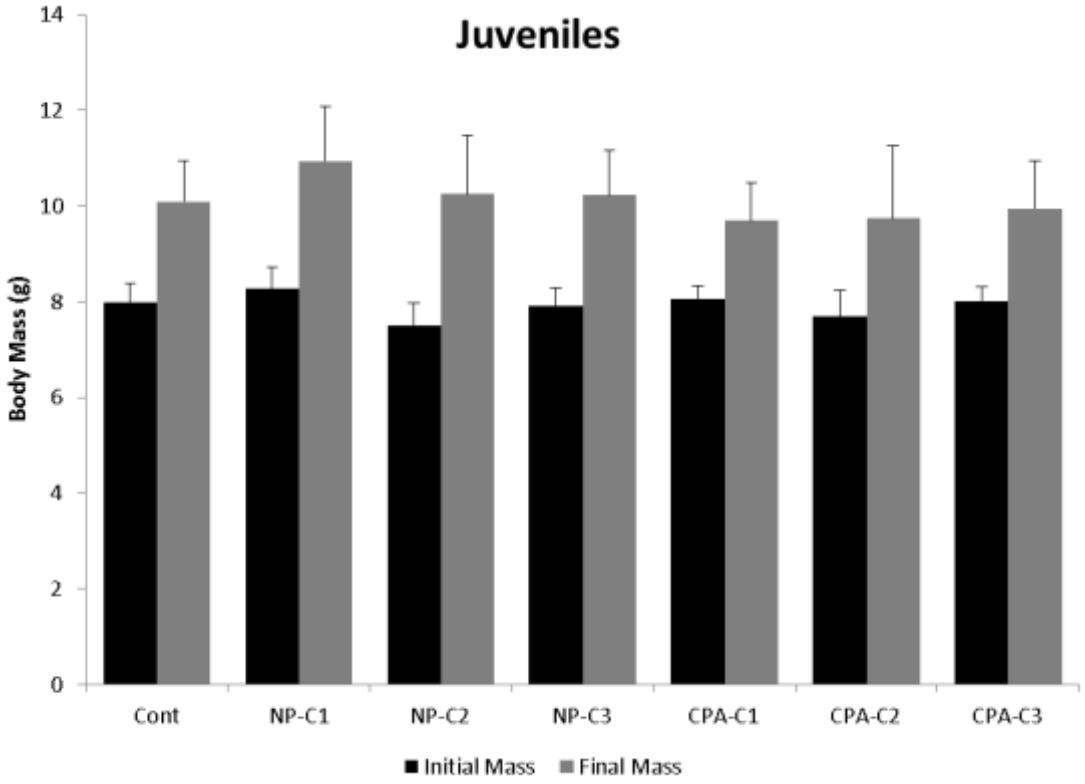

Figure 10 [2]: Mean body mass (g) of the juveniles at the beginning (Initial Mass) and the end (Final Mass) of the experiments for control (Cont) and NP- and CPA-treated groups. No statistical differences were observed between the groups at both times-points. Mean ± SE.

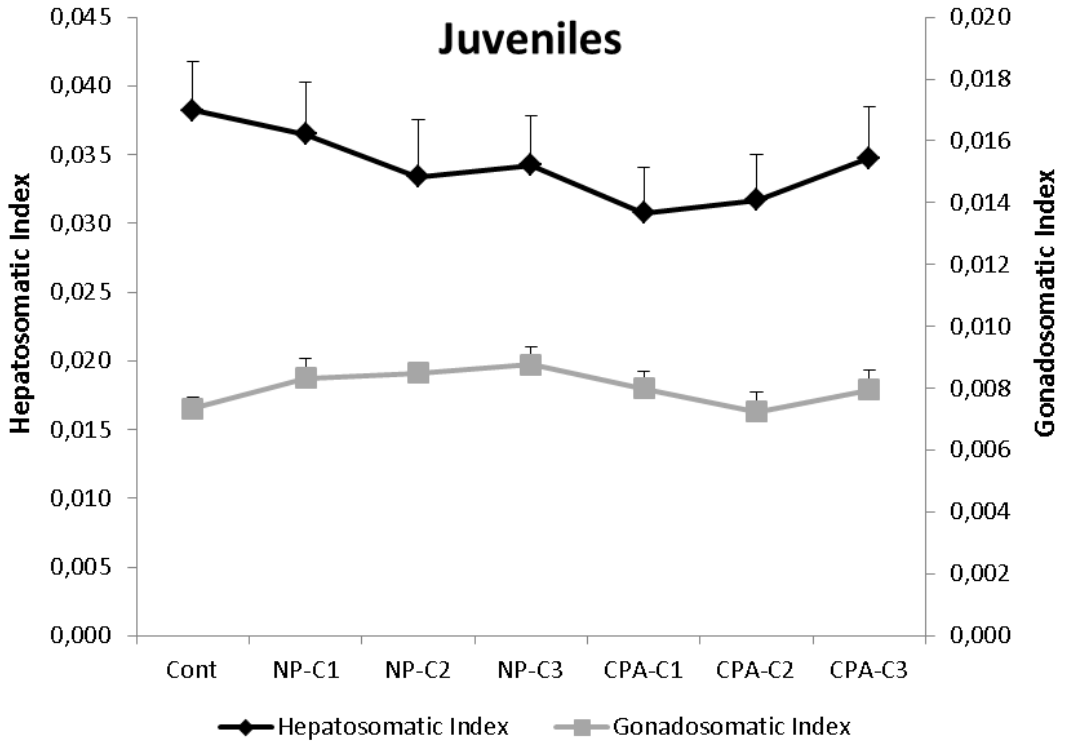

Figure 11 [3]: Hepato- and gonadosomatic indexes calculated for the juveniles of control (Cont) and NP- and CPA-treated groups. No statistical differences were observed between the groups. Mean ± SE.

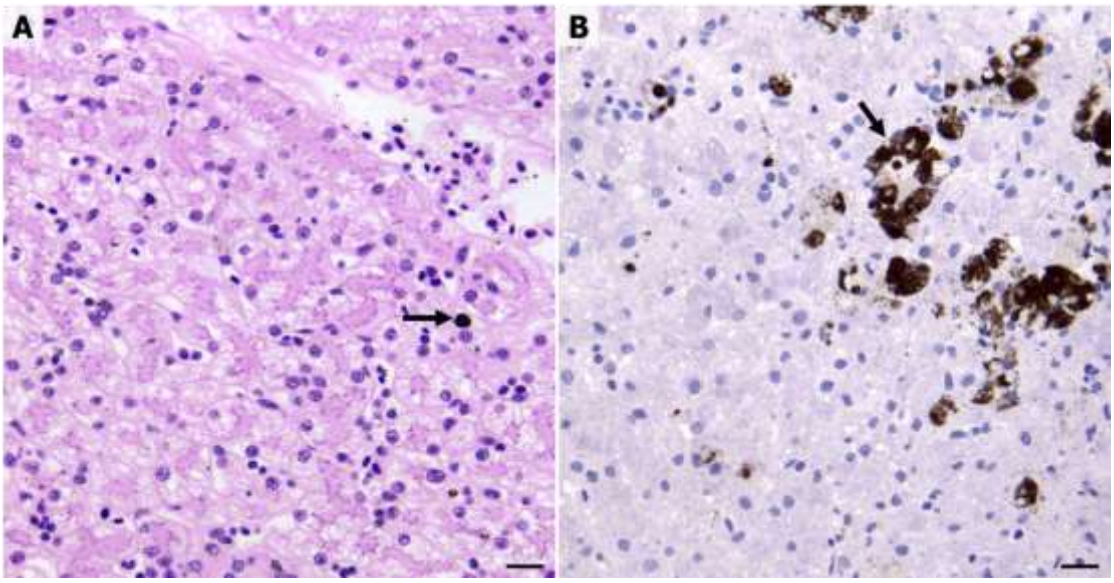

Figure 12 [4]: Liver histological section of a tadpole (**A**) and a juvenile (**B**) *R. catesbeiana*, both from control group, evidencing the difference in melanomacrophages abundance (arrow) between them. While in tadpoles these cells are almost absent, in juveniles they are much more common. Staining: H/E. Scale bars: 25 μm.

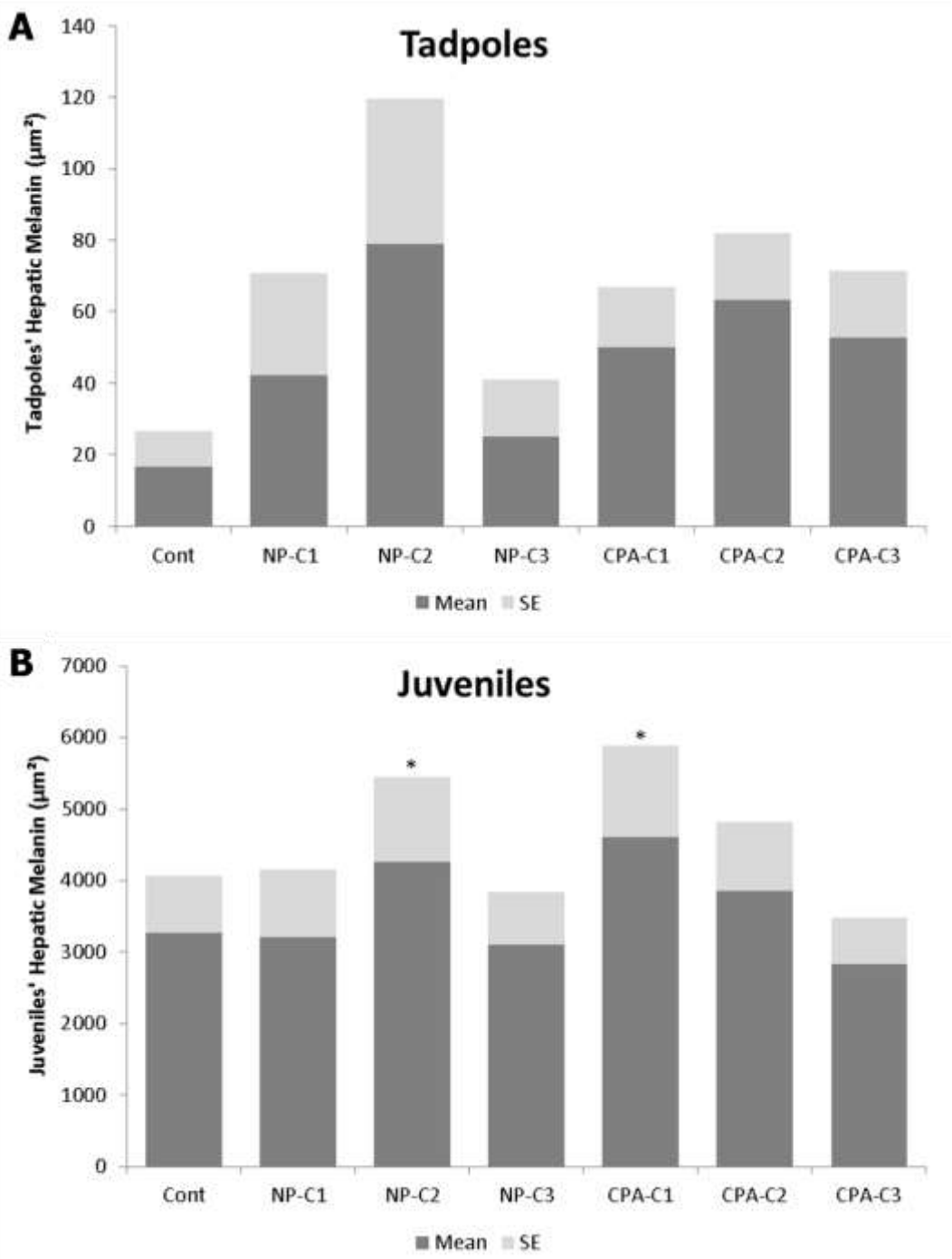

Figure 13 [5]: Mean melanin pigmented area in tadpoles' (**A**) and juveniles' (**B**) liver (µm²) for control (Cont) and the three NP- and CPA-treated groups. Statistical differences were only observed for juveniles (NP-C2 and CPA-C1), indicated by asterisk (*). Mean ± SE.

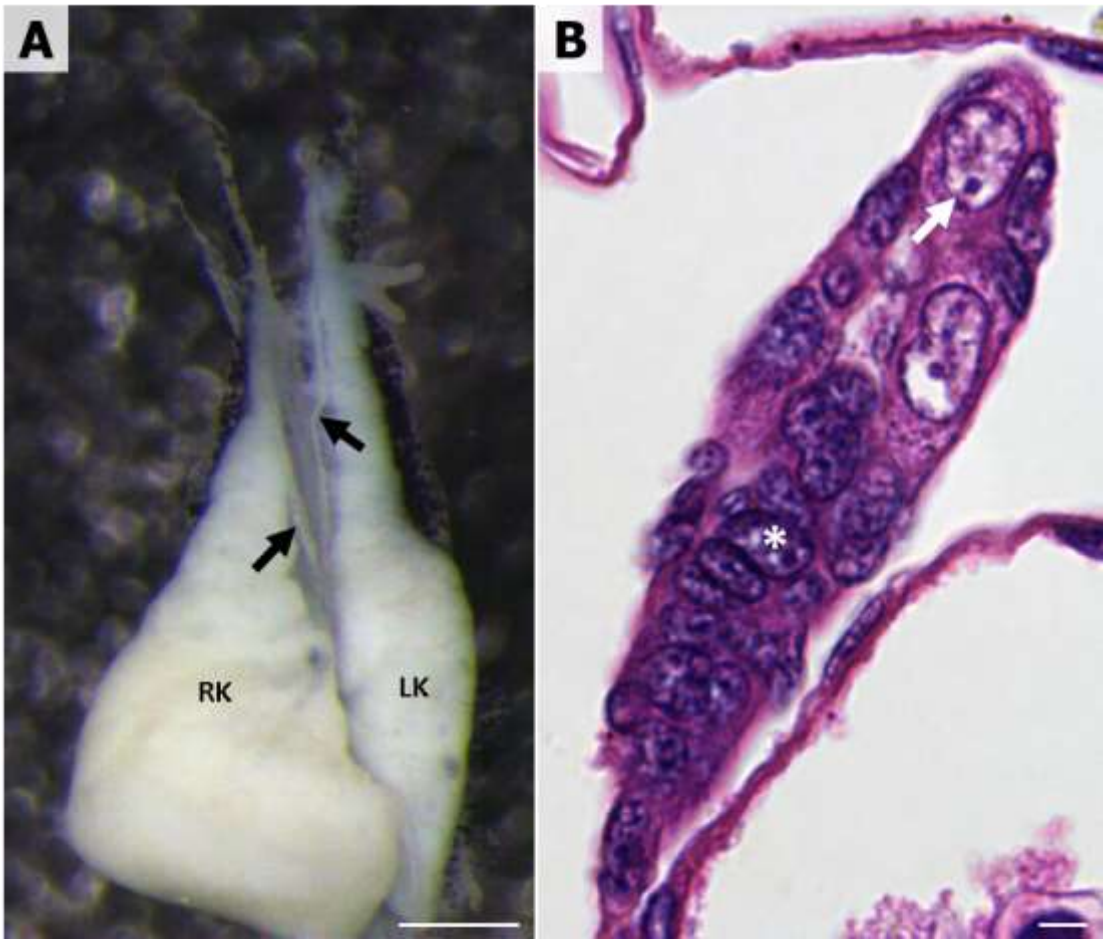

Figure 14 [6]: Gonadal primordium of *R. catesbeiana* tadpoles (Gosner stage 25). **A.** Dissected right (RK) and left kidneys (LK) with the undifferentiated gonads (black arrows) associated with them. **B.** Transversal histological section of the gonadal primordium, showing the primordial germ cells (white arrow) and the somatic cells (*). Scale bars: 0,5 mm (A) and 5 μm (B). Staining: H/E (B).

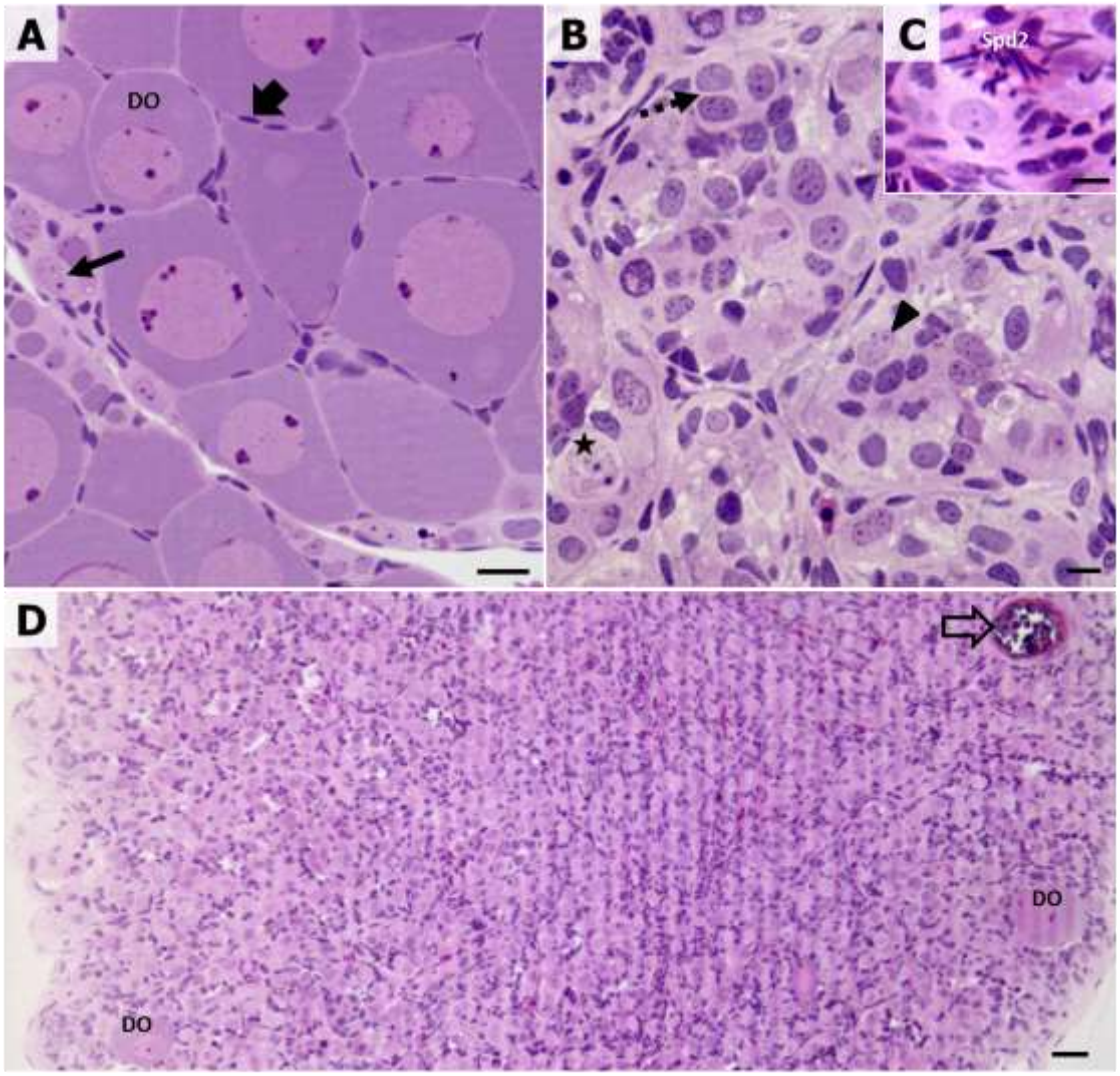

Figure 15 [7]: *R. catesbeiana* juveniles' gonadal histomorphology. **A.** Typical ovary, composed mainly by diplotene oocytes (DO) surrounded by follicular cells (large arrow), but some oogonia (thin arrow) can be found occasionally in the peripheral region. All the ovaries observed were in primary development. **B.** Typical testis, most of them showing only primary (★) and secondary spermatogonia (arrowhead) and primary spermatocytes (dotted arrow). **C.** Secondary spermatids (Spd2), rarely found in the testis of a few animals. **D.** Testis with retention of some diplotene oocytes (DO), including one atretic oocyte being reabsorbed (leaked arrow). Scale bars: 25 μm (A), 10 μm (B and C) and 40 μm (D). Staining: H/E.

Table 3 [1]: Sex categorization of *R. catesbeiana* juveniles from control and NP- and CPA-treated groups, indicating the number of females and males found in each group (*N*=12). The G and p values indicate the statistical comparison (G test) between each treated group to control. No significant difference was observed in any group.

	Control	**NP-C1**	**NP-C2**	**NP-C3**	**CPA-C1**	**CPA-C2**	**CPA-C3**
Female	10	10	10	9	11	7	9
Male	2	2	2	3	1	5	3
G	-	0	0	0.25	0.39	1.86	0.25
p	-	1	1	0.61	0.53	0.17	0.61

6. CAPÍTULO 3: Manuscript in preparation to be submitted to *Environmental Research*.

Leukocyte responses to Nonylphenol and Cyproterone Acetate in *Rana catesbeiana* (Anura) tadpoles and juveniles

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Abstract

The amphibians are worldwide threatened with extinction and one of the global causes is environmental pollution. Several toxicants can interfere in immune system of these animals making them more susceptible to diseases, which is another factor favorable to their population decline. The contaminants 4-nonylphenol (NP), a detergent with estrogenic properties, and Cyproterone Acetate (CPA), an antiandrogenic medicine, are known to impair immune system of some vertebrates, but in anurans it is understudied. In this study we aimed to evaluate the effects of both NP (at 1, 10 and 100 μg/L) and CPA (at 0.025, 0.25 and 2.5 ng/L) in leukocytes population of peripheral blood from tadpoles and juveniles *Rana catesbeiana*. We analyzed lymphocytes, neutrophils, basophils, eosinophils, monocytes and thrombocytes in a proportional count and compared between the groups. The experiments lasted 28 days and the experimental conditions were exactly the same for both stages (varied only the water volume). The tadpoles showed decreased lymphocytes and increased neutrophils at the lowest dose of NP; and increased eosinophils at the two highest doses of CPA. It indicates that the NP is suppressing the adaptive response of lymphocytes and stimulating the innate general response of neutrophils, while the CPA induce the action of the granulocytes eosinophils, which are involved with response against toxic effects. The juveniles showed more expressive responses, reducing neutrophils in all treatments with both compounds and also increasing thrombocytes in all treated groups, except at the highest dose of CPA. Also, CPA at all dosages increased basophils. The neutropenia is possibly a toxic effect of the compounds, which are compromising the innate responses of these animals. The thrombocytosis indicates that vascular impairments should be occurring, since these cells are involved in coagulation and avoiding hemorrhage processes. The increase in basophils by CPA shows that it is even more aggressive than NP, inducing the response of this granulocyte, which has similar function to eosinophils, acting against toxicants and hypersensitivity reactions. Thus, our study showed that NP and CPA can compromise the immune cells from tadpoles and juveniles *R. catesbeiana*, which could make them more susceptible to diseases and contribute to their decline.

Key words: Immune System; Innate Responses; Adaptive Responses; EDCs;

6.1. Introduction

The amphibians' population decline has been discussed since 1989, when this theme was addressed at the First World Congress of Herpetology (STUART et al., 2004). There are several factors contributing to this decrease, such as exotic species introduction, infectious diseases (especially chytridiomycosis), environmental alterations like habitat fragmentation, pollution and global warming (WAKE; VREDENBURG, 2008; HAYES et al., 2010). The amphibians have several characteristics that make them susceptible to these alterations, such as complex life cycle, ecothermia and high permeability, which make them good bioindicators to environmental change (DUNSON; WYMAN; CORBETT, 1992).

Pollution is one of the five ultimate causes of amphibians' decline, which are probably the most influential factors on a global scale (HAYES et al., 2010). Also, the concern about contamination by endocrine disrupting chemicals (EDCs) is increasing recently due to its ability to act in very low doses (BILA; DEZOTTI, 2007). There are several studies showing that EDCs pollutants can decrease immunological responses (LINZEY et al., 2003; CHRISTIN et al., 2004; FORSON; STORFER, 2006a; FORSON; STORFER, 2006b), making them more susceptible to diseases, which is another ultimate cause contributing to amphibian decline (HAYES et al., 2010).

The leukocytes are involved in innate and adaptive immune responses (JUNQUEIRA; CARNEIRO, 2004) and, in amphibians, their morphology and function are similar to other vertebrates (ALLENDER; FRY, 2008). The lymphocytes are the most common cells in amphibians' peripheral blood and, together with monocytes, constitute the agranulocytes cells, representing 80% of the leukocytes count (ALLENDER; FRY, 2008; ARIKAN; ÇIÇEK, 2014). The other white blood cells are eosinophils, basophils and neutrophils (or heterophils), which are called granulocytes, and we can also found thrombocytes in amphibians' blood, that are cells with functional similarities to the mammals' platelets (ALLENDER; FRY, 2008; ARIKAN; ÇIÇEK, 2014).

Exposure to pesticides mixtures containing the EDC atrazine already showed to impair the proliferation of lymphocytes and splenocytes in *Rana pipiens* (CHRISTIN et al., 2004). Atrazine, as well as 4-Nonylphenol (NP) and Bisphenol A (BPA), are also immunotoxic to fishes (YIN et al., 2007; SHELLEY et al., 2012). NP is an estrogenic EDC used mainly in detergent production, found in water bodies from undetectable levels to 644 μg/L (VAZQUEZ-DUHALT et al., 2005). This compound already showed that affect immunological parameters in fish (HÉBERT et al., 2009; SHELLEY et al., 2012; XU et al.,

2013), birds (RAZIA et al., 2006) and mammals (SAKAZAKI; UENO; NAKAMURO, 2002; XIA et al., 2013), but in anurans it is poorly studied. The antiandrogen medicine Cyproterone Acetate (CPA) also showed to be immunotoxic to mammals (ABOUDKHIL et al., 1991), but studies with amphibians immunology were not found. The environmental levels of CPA was only predicted with calculations (GREEN et al., 2005), but some of the most sold contraceptives have this component in the formula.

Thus, in this study we aimed to evaluate the effects of NP and CPA on the leukocytes count of peripheral blood of *Rana catesbeiana* individuals in two stages, tadpoles (Gosner stage 25) and juveniles (10-days post metamorphosis) after chronic exposure of 28 days. There are no studies evaluating immunological aspects in anurans after exposure to these compounds, but in other vertebrates they induced responses. Therefore, we expect some alterations in leukocytes count, probably suppressing lymphocytes, which are part of adaptive immune system, and stimulating the cells that participate in the innate response.

6.2. Metodology

Animals

We used 72 tadpoles of *R. catesbeiana* at stage 25 (Gosner, 1960), obtained from Centro de Aquicultura of Unesp – Jaboticabal (CAUNESP). The experiments with tadpoles were made in two parts, each one with 36 animals, but we randomly chose 20 from each for the blood analyses. We also obtained from CAUNESP 84 juveniles of the same species, 10 days after metamorphosis and 7.92 g mean body mass. The experimentations were approved by Ethics Committee on the Use of Animas (CEUA) from Instituto de Biociências, Letras e Ciências Exatas (Ibilce – Unesp), protocol number 128/2015.

Experimental Design – Tadpoles

Due to the volume of water that was needed, the experiments with tadpoles were separated in two parts: first the animals were exposed to NP (4-Nonylphenol, Ref: 46405, Sigma-Aldrich), then to CPA (Cyproterone Acetate, Ref: C989100, TRC Canada). The acclimation period before the experiments lasted seven days and all the experimental conditions were exactly the same for both experiments (described below).

The 36 tadpoles were organized in four groups for each experiment: one control group (Cont-NP or Cont-CPA) and three different concentrations for NP and CPA. For NP we used environmental concentrations, considered of low, medium and high contamination

(VAZQUEZ-DUHALT et al., 2005). The chosen dosages were 1 μg/L (NP-C1), 10 μg/L (NP-C2) and 100 μg/L (NP-C3), respectively. The CPA concentration was based on predictions made by Green et al. (2015) and pilot tests, so we used the dosages of 0.025 ng/L (CPA-C1), 0.25 ng/L (CPA-C2) and 2.5 ng/L (CPA-C3).

Each experimental group was composed of three replicates, which consisted of a plastic container (capacity: 5 L) containing three tadpoles in each and 3 L of dechlorinated water (1 L/animal), totalizing a sample size of 9 animals per group. We maintained the water proportion of 1 L/animal even when some animal died to avoid any interference. The period of experimentation was 28 days, we changed water each 48h, same interval that food was supplied (CAUNESP developed a protein-based formula specific to *R. catesbeiana*). In these experiments we used mineral water (Hidroleve ®), which was tested for physicochemical and bacteriological properties. The room temperature and photoperiod was measured during the experiments and it was a mean of 26ºC and 14/10h light/dark.

The euthanasia of the animals was made in benzocaine (5 g/L), and 20 animals were randomly selected from each experiment (N=5) for the blood analysis. To collect the blood we had to cut off the tadpoles' head with a razor, because they were still too small at the end of the experiment to collect blood by puncture. We put a drop of blood onto glass slides and immediately make smears to avoid clotting.

Experimental Design – Juveniles

The procedures made with juveniles were exactly the same as described for tadpoles, but we added one replicate per group (N=12). The only difference in experimental design is regarding the water volume, due to the life habit of the post-metamorphic animals that is semi-terrestrial. Therefore, we used 200 mL of water in each container, so the animals could be in contact with both the surface and the water body. After the experimental period (also 28 days), the blood collect and euthanasia was different from tadpoles. We used xylocaine ® (lidocaine) to anesthetize the inner thigh region, then we removed the excess of anesthetic with cotton and collect the blood from the femoral vein with heparinized syringes and needles. We dropped the blood immediately in glass slides to make smears and the animals followed to euthanasia in benzocaine (5 g/L).

Leukocytes Count

The blood slides were dried for 20 minutes, fixed in methanol at 4ºC and stained with Rapid Panoptic (according to manufacture's instructions). We analyzed the white blood cells using a light microscope (Leica DM 4000 B) under 100x magnification. We counted 100 leukocytes and, after that, we calculate the proportion of each kind: lymphocytes, neutrophils, eosinophils, basophils, monocytes and thrombocytes.

Statistical Analysis

We model the ratio of leukocytes counts using a Generalized Linear Model (GLM) with distribution of binomial type and *log* link function including the "Treatment" (categorical predictor, 7 levels) and the "Stage" of the animal (categorical predictor, two levels) and their interaction. We tested model assumptions using diagnostic plots in the R (Team Core, 2017) package *sjplot* (LÜDECKE, 2016). Residuals had homogeneity of variance and normal distribution.

6.3. Results

The morphological aspects of the cells, as expected, were similar to other vertebrates (Figure 16 [1]). However, there were differences between the leukocytes proportion from tadpoles and juveniles control groups, indicating that they would probably respond differently to the exposures (Figure 17 [2]). The lymphocytes were the unique cells that did not vary between the stages ($p = 0.16$). The thrombocytes were higher in juveniles ($p = 0.04$), but in both stages the frequency was very low. All granulocytes cells and the monocytes were higher in tadpoles than in juveniles (neutrophils: $p = 0.04$; basophils, eosinophils and monocytes: $p < 0.01$).

Both compounds tested in this study, NP and CPA, induced alterations in leukocytes count of *R. catesbeiana* tadpoles and juveniles. The agranulocytes cells were less affected (Figure 18 [3]) than the granulocytes (Figure 19 [4]), especially by CPA. Also, thrombocytes were altered by both compounds, only in juveniles (Figure 20 [5]).

In tadpoles, the NP caused a decrease in lymphocytes and an increase in neutrophils at the lowest concentration ($p < 0.01$ for both) The CPA exposure, in turn, increased eosinophils at the two highest concentrations (CPA-C2: $p < 0.01$; CPA-C3: $p = 0.01$). The basophils, monocytes and thrombocytes did not vary with any treatment in tadpoles. Monocytes were the less recurrent cells.

In juveniles the response was more evident than in tadpoles. The NP caused a decrease in neutrophils and an increase in thrombocytes in all concentrations ($p < 0.01$ for all comparisons). The CPA treatment also decreased neutrophils and increased basophils in all concentrations ($p < 0.01$ for all comparisons). The thrombocytes were affected by CPA-C1 and CPA-C2 treatments ($p < 0.01$ for both), decreasing their count. The lymphocytes, eosinophils and monocytes did not show any alteration with treatments in juveniles. As occurred in tadpoles, the less frequent cells were monocytes.

6.4. Discussion

This study showed that tadpoles and juveniles *R. catesbeiana* have different patterns of leukocytes count in peripheral blood, but are both affected by NP and CPA exposures. In the tadpoles the responses occurred only in the lower concentration for NP, but in all dosages for CPA. The juveniles were more responsive, probably due to their better immunocompetence, since they were more developed individuals.

The lymphocytes were the most frequent cell observed in both stages analyzed in this study and did not vary between them. These cells are part of the adaptive immune system, responding to stressors with expression of specific antigens (ROBERT; OHTA, 2009). Therefore, it is reasonable that this is the most common white blood cell. The granulocytes are part of the innate immune system and are related to responses against toxic effects, hypersensitivity ractions and inflammation (JUNQUEIRA; CARNEIRO, 2004; ROMANOVA; EGORIKHINA, 2006). The monocytes are mainly related to bacterial infections and can also act as antigen presenting cells (APCs) (CAMPBELL; ELLIS, 2007; ROBERT; OHTA, 2009). In our study, the proportion of all granulocytes cells and monocytes were higher in tadpoles than in juveniles. In *X. laevis*, the lymphocytes takes 12 days post fertilization to emerge in peripheral blood and, until this time, the innate responses have to be able to protect the tadpoles (ROBERT; OHTA, 2009). These results, togheter with ours, indicate that the immune responses become more specialized with the animal development, increasing the production of cells with more efficient defense mechanisms, such as lymphocytes and, as a compensatory effect, the count of the others leukocytes reduce, as observed in juveniles.

The thromobcytes have similar functions to platelets from mammals, being related to blood coagulation (ALLENDER; FRY, 2008). They usually occur in aggregates due to their function in prevent hemorrhage (JUNQUEIRA; CARNEIRO, 2004). In our study, the mean

percentage of thrombocytes found in tadpoles was 2% while in juvenilies it was 4.42%. Although this difference is statistically significant, this significance is probably an occasional coincidence, since these proportions are both very low and would possibly not exhibit functional responses in any life stages.

Analyzing tadpoles, we observed a decrease in lymphocytes and increase in neutrophils count after exposure to NP at 1 μg/L. This same result was observed in bullfrog tadpoles exposed to UV radiation, probably as a response to inflammatory processes (FRANCO-BELUSSI; FANALI; DE OLIVEIRA, 2018). Also, the NP already showed impairment of T lymphocytes proliferation in *Rana pipiens* (CHRISTIN et al., 2004). Lymphocytes and neutrophils have distinct functions: the first is related to adaptive, specific responses (ROBERT; OHTA, 2009), while the second is related to innate immune system and, when in the target tissue, has phagocytic and cytotoxic functions (JUNQUEIRA; CARNEIRO, 2004; ROBERT; OHTA, 2009). The presence of estrogen receptors (ERα) in lymphocytes B and T was confirmed in mouse, suggesting that estrogenic EDCs could suppress their proliferation through it (SAKAZAKI; UENO; NAKAMURO, 2002). Thus, the decrease of these cells could be possible caused by NP acting via ERα in the tadpoles, while the increase in neutrophils should be a primary mechanism of innate response due to contamination consequences.

The tadpoles' exposure to CPA at 0.25 and 2.5 ng/L showed an increase in eosinophils. These cells are mainly related to hypersensitivity reactions, been recruited to areas with inflammation (JUNQUEIRA; CARNEIRO, 2004). Anurans from polluted areas, compared to other from preserved areas, showed increased counts of eosinophils and basophils, which indicates a try to neutralize the effects of the toxic agents (ROMANOVA; EGORIKHINA, 2006). Thus, the CPA is possibly causing somatic adverse effects in the tadpoles, which triggers this increase in eosinophils count to counteract this action. It could be an adaptive mechanism for living in contaminated environment (FOURNIER et al., 2005).

In the juveniles, both NP and CPA decreased neutrophils proportion in all tested dosages. On the other hand, the thrombocytes increased in almost all the treatments (except CPA-C3) and the basophils increased in all CPA exposures. Neutropenia was already observed in amphibians from polluted or agricultural areas, indicating disorders caused by the toxicants (CABAGNA et al., 2005; ZHELEV; POPGEORGIEV; ANGELOV, 2013). When leucopenia is associated with granulocytosis, it is even more indicative of immune system weakening (CABAGNA et al., 2005; ZHELEV; POPGEORGIEV; ANGELOV, 2013). The

basophils, as the eosinophils, are related to hypersensitivity, and the histamine present in the granules is released in the target tissue to recruit eosinophils and neutrophils to aid in immune responses (JUNQUEIRA; CARNEIRO, 2004). Both alterations in neutrophils and basophils were observed in CPA treatments in juveniles, which indicate high toxicity of this compound, severely compromising the immune system of these animals.

The thrombocytes were affected by NP and CPA in juveniles. Little is known about these cells in anurans, but as stated before, they act in prevention of hemorrhagy and blood coagulation processes (JUNQUEIRA; CARNEIRO, 2004; ALLENDER; FRY, 2008). Thus, the increase observed in thrombocytes after exposure to pollutants indicates that these compounds could be causing vascular impairments, stimulating the production of these cells. This should be further investigated so we can do more assertive statements about the relation between the compounds and the vascular components in anurans.

6.5. Conclusion

Taken together, our results showed that NP and CPA are harmful for both innate and adaptive immune cells from tadpoles and juveniles *R. catesbeiana*. The tadpoles showed milder responses, probably due to their less effective immune system that is still under development, also indicated by the higher proportion of innate immune cells at this stage. The juveniles, in turn, showed an evident neutropenia and thrombocytosis in almost all treatments, indicating that the compounds are immunotoxic and are probably causing vascular disorders. The CPA also increased granulocytes proportion in tadpoles (eosinophils) and juveniles (basophils), which is possibly a mechanism to neutralize the toxic effects of the compound. These impairments in immune system could make the animals more susceptible to infections by pathogens, which could culminate in spreading diseases or even in populations' dissemination.

6.6. Acknowledgments

We would like to thank the entire Anatomy Lab team for their technical support during the experiments.

6.7. Funding Sources

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) – Financing Code 001 and LSG doctoral fellowship, a research

grant to CO provided by São Paulo Research Foundation, FAPESP (#2013/02067-5, #2015/12006-9 and #2018/01078-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (#305081/2015-2). LFB received post-doctoral fellowship from FAPESP (Process 2014/00946-4).

6.8. References

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6.9. Figures and Tables

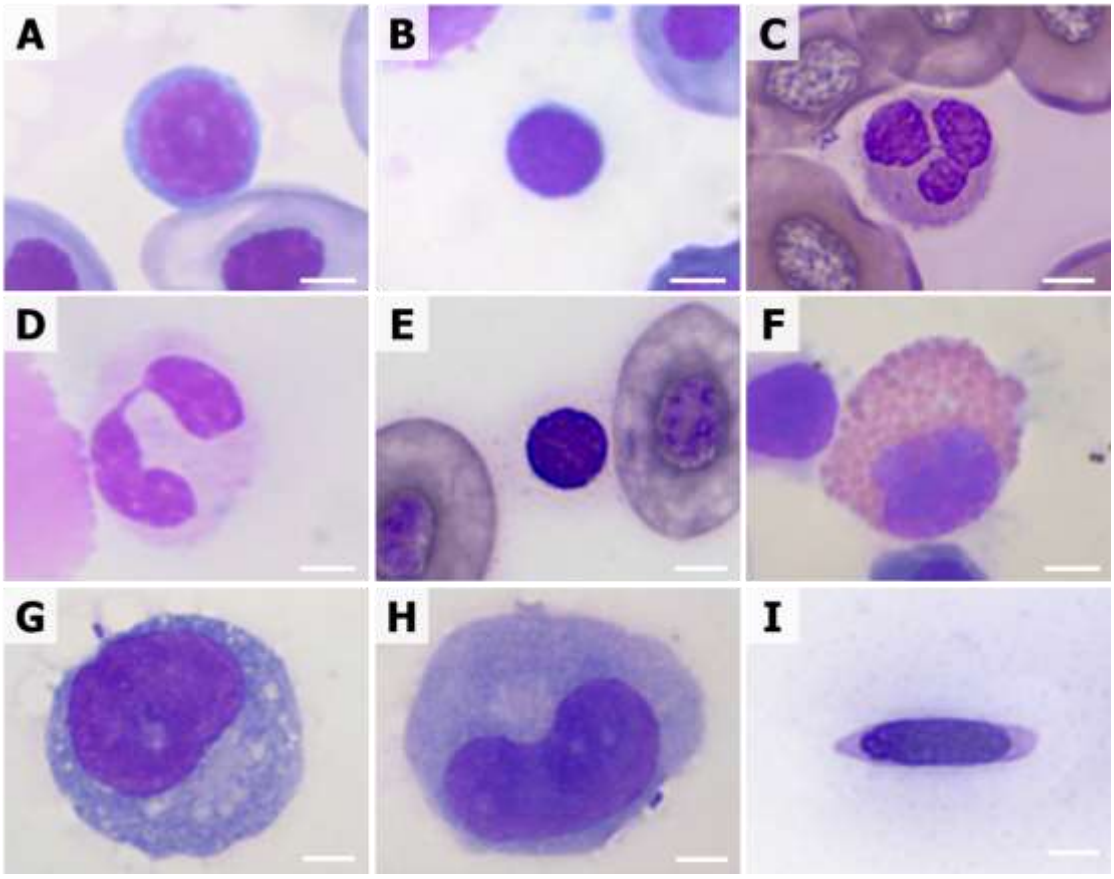
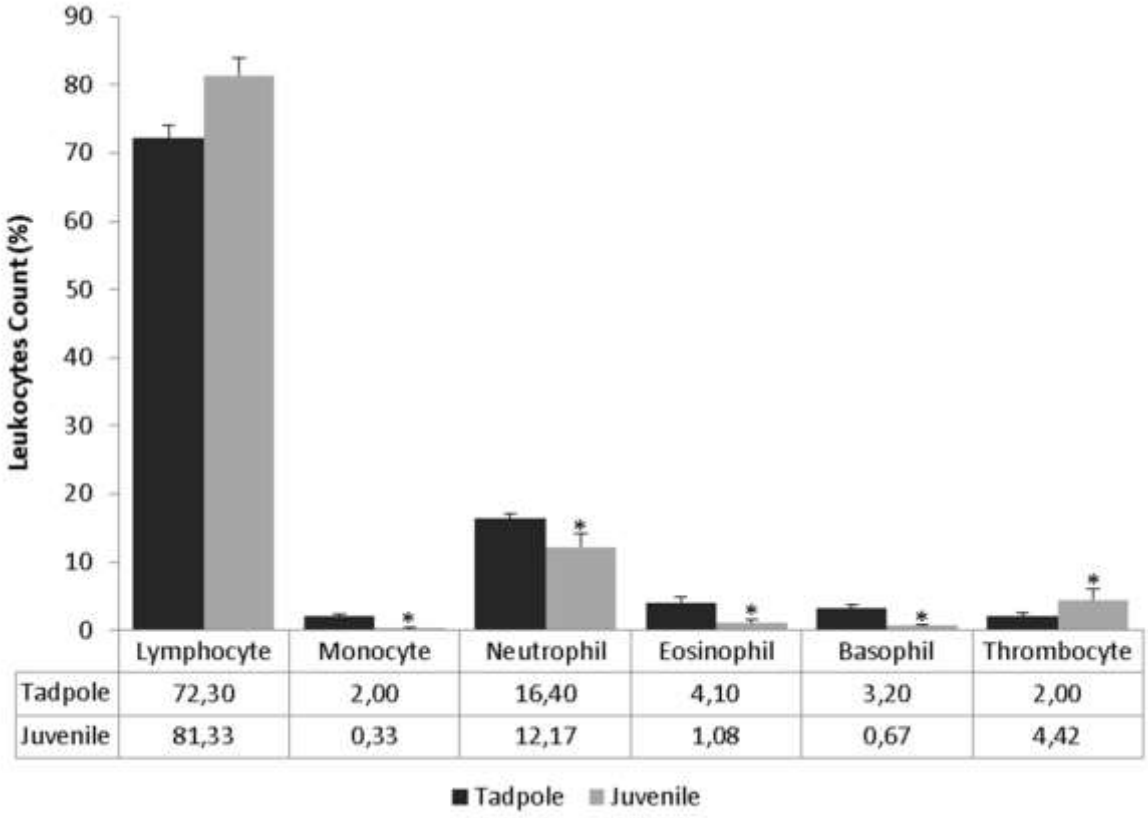


Figure 16 [1]: Leukocytes of tadpoles and juveniles *R. catesbeiana*. **A.** Large lymphocyte, showing little cytoplasm in bluish. **B.** Small lymphocyte, with the nucleus occupying almost the entire area of the cell. **C/D.** Neutrophils with tri-lobed nucleus (C) and bi-lobed nucleus (D). **E.** Basophil, with the nucleus covered by intense basophilic granules. **F.** Eosinophil, showing big eosinophilic granules. **G/H.** Monocyes, evidencing the possible vacuolization of the cytoplasm (G) and the kidney-shape of the nucleus (H). **I.** Thrombocyte, similar to a small lymphocyte but with elongated or fusiform shape. Staining: Rapid Panoptic. Scale bar: 5µm.



	Lymphocyte	Monocyte	Neutrophil	Eosinophil	Basophil	Thrombocyte
Tadpole	72,30	2,00	16,40	4,10	3,20	2,00
Juvenile	81,33	0,33	12,17	1,08	0,67	4,42

Figure 17 [2]: Leukocytes count (%) comparing both life stages, tadpoles and juveniles, in control groups. The asterisk (*) represents statistical difference. Graph: Mean ± SE. Table: Mean.

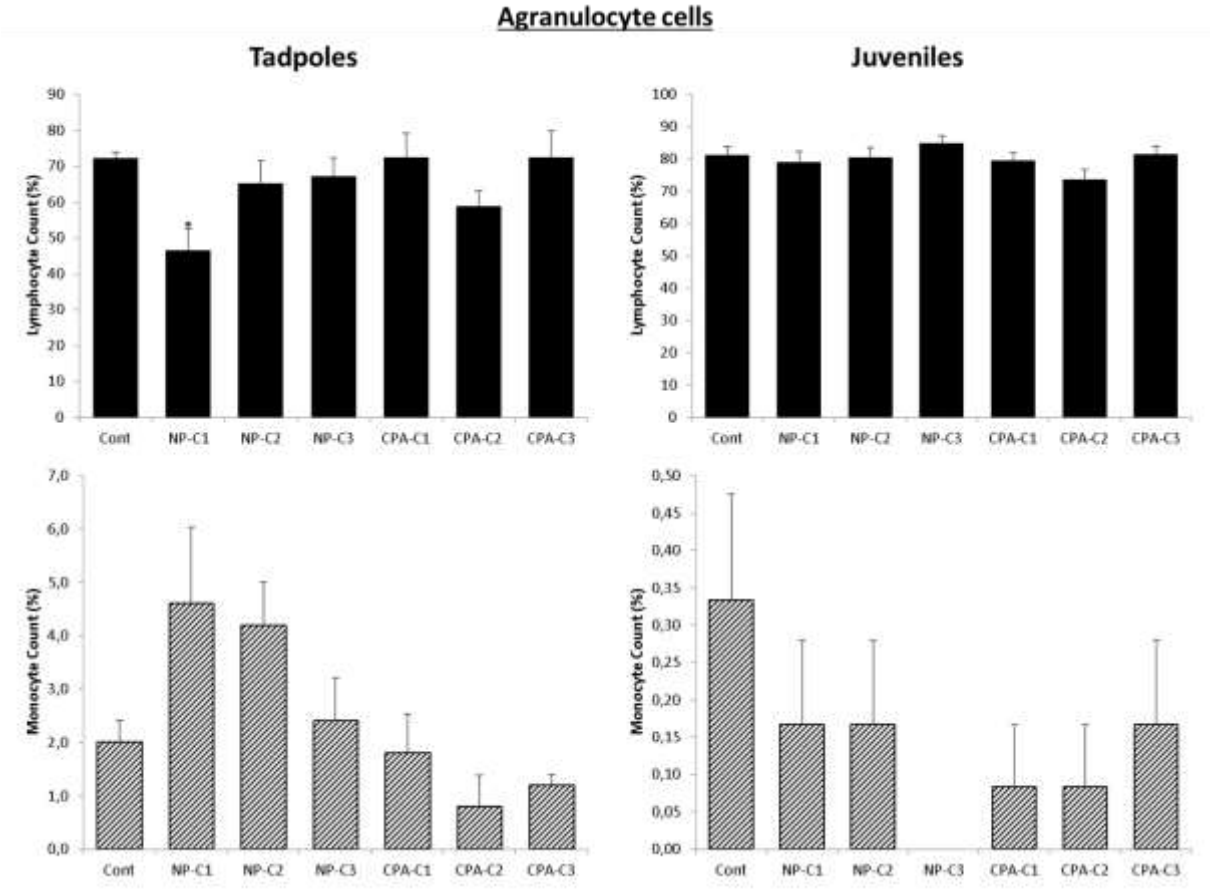


Figure 18 [3]: Tadpoles' (left column) and juveniles' (right column) proportional count (%) of the agranulocytes cell, lymphocytes (top graphs) and monocytes (bottom graphs), for control group (Cont) and each treatment with NP and CPA. The asterisk (*) represents statistical difference. Mean ± SE.

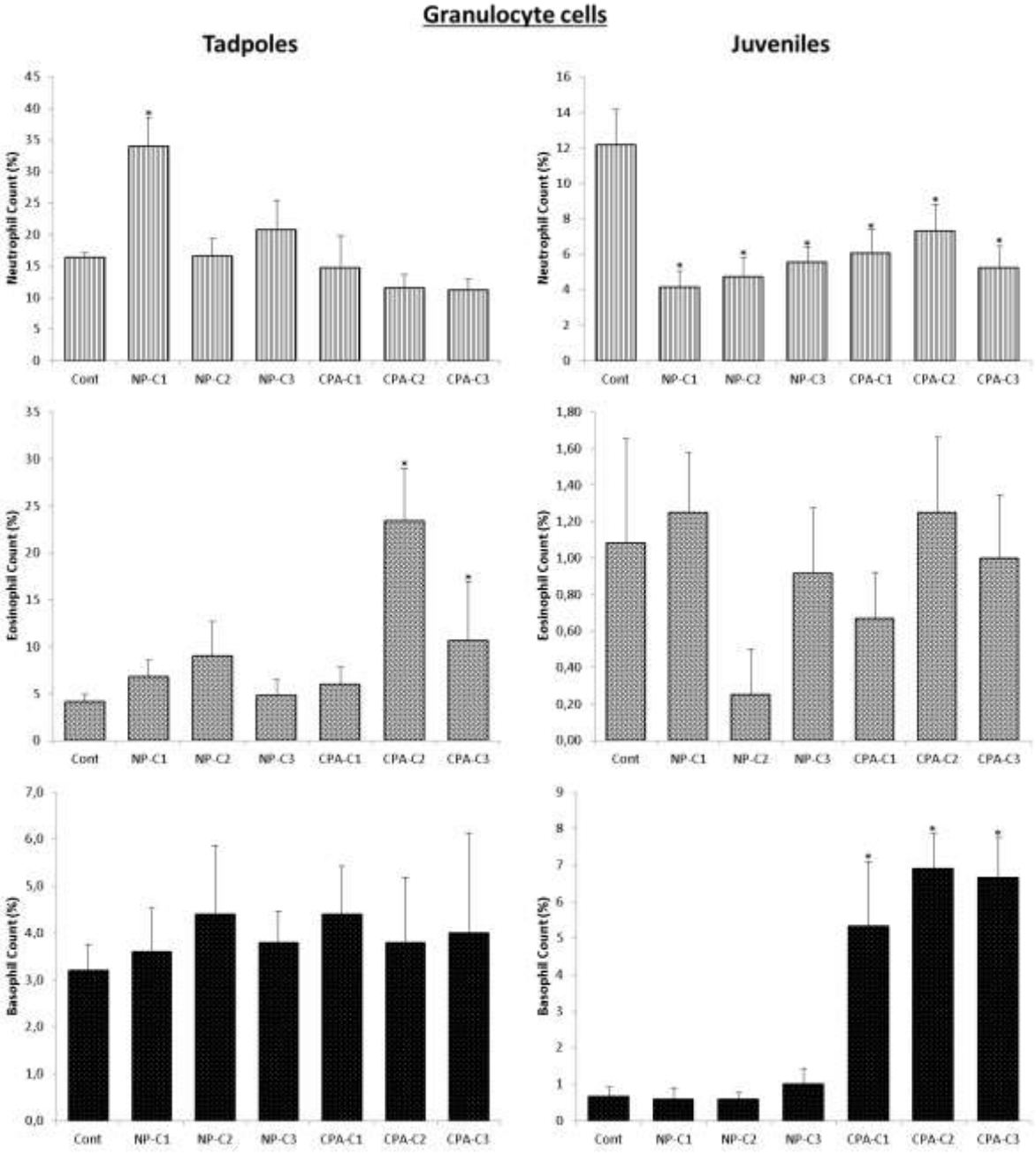


Figure 19 [4]: Tadpoles' (left column) and juveniles' (right column) proportional count (%) of the granulocytes cell, neutrophils (top graphs), eosinophils (middle graphs) and basophils (bottom graphs), for control group (Cont) and each treatment with NP and CPA. The asterisk (*) represents statistical difference. Mean ± SE.

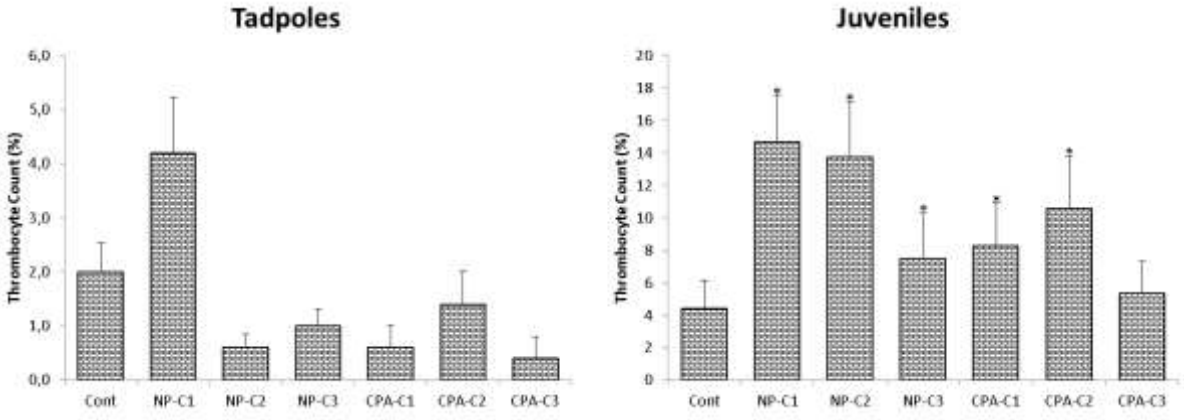


Figure 20 [5]: Tadpoles' (left columns) and juveniles' (right columns) proportional count (%) of thrombocytes for control group (Cont) and each treatment with NP and CPA. The asterisk (*) represents statistical difference. Mean ± SE.

7. CONCLUSÕES GERAIS

Neste trabalho pudemos observar que tanto o NP quanto o CPA apresentaram efeitos tóxicos aos girinos e aos jovens de *R. catesbeiana*. Os efeitos mais evidentes foram os relacionados à defesa do organismo, o que indica uma tentativa de neutralização dos efeitos deletérios. Os animais em estágio iniciais de desenvolvimento se mostraram mais suscetíveis às exposições, visto que tiveram efeitos genotóxicos mais intensos, enquanto as respostas protetivas, como a das células imunológicas e dos melanomacrófagos hepáticos (também relacionados à resposta imune inata) não foram muito eficientes nas respostas contra os agressores. Isso pode estar relacionado a fatores intrínsecos ao desenvolvimento do animal, à espécie ou mesmo ao longo tempo de exposição aos contaminantes e, desta maneira, possíveis respostas imunes iniciais não foram detectadas nesta fase. Os jovens, por sua vez, apesar de apresentarem maior imunocompetência, também sofreram danos genotóxicos e imunológicos, como aumento das anormalidades nucleares nos eritrócitos, aumento da pigmentação, neutropenia e granulocitose, principalmente causados pelo CPA. Já as gônadas não foram afetadas pelos tratamentos, possivelmente devido à eficiência dos mecanismos de defesa do organismo que foram ativados, como foi observado nas outras análises realizadas. Dessa maneira, nós concluímos que ambos os compostos apresentam efeitos deletérios à espécie em questão, *R. catesbeiana*, tanto nos girinos quanto nos jovens, causando principalmente danos citotóxicos mesmo em concentrações baixas, a níveis de μg/L (NP) e ng/L (CPA). Tratando-se de uma espécie modelo bastante resistente, estes compostos demonstram-se potenciais prejudiciais para a anurofauna.

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