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Cassiano Ricardo Schavinski

EFEITOS DA EXPOSIÇÃO ISOLADA E COMBINADA DE LARVAS DE ANFÍBIO *Boana curupi* AO PESTICIDA TRICHLORFON E À RADIAÇÃO ULTRAVIOLETA

Santa Maria, RS
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de Mestre em Bioquímica Toxicológica.

Orientador: Prof.º Dr.º André Passaglia Schuch

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2020

DEDICATÓRIA

À vó Morena e a Renann Toigo Kuhn (In Memoriam).

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“[...] Come forth into the light of things,
Let Nature be your teacher”.

The tables turned
de William Wordsworth.

RESUMO

EFEITOS DA EXPOSIÇÃO ISOLADA E COMBINADA DE LARVAS DE ANFÍBIO *Boana curupi* AO PESTICIDA TRICHLORFON E À RADIAÇÃO ULTRAVIOLETA

AUTOR: Cassiano Ricardo Schavinski
ORIENTADOR: André Passaglia Schuch

O declínio da biodiversidade global afeta principalmente o grupo dos anfíbios e diversos fatores são apontados como catalisadores deste processo. Entretanto, estima-se que este declínio se dá provavelmente devido a interação de múltiplos fatores. Dentre os agentes de ordem química, destacam-se os agrotóxicos. O trichlorfon (TCF), um organofosforado tóxico, é amplamente utilizado em culturas agrícolas, porém seu uso pode afetar a saúde de organismos não-alvo. Outro agente catalisador é a radiação ultravioleta (UVR), um fator exógeno causador de alterações genéticas. Diante deste cenário, analisamos os efeitos estressores gerados pela exposição isolada e combinada ao TCF (0,5 μg/L, dose ambiental; e 50 μg/L, 100-vezes) e à radiação ultravioleta (184,0 kJ/m^2 de UVA e 3,4 kJ/m^2 de UVB, as quais correspondem a 5% da dose diária) em larvas de anfíbio da espécie *Boana curupi* (Anura: Hylidae) por 24 horas. Nos tratamentos combinados com UVR e TCF, nós adotamos três diferentes momentos de irradiação das larvas a partir do início da exposição aguda (0h, 12h e 24h). Nós avaliamos a sobrevivência das larvas, alterações na condição corporal, indução de células apoptóticas, peroxidação lipídica (LPO), proteína carbonil (PC), glutationa S-transferase (GST), tióis não-proteicos (NPSH) e acetilcolinesterase (AChE), além da indução de danos no DNA genômico dos indivíduos. A radiação UVB resultou em alta mortalidade, assim como indução de apoptose. Ambos agentes estressores aumentaram os níveis de LPO, PC e da atividade de AChE, além de diminuir a atividade de GST. A respeito das exposições combinadas, o efeito mais notável foi observado na interação entre UVB e TCF, a qual surpreendentemente aumentou a sobrevivência das larvas e reduziu a indução de danos de DNA. Nossos resultados reforçam a sensibilidade de *B. curupi* diante da radiação ultravioleta, além de demonstrar a complexidade de respostas diante da interação entre a radiação UVB e o TCF, a qual pode estar relacionada a ativação de alguma vida de reparo de DNA.

Palavras-chave: declínio de anfíbios, doses subletais, organofosforados, dano de DNA.

ABSTRACT

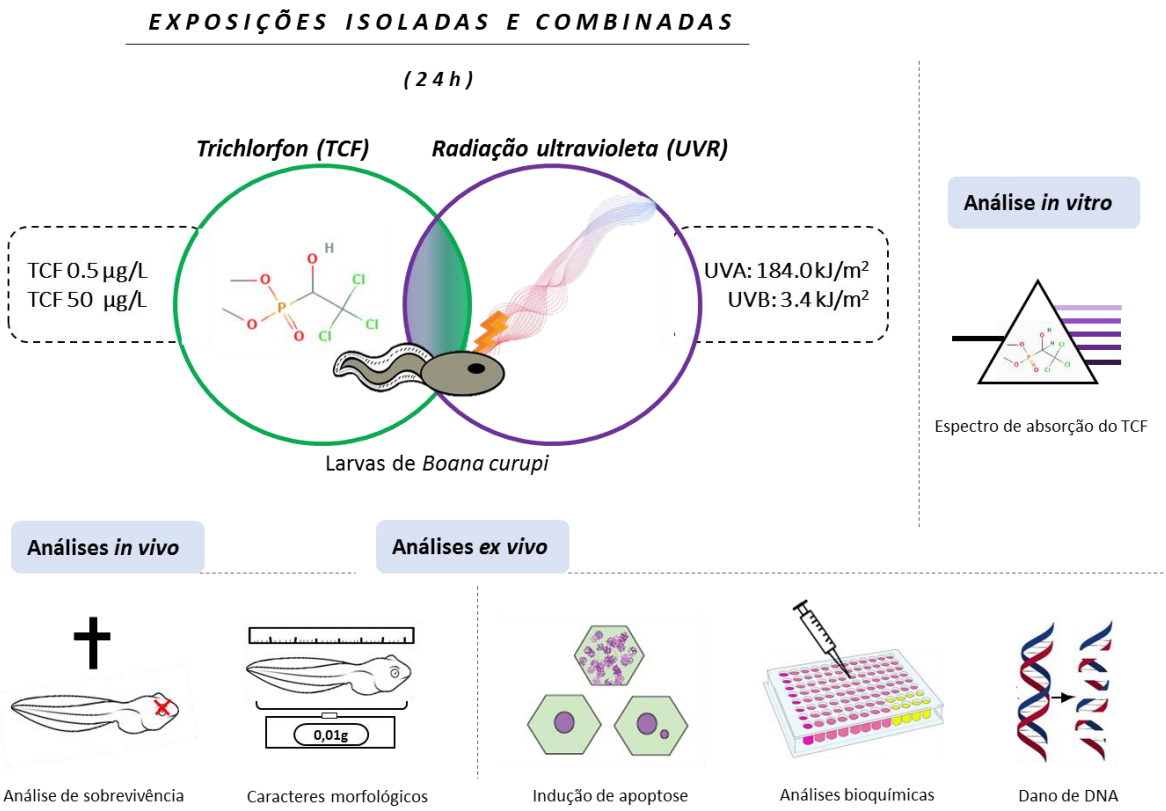
EFFECTS OF ISOLATED AND COMBINED EXPOSURE OF AMPHIBIAN *Boana curupi* LARVAES TO TRICHLORFON PESTICIDE AND ULTRAVIOLET RADIATION

AUTHOR: Cassiano Ricardo Schavinski
ADVISOR: André Passaglia Schuch

The decline in global biodiversity affects mainly the amphibian group and several factors are identified as catalysts of this process. However, it is estimated that this decline is probably due to the interaction of multiple factors. Among chemical agents, pesticides stand out. Trichlorfon (TCF), a toxic organophosphate, is widely used in crops, but its use can affect the health of non-target organisms. Another catalyst agent is ultraviolet radiation (UVR), an exogenous factor that causes genetic alterations. In view of this scenario, we analyzed the stressful effects generated by isolated and combined acute exposures to environmental doses of trichlorfon (TCF) pesticide (0.5 μg/L and 50 μg/L), and ultraviolet radiation (UVR) (184.0 kJ/m² of UVA and 3.4 kJ/m² of UVB, which correspond to 5% of the daily dose) in tadpoles of the species *Boana curupi* (Anura: Hylidae). In the combined treatments, we adopted three different moments of tadpole irradiation from the beginning of the acute exposure to TCF (0h, 12h, and 24h). We evaluated tadpole' survival, change in morphological characters, cell-induced apoptosis, lipid peroxidation (LPO), protein carbonyl (PC), glutathione S-transferase (GST), non-protein thiols and acetylcholinesterase (AChE), as well as the induction of genomic DNA (gDNA) damage. UVB alone results in high mortality, as well as high level of apoptosis induction. Both UVA, UVB and TCF increase LPO, PC, and AChE, and also decrease GST activity. Regarding co-exposure, the most notable effect was observed in the interaction between UVB and TCF, which surprisingly decrease UVB-induced tadpole mortality, apoptosis, and gDNA damage. Despite these results reinforce the UVB-sensitivity of *B. curupi*, it indicates a complex response in face of the interaction with TCF, which may be related to activation of DNA repair pathways and/or inhibition of apoptosis decreasing UVB-induced tadpole mortality.

Keywords: amphibian decline, sublethal doses, organophosphates, DNA damage.

GRAPHICAL ABSTRACT



LISTA DE ABREVIATURAS E SIGLAS

6-4PP	6-4 pirimidina-pirimidona
8-oxoG	7,8-dihidro-8-oxiguanina
AChE	Acetilcolinesterase
ANVISA	Agência Nacional de Vigilância Sanitária
AP-1	Proteína ativadora 1
ATP	Trifosfato de adenosina
Bd	*Batrachochytrium dendrobatidis*
BER	Reparo por excisão de bases
CAT	Catalase
CDNB	1-cloro-2, 4-dinitrobenzeno
ChE	Colinesterase
COX-2	Ciclo-oxigenase-2
CPD	Dímeros de pirimidina ciclobutano
DDT	Diclorodifeniltricloroetano
DDVP	Dichlorvos
DewarPP	Isômero de valência Dewar
DFP	Diisopropilfluorofosfato
DNA	Ácido desoxirribonucleico
DNPH	2,4-dinitrofenilhidrazina
DSB	Quebra de fita dupla
DTNB	5,5'-ditiol-bis- (2-nitrobenzoico)
ERNs	Espécies reativas de nitrogênio
EROs	Espécies reativas de oxigênio
gDNA	DNA genômico
GS-DNB	GS-dinitrobenzeno
GSH	Glutationa
GSSG	Dissulfeto de glutationa
GST	Glutationa S-transferase
HIF	Fatores induzíveis por hipóxia
HR	Recombinação homóloga
IL-6	Interleucina 6
IL-8	Interleucina 8

IUCN	International Union for Conservation of Nature
LPO	Peroxidação lipídica
MAPK	Proteína-quinases ativadas por mitógenos
MDA	Malondialdeído
MN	Micronúcleo
NER	Reparo por excisão de nucleotídeos
NF-κB	Factor nuclear κappa B
NPSH	Tióis não-proteicos
Nrf2	Fator nuclear 2
OP	Organofosforado
PC	Proteína carbonil
PET	Parque Estadual do Turvo
RS	Rio Grande do Sul
SD	Rato Sprague-Dawley
SINDIVEG	Sindicato Nacional da Indústria de Produtos para Defesa Vegetal
SNAP	Sistema Nervoso Autônomo Parasimpático
SNAS	Sistema Nervoso Autônomo Simpático
SNC	Sistema Nervoso Central
SOD	Superóxido dismutase
SSB	Quebras de fita simples
STAT	Transdutores de sinal e ativadores de transcrição
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TCA	Ácido tricloroacético
TCF	Trichlorfon
TCR	Reparo acoplado a transcrição
TF	Fator de transcrição
TNF-α	Fator de necrose tumoral alfa
UC	Unidade de conservação
UVA	Radiação ultravioleta A
UVB	Radiação ultravioleta B
UVR	Radiação ultravioleta

SUMÁRIO

1 INTRODUÇÃO

O equilíbrio das atividades biológicas de um organismo está relacionado com a qualidade do ambiente em que o mesmo vive, sendo este fator uma condição necessária para o estabelecimento e a manutenção da vida (MAGALHÃES, FERRÃO, 2008). A capacidade de sobrevivência, crescimento e reprodução dos seres vivos também está intrinsicamente relacionada a faixa de tolerância a situações de estresse e à capacidade de transpor as possíveis dificuldades provenientes de fatores externos (MAGALHÃES, FERRÃO, 2008; MELSTAD, SHAFFER, 2015). Contudo, nas últimas décadas, muitos ambientes naturais estão sobrecarregados de múltiplos estressores de ordem física, química e biológica oriundos das atividades antrópicas (CHAPIN et al., 2000). Esses estressores, quando não biotransformados, afetam negativamente a biodiversidade taxonômica, funcional e genética, alterando os serviços e as funções do ecossistema (LAURETO et al., 2015). O acúmulo de modificações ambientais pode ser tão profundo a ponto de torna-lo um dos grandes motivos para o declínio global da biodiversidade (MACCRACKEN, 2002). Neste cenário, os anfíbios são apontados como um dos grupos de organismos vivos mais ameaçados, seguido pelas plantas coníferas e pelos recifes de corais (IUCN, 2020). A Lista Vermelha de Espécies Ameaçadas da *International Union for Conservation of Nature* (IUCN), estima que dentre as 6.857 espécies de anfíbios registradas pela instituição, cerca de 41% (33–53%) são categorizadas como vulneráveis, ameaçadas ou criticamente ameaçadas de extinção (IUCN, 2020). Pouco se entende sobre a dinâmica dos diversos elementos envolvidos na aceleração do declínio dos anfíbios, o qual é denominado "enigmático" por muitos pesquisadores (ALTON, FRANKLIN, 2017; MELSTAD, SHAFFER, 2015).

Entretanto, por mais que ainda não exista um consenso sobre a real causa do declínio das espécies de anfíbios, muitos fatores são apontados como catalisadores desse processo. Entre eles, destacam-se a perda, a fragmentação e a modificação dos habitats naturais (MACCRACKEN, 2002; MELSTAD, SHAFFER, 2015; ROCHA et al., 2020), o aumento da incidência da radiação UVB na superfície terrestre (LONDERO et al., 2019; SCHUCH et al., 2015a; SCHUCH et al., 2015b), a mudança climática global (BRUM et al. 2013), as contaminações ambientais decorrentes do uso de fertilizantes químicos, agrotóxicos e outros poluentes (MA et al., 2019; Meter et al., 2019), assim como a introdução de espécies exóticas/invasoras (CHAPIN et al., 2000), além de doenças fúngicas como a causada pelo patógeno *Batrachochytrium dendrobatidis* (*Bd*) (FISHER, GARNER, 2020; ALTON,

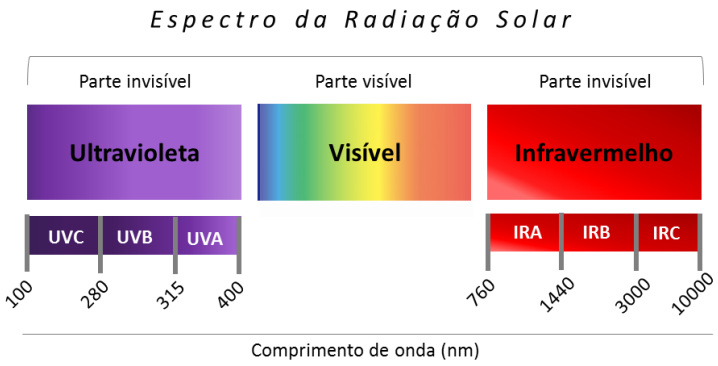
FRANKLIN, 2017). A compreensão da ação destes agentes estressores e as respostas dos anfíbios frente a um quadro de exposição é uma peça chave para adoção de medidas de conservação aos anfíbios e à outros grupos de animais em vulnerabilidade (ALTON, FRANKLIN, 2017; MELSTAD, SHAFFER, 2015).

Cabe ressaltar que com o aumento da influência das atividades antrópicas nos ambientes naturais, os organismos estão cada vez mais expostos a diversos agentes estressores simultaneamente. Isto indica que, provavelmente, o declínio da biodiversidade global ocorra devido a interação de múltiplos fatores (ALTON, FRANKLIN, 2017). Em vista disso, a pesquisa desta dissertação elenca a investigação ecotoxicológica de dois fatores catalisadores do declínio de anfíbios, utilizando como modelo experimental larvas de anfíbios da espécie *Boana curupi*, uma representante da anurofauna especialista de ambiente fechado do estado do Rio Grande do Sul, a qual é utilizada como modelo animal no Laboratório de Fotobiologia (LIPINSKI et al., 2016; LONDERO et al., 2019). O primeiro destes fatores é a radiação ultravioleta, um agente estressor de ordem física, e o segundo é o agrotóxico organofosforado trichlorfon, um agente de estressor de ordem química.

1.1 CONSIDERAÇÕES SOBRE A RADIAÇÃO ULTRAVIOLETA

A radiação ultravioleta (UVR) é um agente físico natural que pertence a uma das séries de ondas do espectro eletromagnético emitido pelo sol. O espectro ultravioleta compreende os comprimentos de onde entre 100 e 400 nanômetros (nm) e é subdividido em três faixas de acordo com os efeitos fotoquímicos nos organismos vivos. São elas: a radiação do tipo C (UVC – 100 a 280 nm), a radiação do tipo B (UVB – 280 a 315 nm) e a radiação do tipo A (UVA – 315 a 400 nm; Figura 1) (LONDERO et al., 2019). Dentre estas faixas, os comprimentos de onda referentes a UVC são completamente absorvidos pela camada de ozônio, a qual se localiza na atmosfera entre aproximadamente 15 e 35 km de altitude em relação a superfície terrestre (VAN DER LEUN, 2004). Quanto as outras faixas emitidas pelo sol, aproximadamente 90-95% e 5-10% dos comprimentos de onda de UVA e UVB, respectivamente, passam pela troposfera e atingem os organismos vivos (SCHUCH et al., 2017).

Figura 1 – Espectro eletromagnético de ondas emitidos pela radiação solar, destacando as subdivisões das faixas correspondentes a radiação ultravioleta, luz visível e faixa do infravermelho.



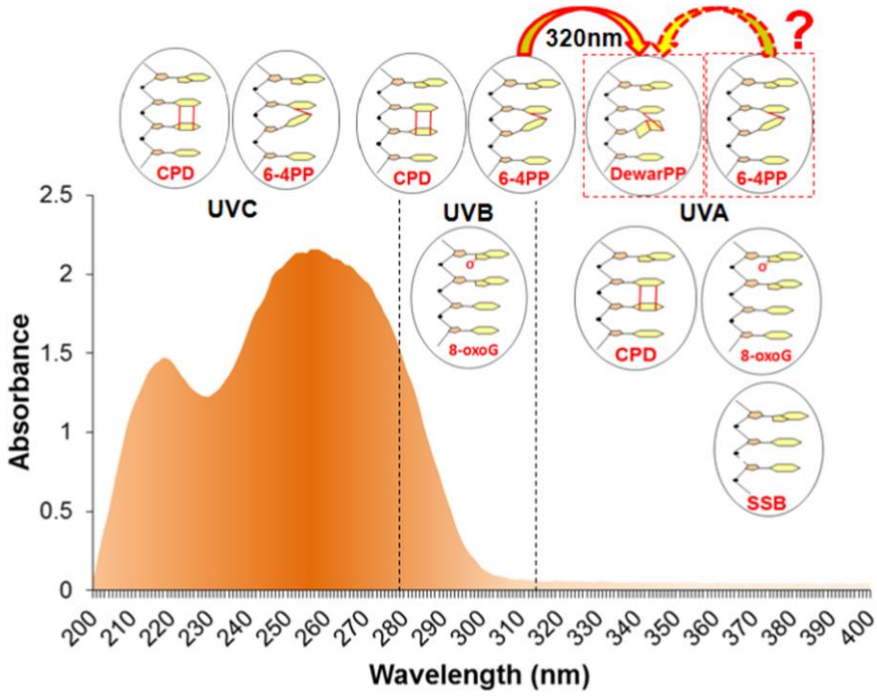
Fonte: O autor.

É tênue o limiar entre os efeitos benéficos e adversos da radiação ultravioleta para a saúde humana e animal. Por um lado, a radiação solar é fundamental para o aporte da vida na Terra, pois esta é a principal fonte de energia utilizada no processo de fotossíntese pelos produtores primários. Além disso, a radiação UVB é a reguladora metabólica para a síntese de vitamina D endógena, importante hormônio esteroide envolvido na fisiologia osteomineral, na modulação da autoimunidade, no controle da pressão arterial e em outros processos de homeostasia celular (CASTRO, 2011). Por outro lado, este agente físico é também considerado um fator exógeno de alterações genéticas devido ao seu potencial genotóxico (BATISTA et al., 2009; SCHUCH et al., 2017). Isso ocorre porque o DNA é o principal alvo da radiação UV devido a estrutura fisicoquímica dos ácidos nucléicos, os quais atuam como cromóforos celulares (BATISTA et al., 2009; VINK, ROZA, 2001). Nesse sentido, as lesões nas moléculas de DNA causadas pela radiação UV põem em risco a estabilidade genômica dos organismos vivos, já que o DNA é o responsável pela manutenção e transmissão da informação genética ao longo das gerações (HOEIJMAKERS, 2001). Sendo assim, embora tanto a radiação UVA quanto a radiação UVB sejam importantes para alguns processos que auxiliam na manutenção da vida, quando absorvida pelas células, podem provocar danos nas estruturas moleculares, causando principalmente lesões no DNA.

Os danos nas biomoléculas celulares provocados pela radiação UV ocorrem por meio de duas vias (BATISTA et al., 2009). A primeira é por meio de absorção direta de fótons de alta energia da radiação UV pelas bases nitrogenadas (BATISTA et al., 2009). As fotolesões geradas nessa absorção decorrem principalmente da dimerização entre pirimidinas adjacentes no DNA, e os fotosprodutos formados são predominantemente dímeros de pirimidina

ciclobutano (CPD) e 6-4 pirimidina-pirimidona (6-4PPs) (SCHUCH et al., 2017). Os CPDs são formados por duas ligações covalentes entre os átomos de carbonos C5 e C6 de bases nitrogenadas vizinhas que, quando excitadas, se reconfiguram formando um anel ciclobutano (VINK, ROZA, 2001). Já os 6-4PPs são formados por uma ligação covalente entre os carbonos C6 e C4 de duas pirimidinas adjacentes (DOUKI, SAGE, 2016). Além disso, a lesão 6-4PP já formada pode dar origem a um terceiro fotoproduto conhecido como isômero de valência Dewar ou fotoproduto Dewar (DewarPP), caso ocorra a absorção de um segundo fóton (DOUKI, SAGE, 2016). A segunda via de dano nas biomoléculas provocadas pela UVR se dá através de uma via indireta. Isso ocorre porque a mudança na distribuição eletrônica nos cromóforos celulares, gerada por um estado de foto excitação, produz energia que pode ser direcionada a um oxigênio molecular ou a outro radical orgânico (BATISTA et al., 2009). Esta transferência de energia pode formar espécies reativas de oxigênio (EROs), espécies reativas de nitrogênio (ERNs) e outros radicais livres que são capazes de atingir e danificar diversas estruturas celulares, assim como biomoléculas que são suscetíveis a oxidação (BATISTA et al., 2009; SCHUCH et al., 2017). A Figura 2 apresenta o espectro da radiação ultravioleta e os principais tipos de lesões de DNA formados em cada faixa de comprimento de onda específico.

Figura 2 – Espectro de absorção da radiação ultravioleta pela molécula de DNA e os principais tipos de lesões de DNA induzidos por cada espectro.



Fonte: SCHUCH et al., 2017.

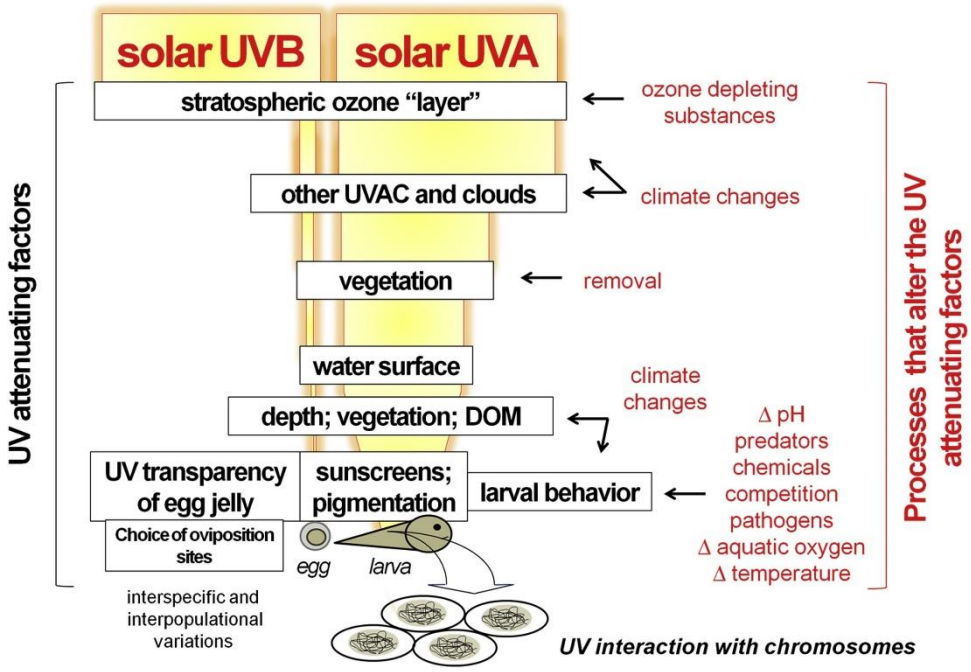
Os radicais livres formados pela ação da radiação ultravioleta atuam no DNA oxidando preferencialmente a base guanina, resultando no fotoproduto 7,8-dihidro-8-oxiguanina (8-oxoG). A interação de EROs com o DNA também pode resultar na formação de quebras de fita simples (SSB) (SCHUCH et al., 2017). Consequentemente, a presença de fotoprodutos no DNA resulta na distorção da dupla-hélice seguida da parada da polimerase diante da lesão (SCHUCH et al., 2017), resultando no bloqueio da transcrição gênica ou de um evento replicativo (BLAUSTEIN et al., 1994). Além do mais, a presença dos fotoprodutos CPD, 6-4PP e 8-oxoG no DNA possuem propriedades mutagênicas, pois podem induzir a transição de bases nitrogenadas nas células filhas devido a erros no pareamento durante a replicação da lesão (SCHUCH et al., 2017). O acúmulo dessas mutações pode ativar proto-oncogenes e inativar genes supressores de tumor, aumentando o risco de surgimento de cânceres (HOEIJMAKERS, 2001). Estas lesões podem ocasionar diversos efeitos biológicos danosos aos organismos vivos, entre eles eritema, queimadura de pele, processo inflamatório, imunossupressão, fotoenvelhecimento, mutagênese, carcinogênese, tumorigênese, morte celular via apoptose, além de outros processos de oxidação e disfunções metabólicas (LONDERO et al., 2019; SCHUCH et al., 2017; VINK, ROZA, 2001). Entretanto, os organismos vivos contam com vias complexas de proteínas que removem os danos do genoma e auxiliam na manutenção da integridade da informação genética.

Diversas vias de reparo de DNA se desenvolveram ao longo da evolução das espécies e estão presentes em organismo procariotos e eucariotos a fim de remover uma diversa gama de lesões (FELTRIN, et al., 2020). Dentre estas vias, o reparo por excisão de nucleotídeos (NER) possui papel fundamental por possuir grande versatilidade para reverter lesões de DNA causadas pela radiação UV, mutagênicos ambientais, drogas quimioterápicas, além de lesões resultantes de processos endógenos, como por exemplo as oriundas das EROs (FELTRIN et al., 2020). Assim como o NER, o reparo por excisão de bases (BER), a recombinação homóloga (HR), e o sistema de reparo *mismatch* são alguns outros exemplos de sistemas complexos que atuam com a finalidade de reparar a ação de agentes genotóxicos (HOEIJMAKERS, 2001). Além dessas vias de reparo, muitos organismos contam também com um mecanismo de fotorreativação para reparar os principais danos de DNA provocados pela UVR, o qual utiliza enzimas de reparo chamadas de fotoliases. Estas enzimas atuam absorvendo fotóns de luz UVA e azul (300 – 500 nm) como co-substrato a fim de empregar esta energia para remover lesões do tipo CPD e 6-4PP do DNA (SANCAR, 2008; VINK, ROZA, 2001). Este mecanismo de

fotorreparo está presente no clado dos anfíbios, os quais utilizam as fotoliases para contornar os malefícios gerados pela exposição à radiação UV (BLAUSTEIN et al., 1994).

Apesar da existência dos mecanismos de reparo de DNA, os organismos podem apresentar consequências danosas a partir da exposição à radiação ultravioleta. As larvas de anfíbios expostas a radiação UV, por exemplo, têm sua performance afetada (LONDERO et al., 2019). Isso ocorre porque a radiação UV pode diminuir a eficiência alimentar das larvas, induzir a redução de massa corpórea, deformar as estruturas queratinizadas do aparato bucal (LONDERO et al., 2017), aumentar a frequência de micronúcleos (MN) em comparação a indivíduos não irradiados, além de resultar em malformações corpóreas (SCHUCH et al., 2015), diminuir a capacidade locomotora e diminuir a espessura da camada epidérmica em função de um aumento de apoptose (SANTOS et al., 2018). O desencadeamento destas consequências está relacionado com a dose de exposição dos anfíbios a radiação UV no ambiente natural, o qual, por sua vez, possui múltiplos fatores que podem atenuar ou potencializar esta exposição, como exemplificado no diagrama da Figura 3, que sumariza os principais fatores que influenciam na incidência de radiação UV para os anfíbios e demais organismos aquáticos.

Figura 3 – Diagrama representativo dos diversos fatores e processos que influenciam na incidência da radiação ultravioleta sobre as larvas de anfíbios.



Fonte: LONDERO et al., 2019.

1. 2 CONSIDERAÇÕES SOBRE O AGENTE QUÍMICO TRICHLORFON

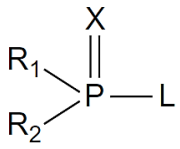
Assim como a radiação ultravioleta, os agentes químicos são considerados estressores ambientais devido aos seus impactos na fauna e no ambiente. Consequentemente, também estão inclusos entre a gama de fatores apontados como catalisadores do declínio dos anfíbios (ALTON, FRANKLIN, 2017). Dentre estes agentes, os agrotóxicos merecem destaque devido a seu uso massivo nas atividades agrícolas. Atualmente, há na Agência Nacional de Vigilância Sanitária (ANVISA) o registro de 496 monografias autorizadas de ingredientes ativos destinados ao uso agrícola, domissanitário, não agrícola, em ambientes aquáticos e como preservantes de madeira (ANVISA, 2020). A partir destes princípios ativos são elaborados produtos técnicos, pré-misturas e produtos formulados para comercialização em larga escala. Desde 2008 o Brasil ocupa a posição de maior consumidor de agrotóxicos no mundo (RUBBO, 2017) e, como demostram os dados do Sindicato Nacional da Indústria de Produtos para Defesa Vegetal (SINDIVEG), em 2019 houve um aumento de 5,9% na aplicação de defensivos agrícolas em relação a 2018, totalizando 993.270 toneladas empregadas em 1.576.710 hectares de área produtiva (SINDIVEG, 2020).

A utilização dos agrotóxicos oferece riscos como a contaminação dos solos agrícolas, das águas superficiais e subterrâneas, dos organismos aquáticos e terrestres, além do risco de contaminação dos próprios alimentos oriundos da produção agrícola (KATSIKANTAMI et al., 2019; PUNDIR et al., 2019). A saúde humana também é susceptível a intoxicação, seja por via direta durante a exposição ocupacional de trabalhadores e produtores rurais, por exemplo, ou por via indireta, através do consumo de água ou de alimentos com teores de agrotóxicos acima dos limites permitidos pela legislação brasileira (BOMBARDI, 2017; KATSIKANTAMI et al., 2019; MAGALHÃES, FERRÃO, 2008; SANTOS et al., 2019).

Cabe ressaltar que os efeitos adversos dos agrotóxicos estão relacionados tanto com a concentração destes compostos no ambiente, como com a capacidade de sua retenção nos compartimentos ambientais (solo, água e atmosfera), além das características físico-químicas das moléculas (SPADOTTO et al., 2004). Dentre os grupos químicos dos agrotóxicos, os organofosforados (OP) são moléculas amplamente utilizadas como pesticidas devido sua elevada toxicidade. Seus representantes podem ter origem natural ou sintética e são compostos derivados principalmente do ácido fosfórico (H_3PO_4) ou do ácido fosfônico (H_3PO_3) (KRIEGUER, 2010; SANTOS et al., 2019). A substituição dos átomos de hidrogênio destes

ácidos por grupamentos orgânicos, resulta na estrutura química básica dos organofosforados, como ilustra a Figura 4.

Figura 4 – Estrutura química básica dos compostos organofosforados. O grupamento L é chamado de grupo de saída pois é o substituinte mais reativo e mais suscetível a hidrólise. R1 e R2 são comumente grupos alcóxi, alquil, arila, alquiltio ou alquilamino. O Radical X pode ser um oxigênio ou enxofre.



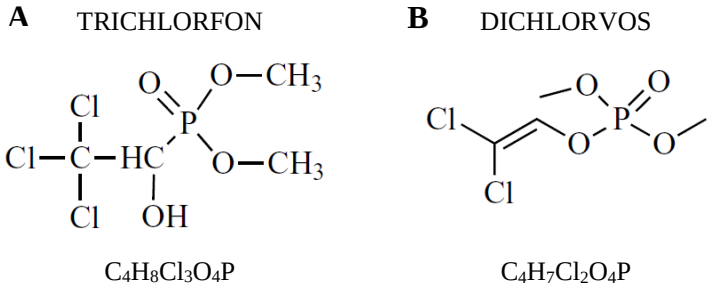
Fonte: KRIEGUER, 2010.

O trichorfon (TCF) – 2,2,2-trichloro-1-dimethoxyphosphorylethanol, comumente conhecido como metriphonate, chlorophos ou foschlor (NCBI, 2020), é um OP enquadrado na classe II (altamente tóxico) e possui propriedades inseticida, acaricida e anti-helmíntico (ANVISA, 2008). Possui uma ampla utilização na aquicultura de peixes contra endo e ectoparasitas, como por exemplo o piolho do mar (*Lepeophtheirus salmonis*), também chamado de lêndea do salmão (ANVISA, 2008; GAO et al., 2016; LI et al., 2017). Na medicina veterinária o TCF é empregado para controlar larvas e piolhos do gado (CHANG et al., 2013). No setor agrícola e domissanitário, o TCF tem a finalidade de erradicar lepidópteros, percevejos, formigas, moscas, baratas, larvas no geral e outros organismos considerados pragas (BINGHAM et al., 2012; LOPES et al., 2006). Na agricultura a aplicação foliar do TCF é indicada para os principais produtos agrícolas cultivados no país, como a soja, cana-de-açúcar, café, milho, algodão, arroz e cacau, além de diversas outras leguminosas, frutas e vegetais (ANVISA, 2008). Vale acentuar que o TCF também já foi utilizado com fins terapêuticos para tratar esquistossomose e também para doença de Alzheimer (POHANKA et al., 2011).

A respeito das propriedades moleculares podemos dizer que o TCF é um composto cristalizado, solúvel em água (1–5 g/100mL à 21ºC) e em solventes orgânicos, apresenta baixo peso molecular (257.44 g/mol) e densidade relativa igual a 1,73 (BINGHAM et al., 2012; NCBI, 2020). A principal via de síntese do TCF se dá através da adição de um dimetil fosfato ($C_2H_7O_3P$) ao haleto orgânico chamado tricloroacetaldeído (C_2HCl_3O) (HOFER, 1981), mesmo composto químico utilizado para a produção de outros agrotóxicos como o organoclorado DDT (diclorodifeniltricloroetano) (BINGHAM et al., 2012). Além disso, a molécula do TCF apresenta vários centros reativos, como por exemplo, o grupo metil, que reage com nucleófilos fracos, conferindo ao TCF a propriedade de um razoável agente metilante (HOFER, 1981)

(Figura 5.A). Outro centro reativo é o grupo hidroxi (OH), sendo que diversas reações de substituição da hidroxila por grupos alquil (C_nH_{2n+1}), alcóxi (R_{alquil}–O) e silil (R_3Si), por exemplo, conferem fortes propriedades inseticida a molécula (HOFER, 1981).

Figura 5 – A Estrutura química e fórmula molecular do agrotóxico Trichlorfon (TCF). **B** Estrutura química e fórmula molecular do composto Dichlorvos (DDVP).



Fonte: Adaptado de BINGHAM et al., 2012.

Após a aplicação dos agrotóxicos, alguns processos naturais podem determinar o comportamento dessas moléculas no ambiente. Podemos citar ao menos três processos: i. a transformação, isto é, a degradação de ordem química, física ou biológica (toxicocinética); ii. a retenção, ou seja, a adsorção das moléculas aos compartimentos do ambientais; e iii. o transporte, que se dá através da deriva, volatilização, lixiviação ou carreamento superficial (RUBBO, ZINI, 2017; SPADOTTO et al., 2004). Particularmente, a molécula de TCF é rapidamente transformada. Sua meia vida é de aproximadamente 57 horas em ambientes aquáticos (e o principal metabólito resultante da sua conversão é o dichlorvos (DDVP – 2,2-dichloroethenyl dimethyl phosphate), um composto oito vezes mais tóxico que o TCF (Figura 5.B) (GAO et al., 2016; MA et al., 2019; HEM et al., 2010; POHANKA et al., 2011). No meio ambiente, as vias de degradação do TCF são a demetilação, a clivagem da ligação fósforo-carbono e a hidrólise do éster (LOPES et al., 2006). Esta degradação pode ocorrer através de hidrólise, fotólise ou da ação de plantas e microrganismos, a qual é favorecida em meio aquoso com pH entre 6 a 8 (ANVISA, 2008; HEM et al., 2010; TALEBPOUR et al., 2006).

Os organofosforados em geral, incluindo o TCF e seu subproduto dichlorvos, apresentam como principal mecanismo tóxico a inibição de moléculas da classe das colinesterases (ChE) (COCKER et al., 2002). Esses compostos são rapidamente absorvidos independentemente da via e exposição. Ao entrar em contato com a pele, o TCF atinge a corrente sanguínea e prontamente é distribuído para os tecidos, podendo ultrapassar a barreira

hematoencefálica e provocar manifestações neurológicas (COCKER et al., 2002; HEM et al., 2010; POHANKA et al., 2011). O principal mecanismo de ação do TCF nos organismos se dá através da interação do grupamento fosfo (P=O) da molécula com o centro esterásico da enzima acetilcolinesterase (AChE), inibindo assim a sua ação catalítica de forma irreversível por impedimento espacial (LI et al., 2017). Essa inibição resulta no acúmulo de acetilcolina não hidrolisada, interrompendo a transmissão do impulso nervoso que afeta as sinapses colinérgicas do Sistema Nervoso Autônomo Parasimpático (SNAP), seguido de estimulação dos receptores nicotínicos e muscarínicos, acometendo então o Sistema Nervoso Autônomo Simpático (SNAS) e por fim o Sistema Nervoso Central (SNC) (DOS SANTOS et al., 2007; SANTOS et al., 2019). Consequentemente, a inibição sistêmica da enzima AChE resulta em intoxicação colinérgica, a qual está associada a diversos efeitos no organismo como neurotoxicidade, imunotoxicidade, carcinogenicidade, desregulação endócrina e alterações no desenvolvimento dos indivíduos (ANVISA, 2008). Além disso, exposições ao TCF podem desencadear alterações metabólicas e amplos distúrbios como estresse oxidativo, alteração no sistema de defesa antioxidante e redução na imunidade (LI et al., 2017; BALDISSERA et al., 2019). Em anfíbios, este composto também está relacionado com a formação de lesões hepáticas (LI et al., 2017), redução na sobrevivência, anormalidades morfológicas e nuclear, além da formação de dano de DNA (MA et al., 2019).

Acerca da legislação referente ao agrotóxico TCF, no ano de 2008 a ANVISA realizou uma reavaliação toxicológica deste composto que culminou na Resolução nº. 37 de 16 de agosto 2010. Essa resolução proíbe em território nacional a importação, a fabricação e a comercialização do princípio ativo TCF e de seus produtos derivados para fins agrícolas devido a sua alta toxicidade e efeitos adversos (BRASIL, 2010). Contudo, de acordo com Rocha et al. (2020), análises de amostras de água coletadas em córregos do Parque Estadual do Turvo (PET) indicaram a presença de 28 agrotóxicos, sendo o TCF o inseticida com maior frequência de detecção. Outro composto detectado em amostras de água bruta e/ou tratada para consumo humano na maioria dos municípios do RS foi o inseticida DDT (diclorodifeniltricloroetano) que, assim como TCF, foi banido para utilização no Brasil no ano de 2009 (RUBBO, 2017). Estes dois exemplos demonstram que mesmo após a proibição, certas moléculas banidas continuam sendo utilizadas. Tal uso, além de ilegal, representa um grave problema ambiental no país, pois coloca em risco a vida de inúmeros animais nativos, como também da própria população.

1.3 ANFÍBIOS: UM MODELO EXPERIMENTAL IMPRESCINDÍVEL

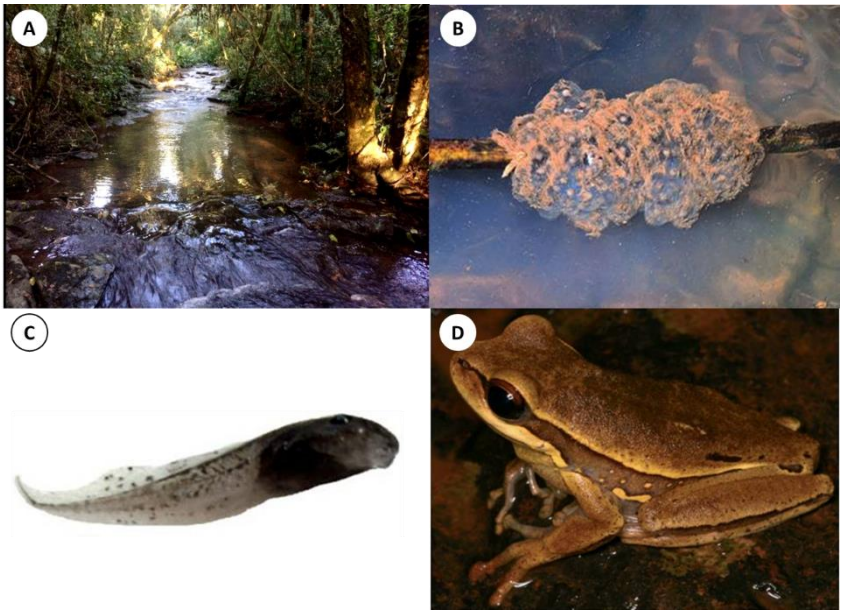
A vulnerabilidade dos anfíbios, expressa através do declínio da biodiversidade dos seus representantes nas últimas décadas, os coloca como um importante grupo que se tornou alvo de estudos de cunho ecotoxicológicos. A utilização de espécies de anfíbios como modelo experimental aliado a realização de análises *in vitro* e *in silico* são de suma importância para a compreensão dos efeitos adversos de agentes estressores que apresentam grande potencial tóxico, como a radiação ultravioleta e os compostos organofosforados. Apesar da utilização de anfíbios nativos na realização de pesquisas científicas parecer contraditória, ela é justificada por fornecer subsídios para compreender os fatores que contribuem para o declínio deste grupo. Essa utilização também vai ao encontro do Plano Nacional Para Conservação de Répteis e Anfíbios Ameaçados da Região Sul Do Brasil (ICMBIO, 2012) que tem como um dos objetivos subsidiar a manutenção de espécies de anfíbios e ampliar e difundir o conhecimento que auxilie a conservação da herpetofauna, fomentando assim a pesquisa científica.

Os anfíbios apresentam algumas características que os tornam propícios para serem utilizados como modelo de experimentação animal na investigação da ação de agentes tóxicos (LONDERO et al., 2019). Uma delas é que um grande número de espécies apresenta um complexo ciclo de vida bifásico, com uma fase embrionária e larval em ambiente aquático e uma fase pós-metamórfica em ambiente terrestre (MELSTAD, SHAFFER, 2015). Outra característica é referente ao fino extrato córneo e a alta permeabilidade da sua pele, a qual possibilita a realização de trocas gasosas e osmorregulação em indivíduos adultos (SANTOS et al., 2018). Além disso, a baixa mobilidade durante a fase embrionária e/ou larval amenta a relação intrínseca dos indivíduos com o ambiente aquático, o que aumenta a sua suscetibilidade a algum fator externo poluidor (BRUM et al., 2013). Estas características demonstram a vulnerabilidade desses indivíduos e o pequeno limite de tolerância às alterações no seu habitat frente aos agentes estressores, o que os tornam ótimos bioindicadores de qualidade ambiental (MAGALHÃES, FERRÃO, 2008).

No presente trabalho, foi utilizado como modelo experimental larvas de anfíbio da espécie *Boana Curupi* (GARCIA, FAIVOVICHI & HADDAD, 2007) (ANURA: HYLIDAE). Este hilídeo possui distribuição reduzida que compreende o noroeste da Argentina até regiões de florestas estacionais nos remanescentes florestais de Mata Atlântica no sul do Brasil (LONDERO et al., 2017; GARCIA et al., 2007). Os indivíduos adultos são arborícolas, isto é, ocupam a vegetação marginal de pequenos riachos no interior ou na borda de ambientes

florestados e são restritos a ambiente cobertos por vegetação (ROCHA et al., 2020; LIPINSKI et al., 2016). A *B. curupi* apresenta atividade reprodutiva sazonal entre os meses quentes de novembro a abril. Sua desova é posta sob a lâmina d'água aderida a pedras ou vegetação e possui característica gelatinosa, uma estratégia de proteção natural (GARCIA et al., 2007; LIPINSKI et al., 2016; LONDERO et al., 2019). A Figura 6 ilustra um sítio reprodutivo em um ambiente de riacho com cobertura vegetal preservada, propício para o desenvolvimento de *B. curupi* (A), uma desova posta sob a lâmina d'água (B), um indivíduo na forma larval (C) e um indivíduo na forma adulta (D).

Figura 6. A Ambiente de riacho. **B** Desova de *B. curupi*. **C** Representação de um indivíduo na fase larval. **D** Representação de um indivíduo adulto.

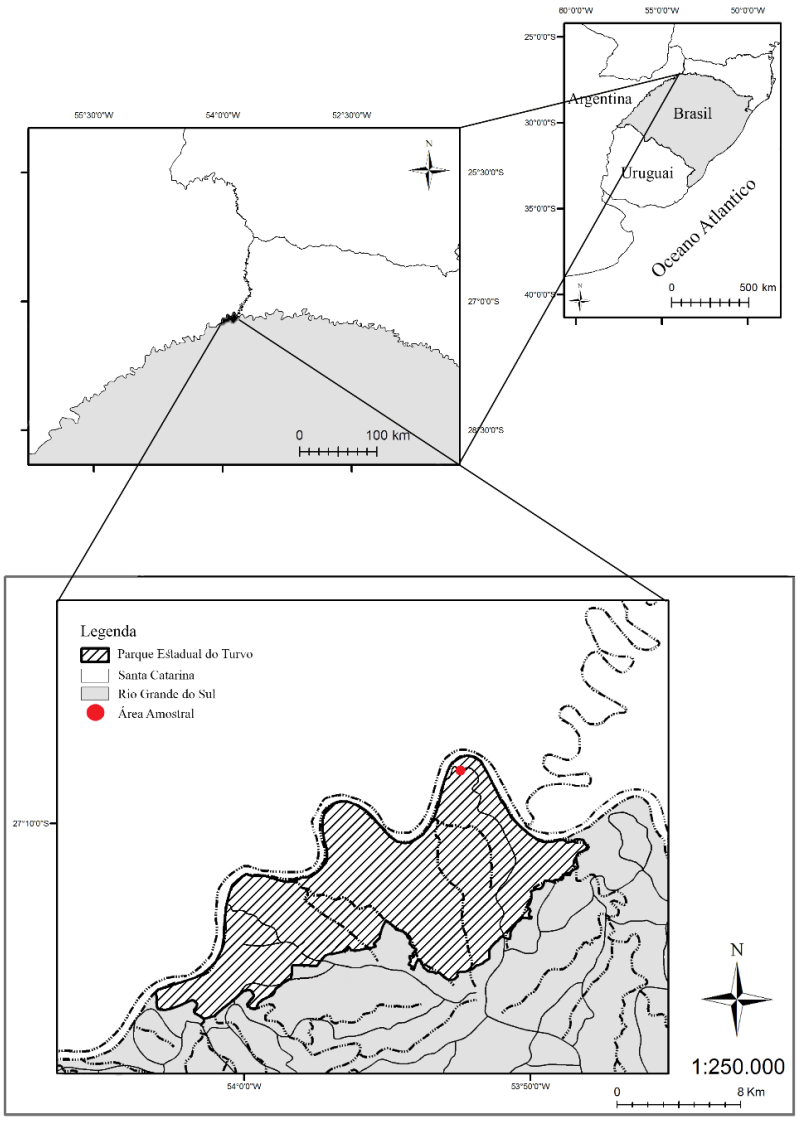


Fonte: Acervo pessoal de Marcelo Carvalho da Rocha.

Uma grande população de *B. curupi* presente no Rio Grande do Sul (RS) se encontra nos riachos do Parque Estadual do Turvo (PET), uma Unidade de Conservação (UC) de Proteção Integral situada no município de Derrubadas, extremo noroeste do estado. O Parque abrange 17.637,5 hectares e possuiu perímetro de aproximadamente 90 km, o qual faz divisa com a República da Argentina e com o estado brasileiro de Santa Catarina, através do Rio Uruguai (Figura 7) (RIO GRANDE DO SUL, 2005). O Parque Estadual do Turvo preserva uma amostra considerável de Floresta Estacional Decidual, sendo este o último grande fragmento desse tipo de vegetação no estado (RIO GRANDE DO SUL, 2005). Esta situação corrobora

com a conjuntura da Mata Atlântica e demais biomas brasileiros, que continuam a sofrer com as altas taxas de desmatamento, principalmente devido a conversão de terras para expansão de áreas destinadas a pastagem e a produção de *commodities* agrícolas (SEYMOUR, HARRIS, 2019).

Figura 7. Localização do Parque Estadual do Turvo. O círculo vermelho indica a localização do riacho no qual foram realizadas as coletas de desovas de *B. curupi* para este trabalho.



Fonte: Acervo pessoal de Álvaro Mainardi.

A pressão sobre a Mata Atlântica, que perdeu aproximadamente 88% de seu habitat natural, impacta fortemente a vida dos anfíbios deste bioma (SCARANO, CEOTTO, 2015) Estudos sugerem que 30% das espécies endêmicas de anuros da Mata Atlântica sofrem um declínio populacional devido a supressão da mata ou mudanças na floresta nativa

(ETEROVICK et al., 2005; MACCRACKEN, 2002; ROCHA et al., 2020). A espécie *B. curupi*, por sua vez, está classificada na categoria *least concern* (menor preocupação, em tradução livre) de acordo com a Lista Vermelha de Espécies Ameaçadas da IUCN (ANGULO, 2020). Porém, de acordo com a Lista Nacional Oficial de Espécies da Fauna Ameaçadas de Extinção do Ministério do Meio Ambiente e com o Decreto nº. 51.797, de 8 de setembro de 2014 do Estado do Rio Grande do Sul, os indivíduos da espécie *B. curupi* estão respectivamente classificados como vulneráveis e em perigo (MINISTÉRIO DO MEIO AMBIENTE, 2014; ESTADO DO RIO GRANDE DE SUL, 2014). Estas categorias de ameaça indicam que a *B. curupi* não se encontra criticamente em perigo, mas corre um risco alto de extinção na natureza.

Por fim, em relação aos apontamentos levantados até aqui, podemos considerar dois pontos fundamentais. São eles: i. a importância de realizar pesquisas científicas a respeito dos efeitos oriundos de doses ambientais do agrotóxico TCF, já que este composto é uma ameaça tangível às espécies de anfíbios como *B. cuupi*, e ii. a importância de compreender os efeitos da radiação UV nesta espécie restrita a ambientes de mata preservada, pois os efeitos maléficos deste agente físico podem ser letais, além de ser uma consequência imediata da remoção de mata ciliar pelo desmatamento (LIPINSKI et al., 2016; ROCHA et al., 2020). Cabe ressaltar que a adoção de doses mensuradas no habitat de *B. curupi* para a realização dos testes garante uma compreensão mais próxima dos fenômenos que possivelmente ocorrem no meio ambiente, uma vez que os animais silvestres e até mesmo os seres humanos, normalmente estão expostos a doses baixas de agroquímicos e de outros agentes estressores como a radiação UV. Desta maneira, esta pesquisa visa entender os mecanismos de ação tóxica no plano molecular, celular e a nível de organismo em larvas de *B. curupi* expostas à ação isolada e combinada ao agrotóxico trichlorfon e à radiação ultravioleta (UVA e UVB), a fim de compreender a interação destes dois agentes estressores com esta espécie sentinela ambiental.

A presente dissertação se estrutura em cinco seções. Na seção um consta a revisão bibliográfica em questão a respeito dos temas abordados neste trabalho. Na seção dois se encontram os objetivos da dissertação. A seção três apresenta o manuscrito elaborado em forma de artigo científico a ser submetido para publicação, o qual trata da metodologia, resultados e discussão a respeito das análises realizadas neste trabalho. A seção quatro conclui o trabalho, apontando perspectivas futuras relacionadas a continuidade desta pesquisa. Na última seção encontram-se as referências citadas na seção introdutória que não foram incluídas nas referências do manuscrito da seção três.

2 OBJETIVOS

Este trabalho objetiva analisar os efeitos gerados pela ação isolada e combinada do agrotóxico trichlorfon e da radiação ultravioleta em larvas de anfíbios da espécie *Boana curupi*, para compreender a dinâmica e a interação de estressores ambientais nos impactos gerados na anurofauna.

2.1 OBJETIVOS ESPECÍFICOS

Em larvas de *B. curupi* expostas de forma isoladas e combinadas ao Trichlorfon e à Radiação Ultravioleta, iremos verificar:

- a sensibilidade dos organismos a partir da análise de sobrevivência *in vivo*;
- a variação de caracteres morfológicos *in vivo*;
- a citotoxicidade *ex vivo*;
- a atividade das enzimas acetilcolinesterase (AChE) e glutationa S-transferase (GST) *ex vivo*;
- os níveis de marcadores oxidativos *ex vivo*;
- os danos de DNA *ex vivo*;
- o espectro de absorção da molécula de trichlorfon em diferentes concentrações *in vitro*;
- o efeito da molécula de trichlorfon e da radiação UV frente à exposição de células humanas deficientes na via NER de reparo de DNA *in vitro*.

3 ARTIGO 1 – Effects of isolated and combined exposures of *Boana curupi* (Anura: Hylidae) tadpoles to environmental doses of trichlorfon and ultraviolet radiation

Esse artigo será submetido a um periódico classificado como Qualis A1 na Área Ciências Biológicas II da Capes.

EFFECTS OF ISOLATED AND COMBINED EXPOSURES OF *Boana curupi* (Anura: Hylidae) TADPOLES TO ENVIRONMENTAL DOSES OF TRICHLORFON AND ULTRAVIOLET RADIATION

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Keywords: amphibian decline, sublethal doses, organophosphates, DNA damage.

Author Contributions.

Cassiano Ricardo Schavinski: designed and executed the essays described in this work, analyzed the results and wrote the manuscript. Maurício Beux dos Santos: helped in the execution of the essays, as well as in the analysis of the results. James Eduardo Lago Londero: helped in the execution of the essays, as well as in the analysis of the results. Aline Monique Blank do Amaral and Vania Lucia Loro: helped in the execution of biochemical analyses. André Passaglia Schuch: is the study supervisor of this work. Helped in the design of the essays, as well as in the analyses of the results, in the revision of the manuscript, and also obtained the financial support.

Abstract

The biodiversity decline affects mostly amphibian groups, with many driver factors already identified. It is estimated that the amphibian decline is probably happening due to the interaction of multiple factors. In view of this scenario, we analyzed the stressful effects generated by isolated and combined acute exposures to environmental doses of trichlorfon (TCF) pesticide (0.5 µg/L and 50 µg/L), and ultraviolet radiation (UVR) (184.0 kJ/m² of UVA and 3.4 kJ/m² of UVB, which correspond to 5% of the daily dose) in tadpoles of the species *Boana curupi* (Anura: Hylidae). In the combined treatments, we adopted three different moments of tadpole irradiation from the beginning of the acute exposure to TCF (0h, 12h, and 24h). We evaluated tadpole' survival, change in morphological characters, cell-induced apoptosis, lipid peroxidation (LPO), protein carbonyl (PC), glutathione S-transferase (GST), non-protein thiols (NPSH) and acetylcholinesterase (AChE), as well as the induction of genomic DNA (gDNA) damage. UVB alone results in high mortality, as well as high level of apoptosis induction. Both UVA, UVB and TCF increase LPO, PC, and AChE, and also decrease GST activity. Regarding co-exposure, the most notable effect was observed in the interaction between UVB and TCF, which surprisingly decrease UVB-induced tadpole mortality, apoptosis, and gDNA damage. Despite these results reinforce the UVB-sensitivity of *B. curupi*, it indicates a complex response in face of the interaction with TCF, which may be related to activation of DNA repair pathways and/or inhibition of apoptosis decreasing UVB-induced tadpole mortality.

Significance Statement

Amphibians have been experiencing an increased decline in recent decades and many factors are identified as catalysts, including the increased incidence of UVB radiation and environmental contamination by agrochemicals. In this research, tadpoles of a threatened forest-specialist treefrog species were exposed to ultraviolet radiation (UVA/UVB) in combination with low concentration of trichlorfon (TCF) pesticide for 24 hours. Environmental doses measured in the natural breeding site of this species were used to evaluate the harmful effects of these stressors at the molecular, biochemical, cytological, and morphological levels. UVB alone caused high mortality in tadpoles. The striking finding of this work was the absence of tadpole mortality when they were exposed to TCF 24h prior to UVB irradiation, which also conferred an apparent protection against UVB-induced damage in gDNA, proteins and apoptosis induction.

Main Text

Introduction

The human activities have been drastically modified global environment, changing biogeochemical cycles, transforming land, and polluting the environmental compartments (1, 2). Hence, these anthropogenic actions are considered a strong accelerator of biodiversity crisis, which is exemplified by the amphibian

decline worldwide (3). Particularly, the amphibians have characteristics that make them vulnerable to environmental stressors, as the complex life cycle associated with both aquatic and terrestrial environments (4), low mobility during the embryonic and larval phase that increases the intrinsic relationship of individuals with aquatic environment (5), and high skin permeability beyond the thick *stratum corneum*, which enables gas exchange and osmoregulation in adults (6, 7). The IUCN Red List estimates that among the 6,857 amphibian species, about 41% (33–53%) are listed as threatened with extinction (8).

Regarding the causes of amphibian decline, several factors may be contributing to this phenomenon, such as the global climate change (5); the loss, fragmentation, and modification of natural habitats (4, 9, 10); the introduction of exotic/invasive species (1); fungal diseases as chytridiomycosis caused by the pathogen *Batrachochytrium dendrobatidis* (Bd) (11); the increased incidence of ultraviolet radiation (UV) on the Earth's surface (12, 13); and environmental contamination from the use of chemical fertilizers, pesticides and other pollutants (14, 15). Furthermore, these organisms are increasingly exposed to several stressors simultaneously in wildlife, which suggests that amphibian decline is probably happening due to the interaction of multiple factors (3, 16). In this work, we focus on two of these stressors' factors, the solar UV radiation and the organophosphate insecticide trichlorfon.

UV radiation is a physical agent and part of the electromagnetic spectrum of the sun that is divided between three wavebands: UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm) (12). Although UVC is completely absorbed by the atmosphere, approximately 95% and 5-10% of UVA and UVB radiation, respectively, can reach the Earth surface (17). Moreover, the depletion events of stratospheric ozone increase the UVB radiation incidence, as well as its genotoxic effects on living organisms (18). Actually, both UVA and UVB can lead to damage in DNA, proteins, and lipids (17). However, DNA molecule is the main cellular target of UV photons and its absorption induces the production of photoproducts by dimerization reactions of adjacent pyrimidine bases (19). The three main types of photoproducts are the cyclobutane pyrimidine dimers (CPD), the pyrimidine 6-4 pyrimidone photoproducts (6-4PP), and the Dewar valence isomers (DewerPP) (20, 21). On the other hand, UV-induced damage in biomolecules can also occur through the formation of reactive oxygen species (ROS), which can lead the oxidation of the guanine base in DNA forming the 7,8-dihydro-8-oxuanuanine (8-oxoG), beyond the oxidation of lipids, proteins, and others cellular structures (17, 19). As general consequence, the presence of DNA lesions can compromise DNA metabolism, blocking DNA replication and transcription, triggering to the cell-cycle arrest or cell death (12, 22).

However, organisms have effective DNA repair mechanisms to remove DNA damage. The nucleotide excision repair (NER) is a versatile pathway that removes the UV-induced photoproducts and adducts induced by environmental mutagens (23). The amphibians also possess enzymes called photolyases, which can remove CPDs and 6-4PPs from DNA after photoactivation with UVA/visible wavelengths (24). Nonetheless, even with these mechanisms, the harmful effects of UV are associated with the time of amphibian exposure to radiation (25). The tadpoles exposed on UV radiation was their decrease food consumption and body weight beyond the increase of abnormalities nuclear (26), decrease survival

rates and occurrence of malformation (18), further decrease of locomotor performance and severe impacts on their skin (7).

The pesticide trichlorfon (TCF) – 2,2,2-trichloro-1-dimethoxyphosphorylethanol, is an organophosphate (OP) with insecticide , acaricide, and anthelmintic proprieties (27). This compound is wildly used in fish aquaculture against endo and ectoparasites (28, 29), as well as in veterinary medicine to control larvae and lice in cattle (30), and in agricultural and household sector to eradicate pests such as lepidoptera, bedbugs, ants, flies, cockroaches, and larvae in general (31). Regarding the properties TCF molecule, it is slightly soluble in water (1–5 g/100 mL at 21º C), has a low molecular weight (257.44 g/mol), and presents a short waterborne half-life of approximately 57 hours (31, 32). These characters imply in a repeat and indiscriminate use of TCF, mainly by fish farm management (33, 34). TCF molecule is subject to hydrolysis and dehydrochlorination (31) resulting in the insecticide dichlorvos (DDVP), which is approximately eight times more toxic than TCF (28). Upon contact with the skin, the TCF reaches the bloodstream (35) and can cross the blood-brain barrier and cause neurological manifestations (36).

Likewise, both TCF and DDVP induce inhibition of cholinesterase (ChE) class molecules as the main toxic mechanism (37), which is responsible for acetylcholine hydrolysis in choline and acetate at synaptic clefts on cholinergic synapses, neuromuscular junctions, and erythrocytes (38). Ultimately, TCF intoxication could affect the central nervous system and induce complex long-term behavioral alterations (37). To revert this scenario, there is a strong requirement of the liver activity aiming to the detoxification of TCF, leading to the hepatotoxic tendency in organisms exposed to low doses of TCF (14, 27, 29, 39). This suggests that the liver is the major target organ targeted by TCF (27). Moreover, some studies reported that TCF induces oxidative stress, as well as metabolic and enzymatic disturbance in amphibians (14, 29) as well as in fishes (32, 39, 40).

There is a lack of knowledge in ecotoxicology investigations about TCF effects on amphibians, especially in combination with the exposure to solar UV radiation. This gap extends to two other factors. Firstly, the understanding of the interaction of multiple stressors agents in non-target species. Secondly, the comprehension of the sublethal doses effect measured in environmental which represent the real risk of aquatic toxicity. In this sense, the values to the TCF, UVA radiation, and UVB radiation adopted in this research were measured in Turvo State Park (TSP), a conservation unit (CU) which preserve the last large area of Atlantic rainforest in southern Brazil (25). This place shelters the *Boana curupi* species (Anura: Hylidae), a forest-specialist amphibian which is used as the experimental model in this work. Therefore, we aim to understand the effects induced by TCF and UV radiation (UVA and UVB) at the molecular, cellular, and morphological levels in *B. curupi* tadpoles exposed to the isolated and combined action of the pesticide TCF and UV radiation (UVA and UVB). Although the results confirm the hazardous effects of UVB radiation for this forest-specialist amphibian species, the combined exposures of TCF and UVB surprisingly revert tadpole mortality, reduces damage in gDNA and protein, as well as the induction of apoptosis.

Materials and Methods

Animals and experimental design

Five freshly laid egg masses of *Boana curupi* were collected in streams at the Turvo State Park (TSP) (27°13′57.58″S, 53°51′04.58″O, 120–436 m a.s.l.), a conservation unit (CU) located in southern Brazil, during November to April of 2018 (late spring and early winter in the Southern Hemisphere). Immediately after collection, the egg masses were transported to the laboratory at the Federal University of Santa Maria and separately stored in plastic tanks with dechlorinated water (pH 8,27-8,72) at 20±1 °C. The photoperiod was set as 12:12 light: dark and the tanks were kept aerated until the beginning of hatching.

Post hatching, 48 healthy tadpoles on Gosner stages 24-26 (41) were randomly separated to each treatment (17 treatments= 816 tadpoles). These 17 treatments were divided in two set. In the first set, each tadpole was exposed to one single factor alone during acute exposure of 24 hours (5 treatments= CTRL; UVA; UVB; TCF 0.5 µg/L; TCF 50 µg/L). In the treatments of the second set, each tadpole was exposed to two factors combined, being one UV waveband more one TCF pesticide concentration during 24 hours. The combined exposure set included also three moments of tadpoles' irradiation to UV lamps. At the moment one, the tadpoles first received an UV irradiation dose and then were kept in contact with TCF for 24 hours (4 treatments= UVA + TCF 0.5 µg/L(24h); UVA + TCF 50 µg/L (24h); UVB + TCF 0.5 µg/L (24h); UVB + TCF 50 µg/L (24h)). At the moment two, the tadpoles remain in contact with TCF for 12 hours, received an UV irradiation dose, and come back to the same pesticide concentration for more 12 hours (4 treatments= TCF 0.5 µg/L (12h) + UVA + TCF (12h); TCF 50 µg/L (12h) + UVA + TCF (12h); TCF 0.5 µg/L (12h) + UVB + TCF (12h); TCF 50 µg/L (12h) + UVB + TCF (12h)). At the moment three, the tadpoles remain in contact with the pesticide for 24 hours to then received an irradiation dose (4 treatments= TCF 0.5 µg/L (24h) + UVA; TCF 50 µg/L (24h) + UVA; TCF 0.5 µg/L (24h) + UVB; TCF 50 µg/L (24h) + UVB). Figure 1 presents the schematic experimental design of this assay. All tadpoles subjected to acute exposure, independent of set and treatment, were accommodated in individuals' glass test tubes (20cm x 5cm), without feed, with 10mL of dechlorinated water (20 ºC) during 24 hours. After this period, all tadpoles except those intended for survival analysis / morphological characters, were euthanized with Xylocaina® (lidocaine ointment 5%). The study was conducted following the Committee of Ethics in Animal Experimentation of the University (CEUA nº 6776110619).

UV irradiation and trichlorfon pesticide

The UV doses and TCF concentration applied in this work were based on the environmental measurements performed at the Turvo State Park (TSP) by Rocha et al. (10). Concerning UV radiation, the doses used here correspond to 5% of the daily solar UVB and UVA radiation doses measured on a clear-sky summer day or to the period of sun exposure from 11:50h to 12:40h (50 min), with a portable radiometer (EKO® UV Monitor, model MS-211-1, Japan) on the edge of a stream without forest canopy. The UVB dose used in the experiments was 3.4 kJ/m^2 and the UVA dose was 184.0 kJ/m^2. In the Laboratory, the tadpoles were exposed to artificial UV radiation lamps in a Petri dish with dechlorinated water (20 ºC) without

pesticide. Post irradiation, the tadpoles were individually relocated to the glass test tubes. UVB irradiation was performed using a 15 W lamp (T15M, Vilber Lourmat, France) filtered with a polycarbonate sheet to block UVC wavelengths. For UVA irradiation, tadpoles were exposed using a 1000 W lamp (Osram Ultramed FDA KY10s) filtered with a 3 mm-thick glass filter (BG39, Schott Glass, Germany). The amount of UVC contamination for the UVB lamp as well as UVC and UVB contamination for the UVA lamp was below the detection limit. The spectral characteristic of the UV lamps was previously presented by Schuch et al. (13).

Concerning trichlorfon, the concentration of 0.5 µg/L refers to the maximum concentration measured in streams at the State Park of Turvo over a six months period to monitor a complete cycle of agricultural cultivation in this area. This determination was carried out by solid-phase extraction (SPE) with the polymeric sorbent and by gas chromatography and coupled in tandem to mass spectrometry (GC-MS / MS and LC-MS / MS). Moreover, an additional 100-fold concentration (50 µg/L) was also applied to investigate tadpoles' responses to a higher dose. Trichlorfon was obtained from Sigma-Aldrich (Trichlorfon Pestanal 250mg, 98,4% purity, CAS-Nº 52-68-6, Sigma-Aldrich Brazil Ltda.) and dissolved in dechlorinated water (20 ºC)

Tadpoles survival and morphological characters' analysis

At the end of acute exposure, 10 tadpoles per treatment were relocated to individual opaque plastic containers (11 cm x 9 cm) with 200 mL dechlorinated water (20 ºC). The tadpoles were fed with spinach *ad libitum* and two-thirds of the water volume of each container was changed in every two days. Then, the tadpoles' survival was monitored daily until the 14th day for the verification of vital signs. The dead animals were removed and Kaplan-Meier analysis was used to evaluate the survival rates over time according to Goel et al. (42). For analysis of the morphological characters, the mass of tadpoles of this experiment (n=10 / treatment) were weighed with an analytical balance (Shimadzu BL3200H, Japan) and the length was measured with millimeter plate and photographic resources in two different periods: in the first and in the fourteenth day. This measures were analyzed through the scale mass index (SMI) proposed by Peig and Green (43) based on tadpoles' mass-length relationships.

Cell-induced apoptosis

The cytotoxic potential was evaluated *ex vivo* from apoptotic cells count based on Thomas et al. (44) with alterations. Firstly, 3 exposed tadpoles per treatment were individually mechanically macerated with sodium citrate 0.09% solution, fixed with Carnoy 3: 1 methanol and acetic acid solution for 10 min and centrifuge with 640×g. After centrifugation, the supernatant was discarded and the fixing process was repeated four times for then the extracts are stored at -20 ºC until the slides' confections. For analysis, each biological extract was placed in clean glass slides at room temperature. The slides were stained with Giemsa 15% for 15 min, gently washed in running water and dried at 60ºC in the greenhouse. An Olympus BX41 optical microscope with a magnification of 1000x was used to count 1000 cells per glass slide. The apoptotic

frequency was given by the ratio between the number of apoptotic cells in 3000 cells counted in each treatment.

Biochemical analysis

After the treatments, the tadpoles (n=20/treatment) were divided in pools (0.05 g each ± 4 tadpoles) and they were mechanically macerated with 1 mL homogenization buffer (Tris-HCl 50 mM pH 7.5) and then centrifuged at 1400×g for 10 min. The resulting low-speed supernatant (S1) was used to the *ex vivo* biochemical analysis, except to the protein carbonyl content, for which it was used the homogenate without centrifugation. The protein concentration of S1 was determined based on the Bradford method (45). The color was measured spectrophotometrically at 595 nm and the results were expressed as mg/ml. *Lipid peroxidation*: Thiobarbituric acid reactive substances (TBARS) levels were measured as a parameter of lipid peroxidation (n=6 S1/treatment) according to Draper and Hadley (46). The reaction product was determined spectrophotometrically at 532 nm and the results were expressed as nmol of MDA/mg protein. *Carbonyl protein*: Carbonylated protein content was analyzed in samples (n=5 S1/treatment) through the method described by Reznick and Packer (47). The 2,4-dinitrophenylhydrazine (DNPH) was used to form hydrazone, which was spectrophotometrically quantified. Absorbance was measured at 370 nm and results were expressed as nmol of carbonyl protein content/mg protein. *Glutathione S-transferase*: GST activity was measured (n=6 S1/treatment) based on Habig et al. (48), And 1-chloro-2, 4-dinitrobenzene (CDNB) was used as a substrate, which formed GS-dinitrobenzene (GS-DNB) in the presence of reduced glutathione (GSH). The enzymatic activity was measured spectrophotometrically at 340 nm and the results were expressed as nmol of GS-DNB/min/mg protein. *Non-protein thiols*: The NPSH levels were determined in samples (n=6 S1/treatment) according to Ellman (49), and the measurement process was based on DTNB reaction to thiol groups after deproteinization with trichloroacetic acid (TCA). The color reaction was measured spectrophotometrically at 412 nm and the results were expressed as nmol NPSH/g tissue. *Acetylcholinesterase activity*: AChE was measured in samples (n=6 S1/treatment) as described by Ellman et al. (50), with some modifications. The acetylthiocholine was used as substrate and potassium phosphate buffer and 5,5'-dithiol-bis- (2-nitrobenzoic) (DTNB) as a system. The enzymatic activity was determined spectrophotometrically at 412 nm and the results were expressed as nmol/min/mg protein.

DNA damage analysis

The formation of DNA damage was evaluated *ex vivo* through the use assay for genomic DNA (gDNA) damage quantification according to Londero et al (51, no prelo). Initially, 15 tadpoles exposed per treatment were whole and individually mechanically macerated with homogenization buffer (NaCl 0.4M; Tris -HCl 10mM pH8.0; EDTA 2mM pH 8.0) for gDNA extraction step described by Aljanabi and Martinez (52) with some modifications, such as the use 20000×g instead of 10000×g. Furthermore, plus another spin-down of 10 minutes at 20000×g (4º C) was added after the addition of the 500 µl of 70% ethanol in samples

for DNA pellet wash. After this step added, the supernatant was discarded and the pellet was left to dry for 10 minutes at room temperature. Then, the pellet was resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). DNA samples were incubated with RNAse (0.2 µg / µl; 37º C) for 30 min to degrade any RNA molecules still present in the solution. DNA concentration was determined using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA), and all DNA samples were stored at -20º C until the beginning of the experiments. To detect and to quantify damage in gDNA, the samples were submitted to alkaline unwinding under three different specific treatments. The first treatment was neutral treatment for double-strand breaks analysis, where 500ng of isolated gDNA were added to complete 20 µl of a Tris-based buffer solution (pH 7.5). Then, 1 µl of loading buffer (0.25% bromophenol blue, 60% glycerol) was added to the solution. The second treatment was alkaline/neutral treatment for evaluation of alkaline unwinding-sensitive sites, were added to complete 20 µl of the Tris-based buffer solution. Then, 3 µl of 1 M Na_2HPO_4 (pH 12.85) was added to this solution and homogenized slightly for 10 times with micropipette for unwinding DNA and disconnecting DNA fragments from alkaline unwinding-sensitive sites (AU-SSs), especially SSBs. After 15 seconds of adding Na_2HPO_4, 9 µl of 0.1 M HCl were added to the solution, which was slightly homogenized 10 times, and samples were kept in ice for 4 minutes for renaturation DNA. Then, 1 µl of loading buffer (0.25% bromophenol blue, 60% glycerol) was added to the solution. Finely, the third treatment was enzymatic cleavage and alkaline/neutral treatment for the evaluation of additional UV/TCF-induced DNA lesions. 500 ng of gDNA was added to complete 19 µl of the Tris-based buffer solution needed for the action of the enzyme T4-endonuclease-V. We added 1 µl of the enzyme T4-endonuclease V (1 U/µl), which was used to cleave cyclobutane pyrimidine dimers (CPDs) into CPD, and incubated samples at 37º C for 1 h, according to the manufacturer's recommendations (New England Biolabs, UK). Then we performed alkalinization and neutralization steps as described in the treatment above. After that, 1 µl of loading buffer (0.25% bromophenol blue, 60% glycerol) was added to the solution. DNA samples from all treatments were submitted to a neutral 0.8% agarose gel electrophoresis run, which was prepared with DNA intercalating Unisafe dye 20000x, according to the manufacturer's recommendations (Uniscience, Brazil). The electrophoresis was performed at 3 V/cm for 2h30min with the gel immersed in 0.5x TBE buffer (45 mM Tris base, 45 mM boric acid, 1.2 mM EDTA; pH 8.0). We used the lambda DNA/Hind III molecular weight marker in the first well from each row of the gel according to the manufacturer's recommendations (Promega, USA). The gel image was revealed using a UV transilluminator (Amersham Imager 600, GE Healthcare, USA). The average length of the DNA population of a given sample was used to determine the frequency of DNA breaks. First, we applied a log relationship between the DNA lengths of known standard molecular marker bands (expressed as kilobase pair; kbp) and their respective measurements of distance (cm) throughout the electrophoretic migration run. Second, we searched the average value of fluorescence intensity of the DNA population in each sample throughout its electrophoretic migration run using the freeware ImageJ. Third, we defined the distance (cm) in the migration run of each sample that represents the average value of fluorescence intensity. Fourth, we applied the distance value of the average value of fluorescence intensity in the log formula obtained in the first step. Since a given fragment of DNA often

results from one break at each end (2 breaks in total), we applied the average length value of the DNA population to a simple rule of three for determining the average number of breaks per kbp.

Determination of the absorption spectrum of the TCF molecule

The absorbance of the pesticide trichlorfon (0.5; 50 and 500 µg/L diluted in destilated water) was carried out in triplicate using the spectrophotometer (UV-1650pc, Shimadzu Europe). The wavelength of interest comprises both the ultraviolet and the visible light spectrum (ʎ = 200 to 700 nm).

Data analysis

To compare the cumulative percentage of survival curves at each day we used the log-rank and Gehan-Wilcoxon tests through GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, USA). The data of morphological characters, apoptotic induction, biochemical analyses, and DNA damage was analyzed and mutually compared with repeated measures by nonparametric test the ANOVA one-way, followed by post-hoc Tukey's tests using the Statistica 12 software (Stata Statistical Software, TX, USA). Differences among groups were considered statistically significant when probability values were less than 0.05 ($p < 0.05$).

Results

Tadpoles' survival

Figure 2.A shows the survival curves of treatments in which tadpoles were exposed to one specific stressors. The survival of individuals exposed to UVA (95.83%) was similar to the control (CTRL - 100%) and of individuals exposed to UVB decreased considerably in relation to CTRL (23%). Survival for both TCF concentrations, 0.5 µg/L and 50 µg/L, were identical to CTRL (100%). When considering the combined treatments of UVA with both concentrations of TCF, it didn't induce a significant difference in tadpole survival when compared to CTRL, isolated UVA, and isolated TCF 0.5 or 50 µg/L (Figure 2.B). On the other hand, in the combined exposure with UVB and TCF 0.5 µg/L, the UVB + TCF (24h) treatment resulted in a decrease in tadpoles' survival when compared to CTRL (41.67%; $X^2 = 31.91$; $p<0.001$), as well as in the treatment of TCF (12h) + UVB + TCF (12h) (73.33%; $X^2 = 6.24$; $p<0.001$). However, in the TCF (24h) + UVB treatment there was no difference in relation to the CTRL (100%; $X^2 = 0$; $p>0.99$) (Figure 2.C). The same pattern was observed in the combined treatments with UVB and TCF 50 µg/L, where there was a decrease in tadpoles' survival in relation to CTRL in the UVB + TCF (24h) treatment (39.58%; $X^2 = 17.31$; $p<0.001$) and in the TCF (12h) + UVB + TCF (12h) (64.18%; $X^2 = 5.56$; $p<0.02$). Again, in the TCF (24h) + UVB treatment there was no difference in relation to the CTRL (Figure 2.C). Surprisingly, all survival curves of combined treatments of UVB and TCF 0.5 µg/L or 50 µg/L show increase in tadpole survival in comparison

to isolated UVB treatment. Considering the treatments of UVB combined with TCF 0.5 µg/L, there was no significant difference in the UVB + TCF (24h) treatment compared to isolated UVB, although the survival increased about 19% in the combined treatment. On the other hand, there was significant difference in the TCF (12h) + UVB + TCF (12h) treatment (X^2 = 24.93; p<0.001) and in TCF (24h) + UVB (X^2 = 44.14; p<0.001) treatment. Likewise, considering UVB combined with TCF 50 µg/L, there was a significant difference in UVB + TCF (24h) treatment (X^2 = 4.63; p<0.03) compared to isolated UVB, as well as in the TCF (12h) + UVB + TCF (12h) treatment (X^2 = 22.05; p<0.001) and in the TCF (24h) + UVB treatment (X^2 = 44.14; p<0.001).

Morphological characters

Considering the values measured in the first and on the fourteenth day, which are presented by the scale mass index (SMI), we no observed a difference in SMI between the treatments with UVA and UVB compared to CTRL or between them (Figure 3). However, we observed an increase in SMI of tadpoles exposed to TCF 0.5 µg/L compared to CTRL (P<0.0049), but there was no difference between TCF 50 µg/L and CTRL or between both concentrations of the pesticide. On the other hand, there was a significant increase in the SMI of tadpoles exposed to UVA and TCF 0.5 µg/L in the combined treatments of UVA + TCF (24h) (P<0.0064), TCF (12h) + UVA + TCF (12h) (P<0.00028), and TCF (24h) + UVA (P<0.019) in comparison to CTRL. This significant difference in the increase of SMI was also observed when comparing the treatments of UVA + TCF 0.5 µg/L (24h) (P<0.034) and the TCF 0.5 µg/L (12h) + UVA + TCF 0.5 µg/L (12h) (P<0.002) to UVA alone. There was no significant difference between combined treatments of UVA and TCF 0.5 µg/L when compared to TCF 0.5 µg/L alone. When considering the combined treatments with UVA and TCF 50 µg/L, there was no significant difference in comparison to CTRL, isolated UVA or isolated TCF 50 µg/L. Considering the combined treatments of UVB and TCF 0.5 µg/L, there was also a significant increase in the SMI of tadpoles in relation to CTRL in the TCF (12h) + UVB + TCF (12h) (P<0.032) and TCF (24h) + UVB (P<0.00003) treatments. This SMI increase was also observed when comparing the treatments of TCF (12h) + UVB + TCF (12h) (P<0.0001) and TCF (24h) + UVB (P<0.00003) to UVB alone. There was also a significant difference between TCF (24h) + UVB treatment (P<0.038) when compared to TCF 0.5 µg/L alone. When considering the combined treatments of UVB and TCF 50 µg/L, there was a significant decrease in SMI in the treatment of TCF (12h) + UVB + TCF (12h) in relation to CTRL (P<0.032). There was no significant difference between combined treatments with UVB and TCF 50 µg/L when compared to isolated UVB. Furthermore, both treatments of UVA + TCF 50 µg/L (24h) (P<0.002578) and the TCF 50 µg/L (12h) + UVB + TCF 50 µg/L (12h) (P<0.00008) presented significant decrease in SMI values in relation to the isolated treatment of TCF 50 µg/L.

Cell-induced apoptosis

The results shown in Figure 4 indicate that tadpoles exposed to UVA (P<0.0024) and UVB (P<0.00016) alone presented a significant increase compared to CTRL, as well as UVB alone also shows a significant difference in relation to UVA (P<0.0003). There was no significant difference between the CTRL and treatments of TCF 0.5 or 50 µg/L. Likewise, there was no difference in relation to CTRL when comparing the combined treatments of UVA and TCF 0.5 µg/L. However, there was a significant decrease in apoptosis in the treatments of UVA + TCF (24h) (P<0.0004) and TCF (24h) + UVA (P<0.0002) when compared to UVA alone. Regarding the isolated treatment of TCF 0.5 µg/L, there was a decrease in apoptosis in the TCF (24h) + UVA treatment (P<0.046). On the other hand, the combined treatments of UVA and TCF 50 µg/L didn't shown difference in relation to CTRL. However, the treatments of UVA + TCF (24h) (P<0.0036), TCF (12h) + UVA + TCF (12h) (P<0.00016), and TCF (24h) + UVA (P<0.00016) showed a decrease in apoptotic cells in relation to isolated UVA. There was also a decrease in apoptosis in the TCF (12h) + UVA + TCF (12h) (P<0.0012) and TCF (24h) + UVA (P<0.0018) treatments compared to the isolated treatment of TCF 50 µg/L. Considering the combined treatments of UVB and TCF 0.5 µg/L, there was a decrease in apoptotic cells in the treatment of UVB + TCF (24h) (P<0.01) in comparison to CTRL. This decrease was also observed in the UVB + TCF (24h) (P<0.0002), TCF (12h) + UVB + TCF (12h) (P<0.0002) and TCF (24h) + UVB (P<0.0002) treatments in relation to UVB alone. In relation to the isolated treatment of TCF 0.5 µg/L, UVB + TCF (24h) treatment (P<0.003) showed a decrease in the level of apoptosis induction. Regarding combined treatments of UVB and TCF 50 µg/L, there was no significant difference in comparison to CTRL. However, the treatments of UVB + TCF (24h) (P<0.0002), TCF (12h) + UVB + TCF (12h) (P<0.0002) and TCF (24h) + UVB (P<0.0002) were different from isolated UVB, showing lower levels of apoptotic cells. There was no significant difference between the combined treatments of UVB and TCF 50 µg/L with the isolated treatment of TCF 50 µg/L.

Biochemical analysis

Lipid peroxidation:

There was a significant increase in lipid peroxidation between isolated and combined treatments ($F_{(16, 146)} = 13.532$, p<0.0001). The results presented in Figure 5.A indicate that the isolated treatments of UVA (P<0.00004), UVB (P<0.0002), TCF 0.5 µg/L (P<0.00016) and TCF 50 µg/L statically differed from CTRL (P<0.00004). UVB alone also showed a decrease compared to isolated UVA (P<0.049). There was no significant difference between the isolated treatments of TCF 0.5 and 50 µg/L. Considering the combined treatments of UVA and TCF 0.5 µg/L, there was an increase in lipid peroxidation compared to CTRL in the treatments of UVA + TCF (24h) (P<0.00003), TCF (12h) + UVA + TCF (12h) (P<0.00003), and TCF (24h) + UVA (P <0.00003). However, there was no significant difference between these combined treatments with UVA alone and TCF 0.5 µg/L alone. This same pattern is observed in combined treatments of UVA and TCF 50 µg/L in relation to CTRL, with a significant increase in lipid peroxidation in UVA + TCF (24h)

(*P*<0.00003), TCF (12h) + UVA + TCF (12h) (*P*<0.00003) and TCF (24h) + UVA (*P*<0.0001). There was no difference between the combined treatments of UVA + TCF 50 µg/L with UVA alone, although treatments of TCF (12h) + UVA + TCF (12h) (*P*<0.00004) and TCF (24h) + UVA (*P*<0, 00003) had lower lipid peroxidation than that observed in TCF 50 µg/L alone. Regarding the combined treatments of UVB + TCF 0.5 µg/L in comparison to CTRL, there was a significant increase in lipid peroxidation in UVB + TCF (24h) (*P*<0.00003), TCF (12h) + UVB + TCF (12h) (*P*<0.00003) and TCF (24h) + UVB (*P*<0.00003), although there was no significant difference in relation to the isolated treatments of UVB or TCF 0.5 µg/L. When considering the combined treatments of UVB + TCF 50 µg/L, there was a significant difference only in the treatment of TCF (12h) + UVB + TCF (12h) (*P*<0.00005) in relation to the CTRL.

Carbonyl protein:

There was a significant increase in protein carbonylation between the isolated and combined treatments ($F_{(16, 118)} = 7.68$, $p<0.00001$). The results presented in Figure 5.B indicate that the isolated treatments of UVA (*P*<0.0012), UVB (*P*<0.0002) and TCF 50 µg/L (*P*<0.0075) differed from the CTRL. There was no significant difference between the isolated treatments of UVA and UVB or TCF 0.5 and 50 µg/L. Considering the combined treatments with UVA + TCF 0.5 µg/L, there was no difference in protein carbonylation when compared to CTRL. However, when compared with isolated UVA, there was a decrease in carbonyl protein levels in the UVA + TCF 0.5 µg/L (24h) treatment (*P*<0.0044). There was no significant difference between treatments in this group with the isolated treatment of TCF 0.5 µg/L. Considering the comparison between the combined treatments of UVA + TCF 50 µg/L with CTRL, there was no observed significant difference. However, there was a significant reduction in TCF (12h) + UVA + TCF (12h) treatment (*P*<0.013) when compared with isolated UVA. In addition, there was no difference between these combined treatments with the isolated treatment of TCF 50 µg/L. Regarding the combined treatments of UVB + TCF 0.5 µg/L, there was no difference to CTRL nor to isolated TCF 0.5 µg/L. However, we observed a reduction in protein carbonylation in the treatments of UVB + TCF (24h) (*P*<0.0002), TCF (12h) + UVB + TCF (12h) (*P*<0.0002) and TCF (24h) + UVB (*P*<0.0002) in relation to isolated UVB. There was no difference between this group of treatments with isolated TCF 0.5 µg/L. When considering these combined treatments with UVB alone and TCF 50 µg/L, there was a significant increase in relation to CTRL in the TCF (12h) + UVB + TCF (12h) treatment (*P*<0.003). However, we observed a reduction in the treatments of UVB + TCF (24h) (*P*<0.0002) and TCF (24h) + UVB (*P*<0.0002) treatment in relation to isolated UVB. Regarding the TCF 50 µg/L, there was a reduction in lipid peroxidation in the TCF (24h) + UVB treatment (*P*<0.01).

Glutathione S-transferase (GST):

There was a significant difference in GST activity after tadpoles' exposure to stressors ($F(16, 155) = 9.1343$, $p<0.00001$). The results presented in Figure 5.C indicate that the isolated treatments of UVA (*P*<0.00003), UVB (*P*<0.00003), TCF 0.5 µg/L (*P*<0.00003) and TCF 50 µg/L differed from CTRL (*P*<0.00003). There was no significant difference between the isolated treatments of UVA and UVB or TCF 0.5 and 50 µg/L. Considering the combined treatments of UVA + TCF 0.5 µg/L, there was a reduction in

GST activity in comparison to CTRL in the treatments of TCF (12h) + UVA + TCF (12h) (P<0.0001) and TCF (24h) + UVA (P<0.0001). However, there was an increase in the UVA + TCF (24h) treatment (P<0.00003) when compared with isolated UVA, as well as to isolated TCF 0.5 µg/L (P<0.0001). There was no statistical difference between TCF (12h) + UVA + TCF (12h) and TCF (24h) + UVA treatments with isolated UVA and TCF 0.5 µg/L. Considering the combined treatments of UVA + TCF 50 µg/L, there was a decrease in GST activity compared to the CTRL in the treatments of TCF (12h) + UVA + TCF (12h) (P<0.0003) and TCF (24h) + UVA (P<0.00004). The UVA + TCF (24h) treatment showed an increase in relation to isolated UVA (P<0.0008) and also to isolated TCF 50 µg/L (P<0.004). Considering the combined treatments of UVB + TCF 0.5 µg/L, there was a reduction in GST activity in relation to CTRL in the treatments of UVB + TCF (24h) (P<0.00003), TCF (12h) + UVB + TCF (12h) (P< 0.00003) and TCF (24h) + UVB (P<0.00003). There was no difference between this group of treatments to isolated treatments of UVB and TCF 0.5 µg/L. Regarding the combined treatments with UVB and TCF 50 µg/L, there was an increase in the TCF (12h) + UVB + TCF (12h) treatment (P<0.003) in relation to CTRL. However, we observed a reduction in the UVB + TCF (24h) treatment (P<0.0002) and TCF (24h) + UVB (P<0.0002) compared to isolated UVB.

Non-Protein Thiols (NPSH):

There was a significant difference in the detection of NPSH after tadpoles' exposure to stressors ($F_{(16, 146)} = 2.734$, p<0.001). Figure 5.D indicates that there is no difference among the isolated treatments with CTRL nor between UVA and UVB or between TCF 0.5 and 50 µg/L. The combined treatments of UVA + TCF 0.5 µg/L presented a reduction in NPSH levels in relation to CTRL only in the treatment of TCF (24h) + UVA (P<0.001), as well as in relation to isolated TCF 0.5 µg/L (P<0.0002). Both treatments of UVA + TCF (24h) and TCF (12h) + UVA + TCF (12h) did not differ from CTRL, as well as to isolated treatments of UVA and TCF 0.5 µg/L. This same pattern is observed when considered the combined treatments of UVA + TCF 50 µg/L in comparison to CTRL, where there is only a reduction in NPSH levels in the TCF (24h) + UVA treatment (P<0.007). There was no difference among the isolate treatments UVA and TCF 50 µg/L with any combined treatment in this group. Considering the combined treatments of UVB + TCF 0.5 µg/L and UVB + TCF 50 µg/L, there was no significant difference in relation to the CTRL, isolated UVB, isolated TCF 0.5 µg/L, and isolated TCF 50 µg/L.

Acetylcholinesterase (AChE):

There was a significant difference in the activity of the AChE enzyme ($F_{(16, 201)} = 20.715$, p<0.0001). Figure 5.E shows that the isolated treatments of UVA (P<0.00003), UVB (P<0.00003), TCF 0.5 µg/L (P<0.00003) and TCF 50 µg/L presented a significant increase in AChE activity in relation to CTRL (P<0.00003). There was no significant difference between the isolated treatments of UVA and UVB or TCF 0.5 and 50 µg/L. Considering the combined treatments of UVA + TCF 0.5 µg/L, there was an increase in AChE activity compared to CTRL in the treatments of UVA + TCF (24h) (P<0.00003), TCF (12h) + UVA + TCF (12h) (P<0.00003), and TCF (24h) + UVA (P<0.00003). We observed a significant reduction in the TCF (24h) + UVA treatment in comparison to isolate treatments of UVA (P<0.005) and TCF 0.5 µg/L (P<0.004).

Considering the combined treatments of UVA + TCF 50 µg/L, there was a significant increase in AChE activity in comparison to CTRL in the treatments of UVA + TCF (24h) (P<0.00003), TCF (12h) + UVA + TCF (12h) (P<0.0001), and TCF (24h) + UVA (P<0.00003). There was a decrease in the treatments of TCF (12h) + UVA + TCF (12h) (P<0.00003) and TCF (24h) + UVA (P<0.0056) in comparison to isolated UVA treatment. It was also observed a difference between the TCF (12h) + UVA + TCF (12h) treatment (P<0.0001) and the isolated TCF 0.5 µg/L. Considering the combined treatments of UVB + TCF 0.5 µg/L, there was a reduction in GST activity compared to CTRL in the treatments of UVB + TCF (24h) (P<0.00003), TCF (12h) + UVB + TCF (12h) (P<0.00003), and TCF (24h) + UVB (P<0.00003). There was an increase in AChE activity in the treatments of UVB + TCF (24h) (P<0.006), TCF (12h) + UVB + TCF (12h) (P<0.0001) and TCF (24h) + UVB (P<0.02) in comparison to isolated UV. When considering the combined treatments of UVBr and TCF 50 µg/L, there was an increase in relation to the CTRL in the treatments of UVB + TCF (24h) (P<0.00003), TCF (12h) + UVB + TCF (12h) (P< 0.00003) and TCF (24h) + UVB (P<0.00003). However, we observed a reduction in TCF (12h) + UVB + TCF (12) treatment (P<0.004) in relation to isolated UVB.

Damage DNA

There was a significant detection of double strand breaks (DSB) ($c1 = F_{(10, 22)} = 22{,}930$; p<0.0001; P<0.001), of single strand breaks (SSB) ($c2 = F_{(10, 22)} = 32.038$; p<0.001; P<0.001) and CPDs ($c3 = F_{(10, 22)} = 14.206$; p 0.001; P<0.003) in tadpoles exposed to isolated UVB treatment when compared to negative CTRL (Figure 6). Regarding the positive CTRL treatment (isolated gDNA from tadpoles control, which was *ex vivo* irradiated with 3.4 kJ/m² UVB), there was an increase in the detection of DSB (P<0.0002), of SSB (P<0.0002) but a decrease in CPD (P<0.047) compared to UVBr treatment isolated. On the other hand, there was no significant difference in the detection of DNA damage in the isolated TCF 0.5 and 50 µg/L treatment in relation to CTRL, neither between the combined treatments of UVB and TCF 0.5 µg/L or UVB TCF50 µg/L in relation to CTRL. Actually, there was a decrease in the detection of DSB, SSB and CPD, in these combined treatments in comparison to UVB alone.

Determination of the absorption spectrum of the TCF molecule

Both TCF concentrations applied in this work (0.5 and 50 µg/L) did not absorb neither UVB, UVA nor visible light spectrum (ʎ = 200 to 700 nm) (Figure 7).

Discussion

The forest canopy has an important role in the preservation of life and aquatic environment, which is fundamental mainly to forest-specialist anuran species (25). Both the forest coverage and the riparian forest provide a strong photoprotection against UV radiation incidence (25), as well as barriers to agrochemical run-off in streams located in agricultural and forest landscapes (10). Indeed, the Atlantic

Rainforest is critical to protect species against stressors agents and for conservation of treefrogs (13), such as the species *Boana curupi*, which is restricted to well-preserved forest habitats (53). Our results corroborated the sensibility described in *B. curupi* tadpoles exposed to low doses of UV radiation (7, 10, 25, 26). This was confirmed by the high mortality within 24 hours after irradiation with doses corresponding to 5% of the daily solar UVB doses measured on clear-sky summer days in a deforested ambient. UVB radiation reduced approximately 17.3-fold tadpoles' survival than UVA, resulting in the death of 72% of the tadpoles exposed. A previous work demonstrated reduced survival under an environmental dose of UVB radiation in tadpoles of the generalist species *Boana pulchella* (13). In comparison, here we demonstrate increased lethal effects of UVB radiation to a forest-specialist amphibian species of the same genus.

According to the UV-sensitivity hypothesis proposed by Blaustein et al. (16), the harmful effect of UV radiation to amphibians is counteracted by photoprotection through different levels of photolyase activity. Photolyases are DNA repair enzymes that use a blue-light photon as a co-substrate to remove the lesions induced in DNA by UV light (24). This scenario could be associated to the non-reduced survival of tadpoles exposed to UVA radiation (18), probably due to the CPD-photolyase and/or 6-4PP-photolyase activation during UVA irradiation (13). However, in comparison to the generalist treefrog species, the higher vulnerability of *B. curupi* to UVB could be related to the low photolyase activity due to their lifestyle and natural history (25, 26). Moreover, a decrease of NER pathway activity could also reflect a low efficiency to remove photoproducts such as CPDs and 6-4PPs from the double-helix of this forest-specialist amphibian (26).

In addition to reduced survival, UVB and UVA radiation induce detrimental effects in other macromolecules, compromising biological activities and decreasing tadpoles' fitness and performance (12). Also, the scale mass index (SMI) is an important factor to estimate the influence of stressor agents on morphological characters in amphibians (54). However, concerning this aspect, there was no significant difference between UV-exposed tadpoles and non-irradiated tadpoles 14 days after the acute exposure. Nevertheless, the decrease of tadpoles' mass and length induced by UV radiation was already documented (7, 18, 26) and this could be associated to reduced feeding efficiency (26), breaks in the keratinized oral apparatus (labial tooth row) (7), as well as to the guidance of energy to repair DNA damage, such as ATP requirement to perform NER pathway (55).

Conversely, our results show an increase of 1.7-fold and 2.5-fold in the apoptosis induced by UVA and UVB, respectively, reinforcing the UV-induced cytotoxic effects observed in *B. pulchella* tadpoles (13). In this work, we observed a relationship between the increase of apoptosis and high mortality in *B. curupi* induced by UVB radiation, as well as the formation of CPDs, single-strand breaks (SSB) and double-strand breaks (DSB) after 24h of UVB exposure. However, the level of cell-induced apoptosis in UVA-exposed tadpoles was significant when compared to the non-irradiated tadpoles, although it was not high enough to induce a high mortality as reported for UVB radiation in *B. pulchella* (13). In contrast, the apoptotic cells induced by UVA could be attributed, at a low level, to oxidative stress that these wavelengths can generate (19).

Our work is the first to evaluate biochemical targets on *B. curupi* tadpoles exposed to UV radiation and trichlorfon (TCF) pesticide. These results are fundamental to understand the status and metabolic responses of amphibians against these stressor agents. Regarding lipid peroxidation (LPO), both UVA and UVB radiation increased malondialdehyde (MDA) formation in tadpoles. MDA and other lipid hydroperoxides can form protein cross-linkages and DNA adducts which could lead to mutagenic and carcinogenic processes, as wel as to trigger cell death. (17, 19). As shown on embryos of the salamander *Ambystoma maculatum* (56) and *Limnodynastes peronei* tadpoles (57), high concentrations of ROS were produced during UVB exposure. According to Blair (58), lipid peroxidation is intrinsically related to ROS generation, which can damage other biomolecules that are susceptible to oxidation, such as proteins. Indeed, we confirm an increase of carbonylated protein content on *B. curupi* after UVB and UVA exposures. Particularly, protein damage represents a high risk to maintain activities such as DNA repair, regulation of proteome renewal, as well as activation of cellular responses to protect against the several insults resulted from irradiation or exposure to any other stressor agent (59).

The glutathione S-transferase (GST) enzyme is responsible to catalyze the conjugation of tripeptide glutathione to endogenous and exogenous electrophilic compounds, such as toxic hydroperoxides, hydrophobic compounds, and pesticides (60). The decrease of GST activity in *B. curupi* tadpoles represents a maintenance of the cellular redox status against an unbalance due to the stress triggered by UV radiation, coming from its enzymatic role in detoxification (40) and in the antioxidant system (29). However, non-protein thiols (NPSH) levels remain similar between non-irradiated and irradiated tadpoles. In this context, the level of NPSH content, which represents the main non-enzymatic antioxidant cell defense (61), could be associated with the high demand of glutathione (GSH) by a conjugation reaction via GST, resulting in an increase of biosynthesis this thiol-compound in the short-term, which is mediated by an increase of GSH/GSSG ratio (61). Concerning AChE, both UVA and UVB led to an increase of this enzyme activity, which is essential to cholinergic synapsis control.

Furthermore, TCF is a harmful agent to non-target aquatic life organisms, mainly to amphibians due to their high sensitivity to aquatic pollutants (27). *B. curupi* tadpoles exposed to the environmental concentration and to a 100-fold artificial concentration of TCF (0.5 and 50 µg/L, respectively) presented no mortality. However, in *Rana chensinensis* tadpoles exposed to 10, 100, and 1000 µg/L of TCF during 2 and 4 weeks, it was observed an increased mortality after the 5th day, considering the higher concentration, and after the 15th day, considering the lowest concentration (14). This suggests a strong relationship between concentration and time-dependent exposure in tadpole mortality. However, concerning the short waterborne half-life of TCF (approximately 57 hours), chronic exhibitions may present artifacts that do not represent reality in a natural environment. Likewise, our results demonstrate that neither SMI, cell-induced apoptosis, nor DNA damage formation was negatively affected under low concentrations of TCF in comparison with non-exposed tadpoles. However, the same relation between concentration and time-dependent exposure could be applied in this analysis, whereas *R. chensinensis* tadpoles showed a decrease in body weight after 2 weeks of TCF exposure under 1000 µg/L and an increase of micronuclei frequency and DNA damage under the concentrations of 100 µg/L and 10 µg/L, respectively (14).

Moreover, the biochemical markers of tadpoles exposed to TCF concentrations responded in a similar way as irradiated tadpoles. Regarding lipid peroxidation and carbonylated protein, the increase of MDA and carbonyl content was higher in TCF 50 µg/L. The GST activity was reduced concerning non-exposed tadpoles and the NPSH content remains similar to control. Nevertheless, in *R. chensinensis* frogs exposed to 10, 100 and 1000 µg/L during 2 and 4 weeks, it was observed a decrease of MDA content and a non-sensitive response in GST activity (29). Regardless, we showed a sensitivity of *B. curupi* tadpoles to low TCF concentrations owing to an increase in biomolecules damage, such as lipids and proteins. Finally, as well as UV radiation, tadpoles exposed to both TCF concentrations had an increase in the acetylcholinesterase activity, which is the opposite to what is found in the literature, considering that organophosphorus (OP) acts on inactivating AChE irreversibly by a special impediment (14, 27, 29, 30). However, chronic cypermethrin insecticide exposure in *Physalaemus gracilis* tadpoles caused an increase in AChE activity under concentrations of 6 and 10 µg/L, whereas 20 µg/L promoted inhibition of AChE activity (62). This increase was also verified in *Boana pardalis* tadpoles exposed 23 days to 2.40 mg/L of glyphosate, 4.00 mg/L of ametryn, 1.21 mg/L of 2,4-D, and 3.34 mg/L of acetochlor (63).

Therefore, two hypotheses could explain the phenomenon here observed in isolated and combined exposure: (1) the AChE activity increase could be a result of biogenesis and *de novo* protein synthesis after the AChE-inactivation by very low OP concentrations, which would induce a metabolic turnover. In tissue-cultured chicken embryo muscle cells, it was reported a recovery of 60-80% of their AChE activity following irreversible inactivation of the enzyme by an irreversible cell-associated AChE inhibitor, the diisopropylfluorophosphate (DFP) organophosphorus, in a period of 2-4 hours (64–68). Another hypothesis is that (2) the AChE increase could be reflecting a low-grade systemic inflammatory response due to reduced levels of acetylcholine, a molecule that acts on anti-inflammatory signaling in the cholinergic anti-inflammatory pathway (69). Indeed, both TCF and UV radiation can increase the inflammatory responses in organisms, which could be associated with the AChE level increase. The TCF molecule induced inflammation and impairment of the immune response in freshwater silver catfish (70), whereas the UV-induced ROS starts a signaling cascade in order to trigger the inflammatory and immune responses, in addition to an increase of pro-inflammatory cytokines like interleukin-8 (IL-8) and TNF-α (17, 71, 72).

Considering environmental dynamics, amphibians are exposed to multiple stressors simultaneously, which can enhance the toxicity and harmfulness of these agents, such as the increase of CPD lesions on *Xenopus laevis* embryos co-exposed to UVB and endosulfan / α-cypermethrin (73), or the reduced tadpole survival and body mass of *Hyla regilla* treefrogs co-exposed to UVB and nitrate (74). In this context, the present study demonstrates for the first time the responses of *B. curupi* tadpoles to acute co-exposure to TCF pesticide and UVA and UVB radiation. Regarding UVA radiation and TCF pesticide, independently of the concentrations tested, the co-exposure did not affect the survival of *B. curupi* tadpoles, the SMI levels and apoptosis induction. By contrast, TCF 0.5 µg/L and UVA, regardless of the irradiation moment, increased the SMI after 14 days comparing to non-exposed tadpoles. Furthermore, the apoptosis reduction rate has been verified in some treatments considering both TCF concentrations and UVA radiation in comparison to tadpoles exposed to isolated UVA radiation. Respecting biochemical markers, lipid

peroxidation, GST activity, NPSH, and AChE activity responded similarly to tadpoles exposed to stressor agents isolatedly. This was different from protein carbonilation, which decreased in both concentrations concerning isolated-UVA exposure.

Surprisingly, the tadpole responses under the co-exposure between UVB and TCF are the most intriguing because these are the opposite to what we initially hypothesized. The major response was observed in the tadpole survival. The longer the tadpoles remained in contact with TCF previously to the moment of UVB irradiation, the greater was the survival of the tadpoles in comparison to the isolated UVB treatment. In TCF (24h) + UVB treatments, on both TCF concentrations, the mortality was 0%, which was the same observed in non-irradiated tadpoles. The co-exposure between UVBr + 0.5 µg/L TCF increases the SMI after 14 days of exposure, mainly in TCF (24h) + UVB treatment. However, considering UVBr + 50 µg/L TCF, there was a decrease in the tadpole SMI compared to isolated UVB treatment and non-irradiated tadpoles. Differently, the co-exposure between UVB + both TCF concentrations reduced the apoptosis rate in relation to isolated UVB treatment. Regarding biochemical markers, lipid peroxidation, GST activity, NPSH, and AChE activity responded similarly to tadpoles exposed to stressor agents alone, except for the carbonylated protein, whose co-exposure led to a decrease of its content in most of the treatments. Similar to tadpole survival, the results of DNA damage quantification under co-exposure between UVB and TCF in both concentrations was highly unexpected, due to the absence of DSB, SSB, and CPDs on genomic DNA of tadpoles in all these treatments.

The UV-visible absorption spectrum of the TCF pesticide in different concentrations showed that its molecule did not absorb the UVB and UVA wavelengths applied in this work, eliminating the possibility of TCF providing a physical barrier to tadpoles against UVB and UVA radiation. To understand these unexpected responses and elucidate the molecular turnover under very low TCF concentration doses, more sensitive endpoints are required. Nevertheless, we present a hypothesis about the possibilities of molecular interactions. Both UV radiation and TCF alone triggered oxidative stress in *B. curupi* tadpoles and this could be associated to the increase of pro-inflammatory mediators regulated by redox-sensitive transcription factors (TFs), such as the nuclear factor κappa B (NF-κB), activator protein-1 (AP-1), signal transducer and activator of transcriptions (STATs), hypoxia inducible factors (HIFs), and nuclear factor erthroid-2 related factor (Nrf2) (75). It was demonstrated that dichlorvos (DDVP), a subproduct of TCF degradation, administered subcutaneously in Sprague-Dawley (SD) rats upregulates local and systemic inflammatory responses through an increase in the production of inflammatory cytokines (TNF-α and IL-6), as well as in the expression of NF-κB proteins in the lung tissue 48h following DDVP administration (76). NF-κB is a stress-inducible protein complex that acts as a transcription factor and is involved in the regulation of proliferation, apoptosis, inflammation, differentiation, and genes involved in the control of cell cycle (77). Activation of NF-κB results from different signaling pathways and is triggered by ROS, ionizing radiation, DNA damage agents, growth factors, tyrosine kinases, hypoxia, acidic conditions in solid tumors, and a variety of cytokines such as TNF-α (75, 77, 78). Likewise, the redox process triggered by UV radiation also results in the activation of NF-κB proteins and then leads to the production of inflammatory mediators such as cytokines and prostaglandins (17). In normal human epidermal keratinocytes exposed to physiologically

relevant UVA and UVB doses, there was an increase in the activation and nuclear translocation of the functionally active protein subunits of NF-κB (79). Moreover, the role of NF-κB is linked also to the development and progression of cancer, as well as chemo, radio, and cancer therapy resistance due to the induction of anti-apoptotic genes (78, 80).

However, Volcic et al. (80) demonstrated the involvement and stimulatory role of NF-κB in the repair of the most detrimental DNA lesion, the DSB, by enhancing the homologous recombination pathway (HR) spontaneously or in response to genotoxic stress in different cell types. Particularly, we suppose that the co-exposure to very low TCF concentrations prior UVB irradiation could be enhanced NF-Kb expression through an increase of inflammatory mediators triggered by ROS. Herein, the effect of co-exposure is quite evident when concerning the reduction of DNA damage caused by UVB radiation after 24 hours. Altogether, this suggests that such interaction may be upregulating some DNA repair pathway. Additionally, although this result reflects an increase in tadpole survival in a short time, the scenario here presented requires more investigation and for that, we suggest that transcriptomic analysis would be useful to understand these responses and the molecular mechanisms underlying the interaction between UVB radiation and TCF pesticide.

Conclusion

Therefore, our results reinforce the amphibian sensitivity, especially from treefrogs species such as *B. curupi*, to environmental doses of UV radiation, mainly UVB. Our findings corroborate the importance of preserving natural environments and the presence of riparian forest to protect these animals against physical stressors. In addition, we found that there is an increase in the complexity of amphibian responses in face of the interaction of more than one stressor agent, which emphasizes the need to understand the responses of amphibians simultaneously exposed to various environmental pollutants in order to better elucidate the processes involved in amphibian decline**.**

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Figures

Figure 1. Schematic representation of the experimental design of this study. Set 1 represents the treatments in which the tadpoles were exposed to one single factor. Set 2 represents the treatments in which the tadpoles were exposed to two factors concomitantly. *Each UV irradiation moment was performed for both 0.5 µg/L and 50 µg/L TCF concentration.

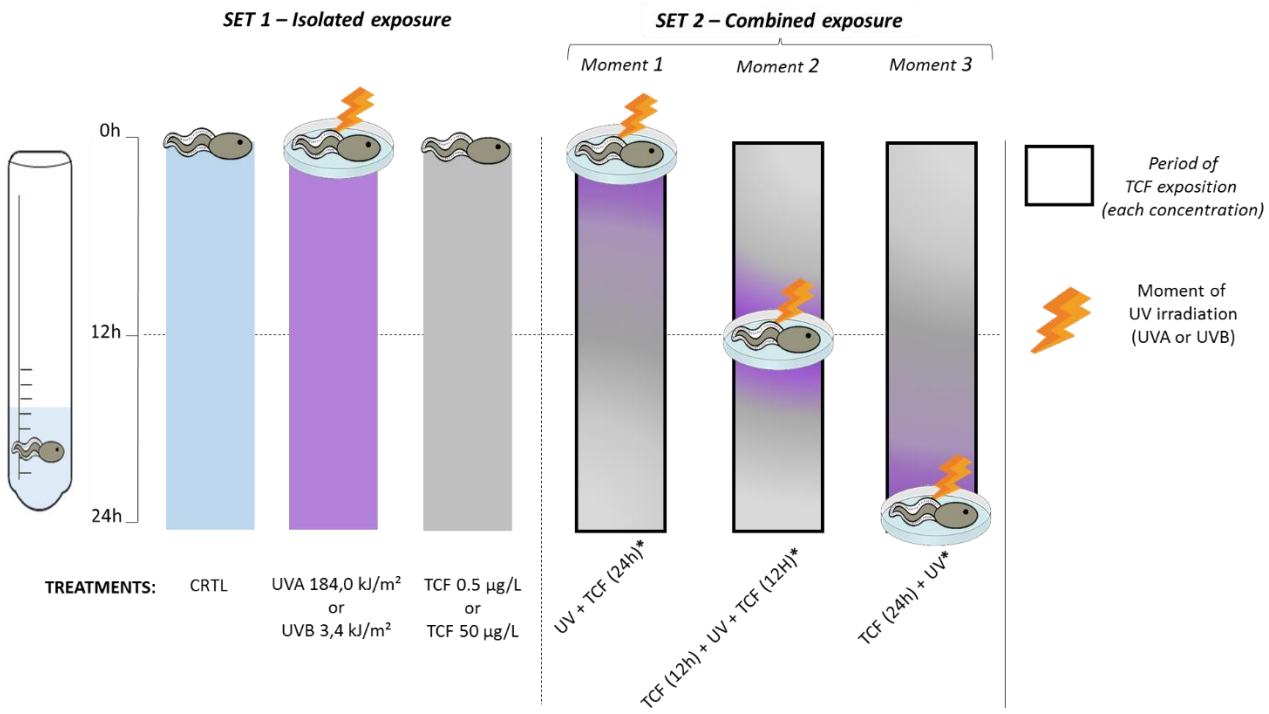


Figure 2. Cumulative percentage of *B. curupi* tadpoles' survival exposed to 184.0 kJ/m² UVA radiation, 3.4 kJ/m² UVB radiation, 0.5 µg/L and 50 µg/L concentration of TCF pesticide isolated and combined, during 14 days, considering the day 1 as the first 24 hours after acute exposures. **A.** Survival curves of individuals exposed to one singly stressor. Log-rank (Mantel-Cox) test, p<0,001. **B.** Survival curves of individuals exposed to UVA + TCF treatments. Log-rank (Mantel-Cox) test p=non-significant **C.** Survival curves of individuals exposed to UVB + TCF treatments. Log-rank (Mantel-Cox), test p<0,001.

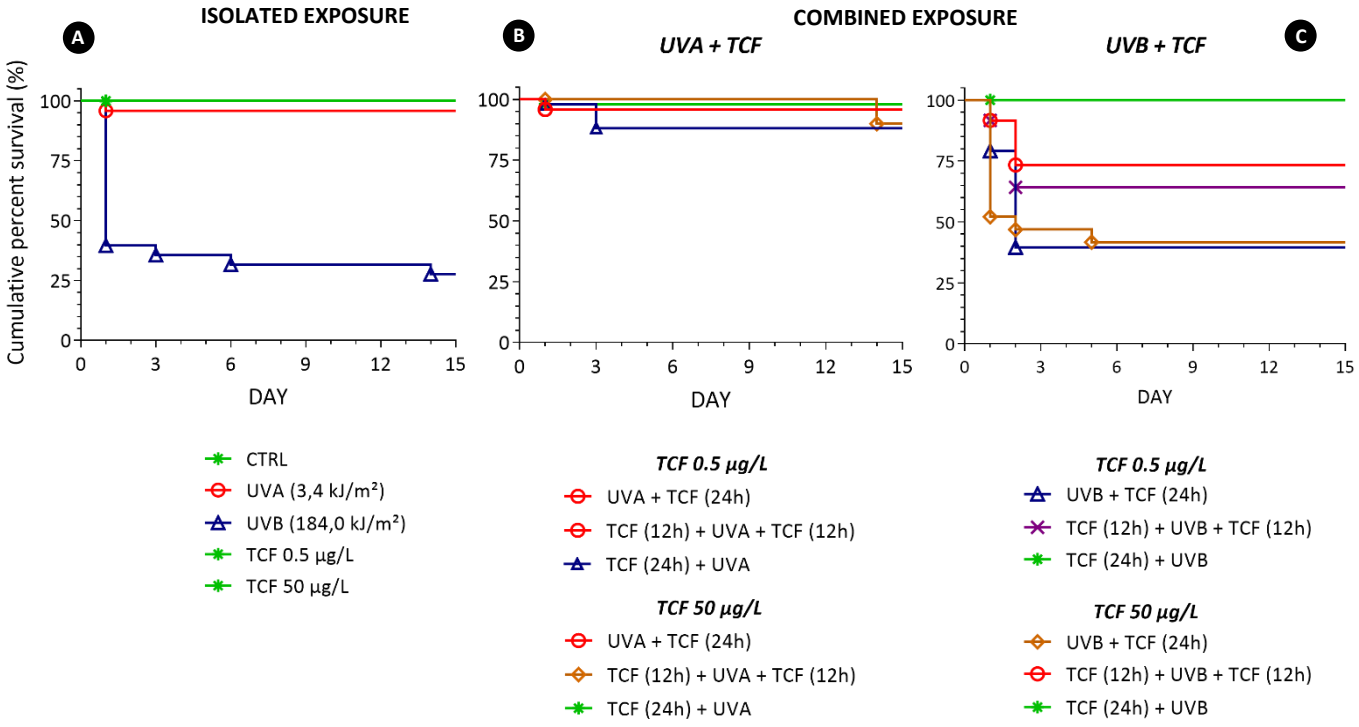


Figure 3. Scale mass index (SMI) of *B. curupi* tadpoles exposed to 184.0 kJ/m² UVA radiation, 3.4 kJ/m² UVB radiation, 0.5 µg/L and 50 µg/L concentration of TCF pesticide isolated and combined. The bars without border represent the tadpoles mass-length relationships measured after 24 hours of acute exposure (day 1). The bars with border represent the tadpoles mass-length relationships measured after 14 days from of acute exposure. * Statistically significant in relation to the non-treated control. # Statistically significant in relation to the isolated UVA radiation treatment. ‡ Statistically significant in relation to the isolated UVB radiation treatment. & Statistically significant in relation to the isolated TCF 0.5 µg/L treatment. ¥ Statistically significant in relation to the isolated TCF 50 µg/L treatment. ¤ Statistically significant between the treatments of both TCF concentrations combined with UV radiation. (p<0,001).

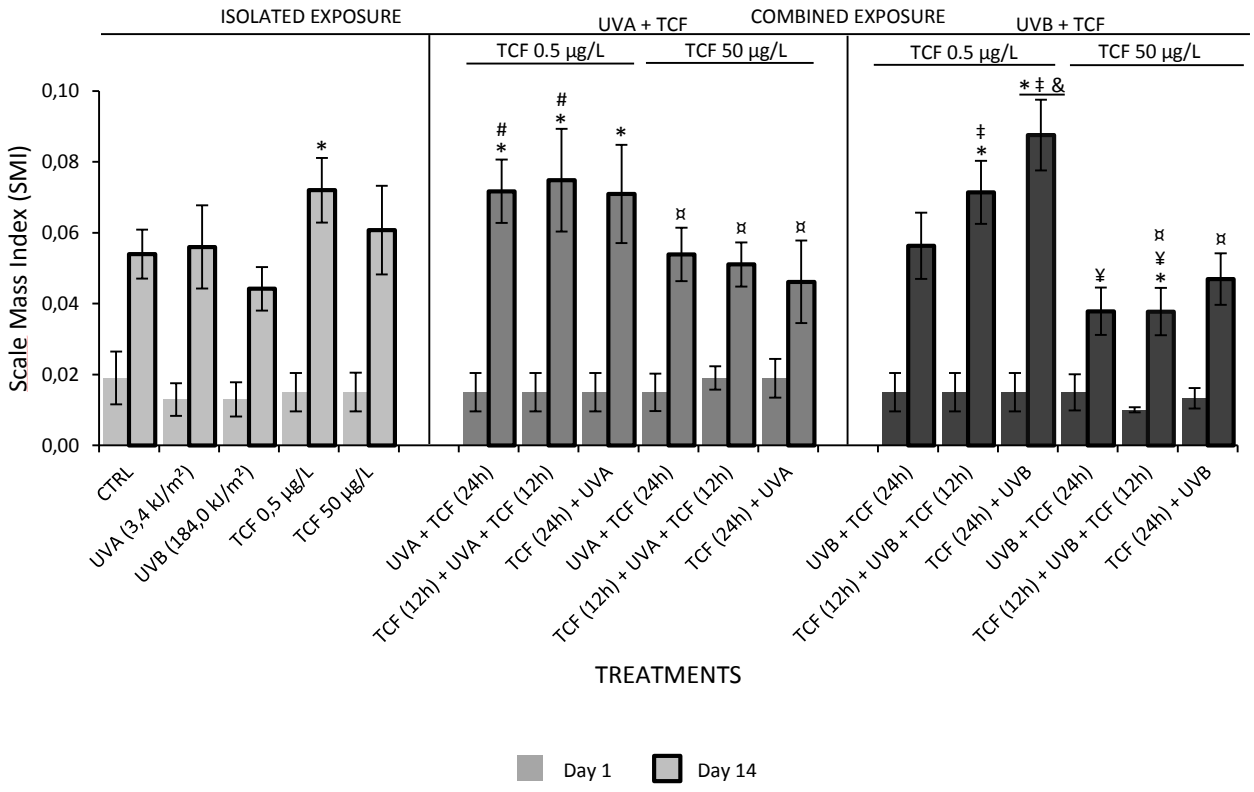


Figure 4. A. Apoptosis induction in *B. curupi* tadpoles exposed to 184.0 kJ/m² UVA radiation, 3.4 kJ/m² UVB radiation, 0.5 µg/L and 50 µg/L of TCF pesticide isolated and combined. Data represent the mean ± standard deviation (s.d.) of three tadpoles analyzed independently in each treatment (3000 cells counted per treatment). **B.** Representative example of normal cells **C.** Representative example of stressors agents-induced apoptosis cells. * Statistically significant in relation to the non-treated control. # Statistically significant in relation to the isolated UVA radiation treatment. ‡ Statistically significant in relation to the isolated UVB radiation treatment. & Statistically significant in relation to the isolated TCF 0.5 µg/L treatment. ¥ Statistically significant in relation to the isolated TCF 50 µg/L treatment. ¤ Statistically significant between the treatments of both TCF concentrations combined with UV radiation. (p<0,001).

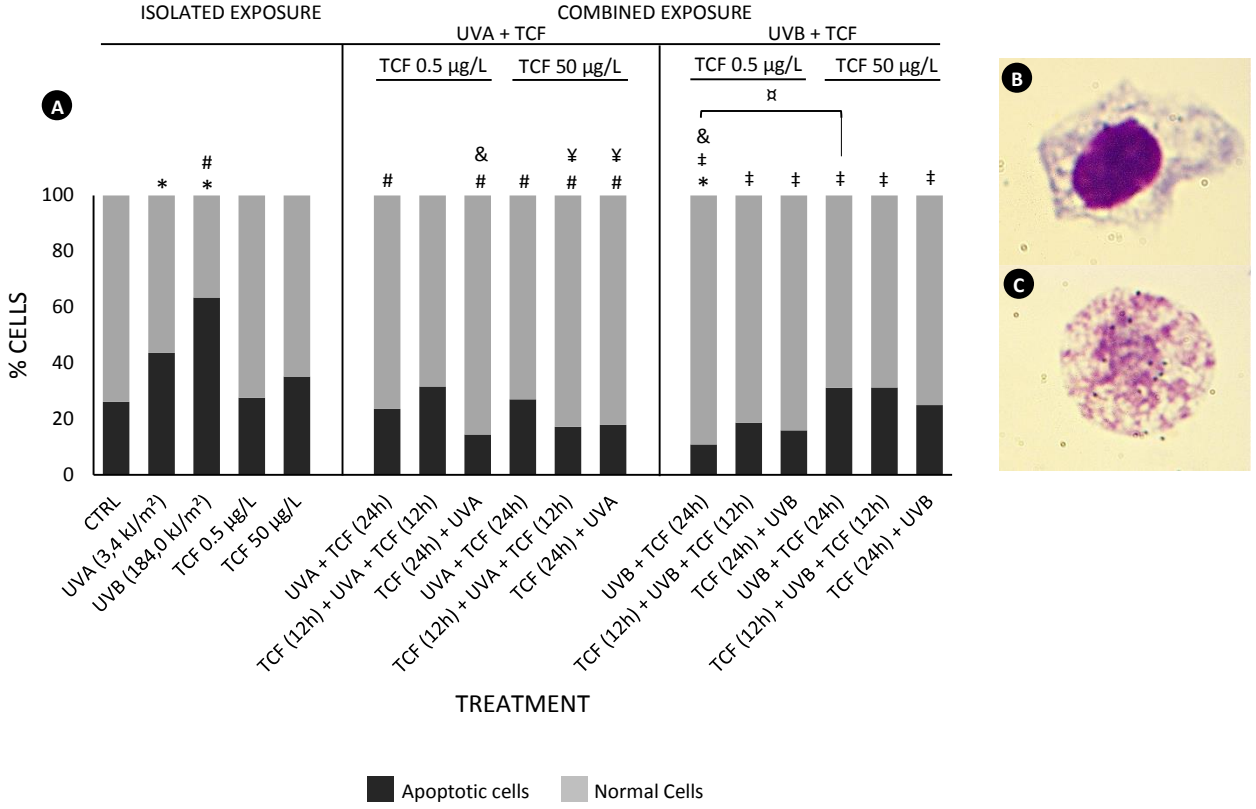
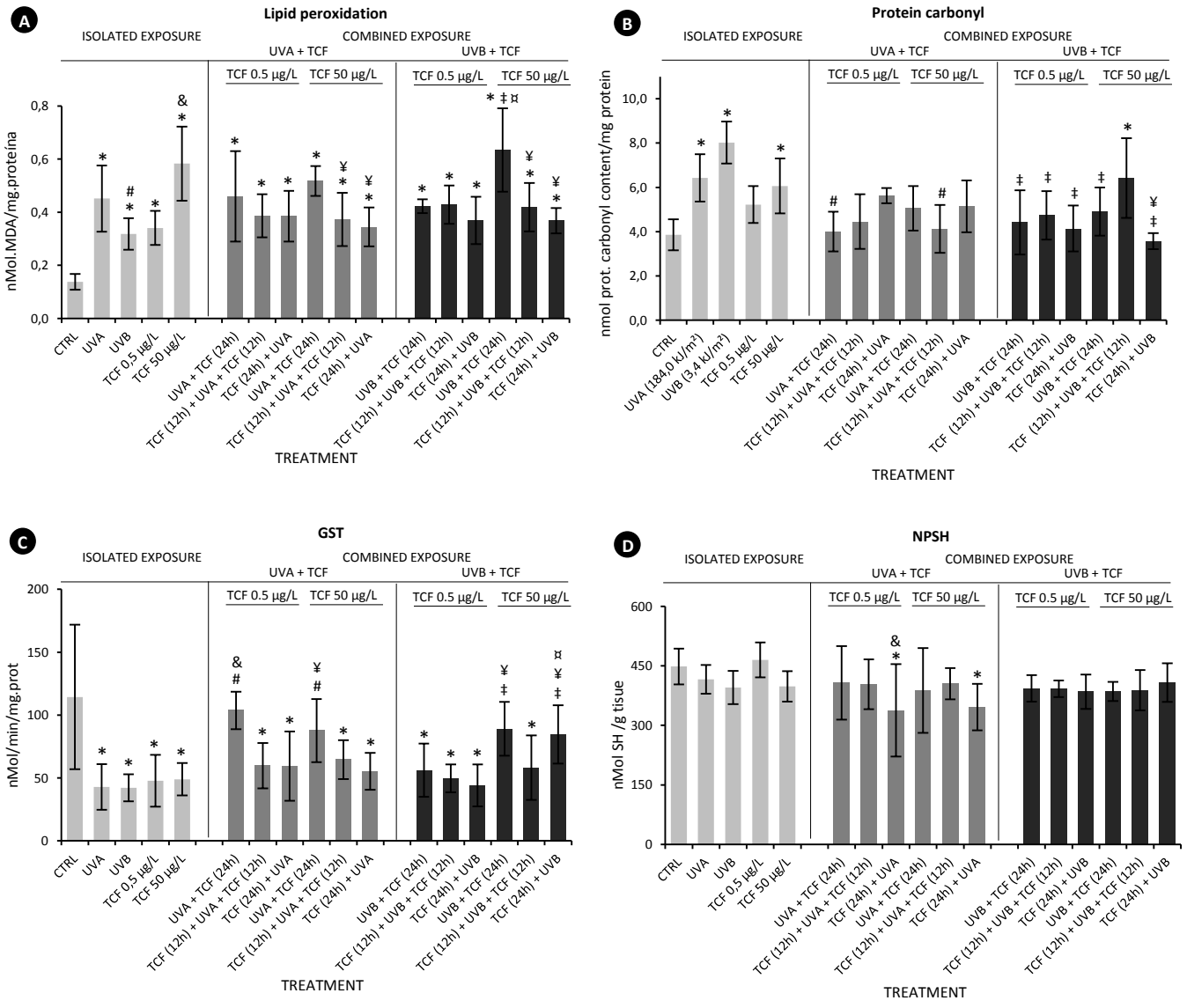


Figure 5. Biochemical analyses in *B. curupi* tadpoles exposed to 184.0 kJ/m² UVA radiation, 3.4 kJ/m² UVB radiation, 0.5 µg/L and 50 µg/L concentration of TCF pesticide isolated and combined. **A.** Amount of malondialdehyde (MDA) in nmol / mg of protein **B.** Amount of carbonyl protein expressed as nmol protein carbonyl content / mg protein. **C.** Glutathione S-transferase (GST) activity expressed as nmol of GS-DNB / min / mg protein **D.** Amount of non-protein thiols (NPSH) in nmol of SH per g of tissue **E.** Acetylcholinesterase (AChE) activity expressed as nmol / min / mg protein * Statistically significant in relation to the non-treated control. # Statistically significant in relation to the isolated UVA radiation treatment. ‡ Statistically significant in relation to the isolated UVB radiation treatment. & Statistically significant in relation to the isolated TCF 0.5 µg/L treatment. ¥ Statistically significant in relation to the isolated TCF 50 µg/L treatment. ¤ Statistically significant between the treatments of both TCF concentrations combined with UV radiation. (p<0,001).



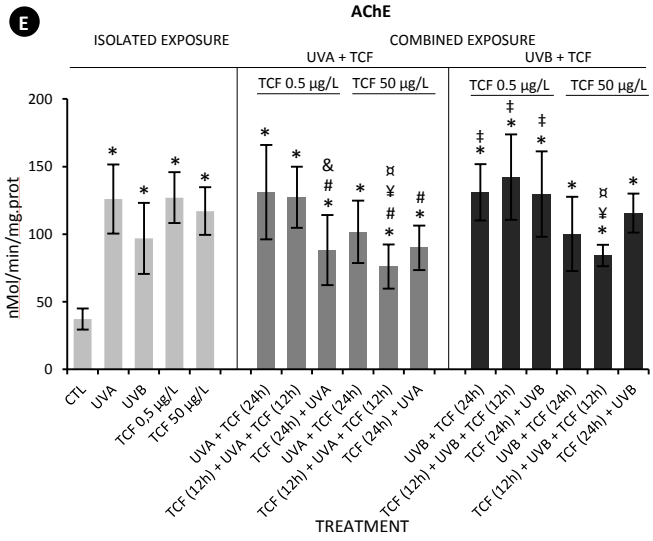

E
AChE
ISOLATED EXPOSURE
COMBINED EXPOSURE
UVA + TCF
UVB + TCF
TCF 0.5 µg/L
TCF 50 µg/L
TCF 0.5 µg/L
TCF 50 µg/L
nMol/min/mg.prot
200
150
100
50
0
CTL
UVA
UVB
TCF 0,5 µg/L
TCF 50 µg/L
UVA + TCF (24h)
TCF (12h) + UVA + TCF (12h)
TCF (24h) + UVA
UVA + TCF (24h)
TCF (12h) + UVA + TCF (12h)
TCF (24h) + UVA
UVB + TCF (24h)
TCF (12h) + UVB + TCF (12h)
TCF (24h) + UVB
UVB + TCF (24h)
TCF (12h) + UVB + TCF (12h)
TCF (24h) + UVB
TREATMENT

Figure 6. Analysis of genomic DNA (gDNA) damage in *B. curupi* tadpoles exposed to 3.4 kJ/m² UVB radiation, 0.5 µg/L and 50 µg/L concentration of TCF pesticide isolated and combined. The positive control refers to isolated gDNA samples of the *B. curupi* tadpoles irradiated *ex vivo* with 3,4 kJ/m² UVB radiation at time 0h. **A.** Electrophoregram of the gDNA samples. **B.** Quantification of DNA breaks per kilobase pair in gDNA. Double-strand breaks (DSBs) were determined through a treatment in which DNA was subjected only to the neutral condition (c1). Alkaline unwinding-sensitive sites (AU-SSs) were determined through a treatment in which DNA was subjected to an alkaline/neutral condition to detect the AU-SSs as well as basal levels of DSBs (c2). T4-endonuclease V-sensitive sites (T4-endoV-SSs = CPDs) were determined through a treatment in which DNA was treated with the enzyme T4-endonuclease-V and subjected to an alkaline/neutral condition to detect the T4-endoV-SSs as well as basal levels of AU-SSs and DSBs (c3). Kbp: kilobase pair. MM: molecular marker. * Statistically significant in relation to the non-treated control. # Statistically significant in relation to the control positive (CTRL +). ‡ Statistically significant in relation to the isolated UVB radiation treatment. (p<0,001).

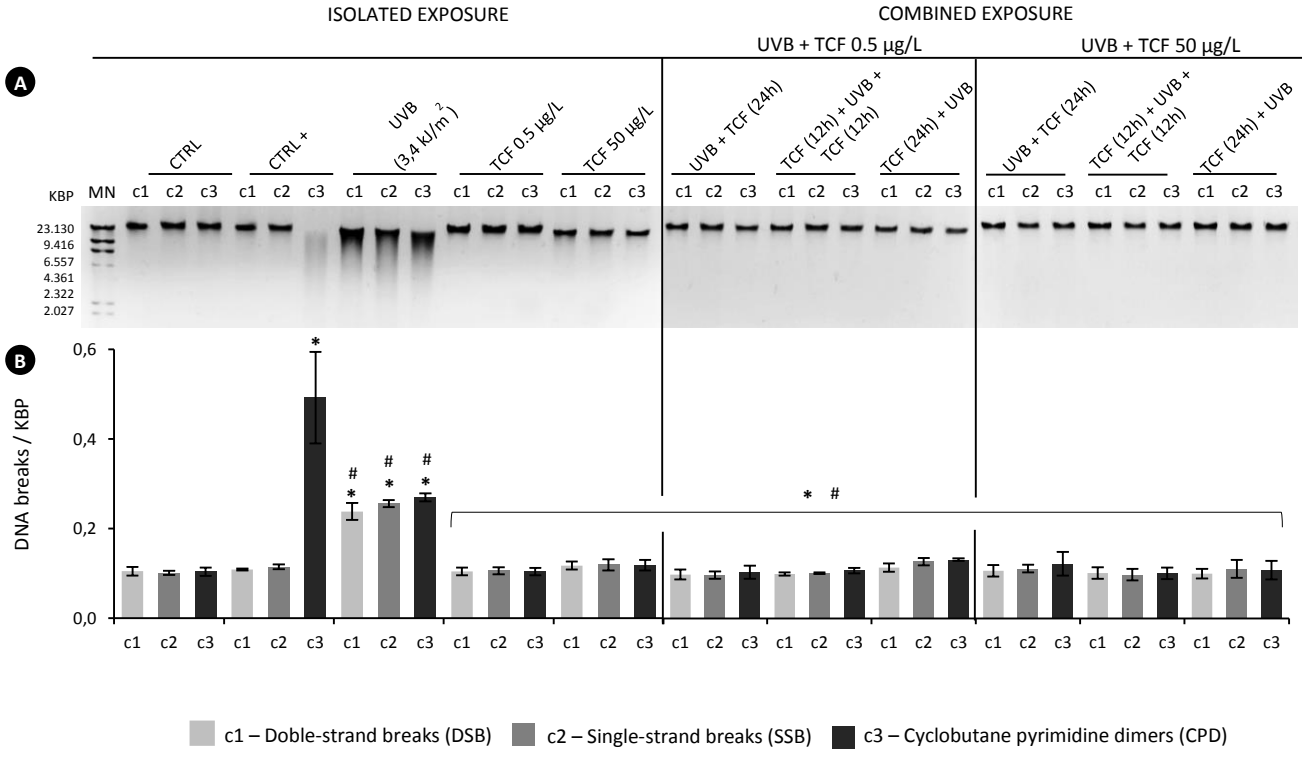
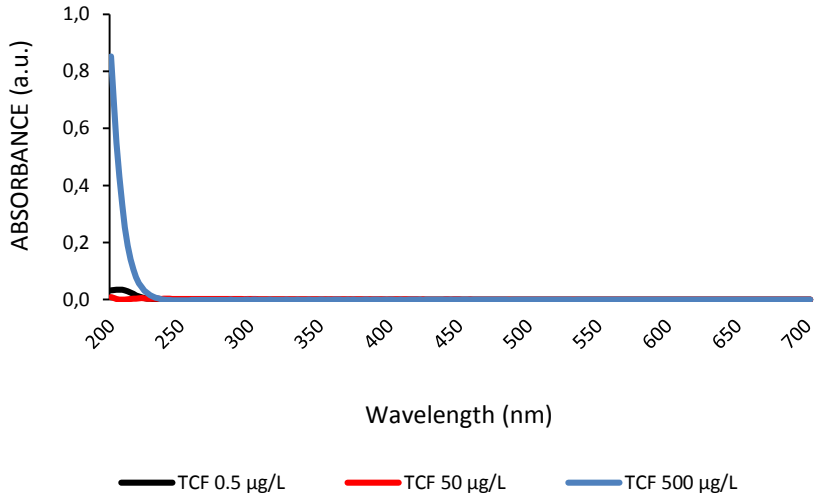


Figure 7. UV-Visible absorption spectrum (ʎ = 200 to 700 nm) of three different concentrations of the Trichlorfon (TCF) pesticide.



4 CONCLUSÃO

Em virtude dos diversos aspectos considerados nesta dissertação, nossos dados contribuem para salientar a alta sensibilidade dos anfíbios à radiação ultravioleta, principalmente de espécies arborícolas como a *Boana curupi.* Esta espécie, restrita a ambientes bem preservados, se mostrou altamente suscetível principalmente à radiação UVB, o que corrobora com a importância de preservar a mata ciliar para proteger estes animais da ação de agentes físicos, como a radiação UV solar. Da mesma forma, de acordo com dados previamente publicados em periódicos acadêmicos internacionais, agrotóxicos como o trichlorfon podem contribuir para a vulnerabilidade dos indivíduos expostos em função do desencadeamento de estresse metabólico e celular.

Mapear a dinâmica dos diversos elementos envolvidos na aceleração do declínio dos anfíbios é uma tarefa bastante complexa e esta complexidade se intensifica ao imaginarmos as interações possíveis quando consideramos os diversos poluentes ambientais presentes no meio natural. Muitos pesquisadores consideram o declínio dos anfíbios um processo enigmático, por ocorrer em ambientes muito bem preservados. Nesta pesquisa, podemos considerar como fator "enigmático" os resultados obtidos da exposição combinada entre UVB e ambas as concentrações de TCF, visto que os efeitos maléficos causados pela exposição isolada da radiação UVB foram muito amenizados quando considerado a interação destes dois agentes. Ademais, a utilização de doses subletais mensuradas no meio ambiente são de suma relevância, ao passo que isto aproxima o entendimento das interações entre anfíbios e os agentes estressores com as situações ocorrentes no ambiente natural. Porém, *endpoints* mais sensíveis são necessários para a mensuração dos impactos resultantes da exposição de *B. curupi* a baixas doses de TCF e, sobretudo, para entender a dinâmica ocorrida durante as exposições combinadas.

5 PERSPECTIVAS

Com base nos resultados obtidos nesta dissertação, fica evidente as possibilidades e a necessidade de novos estudos a fim de compreendermos melhor a interação entre a espécie *B. curupi*, a radiação ultravioleta e o agrotóxico trichlorfon. Diante desta perspectiva, sugerimos a realização de um ensaio considerando a exposição crônica das larvas aos agentes estressores, assim como a adoção de doses mais altas de TCF para análise das respostas biológicas das larvas de *B. curupi*, a fim de confirmar a toxicidade verificada em outros ensaios ecotoxicológicos (LI, et al., 2017; MA et al., 2019). No entanto, em nosso próximo experimento, pretendemos expor células humanas proficientes e deficientes em reparo de DNA a um gradiente de concentração de TCF e doses de radiação UVB para investigar a possível ação deste composto em mecanismos de reparo de DNA e o impacto na sobrevivência dessas células. Por fim, também torna-se necessário a realização do sequenciamento do transcriptoma das larvas tratadas com radiação UV, TCF e principalmente com as exposições combinadas dos dois agentes, para entendermos melhor quais vias estão de fato sendo ativadas (ou suprimidas) e porque isto proporciona o aumento da sobrevivência desses indivíduos após exposição à radiação UVB.

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