

UNIVERSIDADE FEDERAL DO AMAZONAS
INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

**Ecotoxicologia em tempos de emergência climática: Os impactos da
contaminação agroquímica em girinos amazônicos sob estresse térmico**

Guilherme de Azambuja Pereira

Manaus, Amazonas

Fevereiro, 2026

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contaminação agroquímica em girinos amazônicos sob estresse térmico**

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Resumo

O declínio global das populações de anfíbios é impulsionado por múltiplos estressores antropogênicos, com destaque para a poluição química e as mudanças climáticas. Na Amazônia, a conversão de florestas em áreas agrícolas intensificou o uso de pesticidas, coincidindo com projeções de aumento da temperatura regional. Nesse contexto, esta tese investigou os efeitos isolados e combinados do inseticida carbamato metomil e do aquecimento global projetado pelo IPCC sobre girinos de espécies nativas da Amazônia, explorando ao longo de quatro capítulos aspectos relacionados à revisão do cenário atual, toxicidade aguda e crônica, alterações comportamentais e neuroendócrinas, e custos do desenvolvimento larval. No primeiro capítulo, através de uma revisão bibliográfica, diagnosticamos a escassez de dados ecotoxicológicos para espécies amazônicas e discutimos como a fisiologia térmica de ectotérmicos tropicais pode torná-los particularmente vulneráveis à interação sinérgica entre contaminantes e altas temperaturas. No segundo capítulo, determinamos a letalidade aguda em *Osteocephalus taurinus* e *Scinax ruber*. Observamos que o aumento da temperatura (30°C) potencializou significativamente a toxicidade do metomil para *O. taurinus*, corroborando a hipótese da sensibilidade tóxica induzida pelo clima, enquanto *S. ruber* se mostrou mais sensível ao pesticida independente da temperatura, sem alterações na tolerância térmica crítica (CT_{max}) para ambas as espécies. No terceiro capítulo, avaliamos respostas subletais comportamentais e fisiológicas. Enquanto *O. taurinus* exibiu letargia, preferência por substratos escuros e um aumento inesperado na atividade da acetilcolinesterase (AChE) sob estresse térmico e químico, *S. ruber* manteve estabilidade comportamental e enzimática, mas apresentou elevação significativa de cortisol, indicando estratégias de enfrentamento distintas entre as espécies. No quarto capítulo, investigamos os efeitos crônicos em *Leptodactylus knudseni* ao longo da metamorfose. A exposição ao metomil acelerou o desenvolvimento larval, porém resultou em indivíduos menores e com menor massa corporal, evidenciando um *trade-off* energético. Houve inibição da AChE e ativação do sistema antioxidante (aumento de superóxido dismutase - SOD), sugerindo custos metabólicos de desintoxicação. Concluimos que a interação entre pesticidas e aquecimento global afeta os girinos amazônicos de maneira complexa e espécie-específica, variando desde a mortalidade

aguda até custos ocultos no desenvolvimento e na fisiologia, o que demanda a inclusão de variáveis térmicas nas avaliações de risco ambiental para a conservação da biodiversidade na região.

Palavras-chave: Agrotóxico; Amazônia; Anfíbios; Metomil; Mudanças climáticas

Abstract

The global decline of amphibian populations is driven by multiple anthropogenic stressors, most notably chemical pollution and climate change. In the Amazon, the conversion of forests into agricultural land has intensified pesticide use, coinciding with projections of rising regional temperatures. In this context, this thesis investigated the isolated and combined effects of the carbamate insecticide methomyl and IPCC-projected global warming on tadpoles of native Amazonian species. Across four chapters, we explored aspects related to a review of the current scenario, acute and chronic toxicity, behavioral and neuroendocrine alterations, and larval development costs. In the first chapter, through a literature review, we diagnosed the scarcity of ecotoxicological data for Amazonian species and discussed how the thermal physiology of tropical ectotherms may render them particularly vulnerable to synergistic interactions between contaminants and high temperatures. In the second chapter, we determined acute lethality in *Osteocephalus taurinus* and *Scinax ruber*. We observed that elevated temperature (30°C) significantly potentiated methomyl toxicity for *O. taurinus*, corroborating the climate-induced toxic sensitivity hypothesis, whereas *S. ruber* proved to be more sensitive to the pesticide regardless of temperature, with no alterations in critical thermal maximum (CT_{max}) for either species. In the third chapter, we evaluated sublethal behavioral and physiological responses. While *O. taurinus* exhibited lethargy, a preference for dark substrates, and an unexpected increase in acetylcholinesterase (AChE) activity under thermal and chemical stress, *S. ruber* maintained behavioral and enzymatic stability but showed a significant elevation in cortisol, indicating distinct coping strategies between the species. In the fourth chapter, we investigated chronic effects in *Leptodactylus knudseni* throughout metamorphosis. Exposure to methomyl accelerated larval development but resulted in smaller individuals with lower body mass, evidencing an energetic trade-off. There was an inhibition of AChE and activation of the antioxidant system (increased superoxide dismutase - SOD), suggesting metabolic costs of detoxification. We conclude that the interaction between pesticides and global warming affects Amazonian tadpoles in a complex and species-specific manner, ranging from acute mortality to hidden developmental and physiological costs. This underscores the

need to include thermal variables in environmental risk assessments for biodiversity conservation in the region.

Keywords: Amazon; Amphibians; Climate change; Methomyl; Pesticide

Sumário

Abstract.....	8
Introdução geral.....	12
Capítulo 1. A Double Threat to Amazonian Amphibian Larvae: A Review of Toxic Contaminants and Their Interaction with Global Climate Change.....	19
1. Introduction.....	22
2. Amazonia and its frog diversity.....	24
3. Aquatic contamination and effects on anurans.....	26
3.1. Agriculture and the use of pesticides.....	26
3.2. Metals and metalloids.....	34
3.3. Microplastics (MP).....	36
4. Interaction between temperature and contaminants.....	38
5. Implications of deleterious effects for tadpoles.....	41
6. Conclusion.....	43
7. References.....	44
8. List of tables.....	69
Capítulo 2. <i>Rising Temperature Increases Methomyl (Lannate® BR) Toxicity in Amazonian Frog Tadpoles: Impacts on Survival and Thermal Limits</i>	80
1. Introduction.....	83
2. Materials and Methods.....	84
2.1. Tadpole collection and maintenance.....	84
2.2. Determination of lethal concentration (LC ₅₀) of methomyl at different temperatures.....	85
2.3. Effect of sublethal exposure to methomyl at the critical thermal maximum (CT _{max}) in tadpoles.....	86
2.4. Statistical analysis.....	86
3. Results.....	87
3.1. Determination of lethal concentration (LC ₅₀) of methomyl at different temperatures.....	87
4. Discussion.....	87
5. Conclusion.....	91
6. Acknowledgments.....	91
7. Funding.....	92
8. Disclosure of interest.....	92
9. Data availability statement.....	92
10. References.....	92
10. List of Figures.....	100
Capítulo 3. <i>Combined Impacts of Warming and Methomyl on Neurophysiological and Behavioral Responses in Amazonian Frog Tadpoles</i>	103
1. Introduction.....	106

2. Materials and Methods.....	108
2.1. Clutch collection and maintenance.....	108
2.3. Exposure to sublethal concentrations of methomyl at different temperatures	109
2.4. Preparation of alarm substance.....	111
2.5. Behavioral assessment of tadpoles.....	111
2.7. Statistical analyses.....	113
3. Results.....	114
3.1. Survival after sublethal exposure.....	114
3.2. Substrate preference.....	114
3.3. Swimming activity.....	114
3.4. Resting behavior.....	114
3.5. Alarm substance response.....	115
3.6. Acetylcholinesterase activity.....	115
3.7. Cortisol response to stressors.....	115
4. Discussion.....	116
5. Study limitations.....	120
6. Conclusion.....	121
7. Ethics statement.....	122
8. Acknowledgments.....	122
9. References.....	123
10. List of figures.....	135
Capítulo 4. <i>Morphophysiological Impacts of Methomyl and Warming on Frog Tadpoles (Leptodactylus knudseni)</i>	137
1. Introduction.....	140
2. Materials and Methods.....	142
2.1. Tadpoles collection and maintenance.....	142
2.2 Chronic exposure to sublethal concentrations of methomyl at different temperatures.....	143
2.3. Morphological and developmental assessments.....	144
2.4. Preparation of homogenates.....	144
2.5. Antioxidant enzymes.....	145
2.6. Acetylcholinesterase (AChE) activity.....	145
2.7. Statistical analyses.....	145
3. Results.....	146
3.1. Effects of temperature and methomyl on morphometric traits.....	147
3.2. Acetylcholinesterase (AChE) inhibition and antioxidant enzyme modulation under chemical and thermal stress.....	148
4. Discussion.....	148
5. Conclusion.....	153
6. Permissions.....	154
7. Acknowledgments.....	154

8. Disclosure of interest.....	154
9. Data availability statement.....	154
10. List of figures and tables.....	155
Considerações finais.....	167
Referencias bibliográficas.....	169
Anexos.....	173

Introdução geral

Nas últimas décadas, os anfíbios têm apresentado declínios populacionais acentuados em escala global, tornando-se a classe de vertebrados mais ameaçada do planeta (Luedtke et al., 2023). O crescimento populacional e a intensificação da exploração de recursos geram impactos severos, resultando em 41% das espécies atualmente ameaçadas de extinção. Esse declínio dramático reflete a convergência de múltiplos estressores antrópicos (i.e. perda de habitat, contaminação ambiental e mudanças climáticas) (Bishop et al., 2012; Grant et al., 2016; Green et al., 2020). A perda de habitat permanece como a ameaça mais comum, afetando 93% das espécies ameaçadas, impulsionada principalmente pela expansão agrícola. Além disso, as mudanças climáticas emergem como uma ameaça crescente, já impactando 30% das espécies ameaçadas, enquanto doenças e a exploração predatória continuam a pressionar populações vulneráveis (Luedtke et al., 2023). A vulnerabilidade dos anfíbios a esses estressores ocorre devido às suas características morfofisiológicas: ovos sem casca, pele altamente permeável e um ciclo de vida bifásico que inclui uma fase larval aquática e uma fase adulta terrestre na maioria das espécies (Slaby et al., 2019). Durante o estágio larval, girinos apresentam uma morfologia altamente especializada para o ambiente aquático, com sistemas respiratório, digestório e locomotor adaptados exclusivamente a esse meio (MacDiarmid; Altig, 1999). A transição para a forma adulta ocorre através de uma complexa metamorfose, que envolve remodelação tecidual, reorganização fisiológica e alterações comportamentais (Paul et al., 2022; Tata, 2006). Do ponto de vista energético, a metamorfose representa um dos períodos de maior custo metabólico no ciclo de vida dos anfíbios. Durante o estágio larval, os girinos precisam crescer e acumular recursos para o clímax metamórfico. Nesta fase crítica, quando a alimentação cessa e a cauda é reabsorvida, a demanda energética é suprida por diferentes substratos dependendo da espécie, podendo incluir o catabolismo de proteínas da cauda ou, em alguns casos, reservas lipídicas acumuladas (Beck; Congdon, 2003; Shi, 2000; Zhang et al., 2024; Zhu et al., 2024). Esse acúmulo prévio de energia depende diretamente da capacidade do girino de se alimentar continuamente, mantendo taxas eficientes de digestão e conversão dos recursos ingeridos. A formação dessas reservas é vital para o adequado rearranjo estrutural e

fisiológico para metamorfosear de um organismo aquático para um adulto terrestre (Paul et al., 2022; Yaoita, 2019). Qualquer perturbação que reduza a ingestão alimentar, impeça a digestão, aumente a demanda metabólica ou redirecione energia para mecanismos fisiológicos de reparação pode comprometer diretamente o sucesso metamórfico. Embora girinos exibam plasticidade fenotípica na qual conseguem ajustar o tempo de metamorfose em condições adversas (Miner et al., 2005; Steiner; Van Buskirk, 2008), o custo energético do processo é amplamente relatado, frequentemente resultando em juvenis de menor tamanho corporal e menor desempenho (Yagi; Green, 2018). Assim, o equilíbrio entre crescimento e tempo metamórfico é extremamente delicado e dependente da estabilidade ambiental.

Ectotérmicos, os anfíbios são altamente suscetíveis às mudanças de temperatura ambiental. O aquecimento global tem resultado em períodos mais longos de seca, intercalados com chuvas mais intensas, o que altera os regimes de precipitação e aumenta a vulnerabilidade a eventos climáticos extremos, como secas prolongadas e inundações repentinas. O ano de 2023 apresentou anomalias recordes na temperatura média global do ar à superfície em relação ao período pré-industrial, com alta frequência de dias acima de 1,0°C e numerosos episódios próximos ou acima de 1,5°C. Além disso, cerca de 50% dos dias de 2023 registraram temperaturas 1,5°C mais altas do que o período pré-industrial, com uma média global de 1,48°C acima da média histórica (Copernicus, 2023). Com isso, sem medidas urgentes de mitigação, é esperado que o limite de 1,5°C proposto pelo Acordo de Paris seja ultrapassado entre 2021 e 2040 (Masson-Delmotte; Zhai; Pirani, 2021). Modelos climáticos indicam um prolongamento da estação seca e maior frequência de secas severas na bacia amazônica (Marengo et al., 2018), o que reduz a previsibilidade e a duração dos corpos d'água essenciais para o desenvolvimento larval dos anfíbios. Durante os eventos extremos de 2023, foram registradas temperaturas da água entre 38°C e 40°C em ambientes lênticos da região (Braz-Mota; Val, 2024). Assim, podemos inferir que poças temporárias de baixo volume e curta duração, como as utilizadas por diversas espécies de anfíbios, tornam-se ainda mais suscetíveis ao superaquecimento. Em muitos táxons tropicais, observa-se estreita margem de segurança térmica, frequentemente vivendo próximos aos seus limites fisiológicos (Deutsch et al., 2008). Essa condição os torna altamente vulneráveis ao

aquecimento global, visto que qualquer incremento térmico pode deslocar os indivíduos de sua faixa de desempenho ótimo para zonas de estresse fisiológico severo. Essa realidade foi observada durante o desenvolvimento desta tese, onde a severidade da seca de 2023 restringiu a ocorrência de espécies historicamente comuns na região, evidenciando como eventos extremos podem forçar mudanças rápidas na composição das comunidades e, conseqüentemente, nos modelos biológicos passíveis de estudo.

Aliado às mudanças climáticas, a demanda humana trouxe consigo outro grande problema: o uso de agrotóxicos para aumentar a produtividade agrícola. O uso crescente de pesticidas na agricultura tem gerado uma contaminação aquática preocupante. O Brasil é um dos maiores consumidores de pesticidas do mundo (FAO, 2025). Entre 2010 e 2020, as vendas de agrotóxicos na região amazônica cresceram substancialmente, com produtos como o glifosato, o mancozeb e a atrazina sendo amplamente utilizados nas práticas agrícolas.

Na região amazônica um dos agrotóxicos utilizados é o inseticida metomil (IBAMA, 2025). Classificado como um pesticida de extrema toxicidade (Classe I), o metomil é um carbamato sistêmico de ação rápida, amplamente utilizado na agricultura devido à sua eficácia no controle de uma grande variedade de pragas (Van Scoy et al., 2013; IBAMA, 2025). O composto apresenta alta solubilidade em água e uma baixa afinidade por solos, o que favorece sua lixiviação e transporte via escoamento superficial para os corpos d'água adjacentes às áreas de cultivo (Van Scoy et al., 2013; Lin et al., 2020). Esse fator é crítico em regiões tropicais, onde episódios de chuvas intensas podem elevar rapidamente a concentração do contaminante em poças temporárias, habitats essenciais para o desenvolvimento de larvas de anuros.

O metomil age primariamente inibindo a atividade da acetilcolinesterase (AChE), essencial para a transmissão de sinais nervosos nos organismos (Van Scoy et al., 2013). A inibição dessa enzima resulta no acúmulo de acetilcolina, levando a falhas nos sinais nervosos e, em casos graves, pode causar morte (Colović et al., 2013). Nos girinos, essa neurotoxicidade compromete funções motoras básicas, o que pode interferir não apenas no comportamento de natação e fuga de predadores, mas também na sobrevivência e no processo de metamorfose, comprometendo o

desenvolvimento dos organismos (Trachantong et al., 2017). Além disso, em organismos ectotérmicos, a toxicidade de carbamatos como o metomil pode ser exacerbada pelo aumento da temperatura ambiental, que acelera as taxas de absorção cutânea e o metabolismo basal, tornando a interação entre calor e contaminação um desafio fisiológico adicional.

A interação entre estresse térmico e contaminação química configura um cenário crítico para organismos ectotérmicos. O aumento da temperatura eleva as taxas metabólicas, o que não apenas incrementa a demanda energética, mas também pode potencializar a absorção e a toxicidade de pesticidas (Noyes et al., 2009). Para os girinos, o custo energético desviado para a detoxificação do metomil, por exemplo, somado ao metabolismo basal acelerado pelo calor, cria um déficit severo que compromete alocações vitais para o desenvolvimento. Consequentemente, observam-se prejuízos na formação de reservas, na metamorfose e em comportamentos críticos, como a alimentação e a evasão de predadores (Beck; Congdon, 2003; Lajmanovich; Sandoval; Peltzer, 2003). O resultado são indivíduos menores, e com menor aptidão, o que pode gerar repercussões negativas para a dinâmica populacional em ambientes tropicais alterados (Bishop et al., 2012; Venesky; Wassersug; Parris, 2010).

Apesar da gravidade desses efeitos combinados, a literatura ecotoxicológica apresenta uma desproporção histórica, sendo predominantemente voltada para ecossistemas temperados. Essa assimetria gera uma lacuna crítica para a conservação em regiões tropicais (Kwok et al., 2007; Wang et al., 2019). Inferir riscos para a biodiversidade amazônica utilizando modelos ecotoxicológicos desenvolvidos para espécies-teste de clima temperado pode subestimar severamente a vulnerabilidade de espécies que já operam com margens de segurança térmica reduzidas e vivem próximas aos seus limites fisiológicos (Kwok et al., 2007; Pottier et al., 2025). Na Amazônia, dados sobre os efeitos combinados do aquecimento e de contaminantes como o metomil são inexistentes. Ignorar as especificidades fisiológicas dos anuros amazônicos e suas respostas a concentrações subletais impede uma avaliação realista dos riscos ambientais. Consequentemente, há uma urgência em compreender como esses organismos, com suas adaptações ecológicas únicas, responderão ao desafio duplo de um clima em aquecimento e habitats

agricolamente impactados (Wang et al., 2019).

Diante deste cenário, esta tese testa a hipótese de que o aumento de temperatura amplifica os efeitos letais e subletais do metomil em girinos amazônicos. Para isso, foram utilizadas como modelos duas espécies de pererecas (Hylidae), *Osteocephalus taurinus* Steindachner, 1862 e *Scinax ruber* (Laurenti, 1768), e uma espécie de rã (Leptodactylidae), *Leptodactylus knudseni* Heyer, 1972 (Fig. 1).

O. taurinus é um hylídeo de grande porte (machos 71-92 mm, fêmeas 90-101 mm), amplamente distribuído pela Bacia Amazônica e pelas Guianas. A espécie ocorre tanto em florestas primárias quanto secundárias. Sua reprodução ocorre preferencialmente após chuvas fortes, com desovas que chegam a cerca de 2.000 ovos, e são depositadas em forma de película na superfície de poças temporárias (Lima et al. 2008; Frost 2026). Seus girinos possuem corpo globoso e olhos em posição dorsal, característica que favorece a interação com a superfície da água. Ecologicamente, as larvas são caracterizadas pelo hábito oofágico, atuando como predadores em poças isoladas de floresta e bordas de mata (Schiesari et al., 2022).

S. ruber é um hylídeo de pequeno a médio porte (machos 31–37 mm, fêmeas 40–42 mm), com ampla distribuição geográfica que abrange a Bacia Amazônica, as Guianas e partes da América Central. Ao contrário de espécies estritamente florestais, *S. ruber* é comum em áreas abertas, bordas de clareiras e ambientes antropizados, demonstrando grande plasticidade ecológica. Sua reprodução intensifica-se na estação chuvosa, com desovas de cerca de 600 ovos, que são aderidas à vegetação ou às margens de poças temporárias (Lima et al. 2008; Frost 2026). Seus girinos possuem olhos em posição lateral e nadadeiras altas, características que facilitam o deslocamento por toda a coluna d'água. Essas larvas são frequentemente encontradas em poças tanto em áreas de mata quanto em terrenos desmatados, apresentando uma coloração que varia do marrom-claro ao amarelado, com o ventre prateado (Schiesari et al., 2022).

L. knudseni é um leptodactilídeo de grande porte (machos 117–135 mm, fêmeas 113–132 mm), amplamente distribuído pela Bacia Amazônica e Guianas. Com hábitos terrestres e noturnos, a espécie ocorre tanto no interior quanto na borda de florestas. Durante a reprodução, aproximadamente 1.000 ovos são depositados em ninhos de espuma, construídos em bacias escavadas pelos machos próximo a poças

temporárias. Após a eclosão, os girinos completam o desenvolvimento nos corpos d'água (Gascon 1991; Heyer and Heyer 2006; Lima et al. 2008; Frost 2026). Seus girinos são robustos, atingindo cerca de 70 mm de comprimento total, com olhos pequenos em posição dorsal. Ecologicamente, as larvas possuem hábitos bênticos e coloração críptica, camuflando-se no substrato lodoso ou folhoso. Além do hábito oofágico e necrofágico, os girinos apresentam locomoção serpentina, o que lhes permite sobreviver e se deslocar entre a espuma e a lama em ambientes em processo de dessecação (Schiesari et al., 2022).

Essas espécies foram selecionadas com base em sua ocorrência na região de Manaus e na disponibilidade local dos girinos durante o período amostral (entre os anos de 2022 e 2024), o que possibilitou uma abordagem comparativa entre anuros comuns em habitats amazônicos. Adicionalmente, *O. taurinus* e *S. ruber* permitem explorar respostas sob diferentes contextos ambientais, uma vez que foram obtidas em áreas com distintos graus de conservação. Entretanto, a severidade da seca histórica de 2023, intensificada pela sinergia entre o fenômeno El Niño e as mudanças climáticas globais, reduziu drasticamente a detectabilidade de *O. taurinus* e *S. ruber* no período final de amostragem. Esse cenário extremo motivou a inclusão de *L. knudseni* como modelo biológico para as investigações de efeitos crônicos, visto que suas adaptações à dessecação possibilitaram a coleta e o estudo em um período de anomalia climática recorde. Diante deste cenário, a presente tese visa preencher essas lacunas ao investigar os efeitos isolados e combinados do aumento de temperatura e da exposição ao metomil no ciclo de vida de girinos amazônicos.

Para atender a esse objetivo principal, a pesquisa está estruturada nos seguintes capítulos:

Capítulo 1: Revisão do estado atual do conhecimento sobre os efeitos de contaminantes ambientais e sua relação com as mudanças climáticas, em anfíbios tropicais, com ênfase na Amazônia, e identificação das principais lacunas na área.

Capítulo 2: Determinação da concentração letal (LC_{50}) do metomil em girinos de *Osteocephalus taurinus* e *Scinax ruber* a 26,5°C e 30°C, com avaliação dos efeitos da exposição subletal na temperatura crítica máxima (CT_{max}) dessas espécies.

Capítulo 3: Investigação dos impactos do metomil e do aumento de temperatura sobre respostas neurofisiológicas e endócrinas, incluindo a inibição de

acetilcolinesterase e a elevação de cortisol, e como esses fatores afetam o comportamento e a sobrevivência dos girinos de *O. taurinus* e *S. ruber*.

Capítulo 4: Avaliação dos efeitos crônicos do metomil sobre o crescimento, morfologia e defesas antioxidantes em girinos de *Leptodactylus knudseni*, com foco na interação entre estresse térmico e contaminação química.

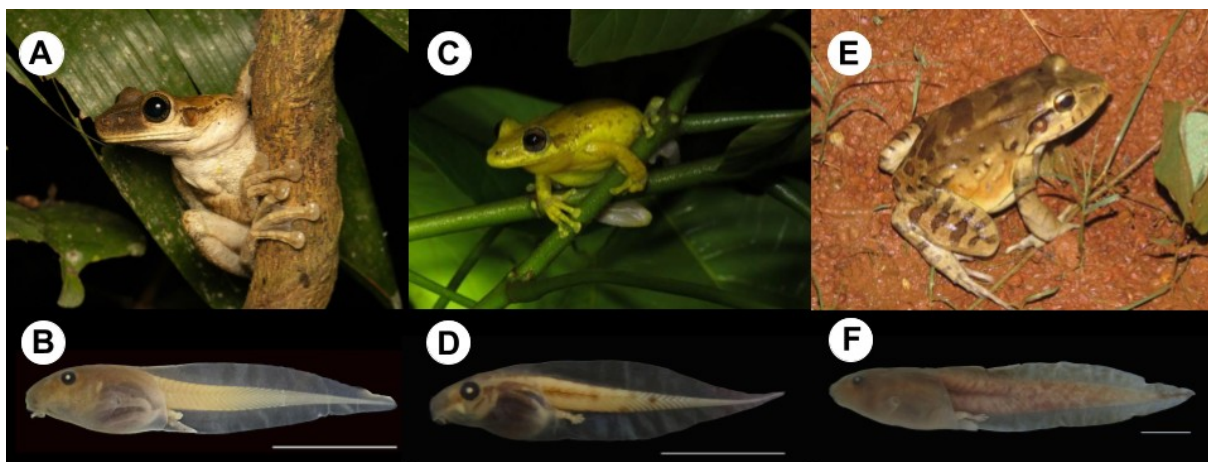


Figura 1. Exemplos adultos e girinos das espécies de anuros utilizados no estudo. **(A-B)** *Osteocephalus taurinus*; **(C-D)** *Scinax ruber*; **(E-F)** *Leptodactylus knudseni*. As barras de escala representam 10 mm. **Fontes:** **(A)** © Sebastian Doak; **(C, E)** © Vincent A. Vos (via iNaturalist). **(B, D, F)**: *Girinos do Brasil* (Rossa-Feres et al., 2024), Anolis Books.

Capítulo 1

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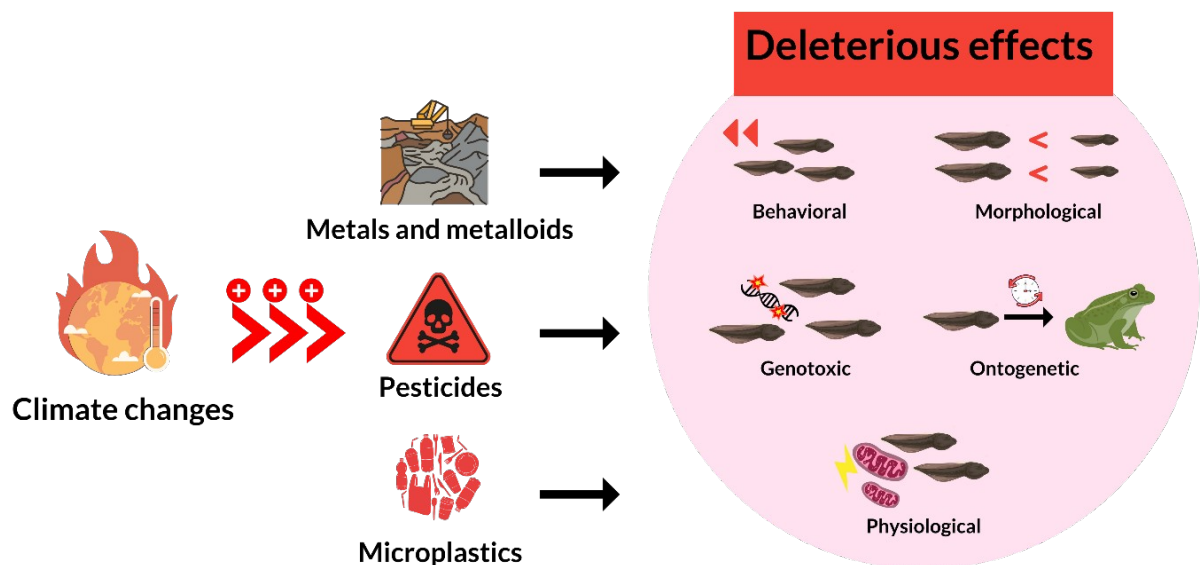
A double threat to Amazonian amphibian larvae: a review of toxic contaminants and their interaction with global climate change

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Graphical abstract. Major categories of negative effects resulting from aquatic contamination in amphibians, with a particular focus on how climate change interacts with contaminants, amplifying their deleterious effects.

Abstract

Amazonia holds global significance due to its essential role in climate regulation and its high biological diversity. However, in recent decades, the region has faced serious threats to its forest structure and rich biodiversity. Human activities, including habitat alterations, pollution, introduction of invasive species, pathogens, and accelerated climate change, have been identified as primary factors contributing to the decline of various populations in these environments. Approximately 76% of amphibian species are estimated to occur in tropical forests, highlighting their significance and the potential for discovering new species. Conversely, knowledge about the effects of environmental contamination in the region remains scarce, which is particularly concerning given the current scenario of climate change. Contaminants such as pesticides and microplastics can induce a range of morphological, physiological, and behavioral alterations in frogs and toads), especially during larval stages. When coupled with climate change, where rising temperatures play a pivotal role, these factors pose intricate challenges for amphibians, intensifying adverse effects and contributing to population declines. Here, we provide a review of the impacts of environmental pollution on various aspects of anuran amphibians, providing insight into the current state of knowledge regarding Amazonia and highlighting knowledge gaps.

Keywords: Ecotoxicology, Metal, Microplastic, Pesticide, Tadpole, Temperature

1. Introduction

The Neotropics harbor the highest amphibian species richness among biogeographical regions (Frost 2023), and Brazil is the world's leading country in amphibian diversity (Guerra et al. 2020). The decline of anuran (frogs and toads) populations has been reported in urban, rural, and pristine areas at least since the 1940s (Bragg 1952; Gardner 2001; Carnaval et al. 2006) throughout the globe and, nowadays, Amphibia is the tetrapod group with the highest proportion of species under extinction risk. Presently, over 40% of the amphibian species are listed as globally threatened in the Red List of Threatened Species (Luedtke et al. 2023). The scientific community gained significant awareness of this issue in the early 1990s during the First World Congress of Herpetology. Researchers from around the globe presented findings on population declines occurring on multiple continents, leading to heightened attention (Green et al. 2020). There is an emerging consensus that the decline of amphibians is intricately linked to the onset of the Anthropocene era and intensified human activity. The factors contributing to the decline of these populations are identified as: i) habitat modification; ii) invasive species; iii) pollution; iv) exploitation; and v) climate change (Bishop et al. 2012; Grant et al. 2016; Balvanera et al. 2020; McRae et al. 2020). The synergistic effects of these impacts exacerbate the already alarming situation for amphibians. Climate change impacts are heightened by alterations in land use, such as deforestation for agricultural production and urbanization. These changes can synergistically interact with pollutants, significantly amplifying their detrimental effects on amphibians. The projected global temperature for the period 2081-2100 is anticipated to be around 1°C to 1.8°C higher than the baseline period of 1850-1900 under the low greenhouse gas emission (GHG) scenario. Under the intermediate GHG emission scenario, the projected temperature increase ranges from 2.1°C to 3.5°C, while under the high GHG emission scenario, it is projected to be between 3.3°C and 5.7°C (Masson-Delmotte et al. 2021). However, without urgent mitigation measures, in all scenarios, the 1.5°C limit proposed by the Paris Agreement is expected to be surpassed between 2021 and 2040 (Masson-Delmotte et al. 2021). In 2023, it has been confirmed as the warmest calendar year on record since 1850. For the first time in a given year, every single day exceeded 1°C above the pre-industrial level average. Moreover, approximately 50% of the days

experienced temperatures 1.5°C higher than the pre-industrial period. The global mean temperature for 2023 stands as the highest on record, with an increase of 0.60°C above the 1991-2020 average and 1.48°C above the pre-industrial average of 1850-1900 (Copernicus Climate Change Service 2023). The heightened vulnerability of amphibians to anthropogenic changes can be attributed to intrinsic morphophysiological characteristics of the group that include pronounced water or moisture dependence due to their unshelled eggs, aquatic larval phase (tadpoles), and highly permeable skin in most species (Slaby et al. 2019). In comparison to other vertebrate groups, the life cycle of anurans is notably complex. It encompasses a wide range of reproductive modes, in which tadpoles can develop in either terrestrial or aquatic environments, or even be absent altogether in certain direct-developing species (Nunes-de-Almeida et al. 2021). The larval phase is considered a critical period in the development of anurans, as tadpoles undergo significant morphological and physiological changes while they transform into their adult form (Yaoita 2019). During the larval stage, tadpoles develop morphological structures suited to the aquatic environment, including fins and external gills (McDiarmid and Altig, 1999). The transformation into an adult tetrapod capable of walking, jumping, and feeding on prey demands extensive external and internal morphological and physiological rearrangements, such as loss of gills and development of lungs, the formation of a urogenital apparatus, the rearrangement and ossification of skeletal elements, and modifications to the digestive tract (Tata 2006; Yaoita 2019). Concomitant with these changes, the highly permeable skin of amphibians, which enables rapid passage of oxygen and water, also facilitates the absorption of xenobiotics.

These foreign substances can diffuse into amphibians at a remarkably faster rate, ranging from one to two orders of magnitude (depending on the species' hydrophobicity), compared to mammals. This increased permeability makes amphibians highly sensitive organisms to anthropogenic activities (Quaranta et al. 2009). While they serve as excellent bioindicators for assessing environmental quality, and recent studies have highlighted the effects of freshwater contamination on physiological, morphological, and behavioral changes in amphibians, there is still a knowledge gap when compared to other freshwater organisms. Deleterious effects of different classes of contaminants have been demonstrated in recent years, with

notable variations in their impacts observed throughout different stages of ontogenetic development, geographic regions, and species (Table 1). While scientific literature frequently focuses on examining the effects of individual contaminants, it is crucial to understand how species in their natural environments, influenced by multiple stressors simultaneously, will respond. In the aquatic environment, anurans are exposed to a mixture of different contaminants, as well as fluctuations in temperature, pH, oxygen levels, and UV radiation. Recent studies have documented both lethal and sublethal effects of contaminants combined with abiotic factors on anurans, including: (i) changes in tadpole hatching rates (Adams et al. 2021), (ii) increased mortality (Vanzetto et al. 2019), (iii) behavioral changes, such as alterations in swimming speed, activity patterns and habitat choice (Sievers et al. 2018), (iv) physiological changes, including shifts in tadpole development time (Adelizzi et al. 2019), which can influence the size of the juvenile individual, as well as metabolic changes, v) morphological alterations, such as reduction in body size (Dulumoni et al. 2020), and structural alterations, both externally (deformities) and internally (changes in internal organs) (Rathanayaka and Rajakaruna 2018) and vi) cellular-level changes, such as an increase in micronuclei in cells (Herek et al. 2021), and modifications in the antioxidant and defense profiles of tadpoles (Rutkoski et al. 2020). These studies highlight the wide range of effects that contaminants combined with abiotic factors can have on anurans, affecting their development, behavior, physiology, morphology, and overall health.

2. Amazonia and its frog diversity

The global importance of the Amazonian biome is widely acknowledged due to its critical role in climate regulation and its biological diversity (Fearnside 2022). Amazonia plays a significant part in the hydrological cycle of South America by recycling wind-borne moisture from the evaporation of the tropical Atlantic Ocean. Approximately 35% of this moisture is recycled within the region, favoring local precipitation, and contributing to the maintenance of the forest ecosystem (Dirmeyer et al. 2009; Zemp et al. 2014). Indeed, Amazonia has a significant contribution to the hydrological cycle beyond its local impact. During the wet season, wind patterns create a flow of moisture from the Andes eastward towards the subtropical regions, providing

water vapor to the Midwest, Southeast, and South regions of Brazil (Arraut et al. 2012). This highlights the role of the biome in providing water resources even to geographically distant areas. Another crucial function of Amazonia is its capacity to store atmospheric carbon, which contributes to the mitigation of greenhouse gasses. It is estimated that the region holds between 350-600 Pg (petagrams) of carbon and experiences an annual loss of 1.1 Pg of carbon per year due to deforestation and forest degradation (Baccini et al. 2012; Silva Junior et al. 2018). This emphasizes the importance of the Amazon Forest for climate regulation.

The importance of Amazonian forests and associated ecosystems go beyond their recognized significance. Unfortunately, intense land use changes over the past few decades have posed a serious threat to the forest's structure, rich biodiversity, and ecosystemic services. Within just 35 years (1985-2021), approximately 44 million hectares (13%) of the native area have been converted for agricultural activities (Projeto MapBiomass 2022). Deforestation in Amazonia has surged to historic levels in recent years, escalating from 4,545 km² in 2018 to 9,696 km² in 2022, representing a more than 143% increase since 2012 (Leite-Filho et al. 2021; PRODES 2023). Anthropogenic landscape changes associated with urbanization, agriculture and cattle ranching have profoundly transformed ecosystems, leading to pervasive landscape and biotic homogenization that negatively impacts the abundance and diversity of amphibian populations and assemblages (Cordier et al. 2021; Fulgence et al. 2022).

Additionally, the region faces a persistent problem of wildfires. The year 2022 has seen a record number of active fires compared to 2021, with only February to April showing lower values due to high precipitation levels. As of September 2022, there have been 41,282 fires, a 147% increase compared to the same period in 2021. The cumulative number of fires in 2022 (87,304) has already been surpassed, reaching a total of 98,646 fires recorded thus far (INPE 2023). These fires, primarily ignited by human activities such as land clearance for agriculture, are facilitated by landscape modifications that elevate temperatures and reduce humidity, creating favorable conditions for the rapid spread of fire.

The characteristics of tropical forests such as Amazonia favor a magnificent diversity of anurans. Approximately 76% of the world's total amphibian species are estimated to inhabit tropical forests (Pillay et al. 2022). In a recent update, the

biodiversity census of the Brazilian Amazon reported 383 frog species in the region (Hoogmoed 2023) – a likely significant underestimate due to the vast area, challenging accessibility, and the potential for sampling bias (Verdade et al. 2011; Vacher et al. 2020). Furthermore, tropical, and subtropical moist broadleaf forests are projected to have the highest percentage of potential future amphibian species discoveries worldwide, particularly due to cryptic taxonomic diversity (Moura and Jetz 2021).

3. Aquatic contamination and effects on anurans

3.1. Agriculture and the use of pesticides

The rapid growth of the global human population in recent decades has led to extensive land use changes and increased demand for food and resources, which have had detrimental effects on amphibians (Balvanera et al. 2020). Agricultural activities play a significant role in these changes, with approximately 38% of the global land surface (five billion hectares) being dedicated to agriculture, of which 1.66 billion hectares are used for food production and 3.32 billion hectares are used for livestock grazing (FAO 2022). Contamination of the aquatic environment can arise from the agricultural sector, which annually consumes substantial quantities of fertilizers and pesticides (Brovini et al. 2021). Between 2000 and 2008, Brazil ranked third in pesticide use, following the United States and China, respectively claiming first and second place (IBAMA 2022). However, Brazil surpassed China in 2009, occupying the second spot (FAO 2022). The sale of pesticides in Brazil has witnessed an exponential growth year after year, with sales increasing by nearly 100% in just 11 years (2010-2021). Sales figures escalated from 361.66 tons in 2010 to 719.50 tons in 2021 (Fig. 1). In the same period, Brazilian Amazonia alone experienced an increase from 67.42 tons in 2010 to 179.36 tons in 2020 (IBAMA 2022). Despite the existence of several recommendations on pesticide use, such as specifying suitable crops, ideal plant stages, climatic conditions, dosage per hectare, and spacing between applications, these guidelines are often disregarded (Kotz et al. 2021).

Pesticides are frequently used indiscriminately, leading to the development of resistance in target organisms. Moreover, the application of pesticides in croplands does not confine their impact to this specific environment; instead, they can disperse

widely (Kjellstrom et al. 2006; Manisalidis et al. 2020). Soil serves as a significant reservoir for pesticides, as it accumulates a significant portion of these compounds during crop application. Soil characteristics, including texture, temperature, and pH, influence its capacity to absorb pesticides, thereby determining their persistence within the environment and their potential mobility into water bodies and other areas (Vryzas 2018). Various processes contribute to the dissipation of pesticides through the soil, such as leaching, runoff, and drainage. Additionally, these chemicals find their way into the aquatic environment through precipitation subsequent to their application in agricultural areas and via air currents during aerial spraying (Casara et al. 2012). The persistence of pesticides in the aquatic environment is further influenced by factors like water temperature, pH, radiation, and microbial community (Vryzas 2018).

The adverse impacts of contaminants on amphibians have been extensively documented (Trudeau et al. 2020), but there is a noticeable bias towards studies conducted in temperate regions (Ghose et al. 2014; Schiesari et al. 2007), particularly in North America (D'Avila et al. 2020; Carrasco et al. 2021). Conversely, there is a scarcity of information on this subject in tropical regions, which is concerning considering that amphibians are a dominant vertebrate group in these areas (Duellman et al. 1999; Ghose et al. 2014; Yang et al. 2022). Research on pesticide effects also tends to focus more on particular families such as Bufonidae, Hylidae, and Leptodactylidae, which alone do not represent the tropical anuran diversity (D'Avila et al. 2020). In Brazilian Amazonia, specifically, there is a significant knowledge gap in terms of ecotoxicological studies involving anurans, which mirrors the overall scarcity of such research in Brazil. Despite the region's high species richness, there is a wide array of pesticides used, totaling approximately 300 different compounds. Notably, the most commonly sold pesticides in Brazilian Amazonia include glyphosate, 2-4,D, mancozeb, and atrazine (IBAMA 2022).

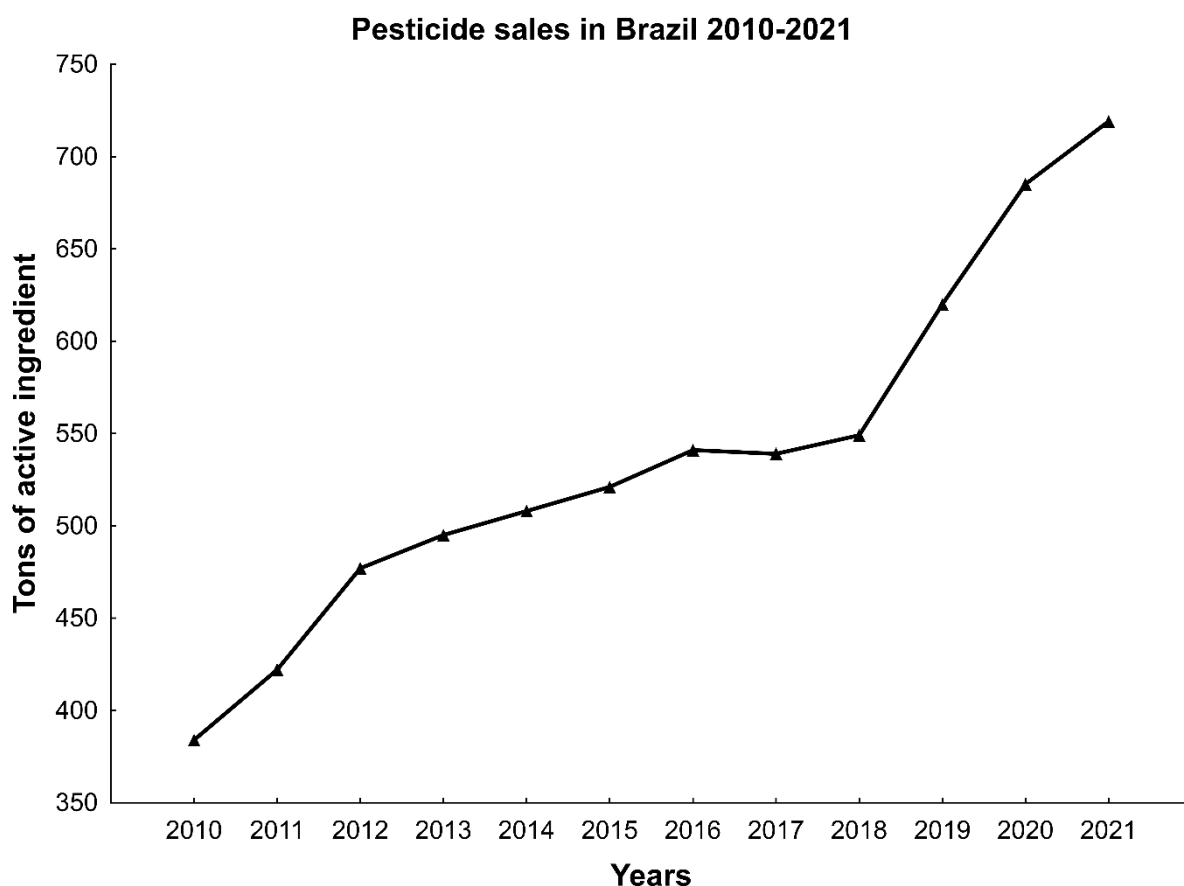


Figure 1. Annual pesticide sales in Brazil from 2010 to 2021.

Glyphosate (GLY)

Glyphosate [N-(phosphonomethyl) glycine ($C_3H_8NO_5P$)] is a synthetic herbicide widely used for its broad-spectrum, non-selective properties, effectively targeting various types of weeds, perennials, monocotyledons, and dicotyledons (Agostini et al. 2020; Peillex and Pelletier 2020). Classified as a class III product hazardous to the environment, glyphosate (GLY) has gained popularity due to its efficacy. It is a highly polar molecule, which contributes to its high-water solubility and facilitates its uptake by aquatic organisms (Williams et al. 2000; Leite-Filho et al. 2021). The global consumption of glyphosate has significantly increased in recent decades, with usage escalating from approximately 56,000 tons in 1994 to over 825,000 tons in 2014 (Benbrook 2016). Forecasts indicate that glyphosate consumption will surpass the milestone of 1,000,000 tons worldwide in the coming years (Landrigan and Belpoggi, 2018; Agostini et al. 2020; Soares et al. 2021). Presently, glyphosate stands

as the most extensively employed herbicide globally, constituting 60% of herbicides within its class (de Maria Serra et al. 2021). Moreover, it holds the distinction of being the most widely sold herbicide in Amazonia (IBAMA 2022).

The presence of elevated concentrations of glyphosate (GLY) in aquatic environments is a global concern. In Brazil, regulatory agencies allow maximum GLY levels of 65 µg/L in class I waters and 280 µg/L in class III waters, which are significantly higher than the limits set by the European Union (0.1 µg/L) (Brasil 2005; Pavan et al. 2021). Class I waters are designated for various purposes, including human consumption after simplified treatment, protection of aquatic communities, primary contact recreation, irrigation of raw-eaten vegetables and fruits, and protection of aquatic communities in indigenous territories. Class II waters can be used for human consumption after conventional or advanced treatment, irrigation of crops, amateur fishing, secondary contact recreation, and animal watering (Brasil 2005). The World Health Organization does not recognize any safe concentration of GLY for human consumption (WHO 2017). However, studies on GLY contamination in Brazil are scarce, with only one study conducted in the Amazon region. In various Brazilian rivers, GLY concentrations exceeding 100 µg/L have been detected in the Passo do Pilão river in the south Brazil, a maximum concentration of 6.80 µg/L in the Pirapó river in the Paraná River basin in the south, and values up to 1.06 µg/L in the Samambaia river sub-basin in the Midwest Brazil (Da Silva et al. 2003; Delmonico et al. 2014; Correia et al. 2020). In the Amazonian biome, water samples collected from the Santarém plateau in Pará state revealed GLY concentrations between 1.5 µg/L and 9.7 µg/L (Pires et al. 2020). The contamination of aquatic environments by GLY has negative effects on tadpoles of various species worldwide. For example, *Microhyla fissipes* exhibited a 10-day median lethal concentration (LC₅₀) of 77.5 mg/L, and exposure to GLY at concentrations of 60 and 90 mg/L suppressed tadpole growth, with more pronounced effects at higher concentrations. GLY also led to transcriptional downregulation of carbohydrate and lipid catabolism and upregulation of amino acid catabolism (Wang et al. 2019). *Xenopus laevis* embryos and tadpoles at Gosner stage 8 and 46 exhibited 96-hour LC₅₀ values of 32.1 mg active ingredient (a.i.)/L and 35.1 mg a.i./L, respectively, with a reduction in embryo length at concentrations of 31-37.6 mg a.i./L (Turhan et al. 2020). Histopathological alterations were observed in

Dendropsophus molitor after one-month exposure to GLY in concentrations ranging from 350 to 750 µg glyphosate acid equivalent (a.e.)/L (Riaño et al. 2020). Studies on Brazilian anurans have shown varied effects. Exposure of *Scinax nasicus* to GLY concentrations between 3.07 and 7.5 mg/L resulted in increased mortality and malformations, with LC₅₀ values of 4.78 mg/L at 24 hours and 2.64 mg/L at 96 hours (Lajmanovich et al. 2003). *Physalaemus cuvieri* and *Boana pardalis* tadpoles exhibited 96-hour LC₅₀ values of 115 and 106 mg a.i./L, respectively (Daam et al. 2019). Malformations and developmental changes were observed in *Rhinella arenarum* tadpoles, including abnormal eye shape, visceral alterations, lack of organization in the gill chamber, bilateral asymmetry, alterations in mouth structure and a reduction in thyroid hormone (T4), resulting in developmental delays (Boccioni et al. 2021). GLY exposure in *Melanophryniscus admirabilis* tadpoles induces oxidative stress, characterized by a decrease in catalase and superoxide dismutase enzyme activity, along with an increase in glutathione S-transferase activity (da Silva et al. 2021). In Brazilian Amazonia, studies have provided valuable data on the effects of GLY on tadpoles of three anuran species (Figueiredo and Rodrigues, 2014). *Rhinella marina* tadpoles exhibited a 96-hour LC₅₀ value of approximately 32 mg/L, while *Physalaemus centralis* tadpoles were found to be more sensitive to acute contamination, with a 96-hour LC₅₀ value of 19.70 mg/L. Chronic exposure to sublethal concentrations, ranging from 25% to 75% of the LC₅₀, mainly resulted in reduced survival for both species. Interestingly, the development and body size of *R. marina* tadpoles were unaffected, whereas *P. centralis* tadpoles exhibited accelerated development and a larger body size. For *Dendropsophus minutus* tadpoles, the LC₅₀ value was determined to be 2.49 mg active ingredient a.i./L. Exposure to a sublethal concentration of 0.28 mg a.i./L for 96 hours resulted in observed DNA damage based on the comet assay (Carvalho et al. 2018). Furthermore, exposure to 520 µg/L of GLY increased the presence of micronuclei in cells and induced histopathological hepatic damage in *D. minutus* tadpoles (Lopes et al. 2021).

2,4-D

2,4-D is a widely used systemic organochlorine herbicide in pre- and post-emergent applications on various crops including soybean, rice, wheat, and corn (Ruiz

de Arcaute et al. 2016). It is the second most sold pesticide in Brazil and is primarily employed for weed control (Barbosa et al. 2015; IBAMA 2022). In Amazonia, it ranks second in herbicide sales (IBAMA 2022). Due to its high solubility in water and low adsorption coefficient, 2,4-D can contaminate surface water and be readily taken up by aquatic organisms (Silva et al. 2007; Gervais et al. 2008; Freitas et al. 2019). The Brazilian legislation allows a maximum concentration of 4 µg/L in class I waters and 30 µg/L in class II waters (Brasil 2005), which aligns with the World Health Organization (WHO) guidelines (WHO 2022). Although 2,4-D contamination has been reported worldwide, there is limited information on its presence in the aquatic environment in Brazil. Studies conducted in the Vacacaí and Vacacaí-Mirim river basins in southern Brazil have detected concentrations ranging from 0.4 to 2.7 µg/L (Marchesan et al. 2010). In Amazonia, concentrations of 3 to 5 ng/L have been found in the city of Manaus, with a frequency of occurrence of 35% in the collected samples (Rico et al. 2022).

Regarding the effects on amphibians, studies have focused on a few species. Tadpoles of *Leptodactylus fuscus* exhibited a 96-hour LC₅₀ value of 28.8 mg/L when exposed to 2,4-D (Freitas et al. 2019). In the case of *Rhinella marina* tadpoles, exposure to 2,4-D resulted in reduced survival, while tadpoles of *Physalaemus centralis* showed higher mortality when exposed to 75% of the LC₅₀. Furthermore, *P. centralis* tadpoles exhibited faster development and larger body size at concentrations of 25% and 50% of the LC₅₀ (Figueiredo and Rodrigues, 2014). The LC₅₀ values were also determined for another South American anuran species, *Physalaemus albonotatus*, which varied with exposure time. The 48-hour LC₅₀ was 1040.2 mg/L, decreasing to 754.2 mg/L at 72 hours and 350 mg/L at 96 hours. Chronic exposure of *P. albonotatus* tadpoles to 2,4-D concentrations of 87.5, 175, and 262.5 mg/L resulted in a significant reduction in survival after 21 days of exposure. Tadpoles exposed to 2,4-D exhibited a shorter larval development time compared to the control group, as well as an increase in malformations affecting the oral disc, intestine, abdomen, and body shape (Curi et al. 2019). Recent studies have also investigated the effects of pesticide mixtures on tadpoles, such as the combination of 2,4-D and glyphosate. Exposure of *Boana faber* and *Leptodactylus latrans* tadpoles to this mixture led to irregular swimming activity, lethargy, convulsions, and morphological damage,

particularly in the mouths of tadpoles. Cellular damage, including increased micronuclei and erythrocyte nuclear abnormalities (the most frequent being lobed nucleus), was also reported (Pavan et al. 2021).

Mancozeb (MZ)

Mancozeb is a widely used fungicide introduced in 1962, belonging to the dithiocarbamate class. It is commonly applied to crops such as soybeans, wheat, beans, and coffee. In Amazonia, mancozeb is the most popular fungicide and ranks third among the top-selling pesticides (IBAMA 2022). However, its misuse is prevalent among farmers in the Manaus region, as they often exceed the recommended dose and number of applications (Römbke et al. 2008). Mancozeb (MZ) exhibits low solubility and persistence in soil. In the aquatic environment, it can be hydrolyzed within 24 hours to seven days. Nevertheless, its degradation product, ethylene thiourea (ETU), is more water-soluble and mobile in soils, and is considered more toxic than MZ itself (Xu 2000). Brazilian regulations allow a maximum concentration of 8 µg/L of MZ in drinking water for human consumption (Brasil 2011). The specific effects of mancozeb on Amazonian species are not extensively documented. However, there are two reports concerning Brazilian tadpoles. Tadpoles of *Rhinella arenarum* exposed to 5 mg/L of MZ for 24 hours did not show mortality, but a 100% mortality rate was observed after 48 hours at the same concentration. Prolonged exposure for 336 days resulted in mortality at lower concentrations ranging from 0.01 to 1 mg/L. Chronic exposure also induced malformations in tadpoles (Asparch et al. 2020). Similarly, *Physalemus henselii* tadpoles exposed to a concentration of 2 mg/L of MZ for 24 hours showed increased expression of the heat shock protein HSP70 compared to the control group. HSP70 is a cellular stress response protein commonly used as a marker to assess temperature and contamination effects on organisms (Azambuja et al. 2021).

Atrazine (ATZ)

Atrazine is a triazine herbicide that was introduced in the 1950s and is widely used for controlling grasses and broad-leaved weeds, particularly in sugarcane plantations. It exhibits high persistence and mobility in soil, which contributes to its

contamination of the aquatic environment. Atrazine has been detected in public water supplies, groundwater, lakes, and rivers (de Albuquerque et al. 2020). Although it is not permitted in the European Union due to its potential for groundwater contamination, in Brazil, it is allowed in freshwater at concentrations up to 2 µg/L (Brasil 2005). In Amazonia, atrazine has been detected with a frequency of 70% in water samples, ranging from 3 to 25 ng/L (Rico et al. 2022). As of 2021, atrazine ranks sixth among the top pesticides used in the biome (IBAMA 2022). The effects of atrazine on anuran species are diverse and have been extensively studied. These effects include changes in biochemical parameters, genotoxic and mutagenic effects, behavioral changes, and abnormalities (de Albuquerque et al. 2020). For example, the species *Dendropsophus minutus*, which is also found in the Amazon basin, has a 96-hour median lethal concentration (LC₅₀) of 36.41 mg/L for atrazine. Although atrazine did not alter cell viability, the comet assay showed that tadpoles exhibited increased DNA damage compared to the control group (Gonçalves et al. 2017). The extent of DNA damage varied depending on the developmental stage, with tadpoles in the early and late stages showing higher occurrence of DNA damage, while those in the intermediate stages showed no significant differences. Additionally, there was an increased frequency of micronuclei in tadpoles of *D. minutus* in the early stages (Gonçalves et al. 2017). Similar effects were observed in tadpoles of *Rhinella diptycha* (at developmental stage 30) exposed to 19 mg/L of atrazine, resulting in an increased frequency of micronuclei and other nuclear abnormalities. Atrazine also induced histopathological lesions in the liver, such as sinusoidal enlargement after 48 and 96 hours of exposure to 12.5 mg/L atrazine, and erythrocyte accumulation in vessels causing hypervascularization after 96 hours of exposure to 1.5 mg/L atrazine. Morphological abnormalities were observed, including abnormalities of the caudal axis, mouth (losses or absence of keratodonts), lateral compression of the body, and invaginations in the dermis (Pérez-Iglesias et al. 2019). Other species, such as *Xenopus laevis*, have shown morphological abnormalities such as intersex gonads, heart malformation, visceral hemorrhage, intestine coiling malformations, curved body axes, and edema (Hayes et al. 2002; Carr et al. 2003; Lenkowski et al. 2008). Tadpoles of *Lithobates pipiens* exposed to ametryn also exhibited smaller size at metamorphosis, delayed gonadal development, and thymus damage (Hayes et al.

2006). Behavioral alterations have been reported for *Physalaemus cuvieri* at concentrations above 0.24 mg/L, and concentrations above 0.1 mg/L have been found to alter larval development time in tadpoles of *Rhinella arenarum* (Brodeur et al. 2009). The sensitivity of species to atrazine varies, with different responses observed among species. When comparing lethal endpoints in a sensitivity-distribution curve, *R. diptycha* exhibited intermediate sensitivity to atrazine compared to other globally distributed species. It was found to be more sensitive to atrazine than *Dendropsophus minutus* and *Physalaemus gracilis* (Rutkoski et al. 2018).

3.2. Metals and metalloids

The expansion of illegal mining in the Amazon poses a significant environmental challenge for amphibians due to aquatic contamination by metals and metalloids, coupled with deforestation associated with these areas (Sonter et al. 2017). The extraction of various minerals in Amazonia contributes to contamination, notably in the extraction of cassiterite and gold, which constitute major sources of sediment in watercourses (Fearnside 2019). During the cassiterite extraction process, substantial quantities of metals and metalloids are released, such as arsenic (As), mercury (Hg), copper (Cu), lead (Pb), and cadmium (Cd). Similarly, in gold extraction, mercury is employed to amalgamate fine gold particles, resulting in the release of tons of this metal into rivers (Moulatlet et al. 2023). Additionally, agricultural systems and solid waste disposal contribute to the introduction of metals into Amazonian aquatic environments, as fertilizers may contain elevated concentrations of these metals (Gupta et al. 2014). Pesticides may also contain metals associated with their compounds, such as manganese and zinc in association with the fungicide carbamate mancozeb.

Similar to pesticide contamination, the impact of mining extends beyond areas directly associated with mining activities, as metals can be transported and deposited through soil and rivers. In the case of the Amazon, during the rainy season, rising water levels transport this contaminated water to marginal regions like puddles and shallows associated with streams, environments frequently utilized by various amphibian species for reproduction. Elevated levels of these metals are currently identified in

various regions of the Amazon basin, both in water and sediment samples, surpassing the limits established by legislation and deemed toxic to aquatic fauna (Ribeiro et al. 2017).

Analysis of samples from the Xingu and Fresco rivers in the state of Pará, Eastern Amazonia, revealed contamination by As, Cd, Cu, Pb, Hg, Chromium (Cr) and Nickel (Ni) in water, soil, and sediment samples (Ribeiro et al. 2017). Particularly noteworthy are the maximum values found for As (0.43 µg/L) and Hg (0.22 µg/L) in water samples, Cr (61.91 mg/kg) and Ni (26.62 mg/kg) in sediment samples, and Cr (339.89 mg/kg), Pb (106 mg/kg), and Ni (40.36 mg/kg) in soil samples, all of which exceeded the values permitted by CONAMA (Brasil 2005). Samples from the Araguari River in the state of Amapá, Eastern Amazon, also exhibited water samples with levels above those allowed by CONAMA for metals such as Cd (9 µg/L), Pb (160 µg/L), Fe (4530 µg/L), Hg (9 µg/L), Cu (11 µg/L), and Zn (4800 µg/L) (Viana et al. 2020). Many anuran species utilize small water formations such as holes in fallen trees, holes in trunks and roots of living trees, fallen palm bracts, in addition to puddles formed on rocky substrates and artificial plastic or metallic containers. Contamination data in these ephemeral environments are scarce, but they can be polluted through airborne metal pollution during ore extraction, as exemplified by Hg in gold mining. Water and sediment samples from these environments, used by a species in French Guiana, *Dendrobates tinctorius*, also revealed high concentrations of Hg ranging from 0.007 to 13.040 ppm (Schlippe-Justicia et al. 2024).

While awareness of contamination in the Amazon exists, there is still limited information regarding the impacts of metals and metalloids on amphibian species in the region. Tadpoles of *Scinax ruber* and *Rhinella granulosa*, exposed to Cu concentrations, exhibited distinct sensitivities, as evidenced by 96 h LC₅₀ values of 23.48 and 36.37 µg/L, respectively. In the case of *Scinax ruber* eggs, the LC₅₀ was 50.02 µg/L (Franco-de-Sá and Val 2014). Similarly, eggs and tadpoles of *Osteocephalus taurinus*, subjected to water contaminated with heavy metals from polluted streams, showed 96h LC₅₀ values of 25.19 and 47.53 mL/L, respectively. Additionally, exposure to the water from the polluted streams induced an increase in larval development time and body size during metamorphosis. Among the identified

metals in the streams, notable ones include Iron (Fe) (3.60 mg/L), Zinc (Zn) (0.13 mg/L), Ni (0.08 mg/L), Manganese (Mn) (0.17 mg/L), Cu (<0.02 mg/L), Cr (0.20 mg/L), Pb (0.19 mg/L), and Cobalt (Co) (<0.05 mg/L). Except for Cu and Co, all exceeded the limits permitted by legislation (Franco-de-Sá et al. 2012). Commonly observed effects in other species facing contamination by metals and metalloids include mortality and varying levels of sensitivity, as well as morphological, physiological, and behavioral alterations (Chen et al. 2009; Thambirajah et al. 2019). For instance, tadpoles of *Bufo gargarizans* and *Lithobates pipiens* exposed to mercury and methylmercury, derived from mercury methylation in water, experienced mortality, thyroid disruptions, liver damage, oxidative stress, and lipid metabolism disorders (Chang et al., 1974; Shi et al., 2018). Tadpoles of *Rana chensinensis* exposed to Cr and Pb, alone or in combination, exhibited malformations, intestinal histological injuries, and reduced activities of digestive enzymes (Zhang et al. 2023). Moreover, metals such as Pb, Cu, Cd, and Zn affected the survival of *Xenopus laevis* tadpoles, declining as the concentration of these metals increased, while for eggs, only Pb, Cu, and Cd reduced hatching success (Haywood et al., 2004). In *Fejervarya limnocharis* tadpoles, exposure to Cd led to concentration-dependent mortality, along with accelerated metamorphosis, reduced body weight and genotoxicity (Patar et al. 2016).

3.3. Microplastics (MP)

Plastics and their associated waste have become a significant environmental concern due to their widespread use and improper disposal. Plastic-based products offer various advantages such as flexibility, corrosion resistance, versatility, durability, and low production cost (Shen et al. 2020). However, the exponential growth in plastic production has led to the accumulation of plastic waste, with approximately 5,800 million tons (Mt) of plastic waste generated between 1950 and 2015. Out of this total, around 800 Mt was incinerated, 100 Mt was reclaimed, and 4,900 Mt was landfilled (Geyer et al. 2017; Yang et al. 2021). Recent years have also shown an increasing trend in plastic production, with 335 Mt produced in 2016 and 367 Mt in 2020 (PlasticsEurope 2021). Microplastics are particles smaller than 5mm and nanoplastics smaller than 1µg, nowadays widely distributed in aquatic aerial and terrestrial ecosystems (Ringwood 2021). They can be classified as primary or secondary

microplastics: primary microplastics are intentionally produced for specific purposes, such as microbeads in personal care products, while secondary microplastics result from the degradation of larger plastic items, including synthetic fibers (Castro-Castellon et al. 2022; Balestrieri et al. 2022). Microplastics exhibit various morphologies, including pellets, fragments, lines, and fibers (Massarelli et al. 2021). While the presence of microplastics has been extensively documented globally, research on microplastic pollution in Amazonian rivers is relatively limited and new. The first record of microplastic pollution in the region was based on sediment samples from the Solimões, Negro, and Amazonas rivers, revealing concentrations ranging from 417 to 8,178 particles/kg dry sediment (Gerolin et al. 2020). Water samples from various rivers in the Amazon region revealed distinct levels of microplastic contamination. Specifically, the Amazon River displayed concentrations ranging from 0.003 to 0.67 mg/m³. In the Tapajós River, the values were measured between 0.53 and 1.9 mg/m³. The Negro River exhibited concentrations from 0.007 to 0.29 mg/m³, with higher values near Manaus. The Tocantins River showed concentrations ranging from 0.31 to 0.33 mg/m³. These findings highlight the variability in microplastic pollution across different water bodies in the Amazon region (Rico et al. 2023).

Once microplastics enter the aquatic environment, they become suspended in the water column and can adhere to algae and substrates. Tadpoles, due to their feeding habits, are prone to ingesting microplastics as they filter phytoplankton from the water and scrape algae from substrates. Microplastics can enter tadpoles' bodies primarily through the mouth and gills. After ingestion, microplastics can accumulate in various organs, including the heart, liver, and intestine. In the intestine, microplastics may accumulate and create a false sense of satiety, leading to reduced food intake and absorption. This can result in deleterious effects such as changes in growth rate, behavior, histopathological damage, erythrocyte changes, and malformations. Furthermore, microplastics can interact with pesticides in the aquatic environment, increasing the toxicity of these chemicals to tadpoles (Lajmanovich et al. 2022). The greatest contributions to the knowledge of the effects of microplastics on Brazilian tadpoles are the studies conducted with *Physalaemus cuvieri*, a widely distributed species in the country. Exposure to environmentally relevant concentrations was able to induce different hepatotoxic and cytotoxic effects (da Costa Araújo et al. 2020). The

accumulation of microplastics in the liver indicates the ability to absorb microplastics from water, and this accumulation induced blood vessel dilation, infiltration, congestion, hydropic degeneration, hypertrophy, and hyperplasia (da Costa Araújo et al. 2020). Microplastics also affected erythrocyte nuclear morphometry (da Costa Araújo et al. 2020) and induced behavioral alterations such as reduced locomotor activity, increased anxiety index, and the absence of the common antipredator response of tadpole aggregation (da Costa Araújo and Malafaia 2020). Studies on *Scinax squalirostris* tadpoles have shown that the activity of metabolic enzymes, specifically carboxylesterases (CbEs) and alkaline phosphatase (ALP), is negatively affected by microplastics (MPs). This indicates significant alterations in physiological and immunological processes, which can lead to a loss of fitness for the tadpoles (Attademo et al. 2022). Furthermore, microplastics can be transferred through the trophic chain. Tadpoles of *Physalaemus cuvieri*, contaminated with microplastics, were preyed upon by juvenile tambatinga fish (*Colossoma macropomum* x *Piaractus brachypomus*). These fish, in turn, were preyed upon by healthy Swiss mice (*Mus musculus*). As a result of this transfer, Swiss mice individuals were found to have an accumulation of microplastics in their livers (da Costa Araújo and Malafaia, 2021). This highlights the potential for microplastics to enter and persist in higher levels of the food chain, posing risks to various omnivore and carnivore consumers.

4. Interaction between temperature and contaminants

Anurans, being sensitive organisms, are greatly influenced by the environmental characteristics of their habitat. The most significant abiotic factor impacting amphibians is temperature, which has been increasing since the end of the industrial revolution, with a more pronounced rise in recent decades. A comparison between the pre-industrial period (1850-1900) and the years 2011-2020 reveals a global temperature increase of 1.09°C, with 1.07°C attributed to intense human activities (Masson-Delmotte et al. 2021). Human activities, such as the burning of fossil fuels and deforestation, contribute to the emission of greenhouse gases (GHGs) into the atmosphere, further exacerbating the global temperature increase (Manabe 2019). Temperature plays a crucial regulatory role in the physiological and behavioral processes of anurans. Fluctuations in temperature on a daily and seasonal basis elicit

adaptive responses that influence various aspects of anuran biology, including locomotor performance, oxygen consumption, digestion, and gas exchange routes (Navas et al. 2008). The thermal characteristics of anurans are defined by thermal performance curves, which include the thermal optimum temperature (T_{opt}), thermal breadth, and critical thermal limits. The Gaussian-shaped curve is bounded by the maximum (CT_{max}) and minimum (CT_{min}) thermal limits. Anurans typically have an optimum temperature for development (T_{opt}) that falls within the CT_{max} and CT_{min} , with temperatures outside this range potentially causing physiological damage or death (Duarte et al. 2012; Katzenberger et al. 2012). The thermal tolerance range of anurans shows a positive correlation with the variation in ambient temperature, with higher latitudes experiencing greater seasonal temperature variations and consequently broader thermal amplitudes. Conversely, amphibians in tropical regions, where seasonal temperature variation is lower, tend to have narrower thermal amplitudes (Deutsch et al. 2008). Anurans employ strategies to mitigate the negative effects of temperature, including phenotypic plasticity and local adaptation.

Phenotypic plasticity allows individuals to express different phenotypes from a single genotype in response to environmental challenges. Plasticity in thermal tolerance is associated with the magnitude of temperature variation, as proposed by the climate variability hypothesis. Consequently, tropical amphibians may be more susceptible to climate change due to their proximity to their optimal temperature range (Janzen 1967; Levins 1968). Although robust data specific to amphibians are currently lacking, an alternative hypothesis regarding the susceptibility of tropical ectotherms suggests that these species already inhabit environments where ambient temperatures are near their upper thermal limits (Vinagre et al. 2019; Nati et al. 2021). These findings suggest that the susceptibility of ectothermic organisms, including amphibians, to climate change may indeed be influenced by the proximity of current temperatures to their upper thermal limits. However, it is important to note that further research specifically focusing on amphibians is needed to validate these trends and understand the unique factors contributing to their vulnerability. Local adaptation, on the other hand, refers to changes in genotype and phenotype that occur after exposure to a stressor, even if these changes do not confer greater fitness in other environments. Individuals with locally adapted traits may have a competitive advantage and higher

fitness within their specific habitat compared to genotypes from other habitats (Kawecki and Ebert 2004).

Thermal thresholds in organisms can be influenced by exposure to contaminants, which is a matter of concern, particularly for thermosensitive organisms. Notably, studies on tadpoles have revealed the impact of specific contaminants on their thermal tolerance. For instance, research conducted on *Rhinella arenarum* tadpoles exposed to chlorpyrifos demonstrated a reduction in their CT_{max} , the maximum critical thermal limit, by approximately 4°C (Quiroga et al. 2019). Similarly, tadpoles of *Physalaemus henselii* exposed to mancozeb exhibited a decrease in their thermal tolerance by 2.41°C (Azambuja et al. 2021). Another commonly observed phenomenon is the synergistic effect of contaminants and elevated temperatures, resulting in increased toxicity. In the case of *Ranoidea citropa* tadpoles, exposure to endosulfan at fluctuating temperatures proved to be more lethal compared to exposure at a constant temperature (Broomhall 2002). This indicates that temperature fluctuations enhance the toxicity of endosulfan in this species. Temperature can also interact with glyphosate, a widely used herbicide. Research on *Boana faber* tadpoles demonstrated that exposure to glyphosate at higher temperatures (32°C) led to increased mortality compared to lower temperatures (25°C) (Alves-Ferreira et al. 2023). This interaction suggests that elevated temperatures intensify the toxic effects of glyphosate on tadpoles. Interestingly, the effects of contaminants on tadpole development and growth can be temperature dependent. Exposure to Tebuthiuron at 25°C accelerated the development of *Aquarana catesbeianus* larvae without affecting their mass gain. However, at 32°C, tadpole development was delayed, mass gain reduced, and total length unaffected (Grott et al. 2022). This illustrates the intricate relationship between contaminants, temperature, and their combined effects on tadpole physiology. These findings underscore the significance of considering the impact of contaminants and temperature on thermosensitive organisms. By exploring these interactions, we can gain insights into the vulnerability and responses of organisms to environmental stressors, facilitating informed conservation and management efforts.

5. Implications of deleterious effects for tadpoles

As previously discussed, contaminants interact in intricate ways with temperature. The combination of contaminants and elevated temperature can induce a spectrum of adverse effects on tadpoles, with documented alterations in morphological, behavioral, and physiological parameters throughout the larval developmental phase. These effects not only lead to immediate lethal consequences but also result in adults with lower fitness, as there is a correlation between tadpole performance and adult fitness. During the larval stage, tadpoles acquire energy and build up sufficient energetic reserves, crucial for the metamorphic climax, a period characterized by profound morphological and physiological changes (Shi 2000; Orlofske and Hopkins 2009). Malformations in tadpoles are frequently observed, particularly in the mouth apparatus. These malformations can involve the total or partial absence of keratodont rows and the loss of the upper jaw sheath (Herek et al. 2020; Samojeden et al. 2022). Changes in eye structure, such as the absence of eyes or lens abnormalities, and abnormally small eyes have also been reported (Sánchez-Domene et al. 2018). Tadpoles may also experience damage to the caudal structure (Da Silva et al. 2020), the gut, and increased edema in the body (Herek et al. 2020). These alterations in mouthparts and intestines, essential for feeding and nutrient absorption, can result in nutrient deficiencies (Venesky et al. 2010; Tolledo et al. 2014). In the larval phase, the degeneration of tadpole mouthparts occurs as adult mandibles develop, leading to a temporary interruption of feeding and requiring the use of energy accumulated during the larval period (Beck and Congdon 2003). Consequently, increased mortality and reduced body size of tadpoles can be observed. Smaller body size is also associated with higher vulnerability to predation, and since tadpole size influences the size of adult individuals, smaller tadpoles are likely to become smaller adults with potentially reduced reproductive success and predator avoidance efficiency (Smith 1987; Beasley 2020; Adams et al. 2021; Samojeden et al. 2022). These effects not only compromise healthy tadpole development but also result in lower energy accumulation during the larval phase (Beck and Congdon 2003). Furthermore, damage to the caudal structure and eye components can alter tadpole behavioral patterns, including reduced effectiveness in predator avoidance and foraging (Lajmanovich et al. 2003; Bach et al. 2016). Changes in tadpole growth and development rates are also

frequently observed, reflecting the overall disruption caused by contaminants and increased temperature.

Tadpoles metamorphosis exhibit high plasticity in their response to adverse environments, enabling them to adjust their developmental rates. However, there is a trade-off between accelerated developmental rates and body growth in tadpoles, with early metamorphosing individuals typically displaying smaller body sizes. As mentioned earlier, smaller tadpole body size translates into smaller adults with lower reproductive success. Contaminants and temperature can induce changes in development and growth by disrupting endocrine function (Liu et al. 2021; Grott et al. 2022). The thyroid gland plays a primary role in initiating metamorphosis in amphibians (Vieira 2010). Under stressful conditions, the levels of stress hormones, such as corticotropin-releasing factor, corticotropin, and glucocorticoids, increase. These hormones act on the hypothalamus-pituitary-thyroid axis, which regulates the production of thyroid hormones (THs) (Denver 2009; Ruthsatz et al. 2019). The main THs are tetraiodothyronine (T4) and triiodothyronine (T3), and they serve as important mediators of tadpole metamorphosis. It is through these hormones that the rate of larval development is established (Vieira 2010). Elevated levels of THs accelerate tadpole development and increase metabolic rate, leading to smaller-bodied metamorphosed tadpoles (Denver 2021). In the long term, these changes in amphibian populations can alter trophic dynamics in many communities. The smaller body sizes of metamorphosed tadpoles can impact their roles as prey or predators within their respective ecosystems, influencing the overall balance of food webs and trophic interactions. In addition to the well-known effects of accelerated development and reduced size of tadpoles in response to pesticides, it is noteworthy that exposures to low concentrations of these chemicals can, conversely, result in an increase in tadpole size (Figueiredo and Rodrigues, 2014). This outcome may be attributed to the phenomenon known as hormesis, which is a biphasic response characterized by stimulation at low doses and inhibition at high doses (Calabrese 2008). The ability of tadpoles to exhibit an increase in body size at lower doses underscores the importance of understanding the underlying mechanisms of hormesis for a comprehensive assessment of the environmental risks associated with pesticide exposure.

Within Neotropical ecosystems, amphibians play a vital role in energy flow and nutrient cycling (Valencia-Aguilar et al. 2013). As adult organisms, they act as important carnivores, primarily preying on insects, worms, and other invertebrates, thus contributing to the regulation of pest populations (Halliday 2008). Similarly, during their larval stage, herbivorous tadpoles play a crucial role in controlling algae populations. Both life stages serve as prey for a diverse array of predators, and any reduction in their biomass can lead to significant changes in ecosystem dynamics (Whiles et al. 2006).

6. Conclusion

Tadpoles face a myriad of environmental stressors that significantly impact their morphology, physiology, and behavior. Among these stressors, temperature plays a crucial role, as it not only influences fundamental processes for amphibians but also interacts with other stressors, potentially magnifying their effects. It is noteworthy that there is a notable lack of ecotoxicological studies on amphibians worldwide, a gap that becomes even more pronounced when considering research conducted in Brazil and the Amazonian region. Existing information examining the effects of contaminants and temperature increase in isolation indicates a wide range of important consequences, including increased mortality rates, the occurrence of malformations and structural damage, as well as biochemical, genotoxic, and neurotoxic effects. However, the limited amount of studies that have investigated the interaction between contaminants and temperature suggest that temperature can enhance the toxicity of contaminants, leading to adverse effects even at lower concentrations. It is crucial to acknowledge that the specific temperature and contamination thresholds required to induce these effects can vary among species. Additionally, in natural environments, other stressors such as oxygen levels, UV radiation exposure, predator presence, food availability, and hydroperiod (the duration and timing of wet and dry periods) also interact with temperature and pollutants.

To gain a comprehensive understanding of the relationship between amphibians, contamination, and temperature, conducting baseline research is imperative. However, this task is challenging due to the vast number of amphibian species, their idiosyncratic responses to environmental stressors, the variety of

contaminants present, and the complex projections related to climate change. Amphibians, highly sensitive to environmental changes, serve as crucial ecological indicators, highlighting the potential impacts of contamination and climate change on biodiversity as a whole.

Therefore, prioritizing research and conservation efforts for amphibians is vital to mitigate the threats they face. The future prospects for amphibians and their habitats are alarming, as the next three decades will be critical in achieving the long-term mitigation goals outlined in the Paris Agreement. In effect since 2016, the agreement aims to limit global temperature increase to well below 2°C above pre-industrial levels and pursue efforts to limit it to 1.5°C. Addressing these challenges and ensuring biological conservation worldwide requires international collaboration and concerted efforts. This necessitates implementing efficient public policies, scientific monitoring, and a global collaborative effort to adopt climate change mitigation strategies that consider the unique vulnerabilities of amphibians. Investments in technologies aimed at reducing CO₂ emissions, such as renewable energies, along with habitat restoration initiatives, are important components of this collective task. By protecting amphibians and their habitats, we can contribute to the overall health and resilience of ecosystems and safeguard the biodiversity upon which we all depend.

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8. List of tables

Table 1. Summary of reported literature results on the main effects of contaminants on amphibian larvae. Abbreviations: GS, Gosner Stage (Gosner, 1960); a.i./L, active ingredient per liter

Contaminant	Species	Family	Larval stage	Tested concentrations	LC50	Effects	Region	Reference
Cadmium (Cd) Lead (Pb) Cd + Pb	<i>Bufo gargarizans</i>	Bufonidae	GS 2-3	control and 108 µg/L control and 204 µg/L control and 106 + 201 µg/L	not evaluated	Morphological: reduced and increased body mass, reduction total length and snout-vent length, reduction bone length of the ilium, decreased maximum jump distance Histological: toward colloid depletion in follicular cells of thyroid gland (TG), reduction and increase TG area, increase in phagophores per TG, decrease in thickness of the epithelial cells, reduction in the colloid area of TG Genotoxic: dysregulation of genes involved in TG action and endochondral ossification	Shaanxi Province, China, East Asia	Chen et al. 2022
Copper (Cu)	<i>Rhinella granulosa</i>	Bufonidae	GS 25	control, 15, 20, 25, 33, 43, 56, 72, 94 ug/L	23.4 ug/L (stream water)	Mortality	Central Amazonia, Brazil, South America	Franco-de-Sá and Val 2014)
GLY 2,4-D	<i>Rhinella marina</i>	Bufonidae	GS 25	control, 4.9, 9.8 and 14.7 control, 128.9, 257.8 and 386.7 mg/L	32 mg/L 282.8 mg/L	Mortality Morphological: increased and decreased body	Southern Amazonia, Brazil,	Figueiredo and Rodrigues 2014

Piroclam Piroclam + 2,4-D				control, 0.12, 0.25 and 0.37 mg/L control, 6.9, 13.9 and 20.9 mg/L	0.3 mg/L 42.4 mg/L	mass	South America	
Glyphosate + predator cue	<i>Anaxyrus americanus</i>	Bufonidae	GS 25	control, 0.1, 1, 5, 10, and 20 mg a.i./L + present/absent predator cue	2.52 mg a.i./L	Mortality	Pittsburgh, USA, North America	Relyea 2004
Glyphosate + temperature	<i>Bufo bufo</i>	Bufonidae	GS 8 GS 21-24	15°C and 20°C: control, 0.5, 1.0 and 1.5 mg a.e./L and a pulse of 0.5 a.e./mg/L	not evaluated	GS 8: Mortality Morphological: body length, body width, tail length and total length of tadpoles at 20°C were increased. Ontogenetic: accelerated development Glyphosate + temperature Morphological: increased body length of herbicide treated tadpoles, body width, tail length, and total length at 15°C. At 20°C body length, tail length, and total length increased, and body width was decreased by 2% compared to no-herbicide control.	Vienna, Austria, Central Europe	Baier et al. 2016

						GS 21-24 Mortality Morphological: Body length, body width, tail length, and total length of tadpoles were increased at 20°C Glyphosate + temperature Ontogenetic: accelerated development		
Cadmium (Cd)	<i>Fejervarya limnocharis</i>	Dicroglossidae	GS 26-28	0.1, 0.2, 0.3, 0.4, 0.5, 1, 2 and 3 mg/L	10-day: 0.32 mg/L 24h: 1.85 mg/L	Mortality Morphological: decreased body mass and higher body length Ontogenetic: accelerated metamorphosis Genotoxicity: increased micronuclei in erythrocytes, DNA damage evidenced by migration of DNA fragments in gels forming a comet-like structure. Decreased DNA content in the comet head region with concurrent increase in the tail region. Increase in the olive tail moment and mean distance of migration in the tail.	Assam, India	Patar et al. 2016
Atrazine	<i>Dendropsophus minutus</i>	Hylidae	GS 25-33 GS 36-39	control, positive control (Cuclophosphamide 40mg/L),	36.4 mg/L	Mortality Genotoxic: increase % of DNA tail damage and	Goiás, Brazil, South America	Gonçalves et al. 2017

			GS 42-43	2.25, 4.5, 9, 18 and positive control		increased micronuclei frequency		
Chlorpyrifos	<i>Acris crepitans</i>	Hylidae	GS 25-27	control, 1, 10, 100, and 200 µg/l with/without sediment	not evaluated	Morphological: reduced snout-vent length Physiological: Cholinesterase activity decreased	Oklahoma, USA, North America	Widder and Bidwell 2008
Chlorpyrifos	<i>Dryophytes chrysoscelis</i>	Hylidae	GS 25-27	control, 1, 10, 100, and 200 µg/l with/without sediment	not evaluated	Morphological: reduced mass Physiological: decreased cholinesterase activity Behavioral: slower swimming	Oklahoma, USA, North America	Widder and Bidwell 2008
Copper (Cu)	<i>Scinax ruber</i>	Hylidae	Eggs GS 25	control, 15, 20, 25, 33, 43, 56, 72, 94 ug/L	36.3 ug/L 50 ug/L (stream water) 15.9ug/L (water from well)	Mortality	Central Amazonia, Brazil, South America	Franco-de-Sá and Val 2014
Water from a contaminated urban stream - Iron (Fe), Zinc (Zn), Nickel (Ni), Manganese (Mn), Copper (Cu), Chromium (Cr), Lead (Pb), and Cobalt (Co)	<i>Osteocephalus taurinus</i>	Hylidae	Eggs GS 25	control, 16, 20, 27, 36, 48, 60, 75, 90 and 117 mL/L	Eggs: 25.19 mL/L Tadpoles: 47.63 mL/L	Mortality Morphological: edema in the abdominal region and increased body size Ontogenetic: accelerated development	Central Amazonia, Brazil, South America	Franco-de-Sá et al. 2012

Cypermethrin (95% purity) Cypermethrin (Commercial)	<i>Boana pulchella</i>	Hylidae	GS 25	control, 0.1, 0.5, 1, 5, 10, 50, 100, 250, 500, 750, 1.000, 2.500, 5.000, 7.500 and 10.000 ug a.i./L	479.7 mg/L 175.2 mg/L	Mortality Behavioral: irregular swimming, delayed response, slow swimming, and immobility Morphological: abnormalities in head and axial, eye, gut, and face. Growth inhibition.	Buenos Aires, Argentina, South America	Agostini et al. 2010
Glyphosate Polyoxyethylene amine (POEA)	<i>Dendropsophus minutus</i>	Hylidae	GS 25	control, 65, 130, 260 and 520 µg/L control, 1.25, 2.5, 5 and 10 µg/L	not evaluated	Genotoxic: All types of genotoxic damage (%DNA in the tail, tail length, and olive tail moment, and increased micronuclei) was observed Histopathological: Vacuolization of the hepatocytes, hyperchromatic nuclei, and presence of spots with inflammatory cells. Presence of edematous degeneration and melanomacrophages were more extensive and more pigmented.	Goiás, Brazil, South America	Lopes et al. 2021
Glyphosate	<i>Dendropsophus minutus</i>	Hylidae	GS 26-31	control, positive control (10mg/L of mitomycin C), 0.28, 1.0, 2.0 and 4.0 mg a.i./L	2.4 mg a.i./L	Mortality Genotoxic: increase in DNA damage for all parameters of the comet: tail lenght, % DNA in the tail and olive tail moment	Goiás, Brazil, South America	Carvalho et al. 2018

Glyphosate + predator cue	<i>Dryophytes versicolor</i>	Hylidae	GS 25	control, 0.1, 1, 5, 10, and 20 mg a.i./L + present/absent predator cue	1.35 mg a.i./L	Mortality	Pittsburgh, USA, North America	Relyea 2004
2,4-D	<i>Leptodactylus fuscus</i>	Leptodactylidae	GS 25	control, 5, 10, 20, 40 and 80 mg/L	28.81mg/L	Mortality	São Paulo, Brazil, South America	Freitas et al. 2019
2,4-D	<i>Physalaemus nattereri</i>	Leptodactylidae	GS 25	control, 20, 40, 80, 160 and 320 mg/L	143.08 mg/L	Mortality	São Paulo, Brazil, South America	Freitas et al. 2019
Cypermethrin Deltamethrin	<i>Physalaemus gracilis</i>	Leptodactylidae	GS 20 GS 25	control, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/L of Cypermethrin control, 0.01, 0.02, 0.03, 0.05, 0.07 and 0.1 mg/L of Cypermethrin and 0.001, 0.0015, 0.003, 0.004, 0.006 and 0.009 mg/L of Deltaethrin	not evaluated	Behavioral: reduction swimming activity, stopped swimming and lethargy, and spasms Morphological: malformation in oral morphology	Rio Grande do Sul, Brazil, South America	Vanzetto et al. 2019
Glyphosate 2,4-D Piroclam Piroclam + 2,4-D	<i>Physalaemus centralis</i>	Leptodactylidae	GS 25	control, 4.9, 9.8 and 14.7 control, 128.9, 257.8 and 386.7 mg/L control, 0.12, 0.25 and 0.37 mg/L	19.7 mg/L 515.7 mg/L 0.5 mg/L 27.9 mg/L	Mortality Ontogenetic: accelerated time to metamorphosis Morphological: increased and decreased body mass	Southern Amazonia, Brazil, South America	Figueiredo and Rodrigues 2014

				control, 6.9, 13.9 and 20.9 mg/L				
Chlorpyrifos	<i>Gastrophryne olivacea</i>	Microhylidae	GS 25-27	control, 1, 10, 100, and 200 µg/l with/without sediment	not evaluated	Morphological: reduced mass and snout-vent length Physiological: decreased cholinesterase activity	Oklahoma, USA, North America	Widder and Bidwell 2008
Carbaryl	<i>Xenopus laevis</i>	Pipidae	GS 8	control, 1, 2, 4, 8, 16 and 24 mg/L	20.28 mg/L	Mortality Morphological: abnormal gut coiling, generalized oedema, cardiac oedema, abdominal oedema, abnormal tail flexure and microphthalmia Histopathological: skeletal muscle lesions consisted of myotomes reduced in size, showing myocytes with disorganized contractile systems and irregular myosepta, coupled with disarranged myocyte apexes, necrotic tissue with vacuolated cytoplasm, pyknotic nuclei and degenerating contractile systems, abnormally developed notochords Physiological: cholinesterase inhibition	Italy, Southern Europe	Bacchetta et al. 2008

Cadmium (Cd) Copper (Cu) Lead (Pb) Zinc (Zn)	<i>Xenopus laevis</i>	Pipidae	Eggs	control (modified Barth solution) for all treatments Cd: 0.5, 0.6, 0.7, 0.8 and 0.9 ppm Cu: 0.1, 0.2, 0.3, 0.4 and 0.5 ppm Pb: 0.5, 0.6, 0.7, 0.8 and 0.9 ppm Zn: 5, 10, 15 and 20 ppm	not evaluated	Decreased embryo hatching and tadpole survival Morphological: body length decreased and malformations (kinking and curling of the tail and edema)	Witwatersrand, South Africa	Haywood et al. 2004
Metamifop	<i>Xenopus laevis</i>	Pipidae	GS 45-46	LC50: control, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 mg/L Sublethal: control and 0.063 mg/L	0.634 mg/L	Mortality Behavioral: increased swirling, side-lying, air-breathing, activity, and use of peripheral area. Inhibited feeding. Morphological: decreased body weight Physiological: decreased triglycerides and total cholesterol and decreased neurotransmitters contents. Oxidative stress. Inhibition of T3 and T4 contents and expressions genes.	Beijing, China, East Asia	Liu et al. 2021
2,4-D	<i>Aquarana catesbeiana</i>	Ranidae	GS 25	LC50: control, 450, 500, 550, 600 and 650 mg/L Sublethal: control, 125, 250 and	574.52 mg/L	Mortality Morphological: decreased growth Ontogenetic: accelerated metamorphosis Physiological: changes in total lipid contents,	São Paulo, Brazil, South America	Freitas et al. 2019

				500 µg/L		total carbohydrates and proteins, and decreased respiration rates Behavioral: reduced speed response to the stimulus		
2-hydroxyatrazine	<i>Aquarana catesbeiana</i>	Ranidae	GS 26-32	25°C and 32°C: control, 10, 50 and 200 ng/L	not evaluated	Physiological: oxidative stress and increased acetylcholinesterase activity Histopathological: in liver was observed increase in cells with vacuolization, mild steatosis, number of melanomacrophages, inflammatory infiltration and cell congestion. In kidney was observed glomerular shrinkage, Bowman's space enlargement, inflammatory infiltrates, and fibrosis.	Santa Catarina, Brazil, South America	Carneiro et al. 2023
Arsenic (As)	<i>Lithobates pipiens</i>	Ranidae	GS 19	control, 10, 20, 150, 500 and 1.000 µg/L	not evaluated	Behavioral: slower swimming	Madison, Wiscosin, USA	Chen et al. 2009
Chlorpyrifos	<i>Lithobates sphenoccephalus</i>	Ranidae	GS 25-27	control, 1, 10, 100, and 200 µg/l + absent/presence sediment	not evaluated	Morphological: reduced body mass Physiological: decreased cholinesterase activity Behavioral: slower swimming	Oklahoma, USA, North America	Widder and Bidwell 2008
Chromium (Cr)	<i>Rana chensinensis</i>	Ranidae	GS 38	control, 104 ug/L (Cr), 50 ug/L	not evaluated	Morphological: reduction in SVL and body	Qinling	Zhang et al. 2023

Lead (Pb) Cr + Pb				(Pb) and 104 ug/L (Cr) + 50 ug/L (Pb)		mass and decrease in relative intestine length. Histopathological: irregular cell nuclei, indistinct cell, and brush-like border were observed in histological structures. Physiological: elevated amylase activity, decrease in pepsin activity and inhibition of lipase activity. Perturbation of intestinal homeostasis: Changes in bacterial richness and diversity, alterations in antioxidant enzymes, and pro-apoptotic gene modifications.	Mountains, Shaanxi Province, China,	
Glyphosate + predator cue	<i>Aquarana catesbeiana</i>	Ranidae	GS 25	control, 0.1, 1, 5, 10, and 20 mg a.i./L + present/absent predator cue	2.07 mg a.i./L	Mortality	Pittsburgh, USA, North America	Relyea 2004
Glyphosate + predator cue	<i>Aquarana clamitans</i>	Ranidae	GS 25	control, 0.1, 1, 5, 10, and 20 mg a.i./L + present/absent predator cue	2.17 mg a.i./L	Mortality	Pittsburgh, USA, North America	Relyea 2004
Glyphosate + predator cue	<i>Boreorana sylvatica</i>	Ranidae	GS 25	control, 0.1, 1, 5, 10, and 20 mg a.i./L + present/absent predator cue	2.17 mg a.i./L	Mortality	Pittsburgh, USA, North America	Relyea 2004
Glyphosate + predator	<i>Lithobates pipiens</i>	Ranidae	GS 25	control, 0.1, 1, 5, 10, and 20 mg	2.46 mg a.i./L	Mortality	Pittsburgh, USA,	Relyea 2004

cue				a.i./L + present/absent predator cue			North America	
Imidacloprid	<i>Polypedates teraiensis</i>	Rhacophoridae	GS 26-28	LC50: control, 18.5, 20, 21.5, 23, 24.5, 26 and 27.5 mg/L Sublethal: control, 0.69, 1.04 and 2.09 mg/L	20.91 mg/L	Morphological: decreased weight and snout-vent length Behavioral: hyperactivity followed by loss of balance, erratic movements, becoming motionless, lying down on the bottom, and leading to dead	Assam, India, South Asia	Dulumoni et al. 2020

Capítulo 2

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Rising Temperature Increases Methomyl (Lannate® BR) Toxicity in Amazonian Frog Tadpoles: Impacts on Survival and Thermal Limits

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Abstract

The increased use of pesticides in tropical regions, combined with rapid warming due to climate change, poses a critical threat to amphibian populations. However, the interactive effects of these stressors on tropical amphibians remain poorly understood. In this study, we assessed lethal and sublethal responses to methomyl in two Amazonian anurans, *Osteocephalus taurinus* and *Scinax ruber*. Tadpoles were exposed to methomyl at two temperatures (26.5 °C and 30 °C), simulating current and projected climate scenarios. Acute toxicity was quantified through LC₅₀, and thermal tolerance was assessed via critical thermal maximum (CT_{max}). Warming significantly increased methomyl toxicity in *O. taurinus*, lowering the LC₅₀ from 96.4 mg/L to 45.9 mg/L at 30 °C. *S. ruber* showed no such effect, with LC₅₀ values of 15.5 mg/L (26.5 °C) and 19.7 mg/L (30 °C). *S. ruber* was approximately six times more sensitive to methomyl than *O. taurinus* across temperatures. Methomyl exposure did not alter CT_{max} in either species, although variability increased in *O. taurinus* at higher concentrations. These findings provide novel evidence of temperature-modulated methomyl toxicity in Amazonian frog tadpoles, highlighting the need to incorporate native species, sublethal endpoints, and climate-relevant stressors in ecotoxicological risk assessments.

Keywords: Aquatic ecotoxicology, Climate change, Lethal concentration, Pesticide toxicity, Thermal tolerance

1. Introduction

Brazil ranks highest globally in pesticide use ^[1] and, in 2022, recorded sales of over 800 tons of pesticides. ^[2] One of the most widely sold pesticides in Brazil is the carbamate insecticide methomyl, which controls various insect species by inhibiting acetylcholinesterase, leading to neurological failure in the target organisms. ^[3,4] Despite its prohibition in many countries due to high toxicity to mammals, birds, fish, and amphibians, methomyl remains approved for use in Brazil. ^[4,5] While no monitoring data exist for methomyl in Amazonian freshwaters, studies from other agricultural regions report low but highly variable concentrations in surface waters and rural wells ^[3,6], and such sporadic sampling often fails to detect short-term contamination pulses following rainfall or runoff. ^[7] Given its intensive agricultural use in Brazil and documented toxicity, potential ecosystem contamination remains a concern. ^[2] Methomyl's toxicity to non-target species is attributed to its chemical properties (high solubility, low molecular weight, and low topological polar surface area - TPSA), which allow it to easily cross cell membranes, including the developing blood–brain barrier, contributing to its neurotoxic effects. ^[3,4,8,9] Previous studies have reported various negative effects of methomyl in aquatic ectotherms, including antioxidant system inhibition ^[10,11], genotoxicity ^[12], tissue injuries ^[13], reduced growth ^[14], neurotoxicity and teratogenicity ^[15] and mortality ^[16]. Furthermore, increasing temperatures due to climate change can exacerbate the toxic effects of these contaminants. ^[17-19] This is because the interaction of both can alter the ability of amphibians to perform effective defensive responses to maintain homeostasis. There is also evidence that temperature interacts with certain pesticides, resulting in increased mortality among tadpoles. ^[16,20,21]

The Intergovernmental Panel on Climate Change (IPCC) climate projections indicate that global temperatures could rise by up to 1.8 °C by 2081–2100 under low greenhouse gas emission conditions. However, this appears increasingly implausible as 2023 was the hottest year since the pre-industrial period. ^[22-24] In the context of climate change, understanding the thermal physiology of anurans is essential. ^[25,26] As ectothermic animals, the primary functions of amphibians, including growth, development, behavior, reproduction and energetic metabolism are influenced by temperature. ^[27-29] The critical thermal maximum (CT_{max}) is a useful and reliable index for understanding thermal tolerance of an individual. ^[30,31] The CT_{max} is defined as the thermal point at which a gradual increase in temperature renders the animal unable to perform its normal locomotor activity (i.e., loss of balance). ^[32] In tropical environments, where temperature fluctuations are smaller than in temperate regions, it is anticipated

that species will experience temperatures closer to their thermal limits ^[33,34], rendering them susceptible to the climatic warming projections put forth by the IPCC. ^[22]

In freshwater systems affected by human activities, tadpoles are continuously subjected to the combined effects of climate change-induced temperature increases and the presence of pollutants. ^[35-38] It is therefore imperative to consider the effects of contaminants under conditions of exposure that more accurately reflect those occurring in natural environments, including variations in water temperature. Furthermore, it is essential to enhance the quantity and quality of data concerning the impacts on tropical amphibian species. The objective of this study was to determine the lethal concentration (LC₅₀) of methomyl (Lannate® BR) at environmentally relevant temperatures (26.5°C) and under IPCC-projected warming (30°C) in tadpoles of two Amazonian anurans, *Osteocephalus taurinus* and *Scinax ruber*. Additionally, we assessed the effects of sublethal methomyl exposure on their critical thermal maximum (Ct_{max}).

2. Materials and Methods

2.1. Tadpole collection and maintenance

Tadpoles of *Osteocephalus taurinus* and *Scinax ruber* at Gosner stage 25 were collected from temporary ponds in Manaus, Amazonas, Brazil. *O. taurinus* from the Adolpho Ducke Biological Reserve (2.916° S, 59.883° W), and *S. ruber* from ponds at the National Institute for Amazonian Research – INPA (3.097° S, 59.987° W). Both species reproduce in temporary ponds, where their clutches are easily accessible, and their larvae are common in natural water bodies. ^[39,40] Collection areas were not affected by land-use changes. Tadpoles were acclimated for seven days in 70 L aquaria (70 × 50 × 30 cm) under controlled conditions (26.13 ± 0.47 °C, 5.72 ± 1.26 mg O₂/L, pH 7.12 ± 0.38) at a density of three tadpoles per liter. Individuals from different clutches were randomly mixed to minimize genetic bias, and animals were fed once daily *ad libitum* with a mix of commercial fish and rabbit food. All procedures followed ethical guidelines and were approved by the appropriate authorities (collection permit SISBio No. 80447-4; Animal Ethics Committee of INPA, protocol No. 83/2023).

2.2. Determination of lethal concentration (LC₅₀) of methomyl at different temperatures

Acute toxicity assays were conducted in glass aquaria (22 × 20 × 18 cm; 6 L) maintained under a 12:12 h light:dark photoperiod. Two temperatures were tested: 26.5 °C, based on field measurements (mean pond temperature = 26.85 ± 1.06 °C), and 30 °C, simulating IPCC SSP3-7.0 projections.^[22] Ten tadpoles (Gosner stage 25–28)^[41] were randomly selected and placed per aquarium. The average body mass was 0.13 ± 0.04 g for *O. taurinus* and 0.05 ± 0.01 g for *S. ruber*.

We used the commercial formulation Lannate® BR (215 g a.i. L⁻¹; 21.5% methomyl w/v). After 30 min acclimation at 26 °C, aquaria were warmed to their target temperature at 0.3 °C/min. Once each aquarium reached its designated temperature (26.5 °C or 30 °C), the calculated volume of the formulation required to obtain nominal concentrations (mg a.i. L⁻¹) was added directly to the water. Solutions were homogenized by continuous aeration. Methomyl is highly soluble and stable under our experimental conditions, with a reported half-life of 25–30 days in freshwater at neutral pH and in the absence of direct UV light.^[3,42]

Concentration ranges were determined from range-finding tests performed separately for each species and temperature, ensuring complete dose–response curves (0–100% mortality) in accordance with OECD and USEPA guidelines.^[43] For *O. taurinus*, the tested concentrations were: 0, 25, 50, 75, 100, 200, and 500 mg a.i./L at 26.5 °C, and 0, 25, 35, 50, 100, and 200 mg a.i./L at 30 °C. For *S. ruber*, the concentrations were: 0, 2, 8, 16, 32, 64, 128, 256, and 512 mg a.i./L at 26.5 °C; and 0, 0.031, 0.062, 0.125, 0.25, 0.5, 1, 2, 8, 16, 32, 64, 128, 256, and 512 mg a.i./L at 30 °C. Each concentration was tested in triplicate (n = 3 aquaria; 10 tadpoles per aquarium).

Temperature was maintained with individual thermostats and remained stable throughout the 96-h exposure period (26.43 ± 0.22 °C and 30.06 ± 0.20 °C; mean ± SD). Dissolved oxygen remained above 85% through continuous aeration. Water pH was also stable across treatments (7.3 ± 0.2; mean ± SD). Water-quality parameters were measured daily using a calibrated multiparameter probe. Mortality was recorded every 24 h. At the end of the experiment, surviving tadpoles were euthanized with MS-222 and fixed in 10% formalin.

2.3. Effect of sublethal exposure to methomyl at the critical thermal maximum (CT_{max}) in tadpoles

Thermal tolerance was assessed using CT_{max} tests after 96 h of sublethal methomyl exposure. Selected concentrations ensured 100% survival at 26.5 °C: 0 (control), 25, and 50 mg a.i./L for *O. taurinus*; 0, 2, and 4 mg a.i./L for *S. ruber*. As the species displayed markedly different sensitivities in the LC₅₀ assays, we adopted species-specific concentration thresholds to avoid introducing mortality-related confounders in the CT_{max} assessment. Nine tadpoles per group (n = 9) were randomly selected for CT_{max} assays. Tests followed protocols by Azambuja et al. (2021) ^[44] and Kochhann et al. (2021) ^[45]. Tadpoles were placed in individual chambers within a 40 × 20 × 30 cm tank containing 24 L of aerated water (>75% DO). The initial water temperature was set to 26.5 °C, matching the exposure temperature. After a 30-min acclimation, temperature was increased at a rate of 0.3 °C/min. The CT_{max} was recorded when tadpoles lost equilibrium and failed to respond to three consecutive hits. ^[46] All individuals recovered within 24 h.

2.4. Statistical analysis

Data normality was assessed using the Shapiro-Wilk test and homogeneity of variances was tested using Levene's test.

Lethal concentration (LC₅₀) values were estimated via probit regression ^[47] using the LC_{probit}() function, and comparisons between temperatures or species were performed using dose-ratio tests with the ratio_{test}() function; both functions are available in the ecotox package. LC₅₀ results are reported as point estimates with 95% confidence intervals (lower CL – upper CL).

Critical thermal maximum (CT_{max}) data for *Osteocephalus taurinus* met parametric assumptions and were analyzed by ANCOVA, with body mass as a covariate. In contrast, *Scinax ruber* data did not meet the assumptions of normality and were therefore analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test with Holm correction for multiple comparisons. CT_{max} are expressed as mean ± SEM.

All analyses were conducted in R version 4.2.2 (R Development Core Team, 2023) with a significance level of p < 0.05.

3. Results

3.1. Determination of lethal concentration (LC₅₀) of methomyl at different temperatures

Species exhibited differential responses to methomyl under varying temperatures. In *Osteocephalus taurinus*, the LC₅₀ significantly decreased from 96.4 mg a.i./L (87.3–109) at 26.5 °C to 45.9 mg a.i./L (34.8–63.5) at 30 °C ($p < 0.001$), indicating enhanced toxicity under warming conditions (Fig. 1 A-B).

In contrast, we found no clear statistical evidence that temperature affected acute methomyl toxicity in *Scinax ruber*: the LC₅₀ was 15.5 mg a.i./L (13.2–18.2) at 26.5 °C and 19.7 mg a.i./L (10.1–42.4) at 30 °C (LC₅₀ ratio test, $p = 0.172$), with the wide confidence interval at 30 °C indicating greater statistical uncertainty around this estimate (Fig 1. C-D).

Inter-species comparison revealed *S. ruber* to be significantly more sensitive than *O. taurinus* at both temperatures ($p < 0.001$). At 26.5 °C, *O. taurinus* exhibited over six times higher tolerance than *S. ruber*.

3.2. Effect of sublethal exposure to methomyl on critical thermal maximum (CT_{max}) in tadpoles

In *O. taurinus*, CT_{max} values were 41.0 ± 0.58 °C (control), 40.2 ± 1.57 °C (25 mg a.i./L) and 39.9 ± 1.14 °C (50 mg a.i./L). When body mass was included as a covariate, methomyl concentration did not significantly affect CT_{max} (ANCOVA: group $F_{2,23} = 1.44$, $p = 0.259$; partial $\eta^2 \approx 0.11$), indicating that any pesticide-induced change in upper thermal limits is likely to be small under our experimental conditions (Fig. 2). In *S. ruber*, CT_{max} values were 42.1 ± 1.14 °C (control), 41.3 ± 1.51 °C (2 mg a.i./L), and 41.3 ± 1.11 °C (4 mg a.i./L), with no statistical differences detected (Kruskal–Wallis, $\chi^2 = 2.65$, $df = 2$, $p = 0.266$) (Fig. 3).

4. Discussion

Our results demonstrate temperature-dependent, species-specific modulation of methomyl toxicity. A 3.5 °C temperature increase, consistent with IPCC SSP3-7.0 projections, significantly reduced the LC₅₀ in *Osteocephalus taurinus*. In *Scinax*

ruber, we did not find clear statistical evidence for a temperature effect on LC₅₀ within the 26.5–30 °C range. Despite this, *S. ruber* exhibited lower LC₅₀ values than *O. taurinus* at both tested temperatures, indicating higher sensitivity to methomyl under our experimental conditions. LC₅₀ values for *S. ruber* (15.5–19.7 mg a.i./L) and *O. taurinus* (96.4–45.9 mg a.i./L) fall within or below the range reported for other anurans. For instance, *Sclerophrys arabica* tadpoles exhibited an LC₅₀ of 14.12 mg/L ^[15], and *Hoplobatrachus rugulosus* showed even greater sensitivity, with an LC₅₀ of 8.69 mg/L. ^[14] *Microhyla pulchra* demonstrated an LC₅₀ of 84.57 mg/L, closer to the values observed for *O. taurinus* in our study, though still lower ^[16]. Conversely, more tolerant species like *Duttaphrynus melanostictus* and *Polypedates megacephalus* showed LC₅₀ values exceeding 300 mg/L. ^[16] This variability underscores the limitations of extrapolating toxicity data from a few temperate or model species.

The increase in methomyl toxicity with rising temperature was observed in *O. taurinus*, with the LC₅₀ decreasing to 45.9 mg/L at 30°C, is consistent with the findings of Lau et al. (2015) ^[16], where the LC₅₀ for *Microhyla pulchra*, *Duttaphrynus melanostictus*, and *Polypedates megacephalus* was reduced between 15 and 60% with increasing temperature. These results support the idea that temperature increases potentiate methomyl toxicity. The differences in sensitivity between species indicate that the response to methomyl toxicity is influenced by the species-specific physiological characteristics.

The climate-induced toxic sensitivity hypothesis provides a framework for understanding the increased toxicity of methomyl at elevated temperatures for *O. taurinus* tadpoles, as it posits that the acceleration of metabolism caused by temperature results in an increased impact of the contaminant. ^[48-49] This aligns with evidence from global change studies showing that rising temperatures enhance pesticide uptake and biotransformation in aquatic organisms, as observed for organophosphates and carbamates in fish and amphibians. ^[50-51] Specifically, higher metabolic rates under warming conditions can intensify the conversion of methomyl into more toxic intermediates, while simultaneously reducing the energy available for detoxification, a pattern consistent with the heightened sensitivity of ectotherms to pollutants under climate change scenarios. ^[52] The increased metabolic rate, due to the increase in temperature, stimulates ventilation rates to increase oxygen consumption in the tadpoles, thereby resulting in higher methomyl absorption rates over time. Moreover, *O. taurinus*, more closely associated with forested environments, where temperatures are typically cooler and more stable than in urban areas due to canopy

cover and evapotranspiration ^[53], may have limited adaptive capacity to cope with such combined stressors.

In contrast, *S. ruber* is frequently associated with highly urbanised habitats, and large Amazonian cities such as Manaus exhibit a pronounced urban heat island, with urban areas consistently warmer than nearby forested or rural sites. ^[54-55] Chronic exposure to these warmer urban microclimates may contribute to the slightly higher baseline CT_{max} we observed in *S. ruber* and help explain its lack of a synergistic response to methomyl and elevated temperature in our experiment, although this remains a tentative hypothesis that requires targeted testing. Nevertheless, *S. ruber* showed consistently lower LC₅₀ values than *O. taurinus* at both temperatures, indicating higher susceptibility to methomyl under our experimental conditions. *S. ruber* tadpoles are considerably smaller, averaging 0.05 ± 0.01 g compared to 0.13 ± 0.04 g in *O. taurinus*, resulting in a higher surface area-to-volume ratio that facilitates contaminant absorption. ^[56-58] The same pattern, where smaller-bodied tadpoles exhibit heightened sensitivity to methomyl, has also been observed in *Microhyla pulchra*, which presented lower LC₅₀ values compared to larger species such as *Duttaphrynus melanostictus* and *Polypedates megacephalus*. ^[16] However, because we did not quantify intraspecific relationships between body mass and survival time, we consider body size as a plausible contributing factor rather than the sole mechanism underlying the higher sensitivity of *S. ruber*, and other species-specific physiological traits (e.g. detoxification and repair capacity) are also likely to be involved. In addition to the overall higher sensitivity of *S. ruber*, mortality at 30 °C showed considerable heterogeneity among replicates, particularly at intermediate concentrations. This shallow and variable concentration–response pattern likely reflects substantial inter-individual variability in acute sensitivity under warm conditions, which in turn contributed to the wide confidence interval around the LC₅₀ estimate at 30 °C. Although this reduces the precision of the point estimate, the dose-ratio test indicated that the LC₅₀ values at 26.5 °C and 30 °C are not statistically distinguishable within the uncertainty associated with these data, supporting our interpretation of no detectable temperature effect on acute toxicity for this species.

Because Lannate® BR contains only 21.5% methomyl and 78.5% co-formulants, our LC₅₀ values represent the integrated toxicity of the commercial product as it is used in the field. Co-formulants may enhance membrane permeability, stability, or bioavailability of methomyl, potentially modifying the observed toxicity. This is ecologically relevant because amphibians in agricultural landscapes are exposed to

the full formulation following rainfall-driven runoff, rather than to the pure active ingredient. Although methomyl was not analytically quantified in the present assays, the compound is highly soluble and stable under controlled laboratory conditions, and studies that performed chemical verification report only small deviations between nominal and measured concentrations over 24–96 h. ^[11,13,16,59,60] These observations support the interpretation that nominal concentrations provide reliable proxies for short-term exposures in experimental settings similar to ours, while future studies would benefit from incorporating time-resolved chemical measurements alongside tests using both the pure active ingredient and different commercial formulations to more precisely disentangle formulation-specific effects.

Despite its neurotoxic mode of action as a reversible acetylcholinesterase inhibitor, we did not detect any statistically significant changes in CT_{max} in either species, suggesting that their acute upper thermal limits remained relatively robust under the present sublethal exposure conditions. In *O. taurinus*, mean values differed by less than 1 °C between control and the highest concentration, indicating that any pesticide-induced change in upper thermal limits is likely to be small. However, the increased among-individual variability in *O. taurinus* at 50 mg a.i. L⁻¹ suggests that more subtle sublethal effects on thermal performance may still occur and would need to be addressed with specific physiological endpoints rather than CT_{max} alone.

Although our experiment focused on a single pesticide and two amphibian species, our findings have broader conceptual implications for ecotoxicological risk assessments in tropical ecosystems. Current regulatory frameworks often rely on temperate model species and fail to capture context-specific stressors prevalent in tropical regions. ^[61] The pronounced increase in methomyl toxicity to *Osteocephalus taurinus* at elevated temperatures illustrates how warming can exacerbate pesticide effects in forest-associated species, a growing concern in the face of ongoing deforestation and habitat fragmentation. ^[53,62] Considering that many Amazonian species are already living near their thermal limits ^[34], even modest warming-induced reductions in tolerance could contribute to population declines. We therefore view our study as a case example that highlights the need to better account for interactions between climate warming and pesticide exposure, which may amplify ecological risks, especially for species with limited thermal plasticity. In this context, integrating native sentinel species into risk assessments, explicitly considering episodic peak exposures (e.g. rainfall-driven runoff events; ^[63-64] and incorporating sublethal endpoints alongside traditional lethality metrics may help improve the ecological relevance and predictive

power of tropical ecotoxicology under climate change scenarios that involve both rising temperatures and enhanced contaminant mobility. ^[65]

5. Conclusion

Our findings reveal species-specific methomyl sensitivities in Amazonian tadpoles: *Scinax ruber* was more sensitive to the pesticide at both temperatures, while *Osteocephalus taurinus* exhibited increased susceptibility under higher temperatures, indicating distinct dimensions of vulnerability to methomyl and warming. These results underscore that ecological traits (e.g., habitat preference, thermal adaptation) critically shape responses to chemical stressors. This study provides new experimental evidence of temperature-dependent pesticide toxicity in tropical amphibians from the Brazilian Amazon, illustrating that current risk assessments, which are typically performed at a single laboratory temperature, may not fully capture temperature-driven changes in species-level sensitivity. Our results highlight that warming can alter toxicity in ways that are relevant for thermally sensitive species such as *O. taurinus*. Given the widespread pesticide use and projected warming trends, we suggest that considering thermal stress when biologically justified could improve the ecological realism of regulatory frameworks and help guide targeted monitoring of more vulnerable species, such as *S. ruber*. Future research should expand on sublethal endpoints and individual variability to refine predictions of climate–chemical interactions in these ecosystems.

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8. Disclosure of interest

The authors report there are no competing interests to declare.

9. Data availability statement

All datasets generated and analysed during this study are openly available in Figshare at the following DOI: 10.6084/m9.figshare.30747389. The repository includes the complete LC₅₀ mortality dataset for both temperatures and species, as well as all CT_{max} measurements used in the analyses presented in the manuscript.

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10. List of Figures

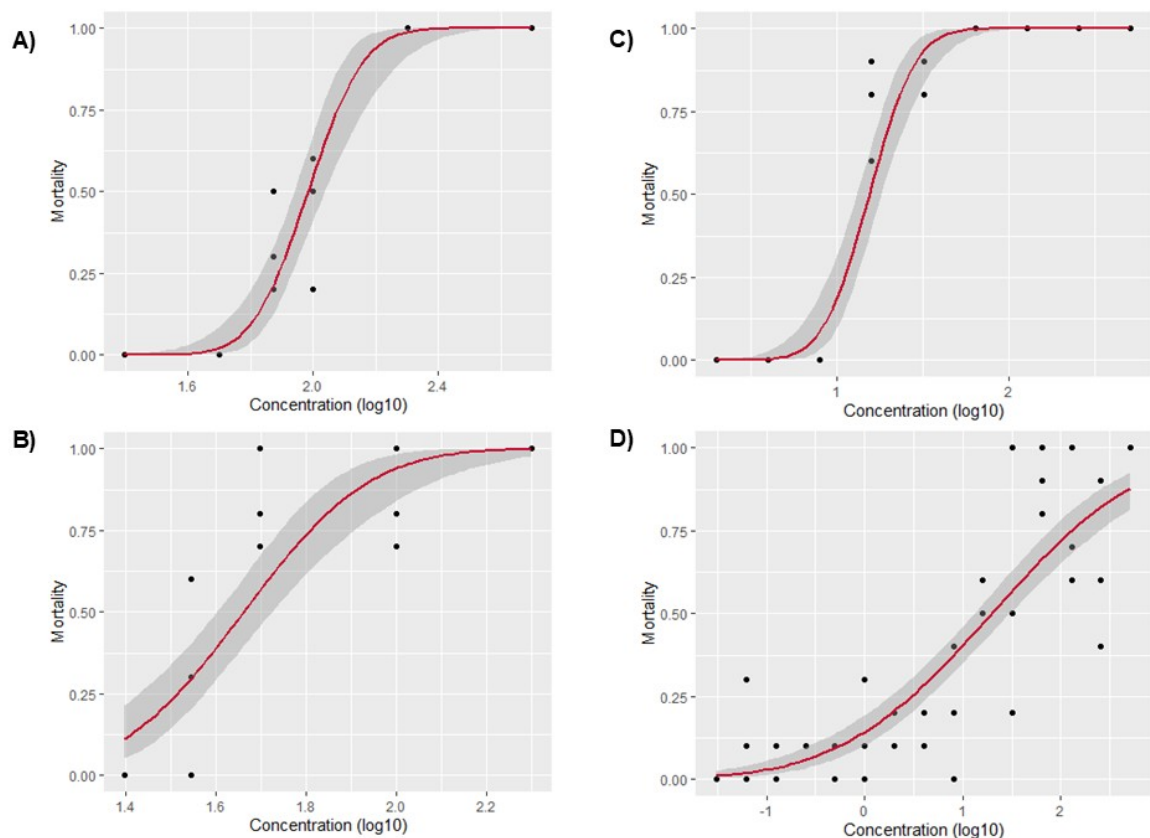


Figure 1. Estimated median lethal concentration (LC_{50}) of methomyl for *Osteocephalus taurinus* at **(A)** 26.5 °C and **(B)** 30 °C, and *Scinax ruber* at **(C)** 26.5 °C and **(D)** 30 °C.

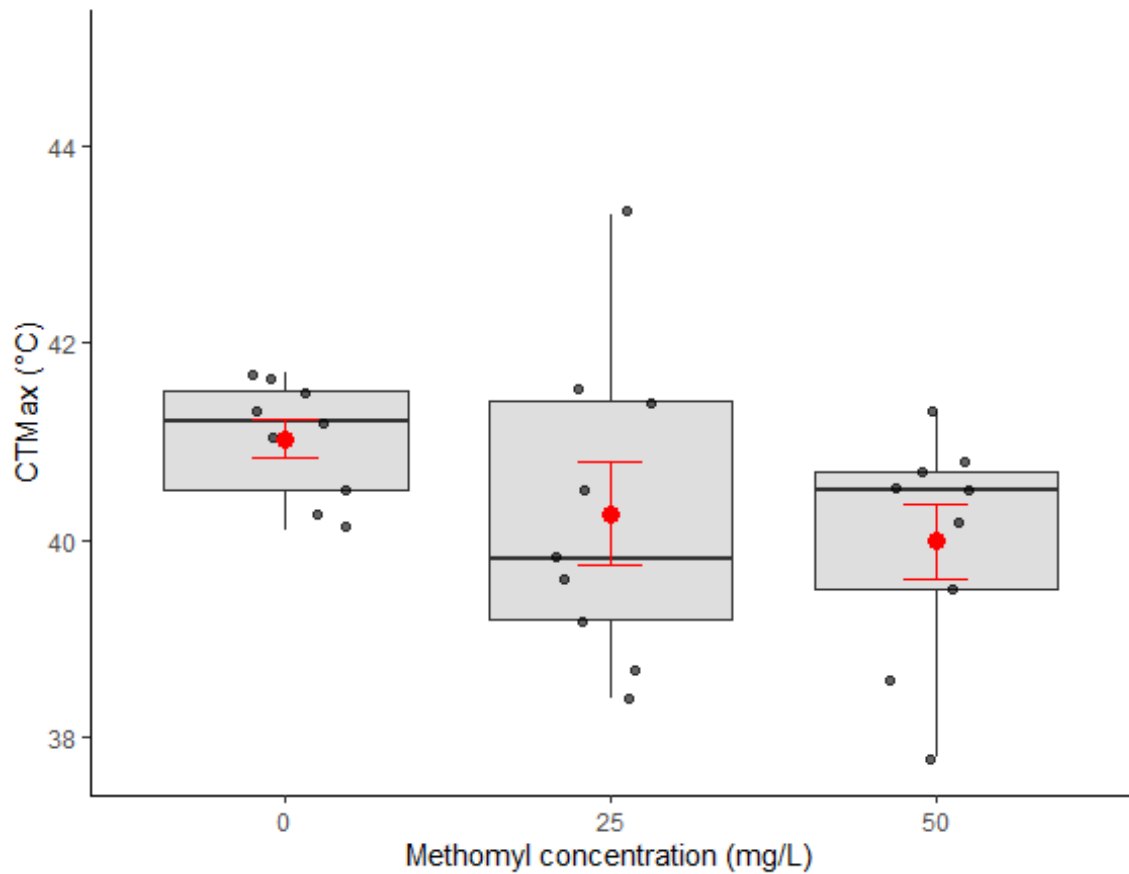


Figure 2. Variation of critical thermal maximum (CT_{max}) of *Osteocephalus taurinus* tadpoles subjected to increasing sub-lethal concentrations of methomyl. Boxplots represent the interquartile range (IQR), with the median indicated by the horizontal line inside each box. Gray points represent observed values. Red points indicate group means, with red error bars representing the standard error of the mean (SEM). No significant difference in CT_{max} was observed among groups (one-way ANOVA, $p = 0.175$; post hoc Tukey, $p > 0.05$).

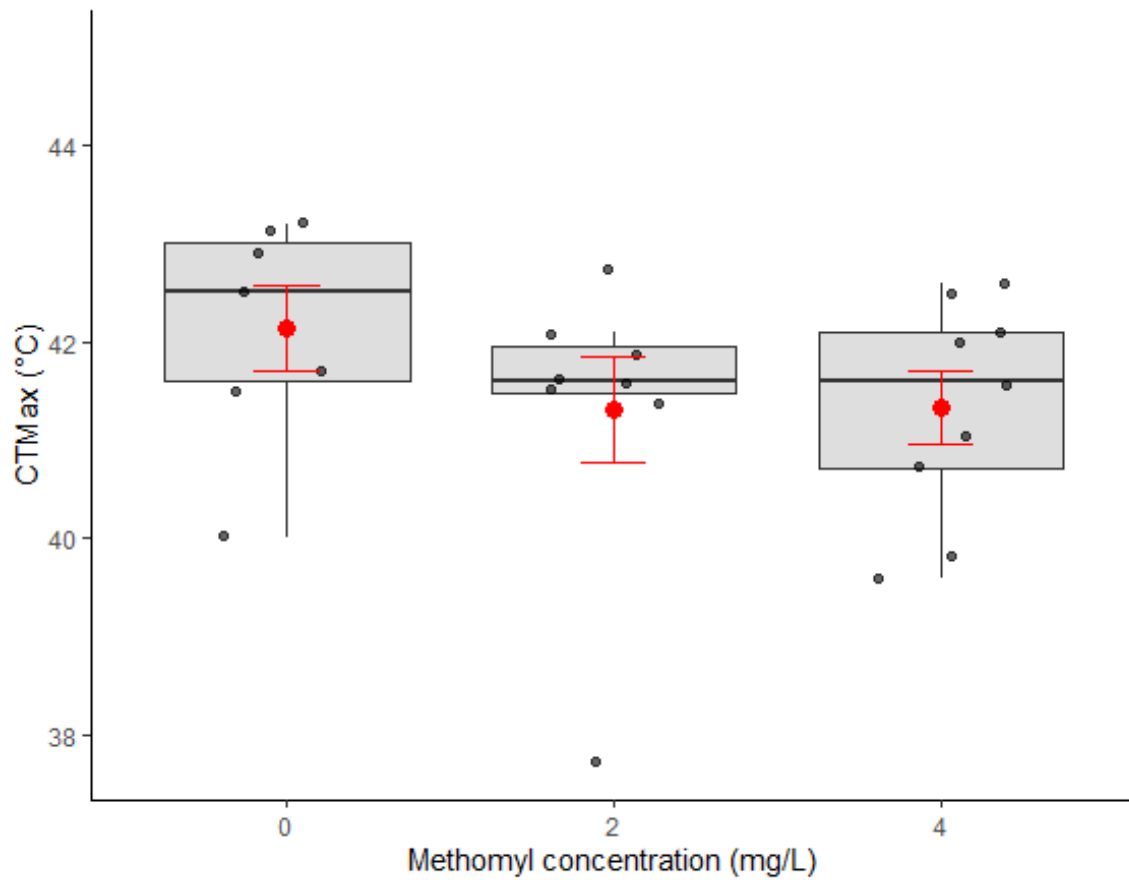


Figure 3. Variation of critical thermal maximum (CT_{max}) of *Scinax ruber* tadpoles subjected to increasing sub-lethal concentrations of methomyl. Boxplots represent the interquartile range (IQR), with the median indicated by the horizontal line inside each box. Gray points represent observed values. Red points indicate group means, with red error bars representing the standard error of the mean (SEM). No significant difference in CT_{max} was observed among groups (Kruskal-Wallis test, $p = 0.2658$; post hoc Dunn, $p > 0.05$).

Capítulo 3

Azambuja G, de Souza SS, Silva CB, Kaefer IL, Val AL, Kochhann D. ***Combined Impacts of Warming and Methomyl on Neurophysiological and Behavioral Responses in Amazonian Frog Tadpoles.*** *Ecotoxicology*.

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Combined Impacts of Warming and Methomyl on Neurophysiological and Behavioral Responses in Amazonian Frog Tadpoles

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Abstract

Amphibian populations are declining globally due to anthropogenic stressors such as climate change and environmental contamination. In Amazonia, where aquatic species already endure conditions near their thermal thresholds, the combined effects of rising temperatures and pesticide exposure pose a threat to their survival and physiological stability. Here, we evaluated the isolated and interactive impacts of elevated temperature and sublethal concentrations of the carbamate pesticide methomyl on behavior, neurophysiology, and endocrine responses in tadpoles of two Amazonian frogs, *Osteocephalus taurinus* and *Scinax ruber*. Tadpoles were exposed to the stressors for 96 hours, and the subsequent assays measured substrate preference, locomotor and antipredator behavior, acetylcholinesterase (AChE) activity, and whole-body cortisol concentrations. *O. taurinus* showed significant behavioral impairments under both stressors, including reduced swimming activity and increased immobility on dark substrates, suggesting antipredator strategies. AChE activity decreased under elevated temperature but showed a pronounced increase under combined exposure to methomyl and heat, while cortisol concentration did not change significantly. In contrast, *S. ruber* maintained stable behavior and enzyme activity, yet exhibited robust endocrine responses, with significant cortisol elevation under both stressors. No significant responses to alarm substances were observed in either species. Our results highlight distinct species-specific responses: *O. taurinus* displayed greater behavioral sensitivity, whereas *S. ruber* showed pronounced endocrine reactivity. These divergent strategies likely reflect ecological and physiological adaptations, emphasizing the importance of multi-stressor and multi-species approaches in risk assessment. This study underscores the vulnerability of tropical amphibians to co-occurring climate and pollution stressors and calls for integrative conservation strategies that consider species-specific traits and ecological histories.

Keywords: anuran larvae, climate change, ecotoxicology, pollution

1. Introduction

Amphibians face unprecedented threats to their survival, with substantial population declines linked to the intensification of human activities (Green et al. 2020; Nolan et al. 2023). The escalating exploitation of natural resources, driven by technological advances and human population growth, has increased anthropogenic pressure on aquatic ecosystems, which are critical habitats for many amphibian species. Amphibians are among the most threatened vertebrates, and their declines are increasingly attributed to climate change and environmental pollution (Balvanera et al. 2020; McRae et al. 2020). Their sensitivity to environmental disturbances makes them valuable bioindicators, owing to their intrinsic morphophysiological traits: (I) eggs that require moist environments, (II) an aquatic larval stage in most species, and (III) highly permeable skin, which facilitates the absorption of contaminants (Slaby et al. 2019). Furthermore, anuran metamorphosis represents a critical period during which specialized larval structures (e.g., gills and tail) are resorbed, while terrestrial organs (e.g., lungs, limbs) develop (Gosner 1960; Yaoita 2019), rendering them particularly vulnerable to environmental disruptions.

In recent decades, pesticide contamination of aquatic ecosystems has exposed non-target species to toxic compounds. Global pesticide usage surged from 2.18 million tons in 2000 to 3.69 million tons in 2022, a 69.68% increase in just 22 years (FAO 2025). These contaminants enter aquatic environments through spray drift during aerial application, direct overspray, surface runoff from heavy rainfall, and soil erosion (Solomon and Thompson 2003; Commelin et al. 2022). In Brazil, one of the most widely sold pesticides is the carbamate insecticide methomyl, with 9.1 thousand tons commercialized in 2023, a 23.6% increase over the past decade (IBAMA, 2025). Methomyl is extensively used to control a broad spectrum of insect pests. Despite being banned in several countries due to its high toxicity to vertebrates, its use is permitted in Brazil (Lin et al. 2020; NCBI, 2024). Its physicochemical properties, including high water solubility (57.9 g/L^{-1}), low sediment adsorption, and extended half-life (6 days in surface water, 25 weeks in groundwater), enhance its environmental mobility, persistence and bioavailability to aquatic organisms (Selvam et al. 2013; Zheng et al. 2023; Dantas et al. 2024). Methomyl exerts its toxic effects by inhibiting acetylcholinesterase (AChE), leading to the accumulation of acetylcholine (ACh) in synaptic clefts, which, in vertebrates, triggers neurotoxic effects such as muscle tremors, spasms, and paralysis (Van Scoy et al. 2013; NCBI, 2024). In non-target

organisms, methomyl is associated with neurotoxicity, endocrine disruption and behavioral impairments. In fish, exposure reduces AChE activity, elevates cortisol concentrations and compromises locomotor performance (Jablonski et al. 2022; Rahman et al. 2022; Li et al. 2024). In tadpoles, pesticide-induced behavioral alterations may impair energy acquisition, a critical factor for proper growth and metamorphosis. Additionally, locomotor deficits could heighten predation risk, thereby reducing survival (Freitas et al. 2017; Liu et al. 2021). Although previous studies have documented neurotoxicity, cellular damage and behavioral impairments caused by methomyl in tadpoles of species such as *Bufo arabicus* (Seleem 2019a,b) and *Hoplobatrachus rugulosus* (Trachantong et al. 2017), the impacts of this pesticide on Neotropical tadpoles remain largely unexplored. This knowledge gap is critical because amphibians in Amazonia play pivotal ecological roles as regulators of trophic dynamics, contributors to nutrient cycling and as indicators of ecosystem health (Valencia-Aguilar et al. 2013; Azambuja et al. 2024). Moreover, the Neotropics harbor unparalleled amphibian diversity, encompassing a wide range of ecological strategies and physiological adaptations that can modulate how different species respond to environmental stressors (Guerra et al. 2020; Azambuja et al. 2024). Comparative approaches are therefore essential to elucidate how distinct lineages cope with multiple stress factors. Previous studies have demonstrated asymmetric responses, where co-occurring species exhibit contrasting behavioral or physiological outcomes under the same contaminant exposure, reflecting differences in life-history traits and evolutionary background (Boone & Semlitsch, 2002; Distel & Boone, 2011a,b; Costa et al. 2024; Henao et al. 2022).

The impact of climate change on tropical ecosystems such as Amazonia is of growing concern, especially for aquatic organisms which already experience temperatures near their thermal limits (Campos et al. 2021; IPCC, 2021). For amphibians, whose thermoregulation depends on environmental conditions, warming directly affects essential physiological processes and behavior (Watkins and Vraspir 2006; Navas et al. 2008). Elevated temperatures may exacerbate physiological dysfunctions, such as locomotion impairment due to increased energy demands (Ruthsatz et al. 2018; Gallo et al. 2020). Amphibian larvae are known to exhibit interactive effects when exposed to multiple environmental stressors, where factors such as predation risk, density, or physicochemical alterations can intensify contaminant toxicity (Boone & Semlitsch, 2001; Relyea & Mills, 2001; Burraco & Gomez-Mestre, 2016). Despite this, the synergistic effects of pesticides combined with

temperature remain poorly studied. One of the few available pieces of evidence shows that rising temperatures enhanced methomyl toxicity in three Asian anuran species (Lau et al. 2015). However, as reviewed by Azambuja et al. (2024), no studies have addressed this interaction in Amazonian species. This is critical because many Amazonian ectotherms already live near their CT_{max} , resulting in narrow thermal safety margins and increased vulnerability to climate stress (Simon et al. 2015; Campos et al. 2021). This knowledge gap is critical, given that the Amazonia region already faces pesticide contamination (Rico et al. 2022; Cezarette et al. 2024) and accelerated warming (IPCC, 2021; Braz-Mota and Luis Val 2024), and these factors may impact behavior, physiology and larval development (Baier et al. 2016; Quiroga et al. 2019, 2024). Therefore, this study evaluates the isolated and combined effects of methomyl and elevated temperature on behavioral, neurophysiological and endocrine parameters in the tadpoles of *Osteocephalus taurinus* (Steindachner, 1862) and *Scinax ruber* (Laurenti, 1768), two Amazonian hylid frog species with distinct ecological backgrounds and environmental tolerances. We predicted that (i) exposure to methomyl, elevated temperature or their combination would impair neurophysiological and endocrine responses, characterized by acetylcholinesterase (AChE) inhibition and cortisol elevation; (ii) such physiological disturbances would lead to behavioral alterations, including changes in substrate preference and reduced swimming performance; and (iii) the ability of tadpoles to detect and respond to alarm substances would be compromised under methomyl exposure, elevated temperature, and particularly their combination, reflecting cumulative neuroendocrine disruption.

2. Materials and Methods

2.1. Clutch collection and maintenance

Osteocephalus taurinus and *Scinax ruber* are distributed throughout the Amazon basin in Brazil. These species reproduce year-round, with peak activity during the rainy season when they lay their eggs in temporary aquatic environments. *O. taurinus* deposits its eggs on the surface of ponds, where they are encased in a gelatinous film, while *S. ruber* deposits its eggs attached to vegetation or along the margins of ponds. *S. ruber* is commonly found in open areas, forest edges and clearings, and is frequently observed in trees and anthropogenic environments (Lima et al. 2008; Frost 2021).

Tadpoles of *O. taurinus* and *S. ruber* were collected at Gosner stages 24–25 (Gosner, 1960) from temporary ponds within the Adolpho Ducke Forest Reserve (2.916° S, 59.883° W), and in a forested area of the National Institute for Amazonian Research (INPA) campus (3.097° S, 59.987° W), Manaus, Amazonas, Brazil. Both locations are surrounded by vegetation cover and lack nearby agricultural activity, as confirmed by land-cover data from MapBiomas (Collection 10, 2025), indicating no direct exposure to pesticide runoff or agricultural contaminants. For each species, tadpoles were collected and transported to the laboratory and kept in 70-L aquaria (70x50x30 cm) with a temperature of 26.13 ± 0.47 °C, oxygen 5.72 ± 1.26 mgO₂ L⁻¹, and pH 7.12 ± 0.38 , at a density of three tadpoles per liter for at least 14 days for acclimation to lab conditions. A 12:12 light:dark cycle was used to maintain the photoperiod, with water renewals occurring every 48 hours. Tadpoles from different clutches were randomized in the aquaria to ensure sample variability. They were fed daily with an *ad libitum* diet comprising a blend of commercial fish feed and rabbit feed, containing 32% and 14% protein, respectively.

2.2. Determination of LC₅₀ values

The LC₅₀ determination was conducted in a separate preliminary study designed to support the present experiment. Acute toxicity tests were performed for both species (*O. taurinus* and *S. ruber*) at 26.5 °C and 30 °C, using the commercial formulation Lannate® as the methomyl source. Ten tadpoles were placed in each aquarium (22 × 20 × 18 cm; 6 L), and each concentration was tested in triplicate. The exposure lasted 96 hours, during which mortality was recorded every 24 hours. LC₅₀ values were estimated for each temperature and species using Probit analysis, following OECD guidelines for acute toxicity tests (OECD, 2025). These LC₅₀ data served as the basis for selecting the sublethal exposure level (10% of the 96-h LC₅₀) used in the present study.

2.3. Exposure to sublethal concentrations of methomyl at different temperatures

Ten tadpoles at Gosner stages 25–28 (Gosner, 1960) were randomly selected and placed into experimental aquaria (22 × 20 × 18 cm; 6 L), with each concentration

tested in duplicate, totaling 20 tadpoles per concentration. Following their transfer, tadpoles were given a 30-minute acclimation period before initiating the heating process. Water temperature was gradually increased from the acclimation baseline (26 °C) to the target values for each aquarium (26.5 °C or 30 °C) at a rate of 0.03 °C min⁻¹, following established protocols (Fan et al. 2021; Azambuja et al. 2021; Kochhann et al. 2021) and representing ecologically realistic warming rates observed in shallow ponds (Terblanche et al. 2011; Turriago et al. 2022). The temperature regimes represented: (1) 26.5 °C, mimicking natural temporary pond conditions, and (2) 30 °C, corresponding to the IPCC SSP3–7.0 projected warming scenario (+3.5 °C global average; IPCC, 2021).

After each aquarium reached its target temperature, the calculated volume of the commercial methomyl formulation (Lannate®) required to achieve the nominal concentrations (mg L⁻¹) was added directly to the water containing the tadpoles. The solution was homogenized by continuous aeration to ensure uniform distribution of the compound. Methomyl is characterized by high solubility, low volatility, and chemical stability under the experimental conditions, with a reported half-life of approximately 25–30 days in freshwater at neutral pH and in the absence of direct UV light (Van Scoy et al. 2013; Dantas et al. 2024). These properties ensured stable exposure concentrations throughout the 96-hour experimental period.

Tadpoles were exposed to 0 mg a.i./L (control) and 10% of the LC₅₀. The sublethal concentration employed corresponded to 10% of the previously determined 96-h LC₅₀ for each species and temperature. This fraction is widely used in ecotoxicological studies to assess sublethal effects without inducing mortality (Al-Asgah et al. 2015; Jiménez et al. 2021; Liu et al. 2021; Uçkun & Özmen, 2021). For *O. taurinus*, the sublethal concentrations were 9.64 mg/L (26.5 °C) and 4.59 mg/L (30 °C). For *S. ruber*, the sublethal concentrations were 1.55 mg/L (26.5 °C) and 1.97 mg/L (30 °C).

Throughout the 96-h exposure, water parameters remained stable. For *O. taurinus*, mean temperature was 26.5 ± 0.12 °C and 30.0 ± 0.20 °C, dissolved oxygen averaged 88.8 ± 2.3% and 88.3 ± 2.6%, and pH was 7.30 ± 0.09 and 7.19 ± 0.07 at 26.5 °C and 30 °C, respectively. For *S. ruber*, mean temperature was 26.5 ± 0.12 °C and 30.1 ± 0.15 °C, dissolved oxygen 88.4 ± 0.8% and 89.0 ± 1.9%, and pH 7.35 ± 0.24 and 7.52 ± 0.24 at 26.5 °C and 30 °C, respectively. All individuals were unfed during the 96-h exposure, ensuring a standardized fasting state.

2.4. Preparation of alarm substance

The alarm substance was prepared from a single naïve tadpole (~85 mg wet mass; not previously exposed to methomyl or temperature variation) of the same species as the tested individuals, ensuring intraspecific specificity. The donor tadpole was euthanized by double pithing of the spinal cord, and the body was manually macerated with a scalpel in 100 mL of distilled water, yielding a final extract concentration of approximately 0.85 mg mL⁻¹. The solution was used immediately after preparation, following established procedures for alarm cue preparation in anuran larvae (Elvidge et al. 2023; Lipkowski et al. 2024).

2.5. Behavioral assessment of tadpoles

Following the 96-hour acute exposure to methomyl and/or temperature, behavioral assays were conducted under controlled environmental conditions. Tests were performed in an isolated and quiet room used exclusively for behavioral trials, minimizing external visual and acoustic disturbances. All assays were carried out during daytime, maintaining the natural photoperiod of the region (12 h light / 12 h dark), and at the same temperatures, pH, and dissolved oxygen levels as the exposure phase to ensure environmental consistency. To standardize exposure duration across individuals, a subset of eight tadpoles per treatment group was randomly selected for testing.

The substrate preference test was conducted in circular arenas (20,5 cm) with 2 cm water depth (420 ml) containing a bipartite black-and-white substrate (50% each), following the protocol adapted from Guimarães et al. (2021). After introducing individual tadpoles into the arena, a 10-minute acclimation period was provided prior to 20 minutes of video recording. Video analysis quantified: (1) time spent in each substrate zone and (2) duration of swimming activity. The alarm response assay was conducted in rectangular tanks (15 × 22.2 cm) with 2 cm water depth (475 mL). Individual tadpoles were acclimated for 10 minutes before sequential additions of (1) 10 mL of distilled water (vehicle control; ≈2.1% of total volume) and, after 10 minutes, (2) 10 mL of the conspecific alarm substance prepared at a ratio of one tadpole per 100 mL immediately before use. Additions were performed slowly using a 10 mL syringe, releasing the liquid along the tank wall over approximately 45 seconds to

minimize hydrodynamic disturbance. Behavioral responses were recorded for 30 minutes in total (10 min baseline, 10 min post-vehicle, and 10 min post-alarm). Video analyses were conducted in BORIS software (Friard and Gamba 2016) to quantify (1) spatial use patterns (center vs. corner occupancy) as stress indicators and (2) changes in swimming activity post-exposure.

Tadpoles were unfed during the 96-h exposure period preceding the behavioral tests, ensuring a standardized fasting state across treatments. Each tadpole was subjected to only one behavioral assay to avoid carryover effects between tests. At the end of each behavioral assay, the tadpoles were anesthetized with MS-222 and euthanized. All the individuals were preserved at -80 °C. Tadpoles from the substrate preference test were later used for acetylcholinesterase (AChE) analysis, while those from the alarm response assay were used for cortisol quantification.

2.6. Biochemical responses

The activity of whole-body acetylcholinesterase (AChE) was measured using the method described by Ellman et al. (1961). Body samples were homogenized (1:4 w/v) in a 0.1 M phosphate buffer with 20% glycerol, pH 7.4, and then centrifuged for 15 minutes at 850 xg at 4 °C. The supernatant was collected and centrifuged again at 12.800 xg at 4 °C. Acetylcholine iodide (9 mM) was used as the substrate, and DTNB served as the color reagent. AChE enzyme activity was measured using a spectrophotometer (SpectraMax M2, Molecular Devices) at a wavelength of 412 nm and was expressed as nmol min⁻¹ mg protein⁻¹.

The total protein content in the whole-body samples was quantified in a spectrophotometer (SpectraMax M2, Molecular Devices) at 595 nm using bovine serum albumin (BSA) as the standard and in accordance with Bradford (1976)

The concentrations of whole-body cortisol were measured using a commercial cortisol ELISA Kit (Cayman Chemical, Item No. 500360). To obtain a homogenized sample, the tadpole was crushed using an ultrasonic homogenizer (Branson Sonifier 450, VWR Scientific). Between tests, the mixer was rinsed with ethanol to avoid cross-contamination. Subsequently, 5 mL of methanol was added as the extraction solvent. The sample was mixed in a vortex (MAXIMIX Plus, Thermolyne) for 30 seconds, placed on an overhead shaker (TE 420, TECNAL) at 180 rpm for 1 hour at room temperature, and centrifuged for 10 minutes at 3500 xg at 7 °C (Centrifuge 5430 R, Eppendorf). The

entire supernatant was collected, evaporated to a concentrate under vacuum (Concentrator Plus, Eppendorf), and reconstituted in 200 μ L ELISA Buffer (1X) from the commercial kit. The assay was developed following the protocol provided by Cayman Chemical (Cortisol ELISA Kit, Cayman Chemical Item No. 500360). Samples were read and quantified in duplicate using a microplate reader (SpectraMax M2, Molecular Devices) at an absorbance of 405 nm.

2.7. Statistical analyses

All the statistical analyses were performed using R software (version 4.2.2). We fitted linear mixed models including an aquarium-level random effect using the lme4 package in all analyses. This hierarchical approach treats the aquarium as the experimental sampling unit and the tadpoles within each aquarium as nested observations, thus accounting for the dependence structure inherent to the design and partitioning variance within and between aquaria. This approach provides insights not only into the variance components but also into the direction of the fixed effects.

However, these results can be unstable due to n and the violation of the normality assumption in some variables of interest. To address these issues, we adopted a two-step inference approach, combining parametric mixed-model results with a nonparametric permutation test using the *permuto* package. This approach provides robust inference under potential violations of normality, small-sample conditions, and unbalanced designs. The permutation test outcomes guided final interpretations.

For activity/inactivity time data, our goal was to examine main effects and interactions between methomyl presence/absence, temperature, substrate preference, and their interactions. Alarm substance responses were evaluated to compare: (1) changes in swimming activity (total distance moved) and (2) shifts in spatial distribution (center vs. corner occupancy) between pre- and post-stimulus periods. In the latter case, a random effect was also assigned to the individual tadpole to account for repeated measurements within subjects. Acetylcholinesterase (AChE) activity and cortisol concentration were also analyzed in relation to methomyl exposure and temperature. All analyses described below were performed separately for *O. taurinus* and *S. ruber*.

3. Results

3.1. Survival after sublethal exposure

No mortality was recorded in any treatment throughout the 96-h exposure period.

3.2. Substrate preference

In this comparative study, we decided to analyze activity and resting time separately, since they perform differently, and summing the total time masks the covariate effects.

All fitted linear mixed models revealed a singular fit, with the variance of the random term (aquarium) estimated as zero, indicating that between-aquarium variance could not be reliably estimated, likely due to the limited number of replicates per treatment, for both activity and inactivity times in the two species, except for *S. ruber* in activity time, where the variance of the random effect was 153.3 (sd = 12.38). This result suggests the absence of significant variability between aquaria within the same treatment.

3.3. Swimming activity

Methomyl exposure and elevated temperature independently reduced swimming activity in *O. taurinus* tadpoles (methomyl: $p < 0.001$; temperature: $p < 0.001$). A significant interaction revealed stronger methomyl effects at 26 °C ($p = 0.002$) (Fig. 1A). In *S. ruber* tadpoles, activity levels were unaffected by methomyl exposure ($p = 0.43$), temperature ($p = 0.49$), or interaction between methomyl and temperature ($p = 0.09$) (Fig. 1B). However, neither species shows a significant preference for either substrate (*O. taurinus*: $p = 0.26$; *S. ruber*: $p = 0.34$).

3.4. Resting behavior

Osteocephalus taurinus tadpoles exhibited substrate-dependent inactivity, spending more time resting on black substrates ($p = 0.002$) (Fig. 1C). In contrast, no other covariate significantly affected inactivity duration for this species. Similarly, no

significant effects were detected in *S. ruber* tadpoles (Fig. 1D).

3.5. Alarm substance response

In this context, all fitted linear mixed models estimated the variance of the random term (aquarium) as zero, except for *S. ruber* in swimming activity, where the variance of the random effect was 3373 (sd = 58.08). This suggests that between-aquarium variance could not be reliably estimated, likely due to the limited number of replicates per treatment, and does not confirm the absence of variability among aquaria within the same treatment.

Following alarm substance exposure, neither swimming activity nor spatial distribution (center vs. edge preference) showed significant changes for *O. taurinus*. Conversely, swimming activity in *S. ruber* tadpoles was significantly affected by methomyl presence ($p = 0.02$), reflected by increased activity time, while spatial distribution and its interaction with activity remained non-significant.

3.6. Acetylcholinesterase activity

The random-effect variance was null in all models, and one tadpole observation was missing in the high-temperature, no-methomyl treatment due to technical issues, leaving only seven individuals in that aquarium. Nevertheless, because all analyses used permutation-based inference, the potential bias due to this minor imbalance was mitigated. Additionally, the null random-effect variance reflects an estimation limitation, rather than the confirmed absence of variation among aquaria.

Regarding AChE modulation in *Osteocephalus taurinus*, a significant interaction between temperature and methomyl exposure ($p < 0.001$) indicated increased activity at 30 °C under methomyl presence, while the baseline group (without methomyl) showed similar activity across temperatures. Separately, only temperature had a significant effect ($p = 0.009$). *Scinax ruber* maintained stable AChE activity across all the treatments (Fig. 2B).

3.7. Cortisol response to stressors

In *O. taurinus*, there are no detectable effects from any of the stressors (Fig. 2C). In contrast, *S. ruber* showed significant increases in cortisol levels in response to

all stressors: high temperature and methomyl exposure significantly increased cortisol levels ($p < 0.001$ and $p = 0.006$, respectively), and their interaction showed a non-additive effect ($p = 0.04$) (Fig. 2D).

4. Discussion

Our results suggest significant interspecific differences in behavioral and physiological responses between *Osteocephalus taurinus* and *Scinax ruber* tadpoles to methomyl exposure and elevated temperature.

These disparities reflect species-specific adaptations to distinct environmental pressures. Among the multiple factors that influence microhabitat selection in lentic environments, substrate coloration may serve as a defense mechanism against visually oriented predators. Preference for low-contrast substrates enhances survival by reducing body visibility, particularly under high predation pressure from the aquatic insects commonly found in these habitats (Kang et al. 2015; Jara 2016). *O. taurinus* tadpoles spent significantly more time resting on dark substrates, indicating a substrate-dependent antipredator strategy. This behavioral pattern suggests a form of crypsis through immobility, enhancing camouflage effectiveness against visually oriented predators. Such microhabitat use minimizes exposure risk in high-predation environments, as dark substrates reduce body contrast in dark-pigmented tadpoles (Espanha et al. 2016; Eterovick et al. 2018; Eterovick et al. 2020). Substrate selection in tadpoles is not solely color-driven but also modulated by perceived predation risk. In temporary ponds, where predator density is high and structural refuges are scarce, larvae often rely on visual concealment strategies such as substrate matching and immobility (Touchon & Warkentin, 2008). Experimental evidence supports this pattern: tadpoles exposed to predator chemical or visual cues preferentially occupy shaded or less exposed areas (Hettyey et al. 2011; Hettyey et al. 2015). Field observations during our collections corroborate this interpretation, as the studied ponds harbored numerous visually hunting predators (e.g., belostomatids, odonate larvae). A similar selection of low-contrast substrates has been reported for *Dryophytes plicatus* (Sánchez-Sánchez et al. 2023) and *Physalaemus gracilis* (Ximenez et al. 2012). Dark substrates may also confer thermoregulatory benefits, optimizing thermal balance for larval development. In contrast, *S. ruber* exhibited no clear substrate preference, possibly reflecting a background-matching strategy based on color plasticity, similar to that observed in *Pelobates cultripes*, which adjusts its pigmentation to track

environmental brightness (Liedtke et al. 2023). Although further research is needed to confirm this mechanism in *S. ruber*, our preliminary field observations suggest that this species may undergo skin pigmentation changes, allowing it to explore a broader range of microhabitats. This plasticity may also be linked to thermoregulatory mechanisms, as observed in *Alytes dickhilleni* tadpoles, which modulate skin pigmentation based on ambient temperature (Rodríguez-Rodríguez et al. 2020). The results suggest that camouflage is not universal among Amazonian tadpoles. For instance, *Dendropsophus minutus* preferred substrates with intermediate contrast, while species such as *Callimedusa tomopterna*, *Leptodactylus fuscus*, *L. knudseni*, and *Rhinella marina* selected lighter substrates that increased contrast with their bodies (Guimarães et al. 2021). These findings highlight the diversity of antipredator strategies among species.

Osteocephalus taurinus exhibited reduced swimming activity under both methomyl exposure and elevated temperature. A significant interaction revealed stronger methomyl effects at 26 °C. In ectotherms, acute stress-induced lethargy is often interpreted as an adaptive trade-off, where individuals redirect finite energy resources away from locomotion and toward essential maintenance and detoxification processes (Sokolova, 2013). The elevated temperature alone increases basal metabolic rate, demanding more energy for basic homeostasis (Gangloff and Telemeco, 2018). When combined with a pesticide, this energetic demand is exacerbated by the costs of biotransformation, excretion, and cellular repair (Sokolova et al. 2012). Thus, the observed reduction in swimming may be a strategic down-regulation of activity to conserve energy in a scenario of heightened demand. However, this energy-saving strategy carries inherent ecological risks. Locomotor performance is critical for foraging and predator avoidance, directly impacting survival, growth and development (Bridges 1997). Swimming activity is closely linked to energy acquisition, as more active tadpoles have a higher likelihood of locating food, thus supporting adequate growth rates (Horat and Semlitsch 1994; Bridges 1997). Several studies have reported reduced locomotor activity in tadpoles exposed to carbamate and organophosphate insecticides, both known to inhibit AChE activity (Bridges 1997; Junges et al. 2017). Methomyl, absorbed through skin, gills, and gastrointestinal tract, reversibly inhibits AChE via carbamylation of the enzyme (Quaranta et al. 2009; David et al. 2017). This mechanism prevents acetylcholine (ACh) breakdown, prolonging its action in cholinergic synapses and triggering neurotoxic and behavioral effects, such as movement inhibition (Sparling and Fellers 2007). Contrary to our expectations,

methomyl exposure did not reduce AChE activity; instead, AChE activity significantly increased at 30 °C when methomyl was present, while tadpoles in the baseline group (without methomyl) maintained similar activity across temperatures. Higher temperatures can enhance enzymatic activity by facilitating enzyme-substrate interactions, accelerating biochemical reactions, in accordance with thermodynamic principles (Lushchak 2011). This is especially relevant for tadpoles, whose metabolic processes are directly influenced by environmental temperature (Gangloff and Telemeco 2018). In this context, elevated temperatures raise metabolic rates, increasing the demand for AChE in order to prevent neuronal overstimulation due to excess ACh in synapses. AChE itself may recover more rapidly from methomyl inhibition under higher metabolic rates, possibly explaining the increased activity at 30 °C. Furthermore, although methomyl is an AChE inhibitor, its effects are reversible and less persistent than those of organophosphates (Fukuto 1990). Thus, higher temperature may accelerate the decarbamylation of AChE, restoring its function. This aligns with recent findings that temperature increases the kinetics of metabolic reactions, potentially accelerating the recovery of enzymatic functions in the presence of inhibitors, as temperature-driven acceleration of toxicokinetic processes has been observed in other organisms exposed to environmental stressors (Grenc et al. 2025). Increased metabolic rates, due to higher temperature, likely enhance the elimination and recovery rates of substances like methomyl, which modifies the enzyme's ability to return to baseline activity more quickly than expected.

However, despite this enzymatic recovery, reduced swimming activity suggests that the response to stressors is not solely linked to AChE function. The lack of correlation between enzyme activity and behavior suggests that other mechanisms, such as increased energetic demands or hormonal imbalances, may also be involved and therefore warrants further investigation. This is consistent with the broader understanding that enzymatic function is not the only determinant of behavioral outcomes in ectothermic species exposed to multiple stressors. While the temperature may enhance enzymatic activity, the resulting behavioral effects can be more complex, influenced by factors such as energy allocation, neurological regulation, and neuroendocrine responses to thermal stress (DeLong et al. 2017). These findings suggest that the relationship between AChE activity and behavior may involve additional physiological responses that go beyond the enzymatic level. *S. ruber* showed no significant behavioral or enzymatic alterations under the same stressors, suggesting a lower susceptibility to methomyl, possibly due to inherently higher

AChE activity. This pattern may reflect biochemical resistance rather than physiological resilience, although both mechanisms could contribute to the observed stability. This adaptive capacity may be related to its historical exposure to anthropogenic environments, where thermal fluctuations and contaminants are more frequent.

The absence of a response to the alarm substance observed across all treatments, including the control group, suggests that the evaluated species may not exhibit typical defensive behaviors triggered by this type of chemical signal, or that the stimulus used was not sufficiently effective or ecologically relevant to elicit a detectable reaction. The use of a single individual to prepare the alarm substance extract may have limited the signal intensity and ecological relevance, as natural variability in chemical cues from different individuals could have influenced the response. Similar findings were reported for *Epipedobates anthonyi* tadpoles, which showed no observable behavioral changes when exposed to injury-related chemical cues, both from individuals of the same species and from different species (Lipkowski et al. 2024). According to the authors, in certain ecological contexts, particularly in small, low-flow aquatic habitats such as temporary ponds, the transmission of chemical signals may be constrained by their low directionality and slow diffusion rate, reducing their effectiveness compared to other sensory modalities such as visual or tactile cues (Lipkowski et al. 2024). This is particularly relevant for the species evaluated in the present study, as they inhabit temporary ponds in Amazonia, where such environmental constraints are likely to influence sensory communication and predator-prey dynamics.

The effects of the stressors on cortisol differed between the two species. Only *S. ruber* exhibited marked elevations in cortisol in response to both temperature and methomyl exposure, as well as a non-additive increase under their interaction. Physiological challenges such as elevated temperature and pesticide exposure can activate the hypothalamic–pituitary–interrenal (HPI) axis in amphibians, triggering a hormonal cascade initiated by corticotropin-releasing hormone (CRH) release from the hypothalamus. CRH stimulates adrenocorticotrophic hormone (ACTH) release from the pituitary, which in turn induces glucocorticoid secretion (e.g., cortisol, corticosterone) by interrenal glands (Santymire et al. 2018; Barough et al. 2025). These glucocorticoids play essential roles not only in acute stress responses but also act synergistically with thyroid hormone (TH) to regulate growth and metamorphosis in tadpoles. This interaction can accelerate metamorphosis in hostile environments as a strategy to escape unfavorable conditions. However, such acceleration often results in

smaller post-metamorphic body size, potentially compromising performance (Denver 2009). During the larval stage, continuous feeding is critical to accumulate energy reserves for metamorphic climax, a phase marked by intense morphological and physiological changes. Stress-induced behavioral changes, such as reduced foraging, can hinder this energy acquisition (Orlofske and Hopkins 2009). Moreover, sustained cortisol elevation may deplete glycogen, protein and lipid stores, reducing the energy available for metamorphosis (Mommensen et al. 1999; Rocha et al. 2010). This aligns with the glucocorticoid-fitness hypothesis (CORT-fitness), which proposes that environmental stressors that promote prolonged increases in glucocorticoid concentrations may ultimately reduce organismal fitness, particularly in scenarios of high energetic demand. The hypothesis underscores the importance of considering not only the presence of an endocrine response, but also its duration and intensity when assessing the ecological and physiological impacts of stress in vertebrates (Bonier et al. 2009). Amphibian species differ in their primary circulating glucocorticoid; while corticosterone predominates in many, some tropical frogs primarily secrete cortisol (Denver 2009; Santymire et al. 2018). Thus, the absence of detectable cortisol variation observed in *O. taurinus* may not indicate the absence of a stress response but rather a physiological difference in the dominant hormone profile, which could possibly reflect a greater reliance on corticosterone. This contrasting pattern, where one species exhibited significant behavioral and biochemical alterations without corresponding changes in cortisol, while the other maintained physiological and behavioral stability yet showed marked cortisol elevation, emphasizes the complexity and species-specific nature of endocrine stress responses in amphibians.

5. Study limitations

Although we adopted a sublethal concentration equivalent to 10% of the 96-h LC₅₀, a conventional metric widely used in ecotoxicology to standardize sublethal assessments, this concentration may not reflect actual exposure levels in Amazonian aquatic environments. Existing monitoring data from other regions indicate that methomyl can occur in surface waters and agricultural wells, but reported concentrations are generally low and highly variable (Carbo et al. 2008; Van Scoy et al. 2013). No information is available for Amazonian water bodies, where pesticide monitoring is sporadic and does not capture short-term contamination pulses associated with rainfall or runoff. This lack of continuous monitoring complicates the

interpretation of ecological relevance because occasional peak concentrations may remain undetected (Chow et al. 2020). Therefore, while the 10% LC₅₀ approach provides a standardized sublethal reference, its correspondence with real exposure scenarios in Amazonian systems remains uncertain. The absence of corticosterone quantification is a limitation that could be addressed in subsequent studies to complement the interpretation of the endocrine effects of methomyl-induced stress. Regarding the alarm substance preparation, it is important to note that there is a wide variety of protocols, and this is an area that needs further standardization and understanding to ensure comparability across studies. Lastly, the linear mixed models showed instability, with random-effect (aquarium) variance estimated as zero in most analyses, and permutation-based inference was used for statistical validation. This acknowledgment is important for properly contextualizing the robustness of the results and preventing overly deterministic interpretations.

6. Conclusion

This study suggests that *Osteocephalus taurinus* and *Scinax ruber* tadpoles exhibit distinct behavioral and physiological responses to combined environmental stressors, including methomyl exposure and elevated temperature. Consistent with our first prediction, both stressors influenced neurophysiological and endocrine pathways, but in species-specific directions. *O. taurinus* exhibited temperature-dependent modulation of AChE activity, with reduced activity at 30 °C and a pronounced enzymatic increase under the combined exposure to methomyl and elevated temperature, whereas no detectable cortisol variation was observed. In contrast, *S. ruber* maintained stable AChE activity but showed significant cortisol elevation in response to both stressors, with a non-additive interaction effect. As anticipated in our second prediction, these physiological shifts were accompanied by behavioral alterations in *O. taurinus*, which displayed reduced locomotion and dark-substrate preference, patterns indicative of antipredator strategies, while *S. ruber* maintained behavioral stability, suggesting higher resilience. Contrary to our third prediction, neither species exhibited significant behavioral responses to alarm cues, indicating that short-term sublethal exposure may have masked chemosensory responses.

These interspecific contrasts likely reflect distinct ecological adaptations and glucocorticoid regulation, with *O. taurinus* potentially favoring corticosterone and *S. ruber* primarily cortisol. Importantly, our study provides the first evidence of how

synergistic stressors affect Amazonian amphibians, a region critically underrepresented in ecotoxicological research. The Amazon harbors unparalleled biodiversity yet faces escalating threats from both climate change and contamination, underscoring the urgent need for region-specific studies that capture species-level vulnerabilities. Furthermore, our results reinforce the importance of multi-stressor approaches in ecological risk assessment, as interactions between contaminants and abiotic stressors can profoundly alter species' responses beyond predictions based on isolated exposures.

Overall, these findings highlight that species-specific traits, particularly endocrine physiology and ecological history, must be considered when evaluating the effects of anthropogenic stressors on tropical amphibians, with direct implications for conservation and management in rapidly changing Neotropical ecosystems.

7. Ethics statement

All the experiments were approved by the Ethics Committee on Animal Use of the National Institute for Amazonian Research (INPA) (process No. 83/2023). The animals were collected and transported under authorization from SISBIO-ICMBio (No. 80447-4).

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10. List of figures

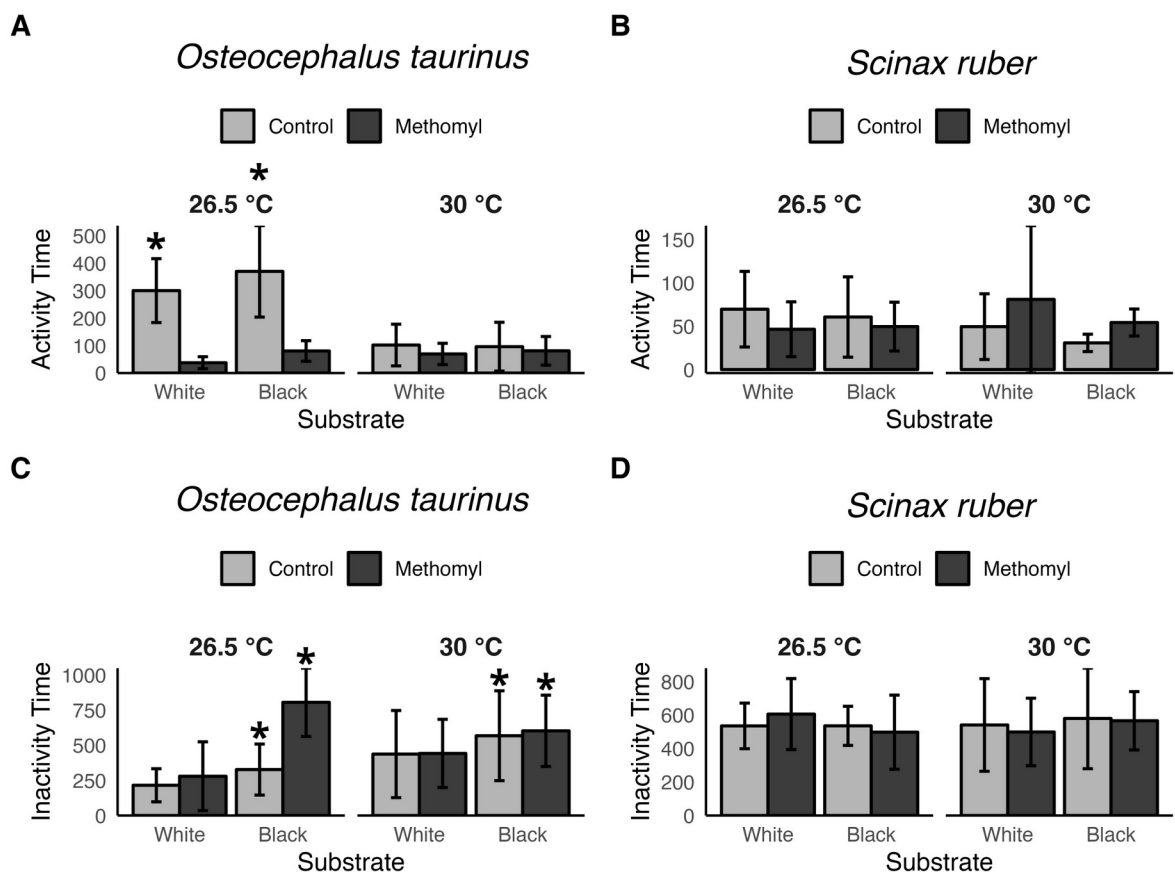


Figure 1. Activity time (A–B) and inactivity time (C–D) of *Osteocephalus taurinus* and *Scinax ruber* tadpoles (in seconds), respectively, on white and black substrates under different temperature and methomyl exposure conditions. Bars represent raw group means $\pm$ SD.

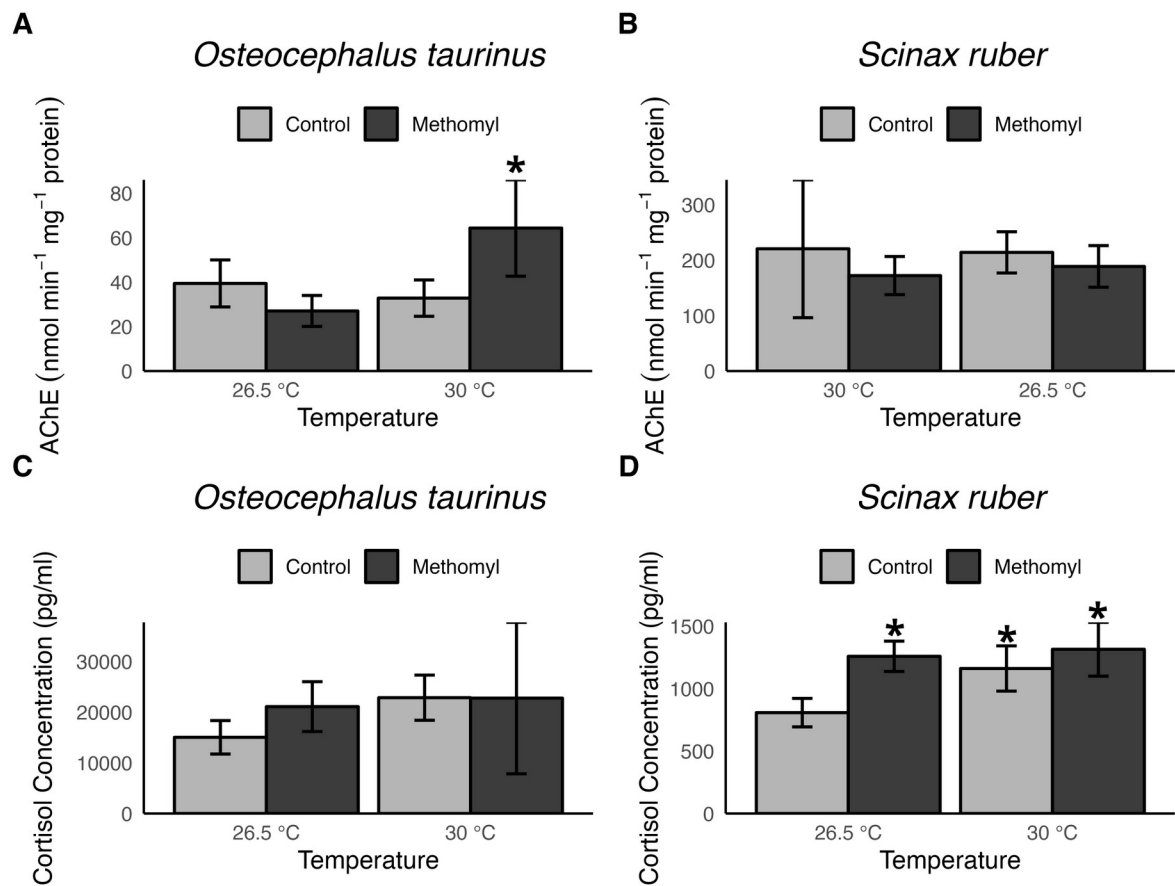


Figure 2. Physiological responses of *Osteocephalus taurinus* (A,C) and *Scinax ruber* (B,D) tadpoles to temperature elevation and methomyl exposure on AChE activity and cortisol concentration. Bars represent raw group means $\pm$ SEM.

Capítulo 4

Azambuja G, de Souza SS, Bezerra AGA, Kaefer IL, Val AL, Kochhann D.
Morphophysiological Impacts of Methomyl and Warming on Frog Tadpoles (Leptodactylus knudseni). *Chemistry and Ecology*.

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Morphophysiological Impacts of Methomyl and Warming on Frog Tadpoles
(*Leptodactylus knudseni*)

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Abstract

Amphibians are particularly vulnerable to environmental stressors due to their permeable skin, ectothermy and complex life cycles. Increase in the use of pesticides and climate change in the Amazon rainforest represent growing threats to native species. This study assessed the individual and combined effects of methomyl, and elevated temperature on tadpoles of *Leptodactylus knudseni*, a common Amazonian frog species. Tadpoles were chronically exposed to methomyl under current (26.5 °C) and projected (30 °C) temperature conditions. We evaluated survival, developmental rate, growth, morphology, neurophysiology and antioxidant responses. Methomyl exposure significantly increased mortality and accelerated metamorphosis while reducing body mass, a combination of effects that suggests endocrine disruption. Morphological analysis revealed systematic reductions in overall body dimensions and tail musculature across all experimental treatments, resulting in a significantly smaller and less robust phenotype, which may compromise post-metamorphic performance. Methomyl significantly inhibited acetylcholinesterase activity and upregulated superoxide dismutase, indicating neurotoxicity and oxidative stress. Although temperature and methomyl acted independently, with no synergistic interactions detected across any response variable, the growing threats posed by agricultural intensification and climate change in the Amazon emphasise the need for integrated ecotoxicological assessments. Such assessments are crucial for developing effective conservation strategies for tropical freshwater biodiversity.

Keywords: Amazonia; amphibian larvae; climate change; ecotoxicology; oxidative stress; pesticide

1. Introduction

Over the past few decades, among the vertebrates, amphibians have experienced one of the most drastic population declines, with approximately 41% of species threatened globally, a scenario that constitutes a biodiversity crisis with profound ecological repercussions [1, 2]. This population collapse is driven by a synergistic combination of anthropogenic factors, among which habitat modification, invasive species, chemical pollution, predatory exploitation and climate change stand out as particularly severe threats [3, 4]. In the tropics, especially in the Brazilian Amazon, which is home to at least 383 known species of anurans and has significant potential for additional taxonomic discoveries [5, 6], these impacts are amplified by intense changes in land use. In the Brazilian Amazon, approximately 12.4 million hectares of native vegetation were cleared in the last decade alone [7]. During this period, land for agricultural use nearly doubled, increasing by over 90% between 2012 and 2022, and reaching more than 7.2 million hectares [7].

Amphibians are considered environmental bioindicators due to their unique common physiological and morphological traits: unshelled eggs, an aquatic larval stage and highly permeable skin, which facilitates contaminant absorption [8]. During the larval stage, tadpoles present specialised morphology for aquatic environments, which is gradually replaced by adult structures throughout metamorphosis [9, 10]. This transition involves drastic changes in the respiratory, digestive, nervous and endocrine systems, making the process highly vulnerable to interference from environmental stressors [11].

Among agricultural pesticides, methomyl, a fast-acting carbamate insecticide, stands out for its potent neurotoxicity and widespread use in tropical agricultural frontiers. Despite being banned in several countries, methomyl remains legal in Brazil, with sales reaching 9.1 thousand tonnes in 2023, a 23.6% increase over the past decade [12]. Notably, approximately 35% of the total pesticide consumption in the country that year occurred in the states that comprise the Brazilian Amazon [12]. Its physicochemical properties, including high water solubility (57.9 g/L), low sediment adsorption, and prolonged half-life (6 days in surface water; up to 25 weeks in aquifers), enhance its mobility, persistence and bioavailability in aquatic ecosystems [13, 14].

Methomyl's toxicity primarily arises from the inhibition of acetylcholinesterase (AChE) activity, leading to acetylcholine accumulation in synapses and disrupted nerve

signalling. In addition to its neurobehavioral effects, some studies suggest that this compound may also interfere with endocrine pathways that are critical for tadpole development [15, 16] and induce oxidative stress, causing imbalances between reactive oxygen species (ROS) production and antioxidant defences [17, 18].

During the larval stage, tadpoles accumulate essential energy reserves to support metamorphosis, particularly during the climax phase when tail resorption occurs and feeding ceases [19, 20]. Metamorphosis involves profound anatomical and physiological changes regulated by thyroid hormones (T3 and T4) through the hypothalamus–pituitary–thyroid (HPT) axis [10, 21]. Environmental stressors, such as pesticides, can disrupt these regulatory pathways, impair oxidative balance and interfere with tissue remodelling, leading to alterations in developmental timing, body size and morphology [22–25]. Such disruptions may compromise survival and post-metamorphic performance, which highlights the vulnerability of amphibian larvae to chemical contamination during critical developmental windows.

Climate change further exacerbates this scenario. Rising global mean temperatures, which have already exceeded 1.48 °C above pre-industrial levels in 2023, with every day of the year exceeding 1 °C and half of them surpassing 1.5 °C [26, 27], impose additional physiological challenges on tadpoles, which are ectothermic organisms whose body temperature depends directly on the environment. Elevated temperatures accelerate metabolism, shorten larval duration and modify the efficiency of physiological and biochemical processes [28–31]. Simultaneously, higher temperatures may enhance pesticide toxicity by increasing solubility, absorption and reactivity [32, 22].

Despite recent advances in characterizing the effects of pesticides on the behaviour and neurophysiology of tropical anurans, three critical gaps remain: (i) the morphological effects of methomyl during metamorphosis are unknown in Amazonian species; (ii) the interaction between thermal elevation and toxicity is underrepresented in environmental risk models; and (iii) few studies assess the capacity of tropical tadpoles to maintain antioxidant defences under combined thermal and chemical stressors.

To address these gaps, this study investigates the chronic effects of methomyl exposure, coupled with projected temperature increases from climate models, on *Leptodactylus knudseni* Heyer, 1972 tadpoles. Specifically, we assessed how these stressors affect: (i) survival, (ii) larval duration and development, (iii) morphology, (iv) neurophysiology via acetylcholinesterase activity, and (v) the antioxidant system

(superoxide dismutase, catalase and glutathione peroxidase). Using an integrative approach, we aim to elucidate the physiological and biochemical mechanisms underlying responses to multiple stressors in a native Amazonian frog species. These findings are critical for advancing ecotoxicological knowledge based on tropical anurans and guiding conservation and management policies in environments under growing anthropogenic pressure.

2. Materials and Methods

2.1. Tadpoles collection and maintenance

Leptodactylus knudseni Heyer, 1972 is an anuran species from the family Leptodactylidae that is distributed throughout the Amazon basin in Brazil, Bolivia, northern Colombia, Venezuela and the Guianas (French Guiana, Suriname and Guyana). In the Adolpho Ducke Forest Reserve (RFAD), near Manaus, Amazonas, Brazil, the species is commonly found near temporary ponds, both in the interior of the forest and along its edges [33]. The reproductive behaviour of *L. knudseni* includes the construction of foam nests by males in shallow depressions excavated near ponds. Each clutch contains approximately 1,000 eggs, which hatch inside the nest before the tadpoles migrate to nearby water bodies for further development. Breeding occurs with a higher frequency of foam nests between October and March. During droughts, tadpoles can survive in mud depressions until the pond is refilled [33].

Tadpoles from eight clutches were collected at Gosner stages (26-26) (Gosner, 1960) from temporary ponds within the RFAD (2.916° S, 59.883°W), Manaus, Amazonas, Brazil. Tadpoles were transported to the laboratory, maintained in 70-L glass aquaria (70 × 50 × 30 cm) and acclimated for 7 days prior to the experiment. Individuals from different clutches were randomly distributed among aquariums to minimise potential clutch effects and ensure that the observed responses represent the natural phenotypic variability of the population, rather than parental bias. The tadpoles were fed daily *ad libitum* with a mix of commercial fish food containing 32% protein.

2.2 Chronic exposure to sublethal concentrations of methomyl at different temperatures

To investigate the interactive effects of temperature increase and methomyl exposure, 30 tadpoles of *L. knudseni* at Gosner stages 25–28 [34] were randomly selected and transferred into 30-liter glass aquaria (40 × 30 × 25 cm) per treatment. Each condition was tested in duplicate, totalling 60 individuals per group. After a 30-minute acclimation at 26 °C, water temperature was gradually adjusted at a ramp rate of 0.03 °C per minute until reaching the experimental values of 26.5 °C or 30 °C, following established protocols [35–37]. These temperatures represented: (1) a control condition (26.5 °C), simulating natural temporary pond conditions in the central Amazon; and (2) an elevated temperature (30 °C), based on the Intergovernmental Panel on Climate Change (IPCC) Shared Socioeconomic Pathways (SSP3-7.0) scenario, which forecasts a + 3.5 °C increase in global mean temperatures [26]. Following temperature stabilization, methomyl was added to the aquaria at concentrations of 9.64 mg a.i./L (26.5 °C) and 4.59 mg a.i./L (30 °C), corresponding to 10% of the LC₅₀ values previously calculated for *Osteocephalus taurinus*, a syntopic species co-occurring with *L. knudseni* in Amazonian temporary ponds. This approach was adopted as a precautionary proxy due to the lack of specific ecotoxicological data for *L. knudseni*. Four experimental groups were established: (i) 26.5 °C without methomyl (control), (ii) 26.5 °C with methomyl, (iii) 30 °C without methomyl (control), and (iv) 30 °C with methomyl. A daily partial water renewal (50% of the volume) was performed, followed by reapplication of methomyl and temperature conditions in the renewed water to maintain consistent exposure concentrations. All the tadpoles were exposed for a period of 28 days and monitored daily throughout the experiment. Water quality parameters were monitored throughout the 28-day experimental period. In aquaria maintained at the control temperature (26.5 °C), the following mean values (± SD) were recorded: temperature = 26.7 ± 0.54 °C, dissolved oxygen = 6.95 ± 0.37 mg O₂ L⁻¹ (86.8 ± 4.49%), pH = 6.48 ± 0.64, and conductivity = 14.7 ± 2.11 µS cm⁻¹. In aquaria at the elevated temperature (30 °C), the values were: temperature = 30.3 ± 0.24 °C, dissolved oxygen = 6.17 ± 0.55 mg O₂ L⁻¹ (81.1 ± 5.89%), pH = 6.84 ± 1.19, and conductivity = 16.1 ± 2.26 µS cm⁻¹.

2.3. Morphological and developmental assessments

When the tadpoles reached Gosner stage 42, they were carefully removed from the aquaria, and the time taken to reach this developmental milestone was recorded. Subsequently, the tadpoles were anesthetized with MS-222, weighed, euthanized and either preserved in 10% buffered formalin for morphometric analyses or frozen at -80°C for subsequent biochemical assessments. Linear morphological measurements were taken from 10 individuals per experimental group under a stereomicroscope using a micrometric lens. The measured parameters included body height (BH), body width (BW), body length (BL), nostril diameter (ND), interorbital distance (IOD), nostril–snout distance (NSD), eye–snout distance (ESD), internasal distance (IND), tail musculature width (TMW), dorsal membrane height (DMH), ventral membrane height (VMH), tail musculature height (TMH) and eye diameter (ED) [38]. A single observer conducted all the morphometric analyses to minimize measurement bias. In addition to morphological traits, final body mass and larval development duration were recorded to assess growth performance and potential treatment-induced developmental effects.

2.4. Preparation of homogenates

Tissue samples were collected from a subset of randomly selected individuals at the metamorphic climax ($n = 5\text{--}10$ per experimental group) for biochemical assays. For the analysis of acetylcholinesterase (AChE) activity, caudal muscle samples were homogenized in phosphate buffer (0.1 M, pH 7.4) containing 20% glycerol, at a 1:10 ratio, and centrifuged at 12,000 g for 20 minutes at 4°C . For the determination of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, liver samples were homogenized in cold buffer containing 200 mM Tris-Base, 1 mM EDTA, 1 mM dithiothreitol (DTT), 500 mM sucrose and 150 mM KCl, adjusted to pH 7.6. The homogenates were diluted at a ratio of 1:10 (w:v) and centrifuged at 9,000 g for 30 minutes at 4°C . The resulting supernatants were used for the enzymatic assays. Total protein content was determined spectrophotometrically at 595 nm, according to the Bradford method, using bovine serum albumin (BSA) as the standard [39].

2.5. Antioxidant enzymes

Catalase (CAT) activity was measured by monitoring the decomposition rate of hydrogen peroxide (H_2O_2) at 240 nm for 60 seconds [40]. The results were expressed in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$. Superoxide dismutase (SOD) activity was determined based on the inhibition of cytochrome c reduction by the superoxide radical at 550 nm and 25 °C [41]. One unit of SOD was defined as the amount of enzyme required to inhibit 50% of cytochrome c reduction, and the activity was expressed as U SOD $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$. Glutathione peroxidase (GPx) activity was determined according to [42], by measuring NADPH oxidation at 340 nm in the presence of 0.95 mM reduced glutathione (GSH) and H_2O_2 . Enzymatic activity is expressed in $\text{nmol NADPH min}^{-1} \text{ mg}^{-1} \text{ protein}$, using a molar extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.6. Acetylcholinesterase (AChE) activity

AChE activity was determined according to Ellman et al. (1961) [43], using acetylthiocholine iodide (9 mM) as the substrate and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as a chromogenic reagent. The increase in absorbance was monitored at 415 nm, and results are expressed as $\text{nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.7. Statistical analyses

All the analyses were conducted in R (version 4.2.2). Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). Mortality data were analysed using Pearson's chi-square test of independence to assess associations between mortality and treatment combinations (temperature $\times$ methomyl exposure).

All the morphometric variables were $\log_{10}$ transformed and then z-score standardized prior to principal component analysis (PCA). PCA was performed to reduce dimensionality and summarize overall morphological variation. The first two principal components (PC1 and PC2) were retained for further analysis. Group-level differences across the four experimental treatments (26.5 °C and 30 °C $\times$ with or without methomyl) were tested using one-way ANOVA on the PC1 and PC2 scores. When significant effects were detected, Tukey's post hoc test was applied. To assess the contribution of each morphometric trait to the multivariate structure, variable

loadings and Pearson's correlation coefficients with PC1 and PC2 were also examined. Additionally, means and standard errors were calculated for each morphometric variable within the treatment groups for descriptive comparison.

Time to metamorphosis was analysed using a two-way ANOVA, considering temperature and methomyl exposure as fixed factors. Final body mass was analysed using a generalized linear model (Gamma distribution, log-link function). Estimated marginal means were compared using Tukey-adjusted contrasts. For the biochemical variables (AChE, CAT, GPx, SOD), two-way ANOVAs (Type III) were conducted to account for the unbalanced design. AChE and SOD data were square-root transformed, while CAT and GPx data were Box–Cox transformed to meet the assumptions of normality and homogeneity of variances. Post-hoc pairwise comparisons were performed using estimated marginal means (emmeans) with Tukey adjustment.

3. Results

Tadpole survival and metamorphosis in the temperature and methomyl treatments

Exposure to methomyl significantly increased tadpole mortality only at 26.5 °C (68.3%). In contrast, mortality remained low in all other groups, ranging from 8.3% (control at 26.5 °C) to 13.3% (control at 30 °C), including the exposed tadpoles at 30 °C (10%). Mortality differed significantly among the treatments, indicating effects of both methomyl and temperature ($\chi^2 = 80.53$, $df = 3$, $p < 0.0001$).

Time taken until metamorphosis was shorter in methomyl-exposed tadpoles (ANOVA: $F_{1,63} = 32.29$, $p < 0.0001$), whereas temperature alone had no effect ($F_{1,63} = 2.04$, $p = 0.16$), and no interaction was detected ($F_{1,63} = 0.01$, $p = 0.92$). Tukey's post-hoc test confirmed a consistent developmental acceleration of approximately 5.7 days under methomyl exposure, regardless of temperature (30 °C: –6.0 days, $p = 0.0004$; 26.5 °C: –5.8 days, $p = 0.002$), with no differences between temperatures under the same pesticide condition ($p > 0.97$) (Fig. 1A). The proportion of individuals that reached stage 42 after 28 days was 23.3% in the 26.5 °C + methomyl group, 23.3% in the 26.5 °C control group, 43.3% in the 30 °C + methomyl group, and 21.7% in the 30 °C control group.

3.1. Effects of temperature and methomyl on morphometric traits

Exposure to an elevated temperature (30 °C) reduced tadpole final mass (stage 42) compared to 26.5 °C (GLM Gamma: $\beta = -0.433$, $t_{63} = -2.25$, $p = 0.028$). Methomyl exposure led to an even greater reduction (GLM Gamma: $\beta = -0.733$, $t_{63} = -3.95$, $p < 0.001$), with no interaction between temperature and pesticide (GLM Gamma: $\beta = 0.350$, $t_{63} = 1.39$, $p = 0.17$). Post-hoc comparisons (Tukey's HSD test) showed that tadpoles exposed to methomyl at 26.5 °C were 48.9% lighter than those not exposed to methomyl at the same temperature ($p = 0.001$), while exposure to 30 °C alone caused a 34.6% reduction, which was not statistically significant ($p = 0.122$) and likely due to higher within-group variation. Combined exposure (methomyl + 30 °C) resulted in additive effects, with tadpoles being 56.1% lighter than those maintained at 26.5 °C without methomyl ($p < 0.0001$), but not different from those subjected to only methomyl ($p = 0.957$).

Tadpoles exposed to both high temperature and methomyl generally exhibited reduced body dimensions compared to the control group that was reared at 26.5 °C without exposure to the pesticide (Table 1). The first principal component (PC1) accounted for 71.3% of the total variance and was interpreted as an overall size axis, as it was strongly and positively correlated with all measured morphometric traits (Pearson's r between 0.65 and 0.95). High positive scores on this axis represent larger individuals with greater body and tail dimensions, while negative scores indicate a reduction in overall larval size. The second principal component (PC2) explained 6.9% of the variance and represented morphological shape variation, primarily reflecting the relationship between interorbital distance ($r=0.37$) and nostril diameter ($r=-0.58$) independent of total size (Figs. 2A-B).

Differences in body shape (PC1 scores) among the treatment groups (Fig. 2A) were confirmed by one-way ANOVA ($F_{3,40} = 4.50$, $p = 0.008$). Post-hoc comparisons (Tukey's test) revealed that the tadpoles reared at 26.5 °C without methomyl differed from those reared at 30 °C both with and without pesticide exposure ($p = 0.016$ and $p = 0.013$, respectively), with a marginal difference compared to those exposed to methomyl at the same temperature ($p = 0.058$). No group differences were detected for PC2 ($F_{3,40} = 1.08$, $p = 0.369$), indicating that treatment effects were mainly captured by PC1.

3.2. Acetylcholinesterase (AChE) inhibition and antioxidant enzyme modulation under chemical and thermal stress

Acetylcholinesterase (AChE) activity was markedly reduced in the tadpoles exposed to methomyl, regardless of temperature. This pattern was supported by the ANOVA, which indicated a strong main effect of the exposure to the pesticide ($F_{1,30}=24.39$, $p < 0.001$), with no significant effect of temperature ($F_{1,30}=0.17$, $p=0.683$) or interaction between temperature and the pesticide ($F_{1,30}=0.02$, $p=0.890$). The post-hoc comparisons confirmed that AChE activity was lower in methomyl-exposed tadpoles compared to their respective controls at both 26.5 °C ($p=0.0076$) and 30 °C ($p=0.0079$), with no differences between temperatures under each pesticide condition (Fig. 3A).

Superoxide dismutase (SOD) activity increased substantially in response to exposure to methomyl, irrespective of temperature. The Type III ANOVA revealed a strong main effect of exposure to the pesticide ($F_{1,31}=27.36$, $p < 0.001$), while temperature ($F_{1,31}=0.25$, $p=0.617$) and the interaction term ($F_{1,31}=0.02$, $p=0.900$) had no effect. The post-hoc comparisons indicated that exposure to methomyl increased SOD activity at both temperatures (26.5 °C and 30 °C), with values approximately 2.9 to 3.6 times higher than in their respective controls ($p \leq 0.006$ for all comparisons) (Fig. 3B).

Glutathione peroxidase (GPx) activity remained stable across all the treatments, with no significant effects of temperature ($F_{1,30}=2.81$, $p=0.104$), pesticide exposure ($F_{1,30}=0.11$, $p=0.748$), or their interaction ($F_{1,30}=0.35$, $p=0.557$) (Fig. 3C).

Catalase (CAT) activity did not vary across the treatments. No effects were detected for temperature ($F_{1,29}=0.07$, $p=0.787$), pesticide exposure ($F_{1,29}=0.22$, $p=0.644$), or their interaction ($F_{1,29}=0.40$, $p=0.530$) (Fig. 3D).

4. Discussion

Chronic methomyl exposure induced drastic changes in tadpole survival across the tested temperatures. Although previous studies suggest that pesticide toxicity can be potentiated by increased temperature [22, 45], such as reported by Lau et al. [32], who observed that methomyl toxicity significantly increases at 30 and 35 °C across different Asian species, following either linear or threshold-dependent patterns, the highest mortality recorded in our experiment occurred at the control temperature (26.5 °C). This outcome was a direct result of our experimental design, where the

methomyl concentrations were based on 10% of the calculated LC50 for the sympatric species *Osteocephalus taurinus*. Since the LC₅₀ for *O. taurinus* reported by Azambuja et al. [38] was significantly lower at 30 °C (4.59 mg a.i./L) than at 26.5 °C (9.64 mg a.i./L), the *L. knudseni* tadpoles were exposed to relatively higher concentrations at the control temperature, explaining the observed survival pattern.

Methomyl exposure significantly accelerated larval development. This response may represent an adaptive strategy to escape from chemical stress, leveraging developmental phenotypic plasticity to favour premature metamorphosis and avoid cumulative damage [46–48]. Tadpole metamorphosis is a complex, endocrine-regulated process involving the transition from an aquatic, gill-breathing, tail-propelled stage to a terrestrial adult form. This transition involves marked morphological changes such as limb development, tail resorption and replacement of gill respiration with pulmonary respiration, as well as internal alterations in the digestive, circulatory and nervous systems [34, 49]. These transformations are primarily mediated by thyroid hormones (T3 and T4), which regulate gene expression for cellular differentiation, apoptosis and tissue remodelling [10, 50]. Environmental contaminants that disrupt this hormonal axis can lead to incomplete, uncoordinated or premature metamorphosis, which compromises post-metamorphic survival and fitness [51–53].

Methomyl is a putative endocrine disruptor that interferes with thyroid-regulated larval development. Endocrine disruptors mimic, block or alter natural hormone production, and affect physiological and behavioural processes in aquatic organisms [54–56]. Experimental evidence supports this hypothesis in *Hoplobatrachus rugulosus* tadpoles, where sublethal methomyl exposure during larval development resulted in a reduced development time, suggesting a premature induction of metamorphosis compatible with the early activation of regulatory hormonal mechanisms [57]. While the acute toxicity of methomyl is documented in amphibians, this study highlights its specific impact on the metamorphic timing of Neotropical tadpoles. Similar patterns of accelerated development have been reported in *Physalaemus nattereri* exposed to the herbicide sulfentrazone [22], suggesting a conserved endocrine dysregulation mechanism in phylogenetically distinct species and different pesticide classes. However, despite its adaptive appearance, contaminant-driven acceleration can impair proper development, reducing body mass, survival and long-term fitness. The accelerated development coincided with significantly lower final body mass in the methomyl-exposed tadpoles, illustrating a

classic life-history trade-off where energy is diverted from growth to expedite metamorphosis [53, 58]. Similar evidence was observed in *H. rugulosus*, whereby sublethal methomyl exposure during metamorphosis not only reduced development time but also resulted in smaller body size in both tadpoles and froglets, reinforcing the association between contaminant exposure, shortened larval periods and impaired growth [57].

In addition to developmental trade-offs, increased antioxidant enzyme activity may impose a metabolic cost. The synthesis, activation and maintenance of antioxidant defences such as SOD require a substantial investment in energy [59, 60]. This metabolic demand may divert energy away from somatic growth, thus contributing to the reduced body mass observed in methomyl-exposed tadpoles. Interestingly, oxidative stress and activation of antioxidant defences have also been linked to developmental acceleration in other amphibians. For example, *Pelobates cultripes* tadpoles exposed to desiccation cues accelerated metamorphosis, accompanied by increased activities of catalase, superoxide dismutase and glutathione peroxidase [61]. This suggests that oxidative stress and redox regulation may be integral components of the physiological adjustments required for rapid development. Although direct causal relationships remain to be clarified, our results raise the possibility that methomyl-induced oxidative stress may have contributed to both the observed upregulation of antioxidant enzymes and the accelerated metamorphosis in *L. knudseni*. Similar body mass and growth reductions have been documented in other species exposed to diverse pesticides. In *Xenopus laevis*, exposure to atrazine resulted in smaller individuals with reduced organs, such as the liver and lipid bodies [62]. North American tadpoles (*Hyla chrysoscelis*, *Rana sphenoccephala*, *Acris crepitans*, *Gastrophryne olivacea*) exposed to chlorpyrifos showed 20–35% mass reductions within four days. In *Rana temporaria*, the fungicide fenpropimorph decreased relative size at metamorphosis by elevating antipredator defence costs [63]. The herbicide metamifop inhibited growth and disrupted hormones in *X. laevis* [16], while 2,4-D delayed metamorphosis and stunted growth in *Lithobates catesbeianus* [22]. Chronic glyphosate, dimethoate, propanil and chlorpyrifos exposure also impaired development and biomass in *Bufo melanostictus* [64], and profenofos + abamectin reduced *Duttaphrynus melanostictus* tadpole length and mass within 15 days [65]. These findings underscore that environmental contaminants, regardless of their mode of action, can compromise amphibian larval growth, with direct implications for their survival and reproductive fitness. Smaller individuals may exhibit lower reproductive

success, reduced locomotion capacity and higher vulnerability to predators. The reduction in average body size can lead to decreased population fecundity and affect population dynamics in the long term.

This additive pattern is reflected in the morphological changes assessed via the PCA. PC1 explained 71% of the variation and was strongly correlated with body traits such as tail musculature width and height, body length and eye diameter. Tadpoles exposed to methomyl and/or elevated temperature showed systematic reductions in these structures, resulting in a shorter and less robust phenotype. Functionally, this has critical ecological implications, as reduced tail musculature and body size are linked to impaired swimming performance, particularly burst speed, which underpins predator evasion and foraging efficiency during the larval stage [66, 67]. Early-life stressors can also produce carry-over effects: smaller size at metamorphosis may persist into the juvenile stage and is associated with lower activity levels, reducing dispersal ability and fitness in terrestrial environments [68]. In addition, pesticide exposure has been reported to cause deformities and behavioural alterations that further compromise locomotion and survival [69–73]. Similar morphological impairments have been associated with reduced swimming capacity in several anuran species, including *Physalaemus cf. cuvieri*, *Leptodactylus latrans*, and *Scinax x-signatus*. Such impairments directly affect predator avoidance and foraging success, ultimately reducing individual fitness and survival [74, 75]. The absence of significant interactions between temperature and methomyl in morphometry, metamorphosis time and body mass analyses reinforce the additive nature of the effects, with both stressors acting independently rather than synergistically.

Methomyl significantly inhibited acetylcholinesterase (AChE) activity, regardless of temperature. This inhibition suggests the direct interference of the insecticide in the cholinergic system, which is consistent with the known mode of action of carbamates. Similarly, Seleem et al. [76] demonstrated that methomyl induces severe neurotoxicity in *Bufo arabicus*, characterized by a marked damage to neuronal cells and overexpression of alpha-synuclein, which is directly linked to neurodegeneration and impaired motor function. This pattern aligns with observations of Widder and Bidwell [69], who reported the inhibition of cholinesterase activity in tadpoles of *Hyla chrysoscelis*, *Rana sphenocephala*, *Acris crepitans* and *Gastrophryne olivacea* exposed to the organophosphate chlorpyrifos, accompanied by a significant reduction in body mass, indicating a systemic neurotoxic effect even in

the short term. Although tadpole studies are scarce, research with other animal models indicates that methomyl can disrupt choline metabolism and reduce AChE activity in a concentration-dependent manner [77,78]. The absence of thermal modulation on this response suggests that, at least at the tested levels, temperature did not intensify the neurotoxic effect of the pesticide. It was expected that the increase in temperature would potentiate the effects of the contaminants, as suggested by several studies [79, 80]. However, the interaction between temperature and the pesticides in modulating AChE seems to strongly depend on the type of compound involved. For example, in *Lithobates catesbeianus*, exposure to the herbicide ametryn did not alter AChE activity, even under heating [18], while the combination of 2-hydroxyatrazine and elevated temperature increased enzyme activity [30]. Thus, our results reinforce that the observed effects were specifically induced by methomyl, without thermal synergy, thus highlighting its direct and independent action as an AChE inhibitor in non-target organisms.

In parallel, we assessed the response of the antioxidant system to exposure to methomyl and temperature variation. The results showed a significant increase only in superoxide dismutase (SOD) activity, with no detectable changes in catalase (CAT) or glutathione peroxidase (GPx). The increase in SOD suggests an increase in superoxide radical ($O_2^{\bullet-}$) generation, while the stability of CAT and GPx indicates that the basal antioxidant system was sufficient to neutralize hydrogen peroxide (H_2O_2). Similar patterns were reported in *H. rugulosus* exposed to methomyl, which showed oxidative stress and liver damage, although without direct evaluation of antioxidant enzymes [56]. Antioxidant responses in amphibians are highly variable and depend on the species, exposure time, type and concentration of the contaminant. In *Lithobates catesbeianus*, for example, ametryn inhibited SOD and CAT only at elevated temperatures [18], while 2-hydroxyatrazine affected only SOD [81]. In *Physalaemus nattereri*, higher temperatures reduced antioxidant activity after eight days of exposure to sulfentrazone, while in *Rhinella schneideri*, enzymes responded quickly in the first few days, returning to basal levels afterward [22]. Furthermore, *Physalaemus gracilis* exposed to cypermethrin showed an increase in SOD and inhibition of CAT [17], while fipronil caused an opposite response in the same species. Bidirectional responses were also described in *Rana nigromaculata* exposed to cyproconazole, though are dependent on the dose [82]. In this study, the observed pattern—a selective increase in SOD and stability of CAT and GPx—indicates a partial activation of the antioxidant system induced by methomyl. The lack of thermal effects or interactions suggests that,

as observed for AChE, the pesticide's action occurred in isolation, without significant modulation by temperature increase. This may reflect a high intrinsic antioxidant capacity of *L. knudseni* during the larval stage, as observed in other tropical species [83,84].

Overall, our results demonstrate that chronic exposure to methomyl, either alone or in combination with elevated temperature, compromises multiple aspects of larval development in *L. knudseni*. We observed consistent effects on survival, growth, morphology and physiology, with predominantly additive patterns. The absence of synergistic interactions between the stressors suggests that, although both are harmful, they act independently at the tested levels. The convergence between morphological and biochemical alterations reinforces the vulnerability of tadpoles to these environmental stressors, particularly in future scenarios of climate change and intensive pesticide use.

5. Conclusion

This study demonstrates the chronic impacts of methomyl exposure on *L. knudseni* tadpoles under different thermal regimes. The results reveal that this pesticide causes significant alterations in development, growth, morphology and biochemical biomarkers, with potential implications for the survival and fitness of individuals in the wild. The lack of synergistic effects with temperature suggests that each stressor independently contributes to impaired larval development. As one of the most biodiverse regions on the planet, the Amazon harbours a remarkable richness of amphibian species, many of which remain undescribed and potentially vulnerable to anthropogenic stressors. Understanding species-specific physiological responses is therefore crucial for safeguarding the functional and evolutionary diversity of this ecosystem. Considering the continued use of pesticides like methomyl in tropical regions and the global-warming scenario, our findings underscore the urgency of integrated ecotoxicological assessments for species of Amazonian wildlife to guide conservation policies and natural resource management in the face of global environmental changes.

6. Permissions

All the experiments were approved by the Ethics Committee on Animal Use of the Brazilian National Institute for Research of the Amazon (INPA) (process No. 83/2023). The animals were collected and transported under authorization from SISBio-ICMBio (No. 80447-4).

7. Acknowledgments

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8. Disclosure of interest

The authors report there are no competing interests to declare.

9. Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

10. List of figures and tables

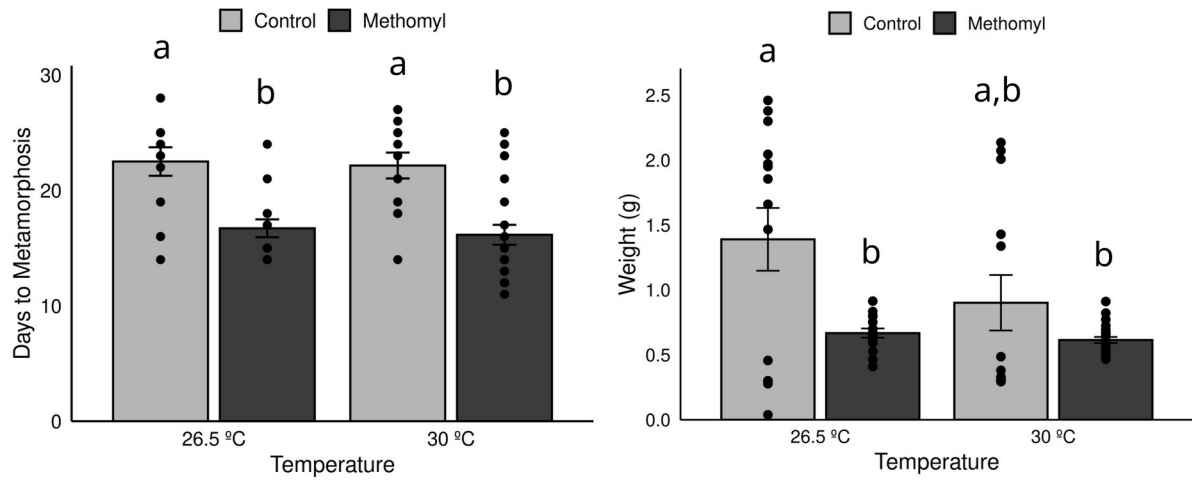


Figure 1. Effects of temperature and methomyl exposure on *Leptodactylus knudseni* tadpoles. (A) Days to metamorphosis under different temperature and pesticide treatments, and (B) Final body mass of tadpoles after chronic exposure. Bars represent means $\pm$ standard error. Letters indicate significant differences between groups based on Tukey's post hoc tests ($p < 0.05$).

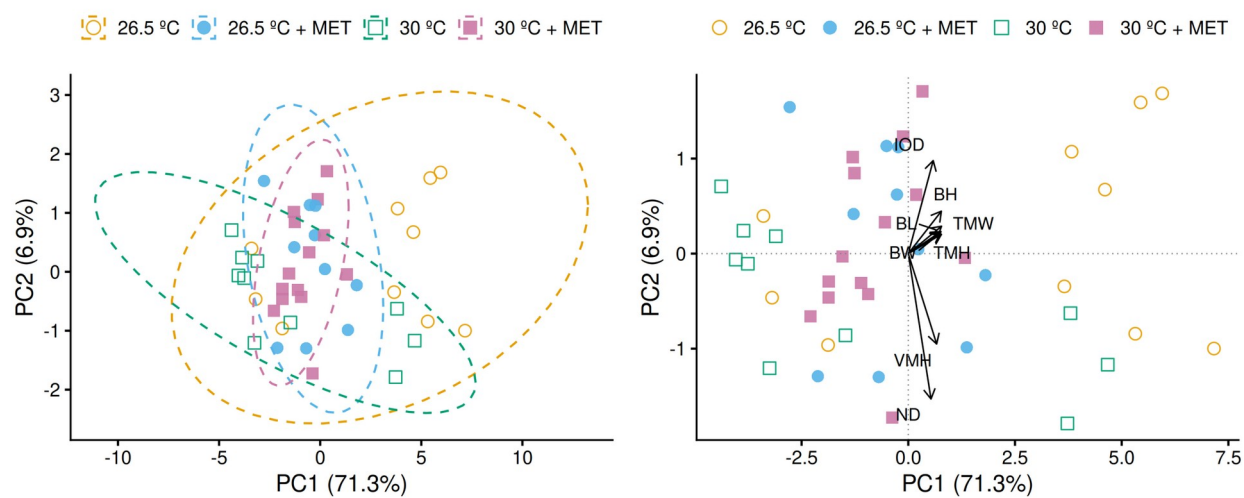


Figure 2. Principal component analysis (PCA) of morphometric traits in *Leptodactylus knudseni* tadpoles exposed to different temperature and methomyl treatments. (A) PCA scatterplot with 95% confidence ellipses for each experimental group. (B) PCA biplot showing individual scores and loading vectors for morphometric variables.

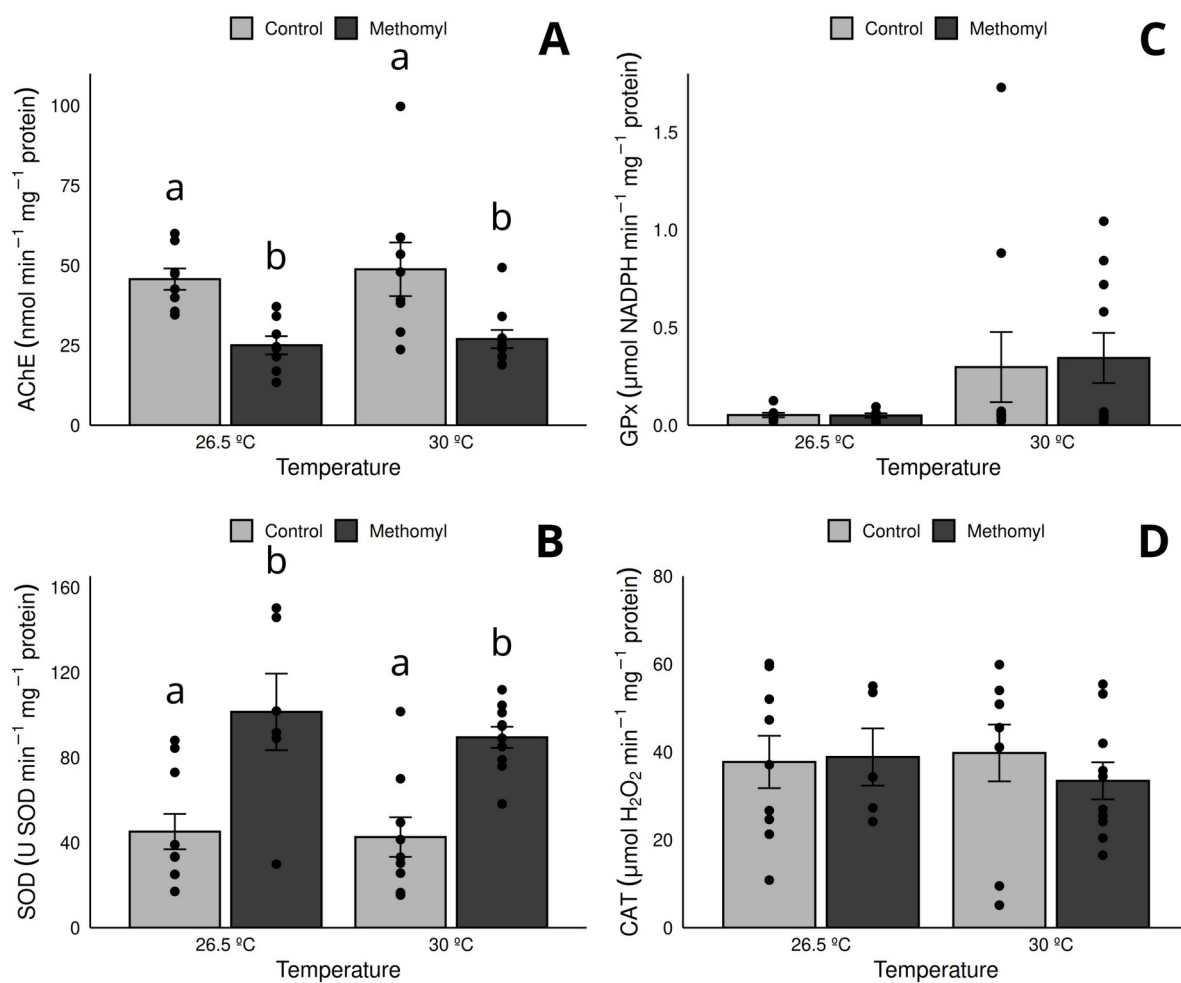


Figure 3. Biochemical responses of *Leptodactylus knudseni* tadpoles to chronic exposure to methomyl and elevated temperature. (A) Acetylcholinesterase (AChE) activity, (B) Superoxide dismutase (SOD) activity, (C) Glutathione peroxidase (GPx) activity, and (D) Catalase (CAT) activity. Bars represent means $\pm$ standard error. Different letters indicate statistically significant differences between groups according to Tukey's post hoc test ($p < 0.05$).

Morphological Trait (mm)	26.5 °C	26.5 °C + methomyl	30 °C	30 °C + methomyl
BH	8.75 ± 0.80	6.63 ± 0.25 (-24.2%)	6.12 ± 0.64 (-30.1%)	6.56 ± 0.17 (-25.0%)
BL	15.96 ± 0.95	12.73 ± 0.34 (-20.2%)	13.30 ± 0.96 (-16.7%)	12.65 ± 0.29 (-20.7%)
BW	9.08 ± 0.74	7.68 ± 0.17 (-15.4%)	6.58 ± 0.65 (-27.5%)	7.32 ± 0.17 (-19.4%)
DMH	2.27 ± 0.24	1.88 ± 0.17 (-17.2%)	1.70 ± 0.26 (-25.1%)	1.55 ± 0.12 (-31.7%)
ED	2.28 ± 0.15	1.77 ± 0.04 (-22.4%)	1.92 ± 0.13 (-15.8%)	1.82 ± 0.06 (-20.2%)
ESD	3.02 ± 0.23	2.45 ± 0.21 (-18.9%)	2.62 ± 0.23 (-13.2%)	2.27 ± 0.08 (-24.8%)
IND	1.91 ± 0.10	1.63 ± 0.05 (-14.7%)	1.67 ± 0.08 (-12.6%)	1.66 ± 0.04 (-13.1%)
IOD	4.30 ± 0.26	3.80 ± 0.09 (-11.6%)	3.23 ± 0.15 (-24.9%)	3.63 ± 0.09 (-15.6%)
ND	0.43 ± 0.05	0.35 ± 0.03 (-18.6%)	0.40 ± 0.05 (-7.0%)	0.34 ± 0.02 (-20.9%)
NSD	1.10 ± 0.15	0.74 ± 0.10 (-32.7%)	0.71 ± 0.10 (-35.5%)	0.76 ± 0.11 (-30.9%)
TMH	4.73 ± 0.49	3.67 ± 0.14 (-22.4%)	3.38 ± 0.44 (-28.5%)	3.38 ± 0.11 (-28.5%)
TMW	5.00 ± 0.54	3.77 ± 0.16 (-24.6%)	3.43 ± 0.43 (-31.4%)	3.60 ± 0.15 (-28.0%)
VMH	1.70 ± 0.20	1.08 ± 0.14 (-36.5%)	1.00 ± 0.18 (-41.2%)	1.12 ± 0.08 (-34.1%)

Table 1. Morphological traits of *Leptodactylus knudseni* tadpoles exposed to methomyl and elevated temperature scenarios. Values are expressed as mean ± standard deviation (SD). Numbers in parentheses represent the percentage of reduction relative to the control group (26.5 °C). Abbreviations: BH (Body Height), BL (Body Length), BW (Body Width), DMH (Dorsal Membrane Height), ED (Eye Diameter), ESD (Eye–Snout Distance), IND (Internasal Distance), IOD (Interorbital Distance), ND (Nostril Diameter), NSD (Nostril–Snout Distance), TMH (Tail Musculature Height), TMW (Tail Musculature Width), and VMH (Ventral Membrane Height).

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Considerações finais

A presente tese integrou abordagens ecotoxicológicas e fisiológicas para elucidar a vulnerabilidade de anuros amazônicos diante da interação entre o aquecimento global e à contaminação por agrotóxicos, mais especificamente o metomil. Podemos concluir que a resposta de girinos tropicais à exposição ao metomil é modulada pelo estresse térmico, embora a magnitude e a natureza dessa modulação variem significativamente entre as espécies, evidenciando a complexidade fisiológica entre espécies.

Diante da exposição aguda, a sensibilidade ao contaminante não é uniforme. Enquanto *Scinax ruber* apresentou alta sensibilidade ao metomil independentemente da temperatura, *Osteocephalus taurinus* evidenciou o fenômeno de toxicidade induzida pelo clima, onde o aumento da temperatura para 30°C potencializou significativamente a letalidade do composto. Curiosamente, a exposição aguda não comprometeu a Tolerância Térmica Crítica (CT_{max}) das espécies.

Com exposições subletais, observou-se que as espécies adotaram estratégias divergentes de enfrentamento. *Osteocephalus taurinus* priorizou a conservação energética através da redução da atividade locomotora e busca por refúgio, acompanhada de uma resposta enzimática compensatória da acetilcolinesterase (AChE). Em contraste, *Scinax ruber* manteve seus padrões de atividade mesmo sob estresse evidenciado pelos níveis de cortisol.

A avaliação dos efeitos crônicos em *Leptodactylus knudseni* nos demonstrou os efeitos clássicos das consequências do custo energético associado à exposição a estressores. A exposição ao metomil induziu uma plasticidade fenotípica caracterizada pela aceleração da metamorfose. Entretanto, o desvio de recursos para a manutenção da homeostase e desintoxicação resultou em girinos com tamanho reduzido ao fim da metamorfose. Conclui-se, portanto, que o estresse químico atua como um fator de seleção que, embora possa permitir a sobrevivência imediata e o escape do ambiente aquático, reduz a qualidade fenotípica dos juvenis, com potenciais repercussões para a dinâmica populacional.

Durante a condução deste trabalho, enfrentamos um cenário que exemplifica a vulnerabilidade dos girinos frente as mudanças climáticas. A severa estiagem de 2023 na Amazônia, impulsionada pelo El Niño, afetou a disponibilidade das espécies para estudo nos corpos d'água, impondo a utilização de *Leptodactylus knudseni* na etapa crônica. Este evento extremo serviu como um alerta de natureza logística e

operacional, reforçando que as mudanças climáticas já impactam a dinâmica das populações, modificando a ocorrência e o tempo de permanência das espécies nos ambientes aquáticos.

Diante da complexidade das respostas observadas e das lacunas ainda persistentes no conhecimento sobre a ecotoxicologia amazônica, os resultados deste estudo apontam para a necessidade de novas investigações. É fundamental que estudos futuros busquem ampliar o realismo ecológico das exposições, incorporando fatores abióticos intrínsecos às poças temporárias, como a hipóxia natural, a redução do volume de água e as oscilações térmicas diárias, bem como a presença de predadores. Adicionalmente, expandir a investigação para outras espécies e contaminantes emergentes na região, como microplásticos e metais pesados, é essencial para compor um panorama mais robusto da vulnerabilidade dos girinos tropicais. Por fim, dado que a exposição crônica suscitou dúvidas sobre a viabilidade populacional a longo prazo, torna-se crucial monitorar os efeitos pós-metamorfose, avaliando se o recrutamento de juvenis com tamanho reduzido compromete a aptidão na fase adulta e resulta em um declínio demográfico real.

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Anexos

Anexo A. Autorização para atividades com finalidade científica (SISBIO), licença nº 80447-4.



Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 80447-4	Data da Emissão: 10/04/2023 18:08:51	Data da Revalidação*: 01/09/2023
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Guilherme de Azambuja Pereira	CPF: 034.273.360-50
Título do Projeto: Avaliação da resposta biológica de girinos expostos à contaminação agroquímica em diferentes temperaturas	
Nome da Instituição: Universidade Federal do Amazonas	CNPJ: 04.378.626/0001-97

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta de indivíduos para o capítulo 1	12/2021	12/2022
2	Coleta dos indivíduos para o capítulo 3	01/2024	12/2024
3	Coleta de indivíduos para o capítulo 2	01/2023	12/2023

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Daiani Kochhann	Orientadora	013.551.930-60	Brasileira

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1	Deve-se observar as as recomendações de prevenção contra a COVID-19 das autoridades sanitárias locais e das Unidades de Conservação a serem acessadas.
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3	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros).
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Autorização para atividades com finalidade científica

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De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Guilherme de Azambuja Pereira	CPF: 034.273.360-50
Título do Projeto: Avaliação da resposta biológica de girinos expostos à contaminação agroquímica em diferentes temperaturas	
Nome da Instituição: Universidade Federal do Amazonas	CNPJ: 04.378.626/0001-97

Observações e ressalvas

9	Esta autorização NÃO exige o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
10	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.

Outras ressalvas

1	1. O volume de coleta se restringe a indivíduos em fase larval/juvenil (girinos). Não está autorizada a coleta de espécimes adultos; 2. Para a eutanásia dos espécimes a serem coletados (removidos do ambiente e sacrificados) o proponente deverá aplicar método ético, devidamente preconizado em protocolo oficial, como, por exemplo, a Resolução CFBio nº 301/2012; 3. Esta Autorização SISBIO não exige o (a) pesquisador (a) de apresentar este projeto para o comitê de ética (CEUA) de sua instituição ou de instituição parceira e só deverá iniciar os trabalhos após aprovação do mesmo pelo comitê.	RAN Goiânia-GO
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Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Fazenda Experimentam UFAM	Manaus-AM	Amazônia	Não	Fora de UC Federal
2	Reserva Florestal Adolfo Ducke	Manaus-AM	Amazônia	Não	Fora de UC Federal
3	Manaus	Manaus-AM	Amazônia	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Captura de animais silvestres in situ	Fora de UC Federal
2	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Atividades ex-situ (fora da natureza)
3	Coleta/transporte de espécimes da fauna silvestre in situ	Fora de UC Federal

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Nome da Instituição: Universidade Federal do Amazonas	CNPJ: 04.378.626/0001-97

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Coleta/transporte de espécimes da fauna silvestre in situ	Osteocephalus taurinus	1680
2	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Osteocephalus taurinus	-
3	Captura de animais silvestres in situ	Osteocephalus taurinus	-
4	Coleta/transporte de espécimes da fauna silvestre in situ	Leptodactylus knudseni	1680
5	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Leptodactylus knudseni	-
6	Captura de animais silvestres in situ	Leptodactylus knudseni	-
7	Coleta/transporte de espécimes da fauna silvestre in situ	Rhinella marina	1680
8	Captura de animais silvestres in situ	Rhinella marina	-
9	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Rhinella marina	-
10	Coleta/transporte de espécimes da fauna silvestre in situ	Callimedusa tomopterna	1680
11	Captura de animais silvestres in situ	Callimedusa tomopterna	-
12	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Callimedusa tomopterna	-
13	Coleta/transporte de espécimes da fauna silvestre in situ	Scinax ruber	1680
14	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Scinax ruber	-
15	Captura de animais silvestres in situ	Scinax ruber	-
16	Coleta/transporte de espécimes da fauna silvestre in situ	Rhinella merianae	1680
17	Captura de animais silvestres in situ	Rhinella merianae	-
18	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Rhinella merianae	-

A quantidade prevista só é obrigatória para atividades do tipo "Coleta/transporte de espécimes da fauna silvestre in situ". Essa quantidade abrange uma porção territorial mínima, que pode ser uma Unidade de Conservação Federal ou um Município.

A quantidade significa: por espécie X localidade X ano.

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Método de captura/coleta (Anfíbios)	Puçá, Peneira

Este documento foi expedido com base na Instrução Normativa nº Portaria ICMBio nº 748/2022. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Página 3/5



Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 80447-4	Data da Emissão: 10/04/2023 18:08:51	Data da Revalidação*: 01/09/2023
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Guilherme de Azambuja Pereira	CPF: 034.273.360-50
Título do Projeto: Avaliação da resposta biológica de girinos expostos à contaminação agroquímica em diferentes temperaturas	
Nome da Instituição: Universidade Federal do Amazonas	CNPJ: 04.378.626/0001-97

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	Universidade Federal do Amazonas	Coleção

Este documento foi expedido com base na Instrução Normativa nº Portaria ICMBio nº 748/2022. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Página 4/5

Anexo B. Certificado de aprovação da Comissão de Ética no Uso de Animais (CEUA/INPA), Parecer Técnico nº 80/2023.

17/05/2023 16:05

SEI/MCTI - 11071487 - Parecer Técnico



MINISTÉRIO DA
CIÊNCIA, TECNOLOGIA
E INOVAÇÃO



INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA
PARECER TÉCNICO Nº 80/2023/SEI-INPA
PARECER CONSUBSTANCIADO E CERTIFICADO SOBRE PROTOCOLOS DE PESQUISAS NO USO DE ANIMAIS
CERTIFICADO DE APROVAÇÃO

Certificamos que a proposta intitulada "**Avaliação da resposta biológica de girinos expostos à contaminação agroquímica em diferentes temperaturas**", registrada com o processo **SEI 01280.000222/2023-91**, sob a responsabilidade de **Guilherme de Azambuja Pereira** - que envolve a produção, manutenção ou utilização de animais pertencentes ao *filo Chordata, subfilo Vertebrata* (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei Nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADA** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) do INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA - INPA.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização:	Início: 01/06/2023 a Final: 30/12/2024
Espécie/linhagem/raça:	Callimedusa tomopterna Leptodactylus knudseni Osteocephalus taurinus Rhinella marina Rhinella merianae Scinax ruber
Número de animais:	10.080
Peso/Idade:	1-2g
Sexo:	M+F
Origem:	LEEM
Jejum:	não se aplica
Inoculação:	não se aplica

Data de liberação do Parecer: **17.05.2023**

Raimunda Liege Souza de Abreu - Presidente CEUA-INPA - PO 230/2022



Documento assinado eletronicamente por **Raimunda Liege Souza de Abreu, Pesquisador(a) em Ciência e Tecnologia**, em 17/05/2023, às 15:52 (horário oficial de Brasília), com fundamento no § 3º do art. 4º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



A autenticidade deste documento pode ser conferida no site <https://sei.mcti.gov.br/verifica.html>, informando o código verificador **11071487** e o código CRC **818958DF**.

Referência: Processo nº 01280.000222/2023-91

SEI-INPA nº 11071487