UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-graduação em Biologia Celular

VICTOR VENTURA DE SOUZA

EFEITOS ECOGENOTÓXICOS E REPRODUTIVOS DA ATRAZINA EM AMBIENTES AQUÁTICOS: AVALIAÇÃO DOS IMPACTOS ATUAIS E FUTUROS

BELO HORIZONTE

2024

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Tese apresentada ao Programa de Pós- Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Doutor em Ciências.

Área de concentração: Biologia Celular

Orientadora: Drª Samyra Maria dos Santos Nassif Lacerda.

BELO HORIZONTE

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
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ATA DE DEFESA DE TESE DO DISCENTE

VICTOR VENTURA DE SOUZA

Às **quatorze horas** do dia **06 de fevereiro de 2024**, reuniu-se, no Instituto de Ciências Biológicas da UFMG,
a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **'"EFEITOS ECOGENOTÓXICOS E REPRODUTIVOS DA ATRAZINA EM AMBIENTES AQUÁTICOS: AVALIAÇÃO DOS IMPACTOS ATUAIS E FUTUROS"'**, requisito final
para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Samyra Maria dos Santos Nassif Lacerda**, após dar a conhecer aos presentes o teor das Normas
Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu- se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas
as seguintes indicações:

Prof./Pesq.	**Instituição**	**Indicação**
Dra. Samyra Maria dos Santos Nassif Lacerda	UFMG	APROVADO
Dra. Elizete Rizzo Bazzoli	UFMG	APROVADO
Dra. Cleida Aparecida de Oliveira	UFMG	APROVADO
Dr. Lázaro Wender Oliveira de Jesus	UFAL	APROVADO
Dr. Ives Charlie da Silva	UNESP	APROVADO

Pelas indicações, o candidato foi considerado: APROVADO

O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que

será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 06 de fevereiro de 2024.**

Drª. Samyra Maria dos Santos Nassif Lacerda (Orient) Drª. Elizete Rizzo Bazzoli

Drª. Cleida Aparecida de Oliveira

Dr. Lázaro Wender Oliveira de Jesus

Dr. Ives Charlie da Silva

Documento assinado eletronicamente por **Samyra Maria dos Santos Nassif Lacerda**, **Professora do Magistério Superior**, em 07/02/2024, às 16:20, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Esta pesquisa foi conduzida no Laboratório de Biologia Celular do Departamento de Morfologia, Instituto de Ciências Biológicas, UFMG, com suporte técnico do Laboratório de Aquicultura da Escola de Medicina Veterinária (LAQUA/EV/UFMG), e em colaboração com o Laboratório de Ecofisiologia e Evolução Molecular do Instituto Nacional de Pesquisas da Amazônia (LEEM/INPA), e com o Laboratório de Biologia Molecular e Reprodutiva do Instituto de Biociências da Universidade Estadual Paulista (UNESP).

Orientadora: Drª Samyra Maria dos Santos Nassif Lacerda.

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Resumo

A Atrazina é o segundo herbicida mais utilizado no Brasil devido ao seu amplo espectro de controle de plantas daninhas e baixo custo, sendo o herbicida mais barato encontrado no mercado. Contudo, a atrazina apresenta efeito residual no solo, devido ao fato de ser lixiviada no período de chuva e tem como consequência a contaminação de sistemas aquáticos. Estudo realizado pelo Ministério da Saúde demonstra que uma em cada cinco cidades brasileiras analisadas apresenta traços residuais de atrazina na água de torneira. Estudos sugerem que a atrazina pode induzir alterações irreversíveis na formação dos tecidos durante o desenvolvimento embrionário, além da diminuição do potencial reprodutivo, distúrbios endócrinos e obesidade. Evidências crescentes mostram que esse herbicida pode apresentar maior toxicidade nas gerações futuras. A partir dessas informações, o escopo desse trabalho é investigar os impactos de concentrações realistas de atrazina encontradas na água potável utilizando bioindicadores aquáticos, além de explorar os efeitos interativos desse herbicida e fatores associados às mudanças climáticas. Ao investigar os impactos de baixas concentrações de atrazina (1, 2, 5, 10, 15 e 20 µg/L), evidenciamos que o herbicida altera o ciclo reprodutivo, a fisiologia e induz aberrações cromossômicas nos três organismos expostos, conforme descrito no capítulo 1. Ao associarmos a exposição aos cenários de mudanças climáticas e atrazina na concentração de 2 µg/L, observamos que a função testicular dos peixes poderá ser afetada drasticamente em cenários futuros. Essa associação comprometeu de forma sinérgica as diferentes populações celulares no testículo, intensificou os impactos na expressão de genes chave e prejudicou a produção dos hormônios sexuais 11-KT e E2. Além disso, a exposição ao futuro cenário climático induziu estresse oxidativo e causou completa imobilidade espermática, conforme descrito no capítulo 2. Esperamos que os resultados encontrados possam nos auxiliar na melhor compreensão do papel dos efeitos da atrazina como moduladores de parâmetros reprodutivos, fisiológicos e mutagênicos nos organismos expostos em cenários atuais e previstos. Isso poderá ter implicações significativas para a tomada de decisões futuras relacionadas às mudanças climáticas e à regulamentação de herbicidas.

Palavras-chave: Concentrações ambientais, herbicida, mudanças climáticas, ecotoxicidade.

Abstract

Atrazine is the second most widely used herbicide in Brazil due to its broad spectrum of weed control and low cost, being the cheapest herbicide found on the market. However, atrazine has a residual effect on the soil, due to the fact that it is leached during the rainy season and consequently contaminates aquatic systems. A study conducted by the Ministry of Health shows that one in every five Brazilian cities analyzed presents residual traces of atrazine in tap water. Studies suggest that atrazine can induce irreversible changes in tissue formation during embryonic development, in addition to reducing reproductive potential, endocrine disorders, and obesity. Increasing evidence shows that this herbicide may present greater toxicity in future generations. Based on this information, the scope of this work is to investigate the impacts of realistic concentrations of atrazine found in drinking water using aquatic bioindicators, in addition to exploring the interactive effects of this herbicide and factors associated with climate change. By investigating the impacts of low concentrations of atrazine (1, 2, 5, 10, 15, and 20 µg/L), we have found that the herbicide alters the reproductive cycle, physiology, and induces chromosomal aberrations in the three exposed organisms, as described in chapter 1. By associating exposure to climate change scenarios and atrazine at a concentration of 2 µg/L, we observed that the testicular function of fish could be drastically affected in future scenarios. This association synergistically compromised the different cell populations in the testicle, intensified the impacts on the expression of key genes, and impaired the production of the sexual hormones 11-KT and E2. In addition, exposure to the future climate scenario induced oxidative stress and caused complete sperm immobility, as described in chapter 2. We hope that the results found can help us better understand the role of the effects of atrazine as modulators of reproductive, physiological, and mutagenic parameters in the organisms exposed in current and predicted scenarios. This could have significant implications for future decision-making related to climate change and herbicide regulation.

Keywords: Environmental concentrations, herbicide, climate change, ecotoxicity.

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INTRODUÇÃO GERAL

Contextualizando a Atrazina e seus efeitos

Os herbicidas são utilizados em diversas plantações com a finalidade de evitar o crescimento e propagação de ervas daninhas que possam competir pelos nutrientes, luz e solo da espécie plantada (Barbosa et al., 2015; Albuquerque et al., 2016; Brovini et al., 2021). A Atrazina (2-cloro-4-etilenodiamino-6-isopropilamino-s-triazina) é um herbicida triazínico seletivo amplamente utilizado para controle de crescimento e o desenvolvimento das plantas invasoras em culturas. O mecanismo molecular de ação da Atrazina envolve sua interação com o sistema fotossintético das plantas (Bai et al., 2015). A atrazina inibe um complexo fotoquímico denominado fotossistema II (PSII), responsável pela transferência de elétrons durante a fotossíntese. Essa inibição resulta na interrupção da produção de energia, levando à nanição e morte de ervas daninhas (Lushchak et al., 2018). Especficamente, a molécula de Atrazina se liga-se à proteína D1, no sítio onde se acopla a plastoquinona (Qb). Essa ligação bloqueia os elétrons da Qa para a Qb e paralisa a fixação de CO_2 (Cordon et al., 2022). O acúmulo de energia na Qa, promove a formação de clorofila tripleto, a qual, ao reagir com o oxigênio, gera o radical livre oxigênio singlete. Este, por sua vez, interage com os lipídios insaturados presentes nas folhas, iniciando o processo de peroxidação. Tal fenômeno pode acarretar danos nas estruturas celulares e interferir nas vias metabólicas de plantas sensíveis. Além disso, a atrazina também afeta o transporte de elétrons nas membranas dos tilacoides, onde ocorre a fotossíntese (Sun et al., 2020). Isso leva a um desequilíbrio na produção de ATP (adenosina trifosfato) e NADPH (nicotinamida adenina dinucleotídeo fosfato), moléculas essenciais para o funcionamento celular e crescimento da normal da planta (Bai et al., 2015).

A Atrazina é um herbicida utilizado há mais de 60 anos em escala mundial, pois é extremamente estável no meio ambiente, possuindo uma meia-vida de aproximadamente 100 dias na água (*Australian Pesticides and Veterinary Medicines Authority*, 2004) e 240 dias no solo (*US Environmental Protection Agency* (EPA), 2003). Com isso, o uso da Atrazina tem gerado diversos debates devido aos seus efeitos em organismos não alvos e, principalmente à sua constante detecção no solo, água potável (de torneira), lençóis freáticos, água de chuva, água superficial, poços artesianos, córregos, lagos, rios, mares e até geleiras em regiões remotas (Barchanska et al., 2012; Sun et al., 2017; Bachetti et al., 2021; Steffens et al., 2022; Urseler et al., 2022; Gagneten et al., 2023). Desde 2004, o uso desse herbicida é proibido em todos os países da União Europeia (UE) e na Alemanha, por

estar provavelmente associado a doenças como Parkinson, câncer de ovário e próstata, e infertilidade (Gagneten et al., 2023).

Em 2019, 15 anos após ser banida, foram encontrados traços de Atrazina em água mineral vendida na França, demonstrando a persistência do herbicida em não ser facilmente biodegradado após a sua utilização (Chen et al., 2019). Invariavelmente, a atrazina ainda continua a ser detectada nas águas costeiras de países da UE, como é o caso do Mar Egeu, que sofre uma considerável influência das correntes de águas provenientes do Mar de Mármara e do Mar Negro. Esses influxos hídricos, por sua vez, perpassam nações não integrantes da UE, e que fazem constante uso do herbicida (Nödler, Licha e Voutsa, 2013; Miladinova et al., 2022).

Isso revela que a proibição da Atrazina em apenas alguns países pode ser ineficaz devido ao seu alto potencial de lixiviação, podendo ser carregada por milhares de quilômetros (Lutri et al., 2022). Na França e na Croácia, por exemplo, foi relatada a presença de traços de Atrazina na urina de mulheres grávidas e de trabalhadores rurais, contudo, nenhuma dessas pessoas teve contato conhecido com o herbicida (Mendaš et al., 2012; Goodman et al., 2014). Países subdesenvolvidos como Brasil, China, Índia e Argentina, empregam a Atrazina em larga escala em sua agricultura (Barbosa et al., 2015; Albuquerque et al., 2016; Brovini et al., 2021). Hoje, o Brasil é o país que mais utiliza esse herbicida no mundo (Salomão, Ferro e Ruas, 2020). De acordo com o IBAMA, no ano de 2021, A atrazina foi o quinto herbicida mais utilizado no país, quando se contabilizou 37.298 mil toneladas.

Muitos fatores influenciam no tempo em que um pesticida permanece no ambiente, tais como luz solar, temperatura, nível de oxigênio, tipo de solo (areia, argila, etc.), pH da água e do solo, e a atividade microbiana (Regar et al., 2019). Além disso, as condições ambientais podem mudar constantemente ao longo do tempo, dificultando estabelecer uma meia-vida única e consistente para um pesticida (Malla et al., 2020). De acordo com Britt et al. (2020), a Atrazina pode causar danos à microbiota essencial em ambientes terrestres e aquáticos. Tendo em vista esses fatores, a presença da Atrazina no meio ambiente pode alterar todo o ecossistema e, consequentemente, modificar seus próprios mecanismos de biodegradação (Aguiar et al., 2020).

A presença de Atrazina no ambiente e a legislação

O herbicida mais detectado no ambiente aquático é a Atrazina (Brovini et al. 2021). Sua presença afeta diretamente os organismos fotossintetizantes sensíveis, tais como plantas aquáticas e algas, o que, como consequência, diminui a presença de oxigênio e

reduz a qualidade da água (Cheng et al. 2023; Hennig et al. 2023). Em 1996, foram realizadas análises para verificar os riscos ecológicos da Atrazina em um microambiente aquático real. Observou-se que invertebrados foram os mais afetados, seguidos por algas e peixes (Solomon et al., 1996).

Cada país possui legislação referente ao uso da Atrazina e determina um valor máximo permitido para água potável ou água superficial (Tabela 1). Até 2004, a União Europeia permitia o valor de 0,1 µg/L de Atrazina e seus metabolitos em água superficial e água para consumo humano. Já a Organização Mundial da Saúde (OMS) admite um nível maior do herbicida, sendo aceito até 100 µg/L para a água potável. A Austrália, país onde estudos relatam impacto reprodutivo severo em marsupiais causados por Atrazina (OMS, 2002), autoriza até 20 µg/L em água para consumo humano. Nos Estados Unidos, é permitido até 3 µg/L, e nos países Brasil, China e Índia é permitido 2 µg/L de Atrazina na água potável ou águas superficiais.

Tabela 1. Valores máximos de concentração permitidos para o herbicida Atrazina em água doce e potável no Brasil e outros países.

País	Atrazina (µg/L)	Referência/Legislação
Brasil	2	Brasil (2005); MS (2011)
Canadá	5	Health Canada (2019)
Estados Unidos	3	US EPA (2000; 2006)
Austrália	20	NHMRC, Australia (2019)
China	2	China (2006)
Índia	2	Índia (2012)
Japão	10	Japão (2003)
União Europeia	Proibido (0,1 até 2004)	UE (2020)
Organização mundial da Saúde	100	WHO (2012)

É importante ressaltar que as concentrações máximas aceitas nos diferentes países funcionam como referência para determinar se a água é segura para consumo. Por várias ocasiões no Brasil foram registrados relatórios indicando concentrações de Atrazina que ultrapassam os limites estabelecidos pelas regulamentações locais (DATASUS 2017). Dados do Ministério da Saúde (2011), por exemplo, revelaram que níveis de Atrazina superiores aos permitidos foram detectados na água da torneira em diversos estados, conforme demonstrado na Figura 2. Se considerarmos os antigos padrões da legislação da UE, todos os estados brasileiros estariam consumindo água contaminada com Atrazina acima do limite autorizado.

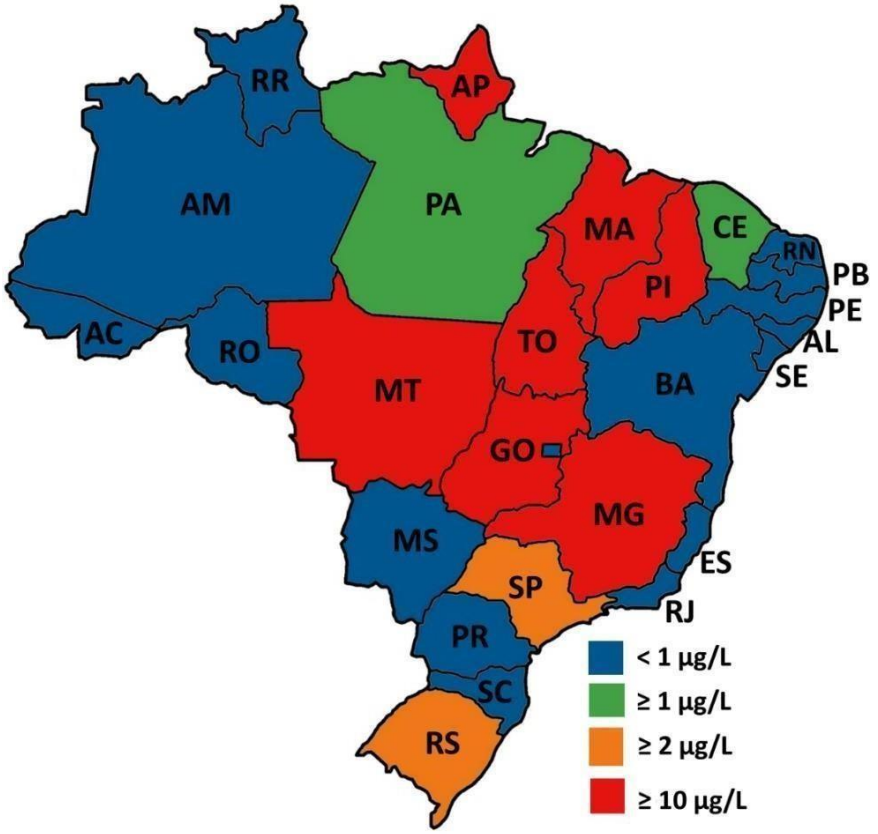

Figura 1. Panorama da presença de Atrazina com base nas concentrações reportadas em águas superficiais, água de poço, água de chuva ou água de torneira nos estados brasileiros. Acre (AC), Alagoas (AL), Amapá (AP), Amazonas (AM), Bahia (BA), Ceará (CE), Espírito Santo (ES), Goiás (GO), Maranhão (MA), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Pará (PA), Paraíba (PB), Paraná (PR), Piauí (PI), Rio de Janeiro (RJ), Rio Grande do Norte (RN), Rio Grande do Sul (RS), Roraima (RR), Santa Catarina (SC), São Paulo (SP), Sergipe (SE), Tocantins (TO), Rondônia (RO) e Pernambuco (PE).

Ao realizar uma análise da literatura cientifica sobre o herbicida Atrazina, nos últimos 10 anos, fica evidente que, baixas concentrações ambientalmente relevantes, podem causar danos reprodutivos em diversos organismos aquáticos (Tabela 2). Apesar disso, esse herbicida ainda é utilizado em grande escala no Brasil e no mundo (IBAMA 2022), o que demonstra a necessidade de uma legislação mais rígida, incentivo do governo a agricultura familiar e desenvolvimento de novas tecnologias em defensivos agrícolas que visem diminuir o impacto no meio ambiente.

Tabela 2. Dados publicados nos últimos 10 anos referentes aos efeitos da Atrazina na função testicular de organismos aquáticos expostos a concentrações realísticas.

Espécie/ Nome popular	Concentração	Exposição	Efeitos observados	Autores
Gasterosteus aculeatus/ Esgana-gato	0.1, 1, 10 e 10 µg/L	42 dias	Presença de ovócitos nos testículos na concentração de 1 µg/L.	Le Mer et al. 2013
Oryzias latipes/ Medaka	0.5, 5 e 50 µg/L	14 ou 38 dias	Espermatogônias anormais.	Papoulias et al. 2014
Bufo bufo gargarizans/ Sapo asiático	0.1, 1, 10 e 10 µg/L	85 dias	População predominante fêmea. Testículo não desenvolvido, oócitos testiculares e redução da porcentagem de células germinativas.	Sai et al. 2014
Danio rerio/Zebrafish	10, 50 e 100 µg/L	3 dias	Aumento do cortisol em todas as concentrações e dias de eutanásia após a exposição ao herbicida.	Kraak et al. 2015
Oryzias latipes/ Medaka	0.5, 5 e 50 µg/L	30 dias	Aumento da expressão da aromatase *Cpy19a1a* e *Cpy11a1*.	Richter et al. 2016
Xenopus laevis/Rã-de-unhas-africana	0.1, 1, 10 e 10 µg/L	90 dias	Redução do índice gonadossomático, degeneração testicular, a expressão de 1165genes foi significativamente alterada com 616 aumentados e 549 diminuídos.	Sai et al. 2016
Oncorhynchus nerka/ Salmão Vermelho	25 e 250 µg/L	21 dias	Redução nos níveis de testosterona no plasma sanguíneo.	Du Gas et al. 2017
Xenopus laevis/Rã-de-unhas-africana	2.5 µg/L	Durante todo período larval	Reversão sexual, redução dos testículos, diminuição da concentração de testosterona.	Hayes et al. 2017
Danio rerio/Zebrafish	2, 10 e 100 µg/L	11 dias	Regulação negativa de genes relacionados à espermatogênese. Reduziu a motilidade, alterou a integridade da membrana e a funcionalidade da mitocôndria dos espermatozoides.	Buatista et al. 2018
Xenopus laevis/ Rã-de-unhas-africana	0.01, 200 e 500 µg/L	90 dias	Redução na porcentagem de mitocôndrias nascélulas de Sertoli e Leydig. Degradação dos túbulos seminíferos, atrofia das gônadas e redução dos níveis séricos de testosterona.	Rimayia et al. 2018

Oryzias latipes/Medaka	5 e 50 µg/L	Durante a determinação do sexo entre 5 e 10 dpf	Redução na contagem de espermatozoides, diminuição de espermatozoides moveis. Aumentode expressão de *Star* e *Fshr*.	Cleary et al. 2019
Acris blanchardi/Rã Grilo	1, 10, 100, e 200 µg/L	51 dias	Presença de ovócitos nos testículos em metamorfose, que também foram observados naprole em idade reprodutiva.	Hoskins et al. 2019
Astyanax altiparanae/ Lambari	0.5, 1, 2 e 10 µg/L	30 dias	Cisto rompidos e aumento de vacúolos.	Destro et al. 2021
Cyprinus carpio/Carpa	4.28 e 42.8 µg/L	125 dias	65% da população sendo peixes fêmeas. Alteroua expressão de miRNAs nos estágios cruciais do desenvolvimento das gônadas da carpa promovendo a expressão de genes com viés feminino.	Wang et al. 2019
Gambusia affinis/ Peixe-mosquito	150 e 1.500 ng/ L	46 dias; eutanásia 30 dias após último dia de exposição	Intersexo, perda da estrutura do cisto levando acélulas espermáticas intersticiais não ligadas e desenvolvimento não sincronizado do cisto.	Matkin, Felgenhauere Klerks, 2021
Hyriopsis bialata/ M*exilhão de água doce*	20 e 200 µg/L	7, 14, 21 e 28 dias	Quebras no DNA, aumento de células germinativas anormais.	Nuchan et ai. 2021
Clarias gariepinus/Bagre-africano	2.5, 25, 250 e 500 µg/L	28 dias	Vacuolização e descamação do epitélio germinativo, interrompeu os componentes histoarquiteturais dos testículos, reduziu o diâmetro das células de Leydig e os níveis de testosterona.	Opute et al., 2021
Acris blanchardi/Rã Grilo	0.5, 5 e 50 µg/L	19 dias	Presença de ovócitos nos testículos	Blanchardi et al. 2022
*Odontesthes bonariensis/*Peixe rei	0.7, 7.0, e 70 µg/L	2 a 6 semanas após a eclosão	Aumento de células germinativas, inversão sexual, aumento da expressãoda*Cyp19a1a*	Carriquiriborde et al. 2023

Os efeitos da Atrazina na esteroidogênese e produção de hormônios sexuais

Atrazina caracteriza-se como potente desregulador endócrino ambiental (EDC), que causa problemas ecológicos e para saúde humana (Almasi et al. 2020; Wang et al. 2020; Galbiati et al. 2021; Barcellos et al. 2022, Lakshmi, 2022; Souza et al. 2023; Vizioli et al. 2023). Os efeitos da Atrazina em diferentes espécies de vertebrados são citados em inúmeros trabalhos, sendo também reportado a desregulação endócrina em peixes (Tabela 2) (Foradori et al., 2018; Graceli et al., 2020; Opute et al., 2020; Stradtman e Freeman, 2021; Wirbisky e Freeman, 2015; Papoulias et al., 2014; Richter et al., 2016; Wirbisky et al., 2016; Yang et al., 2021).

De acordo com o estudo mais recente de Carriquiriborde et al. (2023), ao expor larvas de *O. bonariensis* ao herbicida durante a diferenciação gonadal, a expressão de genes como *Lhb* e *Cyp19a1a* foi desregulada, alterando o desenvolvimento das gônadas dos animais expostos. Os autores ainda descrevem que houve um aumento de células germinativas nos testículos dos peixes em desenvolvimento quando expostos a maior das concentrações testadas (70 µg/L). Na literatura, a Atrazina é conhecida pelo seu potencial de alterar genes citocromo P450 família 19 subfamília A membro 1 (*Cyp19a1*) que codificam a enzima aromatase. Essa enzima é responsável por converter a testosterona em estradiol (E2) (Kazeto et al. 2004; Hecker et al. 2005; Hays et al. 2011; Vasanth et al. 2015; Richter et al., 2016).

Outros estudos sugerem que a exposição à Atrazina pode levar a alterações na expressão dos genes *Cyp19a1* e *Cyp17a1a* em gônadas de peixes. Em algumas espécies, pode supraregular a expressão da *Cyp19a1*, causada por uma maior aromatização da testosterona (Kroon et al. 2014). Efetivamente, diversos estudos descrevem um aumento do hormônio E2 nas gônadas e uma redução da 11-KT em peixes expostos a Atrazina (Tabela 2 e Figura 2) (Spanò et al. 2004; Kroon et al. 2014; Du Gas et al. 2017; Rimayia et al. 2018; Opute et al., 2021; Horzmann et al. 2022).

Figura 2. Efeitos da Atrazina em concentrações realísticas sobre diversas espécies de peixes. Destaque para o aumento na expressão dos genes aromatase citocromo p450, conversão de testosterona em estradiol (E2) e a diminuição da motilidade dos espermatozoides. Autoria própria, 2024.

É importante ressaltar que os efeitos da Atrazina na expressão de genes da via esteroidogênica podem variar entre as espécies de peixes, e devido a fatores como a dose, duração da exposição e condições ambientais. De acordo com Richter et al. (2016), resultados interessantes foram obtidos ao expor peixes medaka (*Oryzias latipes*) e agulha (*Pimephales promelas*) às concentrações de 0.5, 5 e 50 µg/L de Atrazina. *O. latipes* exibiu uma redução na expressão de *Cyp17a1a* quando exposto à concentração mais baixa. Por outro lado, em *P. promelas*, observou-se um aumento na expressão dessa mesma enzima. Chama a atenção a variedade de efeitos que as concentrações ambientais de Atrazina podem desencadear em um ecossistema aquático, especialmente considerando a diversidade de espécies de peixes. Cada espécie pode apresentar uma resposta distinta, como indicado por diversos estudos (Spanò et al., 2004; Kroon et al., 2014; Richter et al., 2016; Du Gas et al., 2017; Rimayia et al., 2018; Opute et al., 2021; Horzmann et al., 2022).

A enzima *Cyp17a1a* desempenha um papel crucial na síntese de pregnenolona e androstenediona, sendo que qualquer interferência em sua expressão ou atividade pode impactar significativamente as funções gonadais e endócrinas (Figura 3). Tais alterações hormonais têm o potencial de comprometer a determinação e diferenciação sexual, a fertilidade, o comportamento reprodutivo, além de afetar a imunidade e a saúde geral das populações de peixes (Filby et al., 2007; Filby et al., 2012; Meng et al., 2019).

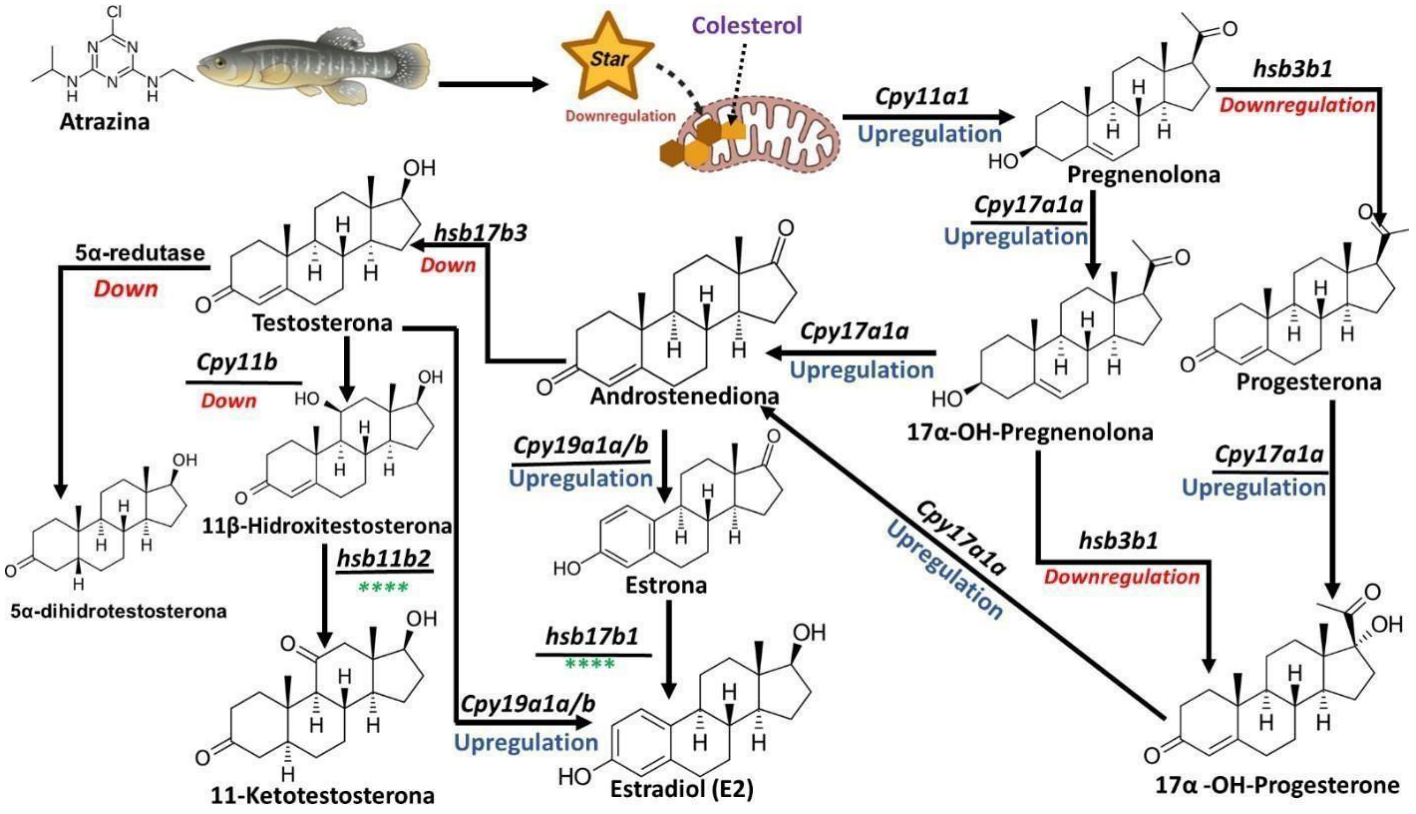

Figura 3. Esquema ilustrativo da via de produção hormonal nos testículos de peixes e os efeitos da exposição a baixas concentraçãoes de Atrazina sobre as enzimas esteroidogênicas. A supraregulação na expressão de *Cyp19a1a/b*, sugere a possível conversão do substrato limitado de testosterona em estradiol, potencialmente resultando na redução da produção de 11-cetotestosterona (11-KT), principal andrógeno dos peixes em níveis plasmáticos e de ativação de receptores. Asteriscos (****) indicam a ausência de informações na literatura sobre a interação entre a enzima da via esteroidogênica e a Atrazina, impedindo inferências sobre regulação positiva ou negativa. Autoria Própria, 2024.

Durante o desenvolvimento de peixes, os genes envolvidos na esteroidogênese e no desenvolvimento das gônadas mostraram ser regulados negativamente com a exposição a Atrazina, dependendo da concentração da exposição (Cleary et al. 2019; Qian et al. 2020; Lent et al. 2021; Carriquiriborde et al. 2023). Com isso, alguns autores indicam uma tendência geral de regulação negativa em machos, incluindo *cyp11a1* e *star* (Figura 3) (Baron et al. 2005; Filby et al. 2007; Leet et al. 2015; Leet et al. 2020).

Os efeitos causados pela Atrazina nos espermatozoides

Os efeitos causados pela Atrazina no espermatozoide podem estar associados a diversos fatores, tais como a repressão de genes relacionados à redução da funcionalidade mitocondrial defesa anti-oxidante e ao estresse oxidativo.

A motilidade espermática em peixes pode variar dependendo da espécie e de outros

fatores ambientais e biológicos. Alguns peixes possuem espermatozoides com movimentos rápidos e altamente direcionais, enquanto outros podem ter espermatozoides que se movem mais lentamente e de forma menos coordenada, indicando assim seu potencial de fertilização (Gallego e Asturiano, 2018). Da mesma forma que nos seres humanos e outros animais, a motilidade espermática é um fator importante na fertilidade dos peixes (Alavi et al., 2019). A capacidade dos espermatozoides de nadarem eficientemente é crucial para que ocorra a fertilização dos oócitos (Castro-Arnau, Chauvigné e Cerdà, 2022).

Os espermatozoides de peixes podem ser uma ferramenta importante para inferir sobre o efeito de contaminantes no ambiente aquático (Bera et al., 2022). A análise da qualidade espermática é comumente usada como um indicador de saúde reprodutiva e pode revelar informações sobre a exposição a herbicidas, como o glifosato e o 2,4D (Bernardi et al., 2022; Peluso et al., 2022). Os peixes são sensíveis a mudanças no ambiente aquático, e muitos contaminantes químicos presentes em rios e lagos podem afetar sua saúde reprodutiva e a produção espermática (Lopes et al., 2022).

Bautista et al. (2018) reportaram uma redução drástica na motilidade e no tempo de motilidade dos espermatozoides em zebrafish pós-exposição com Atrazina, sugerindo que esse herbicida pode reduzir a capacidade de fertilização. Outro achado revelado pelos autores, foi uma redução na funcionalidade mitocondrial do espermatozoide em todas as concentrações ambientais testadas, i.e. 2 , 10 e 100 µg/L de Atrazina.

Fator que também pode causar prejuízo na motilidade e no período de motilidade espermática são danos à membrana celular, e existem relatos na literatura de que a Atrazina gera essas sequelas mesmo em concentrações baixas (Feyzi-Dehkhargani et al., 2012; Mela et al., 2013; Saalfeld et al., 2018). Possuir uma membrana plasmática íntegra no espermatozoide do peixe é essencial para gerar despolarização e iniciar a ativação e a mobilidade para atingir a micrópila do oócito (Saalfeld et al., 2019). Além disso, é relatado que o espermatozoide que possui uma membrana danificada pode apresentar interferências na troca de nutrientes da célula e seu ambiente (Mela et al., 2013), impedindo sucesso na fertilização.

A conversão de testosterona em di-hidrotestosterona (mediador de ação androgênica) é realizada pela proteína SRD5A2. Já o gene CFTR, o qual é expresso na cabeça e na cauda do espermatozoide, apresenta um papel importante na fertilização (Xu et al., 2007) e na motilidade espermática (Hernandez-Gonzalez et al., 2007). Regulação negativa de SRD5A2

e CFTR foi demostrado nas gônadas de peixes expostos à atrazina, podendo ser um dos interlocutores chave para a redução da motilidade e qualidade espermática (Bautista et al. 2018). Uma vez que algumas variantes desses genes já foram descritas como possíveis marcadores genéticos de baixa motilidade espermática (Zhao et al., 2012; Hering et al., 2014), uma repressão dos genes causada pela Atrazina poderia acarretar um atraso na maturação espermática e, consequentemente, na redução na motilidade espermática.

Por fim, como reportado no estudo de Bautista et al. (2018), uma diminuição na expressão de genes que atuam na defesa antioxidante, como SOD2 e GPX4B, juntamente com o gene de reparo de DNA XPC, pode representar vias adicionais que contribuem para os efeitos deletérios causados pela Atrazina nos espermatozoides. Sabe-se que os espermatozoides podem ser significativamente afetados pelo estresse oxidativo (Li et al., 2010). Ao reduzir a expressão da enzima XPC, cuja função é realizar o recrutamento do aparato proteico para reparar danos nas bases do DNA resultante de sua reação com ROS (Nemzow et al., 2015), sugere-se, então, que atrazina pode induzir uma maior suscetibilidade do genoma ao dano oxidativo nas gônadas.

Os efeitos das mudanças climáticas na fisiologia dos peixes

Fatores ambientais dependentes das mudanças climáticas têm um impacto significativo nos ecossistemas aquáticos e podem afetar a fisiologia dos peixes de várias maneiras (Lõhmus e Björklund, 2015). A principal forma é por meio da temperatura da água. As mudanças climáticas e o consequente aquecimento global estão elevando a temperatura média da água em muitas regiões no mundo (Azra et al., 2020). Isso afeta diretamente os peixes, por serem organismos ectotérmicos, ou seja, sua temperatura corporal e fisiologia é influenciada pelo ambiente (Ohlberger, 2013). Aumentos na temperatura podem levar a alteraçãoes na taxa metabólica, crescimento e no desenvolvimento dos peixes (Ohlberger et al., 2012). Também pode afetar a disponibilidade de oxigênio dissolvido na água, levando a condições de hipóxia nos animais (Brander et al., 2007).

Outro fator que pode ocorrer em ambientes aquaticos induzido pelas mudanças climáticas é a modificação da disponibilidade e a distribuição de alimentos (Doney et al., 2012). Por exemplo, elevação na temperatura da água podem alterar a reprodução de algas ou a reprodução de organismos de base na cadeia alimentar (Guo et al., 2016). Isso naturalmente traz consequências negativas para os peixes, afetando sua capacidade de encontrar alimentos adequados e interferindo no seu crescimento e reprodução (Istvánovics

et al., 2020).

Diversos trabalhos descrevem a acidificação do ambiente aquático, já que a absorção de dióxido de carbono (CO_2) pela água do mar resulta em acidificação dos oceanos (Trathan e Agnew, 2010). A acidificação da água também pode afetar a fisiologia dos peixes, especialmente aqueles que têm esqueletos de carbonato de cálcio. O que pode levar a problemas no crescimento e desenvolvimento, bem como na capacidade de resistir a predadores (Lindmark et al., 2022).

As mudanças climáticas podem afetar ainda os padrões de migração e reprodução dos peixes. Alterações na temperatura da água, nos períodos de seca e cheia, e na disponibilidade de alimentos influenciam a migração reprodutiva, bem como o sucesso reprodutivo (Lõhmus e Björklund, 2015), podendo ter implicações para a conservação e a manutenção das populações. Destaca-se que os efeitos das mudanças climáticas na fisiologia dos peixes podem variar dependendo da espécie, do habitat específico e da interação com outros fatores ambientais, incluindo as contaminaçãoes (Hu et al., 2022). O monitoramento contínuo e a pesquisa são essenciais para compreender melhor esses impactos e desenvolver estratégias de adaptação e conservação adequadas (Islam et al., 2022).

A fisiologia reprodutiva, em especial, pode sofrer danos severos causados pelas mudanças climáticas, incluindo efeitos na maturação sexual, na produção de espermatozoides e ovos, na fecundidade e na viabilidade dos embriões e larvas (Lindmark et al., 2022). Por exemplo, temperaturas elevadas podem afetar negativamente a taxa de desenvolvimento dos órgãos reprodutivos e logo, a sincronização entre machos e fêmeas no período de desova (Prakash, 2021). O aumento da temperatura da água, devido às mudanças climáticas, expõe os peixes a níveis mais altos de estresse térmico o que pode também levar a problemas de saúde, como maior suceptibilidade a doenças e parasitas, que, indiretamente afetam as taxas reprodutivas (Azra et al., 2020). Em suma, o potencial das mudanças climáticas de alterar significativamente a reprodução dos peixes, afetando a disponibilidade de habitat para a desova, a disponibilidade de alimentos, a fisiologia gonadal e aumentando o estresse térmico certamente terão consequências negativas para as populações de peixes e para a sustentabilidade dos ecossistemas aquáticos num futuro próximo (Ficke et al., 2007; Harrod et al., 2019).

Raros estudos investigaram o uso de herbicidas associados a mudanças climáticas

na fisiologia dos peixes. Em ambientes naturais, a relação entre herbicidas, temperatura e a fisiologia reprodutiva dos peixes é complexa e pode envolver interações com outros fatores, como demais poluentes, degradação do habitat e introdução de espécies exóticas (Yang et al., 2021). Essas interações podem ter efeitos cumulativos e sinérgicos, tornando os impactos mais significativos do que os efeitos isolados de cada fator (Noyes et al., 2009).

Cenários previstos para 2100 de acordo com Painel Intergovernamental sobre Mudanças Climáticas.

O Painel Intergovernamental sobre Mudanças Climáticas (IPCC), criado e endossado pela Assembleia Geral da ONU em 1988, é uma entidade científica internacional estabelecida pela Organização Meteorológica Mundial (OMM) e pelo Programa das Nações Unidas para o Meio Ambiente (PNUMA). Sua principal missão é avaliar, de forma objetiva e abrangente, informações científicas relacionadas às mudanças climáticas, incluindo suas causas, impactos sociais e econômicos e estratégias de mitigação (https://www.ipcc.ch/).

O IPCC, operando como um fórum intergovernamental que reúne especialistas de diversas áreas, incluindo ciências atmosféricas, oceanografia e geociências, elabora e divulga relatórios periódicos com base na literatura científica mais recente (*Assessment Reports - AR*). Os AR não apenas oferecem uma base crucial para a formulação de políticas e ações relacionadas às mudanças climáticas em escala global e nacional, mas também são reconhecidos como referências fundamentais para compreender o estado atual do clima global e suas implicações futuras (AR6 Synthesis Report: Climate Change 2023).

O IPCC utiliza Modelos Climáticos Globais (GCMs - *Global Climate Models*) para projetar cenários mudanças climáticas para o futuro, isto é, faz-se uma análise robusta a longo prazo das mudanças climáticas até 2100 e além, considerando diversas variáveis e processos. Os GCMs são desenvolvidos por centros de pesquisa em todo o mundo e são baseados em simulações matemáticas e físicas complexas que representam interações entre a atmosfera, os oceanos, a criosfera (gelo e neve), a biosfera e outros componentes climáticos. Com isso, a projeção de cenários climáticos envolve vários passos e considerações (Masson-Delmotte et al. 2021).

Os níveis de gases de efeito estufa (GEE) constituem elementos cruciais nas projeções, e abordam as futuras emissões de gases como dióxido de carbono (CO_2), metano (CH_4) e óxidos de nitrogênio (NOx) (Woodward et al., 2014). Diferentes cenários englobam trajetórias de emissões de GEE distintas, sendo também influenciados por fatores como crescimento populacional, desenvolvimento econômico, adoção de tecnologias e políticas

climáticas (Shukla et al., 2019). Ou seja, os CGMs permite aos cientistas simular como as mudanças climáticas podem evoluir com base em diferentes níveis de emissões de GGE e caminhos socioeconômico. Para garantir a precisão desses modelos, eles são calibrados usando dados observacionais. Isso significa que os resultados dos modelos são ajustados e comparados com dados reais coletados de observações climáticas para assegurar que reproduzam de maneira fiel as condições climáticas existentes (Lahn, 2021).

Especificamente, dá-se o nome de Caminhos de Concentração Representativa (*Representative Concentration Pathways - RCPs*) aos conjuntos de cenários de emissões de CO_2 e outros poluentes atmosféricos utilizados nos GCMs para simular as trajetórias futuras das concentrações desses gases no planeta. Cada RCP é associado a uma estimativa numérica que representa a radiatividade global no ano 2100, conhecida como Força Radiativa (RF), expressa em watts por metro quadrado (W/m^2) (IPCC, 2017). A RF representa a diferença no equilíbrio entre a radiação solar absorvida pela Terra e a radiação térmica emitida de volta para o espaço. Ou seja, a RF indica o quanto a energia radiante recebida do Sol é retida na atmosfera da Terra na presença de GEE e outros componentes atmosféricos. Logo, os GCMs utilizam a estimativa de RF para também prever a variação da temperatura global em relação aos níveis pré-industriais, considerando as diferentes trajetórias de emissões delineadas nos RCPs (IPCC, 2017).

Os quatro principais RCPs estabelecidos pelo IPCC são RCP2.6, RCP4.5, RCP6.0 e RCP8.5, cada um indicando um caminho diferente de emissões e, consequentemente, diferentes potenciais impactos sobre o clima, especialmente sobre a variação da temperatura (aquecimento global). RCP2.6 representa um cenário de mitigação intensa, com emissões de CO_2 decrescentes ao longo do tempo, resultando em uma RF moderadamente baixa até o final do século. RCP4.5 representa um cenário intermediário, com emissões que atingem o pico e começam a diminuir, resultando em uma RF moderada no ano 2100. RCP6.0 reflete um cenário em que as emissões continuam a aumentar até meados do século antes de começarem a declinar, resultando em uma RF relativamente alta em 2100. E RCP8.5 representa um cenário de altas emissões, em que as concentrações de GEE continuam a aumentar ao longo do século, resultando em uma RF alta até o ano 2100 (Alzira and Val, 2017; Wardekker e Susanne; 2019). De maneira preocupante, o cenário RCP8.5 se assemelha consideravelmente às emissões históricas totais acumuladas de CO_2 no mundo. Sua aderência a uma projeção realista se fundamenta na análise das políticas climáticas globais em vigor e nas trajetórias de emissão GEE até hoje reportadas, evidenciando níveis altamente plausíveis de emissões esperados até 2100 e, consequente, de aquecimento

global (Schwalm et al. 2020a e b).

Os cenários projetados pelo IPCC são, portanto, fundamentais para informar políticas e ações em resposta às mudanças climáticas. Eles são e devem continuar a ser usados por cientistas, formuladores de políticas e tomadores de decisão para avaliar o risco climático, planejar adaptações e mitigação, e desenvolver estratégias para enfrentar os desafios decorrentes das mudanças climáticas no futuro.

Objetivos

Objetivo Geral

Investigar e discutir efeitos genotóxicos, reprodutivos e fisiológicos de concentrações ambientais realísticas de Atrazina em ambientes aquáticos, abordando uma visão atual e futura em cenário de mudanças climáticas.

Para isso, o estudo foi desenvolvido em duas etapas: uma com o escopo de avaliar seis concentrações ambientais em três bioindicadores aquáticos *Allium cepa*, *Daphnia magna* e *zebrafish* (Capítulo 1); e outra etapa visando compreender os efeitos interativos da mudanças climáticas e Atrazina na função testicular de zebrafish (Capítulo 2).

Objetivos específicos

Capítulo 1

I. Analisar os efeitos da atrazina por meio de teste de aberrações cromossômicas, citotoxicidade, morte celular e micronúcleo em bioensaio com *Allium cepa*;
II. Descrever os efeitos da Atrazina na reprodução, comportamento e mortalidade do microcrustáceo *Daphnia magna*;
III. Realizar o teste FET utilizando zebrafish para avaliar possíveis alterações morfológicas e fisiológicas após a exposição à Atrazina;
IV. Inferir possíveis impactos ecogenotóxicos da Atrazina.

Capítulo 2

I. Avaliar os indicadores biométricos, morfofisiológicos e reprodutivos em zebrafish machos: comprimento total, peso corporal e índice gonadossomático (IGS) e quantificar a proporção de células germinativas;
II. Quantificar os níveis de 17β-estradiol e 11-ketotestosterona nos testículos;
III. Analisar a expressão de marcadores celulares testicular em resposta à exposição aos cenários de mudanças climáticas e associada à atrazina;

IV. Quantificar a atividade de enzimas antioxidantes e níveis de peroxidação lipídica nos testículos;

V. Avaliar e quantificar os principais parâmetros de qualidade espermática por meio do CASA.

RESULTADOS

CAPÍTULO 1: Ecogenotoxicity of environmentally relevant atrazine concentrations: A threat to aquatic bioindicators

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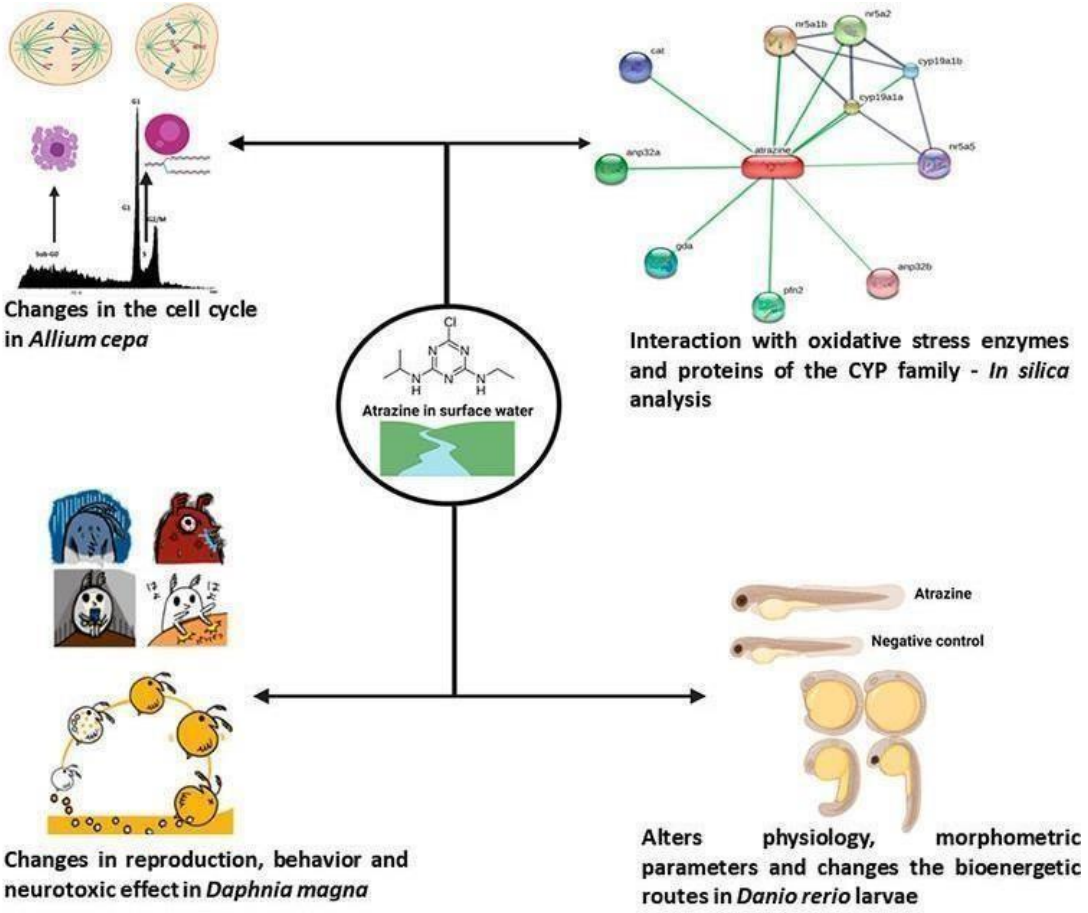
Graphical abstract



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Ecogenotoxicity of environmentally relevant atrazine concentrations: A threat to aquatic bioindicators

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ABSTRACT

Atrazine (ATZ) is a herbicide that is frequently present in surface waters and may result in damage to the health of various organisms, including humans. However, most scientific literature reports injuries caused by ATZ at high concentrations, which are not found in the environment. Therefore, the scope of this study was to investigate the impacts of realistic concentrations of ATZ found in surface waters (1, 2, 5, 10, 15 and 20 μg/L) using the bioindicators *Allium cepa*, *Daphnia magna* and zebrafish (*Danio rerio*). ATZ elicited a genotoxic effect in *A. cepa,* manifested by the induction of chromosomal aberrations, and a mutagenic effect with increased incidence of micronuclei formation, promotion of cell death and reduction in nuclear size revealed by flow cytometry analysis. *D. magna* exposed to 10, 15 and 20 μg/L of ATZ showed significant reduction in body size after 21 days, delayed first-brood release, decreased egg production and total offspring, as well as swimming behavioral changes. ATZ exposure promoted physiological and developmental alterations in zebrafish embryos, including an increased spontaneous movement rate, which led to premature hatching at all concentrations investigated. Increase in total body length, decrease of the yolk sac area, pericardial edema and higher heart rate were also detected in ATZ-treated zebrafish. In summary, environmentally relevant concentrations of ATZ can induce substantial alterations in the three bioindicators investigated. This study evidences the deleterious effects of ATZ on three aquatic bioindicators employing established and current techniques, and may contribute to elucidate the risks caused by this widely used herbicide even at low concentrations and short-to-medium-term exposure.

1. Introduction

The use of pesticides provides control of pests and diseases that affect crops, allowing increased production and reduced economic losses (Savary et al., 2019). In Brazil, agribusiness is centered on monocultures, accounting for 27.4% of the national GDP (CEPEA, 2021). The country has stood out for being one of the largest consumers of pesticides in the world due, in part, to the high agricultural productivity that aims to meet the high demand for food at national and global scales and also the permissive legislation (Brovini et al., 2021; Araújo et al., 2022). Nevertheless, the pending Bill No. 6299/2002, if approved, will loosen the rules for approval and marketing of pesticides, leaving the decision-making power to only the Ministry of Agriculture, Livestock and Supply (MAPA); power that until then is also exercised by the National Health Surveillance Agency (ANVISA) and the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

As a direct consequence of this policy, the high consumption of pesticides has caused impacts to human and environmental health, since active principles and their metabolic derivatives have been frequently reported in the aquatic environment, thus being pollutants found on a

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large scale (Dar et al., 2022a; Dar et al., 2022b; Merola et al., 2022; Ulrich et al., 2022; Saha et al., 2021; Sharma et al., 2021; Syafrudin et al., 2021; Albuquerque et al., 2016; Bernardes et al., 2015). This is the case of Atrazine (ATZ), a selective, organochlorine herbicide from the triazine chemical group and recommended for pre-emergence and early post-emergence control of weeds affecting crops such as sugarcane, corn, and sorghum. Due to its known toxic effects to non-target organisms, which include endocrine disruption, toxicogenicity, and reproductive toxicity (Rodríguez-Robledo et al., 2022; Wang et al., 2022; Blahova et al., 2020), its presence in water is primarily monitored by environmental and human health agencies. In Brazil, the maximum limit allowed in waters intended for water supply and protection of aquatic life as well as in drinking water is 2 μg/L, a value established by Resolution No. 375 of the National Environment Council and by Ordinance No. 2914/2011 of the Ministry of Health. By way of comparison, the maximum limit allowed in water by the European Union, where ATZ is currently banned, is 0.1 μg/L (European Union, 2009).

Studies on ecogenotoxicity have shown that ATZ at environmental concentrations is capable of inducing changes in the plasma osmolality of sodium and chlorine ions, interrupting the release of LH hormone, stimulating the expression of genes related to the biosynthesis of steroid hormones and activating antioxidant enzyme activity (Cleary et al., 2019; Weber, 2013; Paulino et al., 2012; Santos et al., 2012; Hayes et al., 2006). In addition to deleterious effects, ATZ altered the cell cycle, by inhibiting the synthesis of cyclin B and E, arresting cells in the S phase of the cell cycle, ultimately causing changes in the functionality of mitochondria and chromosomal aberrations (Bautista et al., 2018; Felisbino et al., 2018; Cleary et al., 2019; Ventura-Camargo et al., 2016; Wirbisky et al., 2016).

In order to better explore the potential risks of ATZ contamination, this study reports the deleterious effects of environmentally relevant concentrations of the herbicide in water using different bioindicators and multiple biomarkers of toxicity. The cytogenotoxic activity of ATZ was estimated using the chromosomal aberrations test in *Allium cepa*, which correlates strongly with other test systems, such as rodents and human lymphocytes (Nielsen and Rank, 1994; Grant, 1994). Additional data on ATZ interference with cell cycle kinetics and induction of cell death were obtained using the flow cytometry technique, which has been chosen as standard technique by our research group (Fioresi et al., 2020; Andrade-Vieira et al., 2012). Acute and chronic toxicity assays were performed with *Daphnia magna*, through which we further explored the action of ATZ on the swimming behavior of this microcrustacean. From the embryo and larval toxicity test with zebrafish (*Danio rerio*), which has high genetic homology with humans (Pei and Strauss, 2013), we inferred the lethal, cardiotoxic and neurotoxic effects of ATZ, in addition to investigating morpho and physiological alterations. Finally, from bioinformatics analysis, we were able to demonstrate strong interaction of ATZ with different members of cytochrome P450, family 19, subfamily A and oxidative stress-related enzyme in zebrafish.

2. Materials and methods

2.1. Pesticide

Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine, CAS n° 48,085, 99,6% purity, 215.68 g/mol) was purchased from Sigma-Aldrich. The stock solution used was made with: two microliters of acetone solvent to dilute 20 mg/L^{-1} of Atrazine. The following concentrations were tested: 1, 2, 5, 10, 15, and 20 μg/L. This concentration range was established based on the environmentally realistic concentrations found in drinking and surface waters (1, 5 and 10 μg/L) (SISAGUA, 2018; Vieira et al., 2017; Moreira et al., 2012; Wu et al., 2009); and rainwater and groundwater (15 and 20 μg/L) (Moreira et al., 2012; Kolpin et al., 1998) in Brazil and USA. In addition, the Brazilian Resolution n° 375 of the National Environmental Council (CONAMA, 2021) establishes that 2 μg/L is the maximum allowed value for class I and II surface waters (for human supply after treatment and protection of aquatic communities). Such concentration is also established by Ordinance No. 2914/2011 of the Ministry of Health which provides for the control and surveillance of the quality of water for human consumption (BRASIL, 2011). Notably, ATZ concentrations tested in the present study have been also reported in freshwater in other countries (Wang et al., 2020; Hansen et al., 2019; Almberg et al., 2018; Benítez-Díaz and Miranda-Contreras, 2013; Rinsky et al., 2012; Rohr and McCoy, 2010).

2.2. Assays with A. cepa

Seeds of *A. cepa* (2n = 16) of the same batch and variety (Baia Periforme) were used. For all assays, mineral water (Inga´®) and Methyl Methanesulfonate (MMS 0.4 mM) were used as negative and positive control, respectively.

2.2.1. Citogenotoxicity assay

Seeds of *A. cepa* were placed in Petri dishes (150 × 15 mm) lined with filter paper moistened with 5 mL of water for each treatment. The Petri dishes were covered in plastic film to prevent the evaporation of their contents and were incubated at 24°C in the darkness in a growth chamber at 24 ± 1°C for 96 h.

For the microscopy analyses, half of the roots of each concentration were fixed in ethyl alcohol: acetic acid (3:1, *v*/v) and stocked at —20°C. The other half of the roots was transferred to a new Petri dish with a paper filter moistened with mineral water for a 24 h – recovery treatment. Then, the roots were washed three times in distilled water for 5 min, hydrolyzed in 1 N HCl for 8 min at 60°C, and washed again in distilled water, three times. The root tips were submitted to the Schiff's reagent for 2 h in the dark and washed for complete removal of the reagent. The meristems were sectioned on a slide, counterstained with a drop of 2% acetic carmine, covered with coverslip and smashed. The material was analyzed under a light microscope with a magnification of 1000×. For each treatment, 5000 cells were counted (500 cells in 10 slides).

The cytotoxic potential of ATZ was analyzed by calculating the mitotic index (number of cells in mitosis × 100/total number of cells) and by the phase index (number of cells in the particular mitotic phase × 100/total number of cells in mitosis). The genotoxic potential was assessed by counting mitotic and chromosomal abnormalities. Meristematic micronuclei (MN) were counted to determine the mutagenic potential of ATZ at environmentally relevant concentrations. For the analysis of micronuclei in F1 cells (located 1 cm above the meristem), the methodology proposed by Ma et al. (1995) was performed.

2.2.2. Flow cytometry

For flow cytometry, three Petri dishes were prepared for each treatment and 3 meristems per dish were dissected in a dish kept under ice containing 800 μL of LB01 cell lysis buffer. The meristems were crushed with the aid of a scalpel blade, releasing the nuclei in solution. The solution was aspirated through two layers of gauze and collected in a polystyrene tube. The suspensions were filtered with a 45-μm nylon mesh to eliminate most of the residues obtained. The samples were then stained with 50 μL propidium iodide (1 μg/mL) for analysis in the BD FACSCanto II flow cytometer. Frequencies of nuclei in the phases G0/G1, S and G2/M were determined. Sub-G1 particles (cell populations with lower fluorescence intensity than G1 nuclei) were also analyzed. The fluorescence intensity of the nuclei in G1 (Fig 1) was measured. Variations in the DNA content measurement were expressed as the coefficient of variation of G1 (CV^{G1}) = standard deviation divided by the mean. The relative size of the nuclei in G1 was identified by the forward fluorescence detector (forward scatter – FSC) and the complexity (granularity and/or nuclear/cellular density) of the nuclei in G1 by the side scattering light detector (side scatter – SSC).

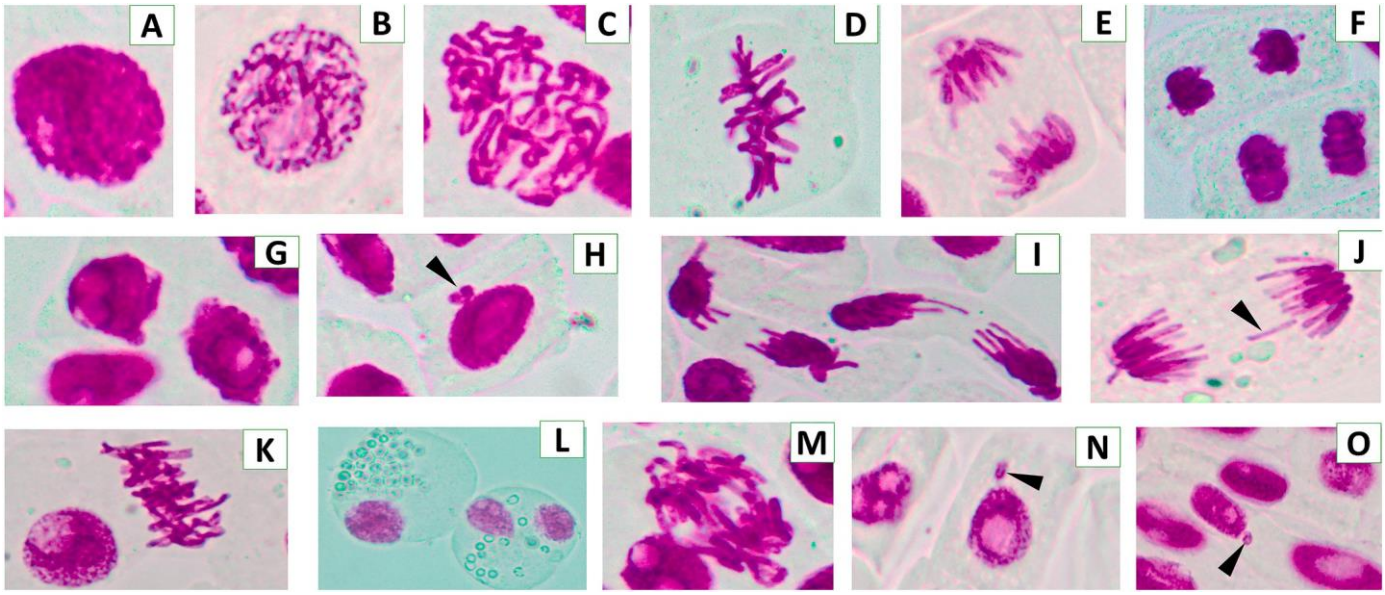

Fig. 1. Cell cycle of *A. cepa*. A–F Normal cell cycle (A. Interphase; B. Prophase; C. Prometaphase; D. Metaphase; E. Anaphase; F. Telophase). G-P Nuclear abnormalities and cell cycle alterations after ATZ exposure (G. Irregular nuclei; H. Nuclear bud (arrowhead); I. Cycle delay; J. Chromosomal loss (arrowhead); K. Chromosomal adherence; L. Cell death; M. Anaphase bridges; N. Micronucleus in meristematic cells (arrowhead); O. Micronucleus in F1 cells (arrowhead).

2.3. Ecotoxicity assays with D. magna

The females of *D. magna* were acquired by the Laborato´rio de Aquacultura (LAQUA) at the Veterinary School of the Federal University of Minas Gerais. For acute and chronic toxicity tests, parthenogenetic females *D. magna* aged <24 h old were individually distributed in glass beakers ($n = 10$) containing 25 mL of different ATZ concentrations and controls (mineral water and ATZ at 1 mg/L were used as negative and positive controls, respectively).

The acute toxicity test was performed according to the ABNT NBR 12713: 2016 protocol. The microcrustaceans were exposed for 48 h under static conditions. After this period, immobilized organisms were counted, and results were expressed as percentage of immobile organisms. The chronic toxicity test lasted 21 days, during which the microcrustaceans were kept in an incubator chamber at 20 ± 2°C and 16 h of light: 8 h of dark photoperiod. *D. magna* was fed daily with the microalgae *Raphidocelis subcapitata*. The system adopted was semi-static, in which the test solution was renewed every two days (Da´vila, 2020). The physical-chemical analysis of the water where the organisms were kept was carried out according to Atique et al. (2020) and the conditions of the assays are shown in Table S1. The observations were made daily from the 9th day. Primiparous age was assessed by recording the parents' age at first reproduction (No, OECD Test, 2012). Fertility was translated into the average number of newborns produced by females. The body diameter of *D. magna* females (parents) was also measured at the end of the experiment. In order to find out whether ATZ-induced disorders in the central nervous system triggering lethargic effect or high excitability, behavioral responses of *D. magna* swimming were evaluated through video recordings on the twenty-first day. For this, a 64MP 1/1.72 in. sensor with 0.8 μm pixels and 26 mm f/1.8 aperture lens camera (DXOMARK Camera-A71 Samsung) was used. Before each recording, there was a twenty-minute wait to avoid abnormal swimming behavior caused by transport and/or light fluctuations. The videos were analyzed in the http://emphazis.org/ software and the following parameters were measured: area covered, swimming speed and total distance covered.

2.4. Fish embryo acute toxicity test

The embryonic toxicity tests in zebrafish (*Danio rerio*) were performed using a modified FET method, OECD guideline 236 (No, OECD Test, 2013). All procedures were performed according to the Animal Health Care Guidelines and were approved by the local Ethical Committee for Animal Research (CEUA-UFMG, Brazil, 353/2018). The females and males of the zebrafish lineage were acquired by LAQUA at the Veterinary School of the Federal University of Minas Gerais, being kept in the Ornamental Fish Laboratory. Adult zebrafish were kept in 20-L aquaria with 10–20 fish each with a sex distribution between female to male of 1:2. The light-dark rhythm was 14:10 h and the water temperature was 27 ± 1°C. For the test, artesian well water (negative control) and 3,4-dichloroaniline (3,4-DCA) at 4.0 mg/L (positive control) were used, as recommended by Lammer (2009).

After spawning, eggs were collected using a grid-covered dish, and only fertilized eggs maximally at the 18-cell stage without obvious irregularities during cleavage were selected for the embryonal toxicity test. Eggs were randomly distributed on microwell plates with 24 wells on each plate, each well containing one embryo, and were exposed to different ATZ concentrations. Twenty eggs were used for each group, the test was performed in duplicate. Half of the test solutions in all groups were replaced daily by gently draining each chamber and adding new solutions slowly to avoid disturbing the embryos. The water temperature during FET was 27 ± 1°C and the daily photoperiod was 12 h of light and 12 h of darkness. Fish were evaluated 24-, 48-, 72-, and 96-h post-fertilization (hpf). The determination of lethality included: embryo coagulation, absence of somite formation, non-detachment of the tail and absence of heartbeat. The parameter of spontaneous movements per minute was analyzed at 24hpf. The analysis of heart beats per minute and hatching rate were performed at 48hpf. Zebrafish larvae at 96hpf were photographed in an optical microscope (Olympus IX-70) at 4× magnification and morphologically analyzed using the ImageJ 1.51j8 program. From the images performed, the following parameters were measured: total length of the larva, height of the head, distance between the myocytes, in addition to the pericardial, yolk sac and eye area.

2.5. In silico analysis

To investigate predicted interactions between ATZ and zebrafish target molecules, network construction and functional annotation enrichment analysis was performed using the STITCH 4.0 Resource (http://stitch.embl.de). The network was built to assess the possible modes

of action of the herbicide, considering the thickness of the lines in the network (thicker lines represent stronger associations). In addition, lines and, for directed edges, different colored arrows represent different types of edges in the action view: turn on (blue), activation (green), inhibit (red), catalysis (magenta), same activity (cyan), and reaction (black) (Kuhn et al., 2007). Statistical significance was determined by the corrected p-value <0.05 using the Bonferroni test. We only consider the shortest paths (allowing a maximum of five interactions with the highest confidence score > 0.8 to ensure a high level of confidence for the interaction).

To assess the effects of potential interactions between ATZ and its possible targets in zebrafish, we use a chemogenomics-based system called ChemDIS-Mixture (Tung et al., 2018), which is available using the STITCH database (Szklarczyk et al., 2016) (Table 1). To allow the inference of ATZ induced effects, possible interaction proteins were extracted, and enrichment analysis was performed based on a hypergeometric test to identify the enriched GO (Gene Ontology) terms with an adjusted p-value <0.05 using the multiple Benjamini-Hochberg correction test.

2.6. Statistical analysis

Statistical analyzes were performed using BioEstat 5.3 software. The evaluation of data distribution in relation to normality was performed using the Lilliefors test, while the homogeneity of variance was assessed using the Cochran test. As the data did not show normal distribution or homogeneity, they were analyzed using the non-parametric Mann-Whitney test ($p < 0.05$). Results were expressed as mean ± standard deviation. CorelDRAW® X7, Biorender (https://biorender.com) and Microsoft Power Point were used for figure design.

Table 1
Functional enrichment analysis for investigating the biological processes involved in the interaction between Atrazine and Zebrafish with their different target molecules.

Molecular function (GO)			
Pathway ID	*Pathway description*	*Count in gene set*	*False discovery rate*
GO 0020037	Heme binding	2	0.0183
KEGG pathway			
Pathway ID	*Pathway description*	*Count in gene set*	*False Discovery rate*
00140	Steroid hormone biosynthesis	2	0.0183
PFAM protein domains			
Pathway ID	*Pathway description*	*Count in gene set*	*False discovery rate*
PF00104	Ligand-binding domain of nuclear hormone receptor	3	0.00443
PF00105	Zinc finger. C_4 type (two domains)	3	0.00443
INTERPPO protein domains and features			
Pathway ID	*Pathway description*	*Count in gene set*	*False discovery rate*
IPR016355	Nuclear hormone receptor family 5	3	2.07e-06
IPR000536	Nuclear hormone receptor ligand-binding domain	3	0.00483
IPR001628	Zinc finger nuclear hormone receptor-type	3	0.00483
IPR001723	Nuclear hormone receptor	3	0.00483
IPR013088	Zinc finger. NHR/GATA-type	3	0.00512
IPR003603	U_2A/phosphoprotein 32 family A. C-terminal	2	0.00702

3. Results and discussion

3.1. ATZ causes genotoxic and mutagenic effects in A. cepa

Table 2 shows the mitotic index and phases index in the root cells of *A. cepa*. ATZ at 10, 15, and 20 μg/L induced significantly higher mitotic indexes compared to negative control. Notably, this effect persisted after the 24 h – recovery treatment when an increase in frequency of cell proliferation was evidenced in all investigated concentrations. Increased and decreased mitotic index indicate disordered cellular proliferation (Leme and Marin-Morales, 2009) due the chemical interaction with specific proteins involved in the progression of the cell cycle, including DNA and RNA polymerases, DNA gyrase, and kinases (Türkog˘lu, 2012).

In addition, ATZ induced changes in the frequency of specific phases of cell division. Regarding continuous treatment, ATZ at 1 and 2 μg/L significantly increased the percentage of cells in telophase. Higher frequencies of cells in metaphase and anaphase were reported after ATZ exposure at 10 μg/L. At 15 and 20 μg/L, ATZ induced a higher frequency of cells in prophase, metaphase, and anaphase compared to negative control (Table 2). Even after 24 h of recovery treatment, an increase in the percentage of cells in prophase was detected for all groups exposed to ATZ. On the other hand, higher frequencies of cells in anaphase were observed in roots exposed to 10 to 20 μg/L of ATZ, while at 15 μg/L the percentage of the cells in the telophase increased significantly. Our findings are in accordance with El-Ghamery et al. (2000) that reported that ATZ may raise the frequency of particular phases of the cell division, mainly prophase and metaphase. Prophase arrest is associated with errors in DNA repair or inhibition of the polymerization of tubulin to the assembly of the mitotic spindle (Grondona et al., 2018; Adeyemo and Ayomide, 2013; Türkog˘lu, 2012). Besides inhibition of the arrangement of the mitotic spindle, metaphase arrest also may be attributed to the inhibition of the complex anaphase promoting complex, which promotes metaphase-anaphase transition (APC/C) (Lub, 2016). Similarly, the anaphase-telophase arrest might indicate the interference of ATZ in proteins involved in the M-phase progression.

El-Ghamery et al. (2000) showed that ATZ is an inhibitor of cell proliferation in root meristems of *A. cepa* and *Vicia faba* when used in high concentrations (3.75 g/L). Ventura and Marin-Morales reported the same effects on the roots of *A. cepa*. Therefore, it is reasonable to assume that the increased mitotic index observed hereafter ATZ exposure might be due to the accumulation of cells in specific phases, caused by a cell cycle arrest.

The genotoxicity of ATZ on plant models has been previously demonstrated (Felisbino et al., 2018; Ventura-Camargo et al., 2016; El-Ghamery et al., 2000). Our findings corroborate this deleterious effect once ATZ caused a significant increase in the frequency of aberrant cells, in both continuous and recovery treatments (Table 3). Although 24 h of recovery were not enough to reduce the genotoxic activity of the herbicide, according to Ventura-Camargo et al. (2016), a period of 48 h of recovery seems to be more efficient.

The frequencies of specific alterations evidenced in *A. cepa* root cells exposed to ATZ are demonstrated in Table 3. Fig. 1 shows representative micrographs of the phases of the normal mitotic cycle of *A. cepa* (Fig. 1A–F) and the main cellular abnormalities identified after 96 h of ATZ exposure, i. e. irregular nuclei (Fig. 1G), an indicative of death cell processes (Leme et al., 2008); nuclear buds (Fig. 1H), frequently derived from chromosomal breaks or losses (Lindberg et al., 2007); laggard chromosomes (Fig. 1I) and chromosomal losses (Fig. 1J) originated from disturbances in the mitotic spindle (Leme et al., 2008); and chromosomal adherence (Fig. 1k) denoting abnormal condensation of the chromosomal fibers, inactivation of the spindles, chromosome breaks and subsequent formation of anaphase-telophase bridges (El-Ghamery et al., 2000; Pathak et al., 1975; McGill et al., 1974). Chromosomal adherence is regularly known to be an irreversible damage, leading to cell death (Fiskesjo¨, 1985). Nevertheless, cells under the death process (Fig. 1L), morphologically characterized as necrotic cells (de Ventura-

Table 2
Cell cycle phase index (%) and mitotic index (%) in *A. cepa* exposed to atrazine, before and after 24 h – recovery.

Time of exposition	Treatments	Interphase	Prophase	Metaphase	Anaphase	Telophase	Mitotic index
	NC	88.66	4.14	3.42	3.46	0.32	64.13 ± 10.13
	PC	85.71	3.96	5.78	3.98	0.57	61.75 ± 9.87
	1 μg/L	87.96	3.85	4.02	3,09	1.08 *	62.5 ± 5.31
	2 μg/L	88.09	3.21	3.77	4.04	0.89 *	67.5 ± 4.40
96 h – continuous treatment	5 μg/L	87.54	3.98	3.85	4.59	0.51	63.5 ± 5.60
	10 μg/L	84.02	4.78	5.26 *	5.02 *	0.92	75.5 ± 7.03 *
	15 μg/L	81.98	6.01 *	6,09 *	5.44 *	0.48	76.87 ± 9.65*
	20 μg/L	81.16	6.12 *	6.78 *	5.58 *	0.46	83.25 ± 6.62*
24 h – recovery	NC	87.11	3.87	4.01	4.12	0.89	65.13 ± 7.32
	PC	86.71	3.75	5.02 *	4.18	0.35 *	63.44 ± 4.11
	1 μg/L	80.62	11.16 *	3.11	4.45	0.66	82.12 ± 3.87*
	2 μg/L	82.47	9.48 *	3.22	4.26	0.57	81.92 ± 1.29*
	5 μg/L	81.89	10.19 *	3.06	4.08	0.78	80.89 ± 6.10*
	10 μg/L	83.13	7.04 *	3.56	5.44 *	0.83	84.87 ± 4.25*
	15 μg/L	82.98	7.56 *	3.52	5.57 *	0.37 *	86.41 ± 6.22*
	15 μg/L	82.98	7.56 *	3.52	5.57 *	0.37 *	86.41 ± 6.22*
	20 μg/L	81.35	8.18 *	4.07	5.89 *	0.51	87.45 ± 5.21*

NC: negative control – mineral water Inga´ ®. PC: positive control – MMS 0.4 −mM. Values followed by * are statistically different from the negative control (Mann-Whitney; $p < 0.05$).

Camargo et al., 2011), as well as anaphasic bridges (Fig. 1M) were confirmed only after the 12 h-recovery treatment in all ATZ groups (Table 3).

In addition to the aberrant cells, micronuclei were also observed in meristematic (Fig. 1N) and F1 cells (Fig. 1O) after ATZ exposure. ATZ at all environmentally relevant concentrations tested induced higher frequency of micronuclei compared to negative control (Table 3). Moreover, we verified that this damage caused by the herbicide may be inherited by the subsequent cell generations. Micronuclei formation can occur because of chromosomal breaks during the process of cell division (clastogenic action), or chromosome loss due to problems in mitotic spindle formation (aneugenic action) (Hintzsche et al., 2017). As most of the aberrations observed in the meristematic cells of *A. cepa* are related to failures of the mitotic machinery, the micronuclei induced by ATZ could probably result from its aneugenic and clastogenic action.

3.2. ATZ disrupts cell cycle kinetics and induces cell death in Allium cepa

The flow cytometry analysis endorsed that ATZ altered the cell cycle progression on *A. cepa* root cells. The deleterious effects observed were induced after continuous treatment and persisted after recovery treatment. The results are shown in Table 4 and representative histograms of the flow cytometry analyzes are depicted in Fig. 2.

The three highest concentrations (10, 15, and 20 μg/L) of ATZ induced an increase in the percentage of the nucleus in the S phase of the cell cycle, although this did not reflect an increase in the percentage of nuclei in G2/M. Freeman and Lane Rayburn (2006) reported that ATZ could raise the percentage of nuclei in the S phase and decrease the nuclei in G2 phase on CHO cell line. In vitro ATZ exposure also promoted the S phase accumulation in HepG2 cells, and this effect was correlated to the downregulation of cyclins E (expressed in late G1 phase) and B (regulates G2/M phases), which induced the deceleration at the end of one cell cycle with subsequent S phase accumulation in the second cycle (Powell et al., 2011). In the current study, observations of delay in DNA synthesis, as well as an arrest in the cycle due to interruption of cell division at specific stages, may support that ATZ retards the cell cycle. Exposure to all concentrations of ATZ significantly increased the frequency of sub-G1 particles which represent cellular and nuclear fragments, chromosomes released in solution or any cellular debris that, in contact with propidium iodide, emit fluorescence. However, they are observed below the fluorescence intensity in G1 as they generally represent particles smaller than an intact nucleus in G1 (Souza et al., 2020; Andrade-Vieira et al., 2012), denoting the occurrence of cell death (Andrade-Vieira et al., 2012). The same effects were observed in analyses of *A. cepa* cells exposed to imidacloprid, iprodione and mixtures of the two pesticides (Fioresi et al., 2020), as well as to further xenobiotics (Ghosh et al., 2016; Andrade-Vieira et al., 2012). Similar to the current study, Fioresi et al. (2020) reported the increase of sub-G1 particles concomitant to the reduction in the frequency of nuclei in G2/M, pointing out that the significant activation of cell death mechanisms can alter the proportion of proliferating cells.

The analysis of FSC (nuclear diameter), SSC (complexity), and fluorescence intensity of the nuclei in G1 (Fig. 1) can also represent indicators of the cell death process (Andrade-Vieira et al., 2012). Decreased FSC was evidenced in treatments with the three highest ATZ concentrations (10, 15 and 20 μg/L). The FSC analysis detects an average value of smaller nuclear diameters than in the negative control, i.e., strongly condensed nuclei, one of the first stages of the cell death process (Andrade-Vieira et al., 2012). ATZ at 10, 15 and 20 μg/L also led to a decrease in the average fluorescence intensity of the nuclei in G1. This effect may also be associated with nuclear condensation that prevents the entry of the dye used to measure the DNA content (propidium iodide). SSC, i.e., the density nuclear was reduced only by the higher concentration of ATZ (20 μg/L). As G1-subparticles, FSC, Fig. 1 and SSC are cell death parameters, the results obtained by flow cytometry support the cytogenetic analysis where the raise of the frequency of aberrant cells, mainly adherence chromosomal (continuous treatment) and necrotic cells (recovery treatment) showed the toxic effect of ATZ on *A. cepa* meristems.

The coefficient of variation of the nuclei in G1 represents how much the flow cytometer is identifying nuclei in this phase of the cell cycle with a variation in the amount of DNA. An increase in this coefficient indicates the presence of nuclei, usually with alterations, such as DNA breaks and generation of lost fragments, loss of chromosomes, adherent chromosomes that hinder the entry of the propidium iodide, among other changes (Fioresi et al., 2020; Andrade-Vieira et al., 2012; Rayburn and Wetzel, 2002). All ATZ concentrations investigated in the present study induced an increase in CV. The observation of chromosomal changes and micronuclei in cytogenetic analysis corroborates the increments in CV reported here, also for all concentrations.

3.3. ATZ inhibit somatic growth and reproduction of Daphnia magna

No concentration of ATZ investigated induced *D. magna* immobility after 48-h exposure. However, ATZ at 10, 15 and 20 μg/L progressively increased the number of days needed for first-brood release, showing an average delay of 1.6, 2.7, and 3.3 days, respectively (Table 5). In addition, ATZ at 5, 10, 15 and 20 μg/L significantly reduced the total number of neonates generated during 21 days of herbicide exposure and inhibited the growth of *D. magna*, leading to a reduction of >15% of the

Table 3
Index of chromosomal aberrations and micronuclei in *Allium cepa* root cells after ATZ exposure for 96 h and after 24 h of recovery.

Treatments	Aberrant cells	Aberrant cells R	MN	MN R	MN F1	MN F1 R	Irregular Nuclei	Chromosomal bridge	Nuclear bud	Chromosomal loss	Delayed telophase	Chromosomal adhesion	Cell death R	Anaphasic bridges R
NC	0.375 ± 0.51	0.21 ± 0.39	012 ± 0.35	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.32 ± 0.52	0 ± 0	0 ± 0	0 ± 0	0 ± 0
PC	31.25 ± 4.26*	28.11 ± 5.63*	7.75 ± 1.83*	3.12 ± 1.12*	3.25 ± 1.16*	5.21 ± 1.19*	0 ± 0	18.37 ± 0.52	4.87 ± 0.99	11.26 ± 0.57	0.76 ± 0.67	6.43 ± 1.82*	0 ± 0	0 ± 0
1 μg/L	24.37 ± 3.33*	8.87 ± 2.64*	7.25 ± 1.16*	0 ± 0	1.25 ± 0.46*	2.03 ± 1.67*	1.85 ± 0.46	5.87 ± 1.55*	1.3 ± 0.11	8.84 ± 0.96*	5.14 ± 1.16*	1.18 ± 0.75	5.12 ± 0.81*	3.75 ± 0.29*
2 μg/L	23.37 ± 2.72*	7.75 ± 1.98*	7.5 ± 1.41*	0.09 ± 0.03	2.37 ± 1.06*	3.06 ± 1.41*	2.5 ± 1.07	6 ± 1.55*	1.03 ± 0.32	7.45 ± 1.04*	5.37 ± 0.95*	1.31 ± 0.51	4.86 ± 0.61*	2.89 ± 1.21*
5 μg/L	18.25 ± 5.09*	10.25 ± 1.98*	9.02 ± 2.39*	0.13 ± 0.58	2.62 ± 0.74*	3.78 ± 2.18*	7.62 ± 1.06*	2.12 ± 1.64	6.37 ± 0.78*	2.36 ± 1.58	3.87 ± 1.64	1.37 ± 1.59	6.63 ± 0.95*	3.62 ± 1.08*
10 μg/L	14.62 ± 3.99*	16.25 ± 2.71*	10.37 ± 2.38*	0.89 ± 0.25*	4.12 ± 1.35*	3.22 ± 1.56*	12.12 ± 3.90*	2 ± 1.19	7.37 ± 2.38*	0.81 ± 0.37	0.5 ± 0.61	1.625 ± 0.74*	8.86 ± 1.77*	7.89 ± 2.14*
15 μg/L	32.37 ± 9.65*	34.5 ± 7.59*	13.87 ± 2.47*	1.01 ± 0.13*	3.5 ± 1.30*	4.22 ± 1.93*	15.62 ± 6.09*	1.62 ± 0.74	13.3 ± 4.02*	0.62 ± 0.51	0.37 ± 0.55	13.62 ± 2.55*	24.86 ± 5.59*	9.64 ± 3.81*
20 μg/L	51.37 ± 5.28*	27.87 ± 8.79*	14.87 ± 2.35*	1.18 ± 0.21*	3.87 ± 3.04*	4.12 ± 1.87*	16.87 ± 1.55*	1.5 ± 0.77	21.7 ± 1.90*	1.5 ± 0.53	0.5 ± 0.59	9.12 ± 2.23*	16.41 ± 3.26*	11.46 ± 1.87*

NC: negative control – mineral water Inga´ ®. PC: positive control – MMS 0.4 −mM. MN: micronuclei. MN F1: micronuclei in non-meristematic cells. R: 24 h recovery. Averages followed by * are statistically different from the negative control (Mann-Whitney; $p < 0.05$).

Table 4
Flow cytometry analysis in meristematic cells of *A. cepa* exposed to ATZ.

Treatments	s.-G1	^{R}s.-G1	G1	^{R}G1	S	^{R}S	G2/M	^{R}G2/M	FSC	^{R}FSC	SSC	^{R}SSC	FI^{G} 1	$^{R}FI^{G}$ 1	CV^{G1}	$^{R}CV^{G1}$
NC	1.81	2.34	64.56	66.89	18.29	17.34	15.34	13.43	100	100	100	100	100	100	2.79	2.90
PC	45.11*	52.67*	40.21*	33.89*	12.34*	8.99*	2.34*	4.45*	87.32*	82.67*	91.21*	90.34*	89.34*	82.34*	8.89*	10.31*
1 μg/L	3.45*	4.22*	65.32	64.32	18.01	17.92	13.22	13.65	98.90	99.75	101.23	100.67	100	101.02	2.34*	2.45
2 μg/L	4.11*	5.79*	64.78	63.32	17.89	17.32	13.22	13.57	99.92	99.78	99.43	99.32	99.89	99.80	3.11*	3.45*
5 μg/L	6.21*	7.11*	62.85	61.21	17.21	16.56	13.73	15.12	100.21	99.89	99.65	101.02	99.56	100.04	4.12*	4.89*
10 μg/L	10.21*	12.32*	58.79*	55.64*	20.31*	19.40*	10.69*	8.64*	96.73*	95.32*	100.21	100.78	95.43*	94.32*	4.89*	5.61*
15 μg/L	13.45*	16.78*	56.80*	55.82*	22.34*	22.11*	7.41*	5.29*	95.73*	94.78*	99.78	99.32	94.34*	93.21*	5.31*	6.78*
20 μg/L	15.61*	22.34*	54.35*	48.35*	23.46*	23.43*	6.58*	5.88*	94.32*	90.31*	95.43*	96.78*	93.41*	90.42*	6.78*	8.79*

s-G1: subparticles G1 (nuclei or fragments with lower fluorescence intensity than G1 nuclei). FSC: nuclei diameter/ complexity. CVG1: nuclei coefficient of variation. NC: negative control – mineral water Ing´a ®. PC: positive control – MMS 0.4 −mM. ^{R} 24 h – recovery treatment. Averages followed by * are statistically different from the negative control (Mann-Whitney; $p < 0.05$).

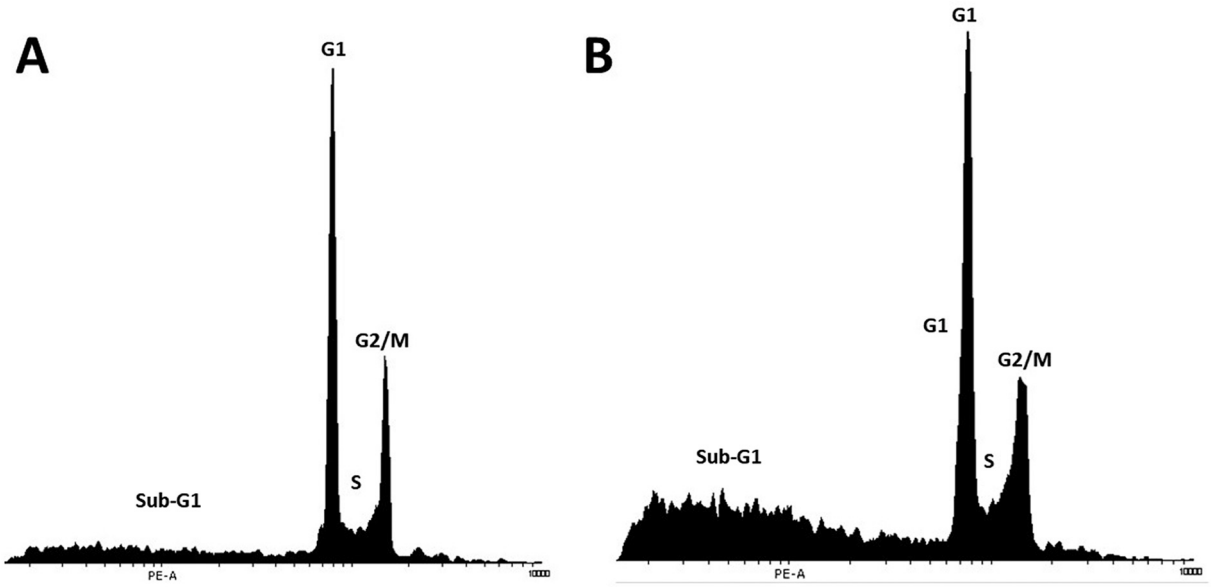

Fig. 2. Representative histograms of flow cytometric analysis on *A. cepa* meristematic cells. A. Histogram showing each cell phase of control group. B. Histogram showing *A. cepa* nuclei at 96 h after ATZ exposure (20 μg/L) following 24 h of recovery. Note in B the increase of subparticles-G1percentage; increase of nuclei in S; decrease in nuclei in G2/M and increase in CVG1. Additionally, in B a shift from the G1 peak to the left is perceived, representing a lower fluorescence intensity compared to control.

Table 5
Chronic test results with *Daphnia magna* exposed to ATZ.

Life cycle variables	NC	PC	1 μg/L	2 μg/L	5 μg/L	10 μg/L	15 μg/L	20 μg/L
Mean size of primiparous	6.84 ± 0.62	4.95 ± 0.54*	6.51 ± 0.62	6.51 ± 0.74	5.67 ± 0.88*	5.62 ± 0.31*	5.79 ± 0.89*	5.55 ± 0.54*
Days for first spawning	9,2 ± 1.33	0	9.3 ± 0.41	9.7 ± 2.74	10.2 ± 1.31	10.8 ± 2.52*	11.9 ± 5.64*	12.5 ± 0.41*
Number of eggs after 21 days	504 ± 63	0	359 ± 44.87	332 ± 41.5	291 ± 36.37*	294 ± 36.75*	260 ± 32.5*	279 ± 34.87*

carapace length compared to the control group (Table 5). Changes in reproductive parameters such as reduction in the hatching of eggs in *P. promelas* and generation a smaller offspring in *D. melanogaster* were evidenced after ATZ exposure at non-environmental concentrations, 0.25 mg/L and 2 mg/L, respectively (Dionne et al., 2021; Vogel et al., 2015). ATZ is able to induce blockage of lipid metabolism, reducing energy production, and it positively modulates detoxification-related enzymes such as glutathione S-transferase, cytochrome P450, and glycosyltransferases as an adaptive response of *D. magna* to chemical stressors (Liu et al., 2022; Sengupta et al., 2017; Schmidt et al., 2017; Sengupta et al., 2016). Under these conditions, it is reasonable to consider that *D. magna* uses its energy reserve prioritizing survival/maintenance and, as a consequence, lacks energy to fully invest in growth and reproduction. Here, fecundity was more sensitive to chronic ATZ exposure than the growth of *D. magna* was. Notably, high concentration of ATZ of the positive control group, i. e. 1 mg/L, completely inhibited parthenogenetic reproduction (Table 5).

ATZ has been identified as a potent endocrine disruptor of different organisms (Wirbisky-Hershberger et al., 2017a, Wirbisky-Hershberger et al., 2017b; Palma et al., 2009) and interferes with the synthesis of hormones essential for sexual maturation and body development (Wang et al., 2022; Zhu et al., 2021; Blahova et al., 2020; McWhinnie et al., 1972). In addition, it is reported that ATZ can modify the metabolic profile of glucose production, diverting energy that would otherwise be used in body growth to cope with the stress caused by the herbicide (Wagner et al., 2017). Accordingly, exposure to ATZ altered the osmoregulation mechanisms in *Prochilodus lineatus*, shifting the mobilization of nutrients and energy to maintain homeostasis (Paulino et al., 2012). Together, these mechanisms could also be responsible for the reduced growth evidenced in *D. magna* exposed to ATZ, even at low concentrations, at the end of the reproduction test.

3.4. ATZ causes abnormal swimming behavior in Daphnia magna

The results of the effects of ATZ on the swimming behavior of *D. magna* are shown in Fig. 3. ATZ at the concentrations of 15 and 20 μg/L reduced the total area covered by *D. magna* (Fig. 3A and B). When analyzing the distance traveled, there was a significant reduction in groups exposed to 15 and 20 μg/L of ATZ (Fig. 3C). Regarding the mean swimming speed (Fig. 3D), it was evident that ATZ at the concentration of 15 μg/L causes a lethargic effect in *D. magna* leading to a decrease in speed, suggesting a possible neurotoxic effect. On the other hand, at the highest concentration tested (20 μg/L) swimming speed remained unchanged, demonstrating that in this group, *D. magna* tend to swim vertically in the water column, however, they remain restricted to a small area in a two-dimensional plane. Although some effects of ATZ show a monotonic linear dose response, other effects frequently do not (Cleary et al., 2019; Bautista et al., 2018; Felisbino et al., 2018; McMahon et al., 2017; Brodeur et al., 2013; Fan et al., 2007; Hayes et al., 2002, 2003, 2006; Larson et al., 1998), as seems to be case of *D. magna* swimming behavior. Specific cellular/molecular mechanisms may explain the nonmonotonic dose-response curves. However, the full range of interconnected biochemical pathways that operate to produce the behavioral response to ATZ exposure is not yet understood.

Changes in swimming behavior associated with area and distance traveled, speed and swimming trajectory occur mainly when aquatic organisms are exposed to environmental pollutants that alter neural functions and consequently their motor coordination (Tkaczyk et al., 2021; Yalsuyi et al., 2021). *D. magna* constantly swim in the water column in order to find food (Fischer et al., 2006), therefore, changes in

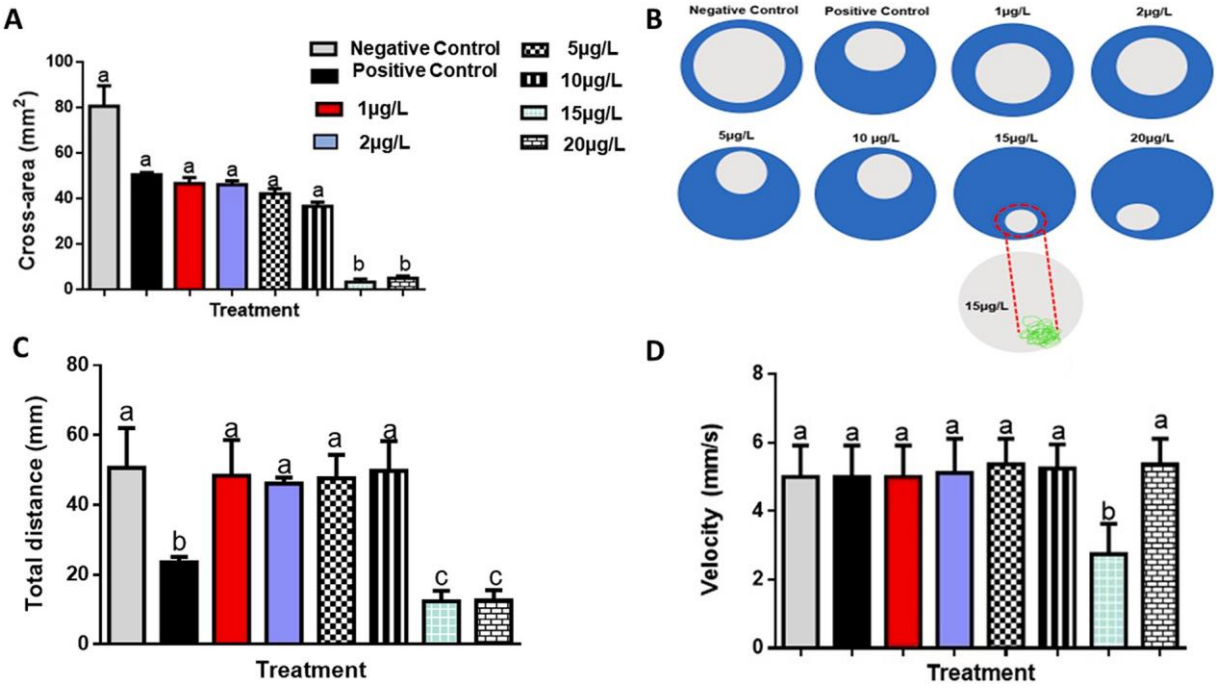

Fig. 3. Data on the swimming performance of *D. magna* 21 days post ATZ exposure. A. Percentage of covered area on swimming by *D. magna*. B. Representative diagrams showing the total specific area explored by *D. magna*. C. Total distance traveled by *D. magna*. D. Velocity of swimming.

swimming style are sensitive and early indicators of toxicity for water quality assessment and can significantly impact survival chance and reproduction (Pawlik-Skowron´ska et al., 2019). Dodson and Stanley (1997) reported that accelerated and continuous movements of *D. magna* attract the attention of fish and may generate greater predation. Marshall (2009) described that under stress conditions *D. magna* changes its swimming style by performing short strokes in an attempt to reach the top of the water column. The vertical swimming movement reported here for the concentration of 20 µg/L, causing the organisms to remain in the same location, could, therefore, be associated with higher energy expenditure and a decrease in the age of first reproduction of *D. magna*.

3.5. ATZ induces developmental and physiological alterations in Danio rerio

Data on mortality in zebrafish embryos and larvae were recorded at 24, 48, 72 and 96 hpf (Table 6). No group treated with ATZ showed differences in mortality rate during this period compared to the negative control. In the present study, we tested environmental concentrations of ATZ far below that required to cause lethality in zebrafish; indeed, according to Wiegand et al. (2000), the LC50 of ATZ at 48hpf is 36.8 mg/L. The results of the hatching rate are also shown in Table 6. A hatching rate above 90% was observed in all treated and control groups, with no differences between them. However, most of the embryos exposed to ATZ at different concentrations hatched before 48hpf, which was not evidenced in the control group. Notably, 91.2 and 89.8% of embryos treated with 15 and 20 µg/L of ATZ hatched between 24 and 48 hpf (Table 6). Consequently, at 72hpf the hatching rate reduced significantly for all ATZ concentrations compared to the control, with the highest rate reported in this period being 8.10% for the 10 µg/L concentration.

Alterations in embryo hatching rate and timing are associated with changes in metabolism and have been observed in zebrafish embryos in response to environmental pollutants (Blahova et al., 2021; Cahova et al., 2021; Liang et al., 2017a, Liang et al., 2017b; Samaee et al., 2015).). Importantly, premature hatching may imply a significant increase in the susceptibility of the newly hatched larvae to various stressors present in the environment (Liang et al., 2017a, Liang et al., 2017b), which may also explain physiological perturbations and morphological abnormalities caused by ATZ during fish development (Barton, 2002).

The morphological parameters investigated in zebrafish larvae are illustrated in Fig. 4A. All concentrations of ATZ significantly increased the spontaneous movement rates of the embryos 24hpf (Fig. 4B). While the negative control averaged 1.2 movements per minute, the concentrations of 1 and 10 µg/L induced 2.8 (lowest response) and 6.3 (highest response) movements per minute, respectively. Assessment of spontaneous movement in zebrafish embryos has been recently proposed as a

Table 6
Mortality results (%) and hatch rate results (%) of zebrafish embryos exposed to ATZ (hpf – hours after fertilization).

	Time	NC	PC	1 µg/L	2 µg/L	5 µg/L	10 µg/L	15 µg/L	20 µg/L
Mortality	24 hpf	2.01 ± 0.62	10.11 ± 3.21*	1.11 ± 0.09	1.44 ± 0.74	1.37 ± 0.52	2.11 ± 0.31	2.25 ± 0.77	2.11 ± 0.88
	48 hpf	1.57 ± 0.59	15.25 ± 1.18*	2.10 ± 0.18	1.45 ± 0.66	2.21 ± 0.21	2.21 ± 0.48	2.19 ± 0.51	2.09 ± 0.62
	72 hpf	1.39 ± 0.52	13.39 ± 4.32*	2.02 ± 0.38	2.28 ± 0.29	2.35 ± 0.14	1.87 ± 0.57	2.01 ± 0.41	2.78 ± 0.44
	96 hpf	1.22 ± 0.3	7.22 ± 3.22*	2.09 ± 0.45	1.39 ± 0.19	1.13 ± 0.30	1.09 ± 0.18	1.81 ± 0.39	2.14 ± 0.91
Hatch rate	24 hpf	0	0	0	0	0	0	0	0
	48 hpf	0	0	83.10 ± 5.55*	85.12 ± 2.85*	88.10 ± 3.18*	84.75 ± 7.99*	89.82 ± 4.55*	91.23 ± 5.32*
	72 hpf	84.20 ± 6.22	71.55 ± 9.01	4.11 ± 1.01*	5.02 ± 1.09*	5.32 ± 0.98*	8.10 ± 2.85*	1.09 ± 0.32*	0.98 ± 0.10*
	96 hpf	8.31 ± 1.41	9.88 ± 2.10	3.59 ± 0.91*	3.01 ± 0.78*	1.55 ± 0.37*	0.69 ± 0.11*	1.01 ± 0.21*	0.59 ± 0.09*

* Are statistically different from the negative control (Mann-Whitney; $p < 0.05$).

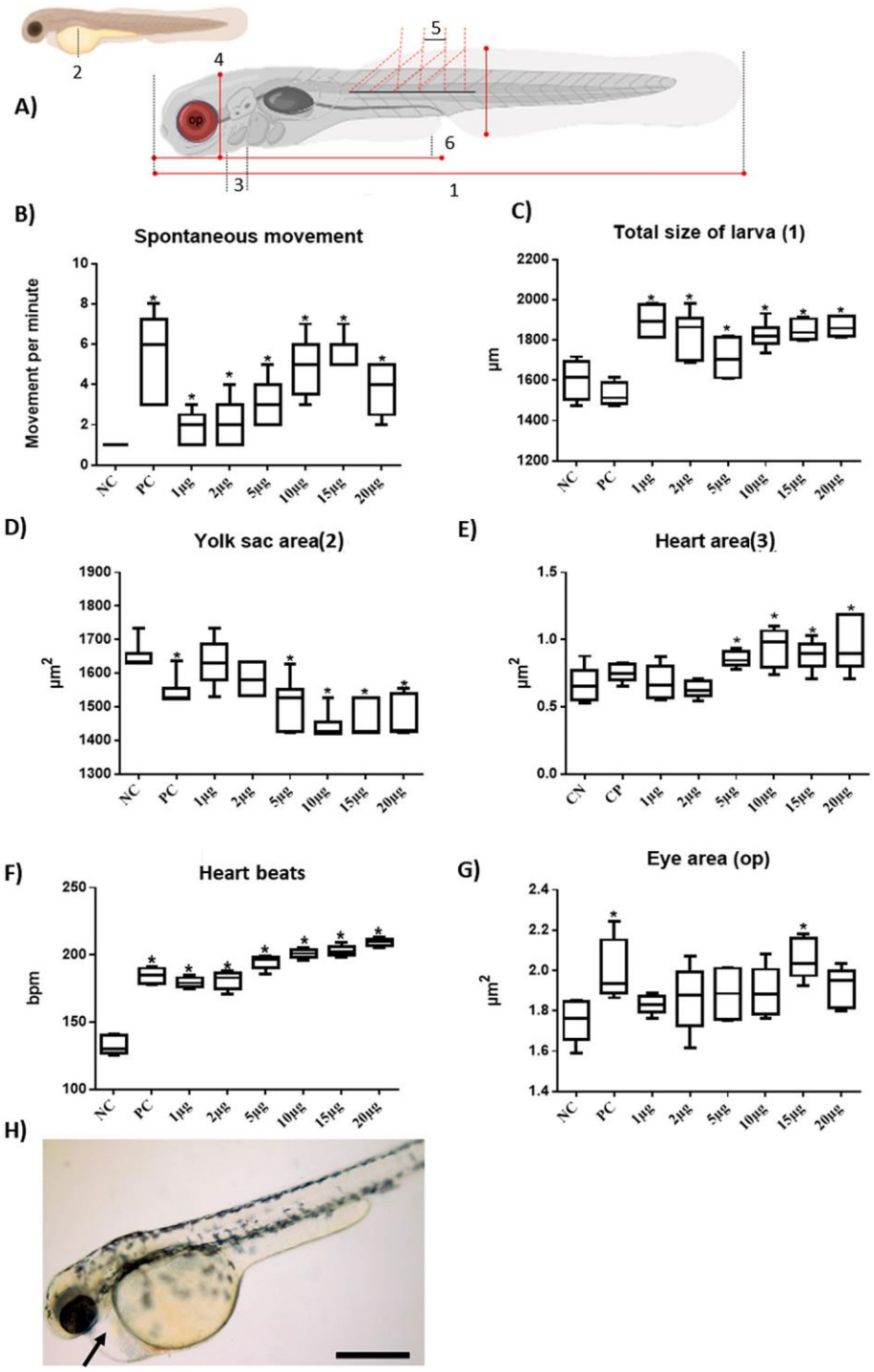

Fig. 4. Morphological and physiological data of zebrafish embryo and larvae exposed to ATZ. A. Ilustration of morphometric analyzes performed on zebrafish larvae at 96hpf. B. Spontaneous movement at 24 hpf. C. Total length of the larvae at 96hpf (A1). D. Yolk sac area at 96 hpf (A2). E Pericardial area at 96 hpf (A3). F. Heart rate of larvae at 96 hpf (beats per minute, bpm). G. Eye area (Op). H. Morphological changes in 96 hpf zebrafish larvae exposed to 20 µg /L of ATZ: yolk sac edema (arrow). NC: Negative control; PC: Positive control; *Significantly different in relation to the NC by the non-parametric Mann-Whitney test, ($p < 0.05$).

neurotoxicity marker in studies on behavioral and motor development changes due to chemical exposure (Soares et al., 2017). Researches have shown that ATZ is an environmental factor that can cause neurological effects (Galbiati et al., 2021; Stradtman and Freeman, 2021; Wang et al., 2015; Bretaud et al., 2004). Accordingly, Wang et al., 2015 reported that Acetyl Cholinesterase (AChE) activity in zebrafish embryos treated with ATZ is downregulated and could impair the innervation efficiency of muscles. Inhibition of the AChE enzyme keeps the nicotinic acetylcholine receptors open for sodium ions to flow into the cells of the nervous system, leading to an action potential and hence potential hyperactivity (Ogungbemi et al., 2020). Our findings might indicate that developmental ATZ exposure can affect neuroactivity and may trigger altered motor response inducing embryo hyperactivity, characterized by higher rates of spontaneous movements, which contribute ultimately to premature hatching (Cheng et al., 2007a, Cheng et al., 2007b; Winnicki et al., 1970).

Fig. 4C shows that all concentrations of ATZ promoted an increase in the size (total body length) of the zebrafish larvae at 96 hpf compared to the negative control. In contrast, there was a significant reduction in yolk sac size in larvae exposed to concentrations of 5, 10, 15 and 20 µg/L of ATZ (Fig. 4D). These findings might indicate an increased metabolization or accelerated utilization of the larvae's sole energy source, contributing to their rapid growth, as suggested in previous studies (Parra et al., 1999). In male rats, ATZ at low concentrations (30 or 300

μg/kg/day) induced an increase in lipid synthesis and intramuscular storage, thereby generating fat accumulation in metabolically active tissue (Lim et al., 2009). Also, exposure of zebrafish embryos to ATZ caused upregulation of the *Crhbp* gene (Wirbisky et al., 2016), which is responsible for stimulating the secretion of preopiomelanocortin-derived peptides, such as α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH). The function of these hormones in vertebrates involves energy homeostasis, food intake, and body weight regulation (Hsieh et al., 2021).

To further investigate whether ATZ developmental exposure elicits cardiotoxicity in zebrafish larvae, the pericardial area was evaluated. As Fig. 4E shows, 5 μg/L or higher concentrations of ATZ exposure led to an increase in the pericardial sac area at 96 dpf (Fig. 4H), indicating a possible alteration in heart morphology and function. Notably, all ATZ-treated zebrafish larvae also exhibited markedly higher heart rate at 96 hpf, increasing the contraction rate by 89% compared to negative control, suggesting that the herbicide may accelerate heart rate and increase cardiac output (Fig. 4F). The increase in heart rate and pericardial area are regularly related to increased metabolic rate, changes in blood pressure, and increased response to external stimuli, aggravating the risk of predation or hampering the individual to seek a favorable environment or food, directly impacting the number of individuals who can reach adulthood (Li et al., 2020).

Regarding other morphological endpoints investigated, such as distance between myosepta and head size (Fig. 4A 4,5), no significative ATZ exposure-related effects were evident in zebrafish larvae, indicating that the herbicide, at low concentration, seems to not elicit alterations or degeneration on muscle development or cause severe neurodevelopmental defects, at least up to 96 dpf. In defiance of these findings, at 15 μg/L of ATZ exposure, we detected an increase in the area of the eyes (Fig. 4G). According to Fan et al. (2007), ATZ exposure can interrupt retinoid signaling and increase the expression of opsin genes, thus impairing the eye development of zebrafish larvae.

3.6. ATZ may interact with zebrafish cytochrome P450 aromatase and catalase

In the bioinformatics analysis, the supposed pathways were explored, integrating ATZ with different proteins in a network of metabolite-protein interaction. According to the STITCH interaction network, ATZ was linked to different metabolic pathways and showed a strong interaction with members of the cytochrome P450 family, family 19, subfamily A (CYP19a1b, CYP19a1a) (Fig. 5), and oxidative stress enzymes catalase (CAT). Whereas in fish, as in other animals, most isoforms of the CYP families 1–4 primarily act on xenobiotic biotransformation (Loerracher and Braunbeck, 2021), isoforms of the CYP families 5–51 predominantly act on endogenous substrates, many of which have critical roles in normal development, maturation and physiological homeostasis, as in the case of neural and gonad aromatases, encoded by the *cyp19a1a* and *cyp19a1b* genes (Guengerich, 2017; Nebert et al., 2013). It has been shown that the antioxidant defense system plays an important role in maintaining cell homeostasis (Burkina et al., 2015; Westphal, 2000). Based on the strong interaction of ATZ with CAT, we speculate that ATZ could elicit changes in the detoxifying system and that the ATZ-related developmental toxicity observed in zebrafish might be related to the excessive oxidative stress. Additional studies are needed to elucidate the mechanisms altered by low concentrations of ATZ exposure during zebrafish development as well as the effects in adulthood after long-term exposure.

4. Conclusion

ATZ is one of the most widely used pesticides in agriculture and, due to its known toxic effects, its presence in the environment is primarily monitored by environmental and human health agencies. However, this study warns that the limit value of ATZ allowed in waters intended for supply, protection of aquatic communities and human consumption, 2 μg/L, caused cytogenotoxic damage in *A. cepa*, and physiological changes in zebrafish embryos, and therefore harmful to water quality bioindicators belonging to different trophic levels.

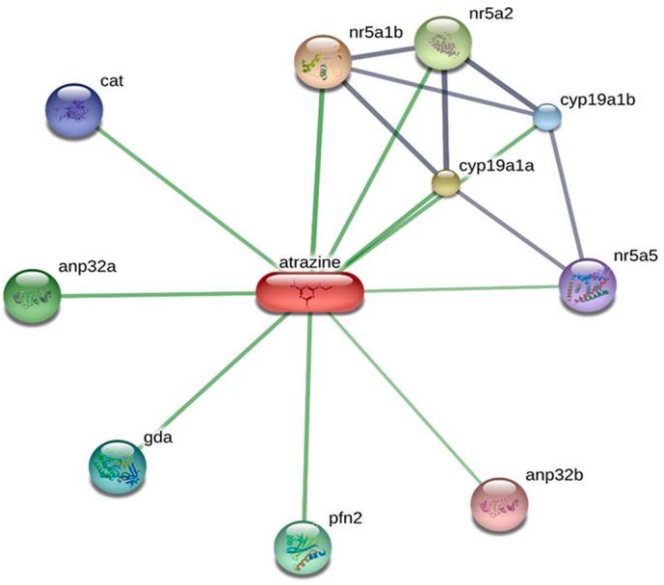

Fig. 5. Network analysis results using the Search Tool for Interactions of Chemicals (STITCH) to explore the interaction between atrazine and zebrafish with their different target molecules. Splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Small nodes: protein of unknown 3D structure.

The presence of concentrations higher than 2 μg/L in the aquatic environment, reported in scientific studies that supported the other concentrations tested here, highlights the indiscriminate use of ATZ in crops, a recurring problem, especially in Brazil. The inefficient regulation of the use of this herbicide hinders the implementation of measures to prevent aquatic contamination, and may contribute to the increase of genetic, physiological and morphological disorders in exposed organisms as reported in this study.

CRediT authorship contribution statement

Victor Ventura de Souza: Writing – review & editing, Formal analysis, Data curation, Visualization. **Tatiana da Silva Souza:** Conceptualization, Investigation, Writing – review & editing, Visualization, Supervision. **Jose´ Marcello Salabert de Campos:** Methodology, Validation, Data curation. **Luiza Araújo de Oliveira:** Methodology. **Yves Moreira Ribeiro:** Methodology. **Daniela Chemin de Melo Hoyos:** Methodology. **Roge´ria Maura Panzini Xavier:** Methodology. **Ives Charlie-Silva:** Methodology. **Samyra Maria dos Santos Nassif Lacerda:** Conceptualization, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2022.105297.

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Table S1. Physicochemical analysis of water exposed to *Daphnia magna*.

Analyze	Analytical Reference	LQ	NC	PC	ATZ
pH	SM 4500H+B	0.1	7.14 ± 0.1	7.35 ± 0.1	7.42 ± 0.2
Temperature			20.3°C ± 0.4	20.1°C ± 0.5	20.3°C ± 0.2
Turbidity	SM 2130B	0.1	0.04 UNT	0.04 UNT	0.09 UNT
Electric conductivity	SM 2510B	0.1	1.13µS/cm	3.53µS/cm	0.86µS/cm
Total solids	SMWW 2540 D	5.0	20 mg/L^{-1}	20 mg/L^{-1}	18 mg/L^{-1}
Total Dissolved Solids	SM 2540 C	1.0	0.53 mg/L^{-1}	1.83 mg/L^{-1}	0.43 mg/L^{-1}
Total Volatile Solids	SMWW 2540 D	5.0	10 mg/L^{-1}	10 mg/L^{-1}	8 mg/L^{-1}
Total Fixed Solids	SMWW 2540 D	5.0	10 mg/L^{-1}	10 mg/L^{-1}	10 mg/L^{-1}
Biochemical oxygen demand	SMWW 5210 B	2.0	2.4 mg/L^{-1}	2.7 mg/L^{-1}	1.8 mg/L^{-1}
Dissolved oxygen	SMWW 5210 B	2.0	5.8 mg/L^{-1}	6 mg/L^{-1}	6.2 mg/L^{-1}
Chlorides	SM 4110 B	5.0	58 mg/L^{-1}	128 mg/L^{-1}	80 mg/L^{-1}
Phosphate	-	1.0	0.06 mg/L^{-1}	0.96 mg/L^{-1}	0.06 mg/L^{-1}
Ammoniacal nitrogen	AMB.007	1.0	1.68 mg/L^{-1}	8.40 mg/L^{-1}	1.12 mg/L^{-1}
Nitrate	-	1.0	0.22 mg/L^{-1}	0.55 mg/L^{-1}	0.35 mg/L^{-1}
Alkalinity	SM 2320 B	5.0	108 mg/L^{-1}	120 mg/L^{-1}	80 mg/L^{-1}

SM - Standard Methods for The Examination of Water and Wastewater, 22nd (2012); NC - Negative control; PC - Positive control; ATZ - Atrazine. * It differs from that authorized by resolution 430/2011 of the National Council for the Environment; UNT: nephelometric turbidity units. HA, A. P. Standard methods for the examination of water and wastewater. **Water Environment Federation, Secaucus, NJ, USA**, 2012.

RESULTADOS

Capítulo 2: Simulated climate change and atrazine contamination can synergistically impair zebrafish testicular function

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Simulated climate change and atrazine contamination can synergistically impair zebrafish testicular function

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Highlights

- Exposure to climate change scenario and ATZ synergistically compromises testicular cell population.
- Climate change exposure amplifies ATZ impact on testicular gene expression.
- Climate change and ATZ synergistically impair 11-KT and E2 production.
- Exposure to climate change scenario induces oxidative imbalance and sperm immotility.
- Interactive effects of climate change and ATZ may cause severe testicular damage and infertility.

Abstract

Elements that interfere with reproductive processes can have profound impacts on population and the equilibrium of ecosystems. Global warming represents the major environmental challenge of the 21st century, as it will affect all forms of life in the coming decades. Another coexisting concern is the persistent pollution by pesticides, particularly the herbicide Atrazine (ATZ), which is responsible for a significant number of contamination incidents in surface waters worldwide. While it is hypothesized that climate changes will significantly enhance the toxic effects of pesticides, the actual impact of these phenomena remain largely unexplored. Here, we conducted a climate-controlled room experiment to assess the interactive effects of the projected 2100 climate scenario and environmentally realistic ATZ exposures on the reproductive function of male zebrafish. The gonadosomatic index decreased significantly in the fish from the extreme scenario. Cellular alterations were observed across all phases of spermatogenesis, leading to a synergistic effect in decreasing sperm production and increasing germ cell sloughing, and cell death. ATZ exposure alone or combined with the effects of climate change, disrupted the transcription levels of key genes involved in steroidogenesis, hormone signaling and spermatogenesis regulation. An additive modulation with decreased 11-KT production and increased E2 levels was also evidenced, intensifying the effects of androgen/estrogen imbalance. Moreover, climate change and ATZ independently induced testicular oxidative stress, upregulation of proapoptotic gene and DNA damage in post-meiotic germ cell, but the negative effects of ATZ were greater at extreme scenario. Ultimately, exposure to simulated climate changes led to complete sperm immotility. These findings indicate that the future climate conditions have the potential to considerably enhance the toxicity of ATZ at low concentrations, leading to severe deleterious consequences for fish reproductive function and fertility. These may provide relevant information to supporting healthcare and environmental managers in decision-making related to climate changes and herbicide regulation.

Key words: climate room, sperm motility; sex hormones; spermatogenesis; herbicide.

1. Introduction

With current demographic trends, ensuring adequate sustenance for the global population of 8 billion individuals is imperative (Behnassi and Mahjoub, 2022). Remarkably, this population is expected to reach approximately 10 billion by 2050 (Chamie, 2023). Providing food security for a growing population remains a fundamental challenge for humanity, and addressing this challenge requires overcoming many obstacles, including changes in diets, conversion of crops to biofuels, loss of agricultural land, and anthropogenic climate change (Lin et al. 2022). Overall, climate uncertainty and potential extreme weather events will pose inherent environmental risks to agricultural production and sustainability (Lesk et al. 2022).

Such hazards, including excessive or unstable temperatures and changes in precipitation patterns and water availability, have been the subject of extensive research (McCoy et al. 2022). Notably, other environmental factors related to anthropogenic climate change, such as high levels of CO_2 emission, may affect biological constraints within global agricultural production, including increased frequency and intensity of insect infestations, weed proliferation, and disease spread (Nguru and Caroline, 2023; Bajwa et al. 2020; Ziska et al. 2018; Dáder et al. 2016; Varanasi, Vara Prasad and Mithila, 2016). Recent estimates of global productivity losses due to pests and pathogens are on average 21.5% for wheat, 30% for rice, 17% for potatoes, and 22.5% for corn (Savary et al. 2019; Shrestha, 2019; Ebert, 2017; War et al. 2016). However, several studies suggest that production risks are likely to worsen due to climate change, increasing the need for pest control measures such as pesticide use (Reza and Gabriela 2022; Heeb, Emma and Matthew, 2019; Ramsfield et al. 2016; War et al. 2016). In this scenario, climate change can also directly lead to reduced pesticide efficacy by altering pesticide metabolism and translocation (Pratibha et al. 2019; Jugulam et al. 2018; Varanasi, Vara Prasad and Mithila, 2016), or indirectly by increasing pesticide detoxification in host-plants (Dubey, Neeraj and Sheo, 2016; Dyer, 2018; Powles, 2018), which will require the use of new pesticides or the intensification of the use of existing pesticides (Matzrafi et al. 2019).

Atrazine (ATZ) is one of the most ubiquitous chlorotriazine herbicides in Latin America, Africa and US widely used in corn fields to control broadleaf weeds and grasses (Gagneten et al. 2022; Lagunas-Basave et al. 2022; Becker et al. 2021; Gabardo et al. 2021; Dias et al. 2020). After rain or irrigation, ATZ is transported horizontally to surrounding water bodies through runoff and vertically infiltrates into the soil, polluting groundwater (Ambriz-Mexicano et al. 2022; Urseler et al. 2022; Correia, Carbonari and Velini, 2020). Due to its heavy use,

as well as its high persistence and mobility in the environment (Rostami et al. 2021; Zhu et al. 2021), ATZ is frequently detected in surface water and drinking water worldwide (Van Opstal et al. 2022; SISAGUA, 2018; Vieira et al. 2017; Ezemonye et al. 2015; González-Márquez et al. 2013; Moreira et al. 2012; Wu et al. 2010; Wu et al. 2009). ATZ is implicated to be an endocrine disrupting chemical (EDC) and, at low environmentally relevant concentrations, has been reported to cause critical neuroendocrine dysfunction in vertebrates (Owolabi and Saratu, 2021). This dysfunction can lead to reproductive disorders, behavioral alterations, and variations in hematological, biochemical, and morphological parameters in fish (De Souza et al. 2023; Destro et al. 2021; Blahova et al. 2020; Nwani et al., 2011; Owolabi and Omotosho, 2017; Paulino et al., 2012; Santos and Martinez, 2012). In addition, ATZ has been associated with harmful effects on the development and sexual reversal of amphibians, as well as alterations in intestinal microbiota (Reichert et al. 2022; Zhao et al. 2021; Hoskins and Michelle, 2018; Gustafson, Jason, and Matthew, 2016; Ji et al. 2016). In mammals, including humans, ATZ has been linked to an increased risk of obesity, disruption of the hypothalamic-pituitary-gonadal axis, and adverse birth outcomes (Yun et al. 2022; Saalfeld et al. 2018; Wirbisky and Jennifer, 2015; Almber et al. 2018). Overall, the evidences strongly suggest that ATZ poses a serious threat to both human health and the aquatic ecosystem.

Through this analysis, it becomes evident that fish represent a group of organisms that can be severely affected by climate change factors and excessive use of herbicides. Temperature, as a key abiotic factor, controls and limits the physiological mechanisms in fish, including reproduction (Islam et al. 2021). As is the case for all organisms, the reproductive system of fish plays a pivotal role in the conservation and perpetuation of the species (Islam, Andreas and Matthew, 2022; Servili et al. 2022; Franco et al. 2020; Mendenhall et al. 2020; Morgan et al. 2020; Oremus et al. 2020). Any disturbance in this system, whether at the molecular, cellular, or systemic level, can have significant implications for maintaining populations and, consequently, for ensuring ecosystem stability. This can encompass changes in mating behavior, decreased fertility, or even a reduction in the number of reproductively active fish, all of which can lead to a decrease in genetic diversity and also increase susceptibility to diseases and environmental changes (Fawole and Shamna et al. 2023; Servili et al. 2022; Manel et al. 2020; Reid et al. 2019; Tamario et al. 2019). Likewise, reproductive changes can directly compromise the success of productivity in fish farming practices (Owolabi and Saratu, 2021).

Efforts to assess how higher-than-optimal water temperature can compromise the reproductive activity, sexual determination, and survival of fish embryos and larvae, have

been made (Kang et al. 2021; Munday, Michael and Ivan, 2019, Geffroy & Wedekind 2020). Nevertheless, rare studies have been designed in climate rooms that allow for more rigorous simulation of environmental impacts on these organisms, which would improve our theoretical understanding of physiological responses to likely future climate changes (Castro et al. 2020; Oliveira and Val, 2017). It is expected that global warming will significantly enhance the toxic effects of herbicides and/or reduce the tolerance of fish to multiple stressors (Hooper et al. 2013; Kim et al. 2010; Noyes et al. 2009; Hani et al. 2019). Notably, meta-analysis studies identified embryos and breeding adult fishes as the most temperature-sensitive stages in the life cycle of fish (Dahlke et al. 2020). Brown et al. (2015) suggested the combination of elevated temperatures and contamination by EDCs is likely to present substantial extinction risks for inbred populations that demonstrate environmental sex determination and differentiation. Nevertheless, the effects of climate changes on the reproductive toxicity of ATZ have been very poorly explored so far.

Considering this background, this study was conducted to unravel the interactive impact of predicted 2100 climate scenario and realistic concentration of ATZ exposure on the testicular function of zebrafish. Climate simulation-based scenarios are routinely used to characterize a range of plausible climate futures (Schwalm et al. 2020, IPCC, 2021). To achieve our outlined scope, we performed a climate room experiment simulating in real time temperature and CO_2 changes in Manaus, Amazonas, Brazil, for the RCP8.5 climate scenario (namely extreme), in accordance with the Sixth Assessment Report of Intergovernmental Panel on Climate Change (IPCC, 2021). Understanding how environmental factors that are dependent on climate change and ATZ contamination affect fish spermatogenesis, sperm quality and fertility may provide valuable information for the risks, management and conservation of fishery resources, as well as future decision-making regarding climate change and herbicide regulation.

2. Materials and Methods

2.1 Herbicide and Fish Exposure

Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine, CAS no. 48085, 100% purity) purchased from Sigma-Aldrich was used in the present study at a final concentration of 2 µg/L of water. The known LD50 values for adult fish species exposed to ATZ range from 2.14 to 42.38 mg/L (Tango et al., 2012; Kleeman et al., 1988; van der Weiden et al., 1994; Khoshnood & Khoshnood, 2014; Nwani et al., 2010; Hussein et al., 1996). Probit analysis revealed that the no-observed-effective concentrations (NOEC) for inducing

zebrafish mortality were 3 mg/L of ATZ (Yan et al., 2015), indicating our work involves a significantly low concentration. This concentration was chosen based on environmentally realistic concentrations found in drinking and surface waters (Wu et al. 2009; Wu et al. 2010; Moreira et al. 2012); and rainwater and groundwater (Kolpin et al. 1998; Moreira et al. 2012) in Brazil (Vieira et al. 2017; SISAGUA, 2018), Mexico (González-Márquez et al. 2013), and Argentina (Van Opstal et al. 2022). In addition, Brazilian Resolution no. 375 of the National Council for the Environment (CONAMA, 2005) establishes that 2 µg/L is the maximum allowed value for surface waters classes I and II (for human consumption after treatment and protection of aquatic communities). Such concentration is also established by Ordinance no. 2.914/2011 of the Ministry of Health, which regulates the control and surveillance of water quality for human consumption (BRASIL, 2011). Notably, the concentration of 2 µg/L tested here has also been documented in freshwater from non-Latin countries and is significantly below the levels of ATZ and its chloro-s-triazine metabolites established in the World Health Organization's Guidelines for Drinking-Water Quality (Rohr et al. 2010; Rinsky et al., 2012; Benítez-Díaz and Miranda-Contreras, 2013; Almberg et al. 2018; Hansen et al. 2019; Wang et al. 2020).

An ATZ stock solution with a concentration of 10 mg/L was prepared by initially dissolving the solute in 2 mL of acetone and subsequently diluting it in one liter of mineral water. The resulting solution was stored in an amber bottle at -20 °C. A volume of 6 mL of the stock solution was used to prepare a working solution for each aquarium, resulting in a final concentration of 2 µg/L.

The zebrafish (*Danio rerio*) used in the present study were obtained from the Aquaculture Laboratory of the School of Veterinary Medicine at the Federal University of Minas Gerais (LAQUA/EV/UFMG) and transported to the Laboratory of Eco-physiology and Evolution located at the Brazilian National Institute of Amazonian Research (LEEM/INPA) where the experiment was conducted under scenarios of climate change. All experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Use - CEUA/UFMG (protocol 353/2018) and by the CEUA of the Brazilian National Institute of Amazonian Research (INPA), Manaus, AM, Brazil, under protocol 085/2017.

Due to the inherently unpredictable nature of future human behavior, which plays a crucial role in shaping the future climate, scenarios are employed to portray a spectrum of possible climate futures and to demonstrate the outcomes of policy decisions (IPCC, 2021). Here, adult male zebrafish were exposed for 28 days (ensuring the occurrence of at least 2

spermatogenic cycles, Leal et al. 2009) to two controlled environmental chambers simulating the current and extreme (Representative Concentration Pathway- RCP8.5) scenarios according to the Sixth IPCC Assessment Report for the year 2100 (AR6) (IPCC, 2021). RCP8.5 scenario was chosen because it closely matches historical total cumulative CO_2 emissions and remains the most appropriate projection until the middle of the century, considering current policies and stated trajectories, with highly plausible levels of CO_2 emissions still expected in 2100 (Schwalm et al. 2020a and b). Six aquariums were placed in both climate simulation rooms, with 2 µg/L of ATZ added to three of them. The current scenario had air temperature and CO_2 levels controlled in real time by a computer based on the conditions of a forested area of the Amazon without human influence, acquired every two minutes by Fieldlogger 512k sensor (Novus LTDA). In the environmental chamber simulating the extreme scenario the air temperature and CO_2 levels were elevated by 6 °C and 900 ppm (AR6- IPCC, 2021), respectively, compared to the current scenario. Fish were artificially acclimated to climate rooms following the procedure described by Gonçalves et al. 2018. Lighting was set to a 12:12 pm cycle, and zebrafish were fed three times a day with Tetra® brand ornamental fish feed containing 30% crude protein throughout the experiment. An overview of the methodology used in the study is presented in Figure 1. The experimental groups were referred to as Current, Current-ATZ, Extreme, and Extreme-ATZ.

To maintain the water quality recommended by Lawrence and Timothy (2012), 50% of the aquarium water volume was replaced every four days. The real concentrations of ATZ in the aquariums and the degradation kinetics were evaluated at each water change using high performance liquid chromatography-mass spectrometry (HPLC-MS/MS) according to the standard protocol established by Bachetti et al. (2021). In addition, daily physicochemical analyses of the water were conducted, evaluating electrical conductivity, water temperature, pH, and dissolved oxygen. The pH was measured using an ultrabasic pH meter UB-10 (Denver Instrument, USA), temperature, electrical conductivity, and dissolved oxygen levels were measured using an 5512-FT oximeter (YSI, USA), and water CO_2 levels were determined by colorimetric assay according to the method described by Boyd and Tucker (1992). After 28 days of exposure, the fish were euthanized by spinal cord section and subjected to biometry. Total length (TL), body weight (BW), and gonad weight (GW) were obtained, and the gonadosomatic index (GSI = GW/BW*100) was calculated. Finally, the gonads were subjected to different investigations.

2.2 Histomorphometry

Zebrafish testes (n=5/group) were fixed in 4% glutaraldehyde, embedded in methacrylate, and stained with toluidine blue for morphometric and pathological evaluations. Structural and cellular components were counted in histological sections. Longitudinal sections with a thickness of 5µm were performed, totaling 6 sections per animal with an interval of 250µm between each section. Using a standardized grid in ImageJ software, the relative proportion (%) of each detected structure/alteration was obtained from the total number of points analyzed per section. The quantification of testicular components was performed in histological sections with a magnification of 400x. The proportion (%) of type A undifferentiated (Aund) and differentiated (Adiff) spermatogonia, type B spermatogonia, spermatocytes, spermatids, spermatozoa, Sertoli cells, Leydig cells, blood vessels, empty seminiferous tubule (ST) lumen, germ cell sloughing, and cell death (pyknotic nuclei) was quantified as described by Leal et al. (2009) and Schulz et al. (2010)

2.3 RNA extraction and quantitative real-time PCR (qRT-PCR)

Testicular RNA (n=6/group) was isolated using TRIzol reagent (Sigma-Aldrich), following the manufacturer's guidelines. Total RNA was quantified using a Nanodrop Lite (Thermo Fisher Scientific, Waltham, MA, USA) and 5 ng of RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis kit (Biorad, USA) following the manufacturer's recommendations. RT-qPCR were carried out using specific primers and non-template control for each pair of primers.

Real-time quantitative PCR (RT-qPCR) was performed to analyze the gene expression of different transcripts related to: i) estrogen or estrogen-related receptors (*esr1*, *esr2b*, *gper-1*); ii) androgen receptor (*ar*) iii) gonadotropin receptor (*fshr*) iv) steroidogenic enzymes (*star;* c*yp17a1*; c*yp19a1*) v) early germ cell and meiotic marker (*pou5f3*, *dazl*; *sycp3*); vi) apoptosis (*noxa*); vii) thyroid hormone receptor (*thrα*).

Oligonucleotides were designed using Primer3 software (Untergasser et al. 2012) and PrimerSelect (DNASTAR®) and sequences are provided in Table 1. Each pair of primers was optimized by conventional and RT-qPCR to set up conditions for similar amplification efficiencies. Product specificity was checked by dissociation curves and product size was visualized by agarose gel electrophoresis (data not shown). Reaction mixture contained 5ng of cDNA, 10 µL of 2X SYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µL of 5 µM forward and reverse oligonucleotides and bidistilled water up to 20 µL. PCR reaction began with a pre-incubation phase of 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 1 min at 63ºC (annealing temperature). Two technical replicates were performed for each

sample and β-actin (*actb2*) and *rna18s* were used as endogenous reference gene for normalizing expression level of target gene using the ΔΔCT method.

2.4 Apoptosis assay

To test apoptosis in zebrafish testes (n=3/group) TUNEL assay (terminal deoxynucleotide transferase-mediated dUTP nick-end labeling) was carried out according to the *In Situ* Cell Death Detection Kit (Roche) instruction. Briefly, 5 µm sections from a paraffin wax-embedded block were deparaffinized and permeabilized by incubation in proteinase K solution (20 µg/ml) for 30 min at 37°C. After two rinses in phosphate-buffered saline (PBS), slides were incubated for 60 min at 37°C in labeling solutions consisting of TdT and fluorescein-conjugated deoxynucleotides in buffer. After three PBS washes, nuclei were counterstained with DAPI, and the slides were mounted with antifade media (ProLong™ Gold Antifade Mountant - Thermo).

2.5 Quantification of testicular estradiol-17β (E2) and 11-ketotestosterone (11KT) levels

To assess E2 and 11-KT levels, zebrafish testes (n=5/group) were stored in liquid nitrogen and then subjected to ELISA assays. The samples were homogenized in an extraction buffer (50 mM Tris-HCl pH 8.0 with 0.002% aprotinin and 1 mM phenylmethylsulfonyl) in a 1:2 tissue: buffer ratio. Then, the extracts were vortexed, centrifuged at 15000 g for 60 min at 4 °C and the supernatant was collected for analysis. To ensure accurate comparative hormone dosage, all samples input were standardized to a concentration of 1mg total protein per mL. Gonadal concentrations of E2, and 11-KT were determined by ELISA, following the manufacturer's guidelines (Cayman Chemical ELISA kit E2 #582251 and 11-KT #582751). The sensitivity of the assays was 0.972 pg/mL (E2 and 11-KT).

2.6 Antioxidant enzymes and lipid peroxidation

Testicular homogenates (n=5/group) were prepared in buffer (pH 7.6) containing (in mM): Tris base 20, EDTA 1.0, dithiothreitol 1.0, sucrose 50, KCl 150. Homogenates were centrifuged at 15,000 g for 20 min at 4 °C, and used to determine catalase (CAT), superoxide dismutase (SOD) and glutathione transferases (GST) activities and lipid peroxidation (LPO) levels (1:10 w/v for GST and SOD and 1:4 w/v for CAT and LPO). To determine the CAT activity, the inhibition rate of H_2O_2 decomposition was monitored at 240 nm (Beutler, 1975), and expressed as µmol H_2O_2 min^{-1} mg $protein^{-1}$. SOD activity was quantified based on the inhibition of cytochrome c reduction rate by the superoxide radical at 550 nm and 25 °C

(Turrens, 1997). Enzyme activity is expressed as U SOD mg protein^{-1}, where 1 U of SOD corresponds to the quantity of enzyme that promoted the inhibition of 50% of cytochrome c. GST activity was determined according to Keen et al. (1976) by measuring the increase in absorbance at 340 nm, incubating reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as the substrates. GST activity is expressed as U min^{-1} mg protein^{-1}.

The LPO levels were quantified in an assay based on the Fe^{+2} to Fe^{+3} oxidation by hydroperoxides in acid medium, in the presence of ferrous oxidation-xylenol orange, at 560 nm (Jiang et al., 1991). LPO concentration was expressed as µmol cumene hydroperoxide mg protein^{-1}. Total protein of testicular samples was determined spectrophotometrically at 595 nm (SpectraMax M2, Molecular Devices Inc., Sunnyvale, CA, USA), according to the colorimetric assay (Bradford, 1976) using a bovine serum albumin (BSA) as standard.

2.7 Sperm parameters

Sperm motility assessments (n=7/group) were performed using the computer-assisted sperm analysis (CASA) method, as proposed by Wilson-Leedy and Ingermann (2007) and Sanches et al. (2013), with the aid of the open-source software IMAGEJ (National Institute of Health, USA). Semen samples were obtained after 20 gentle cuts of the zebrafish testes in a balanced Hank's solution (HBSS) at a 1:50 weight/volume ratio.

The activation process of fresh semen was carried out in microtubes (0.2 mL) using 1 µL of semen and 10 µL of distilled water (0.0 mOsm kg-1). Semen samples from Extreme and Extreme-ATZ group were activated with water at 26ºC and 34ºC. The images were obtained using a light microscope with a 10x objective, coupled with a Basler camera (model acA640-120uc), connected to a computer (Intel Core i7© CPU 2.4GHz, 8GB RAM) running the Microsoft Windows 8© operating system. The videos were captured using the Basler Pylon Camera Software (baslerweb.com) at 100 frames per second (658x492 pixels) in *.avi format, edited with VIRTUALDUB-1.9.0 software (virtualdub.org), and exported as a sequence of images in *.jpg format to a specific directory.

The corresponding images were opened, edited, and compiled using the CASA plugin (University of California and Howard Hughes Medical Institute, USA). The sperm parameters evaluated were: motility rate (MOT%), Curvilinear Velocity (VCL µm/s), Average travel speed (VAP µm/s), Straight Line Speed (VSL µm/s), Straightness (STR %), Oscillation (WOB %), and Progression (PRG µm). The analyses were performed at 5, 15, 30, 45, and 60 seconds after activation, with seven males considered in each treatment in triplicate.

385 **2.8 Statistical analysis**

386 The data were expressed as mean ± standard deviation, except for the qPCR results
387 which were presented as geometric mean ± standard deviation. Mean differences were
388 assessed using a two-way ANOVA (with climate scenarios and ATZ factors discriminated by
389 Tukey's test), with the current scenario as the reference. A significant difference was
390 assumed when $p < 0.05$. The statistical analysis was performed using BioEstat version 5.3
391 software, and the graphs were generated using GraphPad Prism 6 software.
392
393 **3. Results**
394 **3.1 Climate change and ATZ cause mortality and affect testicular biometric parameters**
395 The physicochemical data of the aquarium water are presented in Table 2. The
396 climate-controlled rooms efficiently simulated the climate change scenario predicted by AR6
397 (IPCC, 2021). The temperature and CO_2 exhibited significantly higher levels when compared
398 to the current scenario, while pH and oxygen levels did not differ between the tested
399 conditions. As anticipated, the variations observed in the air of the climatic rooms were
400 reflected in the water of the aquariums (Table 2). The mean values of electrical conductivity
401 were higher in the extreme scenario with the presence of ATZ when compared to the Current
402 group.
403 The ATZ quantification analyses indicated that the concentration during exposure
404 period was 95.05% (1,901 ± 0.02 µg/L) the nominal concentration for Current-ATZ group and
405 91.40 % (1.828 ± 0.05 µg/L) for Extreme-ATZ group. ATZ concentrations remained similar in
406 both scenarios ($p=0.778$), suggesting low degradability and high persistence of the herbicide
407 even under extreme climate change scenario (Table 2). Throughout the experimental period,
408 the ATZ concentration in the control groups (i.e., Current and Extreme), was below the
409 analytical limit of detection.
410 Fish mortality was observed during the experimental period. In the current scenario
411 room, there was a mortality rate of 0.80% and 1.30% among the fish in the Current and
412 Current-ATZ groups, respectively. In the extreme scenario room, mortality rates were notably
413 higher, with 15.4% and 14.80% observed in the Extreme and Extreme-ATZ groups,
414 respectively. Furthermore, deformities in the body axis were observed in 7.30% of fish in the
415 Extreme group and 7.05% in the Extreme-ATZ group.
416 Regarding the biometric parameters, climate changes and/or ATZ exposure did not
417 induce significant differences in the total length and BW of zebrafish (Figure 2b-c). However,
418 anatomical examination revealed that fish from all other experimental conditions distinctly

419 exhibited testes that were thinner and more translucent when compared to males from the
420 Current group (Fig. 2a). Indeed, we evidenced an independent and synergistic effect for
421 reduced testicular weight and GSI in zebrafish exposed to extreme scenario and/or ATZ
422 (Figure 2a, d, e).
423
424 **3.2 Atrazine and climate change alter zebrafish testicular cell populations**
425 To understand the effects of ATZ under climate change scenarios on spermatogenesis
426 and testicular cell population, the proportion of gonadal components was quantified.
427 Testicular parenchyma of zebrafish from the experimental groups are depicted in Figure 3, in
428 which clear histological alterations, such as germ cell apoptosis, germ cell sloughing and
429 reduced luminar diameter, can be observed (Fig. 3c-d). Exposure to ambient ATZ
430 concentration in the current scenario caused an increase in type Aund spermatogonia, blood
431 vessels, empty ST lumen, germ cell sloughing, and cell death rate compared to control. In
432 addition, a lower proportion of Sertoli cells and spermatozoa was observed when compared
433 to Current group (Fig. 3). In the testes of fish submitted to the extreme scenario without ATZ,
434 a higher proportion of types Aund and Adiff spermatogonia, spermatocytes, empty ST lumen,
435 germ cell sloughing, blood vessels and cell death was found when compared to the current
436 scenario. Furthermore, there was a decrease in the amount of Sertoli cells, Leydig cells,
437 spermatids and spermatozoa in the zebrafish testes compared to those exposed to the
438 current scenario (Fig. 3). Finally, in fish exposed to the extreme scenario associated with
439 ATZ, the proportion of types Aund, and Adiff, spermatocytes and blood vessels was also
440 higher when compared to the current scenario, while the population of Sertoli cells decreased
441 in the testes. Compared to Current and Extreme groups, the Leydig cell population
442 decreased, and the proportion of empty ST lumen increased in the testicular parenchyma of
443 Extreme-ATZ fish. Notably, synergistic interaction of ATZ and extreme climatic scenario were
444 evidenced for increased population of Aund, Adiff, germ cell sloughing, blood vessel and cell
445 death. Moreover, ATZ contamination and climate-driven changes exacerbated the decline in
446 sperm production (Fig 3j), worsening the deleterious effects on the spermatogenic processes.
447
448 **3.3 Atrazine and climate change disturb gene regulation of spermatogenesis**
449 The RT-qPCR analyses for sex hormone receptors did not detect alterations in the
450 expression levels of the *esr1* gene among the investigated groups (Fig. 4a). However, we
451 found that exposure to ATZ and/or climate scenarios significantly decreased transcription
452 levels of *esr2a*, also related to estrogen signaling in the nucleus (Fig. 4b), while *esr2b* mRNA

453 levels were increased in the testes of fish from the extreme scenario exposed to ATZ (Fig.
454 4c). Similarly, higher transcription levels of G protein-coupled estrogen receptor (*gper-1*) were
455 evidenced in the Extreme-ATZ group (Fig. 4d). Significant upregulation of testicular *ar* mRNA
456 was observed only in fish submitted to extreme scenario without contact with ATZ (Fig. 4e).
457 With regard to testicular gonadotropin receptor, ATZ and/or climate change exposure
458 significantly increased *fshr* mRNA levels in the zebrafish testes when compared to Current
459 group (Fig. 4f). The alpha thyroid hormone receptor gene (*thrα*) involved T3 and T4 signaling,
460 and which is correlated with *fshr*, also were upregulated in all groups (Fig 4g). In relation to
461 steroidogenic pathways, the expression of gonadal aromatase gene (*cyp19a1*), involved in
462 conversion of androgens to estrogens, was increased in fish of all environmental conditions
463 tested compared to Current group (Fig4 h). In contrast, there was a downregulation of
464 *cyp17a1* transcription levels in the zebrafish testes (Fig4i). Similarly, exposure to ATZ and/or
465 scenarios also reduced mRNA levels of the *star* compared to Current group, with the lowest
466 levels observed in the Extreme-ATZ group (Fig. 4c).
467 We also investigated the effects of ATZ and climate change interaction on specific
468 germ cell genes and found that ATZ exposure in both climate scenarios significantly
469 increased testicular transcription levels of *pou5f3* (Fig. 4k). *pou5f3* gene is expressed in types
470 Aund and Adiff spermatogonia, which is in agreement with the histomorphometric data. The
471 transcript levels of *dazl* gene, a robust type B spermatogonia and pre-leptotene spermatocyte
472 marker, were not altered among the groups (Fig. 4l). Furthermore, upregulation of *sycp3l*
473 transcript, a component of the synaptonemal complex, was evidenced in all tested conditions
474 compared to Current group (Fig. 4m), corroborating the histological parameters.
475
476 **3.4 Climate change and ATZ association enhances testicular apoptosis**
477 To support our data on spermatogenic cell death observed from morphological
478 evaluations we quantified the expression of proapoptotic gene *noxa* and investigated in situ
479 3′-OH end tailing of fragmented DNA in the zebrafish testes. A significant upregulation of
480 *noxa* gene was detected in both groups submitted to the extreme scenario compared to
481 Current group, with additive effect evidence for the Extreme-ATZ group (Fig. 4n). In the testis
482 parenchyma of fish submitted to the current scenario, in the presence or absence of ATZ, a
483 low density of apoptotic TUNEL-positive germ cells was observed (Fig. 5a-f). However, fish
484 from Extreme and Extreme-ATZ groups exhibited high abundance of TUNEL-positive post-
485 meiotic cells, including spermatids and spermatozoa, as clearly demonstrated in Figure 5g-l.
486 Likewise, secondary spermatocytes, Sertoli cells, and Leydig cells TUNEL-positive were

readily detected in the testes of fish from Extreme-ATZ group (Fig. 5j-l). These findings indicate the significant impact of future climate scenario in inducing germ cell apoptosis and sperm DNA damage, which can be exacerbated by ATZ contamination.

3.6 ATZ and/or extreme scenario exposure disturbs sexual hormone production.

The gonadal concentrations of two major sex hormones, E2 and 11-KT, were measured in order to understand the real influence of climate change and ATZ on steroidogenesis and testicular physiology. There was a significant reduction in 11-KT concentrations in all tested conditions compared to the Current group. Specifically, fish exposed to extreme scenario in association with ATZ showed lowest 11-KT ($p < 0.01$) levels (Fig 6a). In relation to testicular E2, an increase in its concentrations was evidenced in all tested conditions, compared to Current group, in agreement with the increased *cyp19a1* mRNA levels in both condition groups. Notably, a clear positive modulatory effect, i.e., enhanced estrogenic activity, was induced by ATZ in combination with extreme scenario exposure, further intensifying the endocrine deregulation.

3.7 ATZ and climate change alter the antioxidant defenses in the testes

The activities of the antioxidant enzymes CAT, SOD, GST, and levels of LPO in the testes of zebrafish from different experimental groups are depicted in Figure 7. Exposure to ATZ in both scenarios induced an increase in CAT activity compared to Current group (Fig. 7a). The activity of SOD increased significantly only in fish subjected to the extreme scenario with ATZ (Fig. 7b). In all tested conditions, there was a reduction in GST enzymatic activity in the testes compared to the Current group, with the lowest levels detected in fish maintained in simulated scenarios, i.e. Extreme and Extreme-ATZ group (Fig. 7c). Finally, we observed an increase in LPO levels in fish of all investigated groups compared to those maintained in the Current group, indicating a potential induction of oxidative damage and cellular injuries in the testes (Fig. 7d).

3.8 Climate change and/or exposure to ATZ compromise sperm quality

None of the fish maintained in the extreme scenario simulation room, whether in contact with ATZ or not (n=7/group), presented viable testicular sperm for post-activation motility measurement through CASA (Table 3). Of note, semen samples of fish originating from the Extreme and Extreme-ATZ groups were activated in medium at 26°C and 34°C, but no motility was observed under both conditions (Table 3, Supplementary movie). Therefore,

521 we evidenced that exposure to the extreme scenario for 28 days resulted in a drastic
522 reduction, or complete absence, of zebrafish sperm motility.

523 Under the current conditions, an increase in the percentage of activated motile sperm
524 was observed in fish exposed to ATZ compared to control fish, starting specifically at 30
525 seconds post-activation, with a mean of 50.8 ± 11.5% versus 22.3 ± 10.9% during this time
526 interval. Motility rates also remained higher at 45- and 60-seconds post-activation in fish
527 exposed to ATZ (Table 3). However, the other kinetic parameters investigated, namely
528 curvilinear velocity, average travel speed, straight line speed, straightness, oscillation, and
529 progression, did not show significant differences between the two groups evaluated in the
530 current scenario ($p > 0.05$; Table 3).

531

532 **4. Discussion**

533 Fish populations are frequently exposed to diverse physical, chemical, and biological
534 stressors, which may synergistically enhance the frequency or intensity of a given effect
535 (Noyes et al. 2009). Notably, environmental pressures will increase with predicted scenarios
536 for climate change (Do Carmo et al. 2023; Fe-Gonçalves et al. 2020), however, interactive
537 effects of climate change-induced extreme temperature and herbicide contamination on fish
538 reproductive biology have been poorly investigated, and therefore their real impacts remain
539 unknown to society and government agencies. Zebrafish is widely used as a prominent model
540 organism in scientific research, owing to its potential to yield findings with substantial
541 implications for other fish species and vertebrates in general (Azra et al. 2023; Meyers et al.
542 2018; Dai et al. 2014). Thus, here we explored the sensitivity of zebrafish to climate change-
543 induced ATZ toxicity focusing on testicular function.

544 At first, we observed a reduction in the GSI of fish kept in the extreme scenario room,
545 with an additive effect when associated with ATZ exposure. High temperature-mediated GSI
546 decrease after long or short exposure has been discussed in the literature for zebrafish
547 (Nóbrega et al. 2010, Vergauwen et al. 2010) and other teleost species (de Siqueira-Silva et
548 al. 2015; Patra et al. 2015; Lacerda et al. 2010; Hani et a. 2019). Still, in accordance of our
549 findings, previous investigations (Vasanth et al. 2017; Wirbisky et al. 2016; Papoulais et al.
550 2014; Nadzialek et al. 2008; Spanó et al. 2004) do not provide evidences for a decrease in
551 GSI in male fish after exposure to low concentration of ATZ. The changes in the proportions
552 of germ and somatic cells, the reduction in sperm production along with the copious apoptosis
553 of testicular cells induced by extreme climatic conditions and ATZ reasonably favored the
554 decrease in GSI. In addition to being conducive to estimating reproductive capacity, the GSI

555 is a significant indicator of the general health status of fish (Martínez-Durazo et al. 2023;
556 Pampanin et al. 2016). Thus, the marked reduction in the testis mass observed in Extreme-
557 ATZ fish provides the first sign that climate change may aggravate the ecotoxicity of ATZ.
558 Subchronic exposure to low concentrations of ATZ, combined with the effects of
559 climate change, could disrupt the transcription levels of genes involved in critical testicular
560 functions, i.e. steroid production and spermatogenesis regulation. ATZ and climate change
561 exposure independently downregulate the expression of the *star* gene, involved in
562 mitochondrial cholesterol transport of Leydig cells. When both conditions are present, an
563 additive effect is observed in inhibiting *star* expression. The lower expression of *star* has been
564 directly linked to a decrease in the production of androgen (Bhat et al. 2021; Li et al. 2021,
565 Fraz et al. 2018), which could be one of the explanations for the significant reduction of the
566 intragonadal 11-KT evidenced here in all treatments, with the lowest levels of detected in fish
567 from Extreme-ATZ group. *star* is mainly involved in the initial stage of steroidogenesis
568 pathway (Zhou, Li and Wang, 2021), and the complete inhibition of its expression can reduce
569 steroid synthesis but does not completely eliminate it (Ahmadifar et al. 2021). We also
570 detected downregulation of *cyp17a1* in all treated groups, with no differences among them.
571 *cyp17a1* plays key roles in hydroxylation of pregnenolone and progesterone (Rajakumar and
572 Balasubramanian, 2020), and its expression has been described in both Leydig and germ
573 cells of zebrafish testes (Hinfray et al. 2014). Previous literature reports conflicting
574 modulations in the *cyp17a1* levels in response to ATZ exposure, depending on the dose
575 tested (Kolaitis et al. 2023, Horzmann et al. 2018; Glisic et al. 2016; Weber et al. 2013).
576 Additionally, studies have failed to detect changes in *cyp17a1* gene expression in zebrafish
577 males submitted to high temperature, potentially due to the difference in period and system
578 of exposure (Nóbrega et al. 2010). Knockout of *cyp17a1* in *ar*-/- male zebrafish causes
579 testicular hypertrophy and enhanced spermatogenesis (Zhai et al. 2022). These data
580 illustrate the complexity of steroidogenic pathways regulation and may justify the inconsistent
581 effects reported for ATZ and temperature-induced changes on testicular endocrine control
582 (Servili et al 2020).
583 Zebrafish gonadal aromatase, encoded by the *cyp19a1* gene is known to be disrupted
584 by environmental relevant exposure to ATZ in various organisms (Cleary et al. 2019; Wang
585 et al. 2019; Vasanth et al. 2017; Wirbisky et al. 2016; 2015). In our study, *cyp19a1* was
586 upregulated in the three different conditions tested. Cytochrome P450 aromatase is an
587 enzyme complex responsible for the synthesis of estrogens by the aromatization of
588 testosterone (Ankleyet al. 2023; Di Nardo et al. 2021; Tokarz et al. 2015). It has been

589 postulated that environmental temperature can act, directly or indirectly, in the regulation of
590 synthesis or activity of aromatase (Vandeputte et al. 2021), particularly during brain and
591 gonadal sex differentiation, when a DNA methylation-mediated control of *cyp19a* gene
592 appears to be involved (Zheng et al. 2022; Blüthgen et al., al. 2013; Liu et al., al. 2013;
593 Navarro-Martín et al. 2011; Fenske and Segner, 2004). The changes in *cyp19a1* transcript
594 levels detected here resulted in functional consequences, leading to a significant increase in
595 E2 in treated fish. The independent negative effects of ATZ or high temperatures on
596 steroidogenesis mediated through alterations of key enzymes have been reported previously
597 in fish (Servili et al. 2023; Servili et al. 2020; Lai et al. 2016; Tse et al. 2015; Anderson et al.
598 2012). But, in association, ATZ and projected climate scenario treatments acted in a
599 potentiating manner to increase E2 levels in the testes of zebrafish, contributing to aggravate
600 the sequel of androgen/estrogen imbalance in the testis function. As a consequence of
601 androgen impairment, the integrity of Sertoli cells and germinal epithelium can be also
602 affected (Liu et al., al. 2020; Johnson, 2014; Lara et al., al. 2019). The potential premature
603 loss of adhesion between germ cells and Sertoli cells, resulting in sloughed and degenerated
604 germ cells, was indeed significantly exacerbated by simultaneous exposure to ATZ and
605 extreme climate conditions.
606 E2 mediates its effects through several intracellular or membrane-associated
607 receptors and their expression has been reported in somatic testicular cells and in haploid
608 male germ cells (Ribeiro et al. 2023; Wang et al. 2022; Liu et al. 2009). Simulated climate
609 change induced significant disturbances in the expression of *esr2a*, *esr2b*, and *gper1* in the
610 zebrafish testis, especially when associated with ATZ. Limited data are available on the
611 mechanisms regulating estrogen receptors abundance in fish testes (Menuet et al. 2022,
612 Amenyogbe et al. 2020), nevertheless it has been reported that thermal stress may be
613 involved in driving gonadal alterations via deregulation of estrogen receptor at transcriptional
614 level (Anderson et al. 2012, van Nes and Andersen 2006). According to Albanito et al. (2015),
615 GPR and ERα seem to mediate the stimulation of gene expression and growth effects
616 induced by ATZ in ovarian cancer cells. Therefore, it is reasonable to hypothesize that in
617 combination, climate change-induced factors and ATZ may also intensify the impairments of
618 testicular hormonal signaling and gene transcription mediated by estrogen receptors, that are
619 pivotal for progression of spermatogenesis.
620 To explore functional changes in the different phases of the spermatogenic process,
621 this study analyzed the proportion of type Aund and Adiff spermatogonia in the zebrafish
622 testes. Our data demonstrate independent and potentiating effect of climate change and ATZ

623 in increasing the proportion of early spermatogonia population. In addition to other functions,
624 E2 stimulates the spermatogonial stem cell self-renewal in adult male zebrafish (de Waal et
625 al. al. 2009) and other teleost species (Miura et al, 1999; Chaves-Pozo et al. 2007), while 11-
626 KT participates in promoting spermatogonia differentiation (Wang et al. 2023; Li et al. 2021;
627 Ribeiro et al. 2021; Postingel Quirino et al. 2021; Hatef and Unniappan 2019; de Castro Assis
628 et al., 2018; Schulz et al. 2010). This may explain the high density and lower differentiation
629 of type A spermatogonia in the testes of ATZ and extreme scenario exposed fish. Accordingly,
630 upregulation of *pou5f3*, that play important roles in the maintenance and self-renewal of
631 undifferentiated spermatogonia (Doretto et al. 2022, Sanchez-Sanchez et al., 2010, Lacerda
632 et al. 2013, Lacerda et al. 2019), were also detected in the testes of exposed fish.
633 Fsh is also a key regulator of zebrafish spermatogonial activity through stimulatory
634 production of growth factors in Sertoli and Leydig cells (Nóbrega et al. 2015, Crespo et al.
635 2016, Safian et al. 2019). Here, we evidenced *fshr* and *thraa* upregulation in the testes of all
636 tested groups. Previous studies suggest that thyroid hormones signaling are associated with
637 the Fsh and Sertoli cells functions (Nitolli et al. 2021, Tovo-Neto et al. 2018). Accordingly,
638 Fsh steroidogenic capacity is increased in the presence of T3 in zebrafish testis, and T3
639 treatment stimulates the mitotic index of Aund and Adiff (Morais et al. 2013, Safian et al.
640 2016). In human granulosa cells, ATZ can suppress FSH-induced steroidogenesis (Pogrmic-
641 Majkic et al. 2018). Therefore, it is plausible that exposure to simulated climate change and
642 ATZ may disrupt Fsh function, either by impairing its production due to failed negative
643 androgen feedback (Harding et al. 2023; de Castro Assis et al. 2019; Kleppe et al. 2017;
644 Nobréga et al. 2015; Ramaswamy, and Weinbauer 2014; van der Stege et al. 2008) or by
645 affecting its signaling in the testes, warranting further specific investigation.
646 In addition to the spermatogonial phase, the meiotic phase of zebrafish
647 spermatogenesis was also affected in climate change scenario and ATZ exposure,
648 particularly in combination, which apparently promoted a delay in the meiotic process. This
649 was indicated by an increase in the amount of primary and secondary spermatocytes, as well
650 as the *sycp3l* upregulation. Studies demonstrate that exposure to high concentrations of ATZ
651 causes disruption in the meiotic progression in mice, leading to persistent DNA double-strand
652 breaks and increased spermatocytes number at zygotene and early pachytene stages (Gely-
653 Pernot et al. 2015). Higher temperatures have been shown to accelerate the duration of
654 spermatogenesis in fish (Postingel Quirino et al. 2021, Vilela et al. 2003, Lacerda et al. 2006,
655 2019, Nóbrega et al., 2010). According to Morgan et al. (2017) temperature influences the
656 frequency and location of crossover events and, at more extreme levels, induces

disturbances in the core structures of synaptonemal complex, that can lead to failures in chromosome pairing, synapsis, recombination and segregation. Nóbrega et al. (2010) reported that zebrafish maintained at a constant temperature of 35°C did not complete spermatogenesis beyond metaphase, while other studies have shown that high temperatures can induce massive germ cell loss and be an effective method of sterilization in sexually mature fish (Siqueira-Silva et al. 2015; Ito et al. 2008). In the view of these findings, the delay or meiotic arrest observed in the testis of fish exposed to extreme scenario and/or ATZ, along with potential alterations in the cell cycle, likely contributed to an increase in DNA abnormalities in zebrafish germ cells. Particularly, heat-induced DNA damage combined with reactive oxygen species (ROS)-mediated oxidative stress, is known to trigger apoptosis in meiotic and post- meiotic germ cells (Sharma et al. 2023; Hirano et al. 2022), leading to decreased sperm production and viability as demonstrated in this study.

The isolated effect of climate change caused a decrease in the biotransformation process, as observed by the reduction of GST activity and resultant oxidative stress, indicating that temperature alone is already a stressful factor for zebrafish testes. In the current scenario, ATZ also reduced the detoxification dynamics (observed by the decrease in GST activity), which contributed to the increase of CAT, indicating a potential reactive oxygen species (ROS) production, and causing oxidative stress, as verified by the increase in LPO. Exposure to ATZ in the extreme scenario was effective in altering the entire antioxidant system of the zebrafish testes. The biotransformation system was diminished, compromising the elimination of ATZ and the defense of cells against oxidative, as observed by the increase in CAT activities in response to H_2O_2 produced by SOD activity, since CAT is responsible for the detoxification of H_2O_2. All the treatments tested here were effective in inducing an increase in oxidative imbalance, verified by the significant increase in LPO. This phenomenon can lead to consequences such as alterations in membrane integrity, fluidity, permeability, and functional loss. Moreover, the byproducts of LPO have been demonstrated to possess mutagenic and carcinogenic properties (West and Marnett, 2006). However, the exposure to ATZ in the extreme scenario showed a significant increase in ROS, verified by the increase in the enzymatic activities of SOD and CAT, generating secondary products, as well as the products arising from LPO itself, which can modify biologically essential molecules, such as proteins and DNA bases compromising the testicular function.

Indeed, climate change and ATZ synergistically upregulate proapoptotic gene in the testis, which was supported by the observation of morphological features typical of cell death in seminiferous tubules, and TUNEL-positive spermatocytes, spermatids and spermatozoa.

Heat stress-induced apoptosis is critically mediated by the generation of ROS and oxidative stress in fish tissue (Castro et al. 2020, Wiens et al. 2017, Madeira et al. 2016). Consistent with our findings, studies have shown that early-stage germ cells exhibit greater resistance to oxidative stress compared to later-stage germ cells (Celino et al. 2011). Considering that sex steroids are also of particular importance for providing a proper environment for fish spermiogenesis (Liu et al. 2021; Milla et al. 2008; Miura et al. 2007), the reduced resistance to oxidative stress, in combination with androgenic dysfunction, could potentially explain the massive apoptosis of haploid germ cells, especially observed in fish exposed to extreme scenario. In addition, our data point out the synergistic modulation in the reduction of sperm production induced by ATZ and climate change, reinforcing that these factors negatively affect (directly or indirectly) the spermiogenic phase and the final maturation of male gametes.

Temperature can significantly influence various sperm parameters in fish species, particularly motility, which is a critical determinant of sperm quality and fertilizing capacity (Bera et al. 2016; Alavi and Cosson 2005). In the present study, fish exposure to simulated climate change scenario resulted in a complete absence of sperm motility, potentially leading to infertility. Here, this deleterious effect does not appear to be related to the temperature of motility-activating solution, as previously reported (Castro et al. 2020; Dadras et al. 2016; Lahnsteiner and Caberlotto, 2012), but is likely associated with impairments in the sperm maturation process and/or storage in the testicular duct. Several factors can influence sperm acquisition of motility and viability in spermatic ducts, such as, seminal plasma pH, osmolality, composition (ions, lipids, protein), and enzymatic and proteolytic activity (Kowalski and Cejko 2019; Tubbs and Thomas, 2008; Lahnsteiner 2007). The physiological basis underlying the disruption of fish sperm maturation and storage due to heat-stress remains poorly explored; however, there are evidences to suggest that this effect could be mediated by alterations in steroidogenesis, particularly in progestin production, and cAMP levels in spermatozoa (Cosson et al. 1995; Miura et al., 1995; Manning and Kime, 1985). Moreover, in the context of male infertility, there is a well-established cohesive pathogenic molecular pathway linking seminal oxidative stress, DNA damage, and apoptosis in spermatozoa (Sharma et al. 2023; Alahmar 2019), which may also underlie the observed absence of sperm motility in zebrafish submitted to climate change scenarios. In the current scenario, environmentally relevant ATZ concentration contributed to extend the sperm motility. Nevertheless, the percentage of spermatozoa in the testes of fish exposed to the herbicide was significantly lower, and the other kinetic parameters of sperm movement remained unaffected. Using subjective evaluation, Bautista et al. (2018), reported harmful effects of ATZ on fish spermatozoa, as

725 denoted by alteration in mitochondrial functionality, and membrane integrity. Evidences
726 indicate that ATZ interferes with the turnover of cyclic adenosine monophosphate (cAMP) by
727 inhibiting the cAMP phosphodiesterase activity and, consequently, increasing the cAMP level
728 in the cells (Suzawa and Ingraham 2008; Kucka et al. 2012; Roberge et al. 2006). cAMP
729 plays a significant role in the acquisition of sperm motility potential (reviewed by Schulz et al.
730 2010), and several axonemal proteins have been identified to be activated by cAMP, either
731 directly or mediated by protein kinases (Avali et al. 2019). This indicates that cAMP may have
732 a regulatory influence on flagellar beating and sperm motility, which can shed some light on
733 our findings with ATZ-exposed zebrafish.
734 In conclusion, our study, comprising detailed histological investigations into the
735 spermatogenic process, analysis of gene expression, steroid hormone production, apoptosis,
736 oxidative stress responses, and sperm quality, provides evidences that exposure to realistic
737 concentrations of ATZ and climate change can independently lead to significant disruptions
738 in zebrafish testicular function. Importantly, our findings point out that the interaction of these
739 factors will aggravate the deleterious effects on fish reproduction, potentially resulting in
740 infertility. While some species may exhibit greater resilience and adaptability to climate
741 change, others may face more significant challenges and risks of extinction, particularly when
742 their reproductive capacity is severely affected, as evidenced in this study. Accordingly, we
743 highlight the potential risks of purportedly safe concentrations of the herbicide ATZ under
744 future climate change scenarios and emphasize the importance of conducting health risk
745 assessments under various environmental conditions. Gaining knowledge of the
746 physiological mechanisms by which global warming and herbicide contamination disrupt fish
747 fertility could significantly contribute to understanding potential alterations in the reproduction
748 of fish in both natural and captive environments in the future, aiding in accurate predictions
749 and decision-making.
750
751 **Declaration of competing interest**
752 The authors declare that they have no competing financial interests or personal relationships
753 that could have appeared to influence the work reported in this paper.
754
755 **Author contributions**
756 **Victor Ventura de Souza**: Writing - review & editing, Formal analysis, Data curation,
757 Visualization. **Davidson Peruci Moreira**: Methodology. **Susana Braz Mota**: Methodology.
758 **Maira da Silva Rodrigues**: Methodology. **Rafael Henrique Nóbrega**: Methodology, review

& editing. **Rebeca Dias Serafim Corrêa**: Methodology. **Daniela Chemin de Melo Hoyos**: Methodology**. Eduardo Antônio Sanches:** Methodology**. Adalberto Luís Val**: Conceptualization, Investigation, review & editing, Visualization, Supervision. **Samyra Maria dos Santos Nassif Lacerda**: Conceptualization, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

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5. Figure Captions

Figure 1. Schematic representation of the methodologies used in a climate-controlled room experiment, describing the Current and Predicted Extreme Scenarios for 2100 (IPCC, 2021), with or without exposure to ATZ. Different aspects of reproductive function in male zebrafish were assessed using histomorphometric and pathological evaluations of the testes, hormonal assays, apoptotic assays, qPCR gene expression analysis, evaluation of antioxidant enzyme

activity, lipid peroxidation, and computer-assisted sperm analysis (CASA).

Figura 2. Biometric analysis of zebrafish exposed to ATZ and simulated climate scenario (IPCC 2021). a) Anatomical examination of zebrafish testes (arrowheads) from the Current, Current-ATZ, Extreme, and Extreme-ATZ groups. Note that testes of fish kept in the current scenario exhibit a whitish appearance due to the presence of semen. In contrast, those exposed to the extreme scenario show more translucent testes with reduced volume. b) Fish total length (TL). c) Body weight (BW). d) Gonad weight (GW) e) gonadosomatic index (GSI). Different letters indicate statistical differences ($p < 0.05$).

Figure 3. Histomorphometric analysis of testicular parenchyma from zebrafish exposed to ATZ and/or an extreme climate scenario. a) Current scenario, high sperm density and organized tissue. b) Current scenario associated with ATZ, decreased sperm abundance, presence of cells with pyknotic nuclei characteristic of cell death (red arrowhead). c) Extreme scenario, observe reduced sperm density and increased cell death (red arrowhead) and germ cell sloughing (yellow arrowhead). d) Extreme scenario associated with ATZ, tissue disorganization, germ cell sloughing (yellow arrowhead), and cell death (red arrowhead), higher density of type Aund spermatogonia and spermatocytes. e) Frequency (%) of type Aund spermatogonia (Aund). f) Frequency (%) of type Adiff spermatogonia (Adiff). g) Frequency (%) of type B spermatogonia (SpgB). h) Frequency (%) of spermatocytes (Spc). i) Frequency (%) of spermatids (Spt). j) Frequency (%) of spermatozoa (Spz). k) Frequency (%) of Sertoli cells (SC). l) Frequency (%) of Leydig cells (LC). m) Frequency (%) of blood vessels (BV). n) Frequency (%) of empty seminiferous tubule (ST) lumen. o) Frequency (%) of pyknotic nuclei (cell death). p) Frequency (%) of germ cell sloughing. Different letters indicate statistical differences ($p < 0.05$). Barr= 20µm.

Figura 4. Relative mRNA levels of testicular genes associated with steroid production, spermatogenesis regulation, germ cell markers and apoptosis. a) *esr*, b) *esr2a*, c) *esr2b*, d) *ar*, e) *gper1*, f) *fshr*, g) *star* h) *cpy19a1*, i) *cpy17a1*, j) *pou5f3*, k) *dazl*, l) *scyp3l*, m) *thrα*. Ct values were normalized with *actb2* and *rna18s* and expressed as relative values of control (Current group) levels of expression. Data are expressed as geometric mean ± SD. Different letters indicate statistical differences ($p < 0.05$) between different treatment conditions.

Figure 5. Fluorescence microscope images of TUNEL staining (green, FITC) indicating

apoptosis in testicular cells of zebrafish exposed to Current scenario (a-c), Current scenario and ATZ (d-f), Extreme scenario (g-i) and Extreme scenario and ATZ (j-l). The nuclei were counterstained with DAPI (blue) and overlay images are shown in the third column. TUNEL-positive reactions were mainly observed in spermatids (Spt) and spermatozoa (Spz). Insets in k and l exhibit TUNEL-positive somatic cells at higher magnification, interstitial cells (red arrowhead) and Sertoli cells (white arrowhead). The interstitium is delimited by spotted lines. Spc= Spermatocytes. Insert scale bars= 10 µm.

Figure 6. Concentration of testicular steroid hormones in zebrafish exposed to ATZ and/or an extreme climate scenario. a) 11-ketotestosterone (11KT). b) estradiol-17β (E2). Data are expressed as pg/mL of buffer solution containing 1 mg of total testicular protein. Different letters indicate statistical differences ($p < 0.05$) between different treatment conditions.

Figure 7. Measurement of antioxidant enzyme activity and levels of lipoperoxidation in the testes of zebrafish exposed to ATZ and/or an extreme climate scenario. a) CAT (Catalase). b) SOD (Superoxide dismutase). d) GST (Glutathione S-transferase). e) LPO (Lipoperoxidation). Different letters indicate statistical differences ($p < 0.05$) between different treatment conditions.

Figure 8. A schematic overview of the main effects of predicted climate scenario and ATZ on zebrafish testis after 28 days exposure. Alterations in the expression of key genes, along with androgen/estrogen imbalance and oxidative stress, could impair germ cell survival and development, ultimately leading to a decline in sperm production and a significant reduction or complete absence of sperm motility.

Supplementary Movie: Illustrative videos for sperm motion assessment via Computer-Assisted Sperm Analysis (CASA). Semen samples from the Current and Current-ATZ groups were activated at 26°C, while samples from the Extreme and Extreme-ATZ groups were activated at two different temperatures: 34°C (temperature of activation medium at 34°C, TAM 34°C) and 26°C (TAM 26°C). Immotile spermatozoa are indicated by arrowheads.

Graphical Abstract

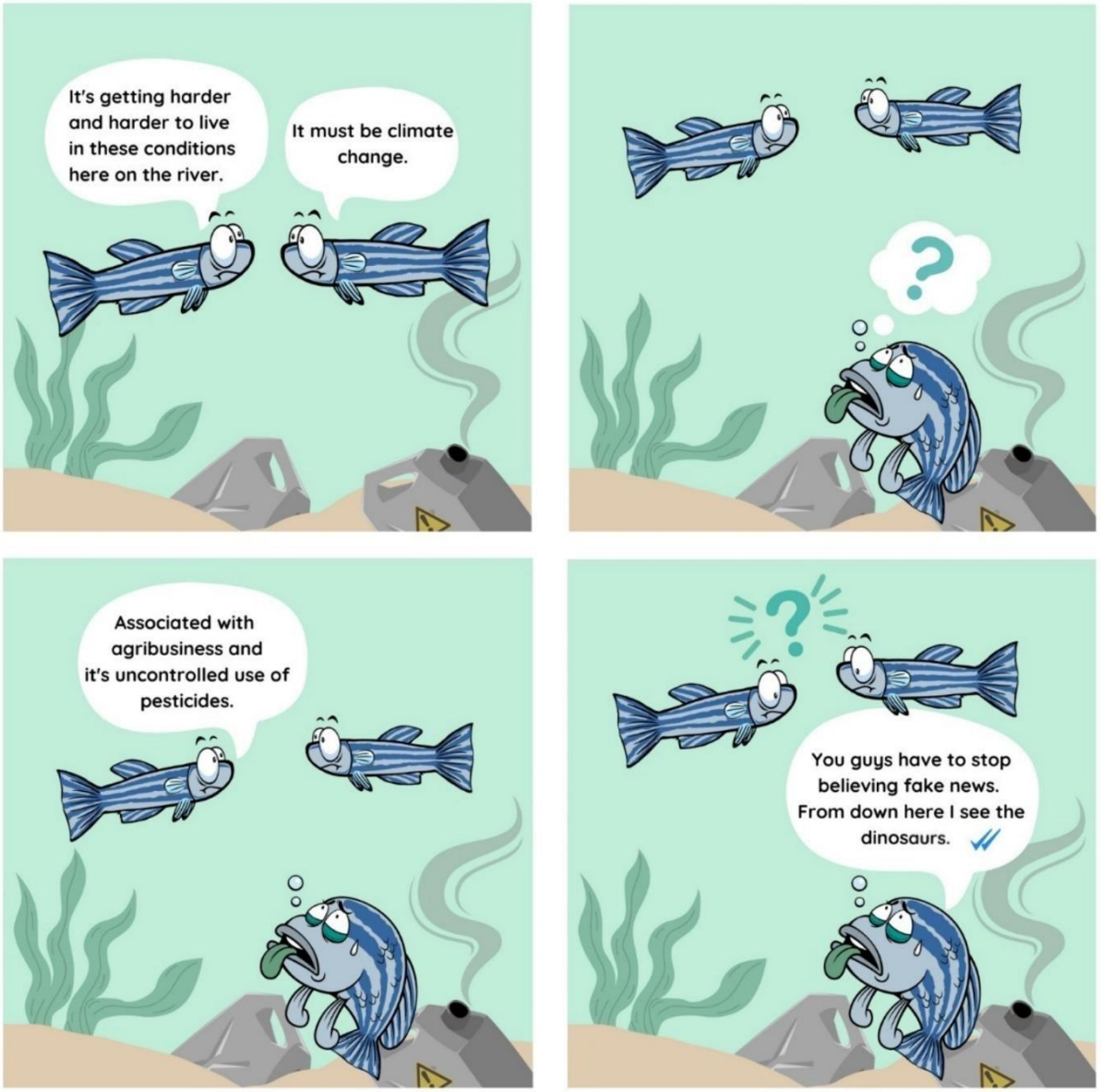


Figure 1

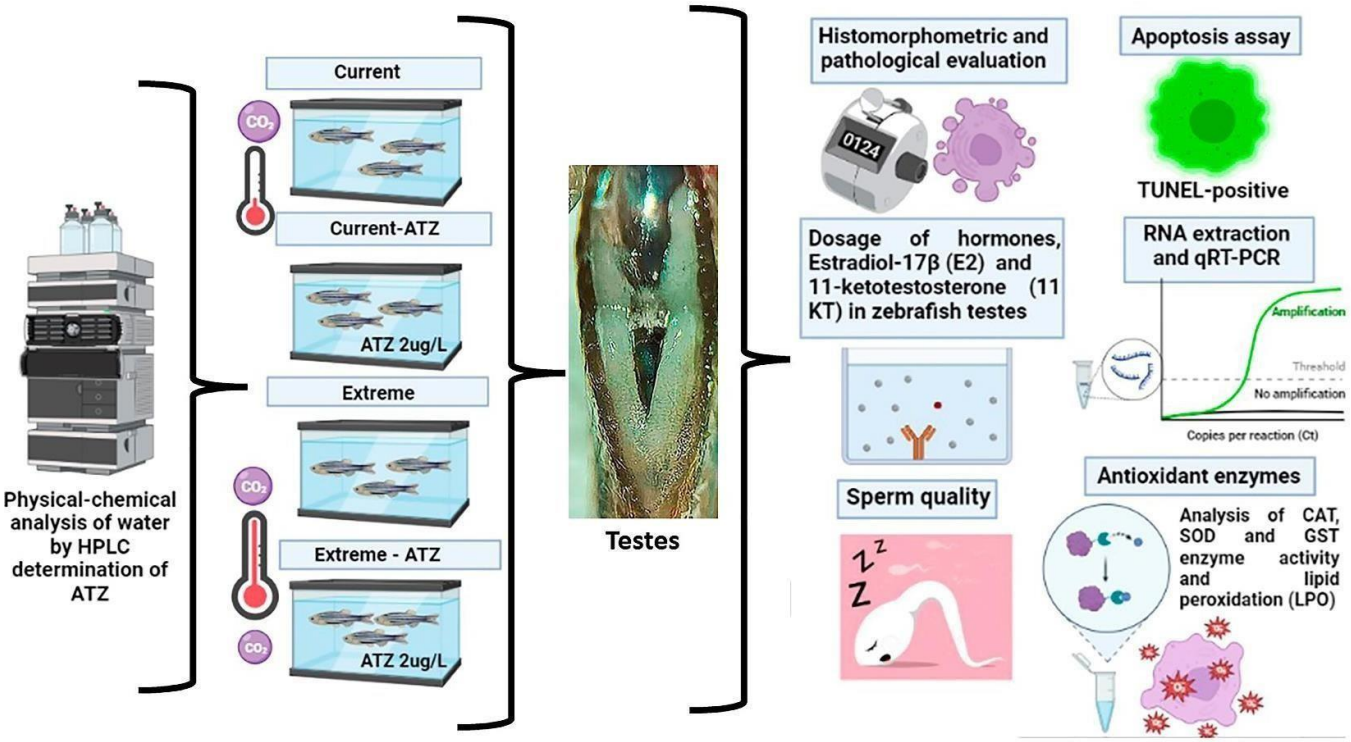
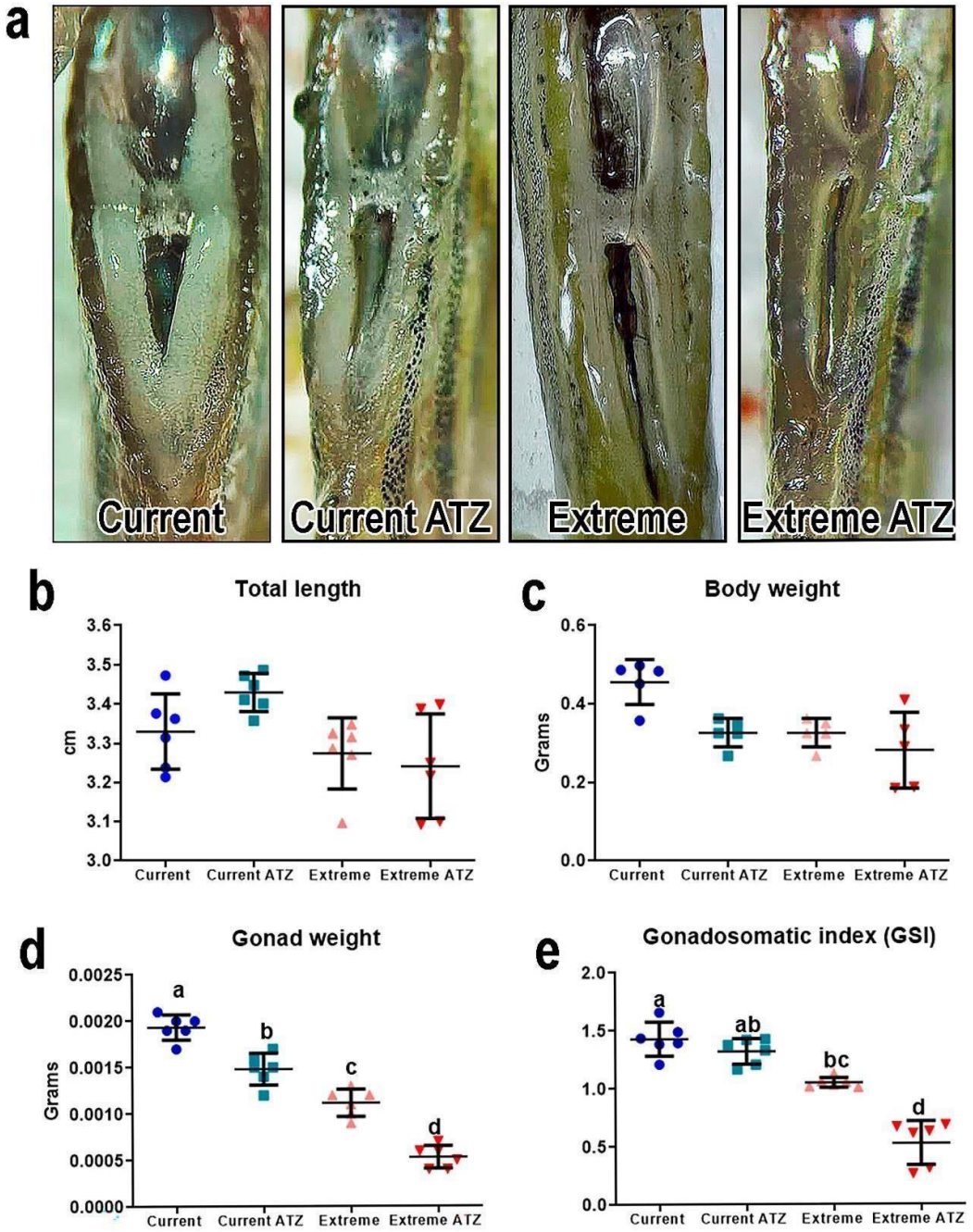

Physical-chemical analysis of water by HPLC determination of ATZ
Current
Current-ATZ
ATZ 2ug/L
Extreme
Extreme - ATZ
ATZ 2ug/L
Testes
Histomorphometric and pathological evaluation
0124
Apoptosis assay
TUNEL-positive
Dosage of hormones, Estradiol-17β (E2) and 11-ketotestosterone (11 KT) in zebrafish testes
RNA extraction and qRT-PCR
Amplification
Threshold
No amplification
Copies per reaction (Ct)
Sperm quality
Antioxidant enzymes
Analysis of CAT, SOD and GST enzyme activity and lipid peroxidation (LPO)

Figure 2


a
Current
Current ATZ
Extreme
Extreme ATZ
b
Total length
cm
3.6
3.5
3.4
3.3
3.2
3.1
3.0
Current
Current ATZ
Extreme
Extreme ATZ
c
Body weight
Grams
0.6
0.4
0.2
0.0
Current
Current ATZ
Extreme
Extreme ATZ
d
Gonad weight
Grams
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
a
b
c
d
Current
Current ATZ
Extreme
Extreme ATZ
e
Gonadosomatic index (GSI)
2.0
1.5
1.0
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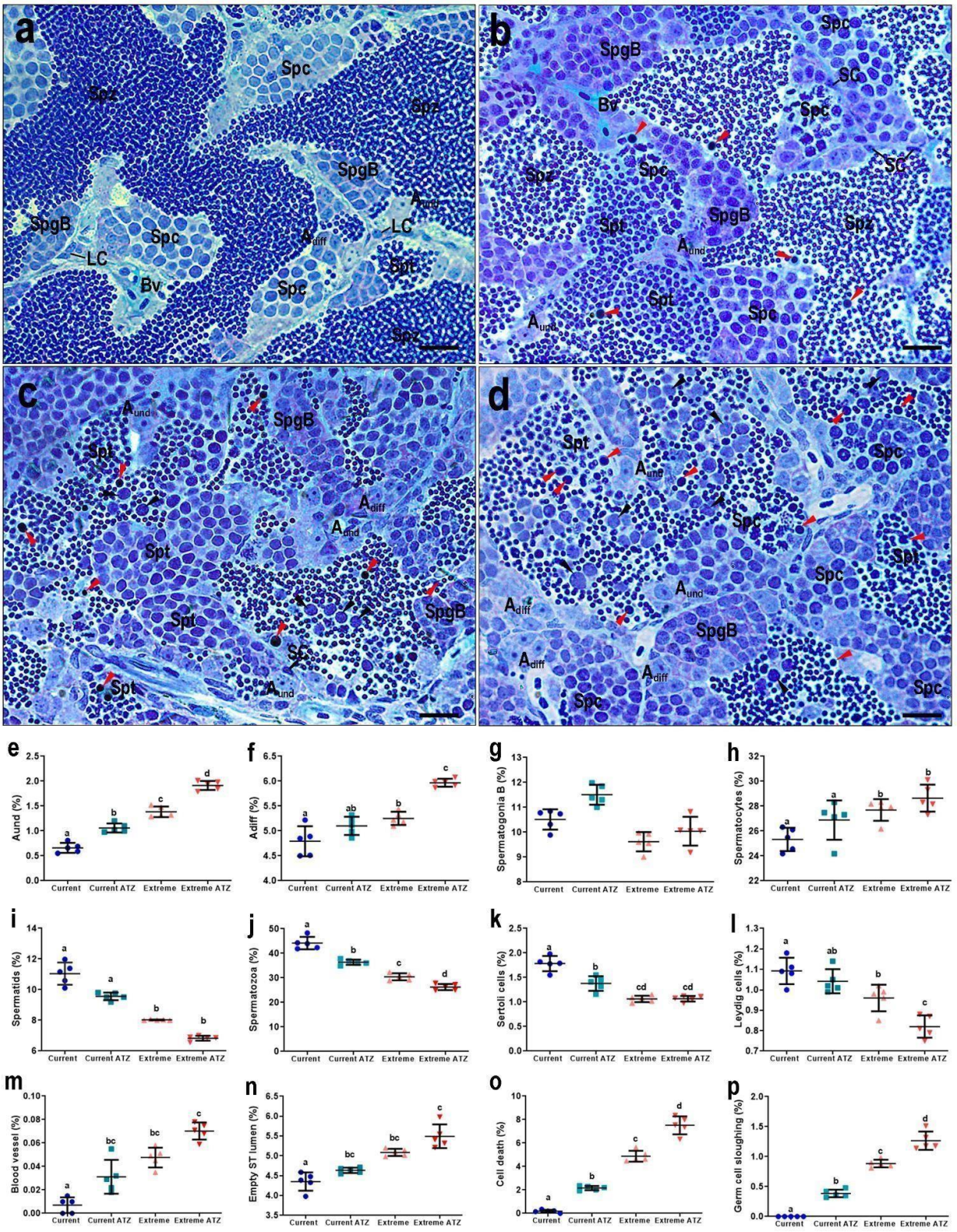
Extreme ATZ

Figure 3


a
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Aund (%)
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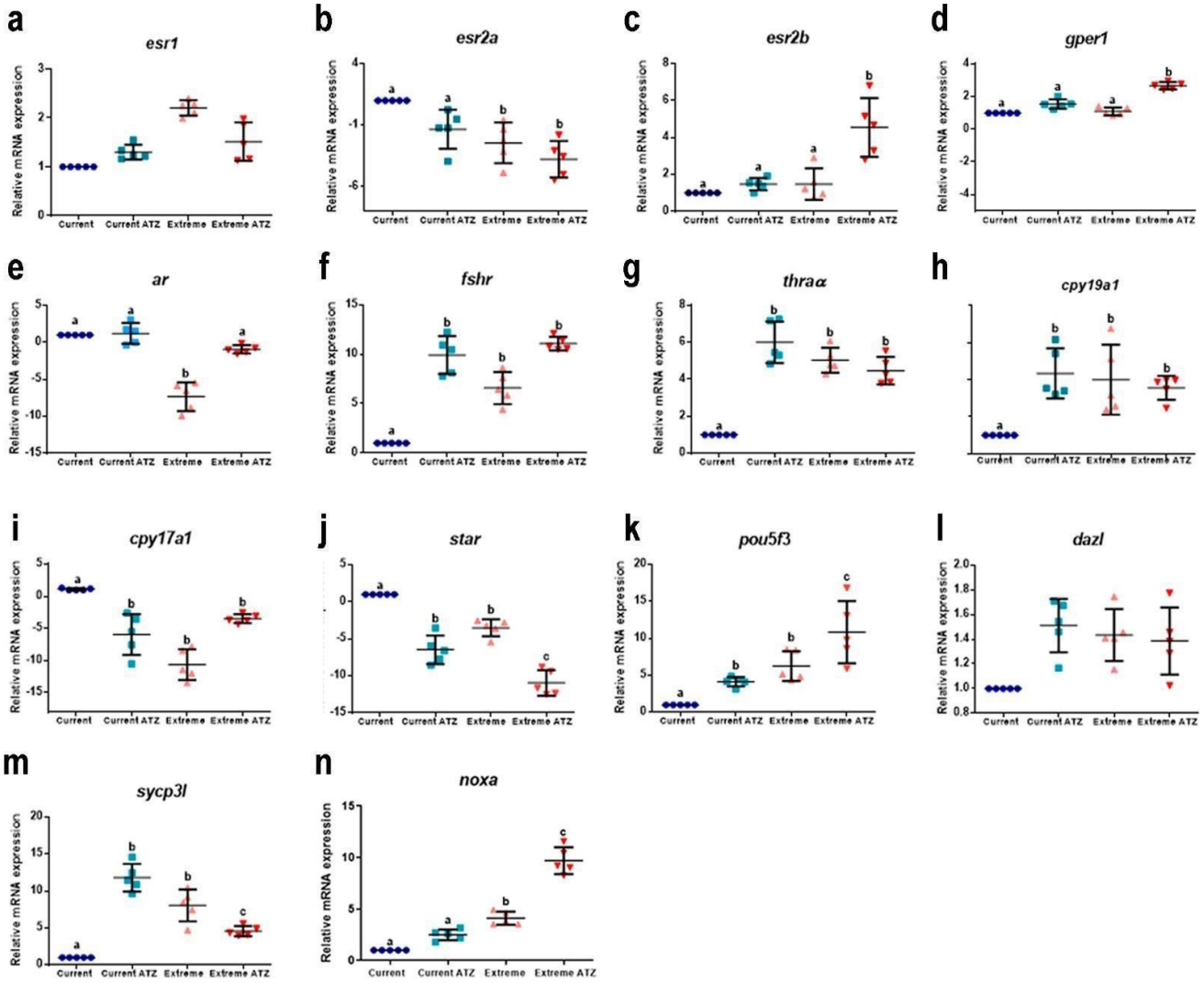
Adiff (%)
g
Spermatogonia B (%)
h
Spermatocytes (%)
i
Spermatids (%)
j
Spermatozoa (%)
k
Sertoli cells (%)
l
Leydig cells (%)
m
Blood vessel (%)
n
Empty ST lumen (%)
o
Cell death (%)
p
Germ cell sloughing (%)
Current
Current ATZ
Extreme
Extreme ATZ

Figure 4


a
esr1
b
esr2a
c
esr2b
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gper1
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fshr
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thraα
h
cpy19a1
i
cpy17a1
j
star
k
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m
sycp3l
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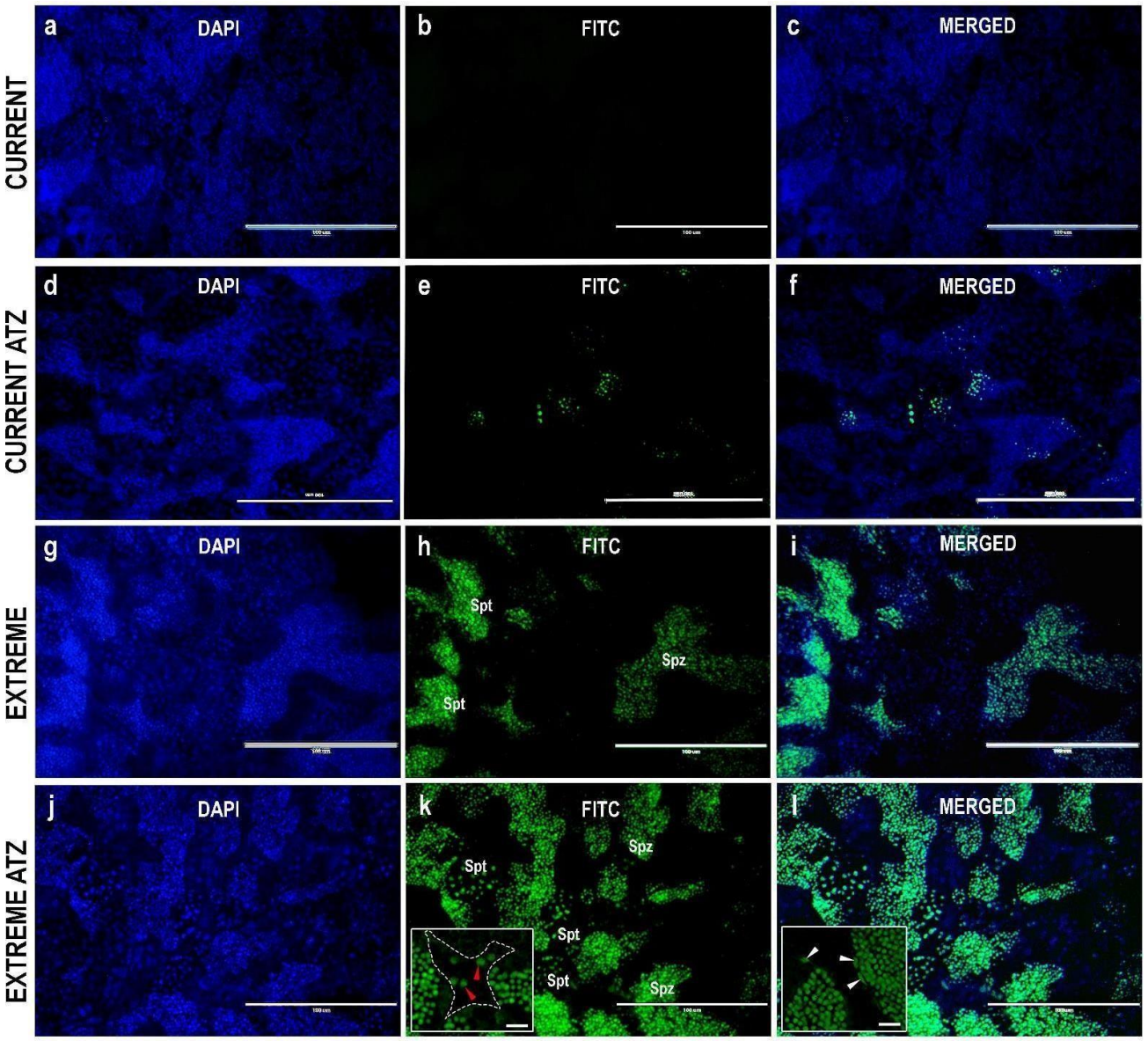
noxa
Relative mRNA expression
Current
Current ATZ
Extreme
Extreme ATZ

Figure 5


CURRENT
CURRENT ATZ
EXTREME
EXTREME ATZ
a
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FITC
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Spt
Spz
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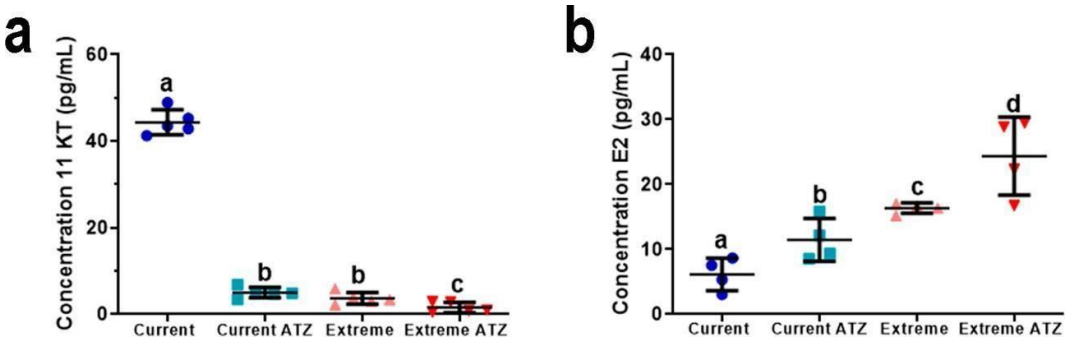
MERGED

Figure 6


a
Concentration 11 KT (pg/mL)
60
40
20
0
a
b
b
c
Current
Current ATZ
Extreme
Extreme ATZ
b
Concentration E2 (pg/mL)
40
30
20
10
0
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b
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Current
Current ATZ
Extreme
Extreme ATZ

Figure 7

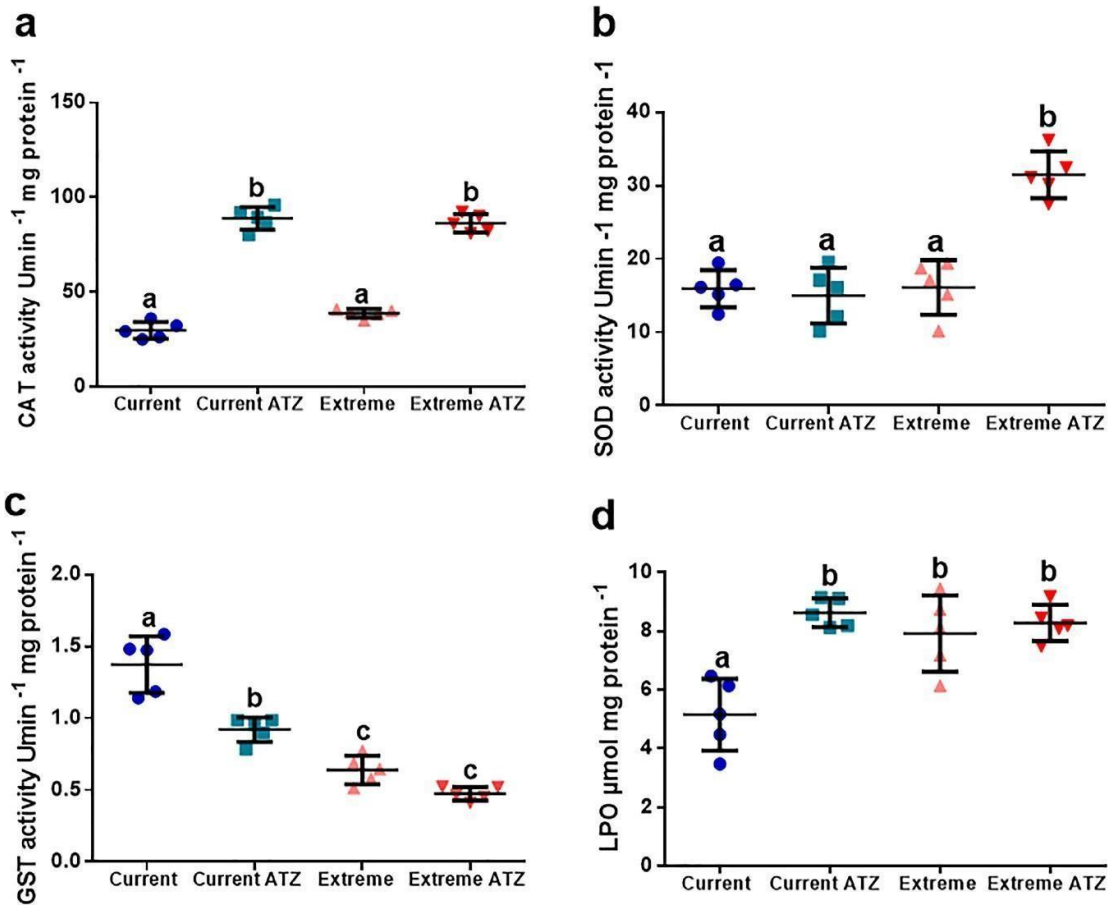
a
CAT activity Umin -1 mg protein -1
150
100
50
0
a
b
a
b
Current
Current ATZ
Extreme
Extreme ATZ
b
SOD activity Umin -1 mg protein -1
40
30
20
10
0
a
a
a
b
Current
Current ATZ
Extreme
Extreme ATZ
c
GST activity Umin -1 mg protein -1
2.0
1.5
1.0
0.5
0.0
a
b
c
c
Current
Current ATZ
Extreme
Extreme ATZ
d
LPO µmol mg protein -1
10
8
6
4
2
0
a
b
b
b
Current
Current ATZ
Extreme
Extreme ATZ

Figure 8

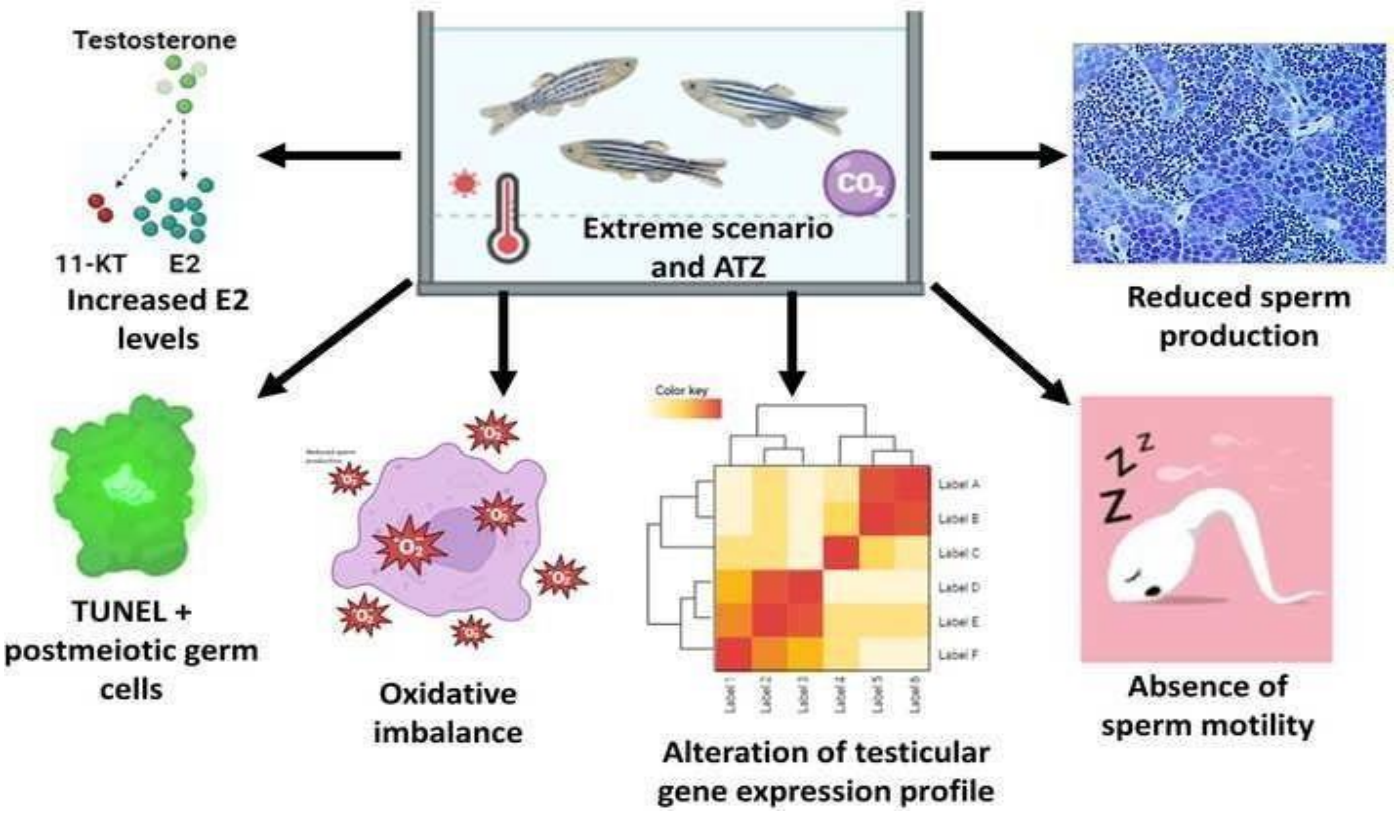
Testosterone
11-KT
E2
Increased E2 levels
Extreme scenario and ATZ
CO_2
Reduced sperm production
TUNEL + postmeiotic germ cells
Oxidative imbalance
Color key
Label A
Label B
Label C
Label D
Label E
Label F
Alteration of testicular gene expression profile
Absence of sperm motility

Table 1. Physical-chemical parameters of aquarium water, and air temperature and CO_2 in experimental microcosm. The two climate rooms were computer-controlled in real-time to simulate current environmental conditions and the extreme climate scenario (RCP 8.5) predicted for the year 2100 (IPCC 2017).

Scenario	**Water**							**Air**	
	Temperature ºC	Ammonia (mg/L)	O^2 (mg/L^{-1})	Electric conductivity	CO_2 (ppm)	pH	Atrazine (µg/L)	Temperature ºC	CO_2 (ppm)
Current	26 ± 3.1[a]	0.008 ± 0.001	6.23 ± 0.93	733.3 ± 21.813µS/cm[a]	9.79 ± 0.60[a]	6.14 ± 0.89	0±0	27.5 ± 2.8[a]	488 ± 6.1[a]
Current-ATZ	26 ± 3.1[a]	0.006 ± 0.002	6.13 ± 0.92	777.1 ± 13.313µS/cm[a]	9.55 ± 0.63[a]	6.32 ± 0.72	1.901± 0.02	27.5 ± 2.8[a]	488 ± 6.1[a]
Extreme	34 ± 4.2[b]	0.009 ± 0.002	5.53 ± 1.0	876.5 ± 34.013µS/cm[a]	29.2 ± 0.91[b]	5.57 ± 1.04	0±0	35 ± 3.2[b]	1.315 ± 120[b]
Extreme-ATZ	34 ± 4.1[b]	0.010 ± 0.004	5.13 ± 0.81	937.2 ± 15.913µS/cm[b]	30.01 ± 0.82[b]	5.21 ± 0.87	1.828± 0.05	35 ± 3.2[b]	1.315 ± 120[b]

Different letters indicate significant differences between groups (two-way ANOVA, $p < 0.05$).

Table 2: Primers used in real-time RT-PCR analysis of male zebrafish gonads after 28 days of exposure to climate change scenarios and ATZ.

Gene	Forward sequence	Reverse sequence	Reference	Group	Target genes (GenBank No.)
esr1	GGTCCAGTGTGGTGTCCTCT	AGAAAGCTTTGCATCCCTCA	Rojo, Lombó and Herráez, 2020	Estrogen receptors	(NM_152959)
esr2b	GTGCCATGACTACGCTTCTG	TCCCTCCTCACACCACACTT	Rojo, Lombó and Herráez, 2020	Estrogen receptors	(NM_174862)
gper-1	TGGCTGTGGCAGATCTTATTC	CAATGGACTGCTGCTCATAGA	Rojo, Lombó and Herráez, 2020	Estrogen receptors	(NM_001128723)
ar	ACGTGCCTGGCGTGAAAA	CAAACCTGCCATCCGTGAAC	Crespo et al. 2020	Androgen receptor	(NM_001083123)
fshr	GATTCTTCACCGTCTTCTCC	TGTAGCTGCTCAACTCAAACA	Santangeli et al. 2015	Gonadotropin receptor	(NM_001001812)
star	CCAAGTGCAGATGACCCCAA	GGAAGGTGTGTGCCCTTGTT	Santangeli et al. 2015	Steroidogenic enzymes	(NM_131663.1)
cpy17a1	GGGAGGCCACGGACTGTTA	CCATGTGGAACTGTAGTCAGCAA	Tovo-Neto et al. 2020	Steroidogenic enzymes	(NM_212806)
cpy19a1	AGATGTCGAGTTAAAGATCCTGCA	TCTACGTTTTCACCCGGTCG	Tovo-Neto et al. 2020	Steroidogenic enzymes	(NM_131154)
pou5f3	GAGAGATGTAGTGCGTGTAT	GCTCGTAATACTGTGCTTCA	Tovo-Neto et al. 2020	Spermatogenesis	(XM_005161103)
dazl	AGTGCAGACTTTGCTAACCCTTATGTA	GTCCACTGCTCCAAGTTGCTCT	Tovo-Neto et al. 2020	Spermatogenesis	(XM_005170072)
sycp3l	AGGCGTTTGACTTCACCGTT	TGAACCTCATTCCCCACACC	Rojo, Lombó and Herráez, 2020	Meiosis/Cell cycle	(NM_001040350)
noxa	ATGGCGAAGAAAGAGCAAAC	CGCTTCCCCTCCATTTGTAT	Fernandes et al. 2018	Apoptosis	(DQ860152)
thraα	GGCGTCCTGTAACTGCTGAA	TCAGCTGCTCTCGACTGACG	Marelli et al. 2017	Thyroid hormone receptor	(NM_131396)
18S rRNA	CATGGCCGTTCTTAGTTGGT	CGGACATCTAAGGGCATCAC	González et al. 2019	Reference gene	(BQ263086)
actb2	CGAGCTGTCTTCCCATCCA	TCACCAACGTAGCTGTCTTTCTG	Riesco and Robles, 2013	Reference gene	(AF057040.1)

Table 3- Zebrafish sperm motility parameters at different periods post-activation from fish exposed to ATZ associated to extreme climate scenario (IPCC 2021).

GROUP	ANALYSIS PARAMETER				
Motility (MOT %)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	35.335 ± 10.41	24.214 ± 11.57	22.323 ± 10.86	15.778 ± 9.44	6.104 ± 3.80
Current-ATZ	41.852 ± 6.30	**41.823 ± 9.72***	**42.026 ± 9.08***	**30.508 ± 10.46***	**14.157 ± 7.66** *
Extreme (35°C)	n/a	n/a	n/a	n/a	n/a
Extreme-ATZ (35°C)	n/a	n/a	n/a	n/a	n/a
Curvilinear Velocity (VCL µm/s)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	135.707 ± 15.18	126.811 ± 8.70	110.696 ± 9.39	88.286 ±10.29	76.133 ± 14.23
Current-ATZ	145.37 ± 14.18	138.277 ± 9.43	112.547 ± 6.18	80.17 ± 4.22	65.88 ± 11.12n/a
Extreme	n/a	n/a	n/a	n/a	n/a
Extreme-ATZ	n/a	n/a	n/a	n/a	
Average travel speed (VAP µm/s)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	104.067 ±21.28	86.437±13.85	69.053 ± 13.12	40.491 ± 8.80	26.235 ± 4.27
Current-ATZ	123.158 ± 33.18	98.13 ± 18.18	77.16 ± 15.10	42.32 ± 5.32	28.14 ± 6.52
Extreme	n/a	n/a	n/a	n/a	n/a
Extreme-ATZ	n/a	n/a	n/a	n/a	n/a
Straight Line Speed (VSL µm/s)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	85.902 ± 17.74	71.773 ±11.41	57.335 ± 10.83	33.972 ± 8.52	21.456 ± 3.67
Current-ATZ	88.33 ± 12.32	78.16 ± 09.15	57.17 ± 17.13	34.458 ± 2.587	29.854 ± 7.98
Extreme	n/a	n/a	n/a	n/a	n/an/a
Extreme-ATZ	n/a	n/a	n/a	n/a	
Straightness (STR %)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	82.435 ± 0.48	83.097 ± 0.63	82.035 ± 0.41	83.998 ± 0.41	82.131 ± 0.71
Current-ATZ	82.874 ±0.47	83.521 ± 0.22	82.879 ±0.32	82.985 ±0.17	83.032 ±0.85
Extreme	n/a	n/a	n/a	n/a	n/an/a
Extreme-ATZ	n/a	n/a	n/a	n/a	
Oscillation (WOB %)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	75.141 ± 7.42	68.454 ± 10.39	62.122 ± 8.31	46.628 ± 8.40	35.760 ± 6.16
Current-ATZ	82.332 ± 4.46	71.584 ± 1.9	68.478 ± 3.12	42.521 ± 5.87	32.851 ± 5.39
Extreme	n/a	n/a	n/a	n/a	n/an/a
Extreme-ATZ	n/a	n/a	n/a	n/a	
Progression (PRG µm)					
	5 seconds	**15 seconds**	**30 seconds**	**45 seconds**	**60 seconds**
Current	260.342 ± 53.24	221.382 ± 38.69	172.464 ± 32.51	102.137 ± 24.42	64.778 ± 10.77
Current-ATZ	277.235 ± 62.88	292.874 ± 72.66	197.552 ± 62.14	98.458 ± 19.55	69.254 ± 12.71
Extreme	n/a	n/a	n/a	n/a	n/a
Extreme-ATZ	n/a	n/a	n/a	n/a	n/a

(*) indicates a statistically significant difference by Student's two-sample t-test ($p < 0.05$). n/a: not available

DISCUSSÃO GERAL

O uso de defensivos agrícolas possui reconhecida importância na produção agrícola mundial por proporcionar o controle de pragas e doenças de plantas, o que permite o aumento da produção e a redução de perdas econômicas (Savary et al. 2019). Essa alta produção acarreta a aplicação de elevadas quantidades de agrotóxicos, frequentemente presente no solo e na água, que mesmo em baixas concentrações pode causar danos para os organismos expostos (Relyea et al. 2005). A contaminação pela Atrazina ocorre pela ação de irrigação e das chuvas, devido ao fato de resíduos desse herbicida presentes no solo agrícola sofrerem escoamento superficial até os ecossistemas aquáticos e, consequentemente, chegarem a água de abastecimento da população. Além disso, os resíduos de Atrazina podem ser acumulados nos tecidos dos organismos aquáticos e bioacumulado ao longo da cadeia trófica, representando um grande risco para a população humana que consome o alimento contaminado (Mondal e Ramen, 2021).

A exposição a poluentes tóxicos, como a Atrazina, acima de um determinado limiar (dose e/ou tempo de exposição específicos ao agente químico) comumente desencadeia uma cascata de respostas biológicas, como reportado nesse trabalho. Importantemente, as respostas iniciais desenvolvidas a nível molecular e celular podem evoluir para disfunções hormonais e reprodutivas, que causam em última análise danos significativos na organização biológica, impactando populações, comunidades e ecossistemas (Relyea et al. 2005). Na primeira etapa do trabalho onde abordamos os efeitos da Atrazina em *A. cepa* onde diversos autores descrevem a ação citotóxica em células meristemáticas de *A. cepa* já foi verificada por outros autores, que relataram seu potencial mitodepressivo (Çavuşoğlu et al., 2011; Mercado et al., 2020; Fattah et al., 2022). Em contrapartida, os resultados deste estudo mostraram o aumento do índice mitótico. Tal resultado pode ser explicado pelo acúmulo de células em prófase promovido por essas concentrações, indicando a inativação de moléculas essenciais para a transição entre a prófase e as próximas fases do ciclo celular. De acordo com Marc et al. (2002), a Atrazina causa alterações na regulação do ciclo celular ao inibir a ativação do complexo CDK1/ciclina B, responsável pela condensação do material genético e pela ativação das proteínas condensinas, que atuam na organização do fuso mitótico, no início da mitose.

Existem vários estudos que demonstram a ação genotóxica e mutagênica da Atrazina em diferentes organismos-teste (Zeljezic et al., 2004; Candioti et al., 2013; Loborde et al., 2020; Fattah et al., 2021; Herek et al., 2021; Zafra-Lemos et al., 2021). Em contrapartida, estudos sobre a genotoxicidade e mutagenicidade do herbicida em concentrações ambientais por meio de bioensaios com vegetais superiores, como o *A. cepa* (Finkler et al., 2020) são escassos. O teste de aberrações cromossômicas e o teste do micronúcleo são amplamente utilizados como indicadores de efeitos genotóxicos e mutagênicas causados por agentes químicos (Heddle et al., 1983; Leme e Marin-Morales, 2009). Aberrações cromossômicas aqui encontradas são caracterizadas como alterações cromossômicas estruturais ou numéricas que podem ocorrer espontaneamente ou devido à exposição a agentes químicos. Por outro lado, os micronúcleos surgem como resultado de danos induzidos por agentes clastogênicos ou aneugênicos facilmente observados como uma estrutura semelhante ao núcleo principal, mas em tamanho reduzido (Leme e Marin-Morales, 2009).

De acordo com os resultados desse estudo mostrou que a Atrazina pode ter efeitos significativos sobre a reprodução e comportamento de da *D. magna*. Em concentrações ambientalmente relevantes, a Atrazina e seus produtos de degradação afetaram negativamente o número de juvenis e o número de ninhadas de *D. magna* em um ensaio de toxicidade crônica (Bordin et al., 2022). Além disso, de acordo com Klementová et al., (2019) a Atrazina pode induzir altas porcentagens de embriões com parada precoce do desenvolvimento, o que pode promover um declínio na população de *D. magna* em todo o mundo. Outro estudo sugeriu que a Atrazina pode ter um efeito antagônico, revertendo os altos níveis de produção de machos induzidos por potentes agonistas juvenóides. Isso pode resultar em uma diminuição da densidade da população de crustáceos e resultar em maior mortalidade (Zavala et al., 2022). Além disso, a inibição do fotossistema II pela Atrazina leva ao acúmulo de espécies reativas de oxigênio (ROS) dentro dos cloroplastos das células da planta. Embora este efeito seja mais relevante para organismos fotossintéticos, ele também pode ter implicações para organismos heterotróficos como a *D. magna*, devido ao fato que o estresse oxidativo pode levar a danos celulares (Han, 2012).

Em relação ao FET realizado em zebrafish organismo modelo popular em pesquisas toxicológicas, demonstamos que a exposição á Atrazina leva a uma variedade de efeitos adversos no desenvolvimento embrionário. Destaca-se a indução de ecloção prematura, geração de edema pericárdio, alteração na obsorção do vitelo, e deformação esquelética. Além disso, de acordo com Utzig et al., (2010) relataram

efeitos teratogênicos na embriogênese, estresse oxidativo com produção excessiva de EROs e genotoxicidade evidenciada por danos ao DNA. No entanto, é importante notar que nem todos os estudos encontraram efeitos adversos significativos da Atrazina em embriões de zebrafish. Por exemplo, um estudo concluiu que a Atrazina não afetou a mortalidade dos embriões tratados, que apresentaram um desenvolvimento normal (Martins, 2023). Esses resultados variados destacam a complexidade dos efeitos potenciais da Atrazina em organismos modelos e a necessidade de mais pesquisas para entender completamente esses efeitos. É crucial considerar a concentração e a duração da exposição, bem como as diferenças individuais e genéticas entre os organismos ao avaliar os efeitos potenciais da Atrazina.

Outro ponto importante a ser abordado, são os vários estudos que sugerem que as mudanças climáticas globais poderão potencializar significativamente os efeitos tóxicos dos contaminantes e/ou reduzir a tolerância de organismos frente a estresses múltiplos (Delnat et al. 2020). De acordo com a resolução 357/2005 do Conselho Nacional do Meio Ambiente - CONAMA (legislação brasileira vigente), o limite máximo de resíduos de Atrazina permitido nos corpos d'água e água potável (água de torneira) é de 2µg/L, sendo essa concentração utilizada no segundo artigo. Dessa forma, as mudanças climáticas e a poluição ambiental são duas das maiores ameaças à biodiversidade aquática. A combinação desses fatores tem sido associada a vários efeitos adversos em peixes, incluindo alterações na produção hormonal, expressão gênica, atividade enzimática e qualidade do sêmen.

As mudanças climáticas têm impactos significativos na vida aquática. Estudos mostram que as mudanças no regime das águas já afetam as espécies de peixes mais comuns na Amazônia central, com mudanças notáveis na abundância de machos adultos, redução do tamanho dos machos ao chegar na maturidade sexual e diminuição de tamanho dos machos (Herrera et al., 2020). Além disso, de acordo com Kirsten (2016) quando expos peixes da espécie *Rhamdia quelen* a Atrazina em concentrações ambientais descreveu uma redução na expressão gênica de receptores hormonais nas gônadas dos animais, tais resultados corroboram os dados aqui apresentados. A Atrazina também tem sido associada a alterações na produção de hormônios esteróides em peixes tais como E2 e 11-KT, como descrito nesse trabalho. De acordo com Opute et al., (2021) a Atrazina afeta a reprodução de peixes, provocando uma diminuição na produção e motilidade dos espermatozoides. Castro et al., (2020), descreveram que as mudanças climáticas podem afetar a motilidade espermática em peixes de várias maneiras. A exposição a diferentes temperaturas (29, 31, 33 e 35°C)

resultou em uma duração mais longa da motilidade espermática a 29°C (50.1 ± 2.70 s), que diminuiu progressivamente quando exposta a 35°C (31.2 ± 1.31 s). Isso sugere que as mudanças climáticas, incluindo o aquecimento da água e a acidificação, afetam significativamente a reprodução de peixes. Além disso, esse efeito pode ser potencializado na presença de um contaminante ambiental.

As mudanças climáticas associado a Atrazina, alterou a atividade de várias enzimas antioxidantes em peixes, incluindo a catalase (CAT), a superóxido dismutase (SOD) e a glutationa S-transferase (GST) (CHOWDHURY e SAIKIA, 2020). Estudos indicam que a exposição salas de mudanças climáticas pode levar a um aumento na atividade dessas enzimas (Islam et al., 2022). Por exemplo, foi observado um aumento na atividade da CAT e da GST nas brânquias de peixes expostos à Atrazina (AKRAM et al., 2021). A CAT e a SOD são enzimas que convertem as ROS em moléculas menos reativas (Chowdhury e Saikia, 2020). A GST, por outro lado, é uma enzima que ajuda a detoxificar compostos estranhos, incluindo pesticidas como a Atrazina ou aumento de temperatura e CO_2 (Akram et al., 2021).

As enzimas CAT, SOD e GST desempenham papéis cruciais na defesa antioxidante celular. Em espermatozoides, cuja função primordial é a fertilização do ovócito, a homeostase redox é vital para garantir a integridade do material genético e a viabilidade celular (Lopes et al., 2021). Segundo Lacy et al., (2022) a CAT, SOD e GST atuam em cascata para neutralizar ROS, que, quando em excesso, podem desencadear danos irreversíveis nas células espermáticas. A CAT é responsável pela decomposição do peróxido de hidrogênio, enquanto a SOD converte o radical superóxido em peróxido de hidrogênio. A GST, por sua vez, participa da conjugação de moléculas de glutationa a compostos tóxicos, promovendo a sua excreção e protegendo as células contra danos oxidativos (Llavanera et al., 2020). Espermatozoides com menor atividade dessas enzimas são mais suscetíveis ao estresse oxidativo, resultando em danos à membrana plasmática e mitocondrial, bem como fragmentação de DNA. Este fenômeno pode comprometer a integridade do DNA dos espermatozoides e, consequentemente, a fertilidade (Lopes et al., 2021).

Em suma, as mudanças climáticas e a exposição à Atrazina representam ameaças significativas para os peixes. Mais pesquisas são necessárias para entender completamente os mecanismos pelos quais esses fatores afetam os peixes e para desenvolver estratégias eficazes de mitigação. No entanto, é importante notar que a resposta exata pode variar dependendo de vários fatores,

incluindo a espécie do organismo, a extensão e a duração da exposição as mudanças climáticas e Atrazina. Portanto, mais pesquisas são necessárias para entender completamente os efeitos das mudanças climáticas associado a um contaminante ambiental.

CONCLUSÕES

- A exposição a concentrações ambientais de Atrazina induziu aberrações cromossômicas e morte celular em células meristemáticas de *Allium cepa*;
- Alterações na reprodução e neurotoxicidade em *Daphnia magna* foi observado em concentrações maiores que 2 µg;
- A exposição aguda à Atrazina altera a fisiologia dos embriões de zebrafish;
- *In sílica*, o uso de Atrazina interage fortemente com proteínas da família CYP;
- Mudanças climáticas associadas à Atrazina comprometem sinergicamente a população de células testiculares e consequentemente a sua função;
- As mudanças climáticas associada ou não a Atrazina causa um distúrbio nos genes que regulam a espermatogênese;
- Mudanças climáticas e a Atrazina induz um desbalanço na produção de 11-KT e E2;
- Exposição a cenário futuro de mudanças climáticas induz a completa imotilidade dos espermatozoides.

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ANEXOS

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RESEARCH ARTICLE

Phytotoxicity and cytogenotoxicity of pesticide mixtures: analysis of the effects of environmentally relevant concentrations on the aquatic environment

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Abstract

In this study, we investigate the toxicity of commercial formulations based on glyphosate, 2,4-D, imidacloprid, and iprodione, in isolation and mixed, on *Allium cepa*. The mixtures consisted of combinations in the lowest (M1), intermediate (M2), and highest concentrations (M3) of each pesticide. We measured physiological (germination rate, germination speed, and radicular length) and cyto-genotoxic (mitotic index and frequency of aberrant cells) parameters. In addition, we analyzed the cell cycle progression and cell death induction by flow cytometry. When applied in isolation, the pesticides changed the parameters evaluated. M1 and M2 inhibited root length and increased the frequency of aberrant cells. Their genotoxic effect was equivalent to that of pesticides applied in isolation. Furthermore, M1 and M2 caused cell death and M2 changed the cell cycle progression. M3 had the greatest deleterious effect on *A. cepa*. This mixture inhibited root length and promoted an additive or synergistic effect on the mitotic index. In addition, M3 changed all parameters analyzed by flow cytometry. This research clearly demonstrates that the pesticides tested, and their mixtures, may pose a risk to non-target organisms.

Keywords Glyphosate · 2,4-D · Imidacloprid · Iprodione · *Allium cepa* · Phytotoxicity · Chromosomal aberrations · Flow cytometry

Introduction

The use of pesticides has increased substantially in Brazil since 2008, following an increased demand for food at the national and global scales (Brovini et al. 2021). In addition, the political-economic model adopted by the country's agrobusiness heavily relies on the use of chemical compounds (Araújo and Oliveira 2017).

The intensive use of pesticides in agriculture has, as a result, the input of it into the water resources, due to soil runoff. Therefore, it is necessary to monitor pesticide levels in the water to protect the environment and people (Barbosa et al. 2015; Albuquerque et al. 2016; Brovini et al. 2021).

In Brazil, Resolution No. 375 of the National Environmental Council (CONAMA 2005) establishes quality standards for water resources to fit their different uses, including protecting of aquatic communities and the water supplied for human use. On the other hand, the Ministry of Health (MS) regulates the pesticide levels allowed in drinking water through Ordinance No. 2,914 of 2011 (MS 2011) and coordinates the National Program for Monitoring the Quality of Drinking Water (Vigiagua). Data from the latter are included in the Sisagua database (Water Quality Monitoring System).

However, CONAMA and MS regulate less than 10% of Brazil's active ingredients approved for use. In addition, different classes of pesticides interact in the environment because of simultaneous or sequential applications, which

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may result in additive, synergistic, antagonistic, or potentiation toxic effects, i.e., different from the responses produced by each chemical substance alone (Fioresi et al. 2020; Finkler et al. 2022).

The herbicides glyphosate (replaced glycine chemical group), and 2,4-dichlorophenoxyacetic (aryloxy alkanoic acid chemical group) are the most commercialized pesticides in Brazil and worldwide (IBAMA 2020). Their water concentrations frequently exceed the limits allowed by the legislation (Aranha and Rocha 2019), raising concern about the adverse effects caused in exposed organisms: acute and chronic toxicity (Rodrigues et al. 2019; Silva et al. 2020; Brovini et al. 2021), oxidative stress, DNA damage, and mutations (Bukowska 2006; Rodrigues et al. 2019; Chaufan et al. 2014). In addition, glyphosate is possibly a carcinogen (IARC 2017), and 2,4-D may cause cancer in humans (IARC 2018).

On the other hand, the levels in the water of the insecticide imidacloprid (chemical group neonicotinoid) and of the fungicide iprodione (chemical group dicarboximide) are unregulated by Brazilian legislation. Nevertheless, the eco-cytogenotoxicity of these compounds has been reported by several studies (Karabay and Oguz 2005; Demsia et al. 2007; Bianchi et al. 2015; 2016; Rodríguez et al. 2015; Iturburu et al. 2018; Aragão et al. 2019; Bernardes et al. 2019; Chaufan et al. 2019; Carneiro et al. 2020; Fioresi et al. 2020; Samojeden et al. 2022).

The environmental persistence of pesticides can be estimated by categorizing their half-lives into three groups: low (less than 16 days), moderate (16 to 59 days), and high (over 60 days). Pesticides with very short half-lives can be problematic if they need to be applied frequently within a short timeframe. Pesticides with longer half-lives are more likely to build up after repeated applications. This may increase the risk of environmental contamination (Hanson et al. 2015). Many abiotic and biotic environmental factors affect how long a pesticide remains in the environment, making it challenging to determine a specific half-life (Hanson et al. 2015). Typically, glyphosate remains in surface waters for a period ranging from 2 to 91 days (Battaglin et al. 2014; Castro Berman et al. 2018). The estimated time it takes for 2,4-D to degrade in aerobic aquatic environments is 15 days, while in anaerobic conditions, it can take anywhere from 41 to 333 days. (NPIC 2011). Imidacloprid breaks down rapidly in water in the presence of light, but it remains persistent in water in the absence of light (Flores-Céspedes et al. 2012). In the dark, it breaks down very slowly at a pH between 5 and 7. In contrast, the half-life is about 1 year at pH 9 (Fossen 2006). The half-life of iprodione in aerobic aquatic environments was estimated to be 9 days, and in anaerobic environments, 7–14 days (US EPA 2007).

In view of the scenario described above, it is necessary to evaluate the adverse effects of these pesticides, isolated and combined, on non-target organisms (Finkler et al. 2022). *Allium cepa* is a plant commonly employed to evaluate water quality. The use of this model organism for the detection of toxic (macroscopic analysis) and cytogenotoxic activity (microscopic analysis) is validated by the International Chemical Safety Program and the United Nations Environmental Program (WHO 1985). *Allium cepa* is highly sensitive (82%) compared to the Ames test and the rodent carcinogenicity bioassay (Rank and Nielsen 1994). In addition, flow cytometry accurately detects changes in the cell cycle kinetics and measures cell death. This technique has been applied to investigate the effects of xenobiotics on plants (Rayburn and Wetzel 2002; Monteiro et al. 2010; Andrade-Vieira et al. 2012; Ghosh et al. 2015; Ghosh et al. 2016; Fioresi et al. 2020; Souza et al. 2023).

This study documents the effects of commercial formulations based on glyphosate, 2,4-D, imidacloprid and iprodione, and mixtures of them, on *A. cepa.* Physiological (germination rate, germination speed, and radicular length) and cyto-genotoxic (mitotic index and frequency of aberrant cells) parameters were analyzed, in addition to cell cycle progression and cell death induction (flow cytometry). Realistic concentrations reported on the freshwater and drinking water, and the maximum values allowed by the Brazilian legislation, were tested (Resolution No. 375/2005 of CONAMA and Ordinance No. 2,914/2011 of the Ministry of Health).

Materials and methods

Pesticides

Stock solutions and test solutions were prepared from the commercial formulations of each pesticide. Pesticides were diluted and homogenized in distilled water. The tested concentrations are expressed as microgram of acid equivalent per liter (herbicides) or microgram of active ingredient per liter (imidacloprid and iprodione).

The original DI Roundup commercial formulation, Monsanto, batch no. 0045 20–63560, was used. It contains 445 g/L (44.5% m/v) of N-(*phosphonomethyl)glycine* diammonium salt) and 370 g/L (37% m/v) acid equivalent of N-(*phosphonomethyl)glycine*), and 751 g/L (75% m/v) of inert ingredients was used. This formulation is classified as hazardous to the environment (class III) but is unlikely to cause acute harm to humans (category 4) (MAPA 2022). The tested concentrations were as follows: (a) 50 µg/L — recorded in tap water (Aranha and Rocha 2019); (b) 65 µg/L — corresponding to the maximum concentration allowed in freshwaters class I and II (CONAMA 2005) and (c) 280 µg/L — maximum concentration allowed in freshwaters class III (CONAMA 2005).

The commercial product Nufarm U46 BR (lot 0800491517499 T/B) containing 806 g/L of dimethylamine salt of 2,4-dichlorophenoxyacetic acid (2,4-D Amine), 670 g/L (67% w/v) of acid equivalent, and 380.9 g/L (38.09% m/v) of other ingredients was used. This formulation is considered hazardous to the environment (class III) and to human health, being classified in category 4 — slightly toxic product (MAPA 2022). The concentrations tested were as follows: (a) 4 µg/L — maximum concentration allowed in freshwaters class I and II (CONAMA 2005), the same value established by Ordinance No. 2,914/2011 (MS 2011); (b) 10 µg/L — found in the tap water of Brazilian municipalities (Aranha and Rocha 2019); (c) 30 µg/L — maximum concentration allowed in class III freshwater (CONAMA 2005).

The fungicide Rovral SC, FMC Química do Brasil LTDA, batch no. 021–17-5475, containing 500 g/L of iprodione (3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxo imidazolidine-1-carboxamide), is classified as dangerous for the environment (class III) and category 5 — unlikely to cause acute damage to humans (MAPA 2022). The tested concentrations were (a) 15.96 µg/L — chosen because it is lower than the other concentrations; (b) 27.14 µg/L — corresponding to the concentration of iprodione found in freshwaters (Sequinatto et al. 2013) and (c) 47.26 µg/L — defined based on the estimated concentration of the active ingredient in freshwaters according to the total number of applications for coffee (Queiroz et al. 2018).

The tested concentrations of the insecticide Warrant® 700 WG, lot no. 3269–16-1154, containing 700 g/kg imidacloprid (1-(6-chlorine-3-pyridynilmethyl)-N-nitroimidazolidim-2-ylidenoamine), were (a) 0.14 µg/L, (b) 1.13 µg/L, and (c) 4.53 µg/L, found in freshwaters and rural wells (Bortoluzzi et al. 2007; Sequinatto et al. 2013). According to the degree of environmental hazard, the formulation is considered hazardous to the environment (class III) and category 4 —slightly toxic to human health (MAPA 2022).

For pesticide mixtures, the lowest (M1), intermediate (M2), and highest concentrations tested (M3) were combined (Bianchi et al. 2016; Felisbino et al. 2018; Fioresi et al. 2020). The concentrations of pesticides were separated into three groups to determine the effects of each one in the mixture (Table 1).

Trials with A. cepa

Seeds of *A. cepa* (2n = 16), periform bay variety (ISLA®, lot no. 127536, germination rate of 98%), not treated with pesticides, were used. In all assays, distilled water was used as the negative control.

Phytotoxicity

The trial was conducted according to the US Protection Environmental Agency (US EPA 1996). For the analysis, 15 seeds of *A. cepa* were arranged in Petri dishes (150 × 15 mm) lined with filter paper with added 4 ml of pesticide solutions and mixtures. The trial was performed in triplicate. Germinated seeds were counted every 24 h until 65% of the seeds in the control group had roots and were at least 20 mm in length. Germination rate (G) was calculated using the formula: G = (N/A) × 100, where *N* is the total number of germinated seeds and *A* is the number of seeds placed for germination. The following formula was used to calculate germination speed: IVG = $(\sum Ni)/(\sum Di)$, where *Ni* is the number of roots germinated on day *i*; Di is number of days for germination. The final length of the radicles was measured with the aid of a caliper.

Cytogenotoxicity

The cytogenotoxicity test was conducted according to Mazzeo et al. (2015). Radicles exposed to pesticides and mixtures, and which measured bout 15 mm, were collected, fixed in methanol + acetic acid (3:1, v/v) and stored in a refrigerator. To remove the excess fixator, the roots were washed in distilled water three times, 5 min each time. The radicles were hydrolyzed in HCl 5N at room temperature

Table 1 Summary of tested concentrations of pesticides and their mixtures

Pesticide	Concentrations (µg/L)	
Glyphosate	50, 65, and 280	
2,4-D	4, 10, and 30	
Iprodione	15.96, 27.14, and 47.26	
Imidacloprid	0.14, 1.13, and 4.53	
Groups		Mixtures
Group 1: lowest concentrations	50 µg/L GLY + 4 µg/L 2,4-D + 15.96 µg/L IPR + 0.14 µg/L IMI	M1
Group 2: intermediate concentrations	65 µg/L GLY + 10 µg/L 2,4-D + 27.14 µg/L IPR + 1.13 µg/L IMI	M2
Group 3: highest concentrations	280 µg/L GLY + 30 µg/L 2,4-D + 47.26 µg/L IPR + 4.53 µg/L IMI	M3

GLY glyphosate, *IPR* iprodione, *IMI* imidacloprid

for 20 min. They were also washed in distilled water three times, for 5 min each time. After this process, the meristematic region of the roots was sectioned on a slide, stained with orcein 2% for 10 min, then covered with coverslip and macerated. Ten slides were prepared for each treatment, and 5000 cells were counted per treatment (500 cells/slides). The slides were analyzed under a light microscope at 400 × magnification. The frequency of dividing cells was determined by calculating the mitotic index (cytotoxic potential). All mitotic and chromosomal abnormalities observed were grouped within the same category: frequency of aberrant cells (Souza et al. 2021).

Flow cytometry

The flow cytometry analyses were conducted in three replicates (three samples) for each treatment, and 12 meristematic regions of *A. cepa* were evaluated *per* replicate. Nuclear suspensions were obtained by the method described by Galbraith et al. (1983). The meristems were sectioned with the aid of a scalpel blade in a Petri dish containing 600 µl of LB01 lysis buffer, in a box with crushed ice. The suspensions with the insulated nuclei were filtered with 45 µm nylon mesh to eliminate waste. These were then colored with 50 µl of propidium iodide (1 mg/L) for analysis in a BD FACSCanto II flow cytometer.

The distribution of nuclei in the different phases of the cell cycle was analyzed: G0/G1 — nuclei with 2C DNA content, S — nuclei with 2 — 4C DNA content, and G2/M nuclei with 4C DNA content. The proportion of nuclei/particles in sub-G1 was measured. This represents condensed nuclei with lower fluorescence intensity, due to the difficult access of the propidium iodide to particles with DNA content smaller than 2C (fragmentation of genetic material due to the process of cell death) (Andrade-Vieira et al. 2012; Moreira et al. 2021). The following were determined: the fluorescence intensity of the nuclei in G1 (IF^{G1}), which allows the estimation of the average amount of fluorescence emitted by the G1 nuclei; the dispersion of the frontal light (FSC — forward scatter), to analyze the diameter/nuclear size, and the lateral dispersion of light (SSC — side scatter), to verify the nuclear complexity. In addition, the coefficient of variation of the nuclei in G1 (CV = standard deviation divided by the mean) was also determined.

Statistical analysis

The Shapiro-Willk normality test was applied, and statistical differences between the control and pesticide concentrations within each experimental group were analyzed using ANOVA with subsequent Tukey test ($p < 0.05$). When the data did not meet the assumptions to perform parametric analysis, the Kruskal–Wallis test was applied, followed by the Dunn's test ($p < 0.05$). GraphPad Prism version 9.4 was used for the statistical analysis and elaboration of the figures.

Results and discussion

Phytotoxicity

The goal of applying phytotoxicity assays is to evaluate whether the chemicals present in the growth medium will cause damage to the development, macroscopic structure, and survival of an organism. In this work, the toxic effects of environmentally relevant concentrations of pesticides, and their mixtures, were analyzed by evaluating the germination rate, germination speed and radicular length of *A. cepa.* The results obtained are presented in Fig. 1.

Inhibition of the germination rate is an indicator of acute toxicity. Among the phytotoxic parameters analyzed, it was the least affected, consistent with the results of another study (Aragão et al. 2017). The herbicides did not affect the germination rate of *A. cepa* (Fig. 1a–c), a monocotyledon plant. Glyphosate and 2,4-D are systemic herbicides of post-emergent action recommended for the control of mono and dicotyledons (glyphosate), or only dicotyledons (2,4-D) (MAPA 2022). Previously, Alves et al. (2021a) had shown that the 2,4-D significantly inhibited the germination rate of the model plant *Lactuca sativa* (lettuce; dicotyledon), but not of *Sorghum bicolor* (sorghum; monocotyledon). In another study, seeking positive controls for phytocytogenotoxicity assays, Alves et al. (2021b) again showed that 2,4-D was effective at inhibiting the germination rate of *L. sativa*, but not of *A. cepa*.

Glyphosate-based herbicides also seem to have different effects on seed germination deleterious (Shuma et al. 1995; Mondal et al. 2017) or little to no effect (Piotrowicz-Cieślak et al. 2010) depending on the concentrations of the chemical, and the plant species tested. Our results contrast with Alves et al. (2021a, b), who showed that 0.01% glyphosate significantly reduced the percentage of germinated seeds of onion and lettuce, but corroborate the findings of Piotrowicz-Cieślak et al. (2010), who attested that 1–2000 µM of glyphosate did not affect the germination percentage of six species — *Lepidium sativum*, *Sinapis alba*, *Sorghum saccharatum*, *Brassica napus*, *Lupinus luteus*, and *Avena sativa*. According to Piotrowicz-Cieślak et al. (2010), therefore, percent seed germination may not be a good phytotoxicity parameter when glyphosate is employed.

Unlike the herbicides, the fungicide iprodione and the insecticide imidacloprid negatively affected the germination of *A. cepa* seeds when in certain concentrations. In group 1, iprodione significantly reduced the germination rate with respect to the control. The effect caused by the fungicide differed statistically from the effect of the glyphosate and 2,4-D

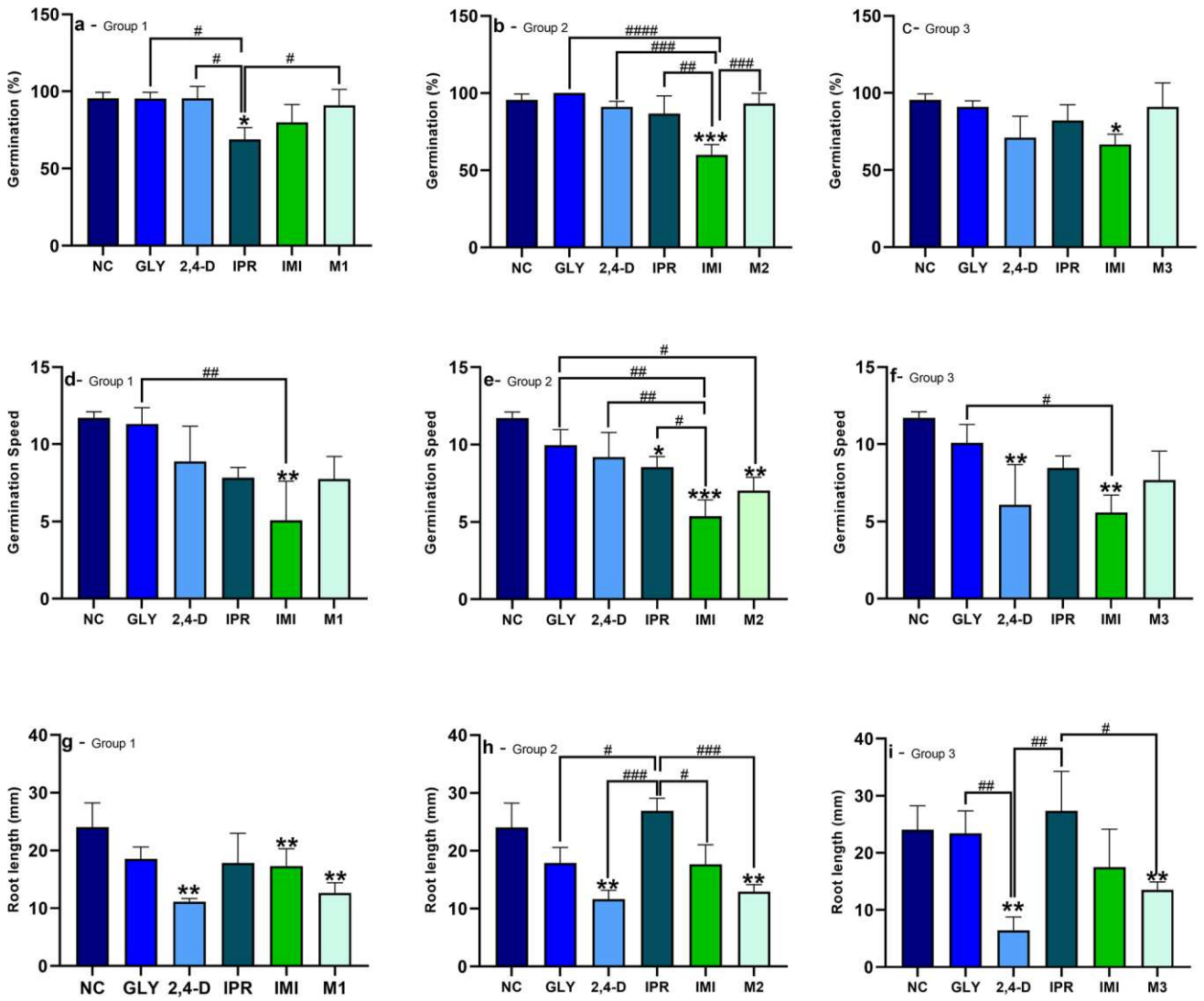

Fig. 1 Phytotoxicity of pesticide glyphosate (GLY), 2,4-D, iprodione (IPR), imidacloprid (IMI), and mixtures (M1, M2, and M3). Group 1: lowest concentrations of each pesticide (50 µg/L GLY, 4 µg/L 2,4-D, 15.96 µg/L IPR, and 0.14 µg/L IMI) and mixture of them (M1). Group 2: intermediate concentrations of each pesticide (65 µg/L GLY, 10 µg/L 2,4-D, 27.14 µg/L IPR, and 1.13 µg/L IMI) and mixture of them (M2). Group 3: highest concentrations of each pesticide (280 µg/L GLY, 30 µg/L 2,4-D, 47.26 µg/L IPR, and 4.53 µg/L IMI) and mixture of them (M3). Asterisks denote statistical significance with respect to the negative control (NC): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 00001$. Treatments followed by number sign differ from each other: $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, and $\#\#\#\#p < 0.0001$

and M1. In turn, M1 did not change the germination rate when compared with the control, indicating that iprodione, when combined with the other pesticides, did not exert an effect (Fig. 1a). Imidacloprid was the only pesticide isolate that inhibited the germination rate compared to the control in groups 2 and 3. In group 2, the insecticide still exerted a greater effect than all other pesticides and mixture M2. In the same manner, M1, M2, and M3 treatments did not differ from the control (Fig. 1b–c).

The results of the germination speed experiments are as follows: in group 1, only imidacloprid at 0.14 µg/L caused an adverse effect compared to the control and glyphosate (Fig. 1d). In group 2, iprodione at 27.14 µg/L and imidacloprid at 1.13 µg/L significantly reduced germination speed in relation to the control. Imidacloprid also reduced germination speed compared to all other pesticides, and therefore caused the greatest adverse effect. The M2 mixture also differed from the control and had an effect similar to isolated iprodione and imidacloprid (Fig. 1e). In group 3, the 2,4-D at 30 µg/L and imidacloprid at 4.53 µg/L reduced the germination speed compared to the control, while M3 did not change this parameter (Fig. 1f).

Unlike germination rate and germination speed, radicular length is considered the most sensitive phytotoxic parameter.

Considering the lowest concentrations of pesticides and M1 (group 1), 4 µg/L of 2,4-D and 0.14 µg/L of imidacloprid and M1 reduced the root length of *A. cepa*. M1 had a comparable effect to that of the isolated pesticides (Fig. 1g). In group 2, the 2,4-D at 10 µg/L and M2 significantly decreased radicular length with respect to the control. The effect of M2 did not differ statistically from the effect of isolated pesticides, except for iprodione (Fig. 1h). In group 3, the 2,4-D at 30 µg/L and M3 resulted in shorter roots of *A. cepa* when compared to the control. The effect of M3 also differed statistically from iprodione (Fig. 1i).

As described above and observed in Fig. 1 h–i, 2.4-D was the only pesticide which, in all concentrations assessed, significantly reduced the length of the roots in relation to the control. These data are consistent with Alves et al. (2021b) and Grabinska-Sota et al. (2003), who experimented with other plant species (*Sinapis alba*, *Lepidium sativum*, *Avena sativa*, *Triticum aestivum*). By mimicking the action of natural auxin, 2,4-D inhibits the production of the hormone ethylene. Thus, abscisic acid is produced, which inhibits cell proliferation and elongation, hindering growth, damaging tissue, and causing cell death (Marcato et al. 2017).

All mixtures also reduced the radicular length of *A. cepa*. Plant root growth depends on cell proliferation and elongation during development and differentiation processes. Therefore, the effect observed in mixtures is an indication that the chemicals in pesticides interfere with the genetic material, mitotic machinery, and cell death induction mechanisms (Fioresi et al. 2020).

Cyto-genotoxicity

The results of the mitotic index analysis are presented in Fig. 2a–c. When in isolation, only the herbicides glyphosate and 2,4-D decreased the proportion of cells in division with respect to the control. According to Türkoğlu (2012), a reduction in the mitotic index indicates interference in the functioning of the enzymes that mediate cell cycle, including DNA polymerase, DNA gyrase, RNA polymerase, and kinases, resulting in inhibition of DNA synthesis or blockade

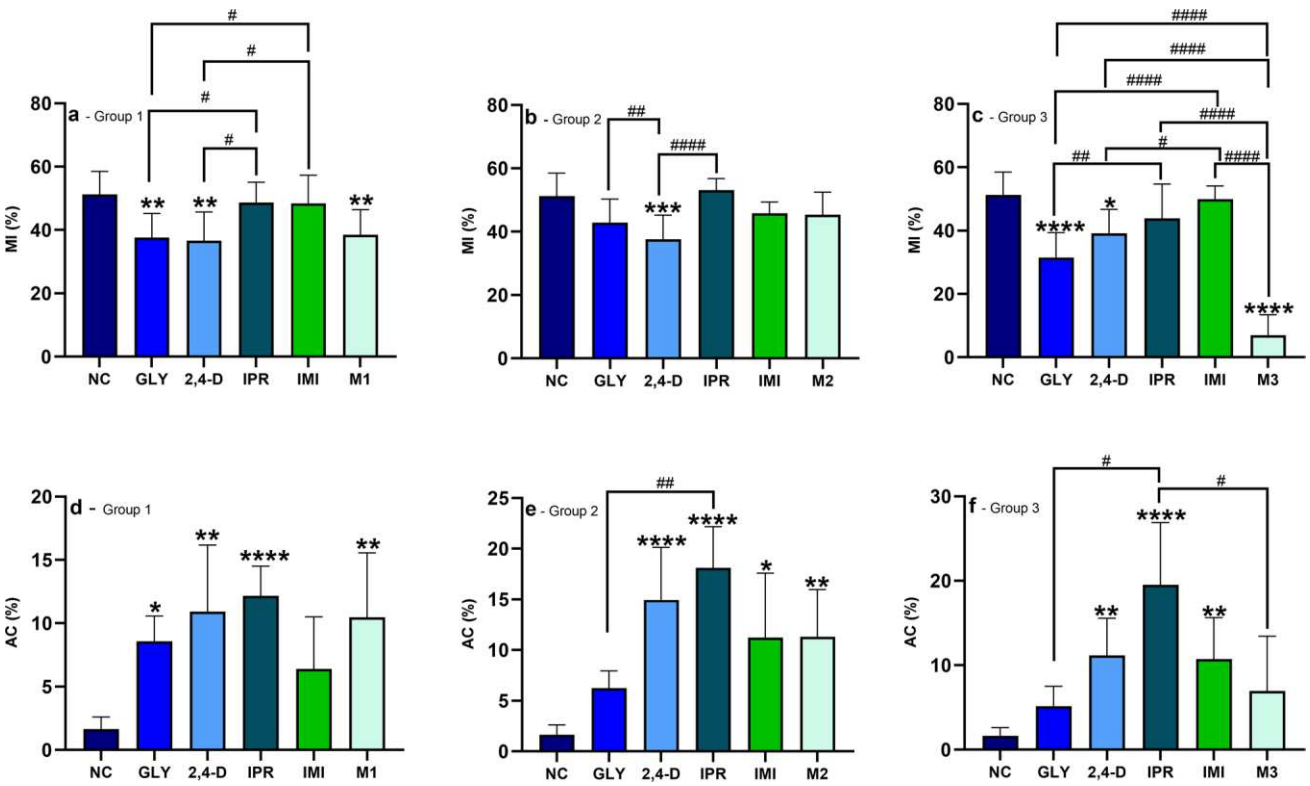

Fig. 2 Mitotic index (**a**–**c**) and frequency of aberrant cells (**d**–**e**) induced by glyphosate (GLY), 2,4-D, iprodione (IPR), imidacloprid (IMI), and their mixtures (M1, M2, and M3). Group 1: lowest concentrations of each pesticide (50 µg/L GLY, 4 µg/L 2,4-D, 15.96 µg/L IPR, and 0.14 µg/L IMI) and mixture of them (M1). Group 2: intermediate concentrations of each pesticide (65 µg/L GLY, 10 µg/L 2,4-D, 27.14 µg/L IPR, and 1.13 µg/L IMI) and mixture of them (M2). Group 3: highest concentrations of each pesticide (280 µg/L GLY, 30 µg/L 2,4-D, 47.26 µg/L IPR, and 4.53 µg/L IMI) and mixture of them (M3). Asterisks denote statistical significance with respect to the negative control (NC): *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. Treatments followed by number sign differ from each other: #$p < 0.05$, ##$p < 0.001$, and ####$p < 0.0001$

of the G1 and G2 phases of the cell cycle and inhibition of microtubule formation.

Considering the lowest concentrations of pesticides (group 1), the effects of the herbicides tested did not differ statistically from each other, but differed from iprodione and imidacloprid ($p < 0.05$) (Fig. 2a). The M1 mixture significantly reduced the mitotic index in relation to the control, and its effect was comparable to that of the isolated pesticides (Fig. 2a). The mitotic index after the M2 treatment did not differ from the control, indicating that the effect of 2,4-D at 10 µg/L was canceled out when associated with other pesticides (Fig. 2b). On the other hand, M3, which combines the highest concentrations of pesticides, drastically reduced the rate of dividing cells concerning the control and also compared to all pesticides when isolated (Fig. 2c). This result indicates that the mixture has an additive or synergistic effect that is more powerful than the pesticides alone. In the additive effect, the toxicity of the mixture is the sum of the individual effects. A synergistic effect occurs when the mixture produces more adverse effects than the isolated chemical substances and the additive effect (Finkler et al. 2022).

The genotoxic activity of pesticides and mixtures was investigated by analyzing the frequency of aberrant cells (Fig. 2d–f). In general, the isolated pesticides, except for imidacloprid at 0.14 µg/L (Fig. 2d) and glyphosate at 65 µg/L and 280 µg/L (Fig. 2e–f), induced a greater genotoxic effect than the control (Fig. 2d–f).

The genotoxicity of pesticides or commercial formulations used in this research has been reported in different studies. Kier and Kirkland (2013) reviewed the genotoxicity of glyphosate and the commercial formulations based on this herbicide, concluding that both have no mutagenic or genotoxic activity on mammalian cells both in vivo and in vitro. In contrast, only high doses of glyphosate caused positive results in non-mammalian systems. In these cases, its genotoxicity was due to the effects of surfactants on commercial formulations and not to the active ingredient itself. In the case of the aquatic biota, Rodrigues et al. (2019) showed that glyphosate, its main degradation product aminomethylphosphonic acid (AMPA), the commercial formulation ATN, and the surfactant polyethoxylated tallow amine (POEA) induced DNA damage on zebrafish larvae. Nevertheless, only the surfactant caused adverse effects in the gonadal cells of the rainbow trout (RTG-2). The difference between the results was explained by the physiological differences among the organisms involved and the fact that those were different species.

In model plants, some studies indicate that glyphosate increases chromosomal aberrations. However, the concentrations tested in those studies are higher than those investigated in the present study: 0.36–7.2 mg/ml (Truta et al. 2011), 5–30 mg/L (Mercado and Caleño 2020), 1.34–13.40 mg/L (Vieira et al. 2022), and 1.56 and 11.66 mg/ml (Finkler et al. 2022).

Bukowska (2006), in his review article, clarified the general mechanisms of action involved in the toxic action of 2,4-D, which involves the induction of oxidative stress, lipid peroxidation, depletion of ATP concentration, NADPH and GSH, modulation of antioxidant system activity, induction of homologous recombination, A → G mutation, chromosomal aberrations, exchange between sister chromatids, breaks in the DNA molecule, and activity-dependent apoptosis and caspases.

When it comes to other pesticides, the data on the toxic effects of iprodione on non-target organisms are scarce, and it is difficult to determine their mechanism of cytogenotoxic action (Chaufan et al 2019). The aneugenic and/or clastogenic effect of the iprodione was reported for some species of model plants (Gadeva and Dimitrov 2008; Aragão et al. 2019; Bernardes et al. 2019; Fioresi et al. 2020). Bernardes et al. (2019) documented the mutagenic activity of iprodione on *A. cepa*. It caused changes in single sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers, indicating the induction of deletions and insertions in the DNA molecule. In turn, Chaufan et al. (2019) also demonstrated that iprodione has different biological targets, being able to induce mitotic abnormalities and micronuclei of aneugenic and/or clastogenic origin. They explained those results as corresponding to an induction in oxidative stress in Hep-2 cells (human larynx epidermoid carcinoma cells).

According to Bianchi et al. (2015), the genotoxic action of imidacloprid is due to the presence of an electronegative pharmacophore (N-nitroimine substitute = NNO_2), located on the nitroguanidine part that binds covalently to the DNA, causing chromosomal damage. Imidacloprid significantly increased the micronuclei index in *Tradescantia* and the F1 cells of *A. cepa* (Rodríguez et al. 2015), and chromosomal aberrations in onion (Bianchi et al. 2016). Karabay and Oguz (2005), Demsia et al. (2007), Bianchi et al. (2015), and Iturburu et al. (2018) also identified imidacloprid as a clastogenic agent.

M1 and M2 also significantly increased the frequency of aberrant cells in relation to the control ($p < 0.01$). This effect was comparable to that exerted by the isolated pesticides (Fig. 2d–e). In group 3, even though 2,4-D at 30 µg/L, the iprodione at 47.26 µg/L and imidacloprid at 4.53 µg/L are genotoxic concerning the control, the frequency of aberrant cells in M3 indicates that it did not induce this effect (Fig. 2f). Iprodione was the only pesticide that, in isolation, induced greater genotoxicity than the mixture (< 0.05) (Fig. 2f). Its effect was reduced in association with other pesticides, indicating an antagonistic effect (Finkler et al. 2022). On the other hand, the low frequency of aberrant cells reported for M3 may also reflect its high cytotoxic action expressed by the inhibition of the mitotic index. A smaller

number of dividing cells may make it impossible to observe mitotic and chromosomal abnormalities.

In Fig. 3, the micrographs represent the phases of the normal mitotic cycle *of A. cepa* and the main abnormalities observed. The frequencies of the specific changes are presented in Table 2. As demonstrated, chromosomal adhesion and micronucleus were the alterations most observed. Chromosomal adhesion is due to abnormal chromatin

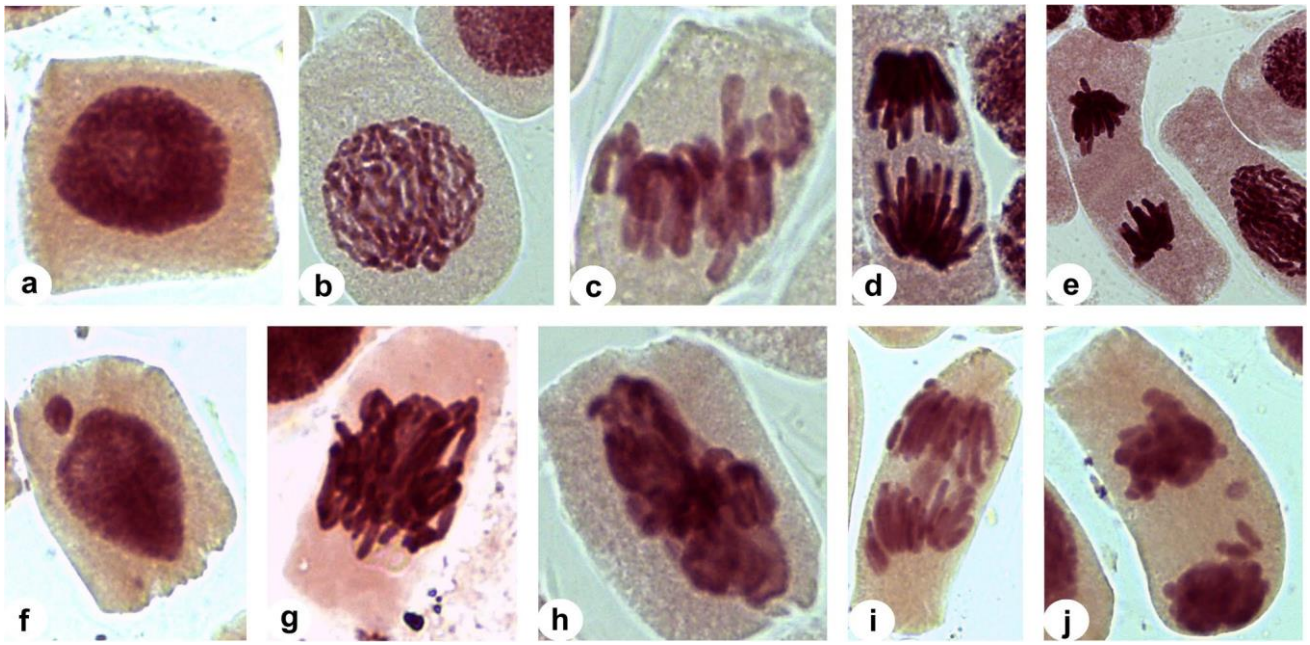

Fig. 3 Phases of the normal cell cycle of *A. cepa* (**a**–**e**) and the main abnormalities observed (**f**–**j**). **a** Interphase. **b** Prophase. **c** Metaphase. **d** Anaphase. **e** Telophase. **f** Micronucleated cell. **g**–**h** Metaphase with chromosomal adherence. **i** Anaphase with chromosomal bridge. **f** Telophase with fragment and loss chromosomal

Table 2 Frequency of each mitotic and chromosomal alteration in root cells of A. cepa exposed to pesticide and mixtures

	Pesticide	CA	CL	CB	CBr	CM	MN	PP
NC		0.30 ± 0.22	0.30 ± 1.21	0.0 ± 0.0	0.46 ± 0.51	0.0 ± 0.0	0.58 ± 0.64	0.0 ± 0.0
Group 1	50 µg/L GLI	**3.86 ± 0.95****	0.36 ± 1.51	0.20 ± 0.35	0.54 ± 0.0	0.24 ± 0.65	2.90 ± 0.35	0.48 ± 0.50
	4 µg/L 2,4-D	**5.46 ± 1.53*****	0.60 ± 0.85	0.0 ± 0.0	1.66 ± 1.65	0.0 ± 0.0	**3.20 ± 1.50***	0.0 ± 0.0
	15.96 µg/L IPR	**4.28 ± 0.82****	0.30 ± 0.25	0.34 ± 0.45	**2.28 ± 0.12***	0.0 ± 0.0	**4.96 ± 0.30****	0.0 ± 0.0
	0.14 µg/L IMI	**3.02 ± 0.91****	0.0 ± 0.0	0.0 ± 0.0	0.88 ± 0.69	0.0 ± 0.0	**3.10 ± 0.50***	0.0 ± 0.0
	M1	**6.26 ± 1.52*****	0.0 ± 0.0	0.0 ± 0.0	1.08 ± 1.52	0.0 ± 0.0	**3.14 ± 0.80***	0.0 ± 0.0
Group 2	65 µg/L GLI	1.60 ± 0.95	0.34 ± 0.35	0.08 ± 0.05	0.40 ± 0.52	0.06 ± 0.09	**3.44 ± 0.98****	0.32 ± 0.10
	10 µg/L 2,4-D	**5.88 ± 0.36****	1.06 ±	0.0 ± 0.0	1.26 ± 1.70	0.0 ± 0.0	**6.74 ± 1.68****	0.0 ± 0.0
	27.14 µg/L IPR	**6.80 ± 0.45****	0.0 ± 0.0	1.42 ± 1.24	2.14 ± 1.05	0.0 ± 0.0	**7.76 ± 1.25*****	0.0 ± 0.0
	1.13 µg/L IMI	**6.12 ± 1.25****	0.0 ± 0.0	0.24 ± 0.35	1.22 ± 0.98	0.36 ± 0.65	**3.26 ± 0.52****	0.0 ± 0.0
	M2	**4.46 ± 0.98****	0.0 ± 0.0	1.06 ± 1.02	1.88 ± 0.95	0.0 ± 0.0	**3.90 ± 0.36****	0.0 ± 0.0
Group 3	280 µg/L GLI	2.02 ± 1.53	0.0 ± 0.0	0.0 ± 0.0	0.28 ± 0.38	0.04 ± 0.08	2.82 ± 1.52	0.0 ± 0.0
	30 µg/L 2,4-D	**6.06 ± 1.35***	0.0 ± 0.0	0.24 ± 0.85	0.54 ± 0.46	0.0 ± 0.0	**4.32 ± 1.26***	0.0 ± 0.0
	47.26 µg/L IPR	**6.92 ± 2.03****	0.40 ± 0.34	0.34 ± 0 .94	**2.94 ± 0.04***	0.34 ± 0.38	**7.00 ± 1.55****	**1.58 ± 0.23***
	4.53 µg/L IMI	**4.78 ± 1.05***	0.0 ± 0.0	0.08 ± 0.46	1.46 ± 0.43	0.0 ± 0.0	**4.42 ± 0.64****	0.0 ± 0.0
	M3	**3.20 ± 0.65***	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.56 ± 0.25	**3.20 ± 0.32***	0.0 ± 0.0

CA chromosomal adherence, *CB* chromosome breakage, *CL* chromosome loss, *CBr* chromosome bridge, *CM* c-metaphase, *MN* micronucleus, *PP* polyploidy. Group 1: lowest concentrations of pesticides. Group 2: intermediate concentrations of pesticides. Group 3: highest concentrations of pesticides. M1, M2, and M3: mixtures. Asterisks denote statistical significance in relation to the negative control (NC): *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

condensation. The intense connections between chromosomes cause cell death (Fiskesjö 1985). This abnormality is, therefore, an initial step in apoptosis. If it persists until the anaphase, chromosomal adhesion can generate bridges and breaks, manifesting as chromosomal aberrations in the next cell generations (Fioresi et al. 2020). Micronuclei result from unrepaired clastogenic and/or aneugenic damage in the parental cells (Fenech et al. 2020).

Flow cytometry

The flow cytometry technique detects minimal differences in nuclear DNA content and makes it possible to quantify the distribution of cells in the different phases of the cycle. Therefore, the method provides relevant information on the effects of chemical substances on the cell cycle. Flow cytometry also can measure cell death induction (Moreira et al. 2021). In this work, the isolated pesticides and mixtures altered the percentage of cells in sub-G1, G1, S, and G2/M. Higher concentrations of pesticides and M3 caused more significant effects. (Figs. 4 and 5).

The intensity of the fluorescence emitted by isolated chromosomes, chromosomal fragments, cell fragments, nuclear fragments, condensed nuclei, and other smaller particles is lower than the intensity that comes from a normal G1 nucleus. These particles are collectively called G1-subparticles (sub-G1) (Andrade-Vieira et al. 2012). Many of these events are by-products of the cell death process, characterized by nuclear condensation with subsequent DNA fragmentation (Andrade-Vieira et al. 2012; Moreira et al. 2021). Except for glyphosate at 65 µg/L, iprodione at 27.14 µg/L (Fig. 4b), and glyphosate at 280 µg/L (Fig. 4c), the tested pesticides considerably increased the frequency of sub-G1 particles concerning the control (Fig. 4a–c). All mixtures also significantly increased the percentage of G1-subparticles compared to the control (Fig. 4a–c). Compared to the isolated pesticides, M1 did not cause a statistically significant result compared with glyphosate, iprodione, and imidacloprid. However, it caused less effects ($p < 0.01$) than the 2,4-D at 4 µg/L (Fig. 4a). The effect of none of the isolated pesticides differed statistically from that of M2 (Fig. 4b). The M3 mixture promoted greater damage than the glyphosate ($p < 0.001$), 2,4-D ($p < 0.05$) and iprodione ($p < 0.0001$) isolates, suggesting an additive or synergistic effect. However, the response induced by this mixture was comparable to the isolated use of imidacloprid (Fig. 4c).

The effect of pesticides and mixtures on the frequency of cells in phase G0/G1 is shown in Fig. 4d–e. The lowest concentrations of pesticides and the M1 mixture failed to change the proportion of cells in this phase (Fig. 4d). The 2,4-D at 10 and 30 µg/L and imidacloprid at 1.13 and 4.53 µg/L increased the frequency of cells in G0/G1 with respect to the control, as did M2 and M3 (Fig. 4e–f). This result indicates that in response to the genotoxic action of 2,4-D, imidacloprid, and mixtures, a cascade of signal transduction was triggered, culminating in a transient interruption of the cell cycle in G0/G1, to allow for repair before progressing to the subsequent phase of the cycle (Fioresi et al. 2020; Moreira et al. 2021). These results also show that no isolated pesticide differed statistically from M2 (Fig. 4e), while the M3 mixture promoted a greater adverse effect than iprodione at 47.26 µg/L ($p < 0.05$) (Fig. 4f).

The lowest concentrations of pesticides and M1 did not interfere with the percentage of cells replicating their DNA (Fig. 4g). Considering the intermediate concentrations, only imidacloprid at 1.13 µg/L reduced the frequency of cells in phase S (Fig. 4h). Glyphosate at 280 µg/L increased the proportion of cells in phase S concerning the control and, in contrast, 2,4-D at 30 µg/L, imidacloprid at 4.53 µg/L and M3 were associated with a decrease in the frequency of cells in this phase (Fig. 4i). The M3 treatment differed statistically from the treatments involving isolated glyphosate, iprodione, and imidacloprid but had a similar effect to 2,4-D (Fig. 4i).

According to Moreira et al. (2021), the accumulation of cells in phase S indicates the blockade of DNA replication by activation of the intra-S checkpoint. Our results are consistent with other studies that reported the same effect for the active ingredient (George and Shukla 2013) and a commercial formulation based on glyphosate (Marc et al. 2004), in vitro and in vivo, respectively. George and Shukla (2013) showed that the accumulation of cells in S was due to increased expression of G1/S cyclins. Marc et al. (2004) presented evidence that the herbicide inhibited the dephosphorylation of the residue of Tyr 15 of CDK1/cyclin B, preventing its activation and G2/M transition because of the interference of the herbicide on DNA replication. In the present study, a direct implication of increasing the percentage of cells in this phase, induced by 280 µg/L of glyphosate, was a significant decrease in the number of cells in G2/M. This result supports the inhibition of the mitotic index. These effects could also partly explain the negative response for the genotoxicity index since mitotic and chromosomal abnormalities are measured on dividing cells.

The effect of pesticides and mixtures on the frequency of cells in the G2/M phase is shown in Fig. 4j–l. Figure 4 j shows that although all pesticides decreased the percentage of cells in G2/M with respect to the control, the M1 mixture did not have the same action. Considering the intermediate concentrations of pesticides (group 2), only 2,4-D at 30 µg/L and mixture M2 reduced the frequency of cells in G2/M (Fig. 4k). And, except for iprodione at 47.26 µg/L, all pesticides decreased the frequency of cells with 4C DNA content. Mixture M3 produced the same effect, comparable to the effect of glyphosate, 2,4-D, and imidacloprid isolated (Fig. 4l).

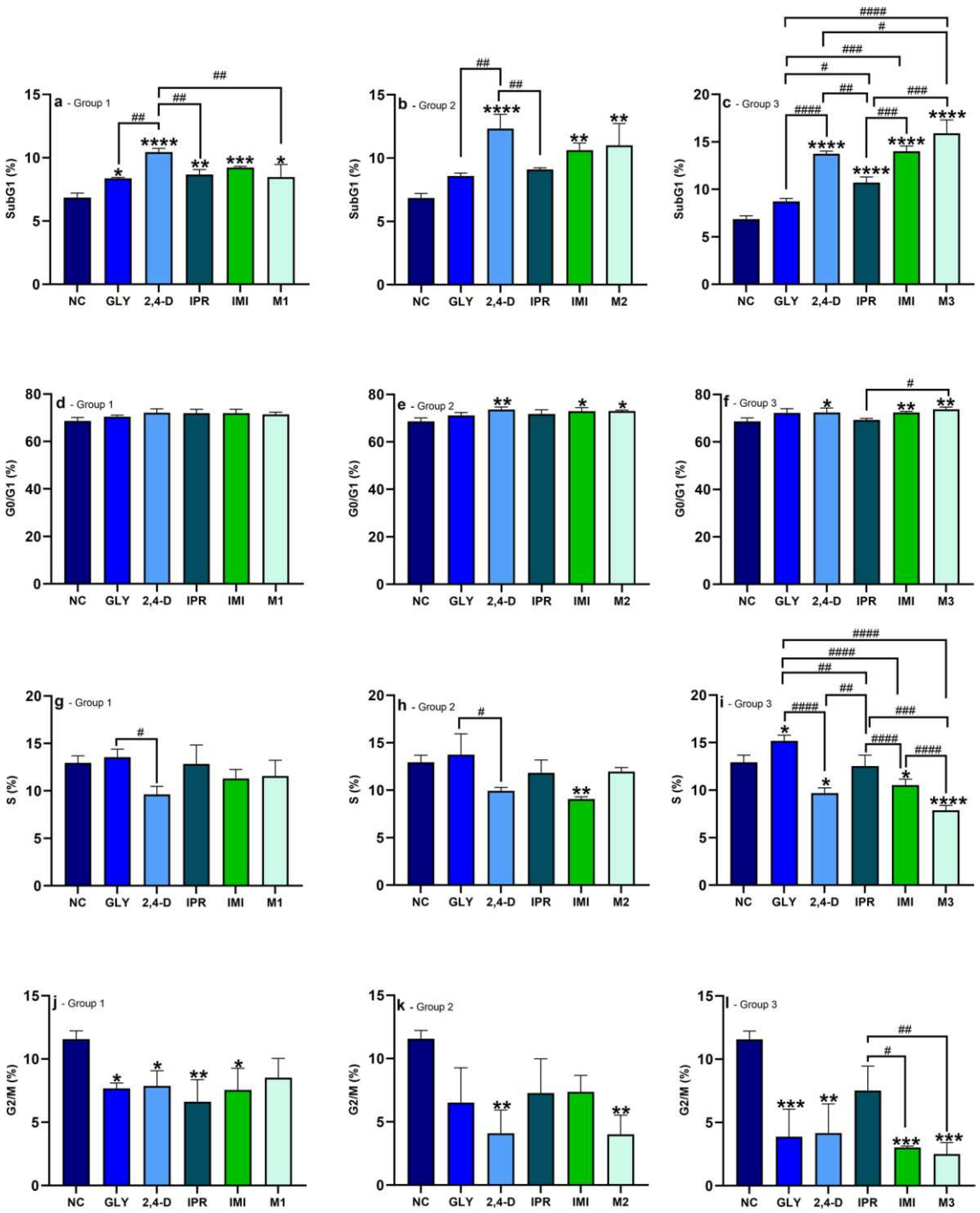

Fig. 4 Frequency of cells in each phase of the cell cycle (subG1, G0/G1, S, and G2/M) of *A. cepa* after exposure to pesticides glyphosato (GLY), 2,4-D, iprodione (IPR) and imidacloprid (IMI), and their mixtures (M1, M2, and M3). Group 1: lowest concentrations of each pesticide (50 µg/L GLY, 4 µg/L 2,4-D, 15.96 µg/L IPR, and 0.14 µg/L IMI) and mixture of them (M1). Group 2: intermediate concentrations of each pesticide (65 µg/L GLY, 10 µg/L 2,4-D, 27.14 µg/L IPR, and 1.13 µg/L IMI) and mixture of them (M2). Group 3: highest concentrations of each pesticide (280 µg/L GLY, 30 µg/L 2,4-D, 47.26 µg/L IPR, and 4.53 µg/L IMI) and mixture of them (M3). Asterisks denote statistical significance with respect to the negative control (NC): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Treatments followed by number sign differ from each other: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ and ####$p < 0.0001$

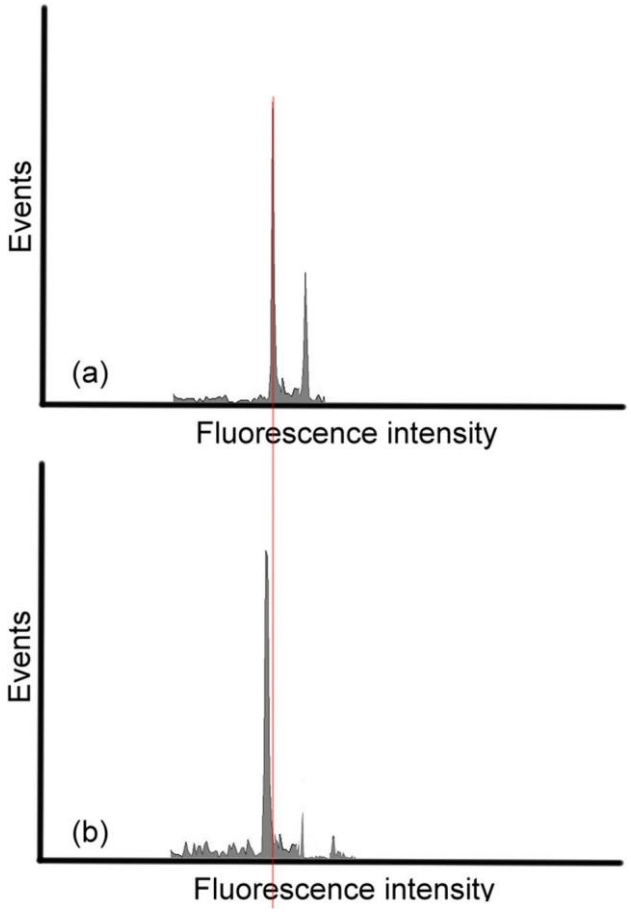

Fig. 5 Representative histograms of flow cytometry analysis. **a** Negative control and **b** M3. Note in the control the low coefficient of variation of nuclei in G1 and a relatively high percentage of nuclei in S + G2/M, in addition to a low percentage of G1 subparticles. M3 altered all parameters analyzed in flow cytometry. Note the decrease in cells entering division (S + G2/M), the increased coefficients of variation of nuclei in G1, and the percentage of G1 subparticles. The red line indicates the position of the negative control G1 peak. Note the shift of this peak to the left after exposure of *A. cepa* root cells to M3

Checkpoint activation is a mechanism that ensures cell survival. Nevertheless, severe lesions activate cell death mechanisms which can also be measured, in flow cytometry, by FSC, SSC, and fluorescence intensity of nuclei in G1 (IF^{G1}) (Andrade-Vieira et al. 2012; Fioresi et al. 2020). Our findings show that pesticides and mixtures modified these parameters (Table 3). Imidacloprid at 0.14 µg/L (group 1), 2,4-D at 10 µg/L and imidacloprid at 1.13 µg/L (group 2), and 2,4-D at 30 µg/L, imidacloprid at 4.53 µg/L, and mixture M3 (group 3) reduced FSC. Furthermore, the intermediate concentrations (group 2) of all isolated pesticides significantly reduced the fluorescence intensity of G1, which is also evidenced in Fig. 5, where the displacement of the G1 peak to the left (lower fluorescence intensity) is shown. However, the effect of M2 did not differ statistically from that of the control. In group 3, this parameter was reduced by the glyphosate at 280 µg/L, the imidacloprid at 4.53 µg/L and the M3 mixture. SSC, which estimates nuclear complexity, was reduced after exposure to iprodione at 15.96 µg/L and imidacloprid at 0.14 µg/L (group 1), glyphosate at 65 µg/L, 2,4-D at 10 µg/L and imidacloprid at 1.13 µg/L (group 2), and 2,4-D at 30 µg/L, imidacloprid at 4.53 µg/L, and mixture M3 (group 3).

In flow cytometry, the light is deflected and refracted as the laser beam passes through the cell. This scattered light is collected by FSC and SSC photodiodes. FSC is associated with cell size. In contrast, SSC reflects cell granularity or internal complexity, for instance, organelle structure and cytoplasm or nucleus condensation (van der Meer et al. 2010; Wlodkowic et al. 2011). Cell shrinkage is characteristic of the initial stages of apoptosis, which results in decreased intensity of the FSC signal, followed by an increase in the SSC signal (condensation and nuclear fragmentation). When the apoptotic cascade advances, the cells become progressively smaller, and the intensity of the SSC signal also decreases. The development of apoptotic bodies is characterized by a decrease in both FSC and SSC signals (van der Meer et al. 2010; Wlodkowic et al. 2011). Nuclear condensation hinders the entry of propidium iodide and, consequently, the average fluorescence intensity of nuclei in G1 decreases (Andrade-Vieira et al. 2012; Souza et al. 2023).

All pesticides and all mixtures increased the CV concerning the control ($p < 0.0001$) (Table 3). Injured cells that have been unrepaired and escape the process of cell death contribute to an increase in the frequency of aberrant cells. In flow cytometry, these cells have been identified by an increase of the CV of the nuclei in G1, which can be associated with alterations that lead to an unequal distribution of genetic material among daughter cells, such as micronuclei and chromosomal adhesions (Bickham et al. 1992; Biradar and Rayburn 1995; Rayburn and Wetzel 2002; Fioresi et al. 2020; Souza et al. 2023). These were the main alterations induced by pesticides isolated and mixtures.

Final considerations

Our study showed that the maximum concentrations of 2,4-D and glyphosate allowed by Brazilian legislation caused toxicogenetic damage in *A. epa*. This result is an alert to other organisms. Brovini et al. (2021) performed an environmental risk assessment focused on three-best-seller pesticides in Brazil. Glyphosate presented the most significant environmental risk due to its elevated environmental concentrations and the toxic effects induced in aquatic organisms (*Daphnia magna*, *Oncorhynchus mykiss*, and *Raphidocelis subcapitata*). Only glyphosate

Table 3 Analysis of forward light scatter (FSC), side light scatter (SSC), fluorescence index (IF^{G1}), and G1 coefficient of variation (CV) after exposure of *A. cepa* to pesticides and mixtures

	Pesticide	FSC	SSC	IF^{G1}	CV
NC		100 ± 0	100 ± 0	100 ± 0	3.08 ± 0.17
Group 1	50 µg/L GLI	100.7 ± 0.14	100.22 ± 0.81	99.89 ± 0.10	**5.54 ± 0.12****
	4 µg/L 2,4-D	98.67 ± 0.51	99.77 ± 0.37	99.77 ± 0.28	**5.59 ± 0.07****
	15.96 µg/L IPR	99.44 ± 1.41	**97.77 ± 0.59****	97.88 ± 0.38	**5.47 ± 0.18****
	0.14 µg/L IMI	**97.78 ± 0.51***	**98.04 ± 0.85***	98.12 ± 0.22	**5.75 ± 0.07****
	M1	99.52 ± 0.35	100.07 ± 0.14	100.15 ± 1.90	**5.82 ± 0.06****
Group 2	65 µg/L GLI	98.10 ± 1.29	**97.15 ± 1.19***	**96.84 ± 0.45****	**5.79 ± 0.08****
	10 µg/L 2,4-D	**97.70 ± 0.51***	**97.41 ± 0.67****	**97.18 ± 1.23****	**5.77 ± 0.13****
	27.14 µg/L IPR	98.18 ± 0.74	98.44 ± 1.03	**97.33 ± 0.12****	**5.90 ± 0.02****
	1.13 µg/L IMI	**97.45 ± 0.49****	**97.15 ± 0.36****	**96.78 ± 0.35****	**5.88 ± 0.10****
	M2	98.49 ± 0.46	99.11 ± 0.11	100.01 ± 1.01	**5.95 ± 0.04****
Group 3	280 µg/L GLI	98.09 ± 0.79	97.70 ± 0.95	**96.67 ± 0.62****	**6.11 ± 0.10****
	30 µg/L 2,4-D	**97.07 ± 1.44****	**97.43 ± 0.11***	97.66 ± 0.35	**5.98 ± 0.07****
	47.26 µg/L IPR	98.27 ± 0.90	98.62 ± 1.31	97.81 ± 0.54	**5.97 ± 0.04****
	4.53 µg/L IMI	**96.97 ± 0.17****	**96.63 ± 0.48****	**96.48 ± 0.28****	**5.92 ± 0.12****
	M3	**96.14 ± 0.75****	**96.88 ± 1.29****	**96.49 ± 2.04****	**6.10 ± 0.11****

Group 1: lowest concentrations of pesticides. Group 2: intermediate concentrations of pesticides. Group 3: highest concentrations of pesticides. M1, M2, and M3: mixtures. Asterisks denote statistical significance in relation to the negative control (NC): *$p < 0{,}05$, **$p < 0.01$, ****$p < 0.0001$

concentrations above 1 µg/L would represent a high risk. In contrast, environmental concentrations of 2,4-D higher than 10 µg/L would cause significant risk. Based on these results, the authors concluded that the maximum values authorized by the legislation do not protect aquatic ecosystems and should be critically reviewed.

The analysis of the adverse effects of the combinations of pesticides is a more realistic scenario of exposure to the isolated pesticides. The results of this study showed that the M3 mixture caused the greatest deleterious effects on *A. cepa*. Therefore, taking M3 as an example, we suggest an association between the induced changes when all parameters used are analyzed together. Thus, radicular growth inhibition can be explained by the cell cycle blockage in G1 and induction of cell death. In turn, this cytotoxic action was confirmed by (a) inhibition of the mitotic index, with the consequent decrease in the frequency of cells in G2/M; (b) increased frequency of cells in the G1 sub-phase; (c) complexity and size of nucleus; and (d) reduction in fluorescence intensity. In general, the interaction of pesticides in the M3 mixture resulted in an additive or synergistic effect for cytotoxicity parameter. Due to it, the total frequency of aberrant cells did not differ from the control. However, micronuclei and chromosomal adhesions were significantly induced, contributing to an increase in CV.

Attention should be given to the M1 and M2 mixtures with the lowest concentrations of pesticides, which triggered a positive response to genotoxicity. Their effect was equivalent to those of pesticides alone, indicating that the activation of checkpoints for DNA repair and cell death, demonstrated in flow cytometry, were not sufficient to maintain cell integrity.

Considering the toxic action of pesticide mixtures investigated in this study, we only found studies on binary combinations in the scientific literature. Finkler et al. (2022) investigated the effects of 2,4-D mixed with glyphosate in two different combinations: environmental concentrations (average concentrations applied in the field) and doses relevant to human health. Both associations caused a significant decrease in the mitotic index of *A. cepa* and a possible additive or synergistic effect due to a considerable increase in aberrant cells with respect to the herbicides applied in isolation. Carvalho et al. (2020) showed, through the comet assay, that glyphosate associated with different acid formulations 2,4-dichlorophenoxyacetic acid (2,4-D, 2,4-D-amine and 2,4-D-ister) increased the genotoxicity index on the erythrocytes of the fish species *Cnesterodon decemmaculatus*. The mixtures glyphosate + 2,4-D and glyphosate + 2,4-D-amine showed a synergistic effect and the mixture glyphosate + 2,4-D-ester had an antagonistic effect.

Fioresi et al. (2020) found that the mixture of iprodione and imidacloprid in field concentrations also induced cytogenotoxic damage *in A. cepa*. We highlight that Brazil's laws do not define the levels of iprodione and imidacloprid allowed in the water. However, non-target organisms can be harmed by these pesticides either alone or when associated with other ones.

Finally, EU directive 2020/2184 sets a limit of 5 µg/L for pesticide mixtures in drinking water. Meanwhile, Brazilian

legislation only establishes maximum values for some isolated pesticides. Hence, the experiments with *A. cepa* aided in comprehending the impacts of pesticide combinations, taking into account significant levels in the water ecosystem.

Author contribution Conceptualization was performed by Tatiana da Silva Souza and Victor Ventura de Souza. Material preparation, data collection, and analysis were performed by Luanna Alves Miranda, Renata Alice Campos, and José Marcello Salabert de Campos. The first draft of the manuscript was written by Luanna Alves Miranda. Writing — review and editing: Tatiana da Silva Souza. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Supervision: Tatiana da Silva Souza.

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Data availability The data that support the funding of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval This is an original article that did not use other information that requires ethical approval.

Consent to participate All authors participated in this article.

Consent for publication All authors have given consent to the publication of this article.

Competing interests The authors declare no competing interests.

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Apresentação ORAL no Congresso Latino Américano de Toxicologia Ambiental, Ocupacional e de Nanomateriais

III Congresso Latino-Americano de Toxicologia Ambiental, Ocupacional e de Nanomateriais – TOXILATIN 2023

Informamos que o trabalho CLIMATE CHANGE EXACERBATES THE TOXIC EFFECTS OF ATRAZINE ON TESTICULAR FUNCTION de autoria de VICTOR VENTURA DE SOUZA, DAVIDSON PERUCI MOREIRA, DANIELA CHEMIN DE MELO HOYOS, EDUARDO ANTÔNIO SANCHES, ADALBERTO LUÍS VAL, SAMYRA MARIA DOS SANTOS NASSIF LACERDA foi aprovado pela Comissão Avaliadora, para apresentação **ORAL** no III Congresso Latino-Americano de Toxicologia Ambiental, Ocupacional e de Nanomateriais, a ser realizado no período de 26 a 29 de setembro de 2023, na cidade de Belo Horizonte/MG/Brasil.

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