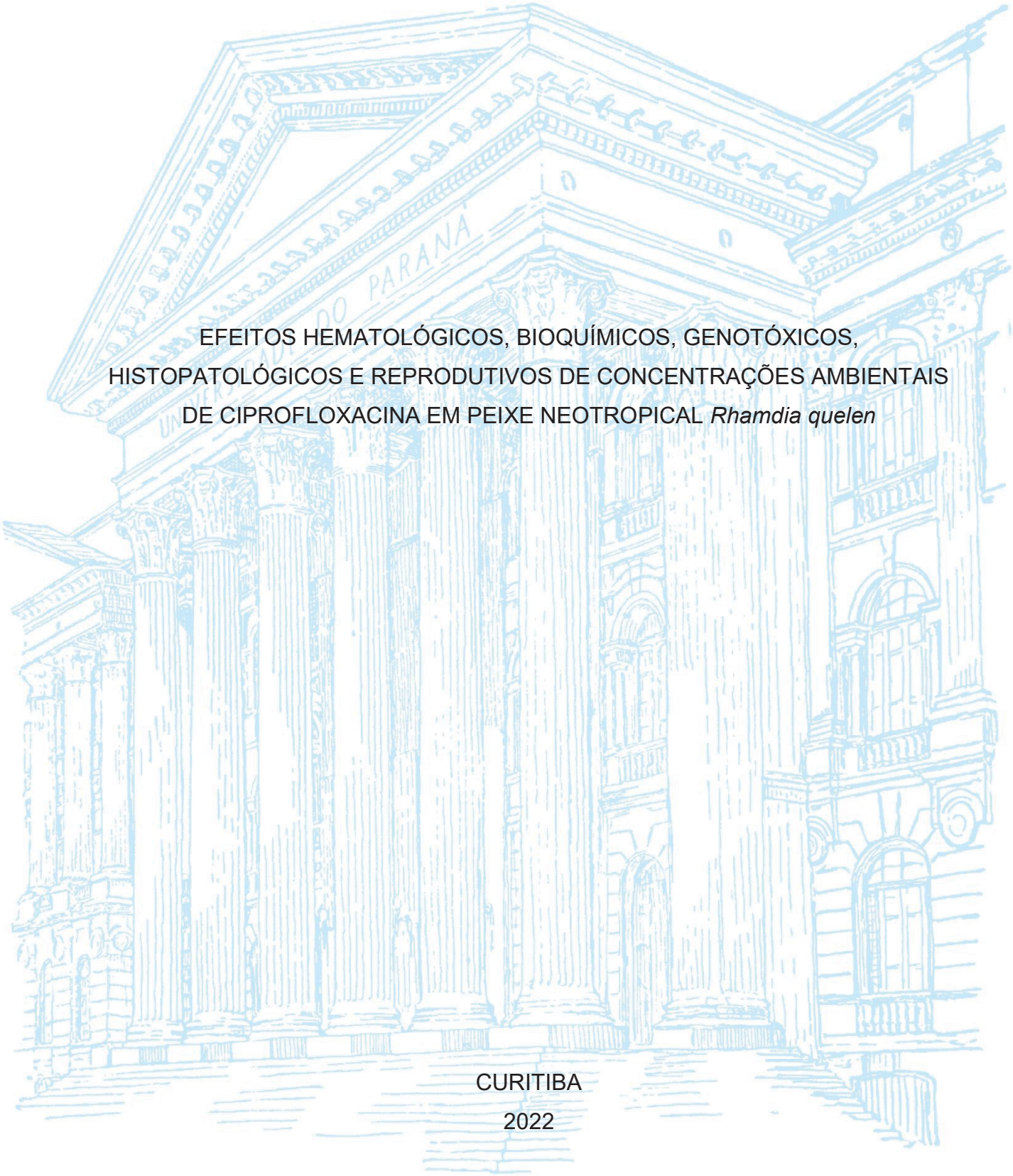
UNIVERSIDADE FEDERAL DO PARANÁ

LUCICLEIDE ÂNGELO SILVA



EFEITOS HEMATOLÓGICOS, BIOQUÍMICOS, GENOTÓXICOS, HISTOPATOLÓGICOS E REPRODUTIVOS DE CONCENTRAÇÕES AMBIENTAIS DE CIPROFLOXACINA EM PEIXE NEOTROPICAL *Rhamdia quelen*

CURITIBA

2022

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Tese apresentada ao curso de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Ecologia e Conservação.

Orientadora: Profa. Dra. Helena Cristina da Silva de Assis

Coorientadores: Prof. Tárcio Teodoro Braga e Profa. Dra. Keite Nogueira

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ECOLOGIA E CONSERVAÇÃO da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LUCICLEIDE ANGELO SILVA** intitulada: **EFEITOS HEMATOLÓGICOS, BIOQUÍMICOS, GENOTÓXICOS, HISTOPATOLÓGICOS E REPRODUTIVOS DE CONCENTRAÇÕES AMBIENTAIS DE CIPROFLOXACINA EM PEIXE NEOTROPICAL Rhamdia quelen**, sob orientação da Profa. Dra. HELENA CRISTINA DA SILVA DE ASSIS, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 19 de Setembro de 2022.

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Dedico essa tese de doutorado à Deus, que me guiou e me deu forças para chegar até aqui, aos meus pais (Lucineide e Assis), irmãos (Louyse e Thiago) e, principalmente, ao meu esposo (Denilson) pelo apoio e compreensão em todos os momentos da minha trajetória acadêmica.

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“Não espere por uma crise para descobrir o que é importante em sua vida.”

(PLATÃO)

RESUMO

A ciprofloxacina (CIP) é um antibiótico comumente encontrado em ambientes aquáticos, porém pouco se sabe sobre seu efeito em organismos não-alvo. O objetivo deste estudo foi avaliar o efeito da exposição prolongada a concentrações ambientais de CIP (1, 10 e 100 $\mu g.L^{-1}$) em peixes machos e fêmeas de *Rhamdia quelen*. Após 28 dias de exposição, os peixes foram anestesiados para retirada de sangue para avaliação de biomarcadores hematológicos, genotóxicos e hormonais. Após a eutanásia, o cérebro foi coletado para análise de biomarcadores bioquímicos e neurotransmissores, o músculo para a atividade da AChE. As brânquias, fígado, gônadas e rim posterior foram removidos para análise de biomarcadores bioquímicos, genotóxicos e histopatológicos. Índices somáticos também foram avaliados. Os resultados mostraram que a CIP causou estresse oxidativo nas brânquias de machos (à 1 e 100 $\mu g.L^{-1}$) e fêmeas (à 1 e 10 $\mu g.L^{-1}$). Além disso, a CIP (à 10 e 100 $\mu g.L^{-1}$) causou danos histopatológicos às brânquias, como o descolamento epitelial da lamela secundária e hiperplasia lamelar com fusão lamelar, de forma concentração-dependente. No sangue, diminuiu o número de leucócitos (à 10 e 100 $\mu g.L^{-1}$ CIP em fêmeas e à 100 $\mu g.L^{-1}$ em machos), aumentou o número de trombócitos (à 100 $\mu g.L^{-1}$ em machos), causou genotoxicidade (10 e 100 $\mu g.L^{-1}$ em machos e em todos os tratamentos na fêmea), houve alterações nucleares morfológicas (à100 $\mu g.L^{-1}$) e apoptose de eritrócitos (em machos à 10 e 100 $\mu g.L^{-1}$, e em fêmeas à 100 $\mu g.L^{-1}$). No cérebro, causou neurotoxicidade (à 10 e 100 $\mu g.L^{-1}$). No fígado, houve estresse oxidativo (à 1 e 100 $\mu g.L^{-1}$), genotoxicidade (nos machos à 1 $\mu g.L^{-1}$) e apoptose de hepatócitos (em todos os tratamentos nas fêmeas e à 1 $\mu g.L^{-1}$ nos machos). O índice histopatológico aumentou, com alterações como infiltração leucocitária, esteatose e foco necrótico (à 100 $\mu g.L^{-1}$). Nas gônadas, a maior concentração de CIP aumentou os oócitos vitelogênicos nas fêmeas e estimulou a espermatogênese nos machos. No rim posterior, CIP causou genotoxicidade (nas fêmeas à 1 e 100 $\mu g.L^{-1}$) e necrose de células renais em fêmeas (à 100 $\mu g.L^{-1}$). Alterou a histoarquitetura normal do parênquima renal, com alterações degenerativas, como infiltração leucocitária, presença de adipócitos no estroma, necrose e extensa área de hemorragia (à 10 $\mu g.L^{-1}$). A 100 $\mu g.L^{-1}$ observou-se a presença de adipócitos no estroma e necrose (nos machos) e necrose (nas fêmeas). Em adição, no tecido renal também houve aumento no Índice de Bernet e da incidência de Centros de Melanomacrófagos (MMCs) após a exposição dos peixes a 10 e 100 $\mu g.L^{-1}$. Houve diferenças nas respostas dos biomarcadores hematológicos, bioquímicos (estresse oxidativo) e de genotoxicidade em relação ao sexo. Portanto, esses dados mostraram que a CIP pode causar efeitos tóxicos à saúde de *Rhamdia quelen*, mesmo na menor concentração, geralmente presente em águas superficiais que podem ser utilizadas para abastecimento público. Houve diferenças nas respostas de biomarcadores hematológicos, bioquímicos e de genotoxicidade em relação ao sexo. Estas alterações podem ter efeitos em nível de organismo, que por sua vez, podem refletir no âmbito de população, podendo causar perturbações ecológicas.

Palavras-chave: Fluoroquinolona. Antibiótico. Biomarcadores. Toxicidade.

ABSTRACT

Ciprofloxacin (CIP) is an antibiotic commonly found in aquatic environments, however little is known about its effect on non-target organisms. This study aimed to evaluate the effect of prolonged exposure to environmental concentrations of CIP (1, 10, and 100 $\mu g.L^{-1}$) in male and female fish of *Rhamdia quelen*. After 28 days of exposure, the fish were anesthetized to remove blood for evaluation of hematological, genotoxic, and hormonal biomarkers. After euthanasia, the brain was collected for analysis of biochemical and neurotransmitter biomarkers, muscle for AChE activity. The gills, liver, gonads, and posterior kidney were removed for analysis of biochemical, genotoxic, and histopathological biomarkers. Somatic indices were also evaluated. The results showed that CIP caused oxidative stress in the gills of males (at 1 and 100 $\mu g.L^{-1}$) and females (at 1 and 10 $\mu g.L^{-1}$). Furthermore, CIP (at 10 and 100 $\mu g.L^{-1}$) caused histopathological damage to the gills, such as epithelial detachment of the secondary lamella and lamellar hyperplasia with lamellar fusion, in a concentration-dependent manner. In the blood, the number of leukocytes decreased (at 10 and 100 $\mu g.L^{-1}$ CIP in females and at 100 $\mu g.L^{-1}$ in males), increased the number of thrombocytes (at 100 $\mu g.L^{-1}$ in males), caused genotoxicity (10 and 100 $\mu g.L^{-1}$ in males and in all treatments in females), there were morphological nuclear alterations (à100 $\mu g.L^{-1}$) and erythrocyte apoptosis (in males at 10 and 100 $\mu g.L^{-1}$, females at 100 $\mu g.L^{-1}$). In the brain, it caused neurotoxicity (at 10 and 100 $\mu g.L^{-1}$). In the liver, there was oxidative stress (at 1 and 100 $\mu g.L^{-1}$), genotoxicity (in males at 1 $\mu g.L^{-1}$) and hepatocyte apoptosis (in all treatments in females and at 1 $\mu g.L^{-1}$ in males). The histopathological index increased, with alterations such as leukocyte infiltration, steatosis and necrotic focus (at 100 $\mu g.L^{-1}$). In the gonads, the highest concentration of CIP increased vitellogenic oocytes in females and stimulated spermatogenesis in males. In the posterior kidney, CIP caused genotoxicity (in females at 1 and 100 $\mu g.L^{-1}$) and renal cell necrosis in females (at 100 $\mu g.L^{-1}$). It altered the normal histoarchitecture of the renal parenchyma, with degenerative alterations, such as leukocyte infiltration, presence of adipocytes in the stroma, necrosis and extensive area of hemorrhage (at 10 $\mu g.L^{-1}$). At 100 $\mu g.L^{-1}$, the presence of adipocytes in the stroma and necrosis (in males) and necrosis (in females) were observed. In addition, in renal tissue there was also an increase in the Bernet Index and the incidence of Melanomacrophage Centers (MMCs) after fish exposure to 10 and 100 $\mu g.L^{-1}$. There were differences in the responses of hematological, biochemical (oxidative stress) and genotoxicity biomarkers in relation to sex. Therefore, these data showed that CIP can cause toxic health effects of *Rhamdia quelen*, even at the lowest concentration, usually present in surface water that can be used for public supply. There were differences in the responses of hematological, biochemical and genotoxicity biomarkers in relation to sex. These changes can have effects at the organism level, which in turn can reflect at the population level, and can cause ecological disturbances.

Keywords: Fluoroquinolone. Antibiotic. Biomarkers. Toxicity.

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LISTA DE ABREVIATURAS OU SIGLAS

AChE - Acetilcolinesterase
ALFAC - Álcool, formol, ácido acético
AMP - Adenosina 3,5 - monofosfato
AMR - Resistência Antimicrobiana
ANVISA - Agência Nacional de Vigilância Sanitária
ATP - Adenosina trifosfato
BChE - Butirilcolinesterase
BHT - Hidroxitolueno butilado
CAT - Catalase
CDC - Centro de Controle e Prevenção de Doenças dos Estados Unidos
ChE - Colinesterase
CIM - Concentração Inibitória Mínima
CIP - Ciprofloxacina
CMHC - Concentração Média de Hemoglobina Corpuscular
CYP450 - Citocromo P450
DA - Dopamina
DE_S - Desreguladores Endócrino
DDT - Diclorodifeniltricloroetano
DNA - Ácido desoxirribonucleico
DNPH - 2,4-dinitrofenilhidrazina
DOPAC - 3,4-dihidroxifenilacético
E2 - 17β-estradiol
EC_{50} - Concentração do fármaco que induz metade do efeito máximo
EC_S - Contaminantes Emergentes
EROD - Etoxiresorufina-O-deetilase
ERO - Espécies Reativas de Oxigênio
ETA - Estação de Tratamento de Água
ETAR - Estação de Tratamento de Águas Residuais
ETE - Estação de Tratamento de Esgoto
FSH - Hormônio folículo estimulante
GPx - Glutationa peroxidase
GnRH - Hormônio liberador de gonadotrofinas

GR - Glutationa redutase
GSH - Glutationa reduzida
GSSG - Glutationa oxidada
GST - Glutationa S-transferase
HPG - Eixo hipotálamo-pituitária-gônadal
HPLC - Cromatografia líquida de alta performance
Ht - Hematócrito
IGS - Índice gonadossomático
IHS - Índice hepatossomático
LH - Hormônio luteinizante
LPO - Lipoperoxidação
MAPA - Ministério da Agricultura, Pecuária e Abastecimento
MNP - Micronúcleo písceo
CSM - Concentração Seletiva Mínima
NA - Noradrenalina
ODS - Objetivos de Desenvolvimento Sustentável
OCDE - Organização para a Cooperação e Desenvolvimento Econômico
OIE - Organização Mundial da Saúde Animal
OMS - Organização Mundial de Saúde
PMD - Permeabilidade Mitocondrial Dependente de Ca2+
RAM - Resistência Antimicrobiana
RBC - Contagem de eritócitos
RNA - Ácido ribonucleico
ROS - Espécies reativas de oxigênio
SCGE - Single-Cell Gel Eletrophoresis
SNC - Sistema Nervoso Central
SOD - Superóxido dismutase
UNESCO - Organização das Nações Unidas para a Educação, a Ciência e a Cultura
VCM - Volume corpuscular médio
VCMH - Volume Corpuscular Médio Hemoglobina
WHO - World Health Organization
5-HIIA - Ácido 5-hidroxiindolacético
5-H - 5-hidroxitriptamina, serotonina

SUMÁRIO

1 APRESENTAÇÃO DA TESE

O presente trabalho contempla o trabalho de pesquisa de doutorado da discente Lucicleide Ângelo do Programa de Pós-Graduação em Ecologia e Conservação da UFPR. Inicialmente, apresenta-se uma introdução geral acerca do assunto no qual a pesquisa está inserida, por conseguinte os objetivos gerais e específicos que nortearam o trabalho, e as hipóteses a serem testadas. Os resultados do estudo geraram dois capítulos referentes à duas publicações futuras.

O primeiro capítulo, intitulado *Hematological, biochemical, genotoxic, and histopathological changes in males na females catfish* (*Rhamdia quelen*) *after exposure to environmental concentrations of ciprofloxacin*, contempla os resultados da investigação do efeito da CIP quanto a neurotoxicidade cerebral, alteração de parâmetros hematológicos e genotoxidade em células sanguíneas, estresse oxidativo, genotoxicidade e alterações histopatológicas em fígado (metabolização) e gônadas. Também foi investigado se a CIP altera parâmetros relacionados à reprodução, como neurotransmissores e hormônios relacionados ao eixo hipotalâmico-hipofisário-gonadal.

O segundo capítulo, intitulado *Effect of environmental concentrations of ciprofloxacin on gills and posterior kidney of male and female fish* (*Rhamdia quelen*), teve como objetivo avaliar o efeito da CIP sob o ponto de vista da absorção e excreção, portanto foram avaliadas as brânquias e o rim posterior quanto aos biomarcadores bioquímicos, de genotoxicidade e histopatológicos.

Por fim, apresenta-se algumas considerações finais abordando os resultados da pesquisa de forma unificada e as recomendações para trabalhos futuros.

2 INTRODUÇÃO GERAL

Resíduos de produtos farmacêuticos têm sido quantificados em ambientes aquáticos em todo o mundo (Miller et al., 2018; Pashaei et al., 2022; Veiga-Gómez et al., 2017) e têm atraído cada vez mais a atenção da comunidade científica (Madikizela & Ncube, 2022; Zhou et al., 2022).

Os antibióticos, uma classe de produtos farmacêuticos, têm recebido atenção crescente devido ao seu amplo uso, distribuição ambiental ubíqua, grande potencial de bioacumulação e possível toxicidade para organismos não-alvo (Madikizela & Ncube, 2022; Rodrigues et al., 2019; Yang et al., 2020a). Além disso, indiretamente representam riscos à saúde humana, pois podem influenciar o desenvolvimento de resistência a antibióticos em patógenos (Guo et al., 2022; Qin and Liu, 2013a).

A resistência antimicrobiana (RAM) é uma grande preocupação de saúde pública em todo o mundo (CDC, 2019; WHO; ECDC, 2022), inclusive no Brasil (ANVISA, 2017; BRASIL, 2018). Estimativas da União Europeia/Espaço Econômico Europeu (UE/EEE) mostram que a cada ano mais de 670.000 infecções são causadas por bactérias resistentes a antibióticos e aproximadamente 33.000 pessoas morrem como consequência direta (WHO; ECDC, 2022). Nos Estados Unidos, 48.700 pessoas morreram a cada ano por resistência a antibióticos (CDC, 2019). Portanto, hoje em dia, a RAM é reconhecida como uma grande ameaça à saúde global (CDC, 2019; WHO, 2017, 2021; WHO; ECDC, 2022).

Estudos têm demonstrado que os antibióticos são considerados contaminantes "pseudo persistentes" devido a sua liberação contínua no meio ambiente (Kazakova et al., 2018; Yang et al., 2017) como resultado de várias atividades humanas, descarga de águas residuais, escoamento agrícola ou descarte inadequado de medicamentos não utilizados. Todas estas vias de entrada associadas à eficiência limitada das estações de tratamento de águas residuais (ETAR) e estações de tratamento de água (ETA) significam que as entradas destas substâncias são contínuas (Gao et al., 2012; Janecko et al., 2016; Liu et al., 2022).

As fluoroquinolonas estão entre os antibióticos frequentemente detectados no ambiente aquático em concentrações relativamente altas que variam de $ng.L^{-1}$ a $\mu g\ L^{-1}$ (Kelly and Brooks, 2018; Teglia et al., 2019). Entre as fluoroquinolonas, a ciprofloxacina (CIP) é um dos antibióticos mais utilizados em todo o mundo (Kelly and Brooks, 2018; Sahlin et al., 2018). É uma fluoroquinolona de segunda geração,

exibindo um amplo espectro de atividade contra bactérias aeróbicas gram-negativas e gram-positivas (González-Pleiter et al., 2013; Sahlin et al., 2018).

Estudos já mostraram que a CIP não é biodegradável (Sahlin et al., 2018) e, também, pode resistir à degradação no meio ambiente (Flach et al., 2018; Xie et al., 2017). A ciprofloxacina já foi detectada em águas superficiais, subterrâneas, águas residuais, efluentes, sedimentos, solo e até mesmo água potável (Gao et al., 2012; Wagil et al., 2014; Xie et al., 2017). Além disso, estudos também detectaram CIP em tecidos de peixes (Beijer et al., 2013; Sehonova et al., 2019; Zhang et al., 2016a).

Na medicina veterinária, embora em alguns países a CIP não esteja mais incluída na lista de antibióticos veterinários, como em países da União Europeia (Idowu et al., 2010), a ciprofloxacina é um metabólito ativo do antibiótico veterinário enrofloxacina (Singha & Ahn, 2016). Portanto, as concentrações de ciprofloxacina presentes no ambiente podem ser provenientes de uso humano e veterinário.

Apesar da disseminação da CIP em ambientes aquáticos do mundo inteiro (Frade et al., 2014; Janecko et al., 2016; Larsson et al., 2007; Mutiyar and Mittal, 2014; O'Flaherty and Cummins, 2016; Pal et al., 2010; Quadra et al., 2017; Riaz et al., 2017), pouco se sabe sobre o efeito da exposição ao CIP na biota aquática. Até agora, poucos estudos foram realizados sobre o efeito deste antibiótico em peixes (Kitamura et al., 2022; Plhalova et al., 2014; Ramesh et al., 2018).

Desta forma, a aplicação de biomarcadores para investigar os efeitos tóxicos de contaminantes ambientais sobre a biota aquática é ferramenta importante. A utilização de biomarcadores em diferentes níveis é essencial para estabelecer sinais de alerta precoce, tendo em vista que os efeitos em níveis hierárquicos superiores (como população, comunidade e ecossistema) são sempre precedidos por mudanças anteriores nos processos biológicos (Van der Oost et al., 2003). Além disso, o uso de biomarcadores é uma ferramenta importante para estimar os efeitos de um contaminante no cenário *One Health*. One Health é uma abordagem colaborativa, multidisciplinar e multissetorial que pode abordar ameaças urgentes, contínuas ou potenciais à saúde na interface homem-animal-ambiente nos níveis subnacional, nacional, global e regional (FAO et al., 2019). A sua utilização permite avaliar a qualidade ambiental e o potencial dos contaminantes ambientais para causar danos aos animais, e predizer os efeitos indesejáveis na saúde humana.

Nesta perspectiva, este estudo pretende contribuir positivamente na aquisição de conhecimento nesta área de pesquisa, investigando os possíveis efeitos de

concentrações ambientais de um antibiótico, comumente presente em corpos hídricos, na saúde de peixes neotropicais. Evidenciando assim, os possíveis efeitos tóxicos para saúde animal, e fornecendo informações que ajudarão a predizer possíveis riscos do ponto de vista ecológicos e *One Health*.

2.1 PRESENÇA DE FÁRMACOS NO AMBIENTE E ASPECTOS LEGAIS

A presença de resíduos farmacêuticos no ambiente está se tornando um problema e uma das maiores preocupações das autoridades nacionais de saúde pública (Comber et al., 2018; Desbiolles et al., 2018; Ebele et al., 2017). A maior fonte da entrada de produtos farmacêuticos no ambiente é o resíduo do uso humano ou veterinário. Uma vez consumidos, os fármacos são metabolizados e parte da dose inicial é excretada inalterada, sendo a maior porcentagem excretada na forma de metabólitos; tanto o composto original quanto os metabólitos são excretados via fezes e urina em humanos e animais (Jjemba, 2006). Em adição, os sistemas de tratamento convencionais não são eficientes na remoção destes compostos (Desbiolles et al., 2018). Desta forma, resíduos de fármacos estão cada vez mais presentes nos ambientes aquáticos e são quantificados em níveis de concentração de $ng.L^{-1}$ à $\mu g.L^{-1}$ em águas de esgoto e vários corpos receptores de água, tais como água doce superficial, águas subterrâneas e água do mar (Desbiolles et al., 2018; Ebele et al., 2017; Kelly and Brooks, 2018; Yang et al., 2017).

Carvalho & Santos (2016) em sua revisão sobre antibióticos em ambientes aquáticos evidenciaram que a classe das quinolonas, está entre as mais analisadas e detectadas em ambientes aquáticos, devido à sua importância para a medicina e persistência no meio aquoso. A ciprofloxacina pertence a classe das quinolonas e é um dos antibióticos mais usados mundialmente (Kelly and Brooks, 2018; Sahlin et al., 2018).Níveis elevados de CIP já foram relatados em vários países como Alemanha, Suíça, Estados Unidos, Japão Polônia, Austrália, Índia, Espanha, Brasil, Itália, França, Grécia, Suécia, Canadá, Paquistão, Coreia do Norte e China (Frade et al., 2014; Janecko et al., 2016; Larsson et al., 2007; Mutiyar and Mittal, 2014; O'Flaherty and Cummins, 2016; Pal et al., 2010; Quadra et al., 2017; Riaz et al., 2017).

Nos quadros a seguir apresenta-se uma média das concentrações ambientais de CIP, conforme pesquisa bibliográfica dos artigos publicado até o momento, divididos em tipos de ambientes aquáticos, sendo águas superficiais (rios) (QUADRO

1), efluentes de estações de tratamento de água potável e águas residuais (QUADRO 2) e efluentes hospitalares ou de industriais farmacêuticas (QUADRO 3).

QUADRO 1- CONCENTRAÇÕES MÉDIAS DE CIPROFLOXACINA EM AMBIENTES DE ÁGUAS SUPERFICIAIS.

Tipos de ambiente	Conc. (µg. L^{-1})	Local	Referência	Média ± Desvio Padrão
Água superficial	0,50	Água superficial na Alemanha	Frade et al. 2014	1,82 ± 3,02
	0,06	Água superficial na Alemanha		
	0,02	Água superficial na Suíça		
	0,03	Água superficial nos EUA		
	6,30	Água superficial na Europa		
	0,57	Água superficial no Japão		
	2,70	Água superficial da Polônia		
	1,30	Água superficial da Austrália		
	0,02	Água superficial na Suíça		
	10,00	Água superficial na Índia	Muttyar Mittal, 2014	
	0,23	Água superficial na Espanha	O'Flarherty, 2016	
	0,12	Água superficial no Brasil	Quadra et al. 2017	

FONTE: Frade et al. (2014); Janecko et al. (2016); Mutiyar & Mittal (2014); O'Flaherty & Cummins (2016) ; Pal et al. (2010; Quadra et al. (2017).

QUADRO 2- CONCENTRAÇÕES MÉDIAS DE CIPROFLOXACINA EM EFLUENTES DE ESTAÇÕES DE TRATAMENTO DE ÁGUA POTÁVEL E ESGOTO.

Tipos de ambiente	Conc. (µg. L^{-1})	Local	Referência	Média ± Desvio Padrão
Afluente e Efluentes de Estações de tratamento de água potável e águas residuais	0,26	Águas residuais na Itália	Frade et al. 2014	5,87 ± 18,23
	0,10	Efluente de estação de tratamento de água na Itália		
	0,06	Efluente de estação de tratamento de água na França		
	0,07	Efluente de estação de tratamento de água na Grécia		
	0,03	Efluente de estação de tratamento de água na Suécia		
	0,12	Efluente de estação de tratamento de água na Suíça		
	0,60	Efluente de estação de tratamento de água na Alemanha		
	0,43	Afluente de estação de tratamento de água na Suíça		
	0,40	Efluente de estação de tratamento de água no Canadá		
	0,16	Efluente de estação de tratamento de água nos EUA		
	0,51	Efluente de estação de tratamento de água na Itália		
	0,36	Efluente de estação de tratamento de água na Holanda		
	0,60	Efluente de estação de tratamento de água na França		
	0,09	Afluente de estação de tratamento de água na República Tcheca	Pal et al. 2010	
	1,10	Efluente de estação de tratamento de água na América do Norte		
	3,35	Efluente de estação de tratamento de água na Europa		
	0,70	Efluente de estação de tratamento de água na Ásia		
	34,60	Águas residuais na Coreia do Norte	Riaz et al. 2017	
	0,35	Águas residuais no Paquistão		
	2,20	Águas residuais no Paquistão		
	82,80	Águas residuais na China		
	0,20	Águas residuais no Brasil		

FONTE: Frade et al. (2014); Pal et al. (2010); Riaz et al., (2017).

QUADRO 3- CONCENTRAÇÕES MÉDIAS DE CIPROFLOXACINA EM EFLUENTES HOSPITALARES E INDÚSTRIAS FARMACÊUTICAS.

Tipos de ambiente	Conc. (µg. L^{-1})	Local	Referência	Média ± Desvio Padrão
Efluentes de hospitais e indústrias farmacêuticas	87,00	Efluente de hospital na Suíça	Frade et al. 2014	104,43 ± 127,06
	14,50	Efluente de hospital na Suíça		
	124,50	Efluente de hospital na Suíça		
	2,30	Efluente de hospital na Alemanha		
	101,00	Efluente de hospital na Suécia		
	15,00	Efluente de hospital na Suécia		
	31,00	Efluente de hospital na China	Muttyar Mittal 2014	
	236,60	Efluente de Hospital na China		
	8,37	Efluente de hospital na Espanha	O'Flaerherty 2016	
	424,00	Efluente industrial no Paquistão	Riaz et al. 2017	

FONTE:Mutiyar & Mittal (2014); Frade et al. (2014); O'Flaherty & Cummins (2016); Riaz et al. (2017).

Em 2015, na Conferência Internacional sobre Gestão de Produtos Químicos (ICCM), a indústria farmacêutica e órgãos não governamentais concordaram que o meio ambiente agora requer proteção contra a “poluição farmacêutica”. Na ocasião, publicaram um artigo enfatizando que a maioria das nações tem controles rígidos sobre os resíduos ambientais, do arsênico ao zinco. No entanto, nenhum limite legal foi estabelecido para controlar a poluição de drogas durante sua fabricação, uso e descarte. (Time to get clean. Nature, 2015).

Adicionalmente, a *Water Framework Directive* da União Européia incluiu produtos farmacêuticos em uma “lista de vigilância” dinâmica com base no potencial de efeitos adversos no ambiente aquático. Esta lista inclui inseticidas, herbicidas, protetor solar, vários antibióticos, alguns hormônios naturais e dois produtos farmacêuticos (17-alfa-etinilestradiol (EE2) da pílula anticoncepcional e diclofenaco), sob a Diretiva de Padrões de Qualidade Ambiental e estão sujeitos a monitoramento europeu (Carvalho and Santos, 2016b).

Em 2017, a Organização das Nações Unidas para Educação, Ciência e Cultura (UNESCO) lançou um relatório sobre “poluentes emergentes na água”. O relatório resume a ocorrência, concentrações e vias de entrada de produtos farmacêuticos no ambiente na região do Mar Báltico (UNESCO, 2017).

Em 2019, a comissão Europeia publicou um relatório tendo como título “Abordagem Estratégica da União Europeia relativa aos Produtos Farmacêuticos no Ambiente”, enfatizando entre outras coisas, que o tratamento de muitas doenças nos seres humanos e nos animais depende do acesso a produtos farmacêuticos eficazes e que isto estaria em risco diante desta problemática ambiental (COM, 2019).

Os estudos têm demonstrado efeitos tóxicos em animais decorrentes da exposição a alguns produtos farmacêuticos detectados em baixa concentração na água e no solo, mesmo em concentrações ínfimas (Elizalde-Velázquez et al., 2022; Fent, 2015; Madikizela and Ncube, 2022; Niemuth et al., 2015)

De modo específico, referente à problemática da crescente ameaça de resistência antimicrobiana, a Organização Mundial de Saúde em 2012 publicou um relatório (OMS, 2012) que contemplava opções de ações para combater este problema. Nos anos seguintes, o tema foi ganhando relevância e outros relatórios foram sendo publicados, como o *Antimicrobial Resistance - Global Report on Surveillance* (WHO, 2014), publicado periodicamente. O tema passou

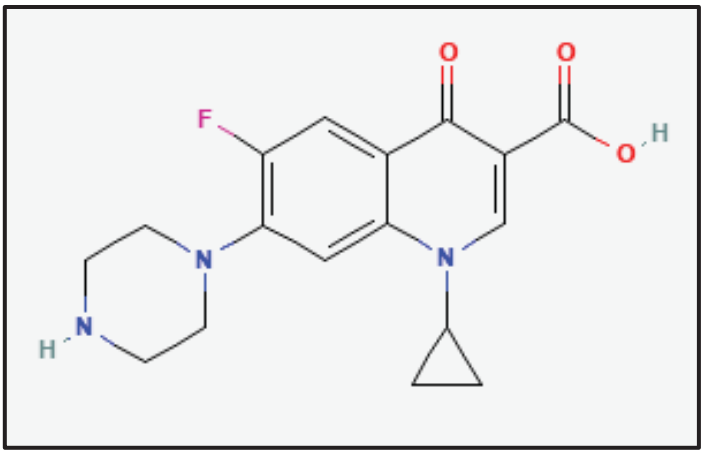
a ser defendido por diferentes instituições como OIE (Organização Mundial da Saúde Animal), OCDE (Organização para a Cooperação e Desenvolvimento Econômico), entre outras. No Brasil, em 2017 foi publicado um Plano Nacional para a Prevenção e Controle da Resistência Microbiana nos Serviços de Saúde com planos estratégicos e operacionais para combater o problema (ANVISA, 2017). Neste mesmo ano, também foi publicada a Instrução Normativa Nº 09, de 21 de fevereiro de 2017 (MAPA, 2017), nela o Ministério da Agricultura, Pecuária e Abastecimento estabelece critérios de amostragem e limites de referência para utilização de antibióticos em produtos de origem animal.

Nesse sentido, percebe-se uma evolução na compreensão desta problemática e na importância da realização de mais estudos nesta área. No entanto, ainda existe uma lacuna sobre os possíveis efeitos dos resíduos de antibióticos na saúde animal, no ambiente e na saúde humana. Desse modo, o presente estudo pretende contribuir com conhecimento na área de toxicologia ambiental e ecotoxicologia, somando-se a muitos outros estudos que ajudaram a formar uma base sólida de conhecimento. Em adição, espera-se contribuir com conhecimento para os tomadores de decisão no aperfeiçoamento de planos de gestão e a criação de legislações específicas.

2.2 CIPROFLOXACINA

A ciprofloxacina (FIGURA 1) é um antibiótico da classe das fluoroquinolonas de segunda geração, amplamente utilizado na terapia de infecções do trato urinário e respiratório leves a moderadas causadas por organismos suscetíveis (Fisher et al., 1989).

FIGURA 1 – ESTRUTURA QUÍMICA DA CRIPROFLOXACINA



FONTE: PubChem CID: 2764. Fórmula molecular: C17H18FN3O3

2.2.1 Mecanismo de ação

Tem como alvo específico a topoisomerase DNA girase tipo II e a topoisomerase IV, interrompendo a replicação, transcrição, reparo e recombinação do DNA em bactérias (Fisher et al., 1989). Já se sabe que as quinolonas interagem de forma diferente com a enzima eucariótica topoisomerase II, principalmente por causa de diferenças na estrutura do DNA, portanto, o potencial de efeitos genotóxicos em eucariotos é consideravelmente menor em comparação com organismos procarióticos (Toolaram et al., 2016). Apesar disso, acredita-se que o mecanismo de ação de CIP em organismos eucarióticos seja o mesmo que em bactérias, resultando em quebras de fita de DNA que, se não reparadas, podem levar a citotoxicidade (Lynch et al., 2003). Exposições repetidas a agentes citotóxicos podem causar danos celulares crônicos, proliferação celular compensatória, hiperplasia, eventualmente originar algum tipo de tumor ou causar a morte celular (Mosavat et al., 2022). No entanto, esse mecanismo em eucariotos é menos claro (Sahlin et al., 2018).

2.2.2 Absorção, distribuição, metabolismo e excreção

A ciprofloxacina é rapidamente absorvida no trato gastrointestinal após administração oral, e o composto sofre metabolismo mínimo de primeira passagem (Al-Omar, 2005). A biodisponibilidade absoluta da ciprofloxacina oral está dentro de uma faixa de 70-80%, sem perda substancial pelo metabolismo

de primeira passagem (Sharma et al., 2010). A ciprofloxacina é amplamente distribuída nos tecidos e líquidos corporais após administração oral e intravenosa. Concentrações mais altas da droga geralmente são encontradas na bile, pulmão, rim, fígado, vesícula biliar, útero, líquido seminal, tecido e líquido prostático, tonsilas, endométrio, tuba uterina e ovários (Al-Omar, 2005).

A ciprofloxacina tem boa penetração em vários fluidos e tecidos do corpo, exceto no sistema nervoso central (SNC), após administração oral. Um nível de droga notável é alcançado no rim, próstata, fígado e pulmão (Sharma et al., 2010). A concentração urinária do fármaco é superior à concentração inibitória mínima, por isso é usado principalmente em infecções do trato urinário (Sharma et al., 2010). O perfil farmacocinético da ciprofloxacina está resumido na TABELA 1.

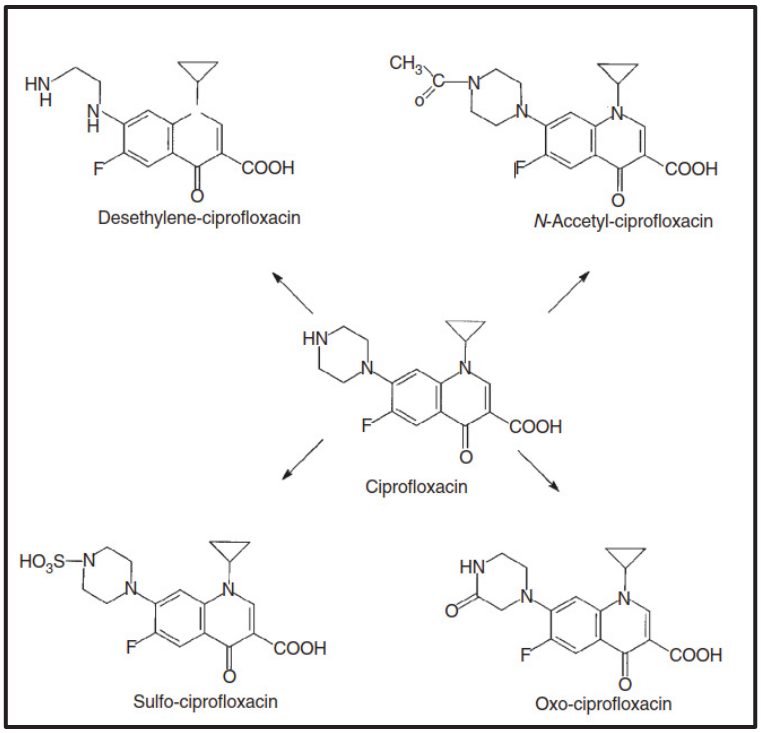
TABELA 1- PROPRIEDADES FARMACOCINÉTICAS DA CIPROFLOXACINA.

Parâmetro farmacocinético	Valor
Meia-vida de eliminação (h)	4,16
Biodisponibilidade oral	70-80%
Concentração máxima da droga no plasma (mg/L)	0,56
Via primária de excreção	Renal
Tempo para o pico (h)	1,1
Depuração renal (L/h)	21,4
Disposição (% of dose)	
Renal	40 – 60%
Fecal/biliar	15
Metabolizada	10 - 15

FONTE: Sharma et al., 2010

A ciprofloxacina é eliminada por mecanismos renais e não renais (Al-Omar, 2005; Sharma et al., 2010). O fármaco é parcialmente metabolizado no fígado pela modificação do grupo piperazinil em pelo menos quatro metabólitos (FIGURA 2) (Al-Omar, 2005; Sharma et al., 2010). Os quatro metabólitos ativos foram identificados, como desetileno ciprofloxacina, sulfo-ciprofloxacina, oxo-ciprofloxacina e N-acetil-ciprofloxacina (Al-Omar, 2005).

FIGURA 2 – CIPROFLOXACINA E SEUS METABÓLITOS



FONTE: (Al-Omar, 2005)

A oxo-ciprofloxacina parece ser o principal metabólito urinário e a sulfo-ciprofloxacina o metabólito fecal primário (Al-Omar, 2005). As atividades microbianas de oxo-ciprofloxacina e N-acetil-ciprofloxacina são comparáveis à norfloxacina, e a desetileno-ciprofloxacina é comparável ao ácido nalidíxico para certos organismos (Al-Omar, 2005). A eliminação da CIP e seus metabólitos ocorre principalmente via renal por filtração glomerular e secreção tubular (Al-Omar, 2005; Sharma et al., 2010). Após a administração oral de uma dose única de 250, 500 ou 750 mg em adultos com função renal normal, 15 a 50% da dose é excretada na urina como droga inalterada e 10 a 15% como metabólitos em 24 horas. 20-40% da dose é excretada nas fezes como droga inalterada e metabólitos em 5 dias (Al-Omar, 2005).

2.2.3 Ecotoxicidade da ciprofloxacina

A ciprofloxacina não está incluída na lista de antibióticos veterinários para venda na União Europeia (EMA, 2015). No entanto, é um metabólito ativo do antibiótico veterinário enrofloxacina (Idowu et al., 2010). Consequentemente,

as concentrações de ciprofloxacina nas águas superficiais podem ter origem tanto no uso humano quanto no veterinário.

Gullberg et al. (2011) mostraram em experimentos de competição pareada com cepas de *E. coli* que a seleção para bactérias resistentes ocorreu até 0,23 $\mu g.L^{-1}$, que foi 100 vezes abaixo da Concentração Inibitória Mínima (CIM) da cepa suscetível. Uma CIM de 0,1 $\mu g.L^{-1}$ foi estimada com base na extrapolação entre os pontos de dados. Da mesma forma, Liu et al. (2011) usaram testes de competição de tipo selvagem e resistente cepas de *E. coli*, que resultou na seleção para resistência a 3 $\mu g.L^{-1}$, aproximadamente 1/5 da concentração de CIM.

Vários estudos mostraram que a ciprofloxacina pode modificar a estrutura da comunidade microbiana (ou seja, abundância e diversidade) na água, sedimento e solo (Cui et al., 2014; Girardi et al., 2011; Gonzalez-Martinez et al., 2014; Näslund et al., 2008). Esses eventos podem inibir os principais processos ambientais mediados por microrganismos, como regeneração de nutrientes, ciclos de carbono e nitrogênio e degradação de poluentes (Girardi et al., 2011). Além disso, estudos já enfatizaram que cianobactérias e macrófitas também são sensíveis à ciprofloxacina com EC_{50} de 36,3 e 174 $\mu g.L^{-1}$ e EC_{10} de 4,47 e 149 $\mu g.L^{-1}$ (Robinson et al., 2001; Sahlin et al., 2018).

Em peixes, nenhum efeito letal foi observado em estudos agudos ou crônicos. Segundo estudo de Zivna et al. (2016), o crescimento dos peixes foi significativamente afetado em 1000 $\mu g.L^{-1}$, com aumento do comprimento e peso de *Cyprinus carpio* (fase inicial). Zivna et al. (2016) também relataram maior taxa de eclosão em todas as concentrações (1-3000 $\mu g.L^{-1}$), desenvolvimento reduzido em alguns estágios de larvas em 1-500 $\mu g.L^{-1}$ e desenvolvimento acelerado em 1000-3000 $\mu g.L^{-1}$. Além disso, Plhalova et al. (2014) e Zivna et al. (2016) investigaram a atividade de alguns marcadores de estresse oxidativo e atividade enzimática em peixe. Os resultados foram dispersos e os efeitos relatados nem sempre foram relacionados à dose-resposta. Ramesh et al. (2021) relataram que o nível de LPO foi elevado nos grupos de tratamento com ciprofloxacina durante todo o período de estudo (exceto Tratamento II de 1,5 $\mu g.L^{-1}$ no décimo dia em tecidos renais). Uma série de alterações histológicas foi observada nos tecidos branquiais, hepáticos e renais dos grupos tratados com

CIP. Em adição, a exposição à CIP causou uma diminuição significativa dos níveis de sódio, potássio e cloreto no plasma do peixe *C. mrigala*.

Em *Rhamdia quelen,* após exposição aguda de 96h à ciprofloxacina foram relatados alterações hematológicas e danos histopatológicos no fígado e rim posterior (principalmente a 100 $\mu g.L^{-1}$) (Kitamura et al., 2022).

Esses dados evidenciam a importância da realização de mais estudos acerca da compreensão dos possíveis impactos negativos da presença de resíduos de CIP em ambientes aquáticos. Curiosamente, observa-se uma carência em estudos que tenham investigado o efeito da CIP em peixes machos e fêmeas. Do ponto de vista ambiental, este tipo de investigação pode evidenciar efeitos fisiológicos que podem resultar em impactos ecológicos, como por exemplo, uma alteração em parâmetros da reprodução pode impactar diretamente a população da espécie estudada.

2.3 MODELO DE ESTUDO

Os peixes são usados como modelos experimentais em toxicologia ambiental, em genética, em pesquisa de câncer, em biomedicina, em neurobiologia, em endocrinologia, em ecologia, em gerontologia, em biologia do desenvolvimento, em aquicultura e em geral como ferramenta para obter informações básicas em biologia (Bolis et al., 2001). A vantagem do modelo animal é representada pelo fato de que as diferentes espécies possuem características específicas que as tornam relevantes para diferentes áreas de estudos científicos e tecnológicos em saúde, como por exemplo para o desenvolvimento de vacinas, medicamentos, kits de diagnósticos e estudos toxicológicos (Bera et al., 2020; More et al., 2021). Entre elas, representatividade regional e local, importância socioeconômica e alimentar, e dependendo de alguns peixes como o *Danio rerio*, similaridade genética com os humanos.

Além disso, os peixes podem ser encontrados em praticamente todos os ambientes aquáticos e desempenham um papel ecológico importante nas teias alimentares aquáticas devido à sua função como transportador de energia dos níveis tróficos inferiores aos superiores (Beyer et al., 1996). A compreensão da absorção, comportamento e respostas de substâncias tóxicas em peixes pode, portanto, ter uma grande relevância ecológica (Beyer et al., 1996).

Desbiolles et al. (2018), realizaram uma investigação sobre a quantidade de artigos científicos que discutem a ecotoxicidade de produtos farmacêuticos (exceto hormônios), classificando-os com base em níveis tróficos e organismos usados. Segundo os autores, dos 80 artigos científicos (1993-2018) apenas 9% utilizaram o peixe. Dentre eles, as espécies mais utilizadas foram o *Oryzias latipes* (3%), *Danio rerio* (3%), *Pimephales promelas* (1%), *Oncorhynchus mykiss* (1%) e *Poecillia reticulata* (1%). Desta forma, salienta-se a carência de informações dentro desta temática, e com espécies de peixes com representatividade local, como o peixe neotropical *Rhamdia quelen*.

2.3.1 Rhamdia quelen

Rhamdia quelen (FIGURA 3), também conhecido popularmente como jundiá, é um peixe teleósteo, da ordem Siluriformes, família Heptapteridae, que possui ampla ocorrência desde o centro da Argentina até o Sul do México (Gomes et al., 2000). Os Silurídeos, também conhecidos como bagres, são um dos grupos de peixes mais representativos da América do Sul (Gutiérrez-Espinosa et al., 2019). É uma espécie que vive em lagos e poços fundos dos rios, preferindo os ambientes de águas mais calmas com fundo de areia e lama, junto às margens e vegetação. Adultos de *Rhamdia quelen* são omnívoros, com uma clara preferência por peixes, crustáceos, insetos, restos vegetais, e detritos orgânicos (Gomes et al., 2000).

FIGURA 3 – PEIXE NEOTROPICAL *Rhamdia quelen.*

FONTE: Próprio autor.

Rhamdia quelen pode suportar invernos frios e pode crescer rapidamente no verão. O seu crescimento aumenta com o incremento da temperatura. Esse crescimento é bastante pronunciado nos primeiros anos de vida (Gomes et al., 2000). A maturidade sexual é atingida quando os machos com 13,4cm iniciam o processo de maturação gonadal e as fêmeas com 16,5cm (Tavares-Dias et al., 2002). Essa maturidade ocorre no primeiro ano de vida, o que favorece muito a reprodução (Montanha et al., 2014a). A taxa de crescimento dos machos é maior do que a das fêmeas até o terceiro ou quarto ano de vida, quando a situação se inverte, pois estas passam a crescer mais rapidamente (Gomes et al., 2000).

Devido suas características têm sido utilizados para a produção em piscicultura intensiva (Barcellos et al., 2001a), inclusive no Brasil. Em sistemas de aquicultura, com uma densidade de dois a quatro peixes por metro quadrado, atingirá 600-800 g de peso corporal em 8 meses (Barcellos et al., 2001a).

Por apresentar representatividade local e importância econômica, a espécie *Rhamdia quelen* tem se destacado como modelo animal de diversas pesquisas em diferentes áreas, entre elas toxicologia ambiental, conforme observado no (QUADRO 4).

QUADRO 4- REVISÃO BIBLIOGRÁFICA DOS ESTUDOS CIENTÍFICOS UTILIZANDO *Rhamdia quelen* COMO MODELO ANIMAL (2000-2022).

Autores	Artigo
Santos et al. (2022)	Sublethal effects of environmental concentrations of caffeine on a neotropical freshwater fish
Vicentini et al. (2022)	Effects of cadmium on the female reproductive axis of a Neotropical fish
Kitamura et al. (2022)	Sublethal biochemical, histopathological and genotoxicological effects of short-term exposure to ciprofloxacin in catfish *Rhamdia quelen*
Fernandes et al. (2021)	Cloning, partial sequencing and 17β- estradiol modulation of hepatic vitellogenin gene of the Neotropical catfish *Rhamdia quelen*
Franco et al. (2021)	The influence of dietary Motore™ supplement on antioxidant status to *Aeromonas hydrophila* infection in *Rhamdia quelen*
Pereira et al. (2020)	The Chelating Mineral on Organic Acid Salts Modulates the Dynamics and Richness of the Intestinal Microbiota of a Silver Catfish *Rhamdia quelen*
Ittzés et al. (2020)	Propagation of Jundiá *Rhamdia quelen* (Siluriformes: Heptapteridae) by applying the ovarian sperm injection method
Lacerda et al. (2019)	Duration of spermatogenesis and identification of spermatogonial stem cell markers in a Neotropical catfish, Jundiá (*Rhamdia quelen*)
Bandeira Junior et al. (2019)	*Aeromonas hydrophila* infection in silver catfish causes hyperlocomotion related to stress
Perussolo et al. (2019)	Integrated biomarker response index to assess toxic effects of environmentally relevant concentrations of paracetamol in a neotropical catfish (*Rhamdia quelen*)

Autores	Artigo
Cunha et al. (2018)	The antibacterial and physiological effects of pure and nano encapsulated Origanum majorana essential oil on fish infected with *Aeromonas hydrophila*
Mathias et al. (2018)	Effects of low concentrations of ibuprofen on freshwater fish *Rhamdia quelen*
Santos et al. (2017)	*Aloysia triphylla* essential oil as additive in silver catfish diet: Blood response and resistance against *Aeromonas hydrophila* infection
Guiloski et al. (2017a).	Paracetamol causes endocrine disruption and hepatotoxicity in male fish *Rhamdia quelen* after subchronic exposure
Ribas et al. (2017)	Inhibition of immune responses and related proteins in *Rhamdia quelen* exposed to diclofenac
Moura Costa et al. (2016)	Characterization, specificity and sensibility of produced anti-*Rhamdia quelen* vitellogenin in Brazilian fish species
Murussi et al. (2015)	Integrated Assessment of Biomarker Response in Carp (*Cyprinus carpio*) and Silver Catfish (*Rhamdia quelen*) Exposed to Clomazone
Muñoz et al. (2015)	Histopathological biomarkers in juvenile silver catfish (*Rhamdia quelen*) exposed to a sublethal lead concentration
Montanha et al. (2014b)	Clinical, biochemical and hematological effects in *Rhamdia quelen* exposed to cypermethrin
Mela et al. (2013)	Effects of the herbicide atrazine in neotropical catfish (*Rhamdia quelen*)
Hernández et al. (2012)	Neuroendocrine system of the digestive tract in *Rhamdia quelen* juvenile: Na immunohistochemical study
Kreutz et al. (2012)	Innate immune response of silver catfish (*Rhamdia quelen*) exposed to atrazine
Ghisi et al. (2011)	Evaluation of genotoxicity in *Rhamdia quelen* (Pisces, Siluriformes) after sub-chronic contamination with Fipronil
Pamplona et al. (2011)	Subchronic effects of dipyrone on the fish species *Rhamdia quelen*
Kreutz et al. (2010)	Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge
Costa et al. (2010)	Vitellogenesis and other physiological responses induced by 17-β-estradiol in males of freshwater fish *Rhamdia quelen*
Pretto et al. (2010).	Acetylcholinesterase Activity, Lipid Peroxidation, and Bioaccumulation in Silver Catfish (Rhamdia quelen) Exposed to Cadmium
Hernández et al. (2009)	Morphology, Histology and Histochemistry of the Digestive System of South American Catfish (*Rhamdia quelen*)
Melo et al. (2008)	Hepatic alterations in the fish *Rhamdia quelen* contaminated with Folidol 600s
Borges et al. (2007)	Changes in hematological and serum biochemical values in jundiá *Rhamdia quelen* due to sub-lethal toxicity of cypermethrin
Borges et al. (2004)	Hematologic and serum biochemical values for jundia´ (*Rhamdia quelen*)
Barcellos et al. (2003)	Haematological and biochemical characteristics of male jundia´ (Rhamdia quelen Quoy & Gaimard Pimelodidae): changes after acute stress
Barcellos et al. (2002)	Plasma steroid concentrations in relation to the reproductive cycle of cultured male *Rhamdia quelen*
Barcellos et al. (2001b)	Steroid Profiles in Cultured Female Jundiá, the Siluridae *Rhamdia quelen* (Quoy and Gaimard, Pisces Teleostei), during the First Reproductive Cycle

FONTE: Próprio autor.

Posto isso, a espécie apresenta características promissoras como bom modelo de estudo e os resultados das análises toxicológicas tem evidenciado a

espécie como sensível à exposição de contaminantes (Perussolo et al., 2019). Por estes motivos optou-se por utilizá-la como modelo animal nesta pesquisa.

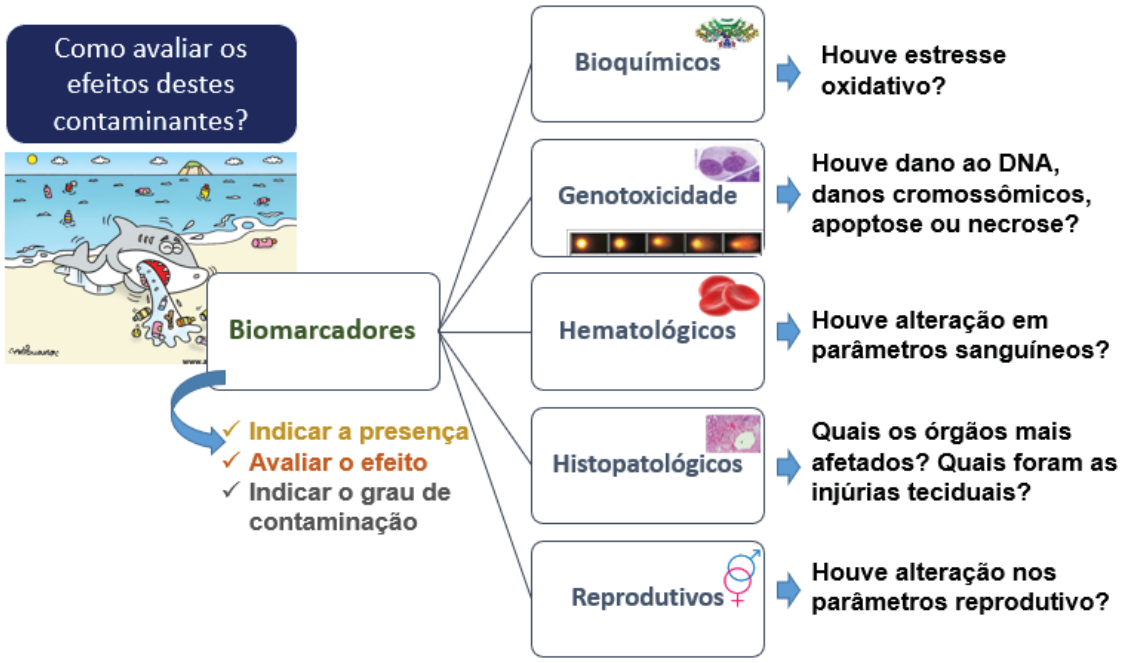
2.4 BIOMARCADORES

Definido o modelo animal outro passo importante no desenvolvimento de uma pesquisa é a escolha de biomarcadores adequados para responder às hipóteses da pesquisa.

Um biomarcador é definido como uma mudança em uma resposta biológica (variando desde respostas moleculares, celulares e fisiológicas até mudanças comportamentais) que pode estar relacionada à exposição ou a efeitos tóxicos de substâncias químicas ambientais, ou da resposta do hospedeiro (CBMNRC., 1987; Peakall, 1994). São de extrema importância, pois efeitos em níveis hierárquicos superiores são sempre precedidos por mudanças anteriores nos processos biológicos, permitindo o desenvolvimento de sinais de biomarcadores de alerta precoce de efeitos em níveis de resposta posteriores (Bayne et al., 1985).

Os Biomarcadores podem ser usados para vários propósitos, dependendo da finalidade do estudo e do tipo da exposição química. Podem ter como objetivos avaliar a exposição (quantidade absorvida ou dose interna), avaliar os efeitos das substâncias químicas e avaliar a suscetibilidade individual (FIGURA 4) (Coelho and Amorim, 2003).

FIGURA 4 – REPRESENTAÇÃO GRÁFICA DA UTILIZAÇÃO DE DIFERENTES BIOMARCADORES NA INVESTIGAÇÃO DE DIFERENTES RESPOSTAS.

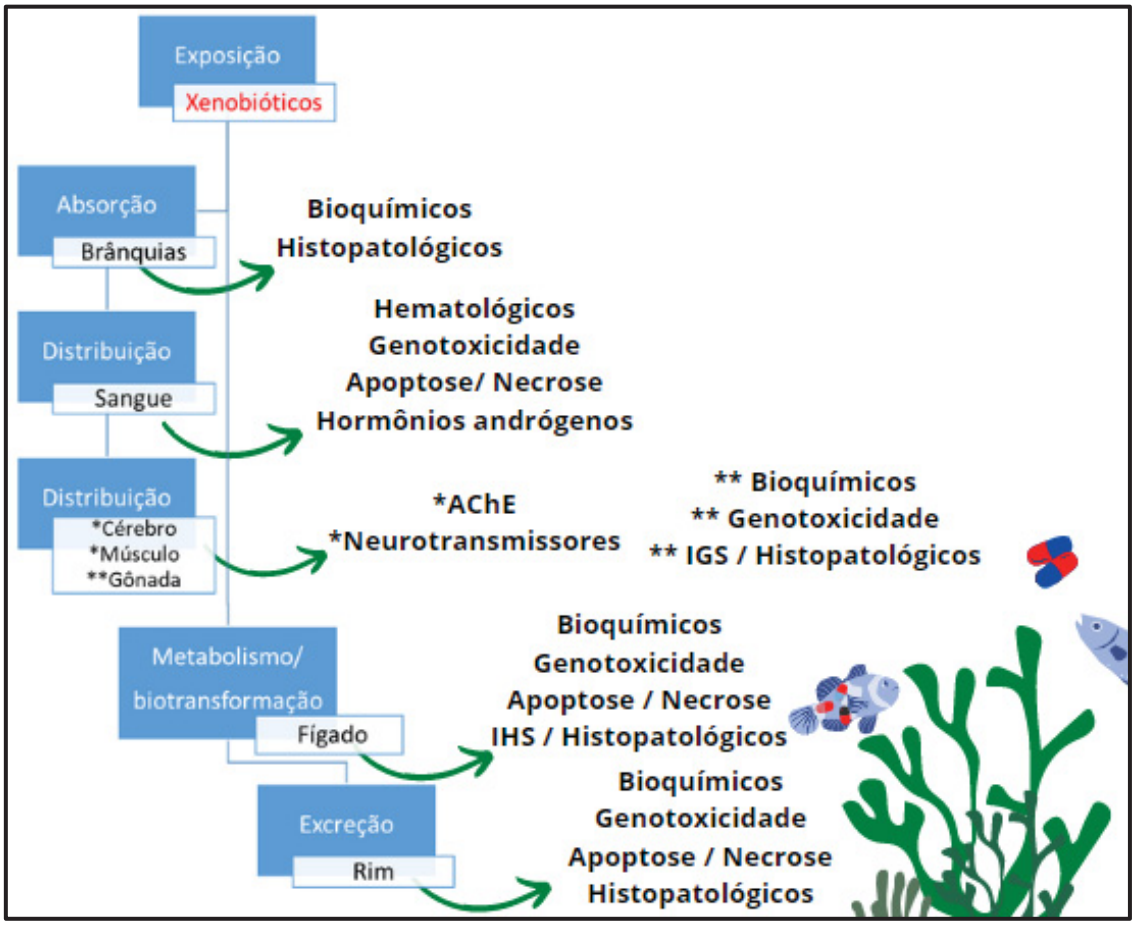


Fonte: Próprio Autor.

A resposta de biomarcadores em peixes tem sido utilizada como uma ferramenta de alerta precoce e confiável do estado de saúde do organismo e seu ambiente circundante. A avaliação de biomarcadores em peixes inclui mecanismos fisiológicos e bioquímicos que ocorrem em tecidos, células, enzimas, moléculas ou fluidos corporais (Van der Oost et al., 2003). As alterações dos parâmetros hematológicos, bioquímicos, genéticos, entre outros, podem ser usadas como um bom modelo de estudo para avaliar a condição de saúde dos organismos aquáticos (Ramesh et al., 2018).

Neste trabalho foram utilizados diferentes tipos de biomarcadores de exposição e efeito (FIGURA 5). A avaliação integrada destes biomarcadores permite uma análise aprofundada acerca da exposição à CIP e dos possíveis efeitos do organismo frente ao contaminante.

FIGURA 5 – ESQUEMA DOS ÓRGÃOS AVALIADOS NA PESQUISA E OS BIOMARCADORES ANALISADOS EM CADA ÓRGÃO.



FONTE: Próprio Autor.

2.4.1 Biomarcadores hematológicos

Muitos parâmetros hematológicos em peixes são biomarcadores para avaliar o estado de saúde, alterações fisiológicas e patológicas que ocorrem em peixes (Ahmed et al., 2020). Os peixes vivem em contato muito íntimo com o ambiente e são, portanto, muito suscetível a alterações físicas, químicas e ambientais que podem se refletir em seus componentes de células sanguíneas (Fazio, 2019).

Os estudos hematológicos das diferentes espécies de peixe são de interesse ecológico e fisiológico uma vez que auxiliam na compreensão da relação entre as características sanguíneas, a filogenia, a atividade física e a adaptabilidade dos peixes no ambiente. Vale salientar que ainda é difícil interpretar os parâmetros sanguíneos devido a variações causadas por fatores

internos e externos, como sexo, tamanho, densidade de estoque e efeitos ambientais (Fazio, 2019).

Karimi et al. (2013) investigaram o efeito do sexo sobre os parâmetros hematológicos da albacora, *Acantopagrus latus*, e relataram que as contagens de glóbulos vermelhos (RBC) foram maiores em peixes machos do que em fêmeas, embora outros parâmetros, como glóbulos brancos (WBC), hematócrito (Ht), volume corpuscular médio (VCM), volume corpuscular médio hemoglobina (VCMH), concentração média de hemoglobina corpuscular (CMHC) e contagem diferencial de leucócitos não mostraram diferença significativa entre os peixes machos e fêmeas. Motlagh et al. (2012) estabeleceram os valores basais dos parâmetros hematológicos para o peixe-lutador-siamês (*Betta splendens*) de um centro de criação de peixes em Gorgan, Irã e relataram que os linfócitos em fêmeas eram significativamente maiores do que em machos. Não foram observadas diferenças significativas nos demais parâmetros hematológicos em relação ao sexo.

Esses dados salientam a necessidade de mais investigações em relação ao efeito do sexo sobre os parâmetros hematológicos, a fim de esclarecer algumas lacunas de conhecimento e fornecer maiores evidências científicas para aferições cada vez mais assertivas na avaliação de possíveis impactos ecológicos de contaminantes ambientais.

Neste trabalho utilizamos como biomarcadores hematológicos, o número de eritrócitos, de trombócitos, leucócitos, o hematócrito e o volume corpuscular médio (VCM).

2.4.2 Biomarcadores bioquímicos

Vários parâmetros bioquímicos em peixes foram testados para suas respostas às substâncias tóxicas e seu uso potencial como biomarcadores de exposição ou efeito. As enzimas envolvidas no processo de desintoxicação de xenobióticos e seus metabólitos, tais como enzimas de biotransformação e enzimas antioxidantes têm sido alvo de pesquisas no campo da toxicologia ambiental (Calado et al., 2017; Guiloski et al., 2017; Kitamura et al., 2022; Perussolo et al., 2019; Pimpão et al., 2007; Silva de Assis, 1998).

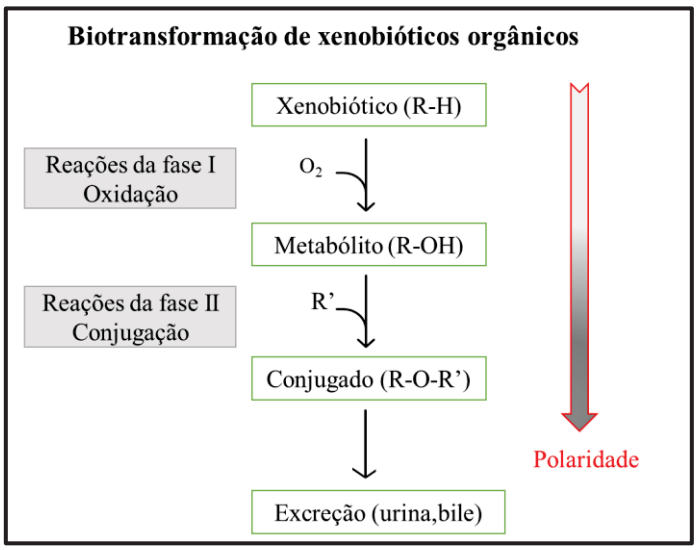
2.4.2.1 Biotransformação

Os organismos vivos possuem diferentes meios de se livrar de substâncias químicas estranhas, denominadas xenobióticos, com potencial toxicidade (Beiras, 2018).

A biotransformação ou metabolismo pode ser definida como uma conversão, catalisada por enzimas, de um composto xenobiótico em uma forma mais solúvel em água, que pode ser excretada do corpo mais facilmente do que o composto original (Santana et al., 2022; Schlenk, 2005; Siroká and Drastichová, 2004). A maioria dos processos de biotransformação ocorre no fígado de vertebrados (Beiras, 2018).

A biotransformação se dá normalmente em duas fases (FIGURA 6). A fase I consiste em reações de oxidação, redução ou hidrólise, e os produtos formados são frequentemente mais reativos que os iniciais. A fase II é um conjunto de reações de oxidação que preparam os tóxicos para serem transformados pelas reações da fase II. Já fase II consiste em reações de conjugação destas substâncias reativas, provenientes da fase I, a fim de serem eliminadas do organismo como substâncias inertes (Schlenk, 2005; Siroká; Drastichová, 2004).

FIGURA 6 - RESUMO DE UM PROCESSO DE BIOTRANSFORMAÇÃO TÍPICO DE UM XENOBIÓTICO ORGÂNICO LIPOFÍLICO. NA FASE I, O PRODUTO QUÍMICO É OXIDADO POR UMA MONOOXIGENASE DEPENDENTE DO CITOCROMO P450 (CYP). NA FASE II O METABÓLITO RESULTANTE É CONJUGADO A UMA MOLÉCULA POLAR NÃO TÓXICA ENDÓGENA. O CONJUGADO RESULTANTE É ADEQUADO PARA ELIMINAÇÃO PELO SISTEMA EXCRETOR.



FONTE: Adaptado de Beiras (2018).

A toxicidade de um xenobiótico pode ser afetada pela biotransformação, que pode ser benéfico (desintoxicação) ou prejudicial (bioativação) para um organismo (Siroká; Drastichová, 2004; Toni et al., 2011). A biotransformação é, portanto, um importante determinante da atividade de um composto, da duração dessa atividade e da meia-vida do composto no corpo (Siroká; Drastichová, 2004).

A fase I da biotransformação envolve oxidação, redução ou hidrólise. Para a maioria dos compostos xenobióticos, as reações de fase I são catalisadas por enzimas microssomais monooxigenase (MO), também conhecidas como sistema oxidase de função mista (MFO) (isto é, citocromo P450 [cyt P450], citocromo b5 [cyt b5] e NADPH citocromo P450 redutase [P450 RED]) (Van der Oost et al., 2003). Portanto, a biotransformação de fase I é catalizada pelos sistemas de citocromos P-450 (Cit P450) e afins, levam a formação de metabólitos intermediários mediante reações metabólicas redutivas e oxidativas. Estas permitem posteriormente a conjugação dos metabólitos intermediários formados, aumentando assim sua polaridade, na chamada fase II (Santana et al., 2022; Siroká and Drastichová, 2004).

Na fase II da biotransformação ocorre a conjugação de compostos eletrofílicos (ou metabólitos de fase I) com GSH ou ácido glucurônico, catalisada pelas glutationas S-transferases (GSTs) (Keen et al., 1976; Schlenk, 2005). As reações de conjugação mais importantes são conjugação de glicuronídeos, sulfoconjugação, acetilação, conjugação de aminoácidos, conjugação de glutationa e metilação.

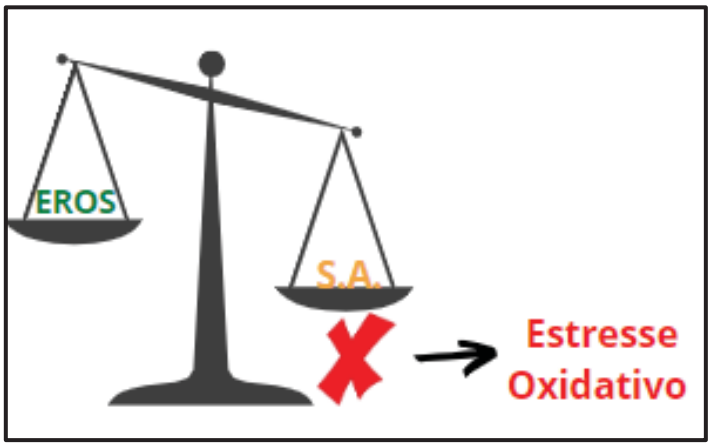
A GSH reduzida é um tripéptido constituído por G-glutamina, cisteína e glicina (Van der Oost et al., 2003). Entre suas funções estão dois papéis em desintoxicações, como um conjugado chave de intermediários eletrofílicos, principalmente via atividades de GST no metabolismo de fase II, e como um importante antioxidante (Gaucher et al., 2018). A GST por sua vez, pertence a uma superfamília multigênica de enzimas diméricas, multifuncionais, principalmente solúveis. Além de suas funções essenciais no transporte intracelular (heme, bilirrubina e ácidos biliares) e na biossíntese de leucotrienos e prostaglandinas, um papel crítico para as GSTs é a defesa contra danos

oxidativos e produtos peroxidativos de DNA e lipídios (Gonçalves et al., 2010; Hayes et al., 2005).

2.4.2.2 Sistema Antioxidante

O estresse oxidativo resulta de um desequilíbrio entre a produção de espécies reativas de oxigênio (ERO) e o sistema antioxidante (S.A.), em favor das primeiras (FIGURA 7).

FIGURA 7 – REPRESENTAÇÃO ESQUEMÁTICA DA ORIGEM DO ESTRESSE OXIDATIVO.



FONTE: Próprio Autor.

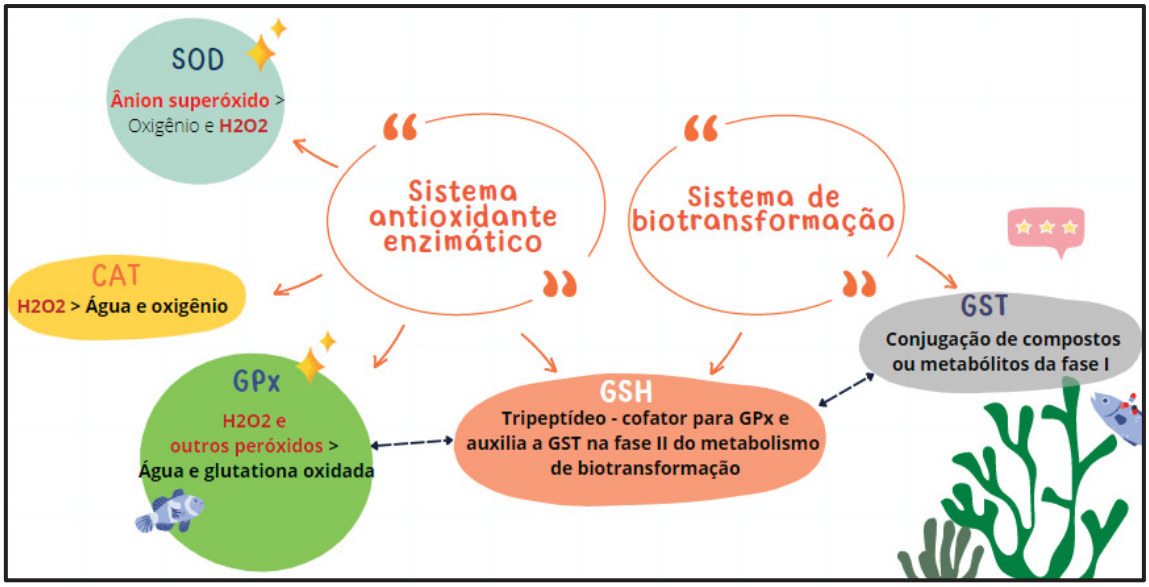
Muitos xenobióticos (ou seus metabólitos) podem exercer toxicidade relacionada ao estresse oxidativo. A toxicidade do oxigênio é definida como efeitos prejudiciais devido às espécies reativas de oxigênio citotóxicas (ERO) (Di Giulio et al., 1989). Esses produtos de redução do oxigênio molecular (O_2) são o radical ânion superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\bullet}OH$), um oxidante extremamente potente capaz de reagir com macromoléculas celulares críticas, possivelmente levando à inativação enzimática , peroxidação lipídica (LPO), dano ao DNA e, finalmente, morte celular (Winston and Di Giulio, 1991).

O radical superóxido é tipicamente o primeiro ERO formado, eventualmente levando à formação de peróxido de hidrogênio e, finalmente, radicais hidroxila que são, quimicamente, os mais prejudiciais das ERO (Birnie-Gauvin et al., 2017). Embora não seja uma espécie radicalar, o H_2O_2 pode passar facilmente através das membranas e tem uma vida mais longa do que a maioria

das espécies radicais derivadas do oxigênio (Birnie-Gauvin et al., 2017). Na presença de íons metálicos de valência variável, o H_2O_2 pode ser convertido no radical hidroxila, altamente reativo, capaz de extrair elétrons e prótons das macromoléculas (ácidos nucleicos, proteínas, lipídios e carboidratos) com as quais entra em contato, gerando mais elétrons desemparelhados e espécies radicais (Birnie-Gauvin et al., 2017).

Para se proteger contra as ERO potencialmente altamente prejudiciais, os organismos possuem um sistema para prevenir ou reparar os efeitos do estresse oxidativo. A prevenção vem na forma de antioxidantes (FIGURA 8), que podem ser enzimáticos, por exemplo, superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX) ou moléculas não enzimáticas, como o ácido ascórbico (vitamina C), glutationa, α- tocoferol (vitamina E), entre outros (Birnie-Gauvin et al., 2017; Santana et al., 2022).

FIGURA 8 – REPRESENTAÇÃO ESQUEMÁTICA DE ALGUMAS ENZIMAS E TRIPEPTÍDEO (GSH) QUE PARTICIPAM DO SISTEMA ANTIOXIDANTE E DO SISTEMA DE BIOTRANSFORMAÇÃO.



FONTE: Próprio Autor.

As alterações nas atividades das enzimas antioxidantes podem fornecer informações de saúde dos peixes sob condição de estresse (Santana et al., 2022). Desse modo, o estresse oxidativo desempenha um papel fundamental na formação das respostas dos peixes às mudanças ambientais, bem como nas estratégias de história de vida (Birnie-Gauvin et al., 2017a; Santana et al., 2022).

A SOD é uma metaloenzima que catalisa a conversão de ânion superóxido ($O_2^{\bullet -}$) em peróxido de hidrogênio (H_2O_2) e oxigênio (O_2). H_2O_2 é posteriormente desintoxicado por dois tipos de enzimas: CAT e GPX (Gao et al., 1998). A CAT é uma enzima contendo hematina que facilita a remoção do H_2O_2, que é metabolizado em oxigênio molecular (O_2) e água (H_2O). A CAT só pode degradar H_2O_2 (Rodrigues et al., 2018). Glutationa peroxidases (GPx) constituem uma família de proteínas que catalisa a redução de H_2O_2 ou hidroperóxidos orgânicos a água ou álcoois correspondentes, usando glutationa reduzida (GSH) como doador de elétrons (Sattin et al., 2015).

A lipoperoxidação (LPO) é um dos principais danos causados pelo estresse oxidativo. Este dano ocorre nos fosfolipídios das membranas celulares, que são regiões muito suscetíveis às reações de oxidação (Birnie-Gauvin et al., 2017a). Quando as ERO reagem com os lipídios de membrana, afetam a função das membranas celulares, causando o aumento da permeabilidade, podendo chegar à ruptura total seguida de morte celular (Winston and Di Giulio, 1991).

2.4.2.3 Neurotoxicidade

Quanto às funções neurais, as enzimas de interesse são as colinesterases (CHE) (Payne et al., 1996). Dois tipos de ChE são reconhecidos; em primeiro lugar, aqueles com alta afinidade para acetilcolina (AChE), e em segundo lugar, aqueles com afinidade para butirilcolina (BChE) (Sturm et al., 2000). O cérebro do peixe contém AChE, mas não BChE, enquanto os tecidos musculares contêm tanto AChE quanto BChE (Sturm et al., 2000). A AChE está envolvida na desativação da acetilcolina nas terminações nervosas, prevenindo disparos nervosos contínuos, o que é vital para o funcionamento normal dos sistemas sensoriais e neuromusculares (Sturm et al., 2000).

Para este estudo foram utilizados os biomarcadores do sistema oxidativo, biotransformação e neurotoxicidade.

2.4.3 Biomarcadores de genotoxicidade

O estresse oxidativo pode levar à instabilidade genética devido a complexos de reparo de DNA não resolvidos (Luzhna et al., 2013). Isso gera

impactos que afetam direta ou indiretamente a integridade do material genético de uma célula (DNA e RNA), podendo levar a efeitos mutagênicos, carcinogênicos ou teratogênicos (Cant et al., 2022).

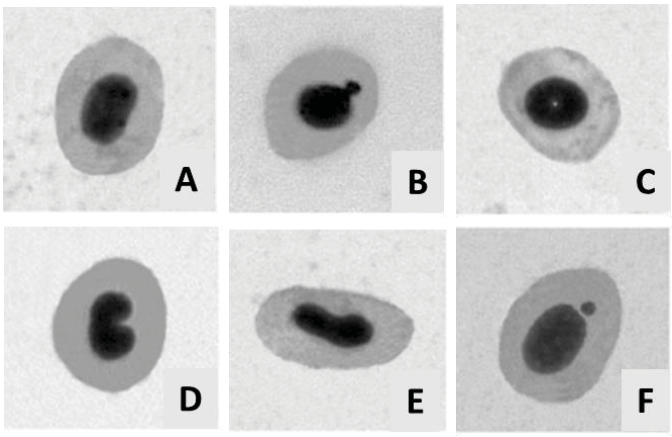
2.4.3.1 Anormalidade nucleares

Essas instabilidades em peixes podem ser facilmente estimadas por várias anomalias/anormalidades nucleares que oferecem informações concretas para danos ao DNA e instabilidade genômica ou cromatina desencadeada por xenobióticos (Cant et al., 2022).

Dentre as anormalidades nucleares, pode-se citar o micronúcleo (MN), um corpo cromínico, resultado de quebras de fitas de DNA e/ou formado pela condensação de fragmentos de cromatina (Trivedi et al., 2021) e a ocorrência de outras alterações eritrocíticas nucleares (AENs). Ambas as alterações são consideradas como um indicador de danos genotóxicos.

Estas outras alterações foram descritas, primeiramente, em eritrócitos de peixes por (Carrasco et al., 1990) e foram classificadas em (FIGURA 9): 1) núcleo segmentado (*blebbed*); 2) núcleo lobulado (*lobed*); 3) núcleo com constrição ou em "forma de rim"(*notched*); 4) apresentar vacúolo no meio do núcleo (*vacuolado*) e posteriormente houve a identificação da presença de células binucleadas. Estes corpúsculos são estruturas encontradas em células em processo de divisão, no qual são compostos por partes de cromossomos ou cromossomos inteiros, que não se mantiveram internalizados no núcleo durante a divisão celular, e formam uma estrutura arredondada, semelhante ao núcleo celular (Carrasco et al., 1990). Estas alterações podem ser avaliadas por meio do teste do micronúcleo Písceo.

FIGURA 9 – ERITRÓCITO NORMAL (A); ERITRÓCITO COM ALTERAÇÃO DO TIPO *NÚCLEO SEGMENTADO (BLEBBED)* (B); ERITRÓCITO COM ALTERAÇÃO DO TIPO *VACÚOLO NO MEIO DO NÚCLEO (VACUOLADO)* (C); ERITRÓCITOS COM ALTERAÇÕES DO TIPO *NÚCLEO COM CONSTRIÇÃO OU EM "FORMA DE RIM"(NOTCHED)* (D, E); ERITRÓCITO COM MICRONÚCLEO (F).



FONTE: Santos (2010).

As anomalias nucleares são formadas principalmente devido à condensação do material genético dentro do núcleo e sob a influência de fatores mitocondriais. Dentre estes, o fator indutor de apoptose é o principal responsável por tal condensação (Trivedi et al., 2021). Além disso, a indução de micronúcleos também indica reparo incorreto de quebras de fita de DNA (Klobučar et al., 2010).

2.4.3.2 Dano ao DNA

A análise de dano ao DNA também é um biomarcador frequentemente utilizado (Bolognesi and Cirillo, 2014). É utilizado através da avaliação de quebras de fita de DNA por meio de um ensaio cometa ou SCGE (Single-Cell Gel Eletrophoresis). Este ensaio possibilita a visualização de quebras de fita simples ou dupla de DNA, que são produzidas sob condições de estresse oxidativo (Azqueta et al., 2011). Um método sensível, confiável e bastante barato (Collins et al., 1997). Além disso, ele precisa de um pequeno número de células e detecta danos em nível de célula única (Bolognesi and Cirillo, 2014).

Neste método, as células são embebidas em gel de agarose em lâminas de microscópio, primeiro são lisadas e depois submetidas à eletroforese. Sob a força do campo elétrico, células com DNA danificado mostram uma migração de fragmentos de DNA do núcleo. A duração da migração indica a quantidade de quebra de DNA e pode ser comparada como uma medida direta do nível de DNA

danificado. O dano ao DNA pode ser estimado tanto por análises microscópicas manuais quanto por análise de pontuação de imagem computadorizada (Bolognesi and Cirillo, 2014). Além disso, outra forma de mensurar o dano é através da razão entre o raio do núcleo e o comprimento da cauda, e os diferentes níveis de danos são então classificados de 0 a 4 (onde 0= nenhum dano e 4= dano máximo, indicando possível apoptose) (Azqueta et al., 2011). O nome cometa provem da cauda que pode ser formada por fragmentos de DNA após a migração, pois pequenos fragmentos tendem a migrar mais rápido que os grandes fragmentos, e quando não há fragmentação o DNA migrará em conjunto, formando uma estrutura circular, chamada de nucleóide.

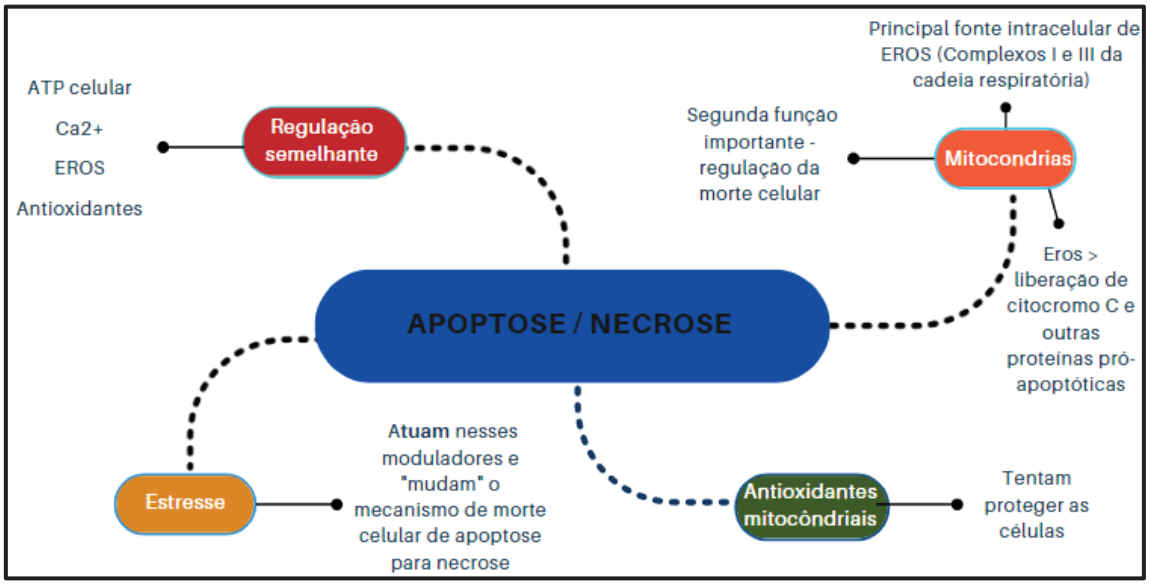
Nesta pesquisa utilizou-se tanto o teste do micronúcleo písceo, quanto o ensaio cometa a fim de mensurar os possíveis efeitos da exposição de *Rhamdia quelen* às concentrações ambientais de ciprofloxacina.

2.4.3.3 Avaliação da frequência de apoptose e necrose

Embora a apoptose e a necrose fossem originalmente consideradas mecanismos totalmente distintos de morte celular, trabalhos mostraram que os processos são regulados por muitos dos mesmos intermediários bioquímicos, mais notavelmente os níveis de ATP celular, Ca2+, espécies reativas de oxigênio e antioxidantes tiol. Além de um certo limiar, parece que as mudanças induzidas pelo estresse nesses moduladores "mudam" o mecanismo de morte celular de apoptose para necrose (FIGURA 10) (Mcconkey, 1998).

Além do papel bem estabelecido das mitocôndrias no metabolismo energético, a regulação da morte celular surgiu recentemente como uma segunda função importante dessas organelas (FIGURA 10) (Ott et al., 2007). Este fenômeno parece estar intimamente ligado ao seu papel como a principal fonte intracelular de ERO, que são geradas principalmente nos Complexos I e III da cadeia respiratória (Ott et al., 2007). As ERO geradas por mitocôndrias desempenham um papel importante na liberação de citocromo c e outras proteínas pró-apoptóticas, que podem desencadear a ativação de caspases e apoptose (FIGURA 10) (Ott et al., 2007). Por outro lado, as enzimas antioxidantes mitocondriais tentam proteger as células da apoptose.

FIGURA 10 – RESUMO SOBRE OS PROCESSOS REGULADORES DA APOPTOSE E NECROSE.



FONTE: Próprio Autor.

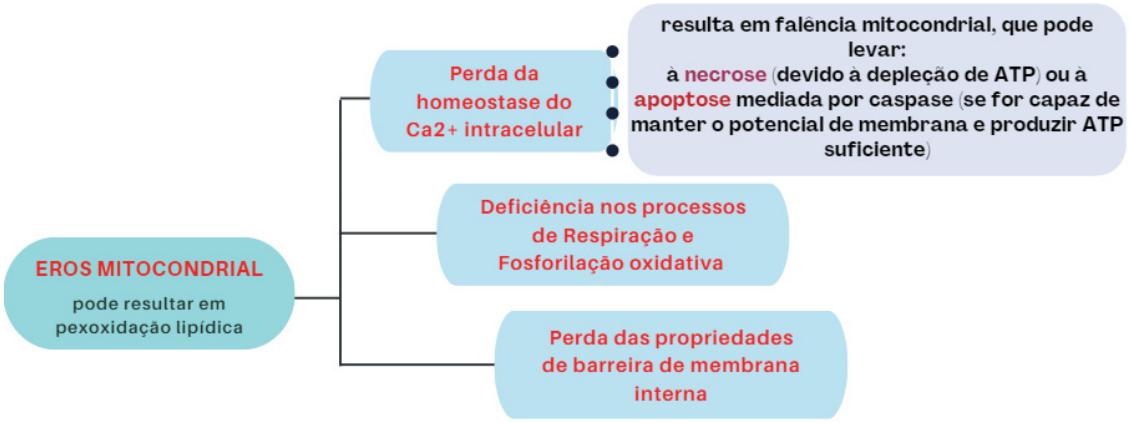
A formação de ERO e a estimulação da peroxidação lipídica nas mitocôndrias podem levar à supressão do metabolismo mitocondrial (Ott et al., 2007). Os peróxidos lipídicos afetam funções mitocondriais vitais, como respiração e fosforilação oxidativa, propriedades de barreira da membrana interna, manutenção do potencial de membrana mitocondrial (ψ) e capacidade de tamponamento de Ca2+ mitocondrial (Albano et al., 1991; Bacon et al., 1993; Zhang et al., 1990). Os produtos da peroxidação lipídica mitocondrial podem prejudicar a função de barreira das membranas, interagindo diretamente com a proteína e/ou indiretamente com as porções lipídicas da membrana (FIGURA 11) (Chen et al., 1995).

Um efeito potencialmente deletério da produção de ERO nas mitocôndrias é a facilitação da transição de permeabilidade mitocondrial dependente de Ca2+ (PMD), que desempenha um papel fundamental em certos modos de morte celular (FIGURA 11). Além da produção de ATP em células aeróbicas, as mitocôndrias desempenham um papel crucial na regulação da homeostase do Ca2+ intracelular (Ott et al., 2007).

Assim, o estresse oxidativo e a homeostase prejudicada do Ca2+ contribuem para o dano celular mediado pela mitocôndria. A PMD resulta em falência mitocondrial, que pode levar à necrose devido à depleção de ATP ou à apoptose mediada por caspase se a indução de PMD ocorrer em uma

subpopulação de mitocôndrias e as organelas restantes ainda forem capazes de manter seu potencial de membrana e produzir ATP suficiente para suportar a apoptose processo (FIGURA 11) (Ott et al., 2007).

FIGURA 11 – REPRESENTAÇÃO ESQUEMÁTICA DE ALGUMAS CONSEQUÊNCIAS DO DESEQUILÍBRIO DA FORMAÇÃO DE ERO MITOCONDIAL.



FONTE: Próprio Autor.

Zhang e Greenberg (2012) publicaram um estudo mostrando a relação da enzima AChE com processos de apoptose celular. Segundo os autores, até o momento, mais de 40 tipos diferentes de células de culturas primárias ou linhagens celulares mostraram expressão de AChE durante a apoptose e após a indução da apoptose por diferentes estímulos. Para eles, já está bem estabelecido que a expressão ou atividade aumentada de AChE é detectada em células apoptóticas após estímulos apoptóticos in vitro e in vivo e, a AChE poderia, portanto, ser usada como um marcador de apoptose. Os autores esclarecem ainda que a AChE não é um iniciador de apoptose, mas as células nas quais a AChE é superexpressa sofrem apoptose mais facilmente do que os controles. Curiosamente, as células com níveis regulados negativamente de AChE não são sensíveis à indução de apoptose e a deficiência de AChE pode proteger contra a apoptose. De acordo com o estudo, diversas questões ainda precisam ser esclarecidas, e mais estudos que abordem a função não clássica da AChE na apoptose são necessários.

Na presente pesquisa utilizamos a frequência de apoptose e necrose para uma avaliação aprofundada do possível efeito da CIP sobre estes processos celulares.

2.4.4 Parâmetros morfológicos e histopatológicos

Os principais parâmetros morfológicos tais como comprimento, peso, índice de condição e índices hepatossomático (IHS) e gonadossomático (IGS) possibilitam analisar quais os órgãos mais afetados e assim, identificar a sensibilidade do organismo em relação aos xenobióticos (Calado et al., 2017; Guiloski et al., 2015, 2017).

Em adição, a análise de parâmetros histopatológicos, tais como atrofia, necrose, edema intercelular ou ruptura das células pilares (em brânquias), hemorragia, edema intercelular, atrofia, necrose, entre outras (no rim), e hiperplasia, necrose, atrofia, infiltração entre outras alterações (no fígado), possibilita obter uma ligação e compreensão entre o grau de exposição a determinado xenobiótico e o efeito desta exposição (Bernet et al., 1999; Kitamura et al., 2022; Ramesh et al., 2021a; Rodrigues et al., 2019, 2017).

As características histopatológicas de órgãos específicos expressam condição e representam impactos endógenos e exógenos integrados no tempo do organismo, decorrentes de alterações em níveis inferiores de organização biológica (Teh et al., 1997). Após as alterações celulares, uma sequência de patologias é observada nos peixes, comprometendo a sobrevivência e, consequentemente, a estrutura da população, afetando o ecossistema (Fry, 1971).

Os órgãos para diagnóstico são escolhidos de acordo com as funções que desempenham. As brânquias estão em contato próximo com a água contaminada, substâncias tóxicas causam danos à sua estrutura e função; o fígado está exposto a substâncias tóxicas, tendo em vista que é o órgão responsável por biotransformá-las; e os rins são expostos durante o processo de excreção (Bernet et al., 1999).

Bernet et al. (1999) estabeleceu a utilização de um índice de lesão para avaliação histopatológica de diferentes tecidos, onde as alterações dos

fotorreceptores foram classificadas em três fatores de gravidade (importância patológica mínima, moderada e acentuada).

Nesta pesquisa, para a avaliação histopatológica das lesões, todos os espécimes foram analisados de acordo com o índice proposto por Bernet et al. (1999) modificado por Mela et al. (2013). Os fatores importantes considerados para cada alteração encontrada foram: (1) importância patológica mínima, que apresenta possibilidades de reversão; (2) importância moderada, reversível em alguns casos e (3) importância patológica grave, geralmente irreversível. De acordo com o grau de ocorrência das alterações, os valores atribuídos às alterações foram: (0) Inalterado, (2) Ocasionalmente (4) Ocorrência Moderada e (6) Ocorrência Grave (difusa lesão). Para calcular o Índice de Lesão (II), a seguinte equação foi usada:

$$II = \sum rp \sum alt \ (a \ X \ w)$$

no qual *pr* é o padrão de reação; *alt* é a alteração; *a* é o valor da pontuação; *w* é o fator de importância.

Posto isto, nesta pesquisa foram realizadas análises histopatológicas das brânquias, fígado, gônadas e rim posterior, a fim de compreender melhor os possíveis efeitos da CIP sobre estes órgãos de acordo com a função que cada um exerce no organismo.

2.4.5 Biomarcadores de alteração do eixo Hipotálamo- Pituitária- Gônada (HPG)

A reprodução e a fertilidade são reguladas por meio de hormônios do eixo hipotálamo-hipófise-gonadal (HPG). O controle desse eixo reprodutivo ocorre em todos os níveis, incluindo o cérebro e a hipófise, e permite a promoção ou inibição da secreção e função gonadal de esteróides sexuais (Acevedo-Rodriguez et al., 2018). Além de orientar o desenvolvimento e a função gonadal adequados, os esteróides sexuais gonadais também atuam em loops de feedback negativo e positivo para regular os circuitos reprodutivos no cérebro,

incluindo os neurônios kisspeptina, modulando assim o status geral do eixo HPG (Acevedo-Rodriguez et al., 2018).

O eixo HPG é regulado por uma variedade de fatores internos e externos (Prasad et al., 2015). O sistema monoaminérgico modula o funcionamento do eixo HPG. Seus neuromediadores, como a dopamina, podem inibir a liberação de LH, enquanto outros, como noradrenalina e serotonina, estimulam a liberação de LH não apenas diretamente, mas também através do eixo GnRH (Hachfi et al., 2012). Dependendo do estágio fisiológico, os esteróides sexuais exercem efeitos de feedback negativo e positivo nos níveis hipofisário e hipotálamo para modular a síntese e liberação de GnRH e GtH (Peter; Yu, 1997; Schulz et al., 1999).

Desta forma, tanto os hormônios esteroidais, neste caso andrógenos e estrógenos, quanto os neurotransmissores envolvidos no eixo HPG podem ser utilizados como biomarcadores para investigação dos efeitos causados por xenobióticos em ambientes aquáticos. O exame da reprodução dos peixes e os potenciais efeitos reprodutivos adversos causados pela exposição química podem servir como uma medida razoável dos potenciais riscos ecológicos (Hedayati; Safahieh, 2012).

Alguns xenobióticos podem ser classificados como desreguladores endócrinos (DEs) que são compostos que alteram as funções do sistema endócrino, podendo imitar, interferir ou bloquear a função de hormônios endógenos e, assim, perturbar a homeostase hormonal normal do corpo (Hedayati; Safahieh, 2012; Saha et al., 2022). Os principais efeitos causados por EDCs em seres humanos incluem redução temporária da contagem de espermatozóides, declínio na qualidade do esperma, ocorrência de câncer de próstata, mama e testículos, proporção sexual alterada, aumento de ovários policísticos, distúrbios cardiovasculares, distúrbios neurológicos, endometriose, deficiências imunológicas e distúrbios da tireoide (Huang et al., 2018; Qiu et al., 2019; Saha et al., 2022; Wang et al., 2017; T. Zhang et al., 2016; Zhao et al., 2019).

Diante disso, de forma a explorar melhor os efeitos no âmbito reprodutivo da exposição de CIP em *Rhamdia quelen* foi investigado as concentrações de um andrógeno (11 – ceto testosterona – 11KT) e um estrógeno (17β estradiol –

E2), além de neurotransmissores como dopamina, noradrenalina e serotonina envolvidos na regulação do eixo HPG.

3 OBJETIVOS

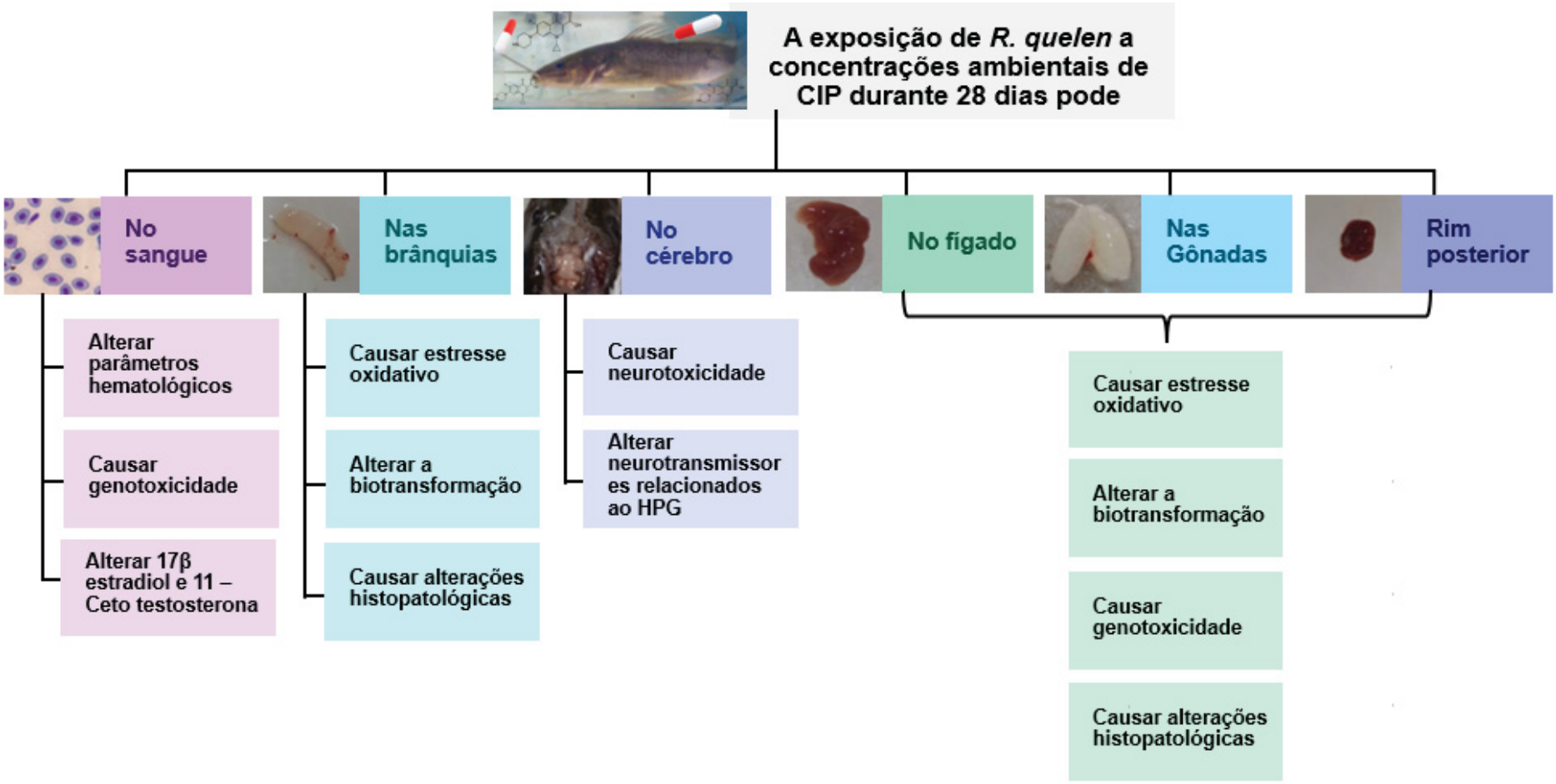
3.1.1 Objetivo geral

Avaliar os efeitos da exposição subcrônica às concentrações ambientais de ciprofloxacina, em machos e fêmeas de *Rhamdia quelen*, por meio de biomarcadores hematológicos, bioquímicos, genotóxicos, histopatológicos e reprodutivos.

3.1.2 Objetivos específicos

- Avaliar se a exposição de *Rhamdia quelen* a concentrações ambientais de CIP (concentrações presentes em ambientes superficiais, efluentes de estações de tratamento de água potável, esgoto e industriais) durante 28 dias causa efeito tóxico na saúde destes animais;
- Avaliar se a CIP causa alterações hematológicas e causa genotoxicidade em células sanguíneas;
- Avaliar se a CIP causa neurotoxicidade;
- Analisar se a CIP causa estresse oxidativo e se altera o sistema de biotransformação nas brânquias, fígado, gônadas e rim posterior;
- Analisar se há danos genotóxicos no fígado, gônadas e rim posterior
- Avaliar se a CIP causa alterações histopatológicas nas brânquias, fígado, gônadas e no rim posterior,
- Investigar se a CIP causa alterações dos índices hepatossomático e gonadossomático;
- Estudar se há alterações nos parâmetros do eixo HPG, como neurotransmissores relacionadas à reprodução e hormônios andrógenos;
- Comparar as respostas dos biomarcadores em relação ao sexo dos peixes expostos a CIP.

4 HIPÓTESES


A exposição de *R. quelen* a concentrações ambientais de CIP durante 28 dias pode
No sangue
Alterar parâmetros hematológicos
Causar genotoxicidade
Alterar 17β estradiol e 11 – Ceto testosterona
Nas brânquias
Causar estresse oxidativo
Alterar a biotransformação
Causar alterações histopatológicas
No cérebro
Causar neurotoxicidade
Alterar neurotransmissores relacionados ao HPG
No fígado
Nas Gônadas
Rim posterior
Causar estresse oxidativo
Alterar a biotransformação
Causar genotoxicidade
Causar alterações histopatológicas

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7 CAPÍTULO I - Hematological, biochemical, genotoxic, and histopathological changes in males and females of *Rhamdia quelen* after exposure to environmental concentrations of ciprofloxacin

Hematological, biochemical, genotoxic, and histopathological changes in males and females catfish (*Rhamdia quelen)* after exposure to environmental concentrations of ciprofloxacin

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HIGHLIGHTS

- CIP caused neurotoxicity, oxidative stress, and genotoxic
- CIP caused histopathological changes such as leukocyte infiltration, steatosis, and necrotic focus in liver
- CIP stimulated gonadal maturation in both sexes
- CIP induced apoptosis of erythrocytes and hepatocytes
- Different responses in male and female

ABSTRACT

Ciprofloxacin (CIP) is an antibiotic commonly used in clinical and its presence and accumulation have been observed in the aquatic environment, but little is known about its effect on non-target organisms. The aim of this study was to evaluate the effects of long-term exposure to environmental CIP concentrations (1, 10, and 100 μg.L^{-1}) in males and females of *Rhamdia quelen*. After 28 days of exposure, the fish were anesthetized for blood collection to evaluate hematological, genotoxic, and hormonal biomarkers. After euthanasia, the brain was collected for analysis of biochemical and neurotransmitter biomarkers. The muscle and brain were analyzed for AChE activity. The liver and gonads were analyzed for biochemical, genotoxic, and histopathological biomarkers. Somatic indices were also assessed. At 100 μg.L^{-1}CIP, it decreased the number of leukocytes, increased the number of thrombocytes (in males), caused genotoxicity, morphological nuclear changes, and apoptosis of erythrocytes. It also caused neurotoxicity in the brain. In the liver, oxidative stress and apoptosis of hepatocytes (in females) were observed. Stimulation of male and female gonad development was observed. At 10 μg.L^{-1} CIP, the number of leukocytes decreased in female animals, and morphological changes occurred in the nucleus of erythrocytes(vacuolated). In male animals, apoptosis of erythrocytes occurred. In addition, it caused neurotoxicity in the brain in both sexes. No lipid peroxidation occurred in the liver. However, increased apoptosis of the hepatocytes was observed in females. Leukocyte infiltration, steatosis, necrosis, and an increase in the Bernet index were observed in males; in females, only an increase in the Bernet index was noted. No lipid peroxidation occurred in the gonads. Even the lowest concentration (1 μg.L^{-1}) also caused adverse effects such as erythrocyte and liver genotoxicity, hepatocyte apoptosis (in females), oxidative stress, and a decrease in somatic indices. Therefore, CIP concentrations present in the environment can cause toxic effects on *Rhamdia quelen*, even at the lowest concentration, which is usually present in surface waters, which can be used for public supply.

Keywords: Biomarkers. Neurotoxicity. Fluorquinoline. Jundiá

7.1 INTRODUCTION

Residues of pharmaceuticals have been found in aquatic environments around the world (Miller et al., 2018; Pashaei et al., 2022; Veiga-Gómez et al., 2017) and have increasingly attracted public concern (Madikizela and Ncube, 2022; Zhou et al., 2022).

Antibiotic represent a class of drugs that are of particular concern in the environment because they can directly lead to adverse effects on nontarget organisms in ecosystems (Yang et al., 2020a). They also pose indirect risks to human health, as they can influence the development of antibiotic resistance in pathogens (Guo et al., 2022; Liu et al., 2022). Antimicrobial resistance (AMR) is a major public health problem worldwide (CDC, 2019; WHO and ECDC, 2022). Estimates from the European Union/European Economic Area (EU/EEA) that more than 670,000 infections are due to the antimicrobial resistant bacteria each year, and approximately 33,000 people die as a direct result(WHO and ECDC, 2022). In the United States, 48,700 people die each year as a result of antimicrobial resistance (CDC, 2019). Therefore, antimicrobial resistance is now considered as a major threat to global health (CDC, 2019; WHO, 2021, 2017; WHO and ECDC, 2022). On the other hand, little is known about the adverse effects on aquatic biota.

Fluoroquinolones are among the antibiotic commonly detected ine aquatic environments at relatively high concentrations ranging from $ng.L^{-1}$ to $\mu g.L^{-1}$ (Kelly and Brooks, 2018; Teglia et al., 2019). Among fluoroquinolones, CIP is one of the most widely used antibiotics worldwide(Kelly and Brooks, 2018; Sahlin et al., 2018).It is a second-generation fluoroquinolone that has a broad spectrum of activity against aerobic Gram-negative and Gram-positive bacteria (Gonzalez et al., 1984b; Sahlin et al., 2018).

Studies have already shown that CIP is not biodegradable (Sahlin et al., 2018) and can also resist degradation in the environment(Flach et al., 2018; Xie et al., 2017). Antibiotic are not completely removed after wastewater treatment, so the aquatic environment is continuously polluted(Yang et al., 2020). In this way, as other antibiotics, CIP has also been detected in surface water, groundwater, wastewater, sewage, sediments, soils, and even drinking water (Gao et al., 2012; Wagil et al., 2014; Xie et al., 2017). In addition, studies have also detected CIP in fish tissues (Beijer et al., 2013; Sehonova et al., 2019; Zhang et al., 2016b). However, little is known about the effects of CIP exposure on aquatic biota. To date, few studies have been conducted

on the effects of this antibiotic on fish(Kitamura et al., 2022; Nie et al., 2008; Plhalova et al., 2014; Ramesh et al., 2021).

In an environmental context, the use of biomarkers at different levels is essential to identify early warning signals because impacts at higher hierarchical levels (such as population, community, and ecosystem) are always preceded by changes in biological processes(Van der Oost et al., 2003). In addition, the use of biomarkers is an important tool for estimating the impact of a pollutant under the One Health scenario. Their use makes it possible to assess the environmental quality and the potential for harm of environmental pollutants to animals and to estimate the undesirable effects on human health.

The aim of this study was to evaluate the toxic effects of long-term exposure to environmental concentrations of CIP in males and females of *Rhamdia quelen*. The hypotheses are that CIP alters hematological biomarkers and causes genotoxicity in erythrocytes. In addition, it causes oxidative stress, genotoxicity, and histopathological changes in the liver and gonads. We also investigated whether CIP causes neurotoxicity and whether it can alter hypothalamic-pituitary-gonadal (HPG) axis parameters. Finally, we investigated whether biomarker responses differ between males and females. Thus, it is expected to contribute knowledge about how these effects can negatively impact ecological terms and emphasize the importance of managing these types of waste.

7.2 MATERIAL AND METHODS

7.2.1 Sampling

Juvenile fish (*Rhamdia quelen*) were obtained from the fish farm of the State University of Western Paraná (UNIOESTE), Toledo, Paraná, Brazil. Males and female fish (weight: 13.66 ± 2.82g; length: 12.04 ± 1.02 cm; mean ± standard deviation), were acclimated for 30 days in glass aquaria (15L capacity; four fish per aquarium) at 27±1 °C, filtered and dechlorinated tap water, and a simulated natural photoperiod (12 h dark:12 h light). We followed the fish density per aquarium recommended by OECD, (2000) and fed the fishes ad libitum once daily during the experiment with a balanced diet suitable for this species (Laguna®, Brazilian Fish, 32% protein). The study was

approved by Ethics Committee for Animal Experimentation of the Federal University of Paraná, under the number 1227.

7.2.2 Chemical analysis

To quantify the presence of CIP during the bioassay, water samples (50mL) were collected from fish bioassay aquariums (one sample per aquarium, five samples per treatment) at specific times of times on the day 0, 1, 2, 7, 14, 21, and 28. Water samples were stored in tubes (Falcon ®) at -20 ° C until the time of analysis.

To study the duration of degradation of CIP in water under the experimental conditions of the bioassay, after acclimation to laboratory conditions, a total of twenty-four fish were divided into two groups, a control group (without CIP) and a treatment group (with 100 $\mu g.L^{-1}$ CIP, D6899 from Sigma Aldrich®). The twelve animals from each group were divided into three glass aquariums (15L). The highest concentration was chosen to facilitate the standardization of antibiotic determination during bioensaio. At this stage, animals were not separated by sex. CIP was added to the aquaria only once, at the beginning of the experiment. Water samples were collected from the aquaria at timepoints 0, 24, and 48hours. They were stored in 50 mL falcon tubes, at - 20 ° C until CIP was quantified.

High-performance liquid chromatography (HPLC) (Waters Alliance®2695) coupled with a multi & fluorescence detector (Waters 2475) and software Empower Pro 2002 was used to quantify CIP in water, according to Kitamura et al. (2022).The mobile phase triethylamine solution 0.4% (v/v) pH 3, acetonitrile and methanol 75:10:15 with a flow rate of 1 mL min^{-1} and 20 µL of sample injection volume. Wavelengths 278 and 453 nm for excitation and emission. Were used a Supelco Analytical Ascentis C18 column (250 × 4.6 mm, 5 µm) at 35 °C.

7.2.3 Fish exposure

After acclimatization to laboratory conditions and the CIP grading study, the animals (n = 80; 35 males and 45 females) were divided into four groups (Control, 1, 10 e 100 $\mu g.L^{-1}$ CIP), 20 animals per group, four fish per aquaria. For the experiment, fish were kept in glass aquaria (15 L), following the recommended biomass (approximate density of 1 $g.L^{-1}$) (OECD, 2000), under the same conditions as for the

of CIP degradation study. Concentrations were selected based on a literature search. In surface and subterranean waters in concentrations varying from 0.018 to 10 $\mu g.L^{-1}$ (Frade et al., 2014; Janecko et al., 2016; Mutiyar and Mittal, 2014; O'Flaherty and Cummins, 2016; Quadra et al., 2017). In effluents from water treatment plants and in wastewater, CIP concentrations ranged from 0.036 to 82.8 $\mu g.L^{-1}$ (Frade et al., 2014; Pal et al., 2010; Riaz et al., 2017), while in hospital and pharmaceutical industry effluents the average concentrations were of 104.43 $\mu g.L^{-1}$ (Frade et al., 2014; Mutiyar and Mittal, 2014; O'Flaherty and Cummins, 2016; Riaz et al., 2017). From the formation of this database, it was decided to use concentrations of 1, 10 and 100 $\mu g.L^{-1}$ CIP.

The bioassay proceeded semi-statically, and every 24 hours, one-third of the water was replaced with the appropriate CIP addition to the aquarium water to obtain the final test concentrations, which corresponded to the result of the CIP degradation study described previously. Control animals were subjected to the same water change schedule without the addition of CIP. The 28-day exposure period was chosen based on OECD Guide Test number 215. This Test Guideline is designed to assess the effects of prolonged exposure to chemicals on the growth of juvenile fish.

The water parameters such as pH (7.05 ± 0.44 units), ammonia (0.31 ± 0.18 ppm), nitrite (0.06 ± 0.18 ppm), dissolved O_2(7.05 ± 0.17 ppm), temperature of the bioassay room (27 ± 1°C) and temperature of aquarium water (20.2 ± 0.6). pH was measured with a pH meter (HANNA Instruments®, model HI98129), ammonia and nitrite with Labcon® test kits, dissolved O_2 and temperature with a probe (HANNA Instruments®, model HI 9146). After 28 days of exposure, animals were anesthetized with benzocaine, and blood was collected from the caudal vein with syringes containing 3% heparin. After euthanasia of the fish by spinal anesthesia, the brain, liver, and gonads were removed and stored at -80°C until biomarker analysis. The weight of the whole body, liver, and gonads weight was determined for calculation of the hepatosomatic index and gonadosomatic index.

7.2.4 Biomarker analysis

7.2.4.1 Hematological biomarkers

Blood smears were stained with May Grünwald-Giemsa-Wright. Total leukocyte and platelet counts were determined manually using the Neubauer chamber

under an optical microscope at 4000x (Tavares-Dias and de Moraes, 2006). The number of erythrocytes was used to calculate leukocytes and thrombocytes: Leucocytes (µL) = counted leukocytes x counts red blood cell (RBC) x 5000, and platelet (µL) = count thrombocytes x RBC x 5000. Hematocrit was determined according toHine (1992). Erythrocyte indices mean corpuscular volume - VCM ((Ht / Er) x 10).

7.2.4.2 Hepatosomatic and gonadosomatic index

Somatic indexes (GSI and HIS) were determined with the formula stated below(Tavares-Dias et al., 2000):

$$GSI = \frac{Gonadweight}{Wholebodyweight} X\ 100$$

$$HIS = \frac{Liver\ weight}{Whole\ body\ weight}\ X\ 100$$

7.2.4.3 Biochemical biomarkers

Brain and muscle samples were homogenized with a phosphate buffer (0.1 M; pH 7.5) at a ratio of 1:10 (mass/volume). The homogenized samples were centrifuged at 1000x g for 20 minutes at 4°C. The supernatants were used for measurement of acetylcholinesterase activity according toEllman et al. (1961), modified for microplates by Silva de Assis (1998).

Livers and gonads were homogenized in phosphate buffer (0.1 M; pH 7.0) at a ratio of 1:10 (m/v), and samples were centrifuged at 15000x g for 30 minutes at 4°C. The supernatants were used to measure the activity of superoxide dismutase (SOD) (Gao et al., 1998), catalase (CAT) (Aebi, 1984), glutathione peroxidase (GPx)(Hafeman et al., 1974), and glutathione S-transferase (GST) (Keen et al., 1976) were used. The concentrations of non-protein thiols/glutathione (GSH)(Sedlak and Lindsay, 1968)and lipoperoxidation (LPO) were determined by the method FOX (Jiang et al., 1992).To calculate the specific activity of the enzymes, total protein concentration was quantified using bovine serum albumin as a standard according to the Bradford method(Bradford, 1976).

7.2.4.4 Biomarkers of genotoxicity

Nuclear morphological changes

The micronucleus pisceo test was performed based on a method described by Ueda et al. (1992). A number of 2,000 erythrocytes were analyzed per animal. One slide per fish was prepared. Staining with giemsa was performed immediately before analysis (Leica® Microscope; model DMLS2) at 1000x magnification. Micronucleus and the other nuclear morphological alterations such as blebbed (B), lobed (L), notched (N) and vacuolated (V) were evaluated according to methodology described by Carrasco et al. (1990) and Çava and Ergene-Gözükara (2005).

DNA damage

For erythrocytes, the comet assay was analyzed as described by Singh et al. (1988), with modifications by Ferraro et al. (2004) and Cestari et al. (2004). Liver and gonads were analyzed according to the above methodology and adjustments described by Ramsdorf et al. (2009). Staining with ethidium bromide (20 $mg.L^{-1}$ - Sigma-Aldrich®) was performed prior to analysis using a Leica epifluorescence microscope (model: DMLS2) with rhodamine filter and 1000x magnification. Per animal, 100 nucleoids were analyzed and classified according to the damage present after the electrophoretic run. According to Azqueta et al. (2011), nucleoids were classified for each type of damage, as follows: 0 (no apparent damage), 1 (low damage), 2 (moderate damage), 3 (severe damage), and 4 (maximum damage). Scores were generated from the classification and quantification of damage, by multiplying the sum of the amount of damage by the value of each class (Koppen et al., 2017).

7.2.4.5 Assessment of ciprofloxacin-induced apoptosis

Quantification of the frequency of apoptosis was performed in blood and liver cells using a morphometric DNA diffusion assay described by Singh (2005, 2000). In this assay, DNA identification was performed with an intense fluorescent dye, YOYO-

1 (Microscopio Leica, model DFC 300). Apoptotic cells showed a granular DNA halo with a cloudy outer border.

7.2.4.6 Histopathological biomarkers

A fragment of liver and gonads were fixed in ALFAC solution (80% alcohol, formaldehyde, and glacial acetic acid) for 16 hours. They were then dehydrated in alcohols (70%, 80%, 90% and 100%), diaphanized in xylol, and plaedin Paraplast®. They were cut into 5 µm thick slices and stained with hematoxylin-eosin (HE). For the histopathological evaluation of lesions, all specimens were analyzed according to the index proposed by Bernet et al. (1999) modified by Mela et al. (2013). The important factors considered for each alteration found were: (1) minimal pathological importance, which presents possibilities of reversal; (2) moderate importance, reversible in some cases and (3) severe pathological importance, usually irreversible. According to the degree of occurrence of the alterations, the values assigned to the alterations were: (0) Unchanged, (2) Occasional Occurrence, (4) Moderate Occurrence and (6) Severe Occurrence (diffuse lesion). The injury indices (II) were obtained after applying a mathematical equation below:

$$II = \sum rp \sum alt \ (a \ X \ w)$$

where *pr* = reaction pattern; *alt* = alteration; a = score value; *w* =important factor.

7.2.4.7 Plasma 17 β-estradiol (E2) and 11 keto-testosterone (11-KT) levels

The collected blood was centrifuged (2000xg for 5 minutes) and stored at -20°C until analysis. Plasma concentrations of the steroid testosterone and estradiol were analyzed by theenzyme-linked immunosorbent assay (ELISA). The 17beta-estradiol ELISA kit was purchased from IBL International® (Hamburg, Germany), and the 11-keto testosterone ELISA kit was purchased from Cayman Chemical® (MI, USA). Testing protocols were performed according to the manufactures recommended procedures. Results were expressed in $pg.mL^{-1}$.

7.2.4.8 Neurotransmitters concentration

Neurotransmitters dopamine, serotonin, noradrenaline and their metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindoleacetic acid (5-HIAA), were quantified by high performance liquid chromatography with electrochemical detection (HPLC-ED). After weighing, the tissue (hypothalamus) samples were diluted in 0.1 M perchloric acid, containing 0.02% sodium metabisulfite (Sigma Aldrich®) and 50 ng/ml of internal standard 3,4-dihydroxybenzylamine hydrobromide (Sigma Aldrich®) and homogenized by ultrasound (Sonics). Samples were centrifuged at 10,000 RPM for 20 minutes at 4°C. The supernatant of the samples was injected into the HPLC (Shimadzu ®) in a volume of 20 µl. The instrument has a reversed-phase C-18 column (Synergi Fusion-RP C-18; 150 x 4.6 mm i.d., 4 µm particles -Phenomenex) with a pre-column (Security Guard Cartridges Fusion-RP, 4 x 3.0 mm) and electrochemical detector (ESA Coulochem III) equipped with a 350 mV guard cell (ESA 5011A) and LC-20AT injection pump (Shimadzu®). The column was maintained at a controlled temperature (25°C). Cell contains two chambers in series: each chamber including a graphite colorimetric electrode, a dual counting electrode and a dual reference electrode. Oxidation potentials were set to 100 mV for the first electrode and 450 mV for the second electrode. The mobile phase used was injected at a rate of 1 mL/min with the following composition: 10 g of citric acid monohydrate (Merck®), 100 mg of 1-octane sulfonic acid (Merck®), 20 mg of ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich®) in 450 mL milli Q water and 10% (v/v) methanol (Merck®), the pH was adjusted to 4. Concentrations of neurotransmitters and their metabolites were calculated by the area under the curve (current x time) interpolated to a straight standard curve obtained from known concentrations of each chemical species analyzed. The unit used to express these species was $ng.mL^{-1}$ of tissue weight, the program used was LC LabSolutions.

7.2.5 Statistical analysis

Data were evaluated according to the assumptions of normality and homogeneity (Shapiro-Wilk and Levene tests, respectively). For data that show the normality assumptions (parametric data), a one-way analysis of variance (ANOVA) was performed, followed by a Tukey test, with results reported as mean ± standard

error. For nonparametric data, the Kruskal-Wallis test was performed, followed by the Dunn and Mann-Whitney paired test, and results were expressed as median ± standard error. Statistical software (R.3.2.2, Team, 2015) was used for analysis, and GraphPad Prism 5.00 (GraphPad Software, Inc.) was used for graphs.

7.3 RESULTS

7.3.1 Chemical analysis

After 24 hours the initial concentration of 100µg.L^{-1} decreased to about50% (49.35 ± 12.45**)**. Based on this result, replacement every 24 hours was chosen to maintain the final Cip concentrations (1, 10, and 100 µg.L^{-1}) during the 28-day exposure.

CIP was not found in the water at the time in the control treatment. The concentration of CIP in the water of the experiment decreased in all groups during the first 48 hours. However, after this period, the concentration remained constant and close to the desired concentration, 1µg.L^{-1} (0.96 ± 0.02), 10 µg.L^{-1} (9.34 ± 1.15), and 100 µg.L^{-1} (93.85 ± 7.9) (**Supplementary material 1**).

7.3.2 Biomarker analysis

7.3.2.1 Hematological biomarkers

In male fish, erythrocytes count, hematocrit, and mean corpuscular volume (MCV) were not altered by CIP. On the other hand, 100µg.L^{-1}increased the number of thrombocytes (59.82%; P = 0.0288) and decreased the number of leukocytes (63.43%; P = 0.0270;

Table 1).In female fish, the number of erythrocytes, hematocrit, mean corpuscular volume (MCV) and the number of thrombocytes were not changed by CIP. However, at 10 and 100µg.L^{-1}decreased the number of leukocytes to 48.70% and 51.28%, respectively (P = 0.0486 and P = 0.0252;**Table 1**).

Table 1

Hematological biomarkers of *R. quelen* male after exposure to ciprofloxacin for 28 days.

		Environmentally relevant concentrations of ciprofloxacin			
		Control	**1µg.L^{-1}**	**10µg.L^{-1}**	**100µg.L^{-1}**
MALE	Erythrocytes (10^6 / µL)	1.93 ± 0.19^{a}	1.98 ± 0.21^{a}	2.34 ± 0.41^{a}	1,99 ± 0.23^{a}
	Hematocrit (%)	22.74 ± 1.26^{a}	23.78 ± 2.15^{a}	21.79 ± 2.17^{a}	24.26 ± 0.93^{a}
	VCM (*f*L)	107.41 ± 7.70 ^{a}	152.17 ± 43.27 ^{a}	101.51 ± 17.76 ^{a}	130.06 ± 25.51 ^{a}
	Thrombocytes (10^3 / µL)	36.06 ± 4.02 ^{a}	28.69 ± 5.28 ^{a}	59.76 ± 5.44 ^{ab}	69.00 ± 12.65 ^{b}
	Leukocytes (10^3 / µL)	25.24 ± 1.71 ^{a}	14.46 ± 3.56 ^{ab}	29.39 ± 8.23 ^{a}	9.23 ± 2,18^{b}
FEMALE	Erythrocytes (10^6 / µL)	1.71 ± 0.08^{a}	2.01 ± 0.13^{a}	1.75 ± 0.16^{a}	1,92 ± 0.11^{a}
	Hematocrit (%)	19.48 ± 0.77^{a}	20.84 ± 0.86^{a}	21.48 ± 1.10^{a}	22.29 ± 1.59^{a}
	VCM (*f*L)	113.49 ± 5.35 ^{a}	105.92 ± 11.09 ^{a}	131.95 ± 12.57 ^{a}	115.20 ± 11.37 ^{a}
	Thrombocytes (10^3 / µL)	48.05 ± 13.73 ^{a}	59.93 ± 6.67 ^{a}	45.58 ± 6.84 ^{a}	47.49 ± 4.76 ^{a}
	Leukocytes (10^3 / µL)	26.26 ± 2.75 ^{a}	22.72 ± 2.89 ^{ab}	13.47 ± 1.76 ^{b}	12.79 ± 2.99^{b}

The results are expressed as mean ± standard error. Different letters indicate significant differences ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

7.3.2.2 Hepatosomatic and gonadosomatic index

The hepatosomatic index (HSI) of *R. quelen* males changed after 1µg.L^{-1}CIP (decrease of 34.30%; P = 0.0075), compared with the control group (**Fig. 1A**). The HSI of females did not change (P = 0.4817; **Fig. 1A**). The gonadosomatic index (GSI) of males was changed after 1µg.L^{-1}CIP (64.32%; P=0.0094), compared with the control group (**Fig. 1B**). The GSI of females did not change (P = 0.8092; **Fig. 1B**).

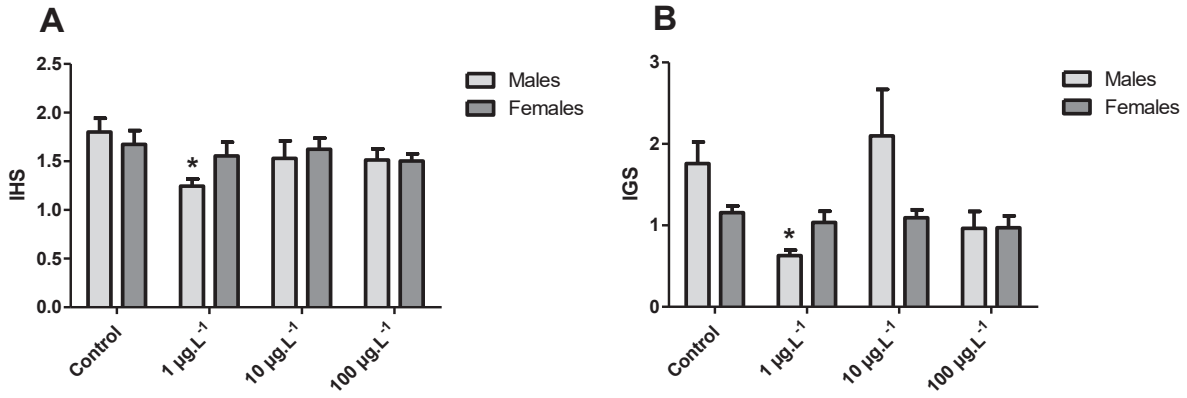


Fig. 1. Hepatosomatic index (A) and gonadosomatic index (B) of *R. quelen* after 28-day exposure to ciprofloxacin. Values are expressed as mean ± standard error. Statistical differences between treatments compared with the control group are indicated by * ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

7.3.2.3 Biochemical biomarkers

In the brain, the activity of the enzyme acetylcholinesterase (AChE) decreased by 73.99% and 86.69% in male fish at10 and 100µg.L^{-1} CIP, respectively (P = 0.0108 and P < 0.001, **Fig. 2A),** compared with the control group. In females, a reduction was also observed in the same treatments, 76.23% and 87%, respectively (P < 0.001 in both treatments; **Fig. 2A**). No change in AChE activity was observed in muscle in either sex (**Fig. 2B**).

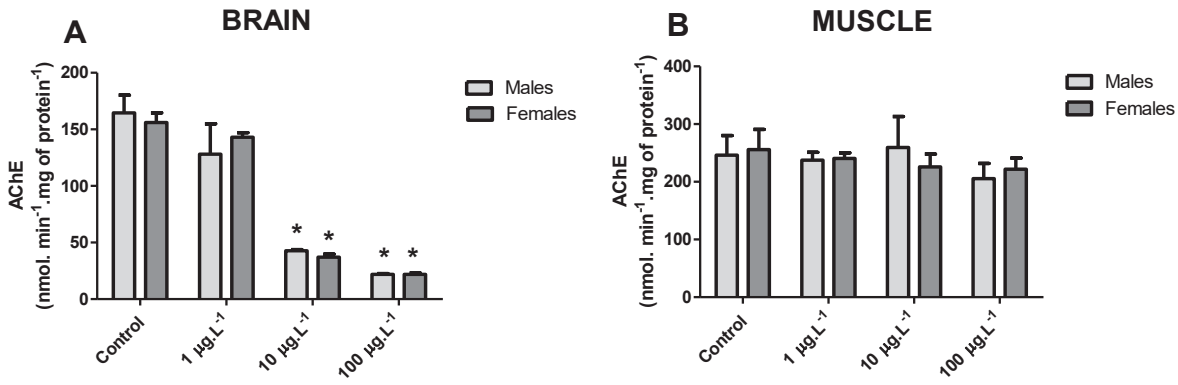


Fig. 2. Acetylcholinesterase activity in the brain (A) and muscle (B) of *R. quelen* after exposure to ciprofloxacin for 28-day. Values are expressed as mean ± standard error. Statistical differences between treatments compared with the control group are indicated by * (P < 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

In male liver, 1µg.L^{-1}CIP increased CAT activity (60.88%; P = 0.0105; **Fig. 3A**) and GSH level (208.13%; P = 0.0020; **Fig. 3E**) and caused lipid peroxidation with an increase in LPO of approximately 295.54% (P = 0.0245; **Fig. 3F**).Exposure to 10µg.L^{-1}decreased GST activity (48.67%; P = 0.0031; **Fig. 3**A), increased CAT activity (77.94%; P = 0.0026; **Fig. 3C**), increased GSH content(190.97%; P = 0.0039; **Fig. 3E**), but no lipid peroxidation was observed (P = 0.9907; **Fig. 3F**). In contrast, after exposure to 100µg.L^{-1}, an increase in LPO was observed (262.52%; **Fig. 3F**), with a decrease in SOD activity (77.14%; P < 0.001; **Fig. 3B**) and an increase in CAT activity(98.02%; P < 0.001; **Fig. 3C**).

In the liver of female fish, exposure to 1µg.L^{-1} did not alter antioxidant system enzymes but caused oxidative stress (LPO increase of approximately 214.30%; P = 0.0119; **Fig. 3F**). Exposure to 10 µg.L^{-1}also did not alter any enzyme, and oxidative stress was not observed. In contrast, exposure on 100µg.L^{-1}CIP decreased SOD

activity (70.95%; P = 0.0088; **Fig. 3B**) and caused oxidative stress (increased LPO by 103.76%; P = 0.0051; **Fig. 3F**).

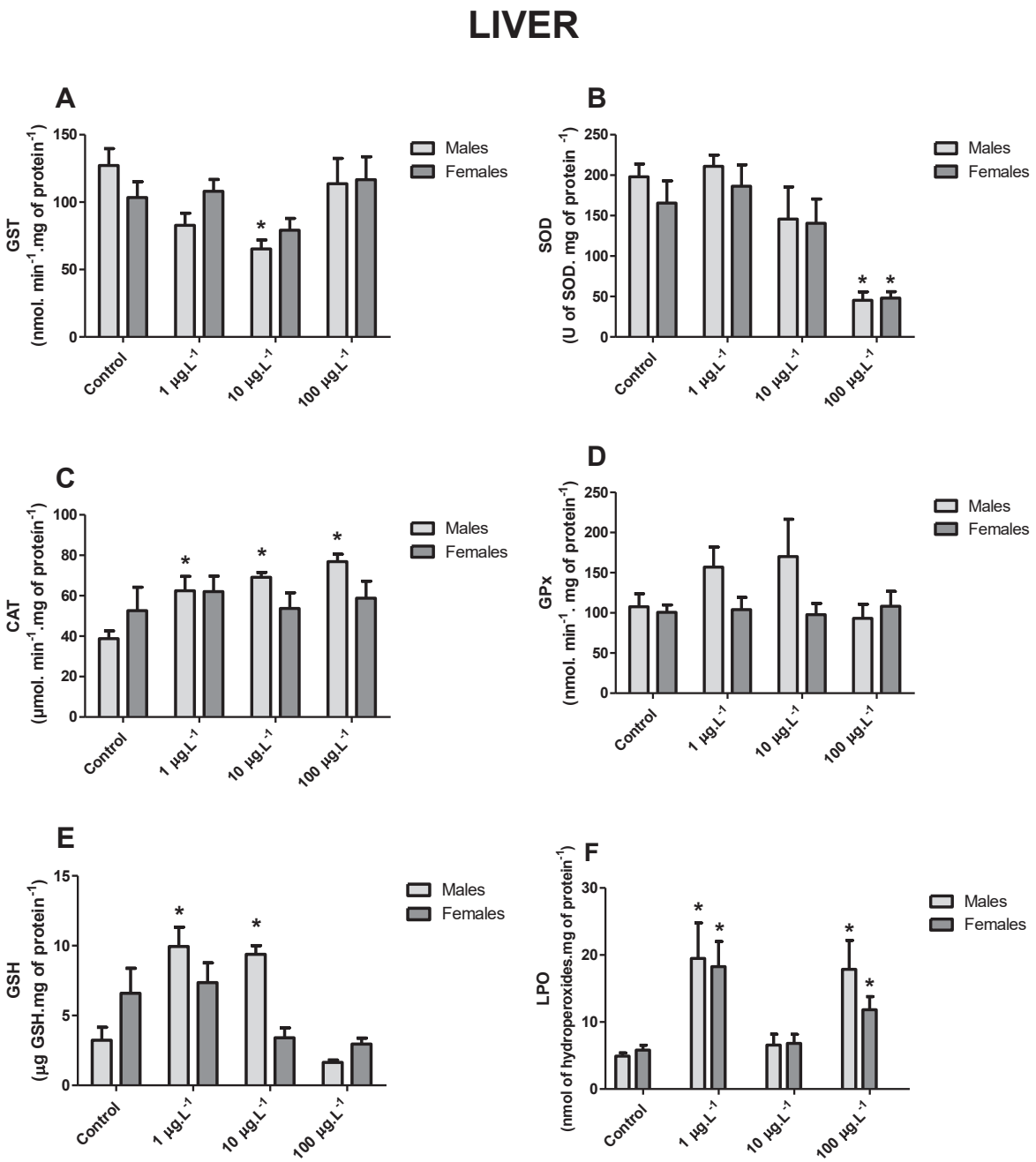


Fig. 3. Biochemical biomarkers in the liver of *R. quelen* males and females after 28-day exposure to ciprofloxacin. Glutathione S-transferase (GST)(**A**), superoxide dismutase (SOD) (**B**), catalase (CAT) (**C**), glutathione peroxidase (GPx) **(D)**, reduced glutathione (GSH) (**E**), and lipid peroxidation (LPO) (**F**). Values are expressed as mean ± standard error. * Indicates statistical differences between exposure groups ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

In the gonads of male fish, CIP at a concentration of 10 µg.L^{-1}increased GPX activity (221.20%, P = 0.0121, **Fig. 4C**) and GSH content (139.58%, P = 0.0300, **Fig.**

4D), but no lipid peroxidation was observed. (P = 0.4351, **Fig. 4E**), compared with the control group. Exposure to the highest concentration (100 µg.L^{-1}) increased GPx by 41.34% (P = 0.0337, **Fig. 4C**). The lower concentration of CIP did not cause changes in the antioxidant system.

In females, CIP only caused an increase in GPX in the gonads by approximately 82.35% (at 100µg.L $^{-1}$, P = 0.0135,**Fig. 4C**).

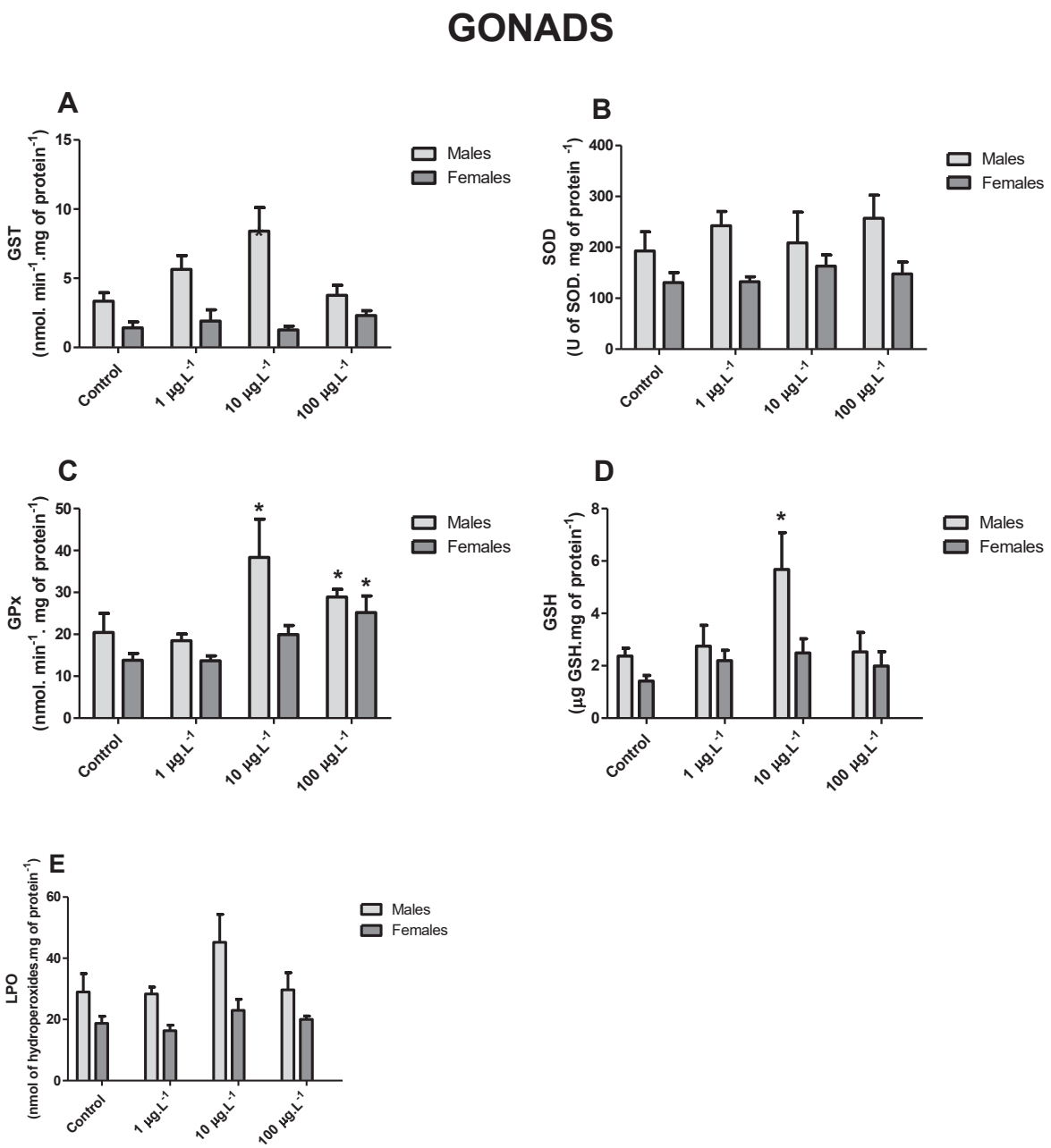


Fig. 4. Biochemical biomarkers in the gonads of male and female *R. quelen* after 28-day exposure to ciprofloxacin. Glutathione S-transferase (GST)(**A**), superoxide dismutase (SOD) (**B**), glutathione peroxidase (GPx) (**C**), reduced glutathione (GSH) **(D**), and lipid peroxidation (LPO) (**E**) Values are expressed as mean ± standard error. * Indicates statistical differences between exposure groups (P < 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

7.3.2.4 Biomarkers of genotoxicity

Nuclear morphological changes

After exposure to CIP, micronuclei were not observed in either in male or female animals. However, some morphological changes were observed, such as blebbed(P = 0.0059), vacuoles (P = 0.0097), and changes in erythrocyte nuclei(P = 0.0106)at 100µg.L^{-1} (**Table 2**). In females, the vacuolated type was observed at 10µg.L^{-1} (P = 0.0212) and 100µg.L^{-1} (P = 0.0171), and nucleated erythrocyte changes were observed at 100µg.L^{-1} (P = 0.0129) (**Table 2**).

Table 2
Mean frequency of nuclear morphological changes and number of erythrocytic nuclear alterations (ENAs) in male and female *Rhamdia quelen* after 28-day exposure to ciprofloxacin.

	Treatments	Blebbed	Lobed	Notched	Vacuolated	Micronuclei	Total ENAS
MALES	Control	2 (0.5 / 2.5)	2 (1/ 4)	3 (2 / 4)	0.5 (0 / 2)	0 (0/0)	8 (6 / 11)
	1 µg. L^{-1}	1 (1 / 2)	1 (1/ 3.5)	1 (1 / 4.5)	1.5 (1 / 2.75)	0 (0/0)	5 (4.25 / 7.5)
	10 µg. L^{-1}	2 (0.5 / 3.5)	2 (0.5 / 3.5)	3 (0 / 4.5)	2.5 (0.25 / 5.5)	0 (0/0)	10 (6.5 / 14.5)
	100 µg. L^{-1}	4 (3.25 / 5)*	4 (3.25 / 5)	5 (3.25 / 7)	4 (1.25 / 6.75)*	0 (0/0)	18 (12.5 / 21.75)*
FEMALES	Control	3 (1 / 4.5)	3 (1 / 4.5)	2 (1 / 3.75)	1 (0 / 3)	0 (0/0)	7 (5.50 / 9.50)
	1 µg. L^{-1}	1 (0 / 2)	1 (0 / 2)	3 (1.5 / 3.5)	1.5 (1 / 3)	0 (0/0)	6.5 (4 / 9.75)
	10 µg. L^{-1}	2 (1 / 4)	2 (1 / 4)	3.5 (0.75 / 5)	4 (1.75 / 5)*	0 (0/0)	10 (6.75 / 12.50)
	100 µg. L^{-1}	3 (2 / 5.75)	3 (2 / 5.75)	4.50 (1.5 / 5.75)	3.50 (2 / 4.75)*	0 (0/0)	13 (9.25 / 17.75)*

The values are given as the medians and first and third quartiles (Q1 and Q3). Statistical differences among treatments compared to the control group are indicated by * (P < 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

Comet test

DNA damage was observed in erythrocytes of male fish at 10µg.L^{-1} CIP (120.37%, P = 0.0059) and at 100 µg.L^{-1}(247.07%, P < 0.001)**Fig. 5A**. In the females at all concentrations tested caused DNA damage, at 1 µg.L^{-1} (139.89%, P = 0.0303), 10 µg.L^{-1}(81.35%, P = 0.0373) and at 100 µg.L^{-1} (204.12%, P = 0.0142) **Fig. 5B**.

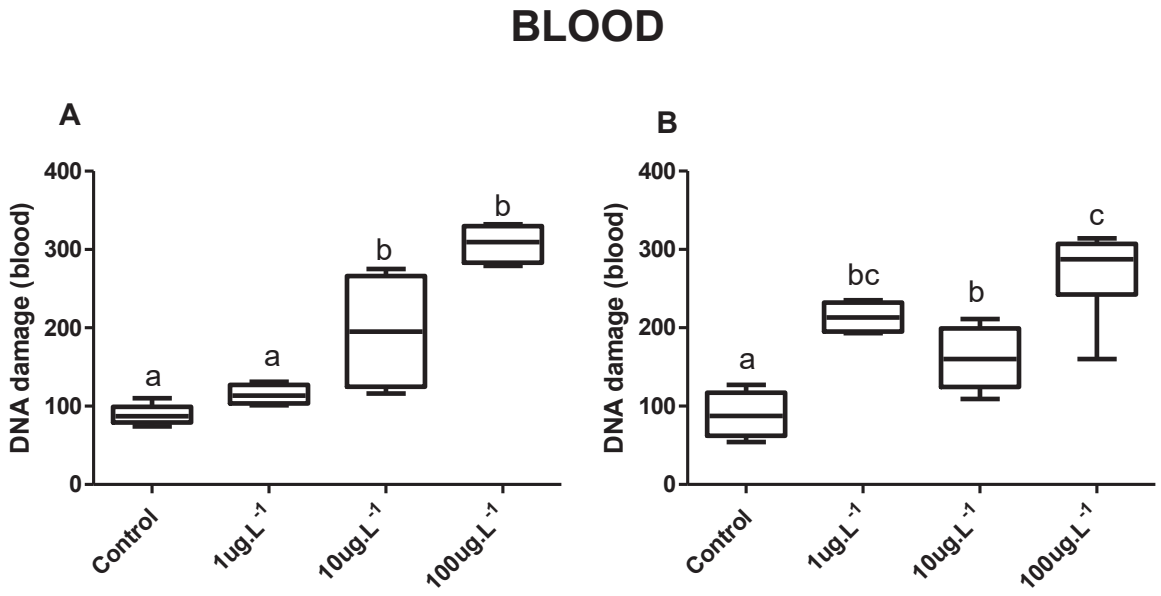


Fig. 5.DNA damage score (median ± interquartile range) in blood of *Rhamdia quelen*, (**A**) male and (**B**) female animals, exposed to three concentrations of CIP for 28-day. Different letters indicate significant differences between treatments (P ≤ 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

In the liver, there was DNA damage in males at exposure to 1µg.L^{-1} (50.69%, P < 0.001, **Fig. 6A**) compared to the control group. There were no changes in females (P = 0.889; **Fig. 6B**).

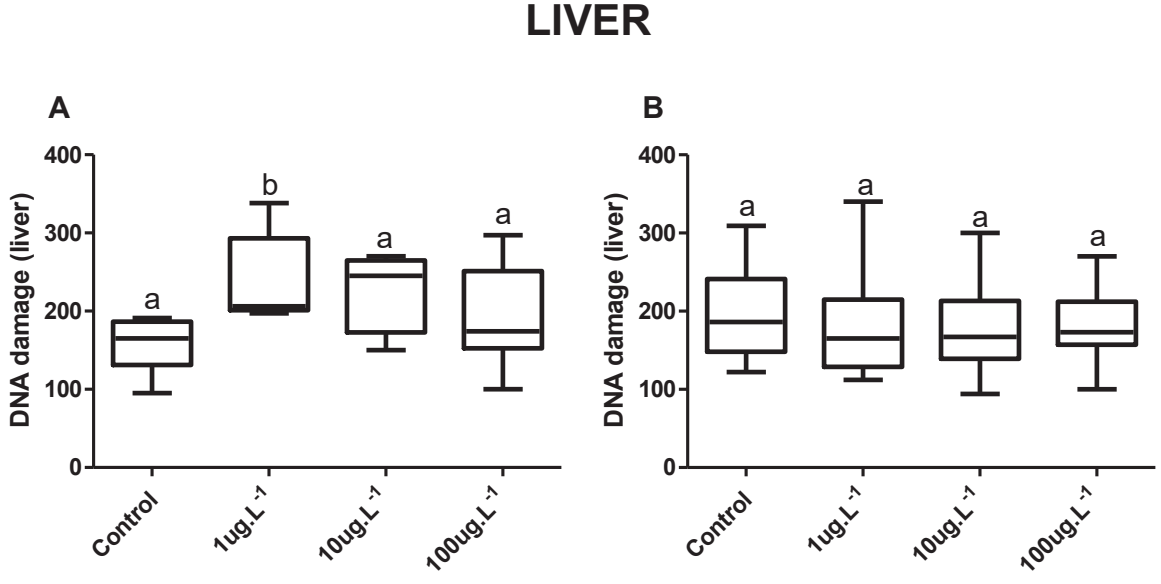


Fig. 6.DNA damage score (median ± interquartile range) in liver of *Rhamdia quelen*, (A) males and (B) females, exposed to three concentrations of CIP for 28 days. “a” and “b” letters indicate significant differences between treatments (P ≤ 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

There was no genotoxicity in male gonads (P = 0.5734) and female gonads (P = 0.6805) (**Supplementary material 2**).

7.3.2.5 Assessment of ciprofloxacin-induced apoptosis

In the blood of male fish, an increase in the frequency of apoptosis was observed after exposure to 10µg.L^{-1}(900%, P = 0.0376) and 100µg.L^{-1}(10.233%, P = 0.0261), **Fig. 7A**. In females, there was an increase in the frequency of apoptosis after exposure to 100µg.L^{-1} (12.971%, P = 0.0046), **Fig. 7A**.

In the liver of male fish there was an increase in the frequency of apoptosis after exposure to 1µg.L^{-1} CIP (554.31%, P = 0.0417, **Fig. 7B**). In females, exposure to the three concentrations of CIP caused an increase in apoptosis frequency (602.78% at 1µg.L^{-1}, P = 0.0029; 367.86% at 10µg.L^{-1}, P = 0.0302; 554.17% at 100µg.L^{-1}, P= 0.0030) (**Fig. 7B).**

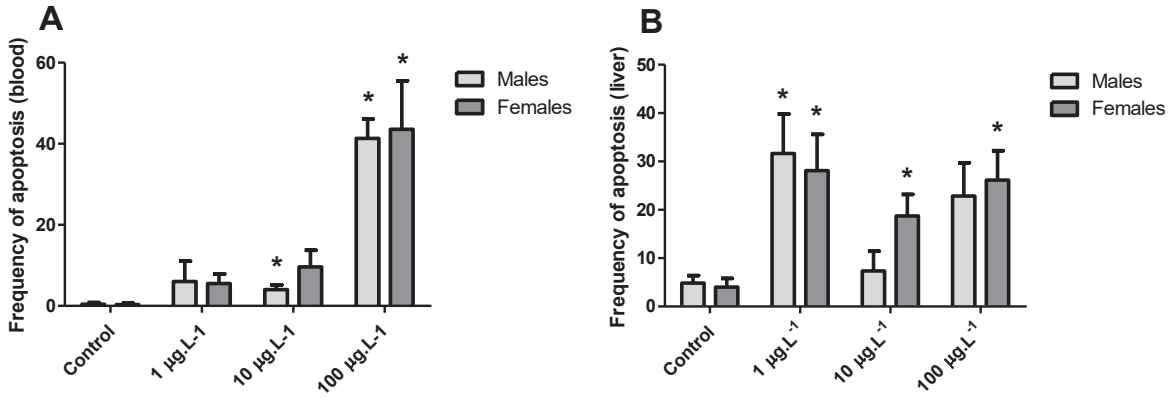


Fig. 7. Frequency of apoptosis in blood (A) and liver (B) of male and female *Rhamdia quelen* exposed to three concentrations of CIP for 28-day. Statistical differences between treatments compared with the control group are indicated by * (P < 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

7.3.2.6 Histopathological biomarkers

The liver tissue of *R. quelen* has a similar description to that of other teleost fishes. The parenchyma is composed of hepatocytes, sinusoidas, and blood vessels (**Fig. 8A**). The histopathological index showed higher values in individuals exposed to 10 and 100 µg.L^{-1}in a dependent concentration (**Fig. 8**). The histopathological damage

observed was leukocyte infiltration (**Fig. 8B**), steatosis (**Fig. 8C**), and necrotic foci (**Fig. 8D**).

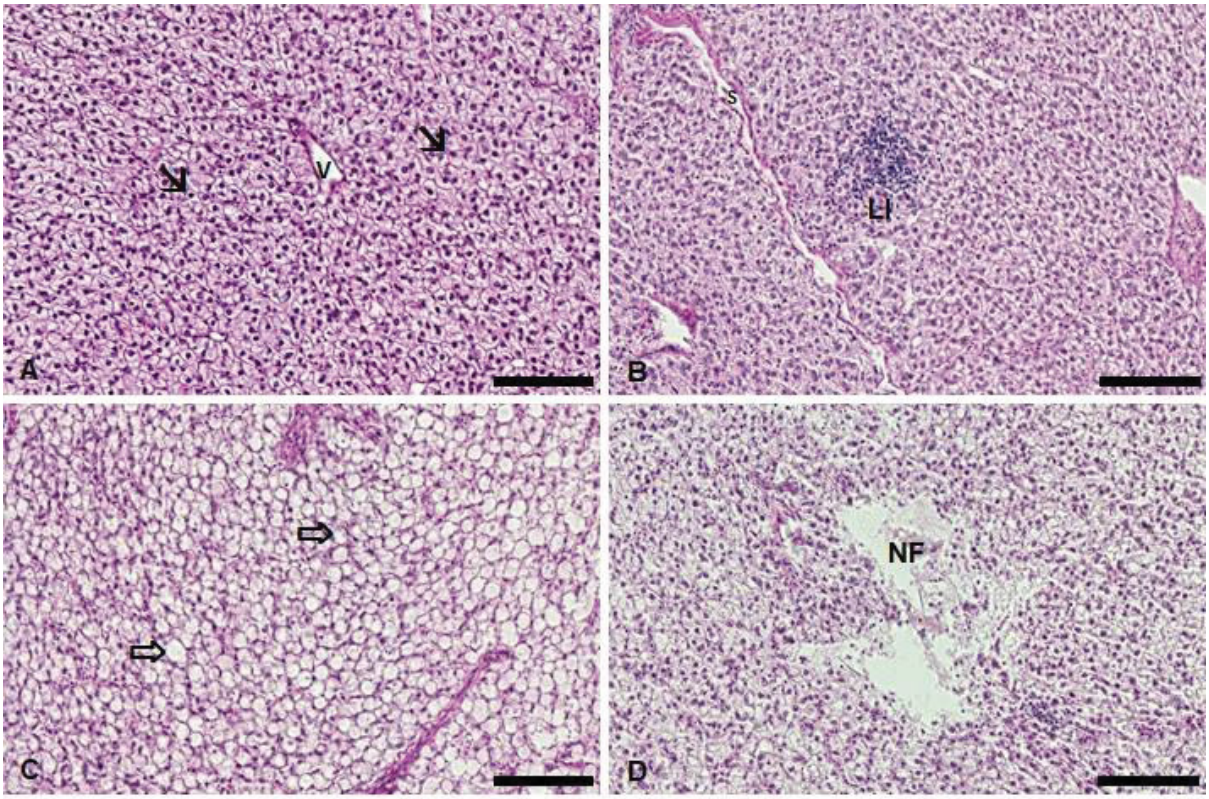


Fig. 8. Histological sections of *Rhamdia quelen,* liver counter stained with hematoxylin/eosin. **(A)** Liver of control group. Blood vessels (**V**) and hepatocyte nuclei (⇲). **(B)**Liver of the exposed group (10 µg**.** $.L^{-1}$). Sinusoid (**S**) and leukocyte infiltration (**LI**). **(C)**Liver of the exposed group (100 µg**.** $.L^{-1}$). Steatosis (⇨). (**D)**Liver of the exposed group (100 µg.L^{-1}). Necrotic foci (**NF**). Scale bar: 100 µm.

Evaluation of histopathological biomarkers separately by sex (**Table 3**) showed that exposure to 10 and 100µg.L^{-1} caused leukocyte infiltration ($P < 0.001$), steatosis ($P = 0.0409$) and necrosis ($P = 0.0034$) in liver tissue in male fish compared to the control group. In females, this damage was observed only in the group exposed to 100µg.L^{-1} (leukocyte infiltration, $P < 0.001$; steatosis, $P < 0.001$; necrosis, $P = 0.0413$). At 10 and 100µg.L^{-1}, CIP caused a significant increase in the Bernet index (in a dose-dependent manner) in both sexes (males, $P = 0.0561$; females, $P = 0.0081$).

Table 3
Mean values (score changes) for histopathologic effects on liver tissue after 28-day exposure of *R. quelen*, males and females, to three concentrations of ciprofloxacin (1, 10, and 100 μg.L^{-1}) and the control group.

		Histopathology Biomarkers			
	Tissue/Treatments	LI	S	N	Bernet Index
MALES	Control	0	0,36	0,36	1,45
	1μg.L^{-1}	0	0	0,4	1,2
	10μg.L^{-1}	2,00**	2,40**	2,80**	14,80**
	100μg.L^{-1}	1,75**	3,25**	2,75*	15,00**
FEMALES	Control	0	0,2	0,4	1,4
	1μg.L^{-1}	0	0,4	0,4	1,6
	10μg.L^{-1}	1,14	1,71	1,43	8,29**
	100μg.L^{-1}	2,00**	2,31**	3,08**	15,54**

Statistical differences among treatments compared to the control group are indicated by * ($P < 0.05$).LI: Leukocyte infiltration; S: Steatosis; N: Necrosis. N = 11 males and 09 females (Control); N = 10 males and 10 females (1μg.L^{-1}CIP); N = 6 males and 14 females (10μg.L^{-1}CIP); N = 8 males and 12 females (100μg.L^{-1}CIP).

The testis from the control fish shows spermatogenic tubules. At the margin of the lobules, spermatogonia are observed. We can see large numbers of spermatocytes (**Fig. 9A).** In the group exposed to 100μg.L^{-1} CIP, an increase in spermatogenesis was observed. In this treatment, sperm increased significantly compared to the control ($P < 0.001$, **Fig. 10B**). Interstitial tissue is observed around the lobules in this treatment (**Fig. 9B**).

In the female ovaries of the control group, the development of oocytes was observed, which presented in perinucleolar oocytes within the cytoplasm and a large, rounded nucleus, characteristic of female oocytes at immature stage of development (**Fig. 9C**). At a dosage of 100μg.L^{-1}, CIP impaired the oogenesis process. An increase in gonadal development was observed with an increase in the number of vitellogenic oocytes ($P < 0.001$, **Fig. 10B**). Vitellogenic oocytes have a larger diameter and hypertrophic follicular cells (at 100 μg.L^{-1}, **Fig. 9D**).Histopathological injury index in testes and ovaries showed higher values in individuals exposed to 100 μg.L^{-1} (**Fig. 10A).**

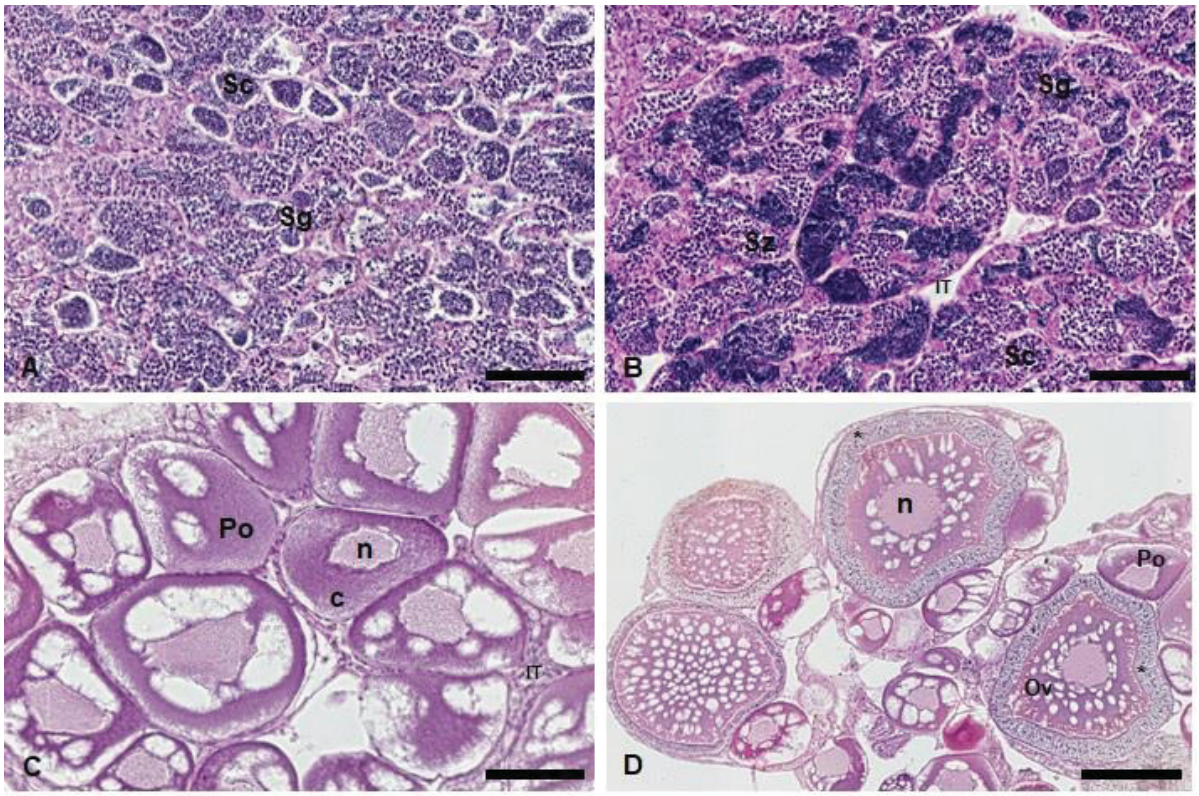


Fig. 9. Histological sections of gonads of *Rhamdia quelen,* counterstained with hematoxylin/eosin. (**A**) Male testis of control group. Spermatocytes (**SC**) and spermatogonia (**Sg**). (**B**) Male testis of the exposed group (100 µg.L^{-1} of CIP). Spermatocytes (**SC**), spermatogonia (**Sg**), spermatozoa (**Sz**) and interstitial tissue (**IT**). (**C**) Female ovary of control group (**C**) Perinucleolar oocytes (**Po**) with different size and shape, with nucleus (**n**) and cytoplasm (**c**). (**D**) Female ovary of the exposed group (100 µg.L^{-1}). Perinucleolar oocytes (**Po**), nucleus (**n**), vitellogenic oocyte (**Ov**) and hypertrophy of follicular cells (*****). Scale bar: 100 µm.

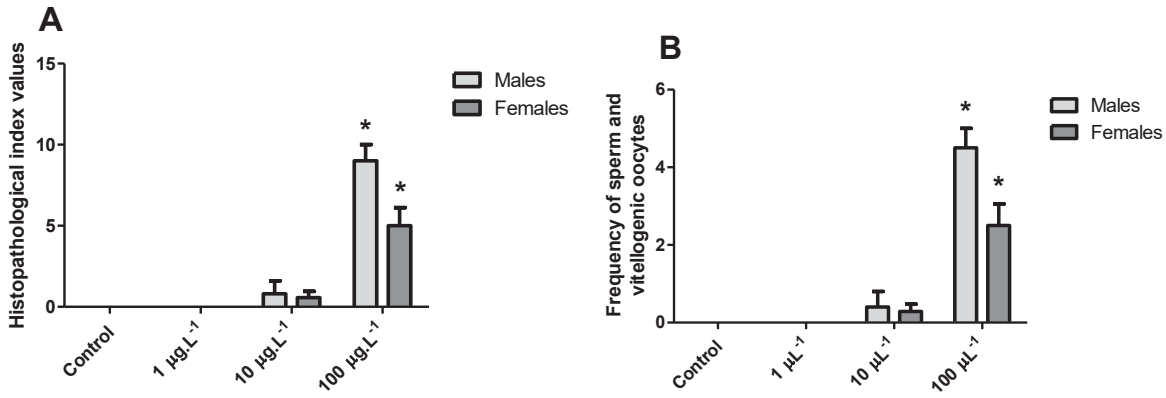


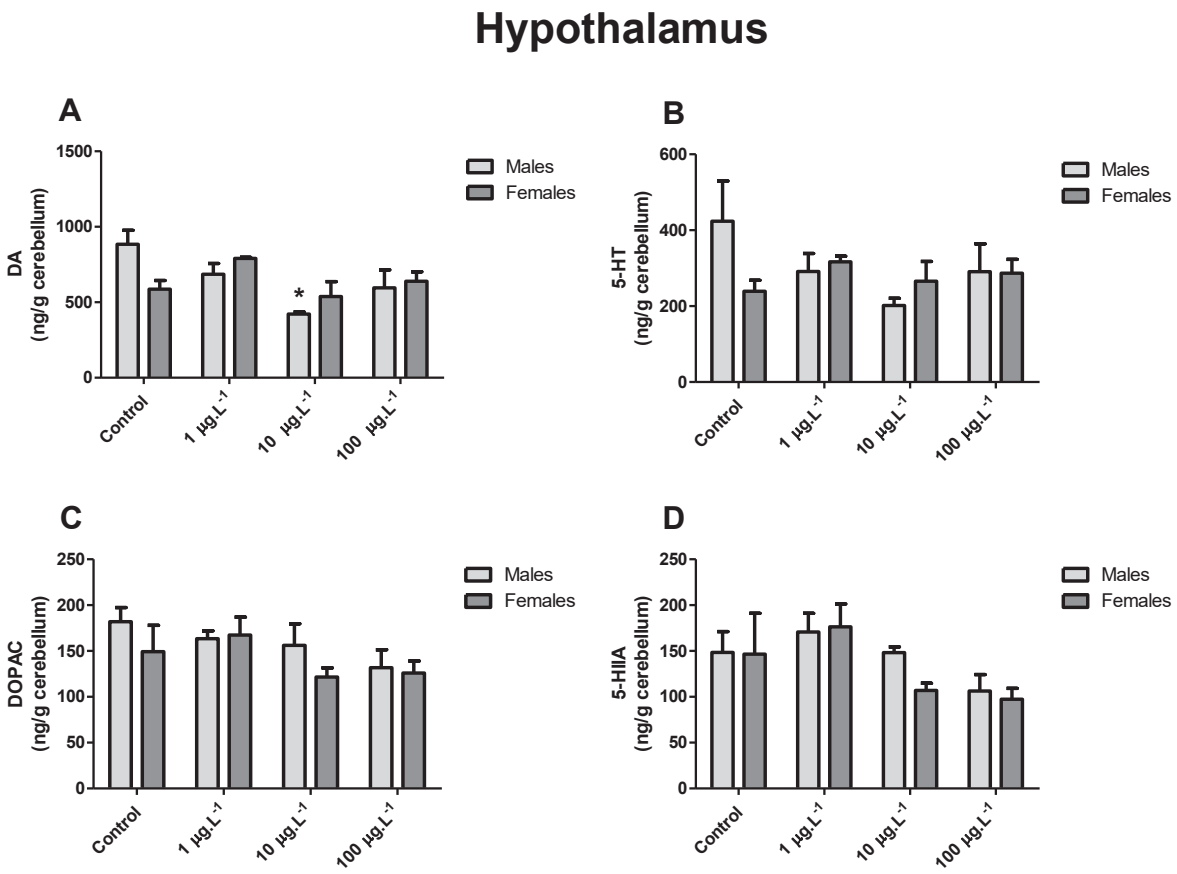
Fig. 10. Histopathological index values in testis and ovaries of *R.quelen* exposed to three concentrations of CIP and control group for 28-day (A) and frequency of sperm and vitellogenic oocytes in R. quelen after exposure to CIP (B). Statistical differences among treatments compared to the control group are indicated by asterisk ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

7.3.2.7 Plasma 17 β-estradiol (E2) and 11 keto-testosterone (11–KT) levels

No changes were observed in the estradiol levels in of males (P = 0.3806) and females (*P* = 0.1579). There was also no change in 11-KT levels in males (P = 0.1900) and females (P = 0.1574) (**Supplementary material 3**).

7.3.2.8 Concentration of Neurotransmitters

In male fish, exposure to 10µg.L^{-1} CIP decreased DA concentrations(52.30%, P = 0.0384, **Fig. 11A**) and increased 5-HIIA/5-HT ratio (81.35% , P = 0.0471, **Fig. 11F**). The values of 5–HT (p = 0.3135), DOPAC (P = 0.1782), 5–HIIA (P = 0.1660), NA (p= 6148), and the DOPAC/AD ratio (P = 0.2751) did not change (**Fig. 11**).In females, CIP increased at 1µg.L^{-1}NA (62.65%, P = 0.0405, **Fig. 11G**). However, CIP did not change the levels of DA(P= 0.1349), 5-HT(P = 0.6534), DOPAC(P = 0.2128), 5-HIAA (P= 0.0852), and the ratio of DOPAC/DA(P = 0.2023) and 5-HIAA/5-HT(P = 0.0568)(**Fig. 11**).



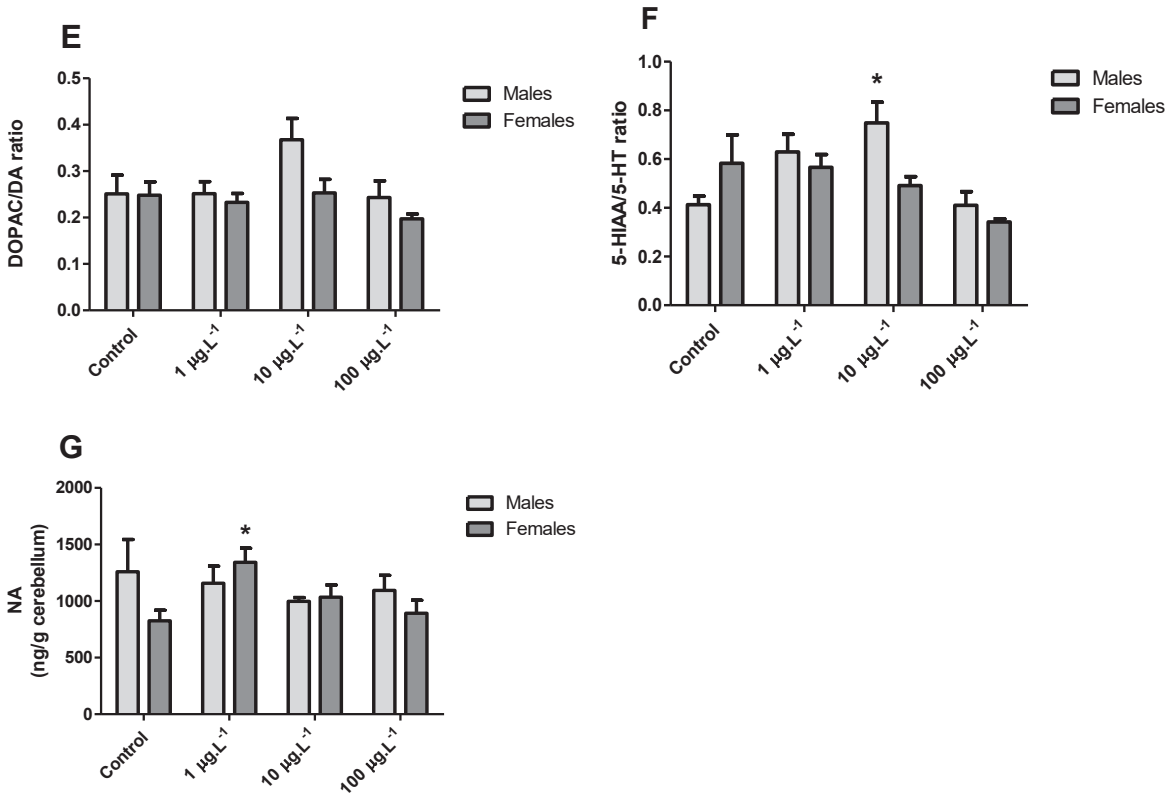


Fig. 11. Effects of CIP exposure on dopamine (DA) (**A**), serotonin 5-HT (**B**), DOPAC (**C**), 5-HIAA (**D**), DOPAC/DA ratio (**E**), 5-HIAA/5-HT ratio (**F**), norepinephrine (NA)(**G**)of male and female *Rhamdia quelen*. Exposure to three concentrations of CIP for 28 days. Values are expressed as mean ± standard error of the mean. Statistical differences between treatments compared with the control group are indicated by asterisk ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

7.4 DISCUSSION

The presence of CIP in aquatic environments, such as rivers used for public supply, can affect the environmental quality, representing a risk to aquatic fauna and human health.

In males, exposure to 100 µg.L^{-1}increased thrombocytes counts and decreased leukocyte counts. In females, 10 and 100µg.L^{-1} caused a decrease in leukocyte count. These data show differences between the sexes. According to Tavares-Dias and Moraes (2004), both are considered defense cells and are included in the white cell group despite their different modes of action. Leukopenia is known to be an important physiotherapeutic measure for harmful substances(Gonçalves et al., 2010). A process of leukopenia mayincrease susceptibility to microbial invasion and allow the spread of infections(Barton and Zwama, 1991; Sumpter, 1997).

According to our results, the decrease in leukocytes in the blood may also be accompanied by an increased presence of leukocytes in the liver, which is characterized by leukocyte infiltration in the tissue. Leukocytes play an important role in the body's defense against xenobiotics through phagocytosis and paper production (Falcon et al., 2008). A study confirming our findings also showed a decrease in blood leukocyte count and liver leukocyte infiltration after 100 $\mu g.L^{-1}$CIP for an acute exposure of 4 days(Kitamura et al., 2022).

Thrombocytes in vertebrates other than mammals, such as fish, are analogues of thrombocytes in mammals. In osteichthyes, these cell fragments are involved in homeostatic processes, including aggregation and release responses to blood vessel damage, and also in the development of the immune response (Stosik et al., 2019). According Witeska et al. (2022) thrombocytes in fish besides coagulation function, have phagocytosis capacity. The increase in thrombocytes count and decrease in leukocyte count detected in our study could be a response to CIP. According to Tavares-Dias and Moraes (2004), there is an inverse relationship between platelet count and leukocyte count. Therefore, the number of thrombocytes increases when the number of lymphocytes decreases(Tavares-Dias and Moraes, 2004).In acute exposure to CIP (Kitamura et al., 2022), no changes in the number of thrombocytes were detected, highlighting that prolonged exposure (28day) to CIP may alter this effect.

Although no micronucleus was observed, the analysis of genotoxicity biomarkers in blood showed that CIP caused morphological alterations in erythrocytes in both sexes (in males at an exposure of 100 $\mu g.L^{-1}$ and in females at 10 and 100 $\mu g.L^{-1}$). The occurrence of nuclear erythrocytic alterations (AENs) is also considered an indicator of gentotoxic damage (Vanzella et al., 2007). In contrast, no nuclear morphological changes were observed in cells during acute exposure (Kitamura et al., 2022), suggesting that damage may occur at these concentrations after prolonged exposure. In contrast, nuclear abnormalities were observed in peripheral erythrocytes of *O. mykiss* after acute and chronic exposure to oxytetracycline(Rodrigues et al., 2016).

Comet assay analysis showed DNA damage to erythrocytes in males in all three CIP contractions and in females only in 10 and 100 $\mu g.L^{-1}$. As reported in the study by Kitamura et al. (2022), acute exposure to CIP at the same concentrations as in the present study also caused genotoxicity.

The main mechanism of action fluoroquinolones is to interfere with DNA synthesis by affecting two enzymes, DNA gyrase (topoisomerase II) and DNA topoisomerase IV. In a bacterial cell, these two topoisomerases act by unwinding the DNA double helix(Champoux, 2001). However, eukaryotic organisms also have topoisomerases that play key roles in many aspects of DNA metabolism. The II DNA type topoisomerase (topo II) is essential for various nuclear processes, such as DNA replication, chromosome segregation, and maintenance of chromosome structure (Bakshi et al., 2001). Topo II is also the cellular target for a variety of clinically relevant antitumor drugs (Bakshi et al., 2001) and for quinolones-class antibiotics(Champoux, 2001). Therefore, the genotoxicity caused by CIP, such as nuclear abnormalities and damage to erythrocyte DNA, also can happen because the CIP can affect the topoisomerases of *Rhamdia quelen*. DNA damage in *Danio rerio* blood cells also was observed after acute exposure to CIP alone and with acetaminophen(mixed) (Elizalde-Velazquez et al., 2022).

Diffusion assays showed that exposure to CIP (at a concentration of 100 $\mu g.L^{-1}$) caused apoptosis of blood cells. Apoptosis, or programmed cell death is characterized by cell shrinkage, chromatin condensation, formation of cytoplasmic opacities, and apoptotic bodies (Ziegler and Groscurth, 2004). Multiple factors are involved in apoptosis, and different apoptotic stimuli converge in a common apoptotic pathway mediated by the mitochondria (Kroemer, 2003; Wang, 2001). Apoptosis via the mitochondrial pathway may be preceded by overproduction of reactive oxygen species (ROS)(Liu et al., 2015; Spierings et al., 2005). Although we did not measure the production of ROS, exposure to CIP may have caused overproduction of ROS by oxidative stress in hepatocytes, as demonstrated in liver. This overproduction of ROS may have been a stressor that triggered intrinsic apoptosis (Ott et al., 2007). CIP is already known to induce cellular apoptosis. Herold et al. (2002) reported that CIP induced apoptosis and inhibited the proliferation of human colorectal carcinoma cells. Growth arrest was mediated by inhibition of DNA synthesis, induction of mitochondrial damage and subsequent apoptosis (Herold et al., 2002). Therefore, ciprofloxacin has been explored as a good candidate for cancer therapies (Sharma et al., 2010). Apoptosis was observed in zebrafish embryos under tilmicos in treatment, with upregulation of apoptosis associated genes such as p53, bcl 2, bax, caspase 3 and caspase 9 (Yan et al., 2019). Caspase 9 is responsible for the intrinsic pathway of apoptosis (McIlwain et al., 2013).

In this study, acetylcholinesterase (AChE) activity was used as a biomarker for neurotoxicity. AChE is involved in the inhibition of acetylcholine in nerve endings, preventing the continuous firing of nerves that is essential for the normal functioning of sensory and neuromuscular systems (Payne, 1976). In this way, AChE is an essential enzyme for maintenance of the nervous system, and its inhibition is considered an indicator of environmental pollution in aquatic organisms (Li et al., 2012). Our data showed a significant reduction in enzyme activity brain AChE at 10 and 100μg.L^{-1} CIP, in both sexes. A similar result was found by Liu et al. (2014), the brain activity of acetylcholinesterase was significantly inhibited by norfloxacin at 0.04 mg.L^{-1} after 4 and 7 days*. C. auratus* (goldfish) exposed for 1, 2, 4 and 7 days treatments with 0.25 and 1.258 mg.L^{-1} ofloxacin and 0.5 mg.L^{-1} of sulfamethoxazole were shown significantly reduced AChE activity on days 4 and 7 (Yang et al., 2019).However, no change in AChE was observed in acute exposure of *R. quelen* to CIP(Kitamura et al., 2022), suggesting that AChE may be inhibited by CIP in the long term. Reduction in the activity of this enzyme may lead to physiological and behavioral changes, which in turn may affect survival, feeding, and reproductive behavior(Smith, 1984; Weis et al., 2001).

Another biomarker used in this study was HSI, a marker of liver stress (Al-Ghais, 2013; Azevedo et al., 2009; Gül et al., 2004; Kumar et al., 2017). In males, exposure to 1 μg.L^{-1} CIP decreased the HSI, while this parameter did not change in females. The pattern of HSI changes suggests that males and females may respond differently to CIP exposure. Other studies also report significant differences in HSI with respect to sex (Flippin et al., 2007; Koporikov and Bogdanov, 2013). In the study by (Flippin et al., 2007), HSI tended to increase with ibuprofen exposure in women, while HIS appeared to decrease in men. In another study (Guiloski et al., 2015b), diclofenac caused a decrease in liver size (HSI) of *H. malabaricus* up to 0.2 μg/kg. Exposure of rainbow trout to diclofenac caused a glycogen depletion in hepatocytes, which may lead to a decrease in HIS (Schwaiger et al., 1997), a similar effect may have occurred in our study. However, no information on the alteration of hepatocytes or HSI due to CIP exposure has been found so far.

In this study, it was observed in the liver of male fish that GST decreased at an exposure to 10 μg.L^{-1}.GST plays an important role in the detoxification of xenobiotics by conjugation with GSH (Al-Ghais, 2013). Our data suggest that CIP impairs phase II detoxification by decrease enzyme activity, which could lead to

possible accumulation of ROS. Acute exposure did not alter the activity of this enzyme (Kitamura et al., 2022). when *C. mrigala* was exposed to CIP (1 and 1.5 $\mu g.L^{-1}$ for 5, 10 and 15) GST activity increased in liver tissue in both ciprofloxacin treatment groups (Ramesh et al., 2021). GST activity increased at ciprofloxacin concentrations of 0.7 and 100 $\mu g.L^{-1}$ in a study of juvenile if *D. rerio* for 28 days (Plhalova et al., 2014), suggesting that the response of GST may be influenced by the exposure time to the xenobiotic and the animal model studied.

In contrast to GST, SOD decreased in both males and females after exposure to 100 $\mu g.L^{-1}$. SOD is an antioxidant enzyme specialized in the conversion of superoxide (O_2^-) to H_2O_2 (Van der Oost et al., 2003). At the same concentration, SOD was also increased in acute exposure, (Kitamura et al., 2022). Exposure of *C. mrigala* to CIP for 5, 10 and 15 day also resulted in an increase in SOD activity in the liver (Ramesh et al., 2021).

CIP caused an increase in CAT activity at the three concentrations in male fish; on the other hand, it was not altered in females. CAT it an enzyme specialized in splitting hydrogen peroxide ($H_2 O_2$) into water (H_2O) and oxygen (O_2) (Van der Oost et al., 2003). Acute exposure also increased the activity of CAT (at 10 and 10 $\mu g.L^{-1}$ (Kitamura et al., 2022) and in *C. auratus* exposed to the norfloxacin (Liu et al., 2014). An increase of catalase also was also observed during long-term exposure of *D. rerio* to norfloxacin (Bartoskova et al., 2014).

GSH levels increased after exposure to 1 and 10 $\mu g.L^{-1}$ (in males), but as in CAT, there was no change in female for this biomarker. GSH is a nonenzymatic component of great importance for the antioxidant system, acting not only as a cofactor for GPx but also having other functions of great importance for the detoxification process, such as in the II phase of metabolism, especially through GST activity, conjugation with electrophilic compounds, or acting as a powerful antioxidant (Van der Oost et al., 2003). There was no change in GSH levels during acute exposure (Kitamura et al., 2022), suggesting that considering that damage may occur at the same concentrations after prolonged exposure to CIP.

CIP caused lipid peroxidation in both male and female animals at the lowest and highest concentrations (1 and 100 $\mu g.L^{-1}$). A non-monotonic U-shaped dose-response curve was observed. Endogenous disruptors are known to act via non-monotonic dose-response curves, and therefore it has been argued that the effects observed at high doses cannot be used to extrapolate to the low dose range (Carnevali

et al., 2018). Non-monotonic curves for biological responses at extremely low concentrations of contaminants have been demonstrated for contaminants such as bisphenol-A and atrazine (Vandenberg et al., 2012). This type of effect has also been accepted for pharmaceutical and personal care products (Wilkinson et al., 2016).Interestingly, in our study we found that CIP (at a concentration of 100μg.L^{-1}) stimulated the development of both female and male gonads, which will be discussed later.

Therefore, our data showed differences in enzymatic responses with respect to sex and possibly in sensitivity to the xenobiotic, but, the effect of lipid peroxidation on liver tissue was the same in males and females. In general, in our study, we observed more changes in the antioxidant system in males than in females in our study.

In Zivna et al. (2016), the effect of ciprofloxacin (1, 100, 500, 1000, and 3000 μg.L^{-1}) in the early life stages–embryo larvae (24 hours after fertilization to day 33 day) of *C. carpio* - and it was found that enzyme activity, antioxidant and biotransformation, and lipid peroxidation could be affected by ciprofloxacin, even at the lowest concentration tested (1 μg.L^{-1}). Ramesh *et al* (Ramesh et al., 2021) studied the effect of CIP on *C. mrigala*, and an increase in LPO was also observed in the treated groups. CIP also induced a significant oxidative stress response in the liver of *D. rerio* after acute exposure (Elizalde-Velázquez et al., 2022). However, LPO concentrations were not significantly affected by acute exposure in the same animal model (*R. quelen*) (Kitamura et al., 2022). This suggests that long-term exposure to CIP can cause oxidative stress in the liver tissue of *R. quelen*.

Lipid peroxidation contributes significantly to the loss of cell function under oxidative stress (Storey, 1997). During oxidative stress, cytotoxic reactive oxygen species (ROS) can react with critical cellular macromolecules, possibly leading to enzymatic inactivation, lipid peroxidation (LPO), DNA damage, and occasionally cell death (Van der Oost et al., 2003).

In the present study, damage to hepatocyte DNA was observed in the liver of male fish at an exposure of 1 μg.L^{-1}, possibly caused by the generation of reactive oxygen species (Yang et al., 2020), considering that oxidative stress was also observed at this concentration and even by the mechanism of action of CIP (Bakshi et al., 2001), as explained previously. In female animals, genotoxicity was not observed with any treatment. Acute exposure of *R. quelen* to CIP (the same concentrations used

in this study) has been reported to damage hepatocyte DNA (Kitamura et al., 2022). These results show that over time *R. quelen* may have adapted to prevent genotoxic damage. In another acute study with *Danio rerio*(Elizalde-Velázquez et al., 2022), CIP also caused damage to hepatocyte DNA at all concentrations tested (0.250, 0.500, and 1 $\mu g.L^{-1)}$. Xenobiotics-induced genotoxicity is mainly due to inhibition of the DNA repair process (Hartwig and Schwerdtle, 2002). Genotoxicity is usually accompanied by changes in physiological and biochemical indicators of fish. Inhibition of transcription of genes involved in inflammation, energy metabolism, and antioxidant responses is also considered an example of genotoxicity detected in liver tissues(Yang et al., 2020).

Exposure to 1 $\mu g.L^{-1}$ CIP increased the frequency of apoptosis in male liver tissue. At a concentration of 100 $\mu g.L^{-1}$ there was an increase of 372.91%, but it was not significant compared to the control group. In the female animals, the frequency of apoptosis increased at all three CIP concentrations. Oxidative stress damage can lead to permeabilization of the outer mitochondrial membrane, resulting in the release of pro-apoptotic proteins and activation of caspase. Cell death occurs frequently in complex multicellular organisms to maintain tissue homeostasis (Spierings et al., 2005). Changes in cellular stress responses play an important role in triggering apoptosis. This event is continuously monitored by mitochondria, which integrate multiple proapoptotic signals into common apoptotic degradation cascades (Ziegler and Groscurth, 2004). To date, only one study (Elizalde-Velázquez et al., 2022) has reported CIP-induced oxidative stress and upregulation of genes related to apoptosis (BAX, CASP3, CASP6, CASP8, and CASP9) and oxidative stress (Nrf1 and Nrf2), further supporting our findings on the hepatotoxicity and genotoxicity of CIP. Liu et al. (2015) investigated the effects of enrofloxacin in grass carp, and the cell damage caused by enrofloxacin was confirmed by apoptosis assays. They showed that apoptosis induced by enrofloxacin in grass carp hepatocytes was dose-dependent.

Histopathological evaluation of liver tissue showed lesions such as leukocyte infiltration (inflammation), steatosis, and necrosis in both sexes at an exposure of 100 $\mu g.L^{-1}$. Data comparable to the acute studies by Kitamura et al. (2022) at an exposure of 100 $\mu g.L^{-1}$ and by Elizalde-Velázquez et al. (2022) at all concentrations tested (0.250, 0.500, and 1 $\mu g.L^{-1}$). In males, these lesions were also observed at an exposure of 10 $\mu g.L^{-1}$. These results confirm the damage caused by CIP exposure and show that although exposure at 10 $\mu g.L^{-1}$ altered the enzymes of the antioxidant system without causing lipid peroxidation, other effects were identified in other biomarkers, such as:

a) morphological changes in erythrocytes of *R. quelen* females, b) DNA damage of erythrocytes (in males and females), c) increase in the frequency of apoptosis (in females). In addition, the injury index showed a dose-dependent relationship (from concentrations of 10 and 100 $\mu g.L^{-1}$, in both sexes). Moreover, it is possible that the increased activity of the antioxidant system reduced or even avoided the damage caused by the excessive production of ROS (Birnie-Gauvin et al., 2017b) due to exposure to CIP. Considering that we had no histopathological lesions when exposed to 1$\mu g.L^{-1}$ CIP, antioxidants play an essential role in the detoxification process and in the regulating of pathophysiology (Qin and Liu, 2013b). The observed steatosis may be a consequence of disorders actions resulting from the action of toxic products and, in several precedes necrosis processes (Robbins and Contran, 2015). The necrosis can have several causes, such as the conversion of a contaminant to a metabolite toxic substance that may include free radicals or reactive oxygen. These lead to peroxidative damage to membrane lipids and hence necrosis. Regarding the leukocyte infiltrate, it can be said that it is a nonspecific response, produced by chemical agents or by pathogens. Its function is to maintain the integrity of the tissue when physical or chemical stimuli affect the the liver tissue (Rocha et al., 2010). So, the presence of an extensive area of leukocyte infiltration in the liver indicate that this tissue was suffering great influence of CIP (Matushima et al., 2006).

In the gonadal tissue, enzymatic changes occurred only at the intermediate concentration (at 10 $\mu g.L^{-1}$). GPx activity and GSH levels increased. There are few of data on the evaluation of gonads of fish exposed to fluoroquinolones. Bartoskova et al. (2014) evaluated the adverse effects of norfloxacin on *D. rerio* in a growth toxicity test. A 28-days exposure to norfloxacin at concentrations ranging from 0.1 $mg.L^{-1}$ to 30 $mg.L^{-1}$ resulted in a significant increase in the activities of glutathione peroxidase, glutathione-S-transferase, and catalase (in the experimental groups exposed to the highest concentrations), whereas no changes were observed in lipid peroxidation.

In the present study, we did not detected DNA damage in the gonads of males and females. In contrast, Liu et al. (2014) reported a strong and positive correlation between the extent of DNA damage in the gonads and the concentrations of norfloxacin (another fluoroquinolone) alone and in combination with sulfamethoxazole during a 7-day exposure. According to the authors, this could significantly affect sperm quality and fertilization ability. It is becoming increasingly clear that DNA damage is associated with and is a reliable predictor of adverse affects on growth, reproduction,

and survival (Ann Rempel et al., 2006), but the threshold for impairment of fish reproduction is not yet known. To date, there are no articles reporting biochemical, genotoxic, and histopathological assessment of male and female gonads, or assessment of neurotransmitters and reproductive hormones following exposure to CIP, as was conducted in this study.

Although we observed no oxidative stress in the gonads as well as DNA damage, histopathological analysis showed that both sexes had altered gonadal tissue after exposure to CIP at the highest concentration. In male exposed to 100μg.L^{-1}, CIP exposure impaired spermatogenesis and stimulated testicular development. Spermatozoa increased significantly compared to the control. In female gonads, CIP impaired the process of oogenesis and stimulated gonadal development. Although we were unable to evaluate vitellogenin, Liu et al. (2014) examined the concentration of vitellogenin after exposure to norfloxacin alone and in combination with sulfamethoxazole. The highest concentration of norfloxacin and two higher concentrations of the mixtures with sulfamethoxazole increased the concentration of vitellogenin in serum. These data confirm our results showing that CIP increase the number of vitellogenic oocytes. These results are important because they show that fluoroquinolones such as norfloxacin and CIP can affect the gonads of fish and potentially affect reproduction; therefore, it is important that further research be conducted to better understand and predict these effects.

However, these effects did not lead to changes in IGS and in the levels of E_2 and 11-KT, possibly we could not follow these changes because the samples were only in the last period of the 28-day bioassay, when the gonads had already developed (Chaves-Pozo et al., 2008). E_2 and 11KT secretion then ceases and the biosynthetic pathway turns to progestin secretion, which initiates the final maturation of the oocytes or testes (Hachfi et al., 2012). Also, in goldfish exposed to bisphenol A, the gonadosomatic index (GSI), hepatosomatic index (HSI) and E2 levels were not affected but an increase in hepatic vitellogenin was induced. The authors suggested that bisphenol A could affect testicular spermatogenesis and cause an alteration in sperm maturation (Hatef et al., 2012). Therefore, it is important to further this investigation of the stimulatory effect of CIP on fish gonads and performed assessments with samples at different time points during the bioassay.

Finally, we also examined some neutrotransmitters. The neuroendocrine mechanism regulates reproduction through the hypothalamic-pituitary-gonadal (HPG)

axis, which is evolutionarily conserved in vertebrates (Okuzawa et al., 2003; Peter, 1982; Schulz et al., 2010, 1999). The HPG axis is regulated by a variety of internal and external factors (Prasad et al., 2015). The monoaminergic system modulates the function of the HPG axis. Its neuromediators, such as dopamine, can inhibit the release of LH, while others, such as norepinephrine and serotonin, stimulate the release of LH not only directly but also through the GnRH axis (Hachfi et al., 2012). Depending on the physiological stage, sex steroids exert negative and positive feedback effects at the level of the pituitary and hypothalamus to modulate the synthesis and release of GnRH and GtH (Peter and Yu, 1997; Schulz et al., 1999).

In the brain of male fish, 10 $\mu g.L^{-1}$ CIP caused a decrease in the levels of DA, suggesting a stimulatory effect on the HPG axis; therefore, an increase in the 5-HIIA/5-HT ratio may have been observed in the animals. It should be noted that at 10 $\mu g.L^{-1}$, a nonsignificant increase in sperm frequency was observed in males and a nonsignificant increase in vitellogenic oocytes was observed in females. However, this was not reflected in hormonal and histopathological changes by the end of the experiment, which may indicate the onset of the process. In females, exposure to 1$\mu g.L^{-1}$ CIP increased NA levels (62.65%). This result could indicate an initial neuroendocrine stimulus. It is possibly that the higher concentration of CIP triggered the development of the gonads of male and female fish at 28-day of exposure, while the lower concentrations did not do so until longer exposure.

7.5 CONCLUSION

The present study showed that long-term environmental concentrations of CIP have adverse effects on non-target organisms, such as brain neurotoxicity. In blood, leukocytes count, blood genotoxicity and erythrocyte apoptosis decreased. In the liver: oxidative stress, genotoxicity, hepatocyte apoptosis and histopathological changes such as leukocyte infiltration, steatosis, and necrotic foci. In addition, it caused the development of female and male gonads. Moreover, it was demonstrated that there was a difference between biomarker responses in relation to sex, especially in hematological, biochemical, and genotoxic biomarkers. These hematological, biochemical, genotoxic, histopathological, and stimulatory effects on reproductive organs may impact the health of these animals and indirectly influence important behaviors such as locomotion, predation, reproduction, and feeding. The results are a

wake-up call for decision makers and need to be given special consideration when formulating legislation and addressing environmental risks.

7.6 DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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7.9 SUPPLEMENTARY MATERIAL 1

Table 4. Mean concentrations of ciprofloxacin (μg.L^{-1}) in water samples from the *R. quelen* exposure experiment to environmental concentrations of ciprofloxacin for 28 days. Data expressed as mean ± standard error.

Experimental groups	Ciprofloxacin concentration (μg.L^{-1})
0 μg.L^{-1}	0.00
1 μg.L^{-1}	0.96 ± 0.01
10 μg.L^{-1}	9.34 ± 0.58
100 μg.L^{-1}	93.85 ± 3.97

7.10 SUPPLEMENTARY MATERIAL 2

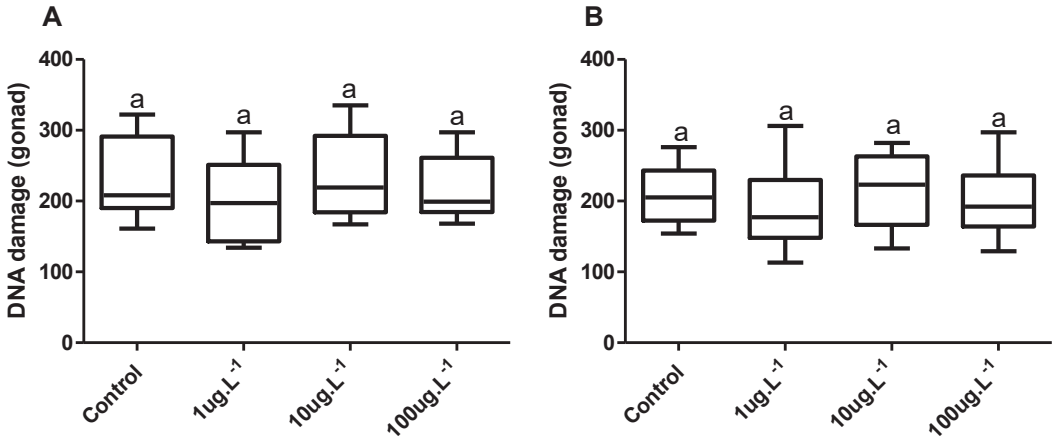

Fig. 12. DNA damage score (median ± interquartile range) in gonads of *Rhamdia quelen*, (**A**) males and (**B**) females, exposed to three concentrations of CIP for 28 days. Different letters indicate significant differences (P ≤ 0.05).

7.11 SUPPLEMENTARY MATERIAL 3

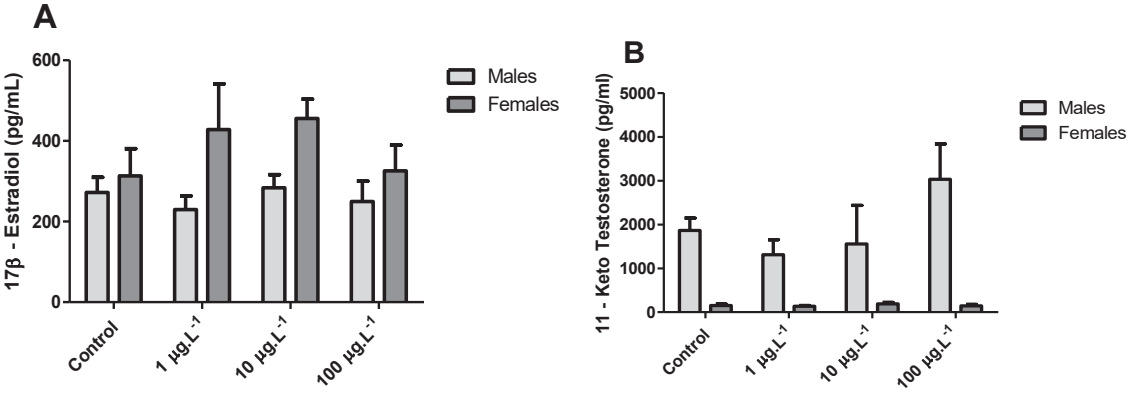

Fig. 13. Plasma levels of 17β-estradiol (A)and 11 – Keto Testosterone (B) of *R. quelen* (A) after exposure to ciprofloxacin for 28 days. Values are expressed as mean ± standard error. Statistical differences among treatments compared to the control group are indicated by * (P < 0.05).

8 CAPÍTULO II - Effects of environmental concentrations of ciprofloxacin on gills and posterior kidney of male and female Neotropical fish (*Rhamdia quelen*)

Effect of environmental concentrations of ciprofloxacin on gills and posterior kidney of male and female fish (*Rhamdia quelen*)

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HIGHLIGHTS

- CIP caused oxidative stress in the gills of *R. quelen*
- CIP caused histopathological changes in the gills and in the posterior kidney
- CIP caused genotoxicity and renal cell necrosis of female *R. quelen*
- MMCs increased in the posterior kidney

ABSTRACT

Antibiotics are commonly found in aquatic environments and their effect on aquatic biota is still unknown. The aim of this study was to evaluate the toxic effects of long-term exposure of *Rhamdia quelen* to ambient concentrations of ciprofloxacin (CIP), focusing on the gills and in the posterior kidney, organs related to absorption, and excretion. After 28 days of exposure, fish were anesthetized, submitted to euthanasia, and the gills and posterior kidney were collected for biochemical and histopathological biomarkers analysis. Biochemical, genotoxic, histopathological biomarkers, evaluation of apoptosis and cell necrosis, and incidence of melanomacrophage centers (MMCs) were analyzed at posterior kidneys. The results showed that CIP caused oxidative stress in the gills of males (at 1 and 100 $\mu g.L^{-1}$) and females (at 1 and 10 $\mu g.L^{-1}$). In addition, CIP (at 10 and 100 $\mu g.L^{-1}$) caused histopathological damage to the gills, such as the epithelial lifting of the secondary lamella and lamellar hyperplasia with lamellar fusion, in a concentration-dependent-manner, in both sexes. In the posterior kidney, CIP caused DNA damage and necrosis of renal cells in females. CIP altered the normal histoarchitecture of renal parenchyma, with various degenerative changes, such as leukocyte infiltration, presence of adipocytes on the stroma, necrosis, and extensive area of hemorrhage (at 10 $\mu g.L^{-1}$, in both the sex). At 100 $\mu g.L^{-1}$ it was observed the presence of adipocytes on stroma and necrosis (in the males), and necrosis (in the females). There was an increase in the Bernet Index after fish exposure to 10 and 100 $\mu g.L^{-1}$, in both sexes. The highest values of the Bernet Index were attributed to fish exposed to 10 $\mu g.L^{-1}$, in both males and females. These concentrations also caused an increase in the incidence of MMCs (higher incidence at 10 $\mu g.L^{-1}$). The results showed that the exposure of *R. quelen* fish to CIP concentrations present in rivers, effluents from water treatment plants, sewage and industrial effluents caused toxicity in the gills and posterior kidney of males and females of Rhamdia quelen, which may compromise the functionality of these organs.

Keywords: Oxidative stress. Genotoxicity. Melanomacrophage centers (MMCs). Nephrotoxicity

8.1 INTRODUCTION

Antibiotics, a class of pharmaceutical products, have received increasing attention due to their widespread use, ubiquitous environmental distribution, great potential for bioaccumulation, and possible toxicity to non-target organisms (Kitamura et al., 2022; Madikizela and Ncube, 2022; Rodrigues et al., 2019, 2017; Yang et al., 2020a). Moreover, the presence of antibiotics in aquatic environments has worried the scientific community, as they can influence the resistance of pathogens to antibiotics (Duarte et al., 2022; Guo et al., 2022; Liu et al., 2022). Antimicrobial resistance (AMR) is a major public health concern worldwide (CDC, 2019; WHO and ECDC, 2022).

Studies have shown that antibiotics are considered "pseudo persistent" contaminants due to their continuous release into the environment (Kazakova et al., 2018; Yang et al., 2017) because of various human activities, the discharge of wastewater, agricultural runoff, or improper disposal of unused drugs. All these entry routes associated with the limited efficiency of wastewater treatment plants (WWTP) and water treatment plants (WTP) mean that the inflows of these substances are continuous (Gao et al., 2012; Li et al., 2012).

Fluoroquinolones are among the antibiotics frequently detected in the aquatic environment at concentrations ranging from $ng.L^{-1}$ to $\mu g.L^{-1}$ (Teglia et al., 2019). Ciprofloxacin belongs to this group and is one of the most used antibiotics worldwide(Kelly and Brooks, 2018; Sahlin et al., 2018). It is a second-generation antibiotic, exhibiting a broad spectrum of activity against gram-negative and gram-positive aerobic bacteria(Al-Omar, 2005; Sharma et al., 2010).

Ciprofloxacin is eliminated by renal and non-renal mechanisms. The drug is partially metabolized in the liver by modification of the piperazinyl group to at least four metabolites (desethylene-ciprofloxacin, sulfo-ciprofloxacin, oxo-ciprofloxacin, and N-acetyl-ciprofloxacin)(Al-Omar, 2005). These metabolites have microbiological activities that are less than that of the parent drug, but may be similar to or greater than that of some other quinolones (Al-Omar, 2005). Ciprofloxacin and its metabolites are excreted in urine by both glomerular filtration and by tubular secretion. Following oral administration of a single 250-, 500-, or 750-mg dose in adults with normal renal function, 15–50% of the dose is excreted in urine as unchanged drug, and 10–15% as metabolites within 24 h. 20–40% of the dose is excreted in feces as the unchanged

drug and metabolites within 5 days (Al-Omar, 2005). In animals, ciprofloxacin also is not completely absorbed or metabolized in vivo(Zhou et al., 2008).

In veterinary medicine, although in some countries ciprofloxacin is no longer included in the list of veterinary antibiotics, as in European Union countries (Idowu et al., 2010), ciprofloxacin is an active metabolite of the veterinary antibiotic enrofloxacin (Singha and Ahn, 2016). Therefore, the concentrations of ciprofloxacin present in the environment may originate from human and veterinary use.

Ciprofloxacin is not biodegradable (Sahlin et al., 2018) and can resist degradation in the environment (Flach et al., 2018; Gothwal and Shashidhar, 2017; Sahlin et al., 2018; Xie et al., 2017). Studies had detected ciprofloxacin in surface water, groundwater, wastewater, effluents, sediments, soil and even drinking water (Wagil et al., 2014; Xie et al., 2017) studies have also measured ciprofloxacin in animals, such as fish (Sehonova et al., 2019; Y. Zhang et al., 2016b). Despite its distribution in the environment, little is known about the effect of exposure on aquatic biota.

So far, few studies have been conducted on the effect of this antibiotic on fish (Kitamura et al., 2022; Plhalova et al., 2014; Ramesh et al., 2021a; Zivna et al., 2016). Carvalho et al., 2022 (article under review) reported that CIP concentrations present in the environment can cause toxic effects on *Rhamdia quelen*, even at the lowest concentration, which is usually present in surface waters and can be used for public supply. The authors showed that CIP caused neurotoxicity, compromised the immune system due to a reduction in the number of leukocytes, and caused genotoxicity and apoptosis of erythrocytes. Oxidative stress was also observed in liver tissue with hepatocyte apoptosis, Leukocyte infiltration, steatosis, and necrosis. The study emphasized that histopathological analysis showed that CIP stimulated gonadal development and that further studies would be needed to investigate the potential of CIP as an endocrine disruptor.

The studies published until the moment do not investigate the effect of exposure to prolonged of CIP in neotropical fish, such as *Rhamdia quelen*, specifically in organs responsible for absorption and excretion.

Thus, the aim of this study was to evaluate the toxic effects of long-term exposure of *Rhandia quelen* to ambient concentrations of ciprofloxacin, focusing on the gills and in the posterior kidney, organs related to the absorption and excretion of xenobiotics. The hypotheses that support this research are that ciprofloxacin causes oxidative stress, genotoxicity, and histopathological changes in the kidney and gills of *Rhamdia*

quelen. Furthermore, it is believed that the exposure of fish to ciprofloxacin causes apoptosis and necrosis in renal cells. It was also investigated if there are differences in the responses of male and female fish biomarkers.

6.2 MATERIAL AND METHODS

8.1.1 Sampling

It was used juvenile fish of the *Rhamdia quelen* species were acquired from the fish farm of the Universidad State of Western Paraná (UNIOESTE), Toledo, Paraná, Brazil. The fish were acclimatized for 30 days in glass aquariums (15L capacity; four fish per aquarium) at 25±1 °C, under 12 h light/dark period, in filtered and dechlorinated water. We followed the fish density recommendations per aquarium as suggested by the OECD (2000). The animals were fed once a day with a balanced diet suitable for this species (Laguna® Brazilian Fish 32). The present research was approved by the Ethics Committee in Animal Experimentation of the Federal University of Paraná, under number 1227.

8.1.2 Chemical analysis

Before the experimental bioassay, a degradation study of CIP (Sigma Aldrich Chemical Corporation and Merck®, Brazil, purity ≥ 98%) was performed to estimate the half-life of the compound in water (under the bioassay conditions) Carvalho et al. (2022), article under preparation. Based on the result of this study, 50% of the CIP was replaced every 24 h to maintain the concentration during the experiment.

During the experimental bioassay, water samples were collected from each group at specific times of times on the day 0, 1, 2, 7, 14, 21, and 28 to quantify the mean concentration of CIP.

The quantifications were performed by High-performance liquid chromatography (HPLC) (Waters 2695 HPLC), coupled to a fluorescence detector (FD Waters multi-fluorescence detector 2475) following Palmada et al. (2000) and Migliore et al. (2003). The fluorescence wavelengths evaluated were 278 nm for excitation and 453 nm for emission. The solvents used in the mobile phase were: phase A) triethylamine 0.4% (v/v); phase B) methanol; phase C) acetonitrile. The calibration curve was made by

use of Analytical-grade Cipro (United States Pharmacopeia, Rockville, MD, USA). The batch of samples included blanks, standards, and fortified samples in triplicate. Recovery rates were 94.4%. The LOD and LOQ were 0.1 and 1.00 $\mu g.L^{-1}$ respectively. Two calibration curves were obtained, one with 5 points, for the samples of 1 and 10 $\mu g.L^{-1}$ of CIP, and another with 6 points for the samples of 100 $\mu g.L^{-1}$. Both calibration curves showed linearity for the analyte ($r2 = 0.999$; $P< 0.0001$) in the domain of expected sample concentration. Each batch of samples included three blanks, three standards, and three fortified samples. Recovery rates were higher than 85%. To the quantification of Cipro concentrations in the water, were used the linear equations: $y = 12105x + 316.82$ (where: y = Cipro concentration and x = area) to curve with 5 points and $y = 11319x -4670.10$ to curve with 6 points.

8.1.3 Fish exposure

After the acclimation period to laboratory conditions, the fish (n = 80), 35 males and 45 females, were divided into four groups (n = 20 per group): control, 1, 10, and 100 $\mu g.L^{-1}$ of CIP. The fish were with an approximate weight of 13.66 ± 2.82g and length: 12.04 ± 1.02 cm (mean data ± standard error). For the experiment, the fish were kept in glass aquariums (15 L, four fish per aquarium, five aquariums per group), following the recommended rate of biomass (OECD, 2000). The concentrations were chosen from a literature review considering the presence of ciprofloxacin residues in three different scenarios: I) in surface waters; II) in effluents from water treatment plants and wastewater treatment plants; III) in effluents from hospitals and pharmaceutical industries, according to a study by Carvalho et al. (2022), article under review.

Exposure was semi-static and every 24h one-third of the water was replaced with the addition of the stock solution (containing 100 mg ciprofloxacin/L), which was added in the appropriate volume to the aquaria water to obtain the final test concentrations. The water parameters were measured in triplicate, throughout the experiment. The values did not differ between groups (pH = 7.05 ± 0.44 units, ammonia 0.31 ± 0.18 ppm, nitrite 0.06 ± 0.18 ppm, dissolved O_2 7.05 ± 0.17 ppm, and aquarium water 20.2 ± 0.6). No mortality was observed during the experiment. The pH was measured using a pH meter (HANNA Instruments®, model HI98129), ammonia and nitrite by Labcon® test kits, dissolved O_2, and temperature using a probe (HANNA Instruments®, model

HI 9146). Control animals were submitted to the same water change schedule without the addition of CIP. We chose the exposure time of 28 days based on the OECD Test Guideline N° 215: Fish, Juvenile Growth Test. After 28 days of exposure, the animals were anesthetized with benzocaine and after were euthanized by spinal anesthesia. The posterior kidney and gills were collected and stored at -80°C until biomarkers analysis.

8.1.4 Biomarker analysis

8.1.4.1 Biochemical biomarkers

The posterior kidney and gills were homogenized in a phosphate buffer (0.1 M; pH 7.0), in a proportion of 1:10 (m/v) and 1:5 (m/v), respectively, and the samples were centrifuged at 15000 x g for 30 min at 4°C. The supernatants were used to measure the activity of the superoxide dismutase (SOD) (Gao et al., 1998) at 450nm, catalase (CAT) (Aebi, 1984) at 240nm, glutathione peroxidase (GPx) (Hafeman et al., 1974) at 340 nm, glutathione S-transferase (GST) (Keen et al., 1976) at 340 nm. Also, were measure the concentrations of the non-protein thiol/glutathione (GSH) (Sedlak and Lindsay, 1968) at 415nm and lipoperoxidation (LPO) using the FOX method (Jiang et al., 1992) at 570nm. The quantification of total protein was determined according to Bradford (1976), with bovine serum albumin (BSA) as the standard. The absorbance went measured at 620 nm.

8.1.4.2 Biomarkers of genotoxicity

DNA damage

The comet assay of posterior kidney was analyzed according to Singh et al. (1988), with modifications made by Ferraro et al. (2004) and Cestari et al. (2004), and the adjustments described by (Ramsdorf et al., 2009). Staining with ethidium bromide (20 $mg.L^{-1}$ - Sigma-Aldrich®) was performed prior to analysis by a Leica epifluorescence microscope (model: DMLS2), with a rhodamine filter and under 1000x magnification. Per animal, 100 nucleoids were analyzed and classified according to the damage presented after the electrophoretic run. According to (Azqueta et al., 2011), nucleoids

were classified for each type of damage, as follows: 0 (no apparent damage) 1 (little damage), 2 (medium damage), 3 (extensive damage), and 4 (maximum damage). Scores were generated from the classification and quantification of damage, using the sum of the amount of damage multiplied by the value of each class (Koppen et al., 2017).

8.1.4.3 Assessment of ciprofloxacin-induced apoptosis and necrosis

The frequency of apoptotic and necrotic cells in the posterior kidney was performed using a morphometric DNA diffusion assay described by (Singh, 2005, 2000). In this test, DNA identification was performed with an intense fluorescent dye, YOYO-1 (Microscopio Leica, model DFC 300). Apoptotic cells showed a granular DNA halo with a cloudy outer boundary. Necrotic cells, on the other hand, show an unusually large homogeneous nucleus with a clearly defined boundary.

8.1.4.4 Histopathological biomarkers

A fragment of the posterior kidney and gills were fixed in ALFAC solution (80% alcohol, formaldehyde, and glacial acetic acid) for 16 h. Then, they were dehydrated in alcohols (70%, 80%, 90%, and 100%), diaphanized in xylol, and included in Paraplast®. They were cut into 5 µm slices and stained with hematoxylin-eosin (HE). For the histopathological evaluation of lesions, all specimens were analyzed according to the index proposed by Bernet et al. (1999) modified by (Mela et al., 2013). The important factors considered for each alteration found were: (1) minimal pathological importance, which presents possibilities of reversal; (2) moderate importance, reversible in some cases and (3) severe pathological importance, usually irreversible. According to the degree of occurrence of the alterations, the values assigned to the alterations were: (0) Unchanged, (2) Occasional Occurrence, (4) Moderate Occurrence and (6) Severe Occurrence (diffuse lesion). The injury indices (II) were obtained after applying a mathematical equation below:

$$II = \sum rp \sum alt \ (a \ X \ w)$$

where pr = reaction pattern; alt = alteration; a = score value; w = important factor.

8.1.4.5 Melanomacrophage centers (MMCs) counting

Afterward routine staining with hematoxylin and eosin, 15 fields on posterior kidney tissues were randomly chosen under the 40X objective. MMCs in each field were recorded in sections using an eyepiece graticule coupled to a light microscope, then, an average count was determined, and results were expressed per mm^2 as described by (Agius and Roberts, 1981).

8.1.5 Statistical analysis

Data were evaluated according to the assumptions of normality and homogeneity using the Shapiro-Wilk and Levene tests, respectively. For data that presented normality assumptions (parametric data), one-way analysis of variance (ANOVA) was used, followed by Tukey's test with the results expressed as mean ± standard error. For non-parametric data, the Kruskal-Wallis test was performed, followed by the Dunn and Mann-Whitney pairwise test, and the results were expressed as median ± standard error. R 3.2.2 statistical software (R.3.2.2, Team, 2015) was used for the statistical analysis and GraphPad Prism 5.00 (GraphPad Software, Inc.) was used to make the figures.

8.2 RESULTS

8.2.1 Chemical analysis

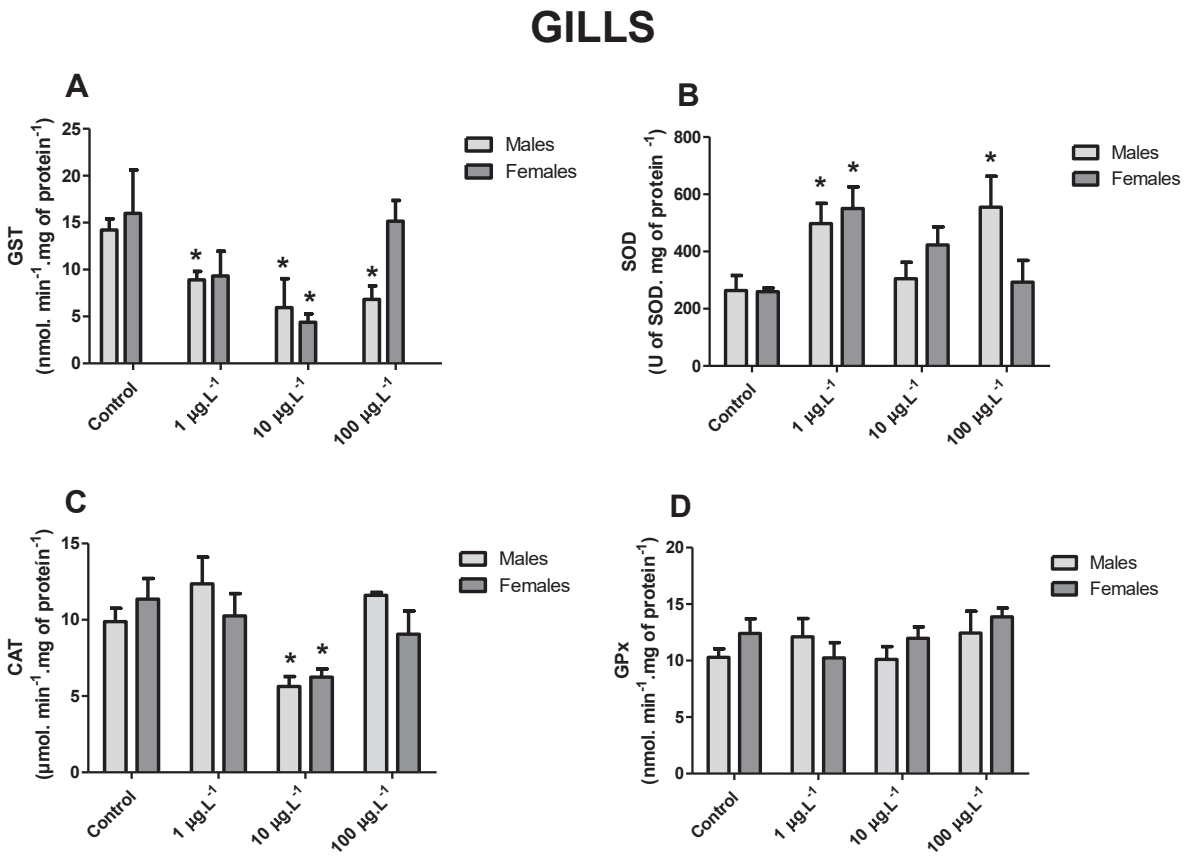
The concentration of CIP measured during the experiment was stable and the values found (mean ± standard error) for the nominal concentrations of CIP during the bioassay were: 00.96 ± 0.01 $\mu g.L^{-1}$ for the concentration of 1 µg. L^{-1}, 9.34 ± 0.58 $\mu g.L^{-1}$ for the concentration of 10 $\mu g.L^{-1}$ and 93.85 ± 3.97 $\mu g.L^{-1}$ for the concentration of 100 $\mu g.L^{-1}$. No CIP concentration in the water from control group.

8.2.2 Biomarker analysis

8.2.2.1 Biochemical biomarkers

In the gills of male fish, exposure to 1 µg.L^{-1} decreased GST activity (37.44%, P = 0.0327, **Figure 1**A), increased SOD activity (88.75%, P = 0.0262, **Figure 1**B) and led to lipid peroxidation (96.94%, P = 0.0105, **Figure 1**E). Exposure to 10 µg.L^{-1} CIP also decreased GST activity (58.14%, P = 0.0139, **Figure 1**A) and in addition decreased CAT activity (43.01%, P = 0.0442, **Figure 1**C), however no lipid peroxidation was observed (P = 0.8965, **Figure 1**E). In contrast, exposure to 100 µg.L^{-1} caused lipid peroxidation (LPO increase by 87.22%, P = 0.0384, **Figure 1**E) with decrease GST (52.01%, P =0.0112, **Figure 1**A), and increase SOD (110.58%, P =0.0122, **Figure 1**B).

In females, exposure to 1 µg.L^{-1} increased SOD activity (117.30%, P = 0.0138, **Figure 1**B) and this was the only altered enzyme. However, oxidative stress was observed (LPO increase by 78.64%, P = 0.0212, **Figure 1**E). Exposure to 10 µg.L^{-1} decreased GST (72.54%, P = 0.0333, **Figure 1**A) and CAT (44.98%, P = 0.0457, **Figure 1**C) activity, and also caused oxidative stress in the gills (LPO increase by 128.52%, P = 0.00119, **Figure 1**E). There was no change in enzymes and oxidative stress caused by exposure to 100 µg.L^{-1}.



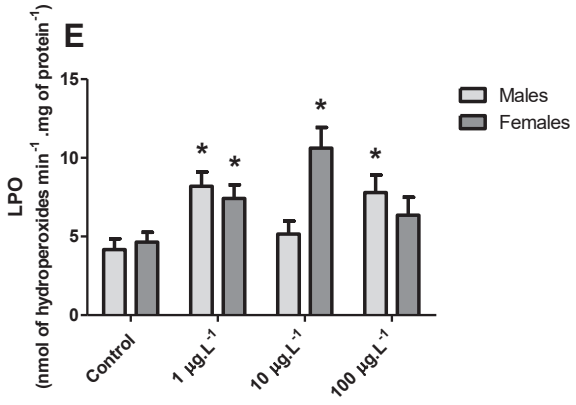
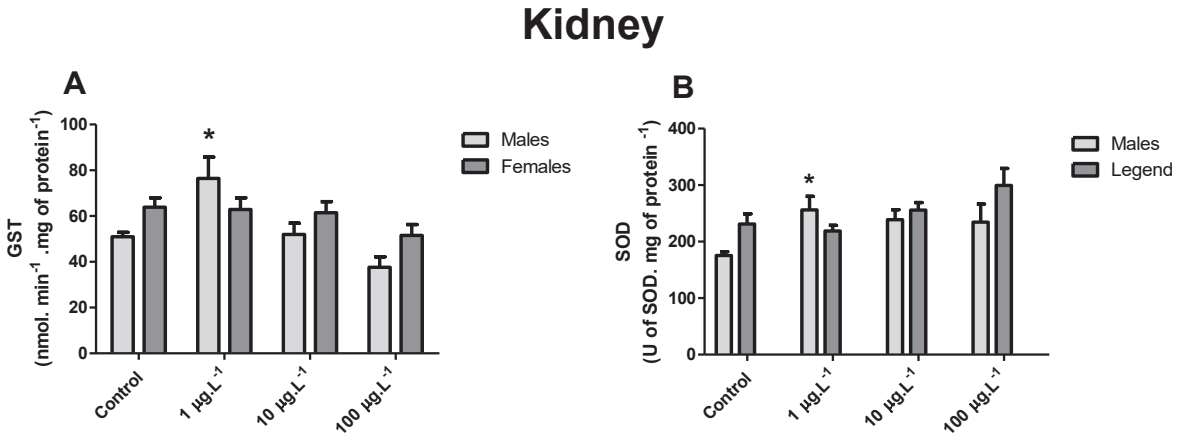


Figure 1. Biochemical biomarkers in the gills of *R. quelen*, males and females, after exposure to ciprofloxacin for 28 days. glutathione S-transferase (GST) (A), Superoxide Dismutase (SOD) (B), catalase (CAT) (C), glutathione peroxidase (GPx) (D), and lipid peroxidation (LPO) (E). Values are expressed as mean ± standard error. Statistical differences among treatments compared to the control group are indicated by * ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

In the posterior kidney of male fish, at 1µg.L^{-1} CIP increased the activity of GST (50%, P = 0.0034, **Figure 2**A) and SOD (46.06%, p = 0.0403, **Figure 2**B). At 10 µg.L^{-1} CIP increased GSH levels (123.79, P = 0.0165, **Figure 2**E). Exposure to 100 µg.L^{-1} did not cause any changes. In females, exposure to 1µg.L^{-1}increased GSH levels (90.73%, P = 0.0199, **Figure 2**E). Exposure to 10 µg.L^{-1} did not cause any changes and at 100 µg.L^{-1} CIP an increase in CAT activity was observed (58.74%, P = 0.0284, **Figure 2**C). The exposure of R. quelen to CIP did not cause oxidative stress in either sex (males, p = 0.6933; females, P = 0.8465).



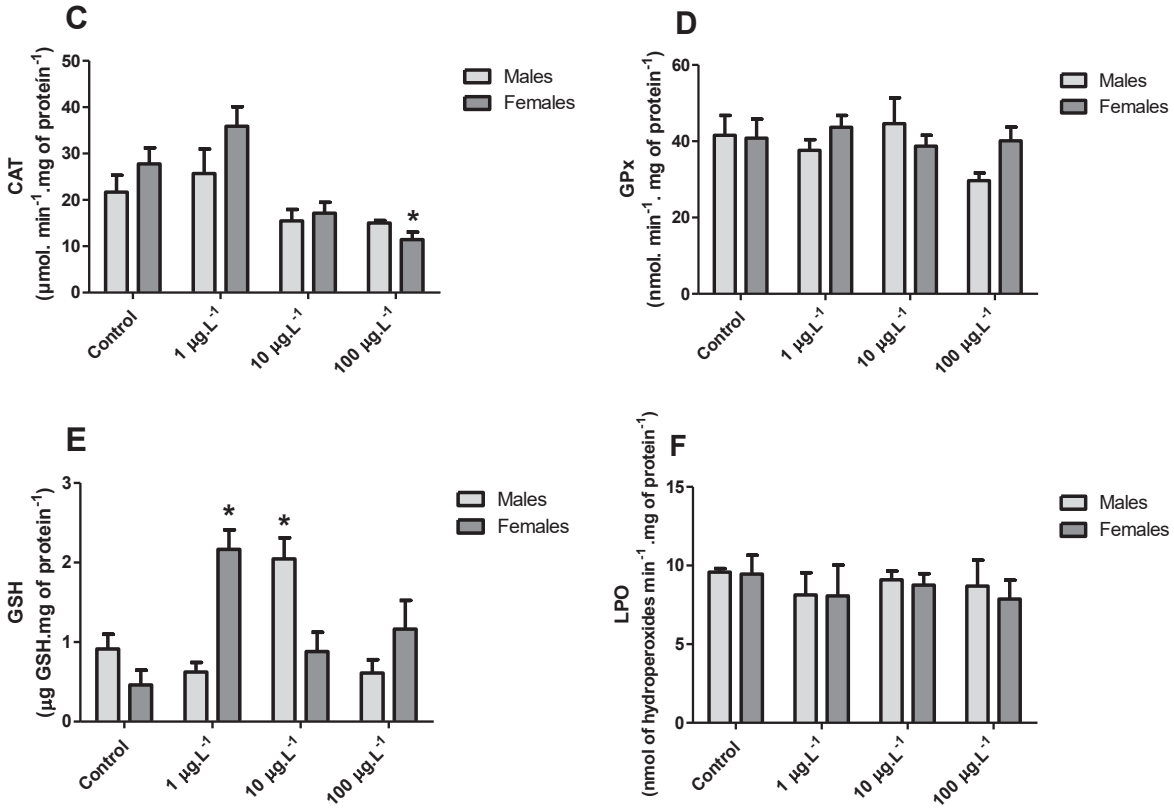


Figure 2. Biochemical biomarkers in the posterior kidney of *R. quelen* (males and females) after exposure to ciprofloxacin for 28 days. glutathione S-transferase (GST)(**A**), superoxide dismutase (SOD) (**B**), catalase (CAT) (**C**), glutathione peroxidase (GPx) **(D**), reduced glutathione (GSH) (**E**) and lipid peroxidation (LPO) (**F**) Values are expressed as mean ± standard error. Statistical differences among treatments compared to the control group are indicated by * ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

8.2.2.2 Biomarkers of genotoxicity

Comet assay

Kidney cell DNA damage was observed only in female fish after exposure to 1 and 100 µg.L^{-1} ($P = 0.0416$ and $P = 0.0111$, respectively), **Figure 3**B.

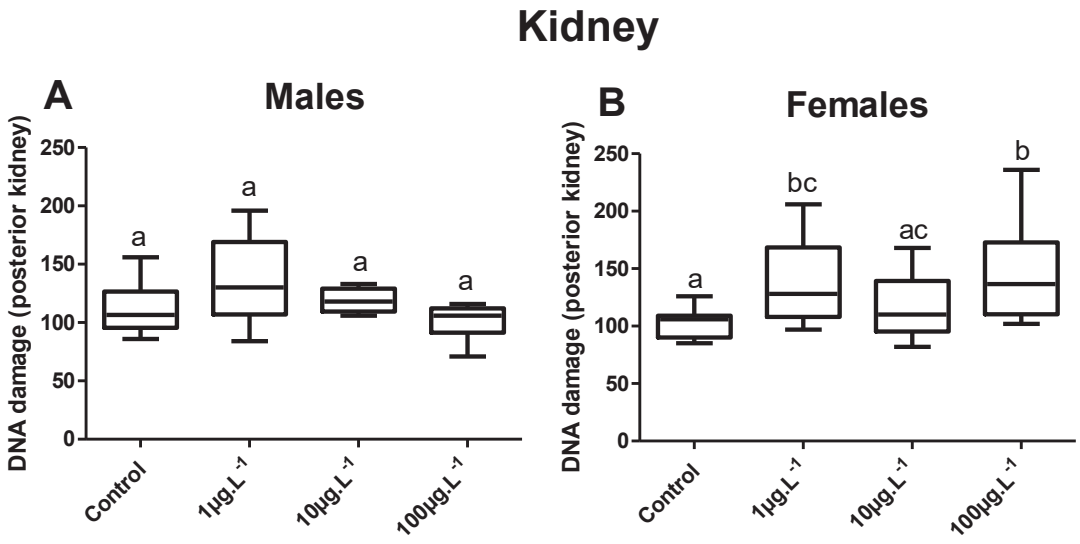


Figure 3. DNA damage score (median ± interquartile range) in posterior kidney of *R. quelen*, males (A) and females (B), exposed to three concentrations of CIP for 28 days. Different letters indicate significant differences ($P < 0.05$). Data expressed as mean ± standard error. N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

8.2.2.3 Assessment of ciprofloxacin-induced apoptosis and necrosis

In kidney tissue, no cells in the process of apoptosis were observed (males, P = 0.4519; females, P = 0.3101). In males, necrosis cells were not observed (P = 0,4519), however, in females, necrosis was observed after exposure to 100µg.L^{-1} (P = 0.0042), **Table 1.**

Table 1

Frequency of apoptosis in blood of *R. quelen*, (**A**) males and (**B**) females, exposed to three concentrations of CIP for 28 days. Different letters indicate significant differences (P < 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

	Posterior kidney		
	Treatments	**Apoptosis**	**Necrosis**
MALES	Control	0,63 ± 0.38^{a}	0
	1µg.L^{-1}	1,20 ± 0.55^{a}	0
	10µg.L^{-1}	2,25 ± 0.95^{a}	0
	100µg.L^{-1}	2,40 ± 2.16^{a}	0
	Treatments	**Apoptosis**	**Necrosis**
FEMALES	Control	0,13 ± 0.13^{a}	0,00 ^{a}
	1µg.L^{-1}	1,56 ± 0.71^{a}	0,38 ± 0.38^{a}
	10µg.L^{-1}	0,57 ± 0.23^{a}	0,07 ± 0.07^{a}
	100µg.L^{-1}	2,45 ± 1.24^{a}	0,73 ± 0.24^{b}

8.2.2.4 Histopathological biomarkers

Histological study of the gills shows a characteristic structural organization of the lamellae in the control fish (**Figure 4**A). The fish exposed to CIP resulted in histopathological changes, such epithelial lifting in the secondary lamella ((**Figure 4**B) and lamellar hyperplasia with lamella fusion ((**Figure 4**C and D).

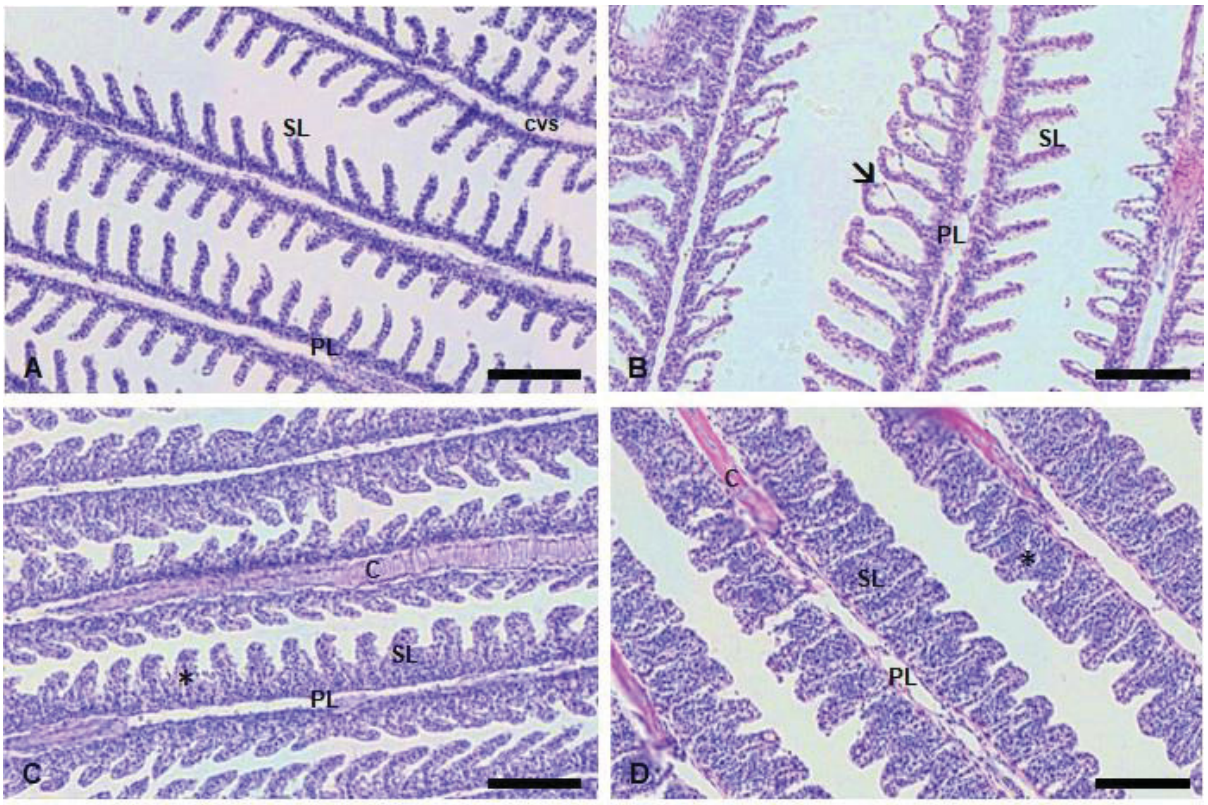


Figure 4. Histomorphological sections in gill filament, sagittal section of *R. quelen*, counterstained with hematoxylin/eosin. **(A)** Gill of control group. Primary lamellae (PL), secondary lamellae (SL) and central venous sinus (CVS). **(B)** Gill of exposed group (100 µg.L^{-1}). primary lamellae (PL), secondary lamellae (SL) and epithelial lifting (⇲). **(C)** Gill of exposed group (10 µg. Ciprofloxacin.L-1). Primary lamellae (PL), secondary lamellae (SL), hyaline cartilage (c) and lamellar hyperplasia with lamella fusion (*). **(D)** Gill of exposed group (100 µg. Ciprofloxacin.L-1). Primary lamellae (PL), secondary lamellae (SL) and lamellar hyperplasia with lamella fusion (*). Scale bar = 100 µm.

The histopathological index in gills showed higher values to individuals exposed to 10 and 100 µg.L^{-1}, in a dependent concentration (**Figure 5**A). Evaluating these lesions by sex, it was possible to observe that the effect of CIP on the gills also had concentration dependent for males and females (**Figure 5**A). At 10 and 100 µg.L^{-1}caused Lifting of the respiratory epithelium and cell hyperplasia in the gill tissue, with similar proportions for both sexes (Table).

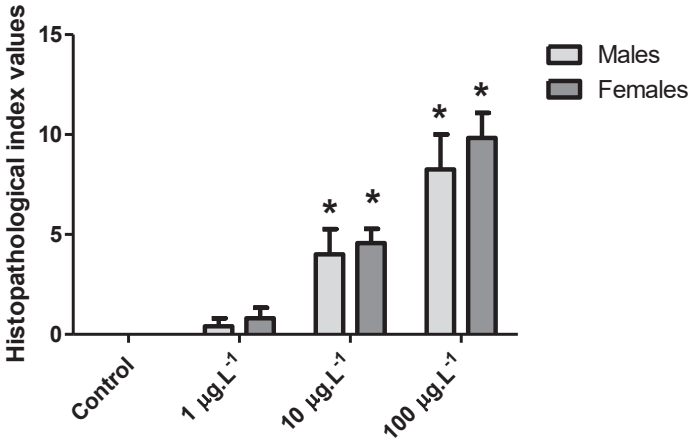


Figure 5. Histopathological index values in the gills of males and females of *R. quelen* after exposure to environmental concentrations of CIP for 28 days. Statistical differences between treatments compared to the control group are indicated by * ($P < 0.01$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

Table 2

Histopathological changes in *R.quelen* gills (males and females) after exposure to environmental concentrations of CIP for 28 days. Statistical differences between treatments compared to the control group are indicated by * ($p < 0.01$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1µg.L^{-1}CIP); N = 6 males and 14 females (10µg.L^{-1}CIP); N = 8 males and 12 females (100µg.L^{-1}CIP).

		Histopathology Biomarkers	
	Treatments	**Lifting of the respiratory epithelium**	**Cell hyperplasia**
MALES	Control	0.00	0.00
	1 µg.L^{-1}	0.00	0.20
	10 µg.L^{-1}	1.20**	2.00**
	100 µg.L^{-1}	2.25**	3.00**
FEMALES	Control	0.00	0.00
	1 µg.L^{-1}	0.00	0.40
	10 µg.L^{-1}	1.14**	2.29**
	100 µg.L^{-1}	2.17**	3.83**

The posterior kidney tissue obtained from control fish showed normal morphology with prominent bowman's capsule, glomerulus, proximal and distal tubules (**Figure 6**A). However, CIP exposure altered the normal histoarchitecture of tissue, with various degenerative changes, such as leukocyte infiltration (**Figure 6**B), presence of adipocytes on stroma (**Figure 6**B), necrosis (**Figure 6**B and D), and extensive area of interstitial hemorrhage (**Figure 6**C) after the exposure at 10 µg.L^{-1} (in both the sex),**Table 3**. In the exposure at 100 µg.L^{-1} was observed just presence of adipocytes on stroma and necrosis (in the males), and necrosis (in the females), **Table 3**. There was an increase in the Bernet Index after fish exposure to 10 and 100 µg.L^{-1}, in both

sexes. The highest values of the Bernet Index were attributed to fish exposed to 10 $\mu g.L^{-1}$, in both males and females,**Table 3**.

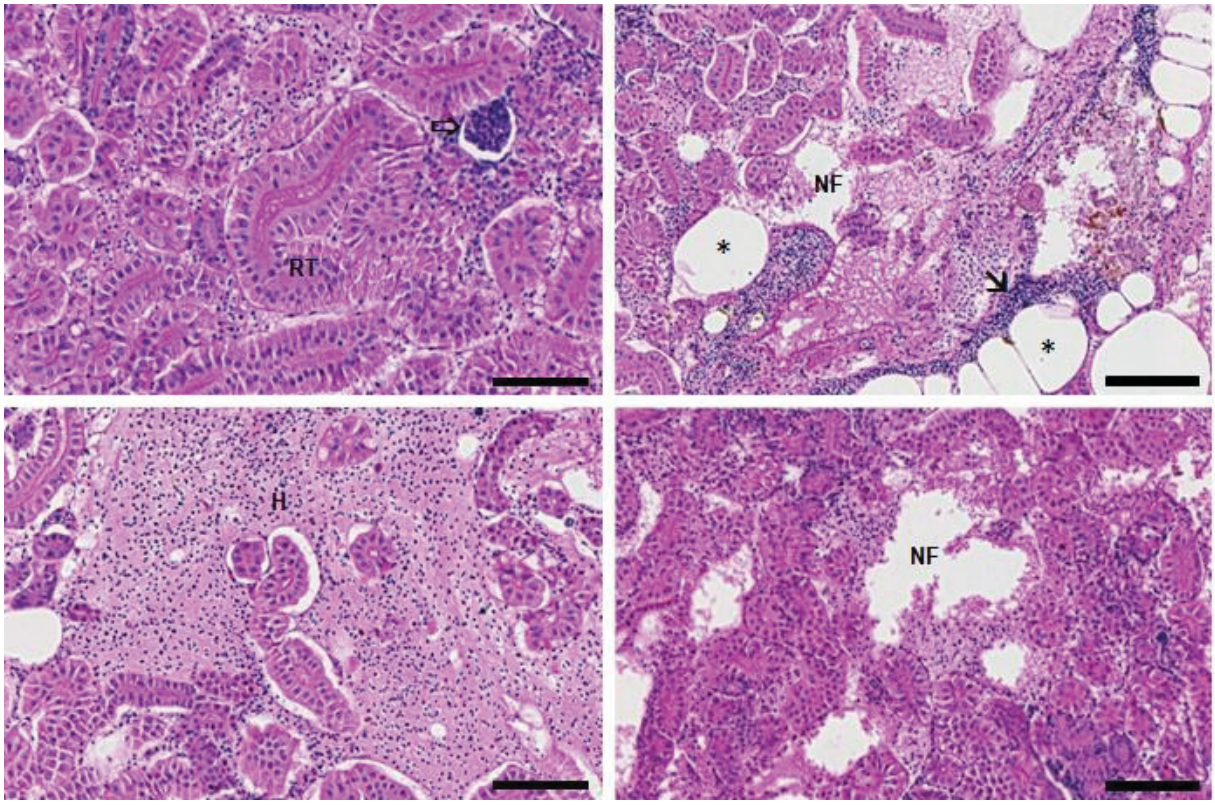


Figure 6. Histomorphological sections in Kidney of *R. quelen*, counterstained with hematoxylin/eosin. **(A)** Kidney of control group. Parenchyma filled with renal tubules (RT) and Glomerulus (⇨). **(B)** Kidney exposed group (10 µg. Ciprofloxacin.L^{-1}). Necrotic focus (NF), leukocyte infiltration (⇲) and adipocytes (*). **(C)** Kidney exposed group (10 $\mu g.L^{-1}$). Extensive hemorrhage area (H). **(D)** Kidney exposed group (100 $\mu g.L^{-1}$). Necrotic focus (NF). (Scale bar = 50 µm).

Table 3
Mean values (score changes) for histopathological effects on kidney tissue after exposure of *R. quelen*, males and females, to three concentrations of ciprofloxacin (1, 10 and 100 µg.L^{-1}) and control group, for 28 days. LI: Leukocyte infiltration; H: hemorrhage; PA: presence of adipocytes on stroma; N: Necrosis. Statistical differences between treatments compared to the control group are indicated by * (P < 0.05) and ** (P < 0.01).

		Histopathology Biomarkers				
	Treatments	LI	H	PA	N	Bernet Index
MALES	Control	0,40	0,00	0,00	0,40	2,00
	1µg.L^{-1}	0,20	0,00	0,00	1,20	4,00
	10µg.L^{-1}	3,60**	2,00**	0,80*	2,80**	20,40**
	100µg.L^{-1}	1,00	0,50	0,75*	2,00*	9,75**

		Histopathology Biomarkers				
	Treatments	LI	H	PA	N	Bernet Index
FEMALES	Control	0,00	0,00	0,00	0,00	0,00
	1µg.L^{-1}	0,40	0,00	0,00	0,40	2,00
	10µg.L^{-1}	3,00**	1,71**	0,86*	2,86**	18,86**
	100µg.L^{-1}	1,00	0,83	0,67	2,00**	10,33**

8.2.2.5 Melanomacrophage centers (MMCs) counting

Incidence of melanomacrophage center (MMC) (**Figure 7**A) in the posterior kidney of *R. quelen* after long-term exposure to ciprofloxacin at doses of 10 (P < 0.001) and 100 µg.L^{-1} (P < 0.001), **Figure 7**B. Exposure to 1 ug.L^{-1} caused an increase in MMC, but not significant (males, P = 0.0599, and females, P = 0.7215).

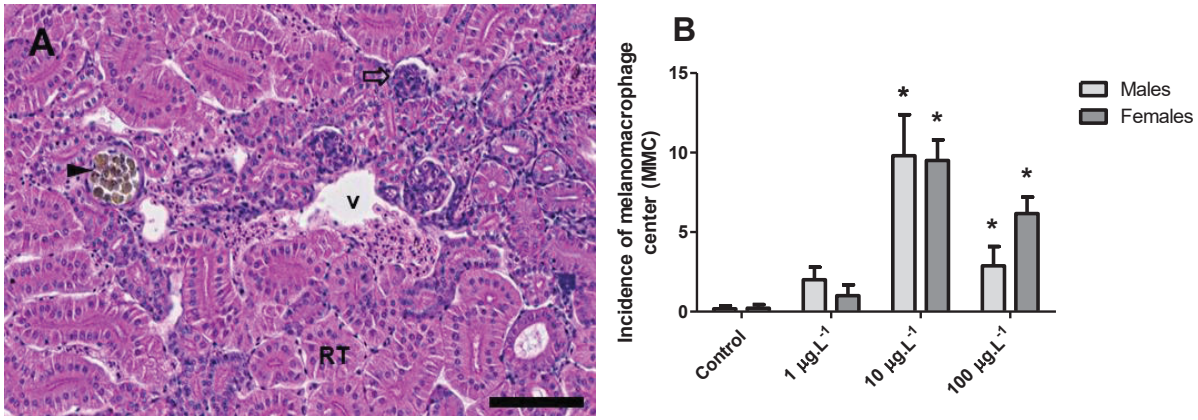


Figure 7. Histomorphological sections in posterior kidney of *R. quelen*, counterstained with hematoxylin/eosin. **(A)** Kidney exposed group (100 µg.L^{-1}). Parenchyma filled with renal tubules (RT) and Glomerulus (⇨). Melanomacrophage center close to a vein (v). (Scale bar = 50 µm). **(B)** Incidence melanomacrophage center (MMC) in the posterior kidney of *R. quelen* after (males and females) after long-term exposure to CIP.

8.3 DISCUSSION

Aquatic environments are contaminated with antibiotics such as CIP. The possible effects of this xenobiotic on the health of non-target organisms are still unknown. In this study, we investigated the effect of CIP on the organs that carry out adsorption and excretion, gills, and posterior kidney. We investigated the possibility of CIP altering the antioxidant system, to cause genotoxicity and histopathology to change in these tissues. In addition, we investigated whether sex influences these responses.

The gill is the first organ to have contact with contaminants in the water (Pereira et al., 2019). Therefore, are quite vulnerable to water pollutants, being considered an efficient indicator of water quality and contamination level (Barja-Fernández et al., 2013; Pereira et al., 2019).

The gills perform several functions such as respiration, osmoregulation, excretion of nitrogenous residues and acid-base balance, so that the functional impairment of the organ can harm the health of fish (Barja-Fernández et al., 2013). In addition, they respond quickly to various chemical agents to overcome physiological stress or injuries that can produce negative effects on the general function of the gills (Pereira et al., 2019).

In this study, the exposure of male fishes exposed to 1 and 100 $\mu g.L^{-1}$ of CIP caused oxidative stress in the gills. Both exposure to 1 and 100 $\mu g.L^{-1}$ of CIP caused a decrease in GST and an increase in SOD activities. Although glutathione-S-transferase has the ability to metabolize reactive oxygen species, detoxification of xenobiotics is its main function (Al-Ghais, 2013). GSTs are central Phase II metabolic enzymes, which catalyze nucleophilic attack via reduced glutathione on nonpolar compounds, protecting cells, organelles, and macromolecules from electrophiles damage and oxidative stress (Ballesteros et al., 2009; Toni et al., 2011). Thus, the decrease of this enzyme activity may have compromised the efficiency of these processes, contributing to the emergence of oxidative stress in the tissue. The SOD, on the other hand, is an antioxidant enzyme specialized in the conversion of superoxide ($O2^{-}$) to H_2O_2 (Ramesh et al., 2018). Therefore, the increase in SOD suggests the production of reactive oxygen species formed after exposure to CIP.

Exposure to 10 $\mu g.L^{-1}$ of CIP, despite not causing oxidative stress in the gill, indicated an alteration of the antioxidant system in the presence of CIP due to the decrease in CAT activity. CATs are hematin-containing enzymes that facilitate the

removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water (Van der Oost et al., 2003). It is a vital enzyme involved in protection against oxidative damage (Qin and Liu, 2013a). Qin and Liu (2013) characterized the harmful effects of oxidative stress induced by CIP and enrofloxacin from the structure and function of catalase (CAT). In their study, the cellular tests firstly confirmed an enhanced oxidative stress in FQs treated erythrocytes from the depletion of GSH contents and decrease of CAT activity, corroborating with our results. Besides, CIP posed more of an oxidative threat than enrofloxacin. During the spectroscopic and computational investigations, both FQs could bind into its central cavity with only one binding site and interact with Arg 65, Arg 362 and His 363 mainly through electrostatic forces. Furthermore, the binding of two FQs not only caused the conformational and microenvironmental changes of CAT, but also inhibited its molecular activity, consistent with the cellular activity measurements.

Interestingly, unlike males that showed oxidative stress at 1 and 100 $\mu g.L^{-1}$ of CIP. In females, CIP led to oxidative stress at 1 and 10 $\mu g.L^{-1}$. In exposure to 1 $\mu g.L^{-1}$ there was an increase in SOD activity, as well as in males. At 10 $\mu g.L^{-1}$ decreased GST and CAT activities. Thus, we observed that CAT activity showed the same behavior of decrease in exposure to 10 $\mu g.L^{-1}$ of CIP in both sexes. In addition, both in males and females the altered enzymes were similar, however the increase in LPO was different between treatments in relation to sex. Therefore, there may be some differences in the response of the antioxidant system as a function of sex. There is a data gap in this type of assessment, so we suggest further investigation of this type of approach in future research.

In an acute exposure to these same concentrations of CIP, with the same bioindicator animal model, CIP did not cause oxidative stress in the gills (Kitamura et al., 2022). However, it increased the activities of antioxidant enzymes (SOD, CAT, and GPx) (Kitamura et al., 2022). Interestingly, a difference can be observed in the behavior of the CAT. In the present study (long-term exposure) a decrease was observed, but in acute exposure, an increase in activity was observed. This suggests that exposure time can influence CAT behavior. Corroborating our results, Ramesh et al. (2021) showed that CAT activity in a freshwater fish (*Cirrhinus mrigala*) was predominantly decreased in ciprofloxacin-treated groups compared to the control group (1 $\mu g.L^{-1}$ and 1.5 $\mu g.L^{-1}$ of CIP - in 5 and 10 days). In agreement with the present study, the increase in SOD activity in the gills was also reported by Kitamura et al. (2022) and Ramesh et

al. (2021). Similar to our results, Ramesh et al. (2021) showed that the level of LPO was elevated in the groups of treatment (1.0 and 1.5 $\mu g.L^{-1}$ CIP) throughout the study period (5, 10 and 15 days). Another antibiotic, Oxytetracycline, widely used in aquaculture worldwide, also caused oxidative stress in gills of a freshwater fish (*Oncorhynchus mykiss*) (Rodrigues et al., 2016).

In the presence of oxidative stress, cytotoxic reactive oxygen species (ROS) can react with critical cellular macromolecules, possibly leading to enzymatic inactivation, lipid peroxidation (LPO), DNA damage and, occasionally, cell death (Van der Oost et al., 2003). Furthermore, previous studies also indicate that oxidative stress can cause damage to different organs (Ogura and Shimosawa, 2014) as observed in this study.

The analysis of histopathological biomarkers showed that exposure of fish to 10 and 100 $\mu g.L^{-1}$ of CIP caused elevation of the respiratory epithelium and cellular hyperplasia in the gill tissue, with similar proportions for both sexes, in a concentration-dependent manner. This shows that, although CIP caused oxidative stress at a concentration of 1 $\mu g.L^{-1}$, both in males and females, this condition did not cause tissue alteration. In contrast exposure to 10 $\mu g.L^{-1}$, which in males did not cause oxidative stress, only alteration of the antioxidant system, it was observed tissue damage. In addition, exposure to 100 $\mu g.L^{-1}$ CIP also did not led to oxidative stress (in females), but caused considerable tissue alteration.

Some factors may have contributed to these histopathological changes in the gills, such as: a) The first contact of the fish with the xenobiotics in water occurred through gills, which may result in structural damages and physiology alterations in those tissues (Rodrigues et al., 2017, 2019); b) The mechanism of action of CIP. The main mechanism of action that fluoroquinolones utilize is the interruption of DNA synthesis by targeting 2 enzymes, DNA gyrase (topoisomerase II) and DNA topoisomerase IV. In a bacterial cell, these 2 topoisomerases act by unwinding the DNA double helix (Champoux, 2001). However, eukaryotic organisms also possess DNA topoisomerase type II that is vital for several nuclear processes, including DNA replication, chromosome segregation, and maintenance of chromosomal structure, as in prokaryotic organisms (Bakshi et al., 2001). In this way, the interference of this drug at DNA levels may reflect on the synthesis of important enzymes and affect future structural processes at higher levels; c) Under stress conditions, acid-base homeostasis is altered, although this situation can be controlled by the gills that produce acid-base secretions; d) This situation can also affect the morphology of the

gill tissue(Rodrigues et al., 2017). Histological changes in gill tissues have been reported in rainbow trout treated with antibiotic (oxytetracycline) (Rodrigues et al., 2017). The authors also stated that antibiotics can inhibit mitochondrial β-oxidation and disrupt the normal respiratory chain, producing reactive free radicals in fish tissue.

In an acute exposure of *R. quelen* to CIP (Kitamura et al., 2022) the authors also did not identify oxidative stress in the gills at 100 $\mu g.L^{-1}$ (as observed in the present study, in the case of females), however fish showed histopathological changes, such as loss of micro-striae and primary lamellar epithelium.

Corroborating our results, a series of histopathological changes, such as lamellar fusion (LF), and epithelial lifting (EL), were identified in the gill tissue of fingerlings exposed to CIP (Ramesh et al., 2021). The authors also identified in this cells, necrosis (N), loosening of the primary gill (LPG), and cytoplasmic vacuolation. In contrast to the study by Ramesh et al. (2021) did not identify lamellar hyperplasia.

Cellular responses such as cell proliferation (hyperplasia) of epithelial filaments have been observed by several authors (Barja-Fernández et al., 2013; Martinez et al., 2004) in fish exposed to contaminants. The hyperplasia is one of the adaptations by which an organism protects its underlying tissues from any toxicant by increasing the number of normal cells (Olurin et al., 2006; Sharma et al., 2021)Sharma et al., 2021, Olurin, Olojo, Mbaka, & Akindele, 2006). Due to rapid cell division, there is an increase in the width of the epithelial layer which results in the fusion of adjacent lamellae, this not only reduces the surface area available for oxygen but also maximizes the oxygen diffusion distance between water and blood (Sharma et al., 2021; Skidmore & Tovell, 1972). In addition, rapid cell growth in the interlamellar region it decreases the surface area for respiration and therefore prevents gas exchange from a more difficult process (Sharma et al., 2021). Its frequency varies according to the type of contaminant and concentration (Pereira et al., 2019). In some cases, changes such as hyperplasia were only observed at high concentrations of the toxicant – for example, specimens of *P. lineatus* exposed to lead – and occasionally lead to fusion of adjacent lamellae and even lamellae of adjacent filaments (Martinez et al., 2004).

Considering all morphometrical changes in gills, i.e., epithelial lifting of the secondary lamella, and lamellar hyperplasia with lamella fusion, the results indicate that fish exposed to CIP can result in physiological consequences, these anomalies could reduce the capability of gills to exchange gases with surrounding environment (Barja-Fernández et al., 2013; Fernandes et al., 2013). Gill cell hyperplasia, lamellar

fusion, and pavement cell (PVC) hypertrophy help to prevent these chemicals from reaching the bloodstream (Cerqueira and Fernandes, 2002) and are considered defensive responses against contaminants, although these changes reduce the respiratory surface area and increase the water-blood diffusion distance, which may impair gas exchange (Cerqueira and Fernandes, 2002; Fernandes et al., 2013).

After its absorption by gills, the CIP is transported to other organs and is excreted by the posterior kidney (Ramesh et al., 2018). For this reason, in our study we also investigated the effect of CIP on the posterior kidney of *R. quelen*.

No oxidative stress was observed in kidney tissue after exposure to CIP. However, in males, we observed that CIP caused an increase in GST and SOD activity (at 1 $\mu g.L^{-1}$) and an increase in GSH levels (at 10 $\mu g.L^{-1}$). In females, an increase in GSH (at 1 $\mu g.L^{-1}$) and a decrease in CAT activity (at 100 $\mu g.L^{-1}$) were observed. These data suggest that exposure to CIP caused the production of ROS, which in turn activated the system antioxidant and possibly the biotransformation system, which were efficient in controlling these ROS, preventing lipid peroxidation in the tissue.

Nevertheless, in females, DNA damage was observed after exposure to CIP at concentrations of 1 and 100 $\mu g.L^{-1}$. In addition, at the highest concentration, cell necrosis was observed. As we did not find oxidative stress in the tissue, these data suggest that the mechanism of action of CIP may have caused this damage, as we have explained previously. However, it is suggested that more studies be carried out, evaluating the influence of sex on these responses, since in male fish, an increase (although not significant) was observed in DNA damage (at 1 $\mu g.L^{-1}$), and also necrosis was not observed.

Contrary to what was observed in the present study, Kitamura et al. (2022), reported oxidative stress in the exposure of *R. quelen* to 100 $\mu g.L^{-1}$ of CIP (which are the same as in the present study). This suggests that time can influence the response of the antioxidant system.

Although males did not show genotoxic damage (such as females at 100 $\mu g.L^{-1}$), exposure to CIP (10 and 100 $\mu g.L^{-1}$) altered the normal histoarchitecture of the kidney, with several degenerative changes, such as leukocyte infiltration, presence of adipocytes in the stroma, necrosis and extensive area of hemorrhage in the renal parenchyma. These changes were observed in both males and females. As opposed to the gills, in the posterior kidney, no concentration-response relationship was observed in the histopathological biomarkers. The highest values of the Bernet Index

were attributed to fish exposed to 10 μg.L^{-1}. Therefore, at this concentration, CIP caused significant damage to fish health. Reda et al. (2013) showed that oxytetracycline induced biochemical alterations that were corroborated by histopathological findings in kidney of *O. niloticus*. In an acute exposure of *R. quelen* to CIP (100 μg.L^{-1}), necrosis, leukocyte infiltration, and the presence of adipocytes in the stroma were also observed (Kitamura et al., 2022), as evidenced in this study.

The histopathological analysis showed significant necrosis in both males and females. The processes of necrosis and apoptosis are regulated by many of the same intermediate biochemical mechanisms, including reactive oxygen species and thiol antioxidants. Beyond a certain threshold, it appears that stress-induced changes in these modulators “switch” the cell death mechanism from apoptosis to necrosis (Mcconkey, 1998).

CIP is already known to induce cellular apoptosis. Herold et al. (2002) reported that CIP induced apoptosis and inhibited the proliferation of human colorectal carcinoma cells. Therefore, due to the topoisomerase inhibitory effect, ciprofloxacin has been explored as a good candidate for such cancer therapies (Sharma et al., 2010). Despite this, in our study we did not find apoptosis of renal cells induced by CIP. However, apoptosis of hepatocytes (at 1, 10 and 100 μg.L^{-1}),) and erythrocytes (at 10 and 100 μg.L^{-1}),) has been reported in *R. quelen* exposure to CIP (Carvalho et al., 2022), article under review. Leukocyte infiltration is the first line of immunological response due to cell death. According to Bernet et al. (1999), this alteration is related to neutralize and destroy the source aggressor, cleaning the tissue, removing the aggressor agent and dead cells, and induce the recovery of damaged tissue. The hemorrhage might be caused due to pre-existing renal tumors or could represent by trauma or blood vessel disorders after pollution exposure (Ezemonye and Ogbomida, 2010). The adipose tissue may directly affect the kidney through its endocrine activity via the production of adipokines. This lipid accumulation in kidney, may lead to glomerular hypertension and increased glomerular permeability, causing a cascade of events that cause kidney damage (Weil et al., 2013).

Analysis of MMCs in the posterior kidney of *R.quelen* showed an increase in the incidence of MMCs after exposure to CIP at the concentration of 10 and 100 μg.L^{-1} in males and females fish. Melanomacrophages (or melanin-macrophages, MMs) are pigmented phagocytes. The Nodular accumulations of closely packed MMs are known as MMCs (Steinel and Bolnick, 2017). Melano-macrophage centres (MMCs) are a

prominent feature of the haemopoietic tissue of all teleosts, being found in the spleen, kidney and, to a lesser extent, the liver (Agius, 1979). The leading hypothesis is that MMCs represent a primitive site of adaptive immune system activation. Besides, the MMC is thought to play dual roles, participating both in immune defenses and normal, non-immunological, physiological processes (Steinel and Bolnick, 2017). Studies have already investigated changes in the size and area parameters of MMs, as well as in their number under different conditions and in various organs (Barst et al., 2015; Jordanova et al., 2016) and different patterns of pigment accumulation in relation to different environmental contaminants (Ivanova et al., 2020).

In an acute exposure of *R. quelen* to CIP (at the same concentrations as in the present study) no MMCs were observed in the posterior kidney after antibiotic exposure (Kitamura et al., 2022). This suggests that the time of exposure to the contaminant is an important factor for the increase in the incidence of MMCs. In another study, Barst et al. (2015) noted an increased area of melano-macrophage aggregates (MA), in the livers and spleens of yelloweye rockfish from Prince of Wales Island, Alaska. The variation in hepatic MA was best described by concentrations of Hg (II) even after accounting for other metals and the age of the fish, demonstrating that MA are excellent biomarkers of Hg exposure. The authors showed positive relationship between MA area and Hg concentration implies a concentration–response relationship whereby cell damage increases with metal exposure.

This study was the first to describe the incidence of MMC in posterior kidney (an organ that, in contrast the anterior kidney, does not present characteristics of hematopoietic tissue) after exposure of fish to CIP. We observed that both sexes had similar proportions, therefore the CIP, in this question, caused the same effect in males and females. Interestingly, the highest incidence of MMC_s was observed at the 10 $\mu g.L^{-1}$ CIP and not at the highest concentration. Therefore, it is interesting that this analysis continues to be included in future studies to try to better understand the influence of concentration on this issue and exposure time.

8.4 CONCLUSION

The present study showed that CIP caused sublethal effects even at low concentration. It caused oxidative stress in the gills, with different responses for males and females. On the other hand, histopathological changes were observed in the

concentration-response relationship in both sexes. Furthermore, CIP caused DNA damage in female kidney cells, with an increase in the frequency of necrosis at the highest concentration. CIP altered the normal histoarchitecture of renal tissue, with an increase in the Bernet Index and also caused an increase in the incidence of MMCs, in both sexes. Thus, we showed that biochemical and genotoxicity biomarkers responded differently in relation to sex, however, MMCs and histopathological biomarkers showed more similar responses between males and females. Therefore, we suggest that further studies can deepen this investigation, analyzing how these effects can be reflected in ecological terms. Finally, *R. quelen* presented itself as a sensitive study model for investigations on the effects of contaminants in relation to sex.

8.5 DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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9 CONSIDERAÇÕES FINAIS

Os resultados mostraram que a exposição de peixes *Rhamdia quelen* à concentrações de CIP presentes em rios, efluentes de estações de tratamento de água, esgoto e efluentes industriais causaram efeitos tóxicos na saúde de machos e fêmeas de *Rhamdia quelen*.

Nos peixes sob exposição à CIP foram observados efeitos subletais desde o nível celular até tecidual, que pode a) torná-los mais suscetíveis a doenças; b) que pode influenciar nas trocas gasosas e, consequentemente, na respiração dos organismos; c) que pode comprometer a síntese de enzimas e proteínas que participam de processos importantes e, por conseguinte, comprometer comportamentos como locomoção, reprodução e alimentação. A exposição à CIP também aumentou o índice histopatológico de Bernet nos órgãos estudados, o que enfatiza a relevância patológica e a potencialidade da lesão de afetar as funções dos órgãos ou a capacidade de sobrevivência dos peixes. Ocasionou aumento na incidência de centros de melanomacrófagos, evidenciando processos de defesas naturais do peixe frente à exposição ao xenobiótico.

A maior concentração de CIP causou maturação das gônadas masculinas e femininas. Não se sabe o impacto (positivo ou negativo) que este efeito poderia ter em termos ecológicos. Assim, sugere-se uma investigação mais aprofundada para predizer estes impactos.

Em adição, nossos resultados também mostraram diferenças nas respostas de biomarcadores em relação ao sexo, principalmente hematológicos, bioquímicos e de genotoxicidade. Ressaltando a importância da investigação dos efeitos da exposição à xenobióticos em relação ao sexo do indivíduo.

Portanto, estas alterações podem ter efeitos em nível de organismo que por sua vez, podem refletir no âmbito de população, podendo causar perturbações ecológicas. Assim, esses resultados ressaltam a importância de um plano de gestão eficiente no controle dos resíduos de CIP no ambiente, e consequentemente, a necessidade de um aprimoramento nos aspectos legais.

10 RECOMENDAÇÕES PARA TRABALHOS FUTUROS

Durante a discussão dos resultados obtidos na pesquisa, evidenciou-se uma carência de dados no tangente às respostas de biomarcadores em peixes comparando machos e fêmeas. Esse aprofundamento é de fundamental importância do ponto de vista ecológico, pois permite fazer aferições mais específicas sobre o efeito de xenobióticos em níveis de organização ecológica. Por exemplo, se for evidenciado que um xenobiótico presente no ambiente causa a diminuição de espermas de indivíduos de determinada espécie, como isso vai refletir na população desta espécie? Ou que um xenobiótico afeta somente as células de defesa de fêmeas quais são os riscos ecológicos desse efeito?

Uma série de produtos químicos mostram diferenças na toxicidade em machos e fêmeas. As diferenças relacionadas ao sexo podem ser devidas a fatores toxicocinéticos ou toxicodinâmicos. As diferenças podem ser baseadas no impacto diferencial das substâncias nas vias de sinalização endógenas (por exemplo, o papel dos estrogênios em mulheres versus homens) e, em alguns casos, diferenças em como homens e mulheres biotransformam a substância (Liu and Pope, 2020). Portanto, é imprescindível a investigação da toxicidade de xenobióticos considerando o sexo como um fator que pode alterar a toxidade do composto.

A segunda sugestão para estudos futuros é considerar amostragens ao longo do período de 28 dias, principalmente para investigar melhor os biomarcadores reprodutivos. Como no presente estudo foi realizada apenas uma única amostragem ao final tempo de exposição, não foi possível acompanhar as mudanças que podem ter acontecido ao longo do processo. Em concordância, nas concentrações menores, por exemplo em 10 $\mu g.L^{-1}$ foi observado o início do aumento na frequência de espermatozoides e ovócitos, porém não significativo, corroborando com as alterações dos neurotransmissores, possivelmente sinalizando o início do processo de maturação das gônadas (que pode ter acontecido mais tardiamente, por ser uma concentração menor CIP). Assim, a sugestão para trabalhos futuros é que o potencial da CIP como desregulador endócrino seja investigado.