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ENSAIOS DE BIOTRANSFORMAÇÃO DA ATRAZINA E AVALIAÇÃO DOS SEUS EFEITOS ESPERMÁTICOS EM PEIXE

TESE DE DOUTORADO

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por

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LISTA DE ABREVIATURAS

11β-HSD2: hidroxiesteróide 11-beta-desidrogenase 2

3β-HSD: 3 beta hidroxiesteróide desidrogenase.

AHR: Receptor aril hidrocarboneto

AMPc: monofosfato de adenosina cíclico

ATP: trifosfato de adenosina, do inglês: adenosine triphosphate

ATZ: atrazina

BaP: benzo[a]pireno

CDNB: 1-cloro-2,4-dinitrobenzeno

CFTR: regulador da condutância transmembranar da fibrose cística

CYP: citocromo P450

CYP11A1: enzima de clivagem da cadeia lateral do colesterol.

CYP19: enzima aromatase

CYP19A1: citocromo P450 família 19, subfamília A, membro 1.

DEA: desetilatrazina

DHT: 5 α-di-hidrotestosterona

DIA: deisopropilatrazina

DNA: ácido desoxirribonucleico.

FSTL3: follistatin like 3.

GPX: glutationa peroxidase

GS-DNB: 2,4-dinitrofenil-S-glutation

GSH: glutationa reduzida

GST: glutationa *S*-transferases

GSTP1-1: GST pi 1-1

H_2O_2 : peróxido de hidrogénio

hCG: gonadotropina coriónica humano

hGST: GST humano

IGF1: fator de crescimento insulina like

INHα: subunidade alfa inibina

LC-MS/MS: cromatografia líquida e espectrometria de massa em tandem

LHβ: polipeptídio beta da hormona luteinizante

NADPH: nicotinamida adenina dinucleótido fosfato

NER: sistema de reparo de nucleotídeo excisado

NR5A1: subfamília nuclear receptor 5, grupo A, membro 1.

O_2 : oxigênio molecular

$O_2^{\cdot -}$: radical ânion superóxido

OCP: pesticidas organoclorados

PAH: hidrocarbonetos aromáticos policíclicos

PCB: bifenilos policlorados

PCDD: dibenzo-p-dioxinas

PCDF: dibenzofuranos policlorados

PDE: fosfodiesterase.

PKA: Proteína quinase A

ERO: espécies reativas de oxigênio

SF1: fator esteroidogênica.

SOD: superóxido dismutase

SRD5A: esteroide 5α-redutase.

SRD5A2: esteróide-5α redutase α-polipeptídio 2

StAR: gene proteína de regulação aguda da esteroidegênesis.

XPC: xeroderma pigmentoso; fator de reconhecimento e reparação de DNA danificado

XRE: Elemento de resposta xenobiótica

αGSU: subunidade α-glicoproteína comum

SUMÁRIO

1. RESUMO

A atrazina (ATZ) é um agrotóxico utilizado na agricultura para controlar ervas daninhas. A sua utilização em larga escala, seu escoamento e lixiviação, tornaram-lhe um dos contaminantes de maior presença no ambiente aquático. Enzimas de biotransformação como o citocromo P450 (CYP) e a glutationa *S*-transferase (GST) participam de sua detoxificação. A atividade destas enzimas pode se mostrar aumentada nos organismos aquáticos expostos a contaminantes ambientais. A ATZ pode ainda causar efeitos nocivos ao nível reprodutivo em mamíferos, anfibos e peixes. Os prováveis mecanismos envolvidos nestes efeitos, incluem a modulação de vias esteroidogênicas pela ATZ, através do incremento da AMPc por inibição da fosfodiesterase, assim como a modução de múltiplas vias bioquímicas fundamentais da gametogênese e ovulação. Outro efeito negativo da ATZ seria a produção adicional de espécies reativas de oxigênio com conseqüente aumento de danos em estruturas celulares e DNA. A presente tese está estruturada em dois capítulos com os seguintes objetivos: 1) estimar a biotransformação de ATZ via GST em camarão e peixes de ambiente contaminado e cativeiro (referência), utilizando três abordagens: ensaio cinético da GST com substrato de amplo espectro (CDNB), ensaio cinético competitivo entre CDNB e ATZ e ensaio cinético de decaímento da ATZ, analisada por cromatografia líquida e espectrometria de massa em tandem (LC-MS/MS); e 2) avaliar efeitos da exposição à ATZ sobre os parâmetros de qualidade espermática e na regulação transcricional de genes-alvo relacionados a estes parâmetros em peixe-zebra (*Danio rerio*). Para o primeiro capítulo, foram utilizados extratos citosólicos de brânquia e hepatopâncreas de camarão *Litopenaeus vannamei* e brânquia e fígado de peixe *Poecilia vivipara*. Ensaios cinéticos com variações nas concentrações de CDNB e ATZ foram realizados a fim de construir gráficos de *Lineweaver-Burk* que permitiram inferir que a inibição causada pela ATZ era do tipo competitivo. A atividade basal estimada por ensaio cinético com CDNB sugeriu maior capacidade de detoxificação via GST em peixe que em crustáceo, maior em fígado que em brânquias e maior em peixe de local contaminado que de cativeiro. Padrões similares foram encontrados quando foi avaliado o decaimento da ATZ por LC-MS/MS. Por outro lado, a capacidade de biotransformação estimada pelo ensaio competitivo, utilizando CDNB e ATZ como substratos de competição, sugere maior atividade de GST com afinidade por ATZ em

crustáceo que em peixe, e maior em peixes de cativeiro que de local contaminado. Aparentemente, os dois primeiros ensaios citados, foram mais apropriados para indicar a capacidade de biotransformação do que o ensaio competitivo. A inespecificidade do ensaio competitivo utilizado pode ter relação com a presença de diferentes isoformas de GST nas matrizes biológicas testadas. Para o segundo objetivo, peixes *D. rerio* foram expostos as concentrações nominais de ATZ de 0, 2, 10 e 100 $\mu g.L^{-1}$ durante 11 dias. As concentrações de 2, 10 e 100 $\mu g.L^{-1}$ ATZ testados causaram diminuição em diversos parâmetros espermáticos, como motilidade, funcionalidade mitocondrial e integridade de membrana, em relação ao grupo controle, porém não houve efeito na integridade do DNA. A repressão da expressão de genes relacionados da espermatogênese (*SRD5A2* e CFTR) e proteção celular (*SOD2, GPX, XPC*) em gônadas de grupos expostos à ATZ sugerem que a ATZ afeta as vias gametogênicas e de proteção na gônada, podendo ser uma das possíveis causas para a redução da qualidade espermática. No fígado, a ATZ ativou a expressão de genes de detoxificação e antioxidante (*CYP1A, GSTP e SOD2*). Fatores de transcrição tais como AHR e NF-κB, podem estar sendo ativados pela presença da ATZ e seus produtos oxidativos, e podem representar possíveis mecanismos de regulação transcricional envolvidos nestas respostas. Em suma, o presente estudo sugere que a capacidade de biotransformação de ATZ via GST é espécie-específica, órgão-específica e maior em peixes que habitam ambiente contaminado. Também sugere que concentrações ambientalmente relevantes de ATZ causam efeitos significativos ao nível reprodutivo em peixes macho, e que estes efeitos podem estar associados à regulação transcricional de genes-chave associados à espermatogênese e proteção celular.

Palavras chaves: reprodução; peixe; atrazina; GST; CDNB

2. ABSTRACT

Atrazine (ATZ) is a substance used in agriculture to control weeds. Its widespread use, drainage and leaching have made it one of the most important contaminants in the aquatic environment. Biotransformation enzymes such as cytochrome P450 (CYP) and glutathione *S*-transferase (GST) participate in its detoxification. The activity of these enzymes may be increased in aquatic organisms exposed to environmental contaminants. ATZ may also cause reproductive harm to mammals, amphibians and fishes. The probable mechanisms involved in these effects include the modulation of steroidogenic pathways by ATZ, through the increase of cAMP by inhibition of phosphodiesterase, as well as the modulation of multiple biochemical pathways fundamental to gametogenesis and ovulation. Another negative effect of ATZ would be the additional production of reactive oxygen species with consequent increase of damage in cellular structures and DNA. The present thesis is structured in two chapters with following objectives: 1) to estimate the biotransformation of ATZ via GST in shrimp and contaminated environment fish and captivity (reference) using three approaches: GST kinetic assay with broad spectrum substrate (CDNB), competitive kinetic assay between CDNB and ATZ, and kinetic assay of decay of ATZ, analysed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS); and 2) to evaluate effects of exposure to ATZ on the parameters of sperm quality and on the transcriptional regulation of target genes related to these parameters in zebrafish (*Danio rerio*). For first chapter, we used cytosolic extracts of gill and hepatopancreas of shrimp *Litopenaeus vannamei* and gill and liver of fish *Poecilia vivipara*. Kinetic assays with varying concentrations of CDNB and ATZ were performed in order to develop *Lineweaver-Burk* plots that allowed to infer that the type of inhibition caused by ATZ was of the competitive type. Basal activity estimated by kinetic assay with CDNB suggested a higher detoxification capacity via GST in fish than in crustacean, higher in liver than in gills and higher in fish from contaminated environment than from captivity. Similar patterns were found when ATZ decay was evaluated by LC-MS/MS. On other hand, biotransformation capacity estimated by competitive assay, using CDNB and ATZ as competition substrates, suggests higher GST activity with ATZ affinity in crustacean than in fish, and higher in captivity fish than in contaminated environment. Possibly, first two trials cited, have more realistically reproduced the biotransformation capacity than competitive assay. The non-specificity of the competitive assay used,

which could have its results masked by GST isoforms that have no affinity for ATZ, but rather for CDNB, could be one of the causes of the contradictory results obtained using this approach. For the second objective, *D. rerio* fish were exposed to nominal concentrations of ATZ of 0, 2, 10 and 100 μg.L^{-1} for 11 days. The concentrations of 2, 10 and 100 μg.L^{-1} ATZ tested caused a decrease in several sperm parameters, such as motility, mitochondrial functionality and membrane integrity, in relation to the control group, but there was no effect on DNA integrity. Repression of expression of genes related spermatogenesis (*SRD5A2* and *CFTR*) and cellular protection (*SOD2, GPX, XPC*) in gonads of groups exposed to ATZ suggest the involvement of ATZ in gametogenic and protection pathways in the gonad, and may be one of the possible causes for the reduction of sperm quality. In liver, ATZ activated expression of detoxification and antioxidant genes (*CYP1A, GSTP* and *SOD2*). Transcription factors such as AHR and NF-κB may be activate by the presence of ATZ and its oxidative products, and may represent possible mechanisms of transcriptional regulation involved in these responses. In summary, the present study suggests that the biotransformation capacity of ATZ via GST is species-specific, organ-specific and higher in fish that inhabit a contaminated environment. It also suggests that environmentally relevant concentrations of ATZ cause significant reproductive effects in male fish, and that these effects may be associated with the transcriptional regulation of key genes associated with spermatogenesis and cell protection.

Key words: reproduction; fish; atrazine; GST; CDNB

3. INTRODUÇÃO GERAL

3.1. O herbicida atrazina e seu impacto ambiental

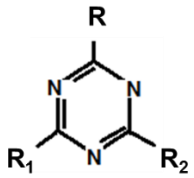
Os agrotóxicos são frequentemente detectados em corpos hídricos superficiais e subterrâneos em todo o mundo, em função da sua ampla utilização em áreas agrícolas e urbanas (de Armas *et al*., 2007). Eles são usados para controlar ervas daninhas, insetos e fungos (Yang *et al*., 2016) e incluem moléculas com diferentes propriedades que lhe conferem diferentes graus de persistência ambiental, mobilidade e o potencial efeito tóxico, cancerígeno, mutagênico e teratogênico ou algum efeito endócrino para vários órgãos não-alvo, incluindo os seres humanos (de Campos Ventura *et al*., 2008; Adeyemi *et al*., 2015).

As triazinas são muito utilizadas como herbicidas no controle pré e pós-emergente de ervas daninhas nas mais variadas culturas (Mei *et al*., 2016), tais como, milho, cana-de-açúcar, sorgo, abacaxi, banana, café, uvas, entre outras. As *s*-triazinas possuem um anel heterocíclico de seis membros onde os átomos de carbono e nitrogênio são simetricamente localizados e os substituintes das posições 2, 4 e 6, constituem-se no diferencial entre as várias formulações disponíveis comercialmente (Hu *et al*., 2013). Devido ao seu uso extensivo, alta persistência, solubilidade em água e absortividade relativamente fraca, eles podem contaminar o ambiente aquático pelo escoamento agrícola, lixiviação e deposição atmosférica e, portanto, são regularmente e mais frequentemente detectados em águas subterrâneas e superficiais (Loos e Niessner, 1999; Jablonowski *et al*., 2013; Marchetti *et al*., 2013).

Na Alemanha Ocidental, das análises de mais de 100 mil amostras de água superficial e subterrânea, as triazinas estão presentes em 80,7% das amostras encontradas, sendo o 59,7% dos casos desta porcentagem o correspondente à atrazina

(ATZ) (Beitz *et al*., 1994). De Armas *et al*. (2007), analisando resíduos de herbicidas em amostras de água e sedimento da sub-bacia do Rio Corumbataí no Brasil, encontrou às triazinas como o grupo de herbicidas de maior ocorrência e presença; com níveis de até 2,7 $\mu g.L^{-1}$ para ATZ. Assim, a detecção frequente de ATZ e seus metabólitos, principalmente deetilatrazina (DEA) e deisopropilatrazina (DIA), no solo, água superficial e subterrânea (Hansen *et al*., 2013; Smalling *et al*., 2015; Sousa *et al*., 2016), justifica o questionamento da segurança em sua aplicação respeitando as boas práticas agrícolas.

Atrazina é um dos herbicidas triazínicos utilizados com maior frequência na agricultura (Fig. 1). Seu nome químico é 6- cloro-N-etil-N'- (1-metiletil)-1,3,5-triazina-2,4-diamina, e o número CAS, 1912-24-9 (Mackay *et al*., 2006). ATZ foi introduzida nos anos 1950, e desde então converteu-se em o herbicida comum na agricultura e setor florestal (Graymore *et al*., 2001), sendo comercialmente disponível individual ou em combinação com muitos outros princípios ativos como glifosato, alaclor e cianazina (Mester and Sine, 2011). ATZ é um herbicida seletivo que inibe o crescimento de ervas daninhas alvo ao interferir com o funcionamento normal da fotossíntese (Brodeur *et al*., 2013). Isso impede as funções de crescimento de uma ampla variedade de plantas, incluindo algumas espécies de algas (Graymore *et al*., 2001).



Composto	R	R_1	R_2
atrazina - ATZ	Cl	$NHCH(CH_3)_2$	$NHCH_2CH_3$
deetilatrazina - DEA	Cl	$NHCH(CH_3)_2$	NH_2
deisopropilatrazina - DIA	Cl	NH_2	$NHCH_2CH_3$
didealquilatrazina - DDA	Cl	NH_2	NH_2
hidroxiatrazina - HA	OH	$NHCH(CH_3)_2$	$NHCH_2CH_3$
deetilhidroxiatrazina - DEHA	OH	$NHCH(CH_3)_2$	NH_2
deisopropilhidroxiatrazina - DIHA	OH	NH_2	$NHCH_2CH_3$

Figura 1. Molécula da atrazina e seus metabólitos.

Depois da sua aplicação, a persistência da ATZ ligada às partículas de solo dependerá do tipo de solo, quantidade de matéria orgânica, quantidade de argila, pH do solo e estrutura do solo (Stagnitti *et al*., 1998; Kookana *et al*., 1998). Em terras agrícolas que possuem drenagem ou são margeadas por riachos perenes, quantidade significativa de ATZ escoam para a água superficial e para a água subterrânea (Davies *et al*., 1994; Graymore *et al*., 2001). Consequentemente, a contaminação destes riachos é inevitável, e concentrações significativas são frequentemente reportadas (Tabela 1). As regiões com lençóis subterrâneos pouco profundos são particularmente susceptíveis à contaminação por ATZ, particularmente em áreas com solos arenosos e argilo arenosos (Lloyd-Smith *et al*., 1999). Isto é acentuado se o solo é pobre em matéria orgânica, uma vez que os processos que facilitam a degradação são principalmente biológicos (Graymore *et al*., 2001).

A degradação biológica no solo ocorre através da atividade de microrganismos e é considerada como o principal processo pelo qual a ATZ é transformada (Steinheimer,

1993; Monard *et al.,* 2011). Os microorganismos utilizam a ATZ como fonte de energia e de nutrientes (Hansen *et al*., 2013). No entanto, a presença de outras fontes de nutrientes pode inibir a degradação deste herbicida. A presença de carbono e nitrogênio mineral no solo limitam a biodegradação de ATZ, devido ao fato de que os microrganismos utilizam preferencialmente aquelas fontes de carbono e nitrogênio, os quais estão mais disponíveis para o metabolismo, em vez de utilizar a molécula de ATZ como fonte de nutriente (Abigail *et al*., 2012). O tempo de meia vida de ATZ em sistemas estuarinos foi de 1-4 semanas (Jones *et al*., 1982; citado por Meakins *et al*., 1994). Sob condições de laboratório, em água destilada e água de rio, ATZ foi completamente degradada depois de 21,3 e 7,3 horas, respectivamente (Mansour *et al*., 1989; citado por Montgomery, 1993). A meia-vida, em água de rio com pH 7,3, foi de 235 dias a 6°C; no escuro a 22°C foi de 164 dias; sob condições de luz de sol foi de 59 dias; 130 dias a 2°C e 200 dias a 22°C na escuridão para água de rio filtrado a pH 7,3; e 169 dias sob condições de luz solar na água de mar com pH 8,1 (Lartiges e Garrigues, 1995).

A degradação da ATZ produz muitos metabólitos, cada um com persistência e toxicidade variável (Graymore *et al*., 2001); mas os principais detectados na água subterrânea e superficial são metabólitos cloro desalquilados, predominantemente DEA e DIA (Nélieu *et al*., 2000). Muitas vezes uma relação 1:1 de ATZ e DEA tem sido detectada (Acero *et al*., 2000). Há cinco processos que determinam a taxa de degradação dentro do solo: hidrólise, adsorção, volatilização, fotodegradação e a mais importante, a degradação microbiana. DEA, DIA e didealquilatrazina (DDA) são formados através da biodegradação, enquanto hidroxiatrazina (HA) e deetilhidroxiatrazina (DEHA) pode ser formada por reações químicas no solo ou biodegradação (Graymore *et al*., 2001).

Tabela 1: Concentrações de ATZ reportados e seus produtos de degradação em corpos de água.

Corpo de água	Lugar	Concentração ($\mu g.L^{-1}$)	Referencia
Subterrâneo	Bacia do Rio Baiyangdian, China	0,4 – 3,29	Ye *et al*. (2001)
Subterrâneo	Franja de Gaza, Oriente Próximo	3,5	Shomar *et al*. (2006)
Subterrâneo	Zona Agrícola de Referencia, México	6,23 – 21,26	Hernández-Antonio y Hansen (2011)
Subterrâneo	Zona agrícola Baixo Sorraia - Portugal	0,05 – 29,0	Cerejeira et al. (2003)
Superficial	Zona Agrícola de Referencia, México	4,62 – 15,01	Hernández-Antonio y Hansen (2011)
Superficial	Córregos e bacias hidrográficas, Tasmânia	<0,01 - 53000	Davies et al. (1994)
Superficial	Laguna costeira, Norte Adriático, Itália	2,4 – 8,2	Carafa *et al.* (2007)
Superficial	Sub-bacia do Rio Corumbataí, Brasil	0,6 - 2,7	de Armas *et al.* (2007)
Superficial	Nordeste Ceará, Brasil	n.d – 15,0	Sousa *et al.* (2015)

Logo que a ATZ foi classificada como um possível carcinogênico humano (Kligerman *et al*., 2000), seu nível máximo permitido em águas superficiais foi fixado em 3 $\mu g.L^{-1}$ pela Agência de Proteção Ambiental (EPA) dos Estados Unidos de América (US EPA, 2001), embora a Comunidade Europeia fixou o limite de 0,1 $\mu g.L^{-1}$ para o mesmo uso da água (OJEC, 1998) e a Organização Mundial de Saúde define limites de 2 $\mu g.L^{-1}$ para ATZ e 100 $\mu g.L^{-1}$ para ATZ mais metabólitos (WHO, 2011). A legislação brasileira, através da Resolução CONAMA 357/05, que define os padrões de qualidade de águas superficiais destinadas ao consumo humano e à proteção a vida aquática, determina o nível máximo para ATZ em 2 $\mu g.L^{-1}$ (CONAMA, 2005).

Pesquisas têm estimado a nocividade da ATZ no peixe. As concentrações letais (LC_{50}) em 96 horas de exposição variam entre 9 e 19 $mg.L^{-1}$ de ATZ para tilápia vermelha (*Oreochromis mossambicus*), tilápia do Nilo (*Oreochromis niloticus*), jundiá (*Rhamdia quelen*), Peixe-lua (*Lepomis macrochirus*) e carpa comum (*Cyprinus carpio*) (Prassad e Reddy, 1994; Hussein *et al*., 1996; Kreutz *et al*., 2008; Bathe *et al*., 1973;

Neskovic *et al*., 1993). Alterações bioquímicas e histopatológicos no tecido do peixe podem ocorrer com exposições longas a concentrações menores de 2 $mg.L^{-1}$ de ATZ (Neskovic *et al*., 1993). No crescimento de peixe-zebra, 0,3 $\mu g.L^{-1}$ foi determinado como a concentração de efeito não observado, enquanto 0,9 $\mu g.L^{-1}$, foi a concentração mais baixa de efeito observado, após 28 dias de exposição (Plhalova *et al*., 2012).

3.2. Biotransformação da atrazina

A ATZ absorvida é assimilada muito rapidamente, seja proveniente do meio aquático ou do alimento. Gunkel e Kausch (1987) sugere que 70% da ATZ consumida pode ainda ser detectada 30 minutos após o alimento ter sido ingerido por peixe. Após esse momento, a quantidade de ATZ detectável em peixes diminui rapidamente dentro de 12 h. O acúmulo de ATZ diretamente a partir da água atinge o ponto de saturação aproximadamente após 6 h. (Huber, 1993). O fator de concentração da ATZ em peixe *Coregonus fera* varia entre dois e cinco e não se altera significativamente mesmo nos casos de exposição prolongada (Gunkel, 1981), o que indica mecanismos efetivos de eliminação. Um equilíbrio é alcançado entre a captação de ATZ e os mecanismos de eliminação (Gunkel, 1981). O peixe *C. feru* e a carpa (*Cyprinus carpio*) atingiram fatores de concentração de 2 a 8 em água com uma concentração de 100 $\mu g.L^{-1}$ de ATZ, sem tendência observável de mudanças nos valores residuais monitorados durante um período experimental de quatro meses em experimentos de campo conduzidos em pequenas lagoas (Gunkel e Kausch, 1987).

No monitoramento do ambiente aquático através de bioindicadores, as brânquias e o fígado do organismo são órgãos-alvo para pesquisa de biomarcadores. As brânquias são utlizadas devido a sua ampla área superficial, localização externa, e seu papel em

funções vitais como a respiração, osmoregulação, excreção de resíduos nitrogenados, e equilíbrio ácido-base (Alazemi *et al*., 1996). Neste órgão ocorre altas taxas de assimilação de contaminantes presentes na água, devido ao fato de que é a principal interface entre o animal e o ambiente aquático (Azevedo *et al*., 2015). Entretanto, o fígado é provavelmente um dos órgãos internos mais importantes para a detoxificação de xenobióticos. Estes compostos absorbidos e transportados via sanguínea são neutralizados pelos hepatócitos (Fernandez-Checa e Kaplowitz, 2005; Izzet *et al*., 2005; Song *et al*., 2000).

No fígado, bem como em outros órgãos, os xenobióticos podem ser submetidos a uma ou duas fases de biotransformação. Na Fase I, um grupo polar reativo é inserido na molécula tornando-a um substrato adequado para as enzimas da Fase II. As enzimas tipicamente envolvidas no metabolismo da Fase I incluem as monooxigenases microsomais citocromo P450 (CYP) e as monooxigenases contendo flavina (FMO); além da álcool e aldeído desidrogenases, amina oxidases, ciclooxigenases, redutases e hidrolases. Todas estas enzimas, com exceção das redutases, introduzem grupos polares na molécula que, na maioria dos casos, podem ser conjugados durante o metabolismo da Fase II (Hodgson, 2010).

As enzimas de Fase II transformam compostos endógenos e xenobióticos em formas mais facilmente excretáveis assim como inativam substâncias farmacologicamente ativas. Estes incluem glucuronidação, sulfatação, metilação, acetilação e conjugação com aminoácidos e glutationa (Hodgson, 2010). Em geral, os conjugados respectivos são mais hidrofílicos do que os compostos originais. As enzimas de metabolização da Fase II são principalmente transferases e incluem: UDP-

glucuronosiltransferases, sulfotransferases, *N*-acetiltransferases, glutationa *S*-transferases (GST) e metiltransferases (Jancova *et al*., 2010).

Em várias espécies de vertebrados, incluído o humano, a reação metabólica dominante na Fase I para ATZ seria a *N*-desalquilação (o grupo metil é oxidado), enquanto as conjugações com glutationa reduzida (GSH) ou glucoronidos de metabólitos da Fase I ou do produto original, seriam as principais reações na Fase II (Figura 2; Adams *et al*., 1990). A exposição de peixes à ATZ causa indução de enzimas que catalisam estas reações. Peixe *Danio reri*o exposto a concentrações que variaram de 0,01 a 1 $mg.L^{-1}$ de ATZ mostraram indução das isoenzimas CYP no fígado (Dong *et al*., 2009). Em peixe *Lepomis macrochirus* a atividade das GSTs no fígado foi aumentada após da exposição a 6 e 9 $mg.L^{-1}$ de ATZ e também em brânquias após a exposição a 9 $mg.L^{-1}$ (Elia *et al*., 2002). O efeito indutor da ATZ também foi mostrado na expressão de genes destas enzimas. Embriões de peixe zebra expostos a 5 e 40 $mg.L^{-1}$ e 0,5 $mg.L^{-1}$ de ATZ, tiveram a expressão de *CYP1A* e *GSTP1* induzidas, respectivamente (Glisic *et al*., 2014); enquanto, o fígado de *Cyprinus carpio L*. expostos a 4,28; 42,8 e 428 $\mu g.L^{-1}$ de ATZ mostrou o mesmo efeito indutor na expressão da *CYP1A* (Xing *et al*., 2014) e *GSTR* (Xing *et al*., 2012).

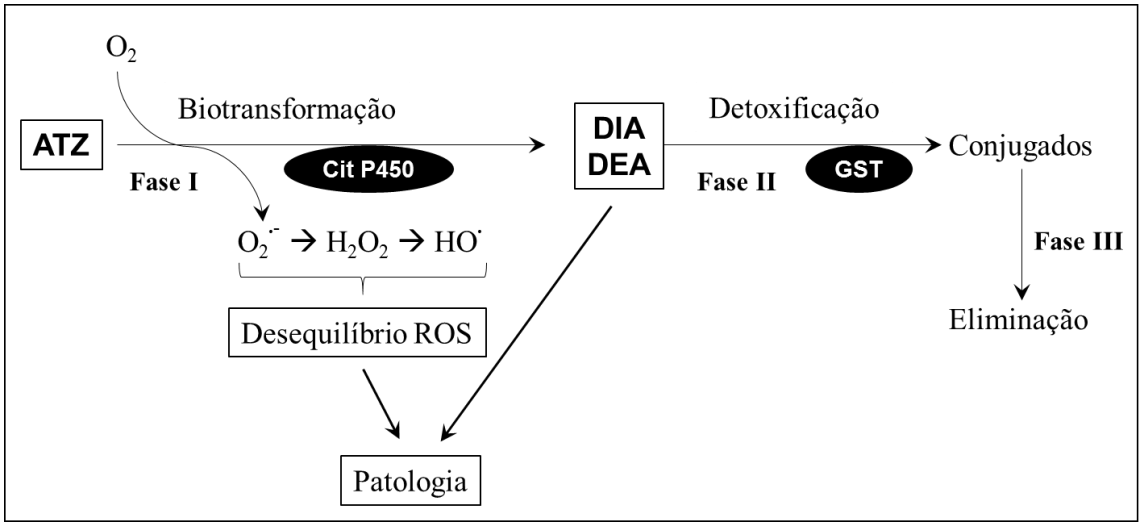


Figura 2. As reações da Fase I e seus produtos monooxigenados são susceptíveis de formarem intermediários reativos capazes de reagir com substituintes nucleofílicos em macromoléculas. Na Fase II, as reações de conjugação por ação de GST reduzem a reatividade de metabólitos da Fase I o seu composto original, aumentando a solubilidade na água. A Fase III refere-se ao efluxo de compostos parentais ou metabólitos por transportadores de membrana. Adaptado de Lushchak, 2012.

Estudos de biotransformação de ATZ em mamíferos reportam monodealquilados *S*-triazina como os principais metabólitos da sua detoxificação através da Fase I. Os metabólitos DEA, DIA e DIHA são detectados de ensaios *in vitro* utilizando microssomas de fígado de ratos Sprague-Dawley, porcos, guinea pig e humanos. Estes metabólitos são resultado de reações de *N*-desisopropilação, *N*-desetilação e 1-hidroxilação catalisadas por muitas isoformas CYP (Lang *et al*., 1996; Hanioka *et al*., 1999). CYP1A2 e CYP2C19 seriam as enzimas principais na produção de DEA e DIA, respectivamente. A contribuição estimada para CYP1A2 e CYP2C19 envolvidas no metabolismo de ATZ foram de 63% e 24% para DEA, e 35% e 56% para DIA (Joo *et al*., 2010). Outros metabólitos de ATZ, tais como atrazina didealquilada, hidroxitrazina e conjugados de ácido mercaptúrico, também são encontrados na urina juntamente com DEA, DIA e ATZ (Buchholz *et al*., 1999; Jaeger *et al*., 1998), evidenciando que a

biotransformação de ATZ na Fase II, tal como a conjugação com GSH, é também uma via significativa da sua detoxificação em seres humanos (Figura 3).

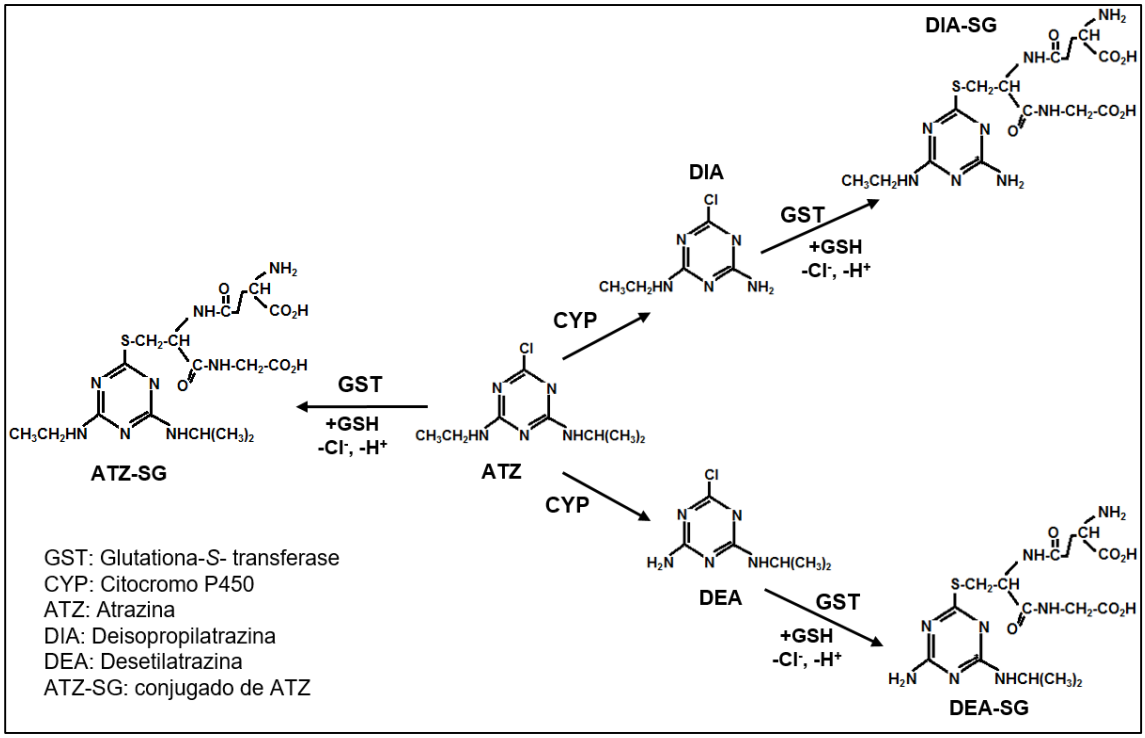


Figura 3. Vias propostas da biotransformação de ATZ em humanos e roedores. ATZ pode ser conjugada diretamente com GSH por ação da GST. A DEA e DIA são monodealquilados formados pela ação de CYP e subsequentemente podem ser conjugados com GSH por ação da GST. Adaptado de Hanioka *et al*., 1999 e Joo *et al*., 2010.

Embora os ácidos mercaptúricos, os conjugados de *N*-acetilcisteína de xenobióticos, tenham sido conhecidos desde o início do século XX, apenas desde os anos 60 a fonte do grupo cisteína (GSH) e as enzimas necessárias para a formação destes ácidos foram identificados e caracterizados (Hodgson, 2010). A partir destas descobertas, desenvolveram-se modelos para estudar a atividade enzimática na Fase II, sendo uma da mais conhecida o ensaio enzimático da GST utilizando o 1-cloro-2,4-dinitrobenzeno (CDNB) como substrato. O modelo está baseado em que todas as GST seriam ativas com o CDNB, sendo a conjugação deste com GSH utilizada para

quantificar-se sua atividade. A conjugacao do grupo tiol da GSH ao substrato CDNB, que produz o conjugado 2,4-dinitrofenil-*S*-glutationa (GS-DNB), permite ensaios espectrofotométricos devido ao aumento da absorvância a 340 nm longitude de onda (Figura 4; Clark *et al*., 1973; Habig and Jakoby, 1981).

A interação de agrotóxicos e as GST, podem ser avaliadas através de ensaios de inibição *in vitro* utilizando CDNB como substrato, e usando, por exemplo, a ATZ como possível inibidor. Baseado neste enfoque, as GST conjugariam GSH com CDNB produzindo conjugados GS-DNB. No entanto, o fato que menos conjugados GS-DNB sejam detectados no ensaio em presença de ATZ significaria a existência de competição pelo sitio ativo na enzima GST pela ATZ e o CDNB. A variação nestas detecções seria um indicativo das variações no conteúdo de isoformas com mais afinidade pela ATZ, se extratos citosólicos procedentes de diferentes órgãos e espécies fossem utilizados nos ensaios. É dizer, enquanto maior inibição na formação de conjugados GS-DNB, maior a afinidade de GST pelo substrato ATZ.

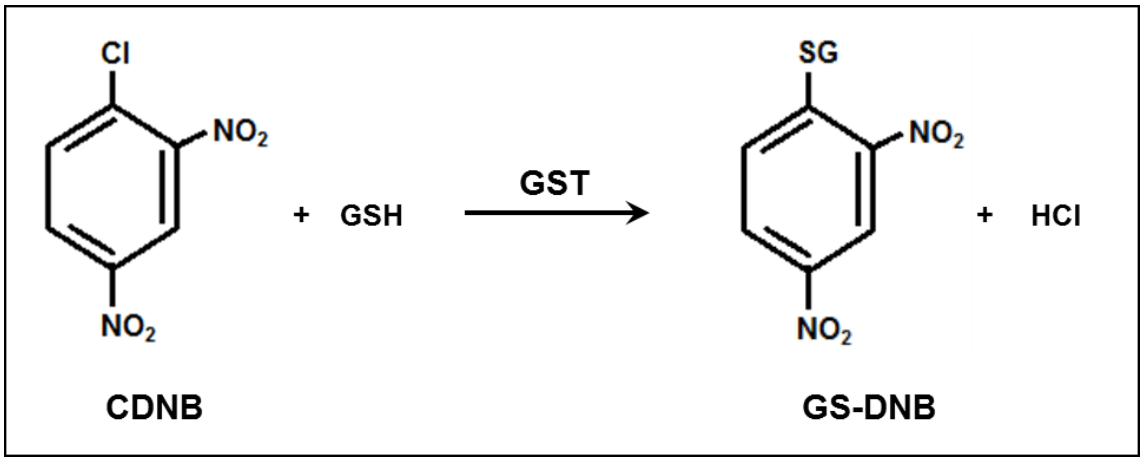


Figura 4. Formação do conjugado GS-DNB. Detoxificação do CDNB através da GST formando 2,4-dinitrofenil-*S*-glutatión (GS-DNB). Adaptado de Hayes e Pulford, 1995.

3.3. As glutationa *S*-transferases

As GST são uma família de enzimas principalmente solúveis, multifuncionais e diméricos (Van der Oost *et al*., 2003). Além de suas funções essenciais no transporte intracelular (heme, bilirrubina e ácidos biliares) e a biossíntese de leucotrienos e prostaglandinas, um papel crítico das GST é a detoxificação celular (Glisic *et al*., 2015). A presença de dois sítios ativos por dímero, com atividades independentes uma da outra, ajudam no cumprimento desta função vital. Cada sítio ativo consiste no mínimo de duas regiões de ligação, um muito especifico para a GSH, e outro sítio de ligação com menor especifidade para os eletrófilos (Danielson e Mannervik, 1985). A reação antioxidante chave conjuga o tri-peptídeo GSH (γ-glutamil-cisteinil-glicina) com um co-substrato hidrofóbico que possui um centro eletrofílico (Oakley, 2011). Para isso, a ativação do átomo de enxofre da GSH ao ânion tiolato (GS^-, um nucleófilo forte que ataca substratos eletrofílicos como carbono, nitrogênio ou enxofre) é necessária, embora os detalhes do mecanismo de ativação variem entre enzimas de diferentes subfamílias (Wu e Dong, 2012). Este tipo de reação normalmente resulta na redução da reatividade do composto e no aumento da sua solubilidade em água como um pré-requisito principal para a subsequente eliminação através dos transportadores de efluxo (Malik *et al*., 2016).

A evidencia que a atividade da GST é criticamente importante em sistemas biológicos está em que evoluíram através de vias convergentes em pelo menos quatro famílias de enzimas estruturalmente distintas (as GST citosólicas, as GST mitocondriais da classe Kappa, as enzimas MAPEG, as proteínas de resistência à fosfomicina) (Atkinson e Babbitt, 2009; Robinson *et al*., 2004; Ladner *et al*., 2004). As GST citosólica são a família mais extensamente estudada e ocorre em todas as formas de vida

celular. As GST citosólica de mamíferos veio a proeminência na investigação biomédica, devido ao papel desempenhado por muitos membros da família em metabolismo de drogas e xenobióticos (Board e Menon, 2013).

As GST citosólicas são divididas em sete famílias (historicamente chamadas classes): as famílias α (alfa), κ (kappa), μ (mu), π (pi), σ (sigma), θ (theta) e ômega (Ω). Um novo sistema de nomenclatura propõe o termo GST para a enzima, precedido pelo uso de uma pequena letra romana para a espécie (m para mouse, h para humanos, etc.) seguida de uma letra romana maiúscula para a família (A para α, K para κ, etc.). As GST de classe Omega normalmente não catalisam as reações de conjugação de GSH pela qual a família GST é bem conhecida. Em contraste, eles catalisam uma gama de redução e reações de tioltransferase onde os resíduos de cisteína são esperados para desempenhar um papel significativo (Schmuck *et al*., 2005; Board *et al*., 2007; Yamamoto *et al*., 2009).

As GST de classe Kappa são também enzimas solúveis com algumas especificidades de substrato que são semelhantes as GST citosólicas e foram originalmente nomeados e considerados como um membro distante da família GST citosólicas (Harris *et al*., 1991). Subsequentemente, a análise da sequência e os estudos estruturais revelaram sua origem evolucionária distinta e antiga (Robinson *et al*., 2004; Ladner *et al*., 2004; Morel *et al*., 2004). As GST classe Kappa parecem ser expressos em mitocôndrias e peroxissomas em mamíferos e em *Caenorhabditis elegans* (Morel *et al*., 2004; Petit *et al*., 2009).

As proteínas procarióticas de resistência à fosfomicina representam outra família de proteínas solúveis que catalisam as reações de glutationa transferase (Rigsby *et al*., 2005). Fosfomicina (ácido (1R, 2S) -epoxipropilfosfónico) é um antibiótico de largo

espectro que é inactivado pela adição de GSH catalisada por FosA dependente de K^+. FosA é uma metaloproteína independente de Mn (II) com semelhanças estruturais com a superfamília de proteínas quelato de oxigênio vicinial (vicinal oxygen chelate: VOC), que inclui a glioxalase I que também usa a GSH como um cofator (Rigsby *et al*., 2005; Armstrong, 2000).

As proteínas MAPEG (proteínas associadas à membrana no metabolismo eicosanóide e glutationa) são a quarta família de proteínas com membros exibindo atividade da glutationa transferase (Jakobsson et al., 1999). A glutationa transferase 1 microsomal (MGST1) é a GST mais extensamente caracterizada dentro da familia MAPEG e constitui 3% da proteína do reticulo endoplasmico no fígado de rato e o 5% da membrana mitocondrial externa (Morgenstern, 2005). Embora o MGST1 seja um trímero ligado à membrana e estruturalmente distinto, ele compartilha a mesma especificidade de substrato, ampla e sobreposta, como os GST citosólicos (Board e Menon, 2013).

3.4. **Indução de GST em ambiente aquático contaminado**

A maioria dos animais, incluindo seres humanos, são expostos diariamente a uma grande quantidade de compostos químicos no ar, água ou alimentos. Alguns destes compostos químicos são moléculas sinalizadores que carregam informações valiosas sobre o ambiente do animal (por exemplo, a presença de alimentos, predadores ou membros do sexo oposto), enquanto outros são tóxicos e devem ser evitados ou eliminados (Hahn, 2002). Para estes últimos, os animais desenvolveram enzimas induzíveis e transportadores para facilitar a biotransformação e eliminação de

xenobióticos encontrados no ambiente (Uno *et al*., 2012; Lushchak, 2012; Burkina et al., 2015).

Os componentes enzimáticos induzíveis para a eliminação de tóxicos incluem as CYP, GST e glucuronosil transferases (Figura 5). Os componentes transportadores deste sistema incluem as proteínas transportadores ABC (ATP binding cassette), que atuam como bombas de efluxo para remover metabólitos de produtos químicos endógenos e xenobióticos das células (Dean *et al*., 2001). O componente sensorial deste sistema consiste em receptores solúveis que regulam a expressão dos genes de biotransformação e transporte em resposta a químicos ambientais. Estes receptores incluem vários membros de família de receptores esteroides/nucleares (Waxman, 1999; Savas *et al*., 1999; Kliewer *et al*., 1999; Honkakoski e Negishi, 2000) bem como o receptor aril hidrocarboneto (AHR).

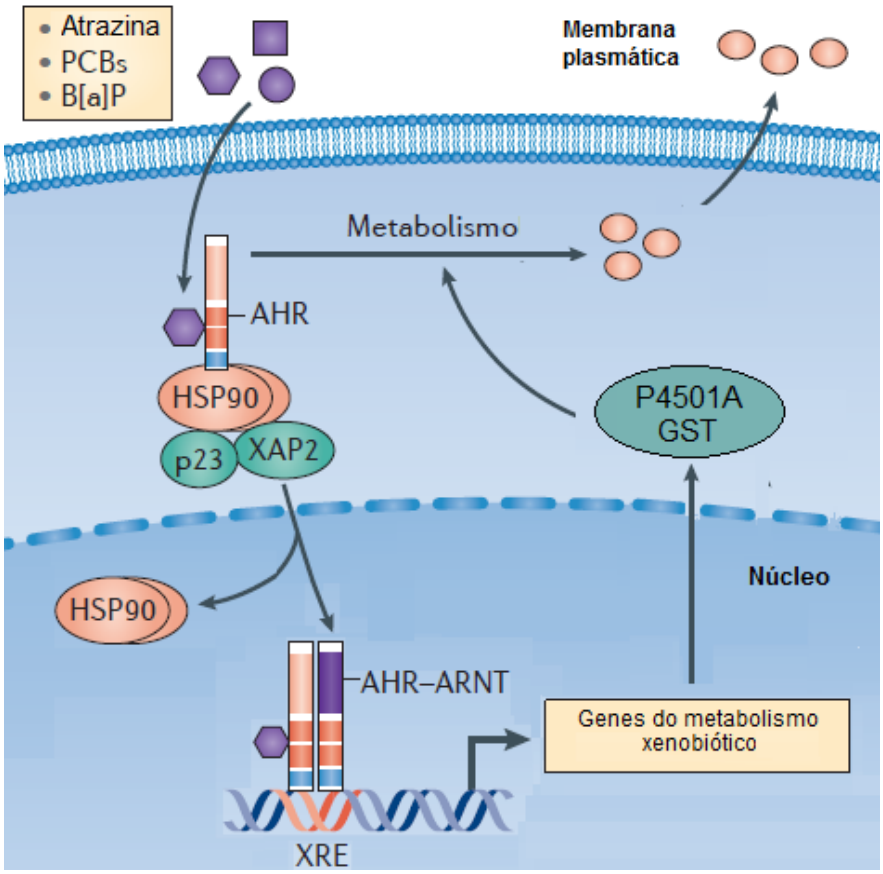


Figura 5. Ativação do AHR por ligantes. Vários ligantes exógenos (tais como benzo[a]pireno (BaP), bifenil policlorinado (PCB) e outros ligantes exógenos podem unir-se ao receptor citosólico de hidrocarbonetos aromáticos (AHR), estimulando assim a translocação para o núcleo, onde as chaperonas são trocadas por o translocador nuclear AHR (ARNT). O dímero AHR-ARNT liga-se a um elemento de resposta xenobiótica (XRE) em *cis* para induzir a transcrição de genes do metabolismo xenobiótico. Adaptado de Bersten *et al*., 2013.

A função adaptativa da AHR é bem conhecida e tem sido estudada há mais de 30 anos. As pesquisas mostraram a potência da 2,3,7,8-tetraclorodibenzo-p-dioxina (TCDD) para induzir efeitos bioquímicos (Poland e Glover, 1974), como a indução na atividade da hidroxilase de hidrocarboneto aromático (AHH) (conhecida por ser catalisada principalmente pelo CYP1A1), assim como estritas relações estrutura-atividade para este efeito (Poland e Glover, 1977; Goldstein *et al*., 1977) e as diferenças

de sensibilidade em ratos (Poland e Glover, 1974). Estas observações levaram à descoberta (Poland et al., 1976) da AHR como um "receptor de indução" que controla a expressão da atividade de AHH. Desde então, o mecanismo pelo qual a AHR regula a indução de enzimas adaptativas, tem sido estudado extensivamente (Whitlock, 1999). AHR é conhecida por reconhecer uma gama ampla de estruturas químicas, incluindo compostos não-aromáticos e não halogenados. Essa promiscuidade é compreensível no contexto dessa função adaptativa (Denison *et al*., 1998).

No meio aquático, os organismos continuamente expostos a xenobióticos desenvolvem estratégias defensivas contra substâncias tóxicas para sobreviver. A sensibilidade e indutibilidade de enzimas CYP e GST sob exposição de muitos contaminantes demonstram o papel vital da Fase I e II na biotransformação de xenobióticos (Ku *et al*., 2014) pelo que são usados como biomarcadores para avaliar a contaminação de um ambiente aquático (Uno *et al*., 2012; Li *et al*., 2008). No entanto, em caso das GST, é bom ter em conta que enquanto algumas classes de GST especificas detoxificam xenobióticos específicos, outro grupo de GST podem compartilhar especificidade por substratos, formando uma rede defensiva contra contaminantes ambientais, drogas antitumorais e produtos de estresse oxidativo (Hamilton *et al*., 2003; Lien *et al*., 2002). Deste modo, utilizar a atividade total de GST em peixes como um biomarcador para avaliar os riscos ambientais parece não viável, além que sua indução tem sido observada em um limitado número de peixes e com resultados conflitantes, onde os contaminantes podem causar indução assim como inibição enzimática (Tabela 2; Henson *et al*., 2001; Van der Oost *et al*., 1996; Gadagbui and Goksoyr, 1996; Burgeot *et al*., 1996). Assim, mais pesquisas sobre este parâmetro poderiam esclarecer isoenzimas específicas que têm uma resposta mais sensível e seletiva aos contaminantes (Van der Oost *et al*., 2003).

Tabela 2: Estudos em laboratório sobre respostas a contaminantes orgânicos traços da GST hepática na Fase II dos peixes.

Especie	Contaminante	GST	Referencia
Cyprinus carpio	PAH (BNF)	=	Riviere *et al*. (1990)
Gadus morhua	PCDD (2,3,7,8-TCDD)	=	Hektoen *et al*. (1994)
Carassius auratus	PCDDs and metals in sediment	+	Chen *et al*. (1998)
Anguilla anguilla	PAH (BNF)	=	Fenet *et al*. (1998)
Fundulus heteroclitus	PAH (BNF) or 2,3,7,8-TCDF	+	Bello *et al*. (2001)
Salmo trutta	Propiconazole	+	Egaas *et al*. (1999)
Clarias anguillaris	PCBs, OCPs	+	Gadagbui and Goksøyr (1996)
Pleuronectes platessa	PCB (Clophen A40)	+	Boon *et al*. (1992)
Salmo gairdneri ou Oncorhynchus mykiss	PCB (Clophen A50), PAH (BNF)	+	Andersson *et al*. (1985)
	PAH (BNF)	+	Celander *et al*. (1993)
	PAH, PCB in sediment extracts	=	Vigano *et al*. (1995)
	PAH (BNF)	=	Fenet *et al*. (1998)
	TCDD, PCB, DDE	+	Machala *et al*. (1998)
Dicentrarchus labrax	PAH (3MC)	-	Lemaire *et al*. (1996)
Sparus aurata	PCB (Arocolor 1254)	+	Pedrajas *et al*. (1995)
	OCP (deielderin), OPP (malathion)	-	Pedrajas *et al*. (1995)
Platichthys stellatus	PAH containing sediments	=	Collier *et al*. (1992)
Lepomis macrochirus	PAH (BaP)	-	Oikari and Jimenez (1992)
Zoarces viviparus	PAH (BNF)	=	Celander *et al*. (1994)

Símbolos e abreviaturas: -, inibição; =, resposta não significativa; +, indução; GST, glutationa *S*-transferase.

Com relação a pesquisas que estimaram a atividade de GST, com substrato CDNB, na biotransformação de ATZ, Abel *et al*. (2004) utilizando hGST das classes alfa, mu, pi e teta, obtido com a tecnologia de DNA recombinante, observou-se que apenas GSTP1-1 mostrou afinidade para conjugar ATZ. Neste mesmo estudo, GSTP acabou por ser a enzima responsável pela biotransformação ATZ-GSH dependente no fígado de rato. Embora a ATZ seja mostrada como substrato especifico para as classes GSTP, Eaton e Bammler (1999) referem que isoformas GST exibem única, mas

frequentemente sobrepostas, especificidade de substrato. Ou seja, embora CDNB seja um bom substrato para hGSTP1-1, é bom ter em consideração que CDNB também é metabolizada por muitos outros hGST. Abel et al. (2004) relata que, devido à especificidade inabitual de ATZ pela GSTP1-1, ATZ poderia ser utilizado como um substrato marcador para detectar a atividade da hGSTP1-1 em tecidos onde múltiplas isoformas GST são expressas.

3.5. Efeitos do herbicida ATZ no proceso reprodutivo

Resultados de muitas pesquisas descrevem tanto os efeitos como a ausência de efeitos em múltiplos níveis de organização biológica nos organismos aquáticos após a exposição a concentrações ambientalmente relevantes de ATZ (Rohr and McCoy, 2010; Solomon *et al*., 2008). A ambiguidade entre estes resultados levou à controvérsia quanto ao risco que a ATZ apresenta em relação à saúde das populações aquáticas. A falta de mecanismos claramente definidos para os efeitos da ATZ contribui para os debates na atualidade (Papoulias *et al*., 2014). Apesar destas incertezas, propõe-se as vias fisiológicas e bioquímicas, envolvendo receptores do sistema endócrino reprodutivo, como alvos da ATZ (Cooper *et al*., 2007; Hayes *et al*., 2011; Suzawa e Ingraham, 2008).

A ATZ afetaria o processo esteroidogênico, resultando em interrupção da função reprodutiva das gônadas do macho e fêmeas (Pogrmic-Majkic *et al*., 2016). Em peixes zebra juvenis, a exposição aguda 2,2 $\mu g.L^{-1}$ de ATZ aumentou a expressão do gene que codifica aromatase, um conhecido gene alvo do receptor nuclear SF-1 (NR5A1); além de incrementar a proporção de fêmeas para os machos quando expostos a 22 $\mu g.L^{-1}$ durante 6 meses (Suzawa e Ingraham, 2008). Em culturas primárias de células

intersticiais de Leydig de rato expostos a ATZ observou-se indução dose dependente de genes-chave envolvidas na esteroidogênese tais como a *StAR, CYP11A1, 3β-HSD*, entre outros (Abarikwu *et al*., 2011, Feyzi-Dehkhargani *et al*., 2012). Também, em linhas de cultura celular de mamíferos, a ATZ induziu alvos SF-1 e outros genes críticos para a síntese de esteroides e a reprodução, incluindo *CYP19A1, StAR, Cyp11A1, hCG, FSTL3, LHβ, INHα, αGSU, e 11β-HSD2* (Suzawa e Ingraham, 2008). Adicionalmente, em ratos machos, a ATZ reduziu a atividade da enzima esteroide 5α-redutase (SRD5A) e inibiu a ligação de 5 α-di-hidrotestosterona (DHT) ao receptor de androgénio (Babic-Gojmerac *et al*., 1989; Kniewald *et al*., 2000).

A fosfodiesterase (PDE), a enzima responsável da hidrólise do segundo mensageiro cAMP para 5-AMP, foi inibida pela ATZ e seus metabólitos, embora a inibição destes últimos fosse menor (Roberge *et al*., 2004). A atrazina aumentou rapidamente os níveis de cAMP em células da pituitária e células Leydig testicular em concentrações dose dependente. As alterações induzidas pela ATZ nos níveis de AMPc foram suficientes para estimular a libertação de prolactina nas células pituitárias e a produção de androgénio em células de Leydig, indicando que atua como um desregulador endócrino tanto em células que segregam hormônio pré-armazenadas por exocitose assim como em células que segregam hormônio novo-síntese (Kucka *et al*., 2012). Neste mesmo estudo determinaram que a ATZ atua como um desregulador endócrino geral por inibição da PDE4 (Figura 6).

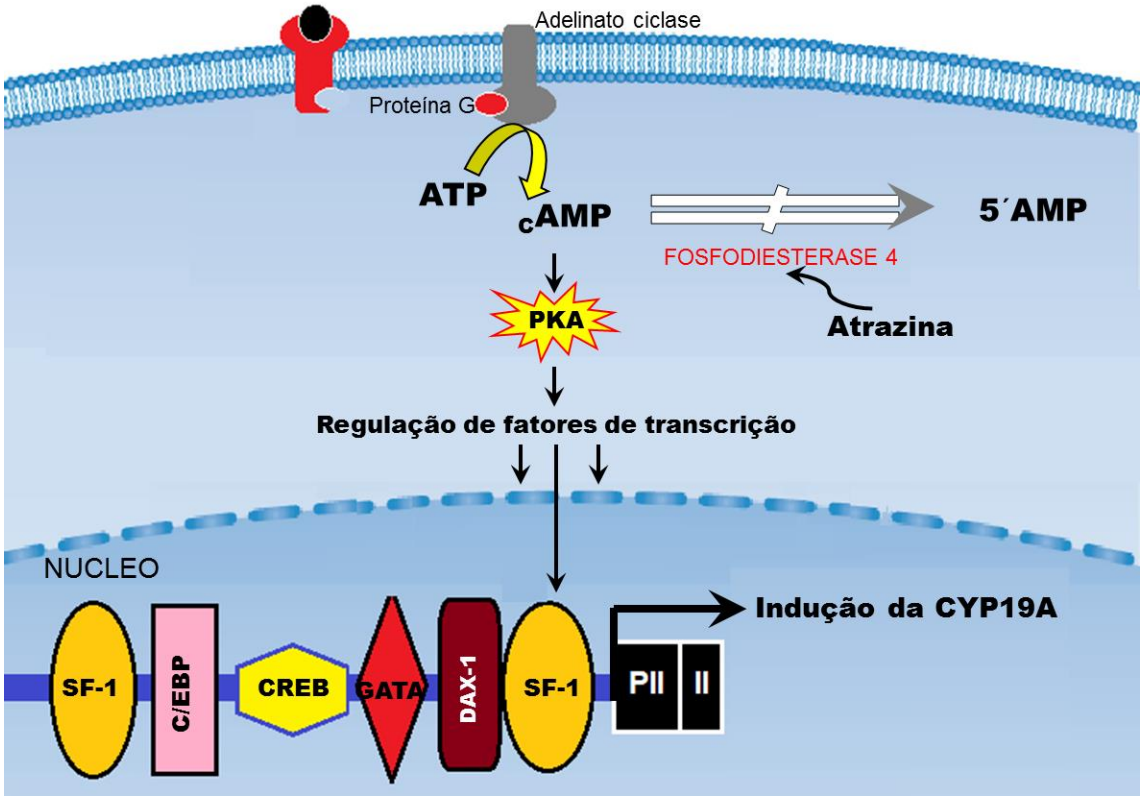


Figura 6. Atrazina inibe a fosfodiesterase 4. A proteína G estimula a adelinato ciclase para produzir grandes quantidades de AMPc a partir de ATP. A continuação AMPc une-se a proteína quinase A e ativaria o fator esteroidegênico 1 (SF-1). A ativação da SF1 dá origem à ligação com o promotor II que é a região reguladora responsável pela expressão da aromatase. Adaptado de Kucka *et al*., 2012.

Os xenobióticos que têm interferência sobre o sistema reprodutivo terminariam afetando a qualidade do espermatozoide (Neubert, 2002). Em coelhos machos, Yousef *et al*. (1995) observaram que a exposição crônica ao herbicida glifosato resultou em uma redução na concentração espermática, acompanhada pelo aumento de espermatozoides anormais ou mortos, e sugerindo-se que estes efeitos seriam devido a efeitos diretos sobre a espermatogênese e/ou efeitos indiretos através do eixo hipotálamo-hipófise-testículo. Em machos *Danio rerio*, a exposição do glifosato, em concentrações de uso comercial, induziu danos no DNA do esperma, na membrana

mitocondrial e na funcionalidade da mitocôndria, que resultaram na deterioração da funcionalidade celular em termos de motilidade e período de motilidade espermática (Lopes *et al*., 2014).

Um estudo focado em efeitos de ATZ sobre espermatozoides reportou aumento de anormalidades mitóticas em espermatogônias de medaka japonês (*Oryzias latipes*) expostos a 0,5; 5 e 50 $\mu g.L^{-1}$ de ATZ (Papoulias *et al*., 2014). Em *Poecilia vivipara*, a proporção de fêmeas em relação ao de machos aumentou pela exposição de 100 $\mu g.L^{-1}$ de ATZ. Nas fêmeas o crescimento folicular foi estimulado nos estágios de nascimento, dois e três meses de vida, e nos machos de três meses de idade foi reduzida a capacidade espermática; com diminuição no número de células espermáticas com DNA íntegro, células espermáticas com mitocôndrias funcionais e na integridade de membrana dos espermatozoides (Quintana, 2012). Além disso, em peixe fêmea *Pimephales promelas* e *Tautogolabrus adspersus*, além da diminuição da produção de ovos pela exposição de ATZ, surpreendeu a carência de respostas dose dependentes e a presença de efeitos a baixa dose de exposição (Mills, 2006; Tillitt *et al*., 2010).

Para descobrir novos caminhos explicativos da diminuição do sucesso reprodutivo ou infertilidade, investigações tem procurado identificar genes que codificam enzimas-chave da espermatogénese ou aqueles que determinam características básicas do sêmen. Hering *et al*. (2014), utilizando polimorfismo de nucleotídeo simples, identificou-se que, entre outros genes, a esteróide-5α redutase α-polipeptídio 2 (*SRD5A2*), o regulador da condutância transmembranar da fibrose cística (*CFTR*) e o fator de crescimento insulina like (*IGF1*) associavam-se significativamente com a mobilidade e a bioquímica do esperma. O papel das enzimas codificadas por estes genes demonstraria a sua importância na espermatogênese. O SRD5A2 converte a testosterona em

dihidrotestosterona, o principal andrógeno envolvido na maturação do esperma (Wilson *et al*., 1993) e manutenção da espermatogénese (Moore and Akhondi, 1996). O polipeptídio CFTR é altamente expresso na cabeça e cauda do esperma; e estaria ligado a funções de fertilização e capacitação espermática (Xu *et al*., 2007 and Hernandez-Gonzalez *et al*., 2007). O IGF1 estimula a esteroidegênese aumentando os receptores de gonadotropina, a expressão de enzimas esteroidegênicas (Lin, 1995; Lejeune *et al*., 1996, Saez, 1994) e atuando como fator de desenvolvimento e diferenciação das espermatogônias, espermatócitos e espermatídeos (Kinner, 1992; Spiteri-Greech and Nieschlag, 1992; Tajima *et al*., 1995).

3.6. Efeitos oxidativos do herbicida ATZ

As espécies reativas de oxigênio (ERO) produzidas na respiração e nas vias enzimáticas intracelulares como a NADPH oxidase e xantina citoplasmática podem interagir com substâncias orgânicas (Rocha *et al*., 2003). Quando as ERO superam a capacidade de tamponamento da célula, a célula entra então no estresse oxidativo, potencialmente levando a danos de DNA/RNA, proteínas e lipídeos. Existem várias adaptações celulares que neutralizam estes efeitos negativos do estresse oxidativo, tais como um tampão de redução de tiol composto por GSH e tiorredoxina e enzimas para remover espécies de oxigénio reduzidas, tais como catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPX) (Hagedorn *et al*., 2012). A SOD, uma das primeiras enzimas de defesa celular antioxidante, converte o ânion superóxido reativo (O_2^{-}) em peróxido de hidrogénio (H_2O_2). Subsequentemente, H_2O_2 é convertida em água pela ação catalítica da GPX e a oxidação concomitante da GSH, um dos mais

importantes antioxidantes não enzimáticos da célula (Van der Oost *et al*., 2003) (Figura 7).

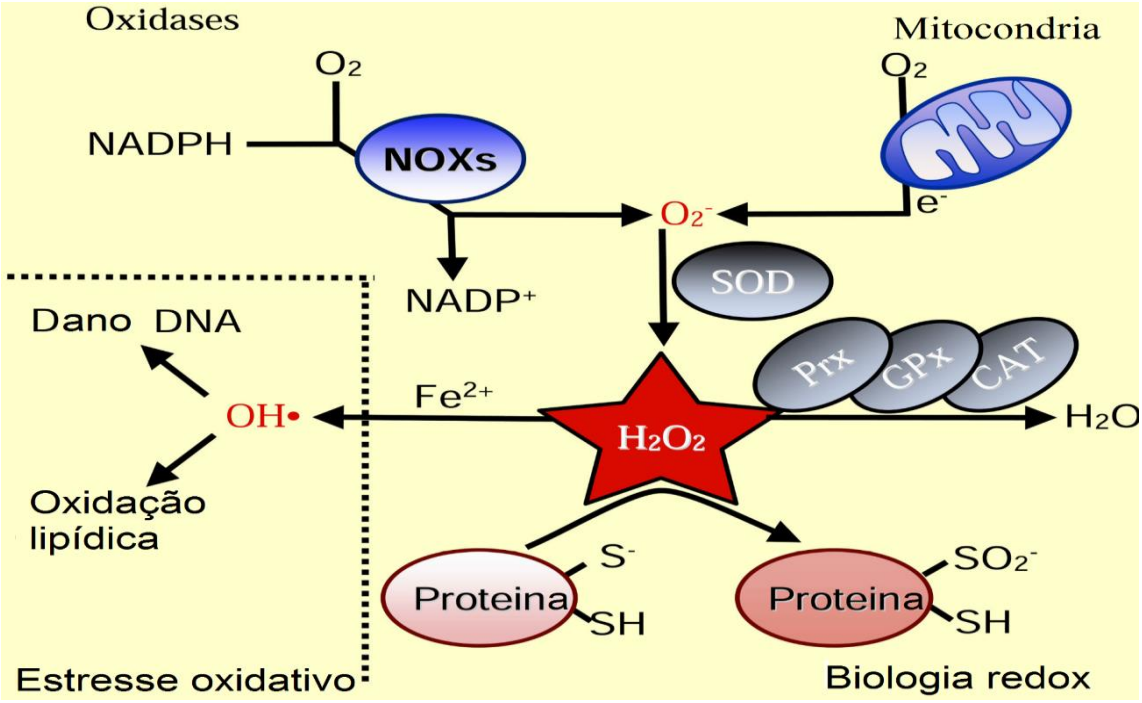


Figura 7. O superóxido intracelular (O_2^-) é produzido principalmente a partir da oxidação do NADPH pelas enzimas oxidase (NOX) ou pelo vazamento de elétrons da respiração aeróbia nas mitocôndrias. O superóxido é rapidamente convertido em peróxido de hidrogênio (H_2O_2) por superóxido dismutase (SODs) específico do compartimento. O H_2O_2 é capaz de oxidar os resíduos de cisteína nas proteínas para iniciar a sinalização redox. Alternativamente, o H_2O_2 pode ser convertido em H_2O por proteínas antioxidantes celulares, tais como peroxirredoxinas (PRx), glutationa peroxidase (GPx) e catalase (CAT). Quando os níveis de H_2O_2 aumentam incontrolavelmente, os radicais hidroxilo (OH) se formam através de reações com cátions metálicos (Fe^{2+}) e danificam irreversivelmente macromoléculas celulares (Schieber e Chandel, 2014)

Estudos em peixes sugerem que a exposição de ATZ induziria ao estresse oxidativo celular e influiria na atividade de enzimas antioxidantes. As concentrações nominais de ATZ (3, 6 e 9 $mg.L^{-1}$) na água aumentou a atividade da SOD e o conteúdo de GSH no fígado de *Lepomis macrochirus* após de 96 h de exposição (Elia *et al*.,

2002). Em peixe zebra *Danio rerio* após exposição com 1, 10, 100 e 1000 $\mu g.L^{-1}$ de ATZ durante 14 dias, a expressão dos genes *SOD, CAT, GP*X e a atividade das enzimas SOD e CAT foram aumentadas (Jin *et al*., 2010). O conteúdo aumentado de malonaldeído (MDA) nos grupos expostos a ATZ refletiriam peroxidacões significativas, principalmente, dos ácidos graxos poli-insaturados da membrana celular (Jin *et al*., 2010). Peixes *Channa punctatus*, após ser expostos durante 15 dias a concentrações subletais de ATZ (1/4 LC_{50}: 10,6 $mg.L^{-1}$, 1/8 LC_{50}: 5,3 $mg.L^{-1}$ e 1/10 LC_{50}: 4,2 $mg.L^{-1}$) mostraram níveis aumentados de MDA e respostas positivas dose dependente da SOD, CAT e glutationa redutase no fígado, pelo que sugerem o uso desses antioxidantes como biomarcadores potenciais de toxicidade associada à exposição de contaminantes em peixes de água doce (Nwani *et al*., 2010).

As atividades aumentadas de enzimas antioxidantes seriam efeito do estresse oxidativo gerado pela ATZ. Os ERO ativariam fatores de transcrição redox sensível tais como NF-κB, AP-1, fator nuclear de células T ativadas e fator indutível de hipoxia 1 (Posen *et al*., 2005; Kaur *et al*., 2006) e induzir atividades enzimáticas (Lopez *et al*., 2000). Uma alta proporção GSH/GSSG é importante para a proteção da célula contra danos oxidativos. A disrupção desta razão pode ativar o NF-κB, por exemplo. Em condições normais NF-κB é mantido inativo pela ligação da sua subunidade inibitória IκB. No entanto, sob condições de estresse, IκB torna-se fosforilada e dissocia-se do NF-κB, que, em seguida, transloca-se para o núcleo e ativa a expressão de genes (Burton *et al*., 2011). NF-κB pode ser ativado em resposta a condições de estresse oxidativo, tais como ERO, radicais livres e irradiação UV (Pande e Ramos, 2005), resultando na ativação de vários genes relacionados à defesa antioxidante (Birben *et al*., 2012).

O estresse oxidativo também pode levar a modificações de DNA. Isto pode acontecer através da degradação de bases, quebras de DNA simples ou dupla, modificações, mutações, deleções ou translocações de purina, pirimidina ou açúcar, e reticulação com proteínas (Birben *et al*., 2012). No entanto, as células desenvolveram mecanismos enzimáticos de reparação de DNA, que reduzem estas mutações e aberrações cromossômicas (Powell *et al*., 2005, Robson *et al*., 1991). Este complexo sistema de reparação pode operar (a) atenuando o ciclo celular que permite o reparo de DNA; (b) ativando eventos de transdução de sinal dos componentes de reparo; e (c) revertendo e excisando danos de DNA através de atividades constitutivas e induzidas (Begley e Samson, 2004). O sistema de reparo de nucleotídeo excisado (NER) é uma destas vias de reparo que é ativado em resposta ao estresse oxidativo, sendo uma de suas enzimas essenciais o fator de reconhecimento e reparação de DNA (XPC). Esta enzima ativa os processos de reparação formando um complexo que inicia o recrutamento de todo o aparelho proteico para a reparação de lesões do DNA (Hoeijmakers, 2001; Nemzow *et al*., 2015).

Ao nível das gônadas, a pouca vascularização deste tecido indica um fornecimento inferior, mas uma intensa competição por oxigênio (Free *et al*., 1976). Uma vez que tanto a espermatogênese (Peltola *et al*., 1994) como a esteroidogênese das células de Leydig (Quinn e Payne, 1984) são vulneráveis ao estresse oxidativo, o menor fornecimento de oxigénio e sua matriz elaborada de enzimas antioxidantes e captadores da ERO garantiria que este órgão não seja afetado pelo estresse oxidativo (Chen *et al*., 2005).

No entanto, a gônada continua vulnerável ao estresse oxidativo tanto pela abundância de ácidos graxos altamente insaturados (em particular, 20:4 e 22:6) como

pela presença de potenciais sistemas geradores da ERO nas células de Sertoli e de Leydig (Hagedorn *et al*., 2012). Nestas células, espera-se que a produção de ERO seja particularmente alta porque, além da cadeia de transporte de elétrons mitocondrial, os ERO também são produzidos como subprodutos de hidroxilações de esteróides pelas enzimas CYP (Hornsby, 1989; Peltola *et al*. 1996). Estudos com cultura de células de Leydig realizados por Quinn e Payne (1985) e Diemer *et al*. (2003) demonstraram que os ERO têm efeitos prejudiciais sobre componentes críticos da via esteroidogénica como as enzimas CYP e StAR.

Um aumento de ERO tem sido associado a espermatozoides anormais ou danificados (Aitken *et al*., 1989; Aitken *et al*., 1994; Rao *et al*., 1989; Iwasaki e Gagnon, 1992; Ball e Vo, 2001), e isso pode ser especialmente verdadeiro em peixes. Por exemplo, a duraquinona, uma substancia química utilizada em nanotecnologia, induziu ERO em espermatozoides de carpa que causaram danos ao DNA no esperma e subsequentemente prejudicaram o sucesso reprodutivo (Zhou *et al*., 2006). A geração de ERO *in vitro* pelo sistema xantina-xantina oxidase reduziu a motilidade espermática (de Lamirande e Gagnon, 1992; Aitken *et al*., 1993; Baumber *et al*., 2001), a viabilidade (Baiardi *et al*., 1997) e aumento da peroxidacão lipídica da membrana a fusão de espermatócito (Aitken *et al*., 1989; Aitken *et al*., 1993; Storey, 1997). O principal responsável destes detrimentos espermáticos *in vitro* seria o peróxido de hidrogênio, apoiado na sua menor polaridade e facilidade para atravessar a membrana plasmática (Halliwell, 1991). Os espermatozoides são células terminalmente diferenciadas que possuem mecanismos de reparo inaccessíveis e limitados (Aitken e Clarkson, 1987; van Loon *et al*., 1991). Como conseqüência, a membrana plasmática perde a fluidez e a integridade que requer para a participação nos eventos de fusão de membrana associada à fecundação (Storey, 1997; Ohyashiki *et al*., 1988; Block, 1991).

O estudo da capacidade de biotransformar ATZ através da estimativa da atividade GST por diferentes enfoques podem ser de ajuda na identificação de estratégias adaptativas contra os xenobióticos desenvolvidas em espécies e condições ambientais diferentes; além de elucidar a GST como biomarcador ambiental para ATZ. Por outro lado, embora os resultados de pesquisas com exposição de ATZ indiquem efeitos nocivos no organismo, principalmente na reprodução, o mecanismo como a ATZ poderia estar afetando estas vias não estão claras. Assim, com a finalidade de ajudar no esclarecimento desses questionamentos, os seguintes objetivos foram planejados:

4. OBJETIVOS

4.1. Objetivo Geral

Avaliar aspectos da biotransformação de atrazina via GST e seus efeitos sobre parâmetros espermáticos e expressão de genes.

4.2. Objetivos Específicos

4.2.1. Estimar e comparar a atividade de GST de camarão e peixes de ambiente contaminado e cativeiro em ensaio cinético utilizando substrato CDNB.

4.2.2. Estimar e comparar a atividade de GST de camarão e peixes de ambiente contaminado e cativeiro em ensaio cinético competitivo entre substratos CDNB e ATZ.

4.2.3. Estimar e comparar a atividade de GST de camarão e peixes de ambiente contaminado e cativeiro em ensaio cinético por decaimento da ATZ.

4.2.4. Avaliar efeitos da exposição de atrazina em parâmetros de qualidade espermática em *Danio rerio*.

4.2.5. Avaliar efeitos da exposição de atrazina na expressão de genes envolvidos na espermatogênese e proteção celular em *Danio rerio*.

5. MANUSCRITO 1

Manuscrito a ser submetido para a revista Aquatic Toxicology.

GST enzymatic assays to estimate atrazine biotransformation in *Litopenaeus vannamei* and *Poecilia vivipara*

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Number of Tables: 1

Number of Figures: 3

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5.1. Abstract

Atrazine (ATZ), an herbicide used for the control of harmful weeds in agriculture, is often questioned because of its ubiquity in the environment and by signs of negative effects on reproduction and animal behavior. ATZ is detoxified through Phase I and II xenobiotics biotransformation pathway, being the cytochrome P450 (CYP) and glutathione *S*-transferase (GST), the main catalytic enzymes, respectively. In organisms living in contaminated aquatic environments, the activity of these enzymes would be induced, and thus used as environmental biomarkers. Thus, the aim of this study was to estimate the biotransformation of ATZ by GST in shrimp and fish in captivity (control) and contaminated environment using three approaches: kinetic assay of GST using substrate CDNB, competitive kinetic assay using CDNB and ATZ, and kinetic assay of ATZ decay analysed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cytosolic extract was obtained from gill and hepatopancreas of *Litopenaeus vannamei* and gills and liver of *Poecilia vivipara* in captivity and contaminated environment. Kinetic assays of varying concentrations of CDNB and ATZ were perform to develop *Lineweaver-Burk* plots and infer whether inhibition caused by ATZ on GST was of the competitive type. Basal activities estimated by CDNB suggest a greater detoxification capacity by GST in fish than in shrimp, in liver that in gills and in fish of contaminated environment than from captivity. Similar patterns were estimated when ATZ decay was analysed by LC-MS/MS. However, the capacity of biotransforming ATZ estimated from competitive kinetic assays suggests greater GST activity with affinity for ATZ in shrimp than in fish and in fish in captivity than in contaminated environment. Assays to determine basal GST activity and decay ATZ would be the approaches that represent the real capacity of biotransformation in the evaluated groups. The conflicting results of competitive kinetic assays would be due to the non-specificity of CDNB metabolized by many GST isoforms and that would mask ATZ conjugations by specific isoforms such as GSTP.

Keywords: atrazine; GST; CDNB; fish

5.2. Introduction

Atrazine (ATZ) is an herbicide used in the control of harmful weeds in agricultural crops, but its low organic degradation and omnipresence in surface water and groundwater has become a threat to the ecosystem (Brodeur et al., 2013; Jeon et al., 2013). Despite being considered low toxic, ATZ would affect reproductive and developmental processes of many species (Stevens and Sumner, 1991; Cooper et al., 2007, Papoulias et al., 2014). In mammals, studies on their biotransformation report monoalkylateds and mercapturates as the main metabolites of their detoxification in Phase I and II of biotransformation. Cytochrome P450 (CYP) would be major enzymes of Phase I catalyzing dealkylating reactions of ATZ and producing deethylatrazine and deisopropylatrazine as the main metabolic intermediates (Lang et al., 1996; Hanioka et al., 1999; Joo et al., 2010). In Phase II, glutathione *S*-transferase (GST) would be the main group of enzymes to biotransform ATZ. GST catalyzes the nucleophilic binding of glutathione (GSH) to ATZ through its electrophilic atom (Hayes et al., 2005), forming deisopropylatrazine mercapturate, deethylatrazine mercapturate and atrazine mercapturate as main conjugates (Buchholz et al., 1999; Jaeger et al., 1998).

From the 60s, after the discovery of mercapturic acid, GSH and enzymes necessary to form this conjugate, models were developed to study the enzymatic activity of GST (Hodgson, 2010). The best known of these study models is cinetic assay using 1-chloro-2,4-dinitrobenzene (CDNB), a relatively good substrate for interacting with several GST isoforms (Habig and Jakoby, 1981) and estimating basal activities of GST (Ensibi et al., 2013). However, investigations using CDNB substrate model showed no altered or slightly altered GST activity in presence of specific xenobiotics (Sturve et al., 2005; Trute et al., 2007). These results possibly would be due to broad specificity for

CDNB conjugation of many GST isoforms, with the exception of omega GST, which would mask conjugations of GST isoforms with specific affinity for specific xenobiotics. Thus, the greater amount of GST conjugating CDNB would often overestimate the specificity of substrates (Eaton and Bammler, 1999).

In aquatic organism, intracellular induction of GST activity is an important cellular mechanism for the removal of xenobiotics, including pesticides, polychlorinated biphenyls (PCB), metals and polycyclic aromatic hydrocarbons (PAH) (Bastos et al., 2013). These contaminants would activate ligand-activated transcription factors such as the aryl hydrocarbon receptor (AHR) that would induce the expression of biotransformation enzyme genes such as *CYP1A* and *GST* (Uno et al., 2012; Van Tiem e Di Giulio, 2011). Among GST isoforms expressed, GSTP would be the highly effective isoform to metabolize PAH (Hu et al., 1997; Nahrgang et al., 2009). Significant alteration of certain isoforms in presence of specific xenobiotics has led to the estimation of isoenzymatic activity as good environmental biomarkers (Arockiaraj et al., 2014; Li et al., 2015). With this approach, and using hGST alpha (A), mu (M), pi (P), and theta (T) cytosolic isoforms, it was estimated that GSTP would be the isoform responsible for biotransformation of ATZ-dependent GSH, being suggested as a useful marker substrate for detecting GSTP activity in tissues with multiple expressed GST isoforms (Abel et al., 2004).

The use of biomarkers in aquatic organisms is considered an effective strategy to obtain information about state of the aquatic environment and the effect of contaminats on living resources (Wu et al., 2014). For this purpose, fish are frequently used in this type of studies due to it play important functions in the food chain, accumulate toxic substances and respond to low concentrations of mutagens (Ensibi et

al., 2013). In South America, *Poecilia vivipara*, characterized by living in contaminated environments with low oxygen content (Chivittz et al., 2016), arise as a potential candidate to be used (Ferreira et al., 2012).

In recent years, shrimp *Litopennaeus vannamei*, a tropical species widely used in aquaculture, has been seriously affected by environmental pollution (Bachère et al., 2000). Although several studies concerning to effects of environmental contaminants have been developed in this shrimp, many aspects this effects on crustaceans, particularly gills and hepatopancreas, remain unclear (Ren et al., 2015).

Investigations of xenobiotics biotransformation carried out in *in vitro* assays have for the purpose of defining the types and mechanisms of reaction and the characteristics of enzymes responsible (Fitzsimmons et al., 2007). Using Phase II biotransformation detoxification enzymes as xenobiotic exposure biomarkers has gained credential for the monitoring of aquatic environment contamination (Ensibi et al., 2013, Ren et al., 2015). Reseach *in vitro* of GST activity in different organs, species and environment may reflect differences in the biotransformation capacity of xenobiotics. Thus, the aim of the present study was to estimate the capacity of GST to biotransform ATZ in gills and hepatic tissue of shrimp *Litopenaeus vannamei* and fish *Poecilia vivipara* in captivity (control) and contaminated environment, through three approaches: kinetic assay of GST using CDNB as substrate, competitive kinetic assay using CDNB and ATZ and kinetic assay by ATZ decay analyzed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS).

5.3. Materials and methods

5.3.1. Sample collection and preparation

Specimens of *L. vannamei* (n: 4, length: 9.0 ± 0.5 cm) and F1 of *P. vivipara* in captivity (n: 15; length: 3.5 ± 0.5 cm), of parents captured in streams located one km to north of the residential area of Cassino beach (Rio Grande, RS, Brazil), were obtained from the laboratory of the Institute of Biological Sciences - FURG; while another group of *P. vivipara* (n: 15; length: 4.7 ± 0.7 cm) were collected from artificial channel that crosses Rio Grande city (RS, Brazil). Chivittz et al. (2016), considers the first and second environment of fish collection as low and high environmental pollution (S2 and S4, in that article). PAH content in sediment of both capture sites confirm wide contamination difference: 66.28 and 3913.97 $ng.g^{-1}$ dry matter, respectively (Chivittz et al., 2016). After capture, shrimps and fishes were anesthetized in a 150 $mg.L^{-1}$ tricaine solution (Sigma, St Louis, MO, USA) for 2.5 min and euthanized by cervical transection. Immediately, samples of gills, hepatopancreas and hepatic tissue were collected and maintained in an icebox. The Ethics Committee on Animal Use (CEUA N ° Pq013/2016 – FURG) approved these procedures. Gill and hepatopancreas of shrimp were homogenized (1:4 w/v) in buffer solution containing 0.5M sucrose, 20 mM Tris-HCl, 1mM disodium EDTA, 1 mM dithiothreitol, 0.15 M KCl and pH 7.6; while fish homogenization buffer contained 50 mM Tris-HCl, 1 mM EDTA and pH 7.6. Next, homogenized samples were centrifuged for 20 min at 20000xg and 4°C. The resulting supernatant or cytosolic fraction was separated into aliquots and stored at -80°C for subsequent analysis of GST and proteins. The following kinetic assay reagents were prepared: reaction buffer 0.1M (KH_2PO_4: 3.402g; K_2HPO_4: 4.355g; ultrapure water: 0.5 L; pH: 7.0), CDNB solution 0.12M (CDNB: 0.0243g; ethanol: 1 mL) and GSH solution 0.1 M (GSH: 0.0307g; reaction buffer: 1 mL).

5.3.2. Kinetic assays of GST using CDNB as substrate

Kinetic assays were performed based on that described by Habig and Jakoby (1981). Briefly, kinetic assay consisted in adding 975 µL of reaction buffer 0.1M (30°C), 10 µL CDNB 0.12M, 10 µL GSH 0.1M, and 5 µL cytosolic extract in a 1 mL quartz cuvette. Each assay was performed in triplicate. Immediately, cuvette content was homogenized by inverting five times and reading of absorbance performed in spectrophotometer (Biomate 3 thermo) at a wavelength of 340nm for three min. According to the following reaction catalyzed by GST: GSH + CDNB = GS-DNB + HCl; GS-DNB conjugate has a strong absorption at 340 nm with molar extinction coefficient (ε) = 9600 $M^{-1}.cm^{-1}$. Its absorption in spectrophotometer is directly proportional at sample activity (Habig and Jakoby, 1981). The absorbance was determined per minute (Δ ABS/min) and enzyme activity calculated (GST/mg protein) with following equation (Monserrat et al., 2006): (Δ ABS (average) * sample dilution) / (9.6 * sample volume (mL) * sample protein concentration ($mg.mL^{-1}$)). Protein concentration of samples, necessary to estimate GST activity was determined using biuret based method for total protein (Total proteins; Labtest Kit, Minas Gerais, Brasil). GST activity was analyzed by two-way analysis of variance (shrimp and fish, gills and hepatic tissue), followed comparing means of groups with Tukey's multiple comparison test with a level of significance of 5%.

5.3.3. Kinetic assays to infer inhibition type of ATZ on GST

Kinetic assays with CDNB and ATZ were performed to develop *Lineweaver Burk* graphics and confer the inhibitory effect caused by ATZ on GST. For that, nine different concentrations of CDNB (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.09, 0.12, 0.15M) and ten concentrations of ATZ (purity> 99%; Sigma-Aldrich, USA) (13.9, 27.8, 41.7,

55.6, 69.5, 83.5, 97.4, 111.3 μM) dissolved in dimethyl sulfoxide (DMSO) were prepared to determine inhibition concentrations ATZ (i.e.: IC_{20}). IC_{20} is a concentration of ATZ that would decrease 20% of GST activity. Preliminary enzymatic assays with concentrations of environmental relevance (≤ 4.64 μM ATZ) did not modify GST activity, while levels greater than 139 μM ATZ did not allow detecting GS-DNB conjugates across wavelength used. Kinetic assay consisted of the addition of 965 μL reaction buffer 0.1M (30°C), 10 μL CDNB (concentration range 0.01 to 0.15M), 10 μL GSH 0.1M, 10 μL ATZ IC_{20} and 5 μL cytosolic extract. Control kinetic assay had the same components, with ATZ exception. The absorbance reading was perform similarly to item 5.3.2. *Lineweaver-Burk* graph was developed using statistical program Prism 6.01 (GraphPad Software, Inc., USA) to visualize the intersection of GST activity with and without the presence of ATZ and determine parameters Vmáx and $\boldsymbol{K_M}$. This method, linearizes equation of Michaelis-Menten in form $1/\boldsymbol{V_o}$ and 1/[S], where the slope of the line is $\boldsymbol{K_M}/\boldsymbol{V_{max}}$ and intersection in axis $1/\boldsymbol{V_o}$ is $1/\boldsymbol{V_{max}}$; while that intersection in axis 1/[S] of the extrapolated line is $1/\boldsymbol{K_M}$ (Voet and Voet, 2013).

5.3.4. Competitive kinetic assays using CDNB and ATZ substrates

This approach utilized CDNB and ATZ as competitive substrates to conjugate with GSH by GST action. These assays consisted of addition of 965 μL reaction buffer 0.1M (30°C), 10 μL CDNB 0.12M, 10 μL GSH 0.1M, 10 μL ATZ (concentration range 13.9 to 113 μM) and 5 μL of cytosolic extract in a 1mL quartz cuvette. Each trial was performed in triplicate. Control kinetic assay had the same components, except ATZ. The absorbance reading was perform similarly to item 5.3.2. Inhibitory effect of ATZ on GST activity was estimate with two equations: 1) GST activity = (initial rate of

reaction inhibitory / initial rate of reaction not inhibitory) * 100; 2) GST activity inhibition = 100 - GST activity.

5.3.5. Kinetics of ATZ decay analysed by LC-MS/MS

For these kinetic assays, ATZ was used as a conjugative substrate of GST. Concentrations lower than 4.6 µM ATZ showed no visible decay of ATZ after three minutes of enzyme activity. The kinetic assays were performed as follows: in eppendorf tubes of 1.5mL capacity was placed 960µL reaction buffer 0.1M (30°C), 10µL GSH 0.1M, 10µL ATZ 4.6µM and 10µL cytosolic extract. Then eppendorf content was homogenised by inverting five times and incubated at 30°C in three time intervals (0, 1 and 3 min). Completed incubation period, eppendorf was placed in a water bath at 80°C, during three minutes, in order to denature the enzymes and stop the reaction. Next, eppendorfs content were filtered with polyethersulfone syringe material for subsequent determination of unconjugated ATZ by LC-MS/MS of accordance with working conditions of Demoliner et al. (2010).

5.4. Results

Statistical differences in basal activity of GST between groups was found (Fig. 1; $p<0.05$). Comparing species, GST activity was higher in fish than in shrimp; while at organ level, in fish, this activity was higher in liver (with tendency for higher activity in contaminated environment fish) relative to the gills. In shrimp, GST activity in gills showed a tendency of greater activity in relation to hepatopancreas. Considering contamination factor, GST activity was higher in fish of contaminated environment than in captivity.

Results of Lineweaver-Burk plot, show changes in inclination of line corresponding to GST activity in presence of ATZ (IC_{20}). These changes of inclination

tend to intercept in Y-axis with line representing GST activity of control group both in gills (Fig. 2A-C) as in hepatopancreas or liver (Fig. 2D-F). Intercepts Y and X, when X and Y are zero, respectively, allow to determine kinetic parameters shown in Table 1, where it is visualized that ATZ would tend to modify the $\boldsymbol{K}_M$ of GST activity in gills and tissue hepatic. Linear model used for estimation of these kinetic parameters was predictive (R^2 close to 1) and significant ($p < 0.05$; Table 1).

Competitive kinetic assays show inhibitory effect of ATZ on GST activity (Fig. 3). The percentage of GST activity inhibition, taking as reference the concentration of ATZ that inhibits 20% of GST activity (IC_{20}), was lower in shrimp than in fish, and in fish in captivity than in fish of contaminated environment. The results of this group of assays showed a behavior of enzymatic biotransformation contradictory to the expected logic.

Kinetic assays monitoring the levels of ATZ after first minute of incubation with citosolic enzymes showed rapid ATZ decay (Fig. 4). ATZ decayed faster in fish than in shrimp, (with exception in gills of captivity fish). Among organs, the ATZ decay varied between 10 and 29% in gills, which was lower than the decay observed in the hepatopancreas - liver (between 12 and 40%). Comparing the contamination factor, the ATZ decay was higher in contaminated environment fish than in captivity. From the first to the third minute of enzymatic activity, ATZ decay in all groups compared was low (0-6%), except for gills in captivity fish, which showed a decrease of ATZ by 9%.

5.5. Discussion

Specific enzymatic reactions of metabolic pathways present in a tissue may be manifest in *in vitro* assays. Also, inoculation of a contaminant in this assays can induce enzymes responsible for its metabolism that helps to deduce the activation or inhibition

of detoxification mechanisms and to estimate the biotransformation capacity of xenobiotics. Thus, the present study estimated and compared GST capacity for biotransforming ATZ in gills and hepatic tissue from different species and environment.

The increased basal GST activity and the rapid decay of ATZ after the first minute of cytosolic enzyme activity suggest that fish would possess greater capacity to biotransform ATZ compared to shrimp. Among species, the activity of metabolic pathways are frequently different. Also, the metabolic activity for a single substrate can vary widely, even within a same species (Wang et al., 2001). GST expression in aquatic animals does not always follow the same pattern and varies with species, tissue, sex and age (Chiou et al., 1997, Hayes and Pulford, 1995). The structure, numbers and aminoacid position of the substrate binding sites vary in different species and forms of GST isoform (Hu et al., 2012; Ji et al., 1997). These modifications of aminoacids of the substrate binding site could lead to decreased affinity and greater flexibility for GST (Hu et al., 2012). These differences can determine the relative sensitivity of a particular species to a given contaminant and represent an uncertainty in efforts to regulate specific chemicals in the environment (Fitzsimmons et al., 2007). Livingstone (1994) estimated, using regression equations, biotransformation rates for BaP tenfold greater in fish (*Cyprinus carpio*, *Lepomis macrochirus*, *Opsanus beta*, *Parophrys vetulus*) than in crustaceans (*Callinectes sapidus, Eohaustorius washingtonianus, Panulirus platyceros, P. argus, Rhepoxynius abronius*).

In fish, increased biotransformation capacity of the liver estimated from kinetic assays with CDNB and by decay of ATZ indicates the great importance of this organ in the detoxification of xenobiotics. In fish, biotransformation enzymes of Phase I and II xenobiotics are mainly distributed in the liver, although it also include the gut, kidneys,

and to a lesser extent gills and the olfactory system (Matsuo et al., 2008). Therefore, this organ is highly recommended in the study of environmental indicators of water contamination (Gül et al., 2004, Cárcamo et al., 2017). However, metabolic comparisons between tissues should be performed with caution. Anatomic relationships between tissues may be an important factor that determines the main route of contaminant exposure (Fitzsimmons et al., 2007). Gill tissue metabolism may limit the absorption of chemicals taken from water (Barron et al., 1989), while biotransformation within the gastrointestinal tract may limit the oral bioavailability of compounds extracted from foods (Van Veld et al., 1988). In either case, presystemic metabolism reduces the amount of the chemical eventually delivered to the liver (Fitzsimmons et al., 2007).

In shrimp, basal GST activity in gill showed trends higher capacity of biotransformation compared to hepatopancreas, suggesting higher gill enzyme activity performing tasks detoxification. Zhou et al. (2009) also reported tendencies of higher basal GST activity in gills than in hepatopancreas of *L. vannamei*. The ATZ decay assays suggests similar impacts of both organs on the metabolism of xenobiotics. While the liver of vertebrates is an organ with specialized functions, the hepatopancreas of crustacean perform more extensive functions (hepatic, pancreatic and intestinal) (Yepiz-Plascencia et al., 2000), so the gills would perform important detoxification tasks that would compensate the many functions developed in hepatopancreas.

Biochemical biomarkers are increasingly being used in ecological risk assessments of aquatic ecosystems to identify incidence of exposure and effects caused by xenobiotics (Wu et al., 2014). Its use has gained in popularity because of its potential as early warning systems of harmful effects on organism (McCarthy and Shugart,

1990). Within a group of enzymes, GST has been indicated as an appropriate biomarker for a great variety of contaminants (Ren et al., 2015, Wu et al., 2014, Carcamo et al., 2017). Its induced activity can be studied as a biomarker of susceptibility related to genetic factors and/or receptors that would indicate an innate or acquired ability of an organism against a contaminant (Van der Oost et al., 2003). The increase basal GST activity and faster decay of ATZ detected by LC-MS/MS in gills and liver of contaminated environment fish compared to those of captivity suggest that fish living in contaminated environment would have activated this mechanism of susceptibility. Along with enzyme inductions, the degree of enzymatic biotransformation would be an important adaptive mechanisms that modulate bioaccumulation, persistence, residue dynamics and toxicity of a chemical in aquatic organisms (Livingstone, 1998).

Sancho et al. (2009) argues that differences in activities of enzymes that metabolize xenobiotics in fish may be used to explain the difference in toxicity. An increased sensitivity to xenobiotic toxicity may be due to low abundance of biotransformation enzymes such as CYP or GST (Küster and Altenburger, 2007). Analogously, the higher GST basal activity and faster ATZ decay in the liver than in gills and in fish of contaminated environment than in captivity suggests a higher content of specific enzymes to biotransform ATZ. In fish, some extrahepatic tissues, such as gills, are strongly involved in the metabolism of xenobiotics (Matsuo et al., 2008). The gills, as an interface organ of organism and environment, and liver, of fundamental importance in the biotransformation of xenobiotics in vertebrates, would be key organs where molecular mechanisms would be developed to compensate adverse effects caused by the presence of contaminants (Fernandes et al., 2009).

High levels of GST activity in native fish may be a response to chronic exposure of contaminants in the environment (Vieira et al., 2017). The induced GST activity in fish of contaminated environment may be due to the inducing effect of environmental contaminants such as PAH, as reported by Chivittz et al. (2016) in the environment where they were captured. These environmental contaminants would be modulating key isoforms like GSTP and would be critical determinants of chemical susceptibility (Henson et al., 2001). Abel et al. (2004) proposed to ATZ as a suitable substrate to determine GSTP activity in tissues with multiple GST isoforms. Thus, in present study, the lower detections of ATZ by LC-MS/MS would be due to greater isoenzymatic activity GSTP.

Inhibition type exerted by ATZ on GST would be of type of competitive inhibition. Kinetic parameters obtained show that ATZ does not affect V_{max} but increases the K_M of GST. This suggests that ATZ decreases the availability of GST to conjugate CDNB and that higher concentrations of CDNB would be required to achieve a determinated rate ($V_{max}/2$) in the presence of ATZ than in its absence (Mathew et al., 2006). In this study, CDNB and ATZ would compete for the same active site of GST, but sufficiently high amounts of CDNB would overcome ATZ. This would explain why the V_{max} does not change, being a measure of speed to infinity (Voet and Voet, 2013). Carletti et al., 2008 demonstrated the ability of ATZ to inhibit isoenzymatic activity of AaGSTP1 and AaGSTR1, reporting it as an effective inhibitor of classical conjugation of CDNB for both enzymes, and suggesting its ability to bind to the active site H of this enzymes.

GST activity was decreased in kinetic assays using CDNB and ATZ as competition substrates. Increased levels of ATZ from 5µM ATZ decreased the detection

of GS-DNB conjugates, suggesting competition of ATZ with CDNB by the active site of GST. However, the results found with this study approach do not fit the expected pattern or index found in kinetic assays with CDNB and kinetic assays with ATZ analized by LC-MS/MS. The higher IC_{20} of ATZ means lower GST activity with affinity for ATZ. These index, suggest that fish would have a lower ability to biotransform ATZ by GST compared to shrimp (with exception of gills in captivity fish). Among organs, the IC_{20} of ATZ estimated for GST of shrimp and fish of captivity were low; but these values were higher in both organs of contaminated environment fish, suggesting their lower capacity to biotransform ATZ. Likewise, considering contamination factor, the lower IC_{20} estimated in captivity fish suggests a higher capacity for biotransformation for ATZ compared to contaminated environment fish.

The conflicting results found with the competitive approach would be due to the masking of GST isoforms with affinity for CDNB but not for ATZ. CDNB is a substrate conjugable by most GST, except for omega isoform; while ATZ would be almost exclusively substrate of GSTP (Abel et al., 2004). Thus, GS-DNB conjugates detected by spectrophotometry represent an integration of the activity of multiple GSTs isoforms, and would not show the true conjugative activity of GST isoforms with affinity for ATZ.

5.6. Conclusion

Briefly, kinetic assays using CDNB substrate and kinetic assays using ATZ as a substrate, would represent the closest reality of GST activities. The patterns of GST activity observed in both approaches show a greater ability of biotransformation in fish than in shrimp, in liver than in gills, and in contaminated environment fish than in captivity. The GST activity was inhibited when ATZ was used as substrate. ATZ

would be substrate of GST and would compete with CDNB for same active site. However, kinetic assays using CDNB and ATZ as substrates showed enzymatic behavior contrary to that observed in kinetic assays to estimate basal GST activity and by ATZ decay analyzed by LC-MS/MS. The conflicting results this approach would be due to a possible greater presence and activity of GST isoforms with affinity for CDNB but not for ATZ that would mask conjugations of GST isoforms with affinity to conjugate CDNB and ATZ.

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5.7. References

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5.8. Figures and table

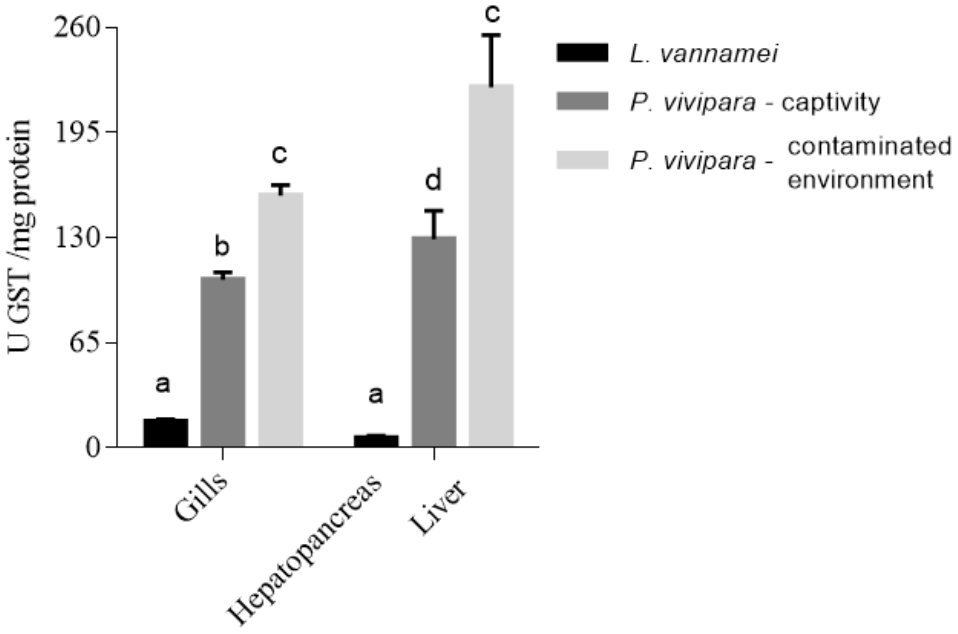
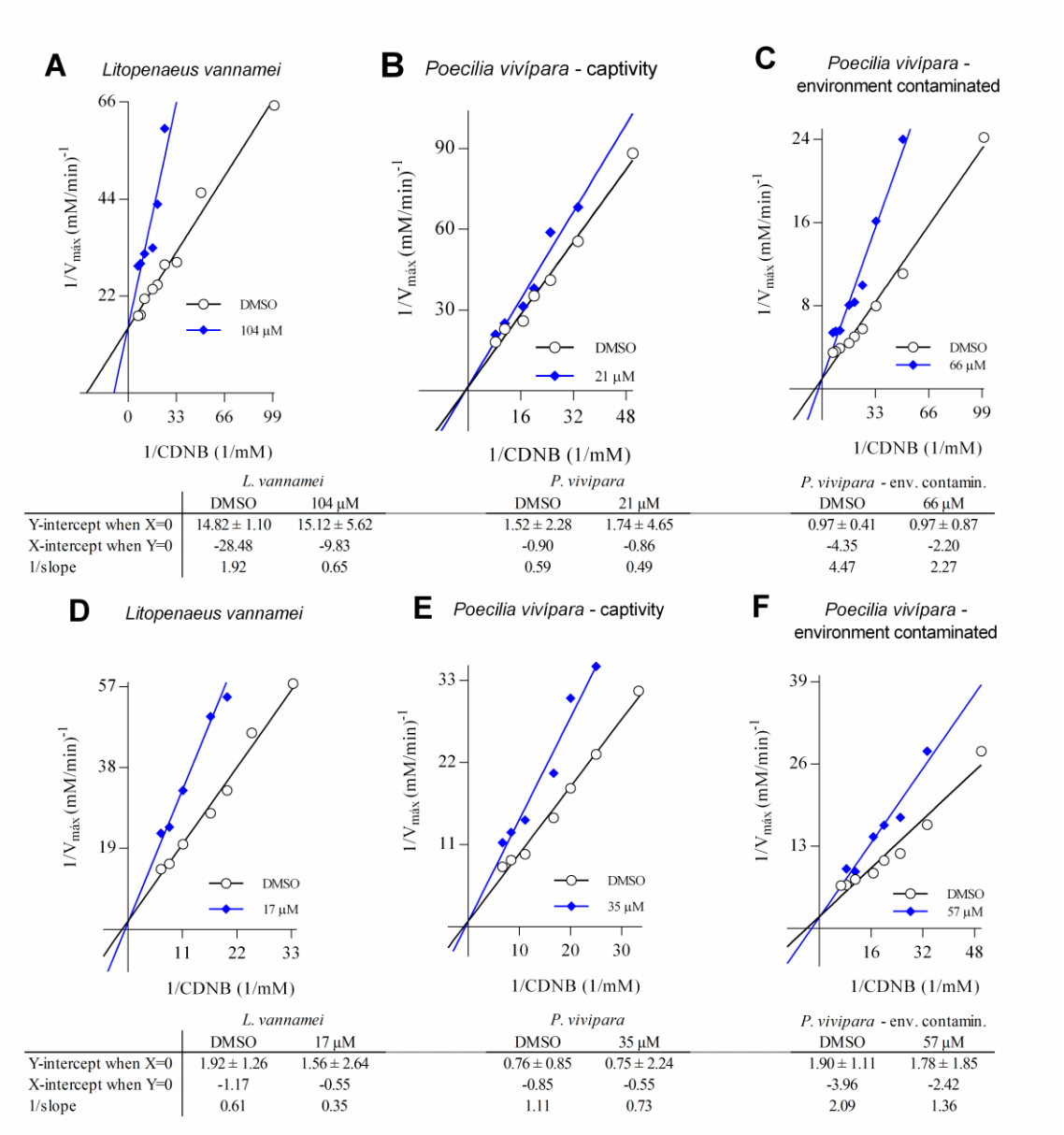


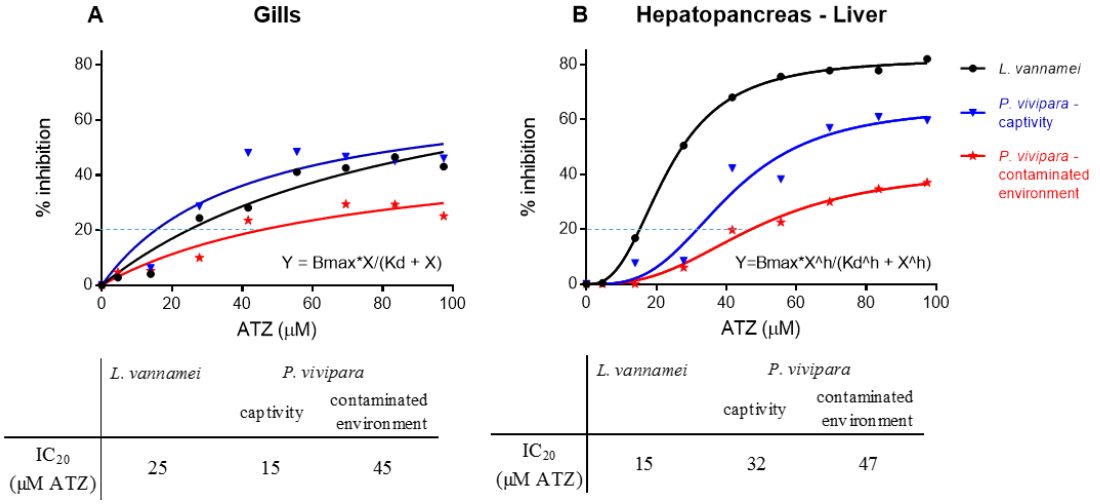
Fig. 1. Basal activity of GST in gills and hepatopancreas of *L. vannamei* and gills and liver of *P. vivipara* in captivity and contaminated environment. The different letters represent significant differences among groups (Two-way ANOVA followed by Tukey-HSD; $p < 0.05$).



	L. vannamei		*P. vivipara*		*P. vivipara* - env. contamin.	
	DMSO	104 µM	DMSO	21 µM	DMSO	66 µM
Y-intercept when X=0	14.82 ± 1.10	15.12 ± 5.62	1.52 ± 2.28	1.74 ± 4.65	0.97 ± 0.41	0.97 ± 0.87
X-intercept when Y=0	-28.48	-9.83	-0.90	-0.86	-4.35	-2.20
1/slope	1.92	0.65	0.59	0.49	4.47	2.27

	L. vannamei		*P. vivipara*		*P. vivipara* - env. contamin.	
	DMSO	17 µM	DMSO	35 µM	DMSO	57 µM
Y-intercept when X=0	1.92 ± 1.26	1.56 ± 2.64	0.76 ± 0.85	0.75 ± 2.24	1.90 ± 1.11	1.78 ± 1.85
X-intercept when Y=0	-1.17	-0.55	-0.85	-0.55	-3.96	-2.42
1/slope	0.61	0.35	1.11	0.73	2.09	1.36

Fig. 2. *Lineweaver-Burk* plot showing inclination changes in the line that represents the activity of GST estimated through reading GS-DNB conjugations in kinetic assays using CDNB (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.09, 0.12, 0.15M) and ATZ (IC_{20}) as substrates and citosolic enzyme extract of gills and hepatopancreas of *L. vannamei* (A,D), gills and liver of *P. vivipara* in captivity (B,E) and contaminated environment (C,F). The intersection of lines on the Y axis indicates similar V_{max}.



	L. vannamei	*P. vivipara* captivity	*P. vivipara* contaminated environment
IC_{20} (µM ATZ)	25	15	45

	L. vannamei	*P. vivipara* captivity	*P. vivipara* contaminated environment
IC_{20} (µM ATZ)	15	32	47

Fig. 3. Inhibited activity of GST (%) estimated through reading GS-DNB conjugates from kinetic assays using CDNB (0.12 M) and ATZ (13.9, 27.8, 41.7, 55.6, 69.5, 83.5, 97.4, 111.3 µM) as competitive substrates. Cytosolic enzymatic extract of gills (A) and hepatopancreas and liver (B) of *L. vannamei* gills and *P. vivipara* in captivity and contaminated environment was used as a source of GST. IC_{20} represents amount of ATZ that inhibits 20% of GST activity. Higher IC_{20} suggest lower biotransformation capacity of GST by ATZ.

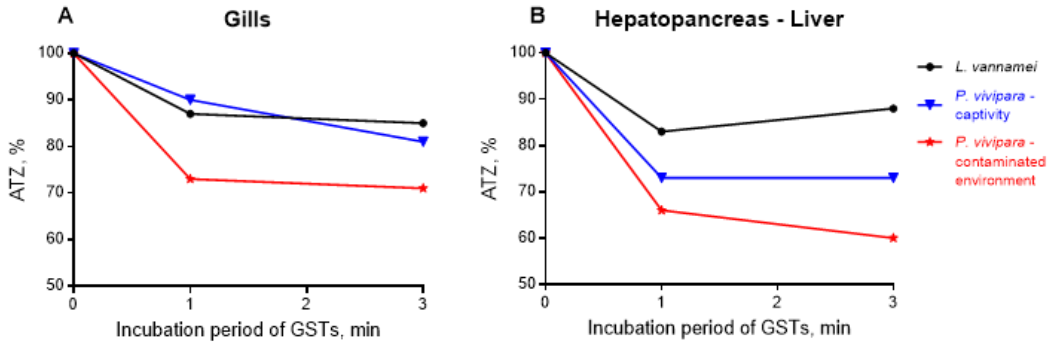


Fig. 4. Decay of ATZ after of its incubation in kinetic assays using cytosolic enzyme extract of gills (A) and hepatopancreas or liver (B) of *L. vannamei* and *P. vivipara* in captivity and environment contaminated.

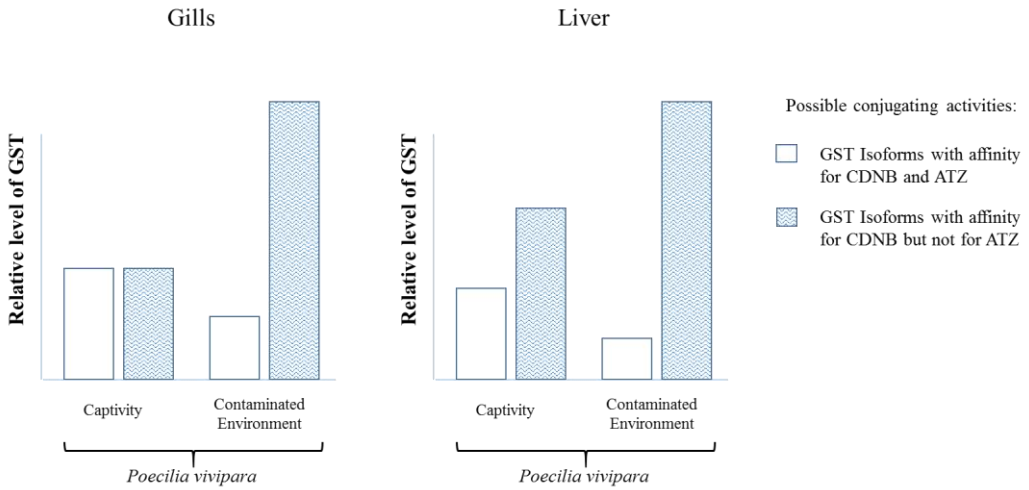
Table 1. Kinetic parameters of GST in presence of ATZ in gills and hepatopancreas or liver of *L. vannamei* and *P. vivipara* in captivity or environment contaminated.

Organ	Specie	Atrazine (μM)	K_M (mM)	$V_{máx}$ (μM/min)	Linear Equation	
					R^2	Validation
Gill	*L. Vannamei*	0	0.035	0.067	0.93	*
		104	0.102	0.066	0.84	*
	P. vivípara - captivity	0	1.109	0.660	0.98	*
		21	1.164	0.575	0.98	*
	P. vivípara - contaminated environment	0	0.230	1.028	0.91	*
		66	0.455	1.033	0.92	*
Liver	*L. Vannamei*	0	0.856	0.521	0.99	*
		17	1.819	0.640	0.99	*
	P. vivípara - captivity	0	1.180	1.312	0.97	*
		35	1.810	1.327	0.93	*
	P. vivípara - contaminated environment	0	0.252	0.526	0.92	*
		57	0.413	0.562	0.94	*

Supplementary data

Kinetic assays	Gills			Hepatopancreas - Liver		
	Litopenaeus vannamei	*Poecilia vivipara*		*Litopenaeus vannamei*	*Poecilia vivipara*	
		Captivity	Contaminated environment		Captivity	Contaminated environment
CDNB substrate	↑↑	↑↑↑	↑↑↑↑↑	↑	↑↑↑↑	↑↑↑↑↑↑
ATZ substrate	↑	↑	↑↑↑	↑	↑↑	↑↑↑
Competitive	↑↑	↑↑↑	↑	↑↑↑	↑↑	↑

Representation of relative GST activities estimated through kinetic assays using CDNB, ATZ and CDNB and ATZ as competitive substrates. GST activities estimated through GS-DNB conjugates and by ATZ decay show patterns of similar activities. The different pattern found in competitive kinetic assays would mask GST conjugations with affinity for ATZ, due to possible higher conjugative activities of GST isoforms with affinity for CDNB.



Possible scenarios that explain the different pattern of GST activity. Representing relative activities of GST estimated through GS-DNB conjugations and competitive kinetic assays would have to: in gills of fish in captivity, conjugations GST isoforms with affinity for CDNB and ATZ would be similar to GST conjugations with affinity for CDNB but not for ATZ. In gills of contaminated environment fish and in the liver of captive fish and contaminated environment, the conjugative activity of GST isoforms with affinity for CDNB and ATZ would be smaller than GST conjugations with affinity for CDNB but not for ATZ. These results would mask conjugative activities of GST isoforms with affinity for ATZ.

6. MANUSCRITO 2

Manuscrito a ser submetido para a revista Aquatic Toxicology.

Atrazine affect sperm parameters and transcription of cellular defense genes in adult male zebrafish

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Number of Tables: 1

Number of Figures: 3

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6.1. Abstract

The herbicide atrazine (ATZ) is widely used in the control of broad leaf weed in agriculture. In fish, ATZ could generate mitotic abnormalities in germ cells and oxidative stress related effects. We evaluated sperm parameters and expression of genes related to sperm quality and cellular defense in zebrafish after 11-day exposure to environmentally relevant ATZ concentrations of 2, 10 and 100 $\mu g.L^{-1}$. All tested ATZ concentrations caused impairment of sperm parameters such motility, mitochondrial functionality and cellular membrane integrity. Those changes occurred together with transcriptional repression of key genes involved in the genesis and maturation of sperm (*SRD5A2* and *CFTR*), antioxidant defenses (*SOD2* and *GPX4B*) and DNA repair (*XPC*) in the gonad, suggesting the possible involvement of gene repression caused by ATZ in the cellular effects observed in the gonad. In the liver, increased expression of key genes involved in the biotransformation of ATZ, such *CYP1A* and *GSTP1* (but not *GSTR*) and increased expression of the antioxidant defense *SOD2*, suggest a general hepatic cellular effort to eliminate ATZ from the fish body by increasing enzymatic defences via transcriptional activation. Our results show that ATZ decreases sperm quality, and its presence in the gonads suppressed the expression of important genes of spermatogenesis and cellular protection; however in the liver, induces the expression of genes that encoding enzymes of xenobiotic transformation and antioxidants, a cellular protection mechanism possibly activated by transcription factors such as AHR and NF-κB, respectively.

Keywords: atrazine; reproduction; fish;

6.2. Introduction

The success of the current agricultural production is based in the massive use of pesticides and herbicides (Brodeur et al., 2013). Atrazine (ATZ) is one of the most common herbicides, used to control weeds in crops such as corn and sugar cane, but its low organic degradability after its application has become in a potential contaminant of surface water and groundwater (Blahová et al., 2013). Regulatory institutions in Brazil, such as CONAMA (2005) recommend 2 $\mu g.L^{-1}$ ATZ as the maximum permitted level in the fresh water. However, ATZ have been found in higher levels in agricultural areas in Brazil (Armas et al., 2007), sufficient to cause adverse effects in aquatic organisms (Zhu et al., 2011; Blahová et al., 2013).

Many studies report that ATZ acts as endocrine disrupter (Hayes et al., 2003; Victor-Costa et al., 2010) but few studies have been performed on the direct effect in animal reproduction. In the male, a first study of the possible adverse effects of ATZ in sperm cells was performed by Papoulias et al. (2014), who reported increases in mitotic abnormalities in spermatogonia to Japanese medaka (*Oryzias latipes*) exposed to 0.5, 5 and 50 $\mu g.L^{-1}$ ATZ. In rats, ATZ decreases sperm motility, the most common parameter used to have an approximation of male reproductive success (Cosson, 2004), and causes increasing number of abnormal and dead sperm (Abarikwu et al., 2010). Current studies are focused on the identification of genes that encode key enzymes of spermatogenesis in order to figure out why the low reproductive success or infertility occurs. Using single nucleotide polymorphism to identify genes that explain the poor sperm motility, steroid 5-α reductase, α-polypeptide 2 (SRD5A2), cystic fibrosis trans-membrane conductance regulator (CFTR) and insulin-like growth factor 1 (IGF 1) evidenced to be significantly associated with motility and sperm biochemistry (Hering et al., 2014). The

role of enzymes encoded by these genes demonstrate its importance in spermatogenesis. The SRD5A2 converts testosterone into dihydrotestosterone, the main androgen responsible for sperm maturation (Wilson et al., 1993) and maintenance of spermatogenesis (Moore and Akhondi, 1996). The CFTR polypeptide is expressed in the head and tail of the sperm which would demonstrate its vital role in fertilization and sperm capacitation (Xu et al., 2007; Hernandez-Gonzalez et al., 2007). Meanwhile, isoform IGF3, besides IGF1, would be the best candidate to mediate the stimulation of spermatogenesis due to only expressed in the gonads, suggesting that it has an important role in the gonads (Wang et al., 2008; Zou et al., 2009).

In the cell, the presence of xenobiotics activates the aryl receptor hydrocarbons (AHR), a transcription factor activated by ligand, inducing expression of biotransformation genes, as cytochrome P4501A (*CYP1A*) and glutathione *S*-transferase (*GST*) (Bock et al., 2013; Garner and Di Giulio, 2012). This protection mechanism of the cell that allow it to adapt to changes in their environment (Denison and Nagy, 2003) has been observed after exposure of ATZ (Xing et al., 2014, Glisic et al., 2014; Xing et al., 2012). Furthermore, exposure of ATZ would generate additional reactive species oxygen (Blahova et al., 2013, Nwani et al., 2010) which alter the redox state of the cell, and would trigger transcription factors redox sensitive as NF-κB that induce gene transcription (Perkins, 2007) involved in responses antioxidants, such as superoxide dismutase (*SOD*), glutathione peroxidase (*GPX*) and *GST* (Kamata et al., 2005; Kaur et al., 2006).

A few studies in mammals reported impairment of reproductive function (Abarikwu et al., 2013; Friedmann, 2002; Stoker et al., 2000) and suggest decreased sperm quality (Betancourt et al., 2006) by effect of ATZ. However, in aquatic species

there are no information on ATZ exposure to environmentally relevant concentrations and its effect on sperm. Zebrafish (*Danio rerio*) is a suitable animal model for toxicological and endocrine disruption studies (Chen et al., 2012; Kanungo et al., 2012; Tu et al., 2013), and its popularity in researchs has risen rapidly due to its physiological similarity with mammals, genetic traceability, size and early development (Segner, 2009). The objective of the present study was to evaluate parameters of sperm quality of zebrafish exposed to ATZ concentrations of environmental relevance (2, 10 and 100 $\mu g.L^{-1}$) during 11 days, besides its effects on transcription of key genes involved in spermatogenic pathways and cellular protection.

6.3. Materials and methods

6.3.1. Animals and treatment

Sixty four male fish (*Danio rerio*) were obtained from a commercial distributor and kept in tanks containing dechlorinated and aerated water at 28 ± 2 °C, pH 7.0 and under a photoperiod of 12 h light: 12 h dark. Fishes were fed ad libitum twice daily with a commercial fish food (Tetra ColorBits). After acclimation for one month, the fish were randomly divided into four groups. In order to remove chloride and pesticide residues, the water of the experiment was previously filtered with Hydronix UDF-10 and CB-25-1005.

The experiment was conducted in four 16 L tanks (16 fish per tank). ATZ (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) and added in the tank of three experimental groups, that received the final concentrations of 2, 10 and 100 $\mu g.L^{-1}$, for 11 days. The final concentration of 0.005% DMSO was maintained in all four tanks, including the control group that does not received ATZ. The water in the four aquariums was renewed every 24 hours, in order to avoid ATZ decay. The

concentration of 2 μg.L^{-1} corresponds to the maximum concentration of ATZ allowed by the Brazilian regulatory agency (CONAMA, 2005) for fresh water. The concentration of 10 and 100 μg.L^{-1} corresponds to sub-lethal concentrations for zebrafish (Plhalova et al., 2012). The water samples (1 mL) in triplicate, barely started the experiment, 1st, 10th and 11th day, were collected before of the daily change of water. These samples were filtered with filters for syringe of polyethersulfone to then be analyzed ATZ concentration by liquid chromatography-tandem mass spectrometry (LC-MS/MS), in accordance with work conditions of Demoliner et al. (2010). The residue of water with ATZ was purified with filters (Hydronix CB-25-1005), based on its good ability to adsorb ATZ (Lladó et al., 2015; Vieira dos Santos et al., 2017), and later thrown in the sink.

6.3.2. Sperm parameters

At the end of the exposure period, the animals were euthanized by cervical transection. The Ethics Committee on Animal Use (CEUA N ° P013/2011 – FURG) approved these procedures. In order to analyze sperm parameters, one of the excised gonad was placed placed into tubes containing 100 μL of Beltsville Thawing Solution (BTS) for subsequent analysis. The tubes were shaken for the release of spermatozeugmatas (sperm bundles). Sperm was released by gently and repeatedly disrupting the spermatozeugmatas with a 10 μL pipette tip. Of this sperm suspension, 200 sperm cells were counted and evaluated with an epifluorescent microscope at 400 X magnification (Olympus BX 51) to determine sperm motility, motility period, mitochondrial functionality, membrane and DNA integrity.

For evaluation of sperm motility, a 10 μL sperm sample was diluted in a 40 μL working solution in an isotonic saline solution, including: 1.7 mM-formaldehyde, 20

μM carboxyfluorescein diacetate (CFDA), and 7.3 μM propidium iodide (IP). Sperm with green fluorescence were considered viable, because their metabolic activity allowed carboxyfluorescein diacetate to accumulate in their cytoplasm, whereas those with heads with either red or red and green fluorescence were classified as nonviable (Harrison and Vickers, 1990). The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red, or red and green fluorescence). Sperm motility period was expressed as the percentage of progressive motile spermatozoa 10 s after activation, and the motility period was comprised of the time (in seconds) between sperm activation and the absence of progressive movement (straight line movement).

Mitochondrial functionality was evaluated after incubation of a 10 μL sperm sample with a 40 μL rhodamine 123 solution (13 μM), at 20 °C for 10 min. Sperm with positive rhodamine staining (green fluorescence) were considered as having functional mitochondria. Conversely, nonfunctional mitochondria were characterized by negative rhodamine staining (sperm with no fluorescence) (He and Woods, 2004). The rate of mitochondrial functionality was determined by the proportion of sperm emitting green fluorescence compared with total sperm (green or no fluorescence).

The membrane integrity of the sperm was examined following the methodology of Harrison and Vickers (1990). For that goal, 5 μL of sample were diluted in 20 μL of saline solution with 1.7 mM formaldehyde, 20 μM carboxyfluorescein diacetate (CFDA) and 7.3 μM propidium iodide (PI). When the spermatozoa membrane was intact, CFDA accumulation occurred. After the hydrolysis of CFDA, carboxyfluorescein was generated along with a corresponding green fluorescence. Sperm with damage in the membrane incorporated PI and emitted a red or red and green

fluorescence. The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red or red and green).

Sperm DNA integrity was evaluated after putting a 45 µL sperm sample in 50 µL TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2). After 30 sec, 200 µL of Triton solution 1 X was added and, 30 sec later, 50 µL of acridine orange was added (2 mg/mL in deionized H_2O). The evaluation was done after 5 min, without exceeding 1 min of slide exposure. Sperm with green fluorescence were considered as having intact DNA, whereas those with red or orange fluorescence were considered as having denatured DNA (Bencharif et al., 2010). The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red, or orange fluorescence).

6.3.3. Gene expression analysis

One of excised gonads, along with liver were preserved in RNAlater® (Ambion) for 24 hours at room temperature and then stored at -80°C. Total RNA was isolated from tissue using TRIzol reagent (Invitrogen), following the manufacturer's recommendations. The quality of RNA was evaluated on agarose gel 1% to evaluate possible degradation and after confirmation of good quality has been treated with DNAse (InvitrogenTM) to avoid contamination of RNA samples with genomic DNA. Total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA) and a mix of oligo-dT and random hexamer primers. First-strand cDNA was amplified by real-time PCR (qPCR) using gene specific primers (Table 1), which were designed from sequences available in the DDBJ/GenBankTM/EBI Data Bank, using the Primer3

online software (Rozen and Skaletsky, 2000). Real-time PCR analyses were performed with GoTaq qPCR Master Mix kit (Promega, Madison, WI, USA) and real-time PCR System 7300 (Applied Biosystems) using the following program: 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative quantification of gene expression in experimental groups was analyzed by the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) using *b-actin* as a housekeeping gene. The *b-actin* gene was chosen after preliminary comparative stability tests with other genes (*g6pd, 18s*) for ATZ exposure data. The qPCR efficiencies for the primer pairs were tested using 1x, 2x, 4x, 8x, 16x and 32x serial cDNA dilutions and the primers were accepted if efficiency were 1.8 - 2.2 (Schmittgen and Livak, 2008).

6.3.4. Statistical analysis

The statistical analyses were carried out in R version 3.0.2. (R Development Core Team, 2014). The normality of the samples and the homogeneity of the variances were tested using the Shapiro–Wilk test and the Levene test, respectively. The parameters considered normally distributed and homoscedastic were tested by analysis of variance (ANOVA), comparing the groups of animals exposed to ATZ (2, 10 and 100 $\mu g.L^{-1}$) and control (DMSO), with comparisons of the means by Tukey's HSD test with a significance level of 5%. The data that did not present normal distribution were submitted to Kruskal–Wallis analysis of variance for non-parametric data, followed by the Dunn all-pairwise comparisons, using a significance level of 5%.

6.4. Results

6.4.1. Atrazine exposure and sperm parameters

The ATZ concentration remained constant and close to nominal concentrations during the experiment. The mean ATZ concentration of water samples in triplicate at

the beginning of the experiment, 1st, 10th and 11th day were zero, 2.58 (± 0.12), 11.58 (± 0.29) and 110.78 (± 1.86) μg.L^{-1} for control group, 2, 10 and 100 μg.L^{-1}, respectively.

The ATZ exposure decreased significantly the sperm quality parameters, with the exception of DNA damage, compared to the control group ($p < 0.05$). Motility, motility period, mitochondrial functionality and integrity of the cell membrane of spermatozoa from fish exposed to 2, 10 and 100 μg.L^{-1} ATZ were lower than shown by the control group (Fig. 1A-E).

6.4.2. Effect of ATZ on gene expression

The expression of *CFTR* and *SRD5A2* genes, but not *IGF3*, were decreased in the gonads of fish exposed to ATZ ($p < 0.01$). The mRNA level *SRD5A2* was lower in 16 and 26 fold in fish exposed to 2 and 100 μg.L^{-1} ATZ; while that *CFTR* was lower by 2.6 fold in group exposed to 100 μg.L^{-1} ATZ, both with regard to the control group (Fig. 2A-B).

The levels of mRNA expression of genes that encoding antioxidant enzymes were decreased in the gonads of male fish exposed to ATZ. Compared with the control group, the expression of *SOD2* decreased by 1.7 and 1.8 fold in groups exposed to 10 and 100 μg.L^{-1} ATZ, respectively ($p < 0.05$; Fig. 2D); while that *GPX4B* expression, decreased by 3.4 and 6.1 fold in groups exposed to 2 and 100 μg.L^{-1} ATZ, respectively ($p < 0.01$; Fig. 2E). Regarding the gene that encoding the DNA repair enzyme, DNA damage recognition and repair factor (*XPC)*, its expression showed a moderate decrease (2.3 fold) in group exposed to 2 and 10 μg.L^{-1} ATZ; and a greater decrease (7.8 fold) in the gonads of fish exposed to 100 μg.L^{-1} ATZ, compared with the control group ($p < 0.01$; Fig 2F).

The transcriptional evaluation of genes involved in the biotransformation of xenobiotics in the liver, showed that *CYP1A* and *GSTP1* were affected by the ATZ exposure ($p< 0.01$). The expression of *CYP1A* was induced by 3.7 fold in the group of 100 $\mu g.L^{-1}$ ATZ (Fig. 3A), while *GSTP1* was induced by 4.4 and 5.4 fold in fish exposed to 2 and 10 $\mu g.L^{-1}$ ATZ (Fig. 3B). The *GSTR* expression was not affected in groups exposed to ATZ ($p> 0.05$; Fig. 3C). Regarding the effect of ATZ in the mRNA level of *SOD2*, its expression was 3.5 fold higher in group 10 $\mu g.L^{-1}$ ATZ compared with the control ($p< 0.01$; Fig. 3D).

6.5. Discussion

Several studies report that ATZ is an endocrine-disrupting chemical which alters the reproductive system (Hayes et al., 2003; Suzawa and Ingraham, 2008; Wiegand et al., 2001; Cooper et al., 2007; Rohr and McCoy, 2010; Papoulias et al., 2014). The measurement of parameters which are indicative of the quality of sperm in an organism exposed to environmentally relevant xenobiotic concentrations would allow us to infer its possible effect on the environment. Consequently, the present study shows that sperm quality parameters decreased in the presence of ATZ, as well as its effect on the expression of key genes in spermatogenesis and cell protection in the gonad and liver of male zebrafish.

In our study, a significant decrease in motility and motility period of sperm indicate that ATZ causes a detrimental effect on sperm, which would lead to important decreasing of fertilizing capacity. Motility and motility period are sperm quality indicators, as they indicate the ability to move toward the oocyte and the time that keeps on moving, respectively (Rurangwa et al., 2004). As far as we know there are no studies of the effect of ATZ on sperm motility in aquatic species. In rats, the exposure

to ATZ cause a dose-dependent decrease in sperm motility and viability, similar to what we observed in fish in the present study (Kniewald et al., 2000; Abarikwu et al., 2010; Farombi et al., 2013).

The decreased sperm motility may be related to decreased mitochondrial functionality that was also observed in all concentrations of ATZ that were tested. The energy produced in mitochondria is vital to sufficient sperm cell motility (Ramalho-Santos et al., 2009). The biochemical mechanism of this effect of ATZ possibly involve its binding to ATP synthase, a transmembrane enzyme that catalyzes the synthesis of ATP (Hase et al., 2008). In our study, this possible effect of ATZ was analyzed using the rhodamine 123 test, a mitochondrial dye transported within actively respiring mitochondria and their accumulation in the mitochondria causes them to fluoresce green (Gillan et al., 2005). Indeed, in our study, mitochondria within spermatozoa of fish exposed to ATZ showed less dye transport capacity to their interior, and analogically, suggests a lower capacity for transport of protons for ATP production. Different studies with vertebrates support the statement that the motility of the sperm cell depends on the functionality of their mitochondria to human (Troiano et al., 1998), equine (Love et al., 2003), rats (Gravance et al., 2001), pigs (Spinaci et al., 2005) and sheep (Martinez-Pastor et al., 2004), and based in our study, it is very possible that this is the case for fishes too.

A damaged membrane may interfere with the exchange of nutrients from the cell and its environment affecting sperm motility (Ramirez et al., 1992). In the present study, the increased damage of the cell membrane coincides with the lower sperm motility observed in fish exposed to ATZ and would be another explanatory factor of this detriment. In the fish, a healthy sperm membrane is vital to generate their

depolarization and initiate mobility (Linhart et al., 2002; Cosson, 2004) to reach the micropyle of the oocyte and fertilize it (Cosson et al., 1999). The greatest damage of the membranes observed would be by oxidative effect of ATZ (Jin et al., 2010) suggesting increased lipid peroxidation in the spermatic membrane. In addition, the oxidative effect of ATZ can attack to chromatin and increase DNA damage (Sharma et al., 2012; Zhu et al., 2011), however, in this study this effect was not observed.

Genes that encoding key proteins of spermatogenesis were evaluated in order to explore possible molecular mechanisms that could be involved in decreased sperm motility caused by ATZ. The enzyme Srd5a2 converts testosterone to dihydroxysterone (DHT). The androgenic activity of DHT is 2-fold higher than testosterone, and the epididymis is highly androgen dependent to complete its transport and storage of spermatozoa before release (Robaire and Hamzeh, 2011). Studies with human semen report decreased sperm motility in patients with srd5a2 deficiency (Imperato-McGinley et al., 1992; Cai et al., 1994). A lower expression of SRD5a2 suggests its lower enzymatic activity and therefore, lower sperm maturation that would be reflected in lower sperm motility. CFTR is a highly expressed protein in the head and tail of the spermatozoa (Xu et al., 2007). Its lower gene expression suggests less availability of its protein in the processes of the spermatogenesis that could also translate into lower motility sperm. Genetic studies based on SNP and immunofluorescence with sperm from livestock and human, report that SRD5A2 (Hering et al., 2014; Zhao et al., 2012) and CFTR (Hering et al., 2014; Jiang et al., 2014) are related to the quality of sperm and could be used as genetic markers of poor sperm motility. The IGF3 is expressed exclusively in gonads (Wang et al., 2008; Zou et al., 2009) and its enzymatic activity would be directly related to sperm motility (Henricks et al., 1998). In the present study, its expression was not affected by ATZ. Information on role of IGF in gonads and

spermatogenesis in teleosts is very limited (Chen et al., 2013). IGF3 would be involved in the regulation of male gonad functions in tilapia, as shown by its lower gene expression in estrogen treatments (Berishvili et al., 2010).

Like another transcription factors, the NF-κB family of transcription factors can activate and repress testicular gene transcription (Lui and Cheng, 2008). Under normal conditions, NF-κB is held inactive by the binding of its inhibitory subunit IκB; but under stress conditions, IκB becomes phosphorylated and dissociates from NF-κB, translocating to the nucleus and activating the expression of antioxidant genes and cytokines (Lui and Cheng, 2008). The oxidative stress generated by ATZ (Jin et al., 2010) may be activating this nuclear transcription factor and modulating gene expression (Lui et al., 2008; Kaur et al., 2006) as suggested by results found In the present study. A repressor effect on the expression of these genes in the gonads, suggests a lower synthesis of these key enzymes of cellular defense against oxidizing agents. The fact that sex cells have DNA coiled that block activation of its antioxidant pathways (Hagedorn et al., 2012), the suppressive effect of expression of antioxidant genes, suggests a greater susceptibility to PUFA oxidation of gonad environment (Lui and Cheng, 2008) and would relate to the greater damage of membranes that would led to lower sperm motilities of fish exposed to ATZ in this study.

The *XPC*, another gene repressed in the gonad of groups exposed to ATZ, encodes the protein that initiates the recruitment of the whole protein apparatus to repair oxidative DNA damage (Nemzow et al., 2015). Repression of its gene expression did not coincide with sperm DNA damage. Spermatogenesis in *Danio rerio* lasts 6 days (Leal et al., 2009) and spermatogonial generations in meiotic and spermiogenic phases may be developing and susceptible to oxidative DNA damage during the time of

exposure of ATZ. However, the absence of females and sexual activity could be factors of inactivation of spermatogenesis, and sperm cells analyzed would be cells synthesized previous to exposure experiment.

While the expression of cellular protection genes was repressed in the gonads of groups exposed to ATZ, in the liver these genes were induced. Increased expression of *CYP1A* and *GSTP1* suggests that ATZ would be activating AHR receptor and inducing expression of genes of enzymes that increase its biotransformation, decreasing its toxicity and increasing hydrophilicity for elimination (Garner and Di Giulio, 2012; Girolami et al., 2016). In fish *Cyprinus carpio* exposed to 4.28µg.L^{-1} ATZ showed induction in the expression of CYP1A (Xing et al., 2012), while in the present study significant induction was only seen in liver of fishes exposed to 100µg.L^{-1} ATZ. *Danio rerio* embryos exposed to 500µg.L^{-1} ATZ induced expression of GSTP1 (Glisic et al., 2014), while exposures of 4.28 and 428µg.L^{-1} ATZ in *Cyprinus carpio* showed hepatic GSTR induction (Xing et al., 2014). Abel et al. (2004) using hGST alpha, mu, pi, and theta isoforms *in vitro* assays found that only pi isoform (GSTP) had affinity for biotransforming ATZ. Carletti et al. (2008) found that ATZ inhibited the specific activity of AaGSTP1 and AaGSTR (GST rho) in 50 and 70%, respectively, at a concentration of 24mg.L^{-1} ATZ. GSTR is an isoform that has been identified only in fish (Fu and Xie, 2006), and would be a more isoform with capacity to biotransform ATZ; however, the expression of GSTR in the present study was not affected in groups exposed to ATZ.

Another gene of cell protection induced in liver of groups exposed to ATZ was *SOD2*. ATZ exposure studies in fish also found gene induction (Jin et al., 2010) and induced SOD2 activity (Jin et al., 2010; Nwani et al., 2010) suggesting activation of

nuclear receptors as NF-KB, possibly by effect of additional ROS generated by ATZ, which would induce gene expression of the antioxidant system.

It should be noted that presystemic metabolism reduces amount of chemical entering the liver, the main organ of xenobiotic metabolism (Fitzsimmons et al., 2007). Gill tissue metabolism would limit absorption of chemicals taken from water and biotransformations in gastrointestinal tract would limit oral availability of chemicals (Barron et al., 1989; van Veld et al., 1988). In this study, these factors plus the possible induced activity of hepatic enzymes that biotransform ATZ, would be limiting the entry of ATZ into the gonads. However, still all with these detoxification strategies, ATZ caused negative effects at gonadal level.

6.6. Conclusion

The results show that ATZ negatively affected the quality of zebrafish spermatozoa. Motility and sperm motility period were decrease in groups exposed to ATZ. Lower mitochondrial activity and loss of cell membrane integrity in sperm cells from groups exposed to ATZ means less energy production and inconvenience in transporting nutrients between gonad environment and sperm cell, respectively, suggesting effects of ATZ at subcellular level that would be relate to decreased sperm motility. Repression of key genes of steroidogenic pathways (*SRD5A2* and *CFTR*), antioxidant (*SOD2, GPX4B*) and DNA repair (*XPC*) suggest negative effects of ATZ on spermatogenesis and cell protection system in gonads, respectively; that would also explain decreased sperm motility. In the liver, however, it was found induced expression of genes belonging to the xenobiotic transformation pathway and antioxidant system, evidencing fundamental functions of detoxification and cellular protection of this organ.

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6.8. Figures and table

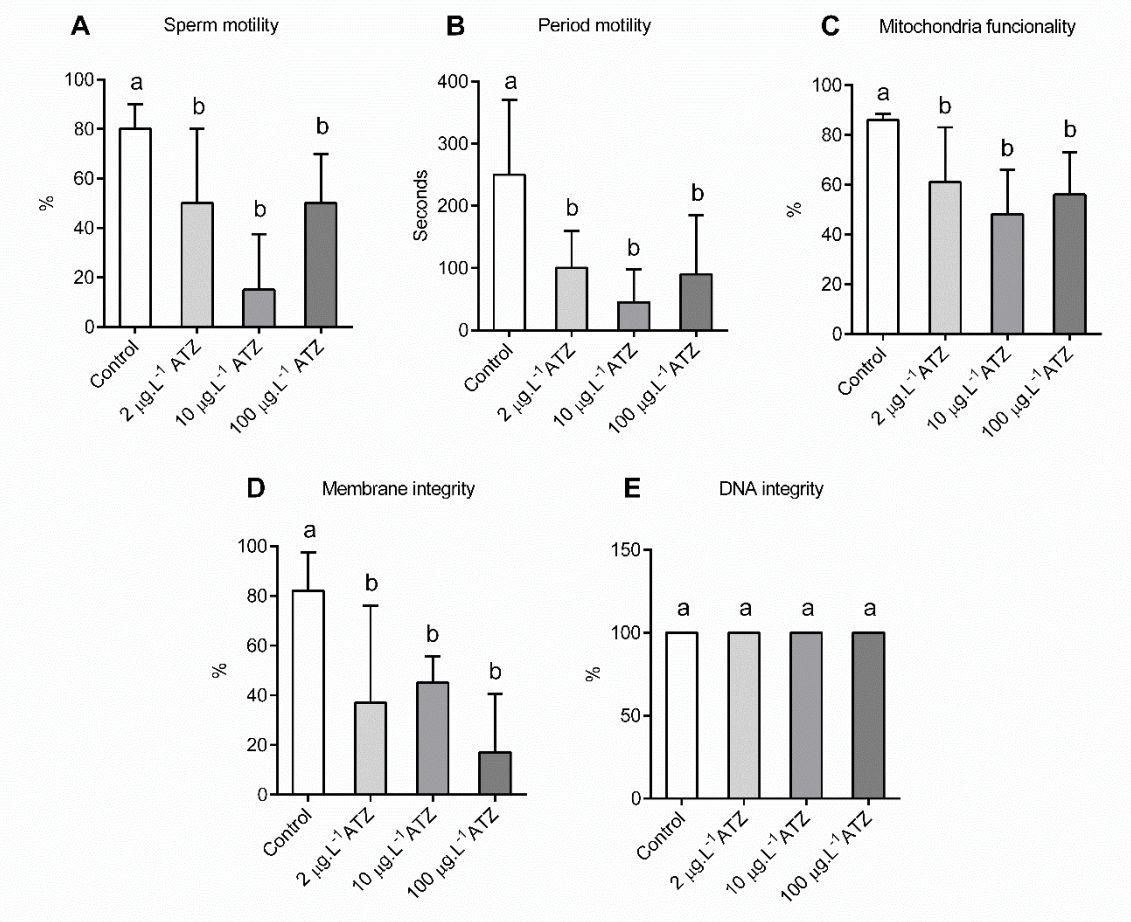

Fig. 1. Effect of atrazine on sperm motility (%) (A), period motility (seconds) (B), mitochondria funcionality (%) (C), membrane integrity (%) (D) and DNA integrity (%) (E) in zebrafish exposed to ATZ (2, 10 and 100 µg.L^{-1}) for 11 days. The values are medians ± IR (n = 7 -13). The different letters represent significant differences among treatments (Kruskal -Wallis analysis followed by the Dunn all-pairwise comparisons; $p < 0.05$).

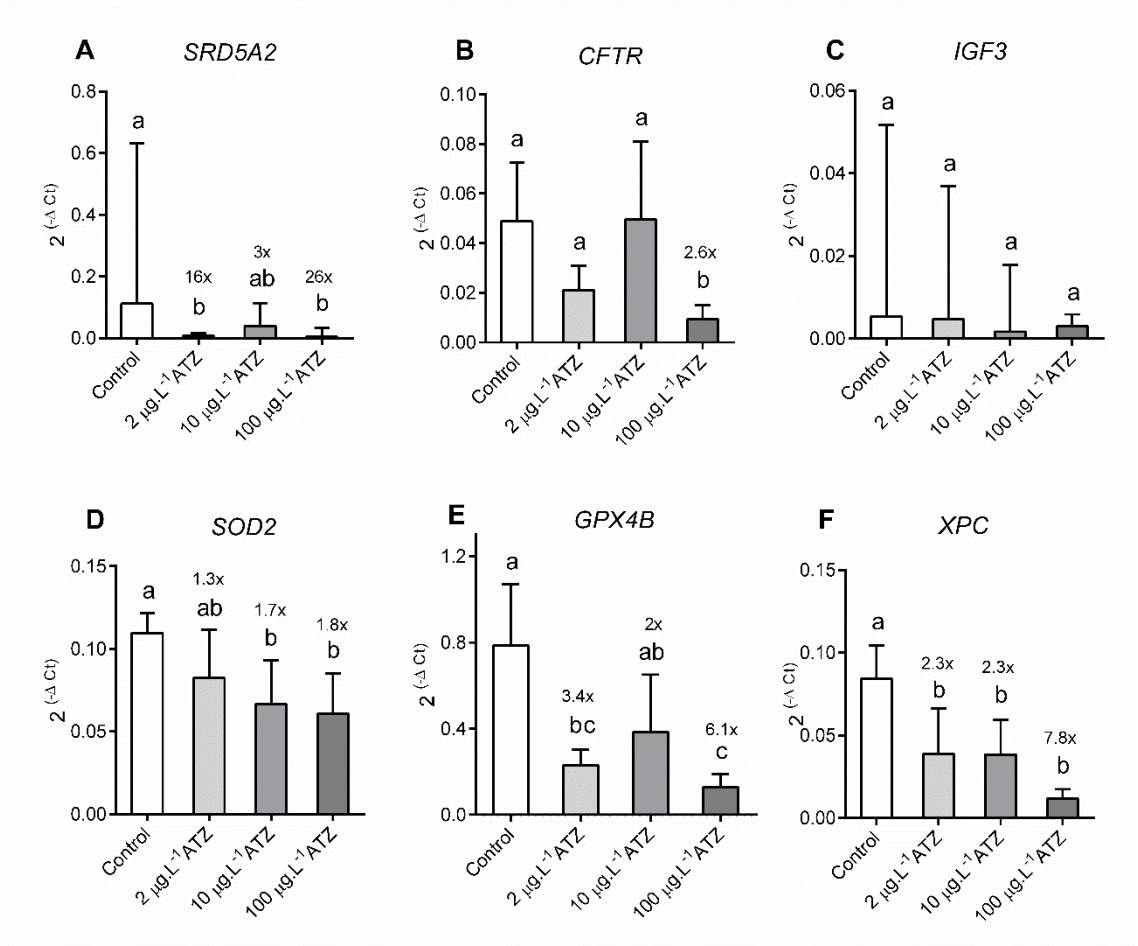

Fig. 2. Gene expression associated with low sperm motility (steroid 5-α reductase, α-polypeptide 2 (*SRD5A2*), cystic fibrosis trans-membrane conductance regulator (*CFTR*) and insulin-like growth factor 1 (*IGF1*); A, B and C; respectively); antioxidant activity (superoxide dismutase (*SOD2*) and glutathione peroxidase (*GPX4B*); D and E; respectively); and DNA repair (DNA damage recognition and repair factor (*XPC*); F) in gonad of zebrafish exposed to ATZ (2, 10 and 100 µg.L^{-1}) for 11 days. Beta-actin was used as housekeeping gene. The values are medians ± IR for A, B and C or means ± SD for D, E and F (n = 3-5). The different letters represent significant differences among treatments (Kruskal -Wallis analysis followed by the Dunn all-pairwise comparisons or one-way ANOVA followed by Tukey-HSD; $p < 0.05$).

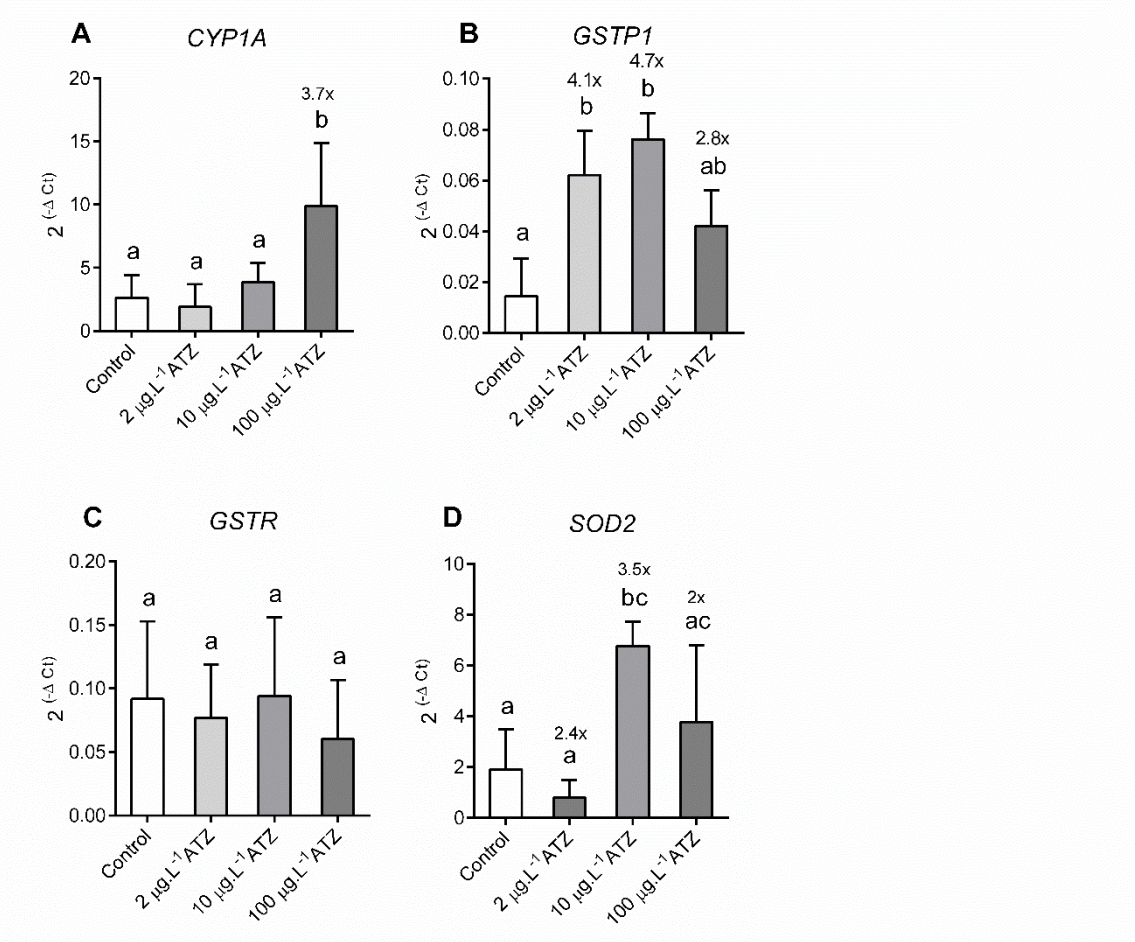

Fig. 3. Gene expression related to the biotransformation (superoxide dismutase (*SOD*), glutathione *S*-transferase pi 1 (*GSTP1*) and glutathione *S*-transferase rho (*GSTR*); A, B and C, respectively) and antioxidant activity (superoxide dismutase (*SOD2*); D) in the liver of zebrafish exposed to ATZ (2, 10 and 100 µg.L^{-1}) for 11 days. Beta-actin was used as housekeeping gene). The values are means ± SD (n=4-5). The different letters represent significant differences among treatments (one-way ANOVA followed by Tukey-HSD; $p < 0.05$).

Table 1. Sequence, product size and location of NCBI access by primers used in RT-qPCR reactions.

Official symbol	Primer sequences	Product length (pb)	Accession number
srd5a2	F: 5′-GCGTACGGACGCTATGTGGA-3′ R: 5′-GCCTGGCAAACTTTCCGTTG-3′	132	NM_001017703.1
cftr	F: 5′-CGGCACCTTTCAGTGGTGTG-3′ R: 5′-GGTTTGGGCGTCTCAGATGG-3′	108	NM_001044883.1
igf3	F: 5′-CACGCTGCGGACGAGAACTA-3′ R: 5′-TTTCCCACGAGAGCGAGGAC-3′	113	NM_001115050.1
sod2	F: 5′- TGGCAAAGGGTGA -3′ R: 5′- CACCGCCATTGGGTGACAGA -3′	114	AY195857
gpx4b	F: 5′-TGCAACCAGTTCGGAAAGCA-3′ R: 5′-GAGCTGCGTCTCCGTTCACA-3′	118	NM_001030070.2
xpc	F: 5′-CGTATCTGCGGCGAATGATG-3′ R: 5′-GCCAACAGGTCAGGCTCACA-3′	130	NM_001045210.1
cyp1a	F: 5′-GCATTACGATACGTTCGATAAGGAC-3′ R: 5′-GCTCCGAATAGGTCATTGACGAT-3′	147	NM_131879.1
gstp1	F: 5′-GAGAACCTGGTGACCTTTGAAGAG-3′ R: 5′-TGTCTCAGCATGGCGTTGGA-3′	125	NM_131734.3
gstr	F: 5′-ACTTCAGCATGGCCGACGTG-3′ R: 5′-ACTGGGCCGATCCTTCACCA-3′	122	NM_001045060.2
actb1	F: 5′- GCTGTTTTCCCCTCCATTGTT -3′ R: 5′- TCCCATGCCAACCATCACT -3′	60	AF057040

7. DISCUSSÃO GERAL

Muitas investigações foram realizadas para estudar atividades GST e inferir capacidades de biotransformação de xenobióticos. No organismo, uma estratégia de defensa que compensa os potenciais efeitos nocivos dos xenobióticos seria a indução da atividade de enzimas chaves das Fases de biotransformação, como as GST. Resultados de estudos, tanto *in vitro* e *in situ*, mostram evidências da sua indução; mas poucos estudos comparam capacidades de biotransformação entre espécies, órgãos e ambiente diferentes. Assim, o primeiro capitulo desta tese estimou *in vitro* a atividade basal de GST através de três abordagens e utilizando extrato citosólico de camarão *L. vannamei* e peixe *P. vivipara* de ambiente contaminado e cativeiro. Isto, com a finalidade de avaliar capacidades de biotransformação interespécie e em condições de diferente impacto ambiental.

A maior atividade basal de GST e o maior decaimento de ATZ analisado por LC-MS/MS no fígado em comparação com as brânquias nos peixes, mostra a maior importância deste órgão na biotransformação de xenobióticos. Em peixe, as enzimas de biotransformação de xenobióticos Fase I e II estão distribuídas principalmente no fígado, embora também incluam o intestino, os rins, e em menor medida nas brânquias e o sistema olfativo (Matsuo *et al*., 2008). Por isso, este órgão é altamente recomendado no estudo de indicadores ambientais da contaminação da agua (Gül *et al*., 2004, Cárcamo *et al*., 2017).

No camarão, a tarefa de detoxificação seria igualmente importante tanto nas brânquias como no hepatopâncreas. Estes resultados coincidiram com os de Zhou *et al*. (2009) que estudaram o efeito do pH sobre a atividade da GST em crustáceo *L. vannamei* e reportaram GST com atividades similares nas brânquias e hepatopâncreas.

Enquanto o fígado de vertebrados é um órgão com funções especializadas, o hepatopâncreas do crustáceo cumpre funções mais amplas (hepáticas, pancreáticas e intestinais) (Yepiz-Plascencia *et al*., 2000), pelo que a brânquia realizaria tarefas importantes de detoxificação que compensariam as muitas funções desenvolvidas no hepatopâncreas.

A maior atividade basal GST e maior decaimento de ATZ analisado por LC-MS/MS observado em peixe de ambiente contaminado sugere a maior capacidade de biotransformar xenobioticos. Isto pode ser devido à presença de contaminantes tais como PAH, tal como detectado por Chivittz *et al*. (2016) no local onde foram capturados estes peixes (S4 no artigo da mesma), que induziriam maiores atividades GST. Peixes em ambientes naturais podem mostrar atividade GST aumentada como resposta a exposição crônica de contaminantes no ambiente aquático (Vieira *et al*., 2017). Os contaminantes ambientais estariam modulando isoformas chaves como GSTP e seriam determinantes críticos da susceptibilidade química (Henson et al., 2001). GSTP conjuga muitos substratos PAH e sua possível atividade induzida por estes contaminantes no ambiente aquático seria detectado em ensaios *in vitro* utilizando ATZ. Abel *et al*., (2004) propõe a ATZ como um substrato adequado na determinação de atividades GSTP em tecido de múltiplas isoformas expressas.

Os ensaios enzimáticos competitivos realizados no estudo sugerem que ATZ é um inibidor competitivo de GST, já que os parâmetros cinéticos estimados em presença de ATZ mostram mudanças no $\boldsymbol{K}_{\mathrm{M}}$ aparente, mas não em $\boldsymbol{V}_{\mathrm{máx}}$. A inibição competitiva de uma enzima é geralmente causada por dois substratos competindo pelo mesmo local ativo; e segundo a cinética enzimática clássica, deve haver uma mudança no $\boldsymbol{K}_{\mathrm{M}}$ aparente, mas não em $\boldsymbol{V}_{\mathrm{máx}}$ (Hodgson, 2010). Isto significaria que o CDNB e a ATZ

estariam competindo pela mesma localidade do sitio ativo da GST. A característica mais distinta de um inibidor competitivo é o fato de que o substrato o inibidor pode ligar-se à enzima, mas não ambos. Como os dois competem pela mesma localidade, o substrato suficientemente alto "vencera" o inibidor. É por isso que Vmax não muda; já que é uma medida da velocidade ao infinito (Substrato) (Voet e Voet, 2013).

Os ensaios competitivos mostram a inibição das GST pela ATZ, quando a atividade foi estimada por conjugados GS-DNB. A partir de quantidades ≥ de 5μM de ATZ no ensaio cinético competitivo, a capacidade de biotransformação foi diminuindo, mas não de acordo com os padrões esperados. Das concentrações inibitórias de ATZ (IC_{20}) por GST pode se interpretar maior capacidade de biotransformação em crustáceo que em peixes, sugerindo maiores níveis de GST com afinidade por ATZ. Enquanto o IC_{20} no camarão e peixe de cativeiro eram baixos e variados para ambos órgãos avaliados, estas concentrações foram maiores nos órgãos de peixes de ambiente contaminado o que sugere que GST destes peixes teriam menor capacidade para biotransformar ATZ.

A conjugação GS-DNB representa uma integração de atividade das múltiplas isoformas GST, com exceção da GST omega (Henson *et al*., 2001). No entanto, em ensaios que incluem substratos de afinidade GST-especifica como ATZ, estimações da atividade GST por conjugações GS-DNB sobrestimaria esta atividade em detrimento das conjugações GST-especifica. Isto explicaria os resultados contraditórios obtidos com o enfoque competitivo.

Em conclusão, as atividades de biotransformação por GST mais realísticas seriam observadas através de ensaios cinéticos que estimam sua atividade com conjugados GS-DNB e em ensaios cinéticos analisando ATZ por LC-MS/MS. Os

decaimentos de ATZ analisados por LC-MS/MS relacionaram-se com atividades da isoforma GSTP. Esta enzima seria a isoforma chave na biotransformação da ATZ, pelo que é sugerido como substrato marcador adequado para estimar sua atividade em tecidos com múltiplas isoformas GST expressas (Abel *et al*., 2004). Os resultados contraditórios encontrados com ensaios cinéticos competitivos seriam explicados por maiores atividades conjugativas de isoformas GST afins a CDNB mas não a ATZ que mascarariam conjugações GST afins a ATZ e CDNB. Assim, a capacidade de biotransformação seria maior em peixes que em crustáceo, em fígado que em brânquias e em peixes de ambiente contaminado que em cativeiro.

O segundo capitulo da tese avalio a qualidade espermática e a expressão de genes chaves da espermatogênese e proteção celular de *Danio rerio* exposto a ATZ, em concentrações de relevância ambiental, durante 11 dias. Os resultados mostram diminuição dos parâmetros espermáticos nos grupos expostos a ATZ sugerindo efeitos prejudiciais no espermatozoide, e na capacidade de fertilização do peixe. A motilidade e período de motilidade medem a capacidade de movimentação do espermatozoide na direção ao ovócito para a fertilização e o tempo em que os espermatozoides se mantem movimentando em linha reta, respectivamente (Rurangwa *et al*., 2004). Não se tem estudos dos efeitos da ATZ na motilidade do espermatozoide em espécies aquáticas, mas em estudos com ratos expostos a ATZ, a motilidade espermática foi diminuída (Kniewald *et al*., 2000; Abarikwu *et al*., 2011).

A motilidade diminuída do espermatozoide de grupos expostos a ATZ estaria ligada com a menor funcionalidade da mitocôndria encontrada no estudo; o que é logico devido a que a mitocôndria fornece energia ao espermatozoide para vários propósitos que incluem a motilidade espermática (Ramalho-Santos *et al*., 2009). Neste cenário, a

ATZ ligaria se à enzima ATP sintase, inibindo os processos de fosforilação oxidativa (Hase *et al*., 2008). Estudos em humanos (Troiano *et al*., 1998), equinos (Love *et al*., 2003), ratos (Gravance *et al*., 2001), porcos (Spinaci *et al*., 2005) e carneiros (Martinez – Pastor *et al*., 2004) confirmam que o estado funcional da mitocôndria tem relação com a motilidade espermática.

O maior dano na membrana espermática de grupos expostos a ATZ seria outro dos fatores que explicariam a baixa motilidade. Em peixes, a integridade da membrana do espermatozoide é vital para atingir a despolarização membranal, pelo que é considerada um fator adicional que controla a iniciação da motilidade (Cosson, 2004). Além disso, a integridade diminuída da membrana espermática apoiaria a hipótese do efeito gerador de ERO da ATZ (Jin *et al*., 2010), que através da utilização de prótons pertencentes aos ácidos graxos poli-insaturados da estrutura da membrana do espermatozoide, alcançariam estabilidade (Jones *et al*., 1979). Adicionalmente, estes possíveis níveis aumentados de ERO gerados pela ATZ atingiriam a integridade da cromatina e aumentariam a frequência de danos no DNA (Sharma *et al*., 2012; Zhu *et al*., 2011). No presente estudo, a integridade de DNA do espermatozoide não foi afetada em nenhum dos grupos de exposição de ATZ. Uma possível explicação deste resultado seria a espermatogênese inibida pela ausência da fêmea, considerando que as multiplicações celulares são as etapas susceptíveis ao dano oxidativo e a células espermáticas, os quais foram avaliadas, possuem DNA fortemente empacotado e protegido (Hagedorn *et al*., 2012).

A repressão da expressão de genes chaves da espermatogênese em grupos expostos a ATZ, sugerem seu envolvimento em mecanismos relacionados com a baixa motilidade espermática e poderiam ser utilizados como marcadores moleculares da

qualidade do espermatozoide. A repressão pela ATZ dos genes *SRD5A2* e *CFTR*, afetariam possíveis mecanismos relacionado com a motilidade do espermatozoide. A conversão de testosterona em hidroxitestosterona pela enzima SRD5A2 e chave no processo de maturação do espermatozoide no epidídimo (Robaire e Hamzeh, 2011). Em quanto a proteína CFTR é altamente expressa na cabeça e cauda do espermatozoide pelo que a repressão da sua síntese enzimática afetaria a espermatogênese (Xu *et al*., 2007). Estudos em homens reportam a sua relação, da SRD5A2 e CFTR, com a qualidade do espermatozoide (Zhao *et al*., 2012; Jiang *et al*., 2013.

A geração de agentes oxidantes por efeito de xenobioticos, como ATZ, ativam vias que codificam proteínas de proteção celular e neutralizam o dano oxidativo (Suzuki *et al*., 2005). No entanto, as células sexuais, possuem o DNA compactado que dificulta o acesso de ativação das suas vias antioxidantes, além de não transcrever DNA ou traduzir RNA (Hagedorn *et al*., 2012), e seria insuficiente para a inativação do ERO adicional gerado pela ATZ. No estudo, a repressão da *SOD2* e *GPX4B*, sugerem a menor codificação destas enzimas antioxidantes, e portanto, maior susceptibilidade de ácidos graxos poli-insaturados do ambiente gônadal a ser oxidados pelo estresse oxidativo gerado pela ATZ guardando relação com o maior dano membranal, e a menor motilidade do espermatozoide encontrado no presente estudo. Enquanto o gene *XPC*, cuja enzima codificada ativa o recrutamento de todo o aparelho proteico na reparação de lesões oxidativas de DNA (Nemzow *et al*., 2015), sua expressão foi diminuída em grupos expostos a ATZ, sugerindo diminuição na proteção celular e maior susceptibilidade ao dano oxidativo de DNA na gônada do peixe. No entanto, células espermáticas de grupos expostos a ATZ não mostraram dano de DNA. Possivelmente, o estresse e a falta de estimulo sexual da fémea tenham inibido a espermatogênese na

etapa experimental, detendo as etapas de divisão celular susceptíveis ao estresse oxidativo.

A presença de ATZ na célula ativaria o receptor AHR e induziria a expressão de genes das vias da transformação de xenobióticos (Denison e Nagy, 2003). No estudo, a expressão da *CYP1A* e *GSTP1* foi induzida no fígado de grupos expostos a ATZ, sugerindo uma maior síntese destas enzimas para suas participações nas vias de detoxificação. Os resultados dos poucos estudos realizados em peixes apoiam esta hipótese. Em *Cyprinus carpio L*, a exposição de 4,28; 42,8 e 428 $\mu g.L^{-1}$ ATZ durante 40 dias induziram a expressão de *CYP1A e GSTR* no fígado (Xing *et al*., 2012, 2014). Em embriões *Danio rerio*, a exposição de 5-40 $m.L^{-1}$ ATZ durante 72 horas aumentou a expressão de *CYP1A*; enquanto, a exposição de 0,5 $mg.L^{-1}$ ATZ durante 48 horas induziu a expressão da *GSTP1-2* (Glisic *et al*., 2014).

Os resultados da presente pesquisa mostram que ATZ induz danos na célula espermática no nível molecular diminuindo a funcionalidade da mitocôndria e a integridade da membrana, além de exercer influência na expressão de genes fundamentais envolvidos na espermatogênese (repressão de *SRD5A2* e *CFTR*) e proteção celular (repressão de *SOD2, GPX4B* e *XPC*); o que teriam refletidos nas menores motilidades e tempo de motilidade espermáticas encontrados. No entanto, a indução de genes de biotransformação (CYP1A, GSTP1) e antioxidante (SOD2) no fígado sugerem a ativação de fatores de transcrição como AHR ou NF-κB nos grupos expostos a ATZ que levariam ao cumprimento de funções vitais de proteção celular, tais como o aumento da biotransformação de ATZ e a neutralização do estresse oxidativo gerado pela sua presença.

A inibição da atividade GST pela ATZ, o rápido decaimento de ATZ em ensaios cinéticos analisados por LC-MS/MS e a expressão induzida do gene GSTP; que são alguns dos resultados dos dois capítulos da presente pesquisa, sugerem a GST como umas das enzimas responsáveis da biotransformação da ATZ em crustáceo e peixe.

8. PERSPECTIVAS

A capacidade da GST para biotransformar ATZ observada pelo seu decaimento em analises de LC-MS/MS, e apoiado por padrões similares de atividades basais GST estimadas, a sugere como uma abordagem que pode ser adequada para estudar atividade de isoforma GST-especifica. Após de conhecida a especificidade de uma isoforma GST na metabolização de um xenobiótico, planejamentos com abordagens similares ao presente estudo poderiam ser feitos para estimar atividade GST-especifica.

Com relação aos resultados da exposição de ATZ, embora os resultados de repressão de genes chaves da espermatogênese e proteção celular sugerem-se como vias explicativas da motilidade espermática diminuída, outras pesquisas são necessárias para descobrir os mecanismos explicativos dos efeitos negativos da ATZ nos parâmetros reprodutivos do peixe. Existem evidencias que o Ca^{2+} estaria envolvido em processos do desenvolvimento de espermatozoides, pelo que o estudo da expressão destes genes, como o canal de Ca^{2+} especifico do espermatozoide (CATSPER) e proteases cisteína Ca^{2+} dependentes o calpaina (CAPN), podem também ajudar nas explicações do efeito prejudicial da ATZ nos parâmetros espermáticos.

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10. ANEXO

COMISSÃO DE ÉTICA EM USO ANIMAL
Universidade Federal do Rio Grande
Pró-Reitoria de Pesquisa e Pós-Graduação - PROPESP
ceua@furg.br http://www.propesp.furg.br

CEUA

PARECER Nº P028/2011

PROCESSO Nº	23116.003244/2011-11
CEUA Nº	P013/2011
UNIDADE	ICB
TÍTULO DO PROJETO	Identificação de biomarcadores CYP1 em peixes de interesse para a ecotoxicologia
NÚMERO DE ANIMAIS	320
PROFESSOR RESPONSÁVEL	Juliano Zanette

PARECER DA CEUA:

Após a análise da resposta às pendências encaminhadas no Parecer Nº P013/2011, o projeto foi considerado APROVADO.

Rio Grande, 15/09/2011.

Prof. Dr. Duane Barros Fonseca
Coordenador da CEUA

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CEUA

CERTIFICADO Nº P065/2016

Certificamos que o projeto intitulado "Ensaios in vitro com enzimas de peixe", protocolo nº 23116.004342/2016-72, sob a responsabilidade de Juliano Zanette - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 24 de agosto de 2016 (Ata 009/2016).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq013/2016
COLABORADORES	
VIGÊNCIA DO PROJETO	30/12/2018
ESPÉCIE/ LINHAGEM / RAÇA	*Cyprinus carpio* (carpa) e guarús (barrigudinhos)
NÚMERO DE ANIMAIS	40 carpas e 200 guarús
PESO/ IDADE	Carpas - 10 g; guarús - 4 g
SEXO	Indeterminado
ORIGEM	Carpas – Toca dos Bichos (CNPJ: 11.960.647/0001-81); guarús – 10 locais com diferentes graus de contaminação ambiental.
ENVIO DO RELATÓRIO FINAL	Janeiro de 2019

Rio Grande, 25 de agosto de 2016.

Med. Vet. Márcio de Azevedo Figueiredo
Coordenador da CEUA-FURG

Controle da CEUA - Parecer(es) prévio(s) relacionado(s) ao processo: P053/2016. Página 1/1