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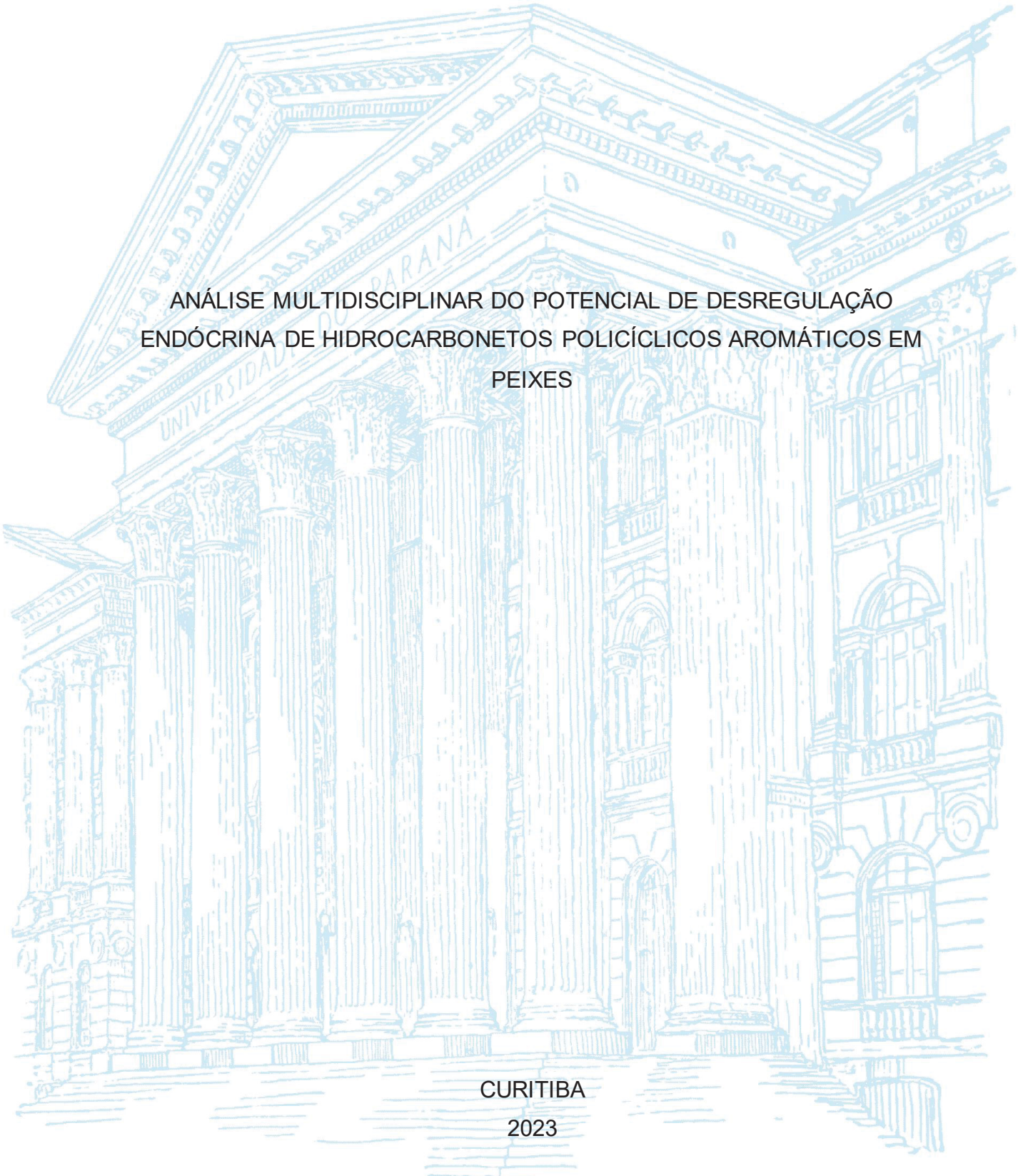
TUGSTÊNIO LIMA DE SOUZA



# ANÁLISE MULTIDISCIPLINAR DO POTENCIAL DE DESREGULAÇÃO ENDÓCRINA DE HIDROCARBONETOS POLICÍCLICOS AROMÁTICOS EM PEIXES

CURITIBA

2023

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# ANÁLISE MULTIDISCIPLINAR DO POTENCIAL DE DESREGULAÇÃO ENDÓCRINA DE HIDROCARBONETOS POLICÍCLICOS AROMÁTICOS EM PEIXES

Tese apresentada ao curso de Pós-Graduação em Biologia Celular e Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Biologia Celular e Molecular.

Orientador: Prof. Dr. Francisco Filipak Neto

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Dedico:

A todos aqueles que me apoiaram e contribuíram para a minha formação durante meu processo de formação enquanto pesquisador

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“E agora José? A festa acabou”

(Carlos Drummond de Andrade)

## RESUMO

Ecossistemas aquáticos estão cada vez mais expostos a poluentes químicos, levantando preocupações sobre seus efeitos na saúde e reprodução de peixes. Este estudo combina descobertas de quatro resumos de pesquisa distintos, lançando luz sobre a relação intricada entre os hidrocarbonetos aromáticos policíclicos (PAHs) e tanto a disrupção endócrina quanto o metabolismo lipídico em espécies de peixes. No primeiro capítulo foi realizado a caracterização da vitelogenina (Vtg) em *Oreochromis niloticus*, enfatizando seu papel como biomarcador para exposição a xenoestrógenos. As diferenças estruturais da Vtg entre as formas químicas, juntamente com insights da bioinformática, aprimoram seu potencial como indicador confiável de disrupção reprodutiva em peixes. Para o segundo capítulo foi analisado a toxicidade de uma mistura complexa de PAHs em hepatócitos primários de *O. niloticus*. A expressão elevada de vtg e receptores de estrogênio sugere uma possível disrupção endócrina devido à exposição. Marcadores de estresse oxidativo fornecem evidências adicionais das respostas celulares aos efeitos tóxicos da mistura. No terceiro capítulo, a análise lipidômica de larvas de zebrafish expostas a PAHs revelou alterações na expressão gênica e nos perfis lipídicos. Essas mudanças refletem o impacto dos PAHs no metabolismo lipídico, com implicações para respostas celulares e possível disrupção de vias de sinalização endócrina. As descobertas sintetizadas enfatizam coletivamente os efeitos abrangentes da exposição aos PAHs em espécies de peixes. Por fim, no quarto capítulo foi empregado modelagem molecular para avaliar o potencial de disrupção endócrina dos PAHs em *Danio rerio*. A correlação entre a estrutura dos PAHs e a afinidade de ligação a proteínas reguladoras endócrinas destaca a complexidade das interações moleculares subjacentes a seus efeitos. Desde alterações estruturais até mudanças na expressão gênica e disrupções lipidômicas, os estudos destacam a complexidade das interações entre poluentes e organismos aquáticos. Essa perspectiva integrada aprimora nossa compreensão dos riscos ecológicos apresentados pelos PAHs e enfatiza a necessidade de abordagens multifacetadas para avaliar e mitigar seus impactos nos ecossistemas aquáticos.



Palavras-chave: Vitelogenina. Reprodução. Cultivo celular. Desreguladores endócrinos. Bioinformática.

## ABSTRACT

Aquatic ecosystems are increasingly exposed to chemical pollutants, raising concerns about their effects on fish health and reproduction. This study amalgamates findings from four distinct research abstracts, shedding light on the intricate relationship between polycyclic aromatic hydrocarbons (PAHs) and both endocrine disruption and lipid metabolism in fish species. The first study delves into the characterization of vitellogenin (Vtg) in *Oreochromis niloticus*, emphasizing its role as a biomarker for xenoestrogen exposure. Vtg's structural differences between chemical forms, coupled with insights from bioinformatics, enhance its potential as a reliable indicator of reproductive disruption in fish. The second investigation examines the toxicity of a complex mixture of PAHs in primary hepatocyte of *O. niloticus.* The elevated expression of vitellogenin and estrogen receptors suggests a potential endocrine disruption due to exposure. Oxidative stress markers provide further evidence of cellular responses to the mixture's toxic effects. In the third study, the lipidomic analysis of zebrafish larvae exposed to PAHs reveals alterations in gene expression and lipid profiles. The fourth study employs molecular modeling to evaluate the endocrine-disrupting potential of PAHs in *Danio rerio*. The correlation between PAH structure and binding affinity to endocrine-regulating proteins underscores the complexity of molecular interactions underlying their effects. These changes reflect the impact of PAHs on lipid metabolism, with implications for cellular responses and potential disruption of endocrine signaling pathways. The synthesized findings collectively emphasize the comprehensive effects of PAH exposure on fish species. From structural alterations to gene expression changes and lipidomic disruptions, the studies underscore the complexity of interactions between pollutants and aquatic organisms. This integrated perspective enhances our understanding of the ecological risks posed by PAHs and emphasizes the need for multifaceted approaches to assess and mitigate their impacts on aquatic ecosystems.


Keywords: Vitellogenin. Reproduction. Cell culture. Endocrine disruptors. Bioinformatics.

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# 1) INTRODUÇÃO

## 1.1) Toxicologia ambiental

O crescente processo de industrialização, bem como o crescimento urbano, tem aumentado consideravelmente o desenvolvimento e emissão de diversos compostos provenientes de processos industriais, agrícolas e de atividades domésticas no meio ambiente (DEBLONDE et al., 2011). Há uma grande preocupação quanto às rotas de exposição, atividade, mecanismo de ação e persistência destes compostos no ambiente, bem como sua relação com os seres vivos.

Sendo assim, com o aumento da concentração ambiental de poluentes, em especial de agrotóxicos e metais, a saúde humana e a biota podem ser afetadas devido ao seu potencial tóxico (BRANDER *et al*., 2016; FREEMAN *et al*., 2016; LIU *et al*., 2016). Além disso, muitos países em desenvolvimento possuem falhas em estabelecer políticas sanitárias, especialmente em áreas de crescimento populacional rápido e descontrolado (PETRIE et al., 2015). Consequentemente, corpos hídricos próximos a grandes centros urbanos ou regiões de intenso cultivo agrícola podem apresentar baixa qualidade, considerando que há uma complexa mistura de contaminantes provenientes de fontes difusas. Porém, apesar da diversidade de procedimentos para o tratamento e reuso de águas residuais de processos industriais, agrícolas e urbanos, a eficácia na remoção de determinadas classes de contaminantes é variável (BOLONG et al., 2009; QIAO et al., 2014).

Geralmente, grande parte desses compostos químicos é classificada como contaminantes de preocupação emergente, termo utilizado para designar compostos potencialmente perigosos, orgânicos ou sintéticos, que apresentaram aumento gradual da sua concentração e detecção no meio ambiente (SAUVÉ e DESROSIERS, 2014; ZENKER et al., 2014). Dentro dessa classe ainda existem alguns compostos químicos categorizados como POPs (*Persistent Organic Pollutants*), os quais são resistentes à degradação ambiental e persistem por muitos anos no solo, sedimento, ar e biota (JACOB, 2013). Apesar de muitos desses contaminantes serem conhecidos e alguns já serem banidos, muitos compostos não possuem legislação ambiental e sanitária vigente para o seu controle, e há escassez de conhecimento acerca de aspectos ecotoxicológicos (MONTAGNER et al., 2017; JACOB, 2013).

As características físico-químicas dos contaminantes influenciam no seu comportamento no ambiente, como transporte e meia-vida, além de permitirem a predição do mecanismo de toxicidade e do potencial de bioacumulação, fatores essenciais para a avaliação de risco e compreensão dos aspectos ecotoxicológicos dessas substâncias (JACOB, 2013; PETRIE et al., 2015). Os POPs são transportados por longas distâncias através do ambiente, fazendo com que eles possuam uma ampla distribuição global, inclusive em regiões onde não se faz uso dessas substâncias (ASHRAF, 2017). Estudos têm demonstrado que esses contaminantes e os seus metabólitos são danosos por provocar efeitos adversos à biota e saúde humana. Desta forma, a presença de POPs em corpos hídricos gera grande preocupação, particularmente porque o tratamento de água empregado nas empresas de saneamento não consegue remover eficientemente esses compostos da água (BOLONG et al., 2009; PETRIE et al., 2015).

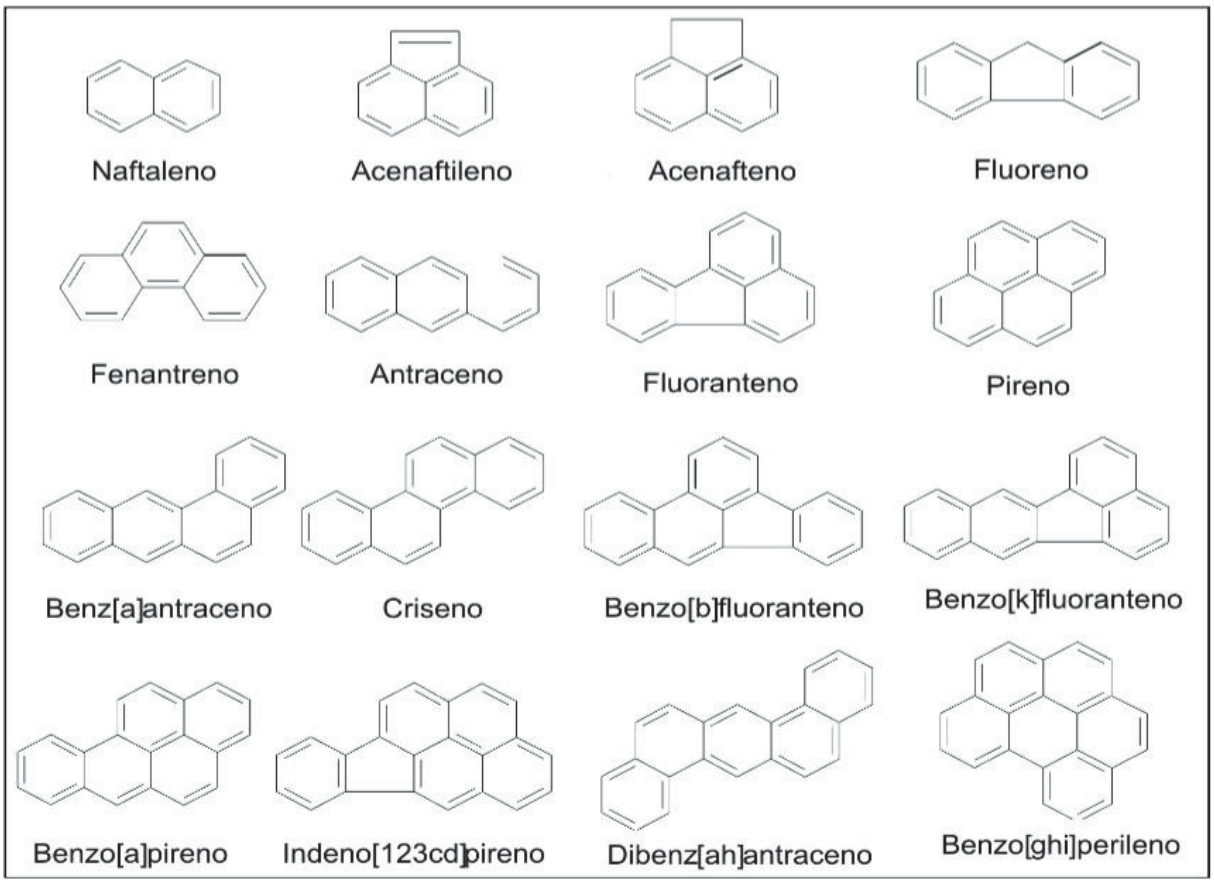
Há uma grande diversidade de classes de contaminantes presentes no solo e em matrizes hídricas devido à grande variedade de atividades antrópicas, como descarte de efluentes brutos ou tratamento inadequado, e escoamento de áreas urbanas e agrícolas (TRAN et al., 2018). Uma série de características químicas também faz com que essas moléculas usualmente sejam de interesse industrial para a produção de solventes, plásticos, fluidos dielétricos e pesticidas (KLEANTHI et al., 2008). Entretanto, apesar de muitos tipos de POPs já serem banidos, é imprescindível considerar a capacidade destes de persistirem no meio ambiente e interferirem nos sistemas biológicos, dado que ainda são detectados no ambiente em concentrações que podem causar efeitos adversos (BARRETO et al., 2020; CARVALHO, 2006). Logo, através do acúmulo desses compostos nos recursos hídricos ou solo, tanto a biota quanto a população humana estão sujeitos ao contato com esses agentes, sendo necessária a realização de estudos de biomonitoramento para avaliar os riscos de exposição e as suas concentrações ambientais (CARVALHO, 2006; CASTRO et al., 2004; GRIZA et al., 2008).

### 1.2) Hidrocarbonetos Policíclicos Aromáticos (HPAs)

Há também outra classe de contaminante que gera preocupação, como os hidrocarbonetos policíclicos aromáticos (HPAs), os quais podem ser de origem pirogênica ou petrogênica (JACOB et al., 2013; WEBER et al., 2011). Os HPAs constituem uma família de mais de 100 compostos orgânicos encontrados como

misturas complexas no ambiente, formados por dois ou mais anéis aromáticos condensados de carbono e hidrogênio (CARUSO e ALABURDA, 2008; MEIRE et al., 2007) (Figura 1).

Figura 1 - Estrutura química dos HPAs




Nessa figura são demonstrados os 16 HPAs prioritários em estudos ambientais de acordo com a Agência de Proteção Ambiental dos Estados Unidos (EPA). Fonte: Meire et al. (2007)

São poluentes de grande importância ambiental e interesse toxicológico por apresentarem propriedades mutagênicas, carcinogênicas, neurotóxicas e atuarem como desreguladores endócrinos em humanos e animais (SCHUG et al., 2015; CARUSO e ALABURDA, 2008). Esses contaminantes são amplamente encontrados em hábitats aquáticos, especialmente em áreas urbanizadas, e a sua concentração hídrica pode variar de 0,03 a 8310000 ng/L (MOJIRI et al., 2019). Muitos estudos têm mostrado que a disponibilidade desses compostos na água promove a bioacumulação em peixes em concentrações de até 4207.5 ng/L, sendo relacionado com os efeitos toxicológicos na biota (MOJIRI et al., 2019). Além disso, há grande preocupação quanto à dinâmica de distribuição dos HPAs no ambiente, especialmente pelo fato de que eles também se acumulam no sedimento e podem

se tornar mais disponíveis e promover danos a organismos bentônicos (MCGRATH et al., 2018). Portanto, é importante considerar as rotas de exposição, atividade, mecanismo de ação e persistência destes compostos no ambiente, bem como sua relação com os seres vivos. Nesse sentido, com o aumento da concentração ambiental dessas classes de contaminantes, tanto a saúde humana quanto a biota podem ser afetadas negativamente devido ao potencial tóxico desses contaminantes (BRANDER et al., 2016; FREEMAN et al., 2015; LIU et al., 2016; YAMAMOTO et al., 2018).

Diversos biomarcadores são empregados para investigar a qualidade de ambientes potencialmente impactados pelo uso de pesticidas ou efluentes urbanos/industriais, como rios cercados por áreas de atividade agrícola (VIANA et al., 2018; BARRETO et al., 2020). Entre eles, as alterações nucleares em eritrócitos e as análises bioquímicas e comportamentais são muito utilizadas, considerando que uma série de pesticidas é capaz de induzir estresse oxidativo, causar alterações comportamentais relacionadas com aspectos neurotóxicos e induzir a expressão de proteínas envolvidas com desregulação endócrina (MARINS et al., 2018; MULLER et al., 2018; PERSCH et al., 2018; SLANINOVA et al., 2009; VIANA et al., 2018; YAMAMOTO et al., 2017; YAMAMOTO et al., 2018). A capacidade de desregulação endócrina promovida por contaminantes ambientais é de grande preocupação, pois pode promover danos reprodutivos a seres humanos e diversas espécies animais, comprometendo o desenvolvimento embrionário e a manutenção da população. Alguns pesticidas organoclorados, bem como os HPAs podem atuar como hormônios sintéticos e xenoestrógenos, causando efeitos estrogênicos em modelos *in vitro* e *in vivo* (FUDVOYE et al., 2014; MARTÍNEZ et al., 2018).

É importante ressaltar que no ambiente natural os organismos geralmente estão sujeitos à exposição a muitos contaminantes que constituem misturas bastante complexas (ARORA et al., 2016; BARRETO et al., 2020; YAMAMOTO et al., 2018), sendo necessário utilizar de ferramentas e experimentos capazes de avaliar respostas toxicológicas em um contexto mais realista. Em humanos, por exemplo, contaminantes isolados e em misturas têm sido associados com desordens hepáticas, neurológicas, reprodutivas e embriológicas, dado a sua capacidade de alterar diversos mecanismos de resposta celular durante o desenvolvimento do organismo (FUDVOYE et al., 2014; NICOLOUPOULOU-STAMATI et al., 2016; SABARWAL et al., 2018; SCHUG et al., 2015). Portanto, o uso indiscriminado de

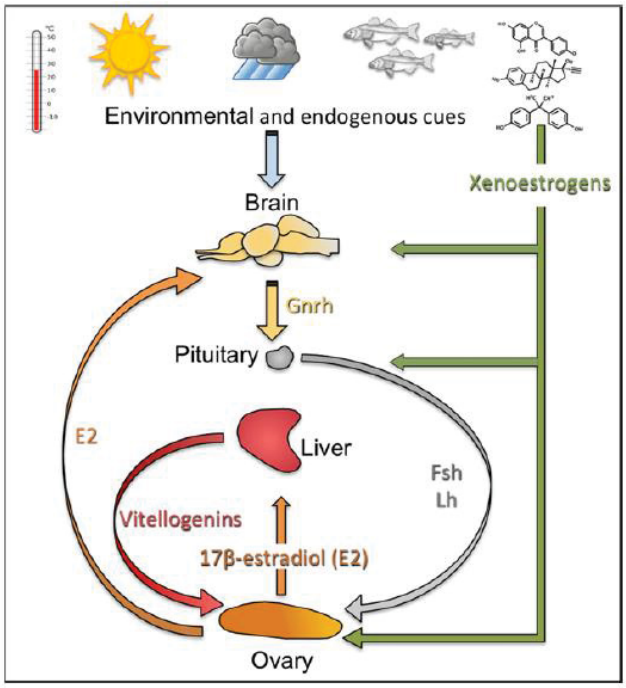
pesticidas e outras classes de poluentes orgânicos, bem como o seu despejo no meio ambiente através de efluentes agrícolas, urbanos ou industriais, pode comprometer diversos níveis de organização biológica. Sendo assim, compreender os processos biológicos sistêmicos, em diferentes níveis de organização fisiológica, envolvendo a resposta do organismo frente ao estresse promovido por misturas complexas de contaminantes ambientais se faz necessário.

### 1.3) Vitelogenina como biomarcador de desregulação endócrina

A vitelogenina (Vtg) é uma proteína evolutivamente conservada em vertebrados ovíparos, sendo precursora das moléculas que compõe o vitelo, a qual é altamente expressa durante a vitelogênese em fêmeas no período reprodutivo, atuando como reserva alimentar no desenvolvimento do embrião (SULLIVAN; YILMAZ, 2017; TOKARZ *et al*., 2015). A Vtg apresenta alto peso molecular, podendo variar de 300 a 600 kDa, e é formada pela união de dois homodímeros polipeptídicos que contêm componentes com fosfato, carboidratos e lipídios, caracterizando a estrutura de uma lipo-fosfo-glicoproteína (SULLIVAN; YILMAZ, 2017). Sua expressão é regulada pela presença de hormônios e, nos indivíduos machos de peixes, a Vtg pode ser expressa devido à presença de desreguladores endócrinos no ambiente aquático (SULLIVAN; YILMAZ, 2017; TOKARZ *et al*., 2015; YAMAMOTO *et al*., 2017).

Diversos fatores ambientais como temperatura e a presença de xenoestrógenos, podem promover uma cascata de efeitos no eixo HPG (hipotálamo-pituitária-gonadal) e modular a síntese de hormonal de 17β-estradiol (E2), necessário para produção da Vtg e proliferação das células germinativas (SULLIVAN; YILMAZ, 2017; TOKARZ *et al*., 2015; YARON e LEVAVI-SIVAN, 2011) (FIGURA 2).

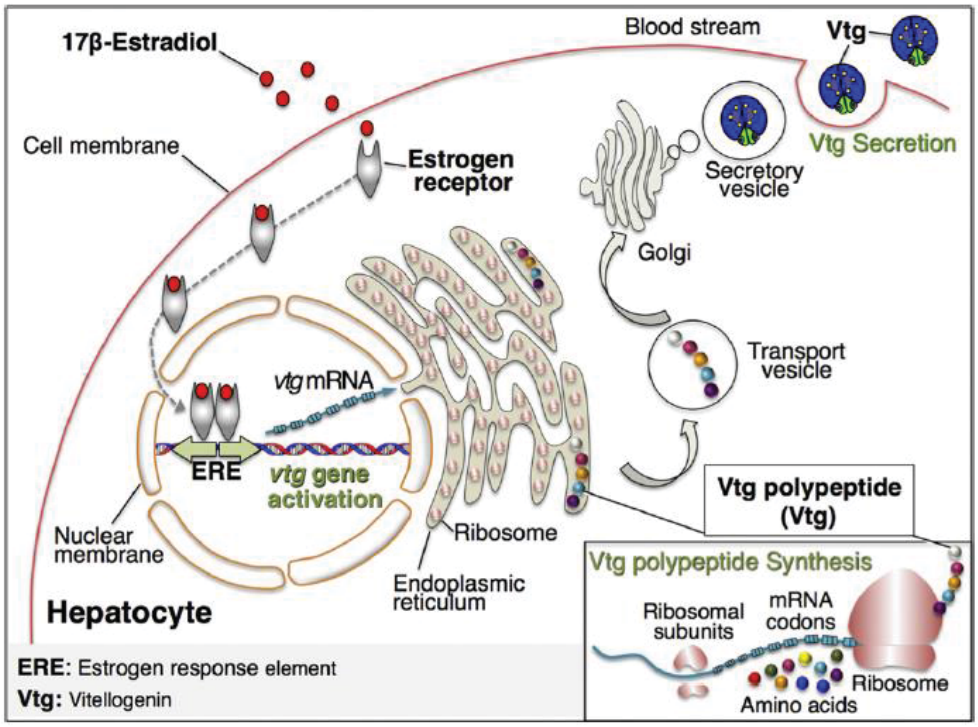
Figura 2 - Eixo HPG em peixes




Vias de regulação da vitelogênese em peixes teleósteos. Fatores abióticos como temperatura, duração do dia, clima e a presença de xenoestrógenos podem influenciar na resposta hormonal promovida pelo eixo hipotálamo-pituitária-gonadal (HPG). GnrH: hormônio liberador de gonadotrofina; FSH: hormônio folículo estimulante; LH: hormônio luteinizante; E2: 17β-estradiol. FONTE: Sullivan e Yilmaz (2017).

A síntese de Vtg ocorre nos hepatócitos e é dependente da interação de E2 com receptores nucleares de estrogênio, sendo que em peixes duas isoformas são conhecidas, a ERα e a ERβ (BABIN *et al.*, 2007; SULLIVAN; YILMAZ, 2017; WANG *et al.*, 2005). O E2 é capaz de se difundir através da membrana plasmática dos hepatócitos e se ligar aos receptores de estrogênio, os quais sofrem alterações estruturais e são translocados para o núcleo. No núcleo, ocorre a dimerização dos receptores, seguida da ligação a regiões do DNA denominadas de **e**lementos **r**esponsivos a **e**strógeno**s** (EREs) e consequente indução da expressão de Vtg e de outros genes (HELDERING *et al.*, 2007; SULLIVAN; YILMAZ, 2017) (FIGURA 3).

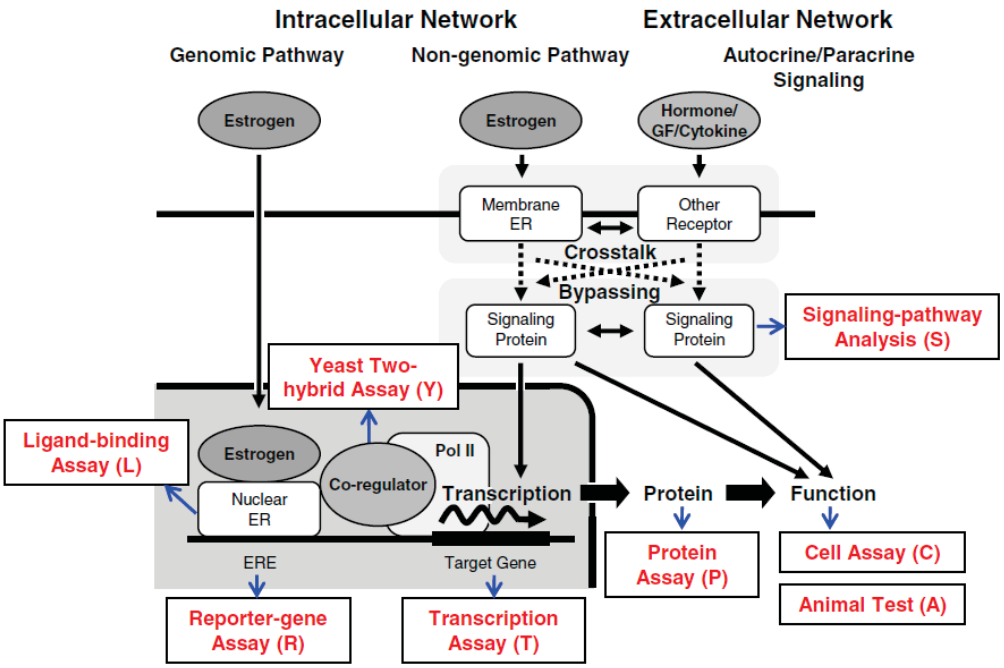
Figura 3 - Expressão de vitelogenina em hepatócitos




Via de produção de vitelogenina em hepatócitos através da ação do 17β-estradiol. O estradiol, presente na corrente sanguínea, se difunde pela membrana plasmática do hepatócito e se liga aos receptores de estrógeno. Em seguida, os receptores são translocados para o núcleo, onde se ligam a regiões envolvidas com resposta estrogênica (EREs) e iniciando a transcrição do gene de vtg. Após, ocorre a tradução do mRNA e secreção da proteína formada para a corrente sanguínea, através do complexo e Golgi. FONTE: Sullivan e Yilmaz (2017).

A resposta estrogênica pode ocorrer em múltiplos níveis, sendo que esses variam de acordo com o mecanismo de ação promovido por determinada molécula sobre a célula, bem como a sua capacidade de ativar os receptores estrogênicos. De acordo com Kiyama e Wada-Kiyama (2015) a rede de resposta estrogênica pode ser classificada em intracelular e extracelular (FIGURA 4). A resposta intracelular corresponde à via clássica (via genômica), envolvendo a transcrição direta de genes-alvo, e a via não-genômica, a qual gera uma cascata de transdução de sinais por meio de proteínas presentes na membrana plasmática (e.g. Proteína G acoplada ao receptor de estrógeno – GPER) (HELDERING *et al*., 2007; KIYAMA; WADA-KIYAMA, 2015; NELSON; HABIBI, 2013). Entretanto, a via extracelular ocorre através de sinais autócrinos ou parácrinos, podendo gerar *cross-talk* entre as vias de sinalização da resposta não-genômica (KIYAMA; WADA-KIYAMA, 2015).

Figura 4 - Vias de resposta estrogênica




Vias de sinalização, *crosstalk*, e rede de respostas para moléculas com atividade estrogênica. Os quadros com letras vermelhas indicam os diferentes níveis onde é possível realizar análises para avaliar o potencial estrogênico de um composto de interesse. FONTE: com Kiyama e Wada-Kiyama (2015).

A partir das diferentes maneiras de modulação de marcadores moleculares da resposta estrogênica, diversos pontos podem ser analisados e empregados para estudos de biomonitoramento ou determinar o potencial desregulador endócrino de contaminantes ambientais. De acordo com o *International Programme on Chemical Safety* da Organização Mundial da Saúde (WHO; IPCS, 2002) um desregulador endócrino pode ser definido como “uma substância exógena ou mistura que altera a função do sistema endócrino e, consequentemente, causa efeitos adversos no organismo, progênie ou subpopulações”. Esses compostos são capazes de interferir no sistema hormonal devido à homologia molecular com hormônios esteroides, alterando a fisiologia reprodutiva do organismo (HAMPL *et al.*, 2016). Além disso, muitos desreguladores endócrinos podem interferir em sítios específicos da produção de hormônios, através da ligação à moléculas transportadoras, alteração da atividade de receptores ou ativando vias de morte celular (HAMPL *et al.*, 2016; KIYAMA; WADA-KIYAMA, 2015).

Neste sentido, diversos trabalhos têm relatado os mais diversos efeitos de desregulação endócrina promovida tanto por metais como por compostos orgânicos, como os agroquímicos, em animais e humanos (CARNEVALI *et al*., 2018; IAVICOLI *et al*., 2009; TAN *et al*., 2009; TONI *et al*., 2017; TOMKINS *et al*., 2018; KIYAMA; WADA-KIYAMA, 2015). Porém, essa resposta pode ocorrer em dois níveis, dado que a exposição a agroquímicos e metais é capaz de induzir e inibir a produção de vtg. Compostos como methoxychlor, diazinon e misturas complexas de vários pesticidas organoclorados aumentam os níveis plasmáticos de vtg, bem como a expressão de genes relacionados com a resposta estrogênica em peixes (BLUM *et al.*, 2008; INUDO *et al.*, 2004; KORKMAZ; DÖNMEZ, 2017). Em contrapartida, o pesticida cipermetrina modula o nível de vtg no fígado (redução: 0,29 para 0,22 ng/mg) e nas gônadas (aumento: 22,4 para 29,5 ng/mg), demonstrando que a atividade de desregulação endócrina pode ocorrer em diferentes órgãos-alvo (SARAVANAN et al., 2016). Já os metais, geralmente, possuem atividade anti-estrogênica, o que pode ser relacionado com seu efeito citotóxico (MONCALEANO-NIÑO *et al.*, 2017). Entretanto, misturas entre diferentes metais ou a co-exposição desses com moléculas orgânicas é capaz de ativar mecanismos de resposta estrogênica, aumentando a produção de vtg (OLIVARES-RUBIO *et al.*, 2016).

Sendo assim, técnicas como o cultivo primário de hepatócitos de diversas espécies de peixes têm sido amplamente empregadas para realizar *screening* de substâncias com potencial atividade estrogênica, avaliando, principalmente, a capacidade desses compostos de induzir a expressão de Vtg (CHEN; CHAN, 2016; TAKEMURA; KIM, 2001; LIU *et al*., 2007; NAVAS *et al*., 2006; PETERSEN *et al*., 2017). Dessa forma, analisar a indução da expressão de Vtg sob diferentes tipos de exposição (*in vivo* e *in vitro*) pode ser uma poderosa ferramenta para identificação de compostos com capacidade de desregulação endócrina, bem como a presença destes em ambientes aquáticos.

### 1.4) Desregulação endócrina e metabolismo lipídico

Durante o processo de metabolização de contaminantes ambientais – em sua maioria via citocromos P450 – tanto as moléculas parentais quanto seus metabólitos podem exercer múltiplos efeitos no organismo, dentre eles a desregulação endócrina (HAMPL et al., 2016; ANZENBACHER et al., 2001). Compostos com esse potencial podem afetar vias biológicas relevantes para a biossíntese e manutenção da

homeostase de hormônios por meio da inibição de proteínas chaves para esses processos, além de interferirem com a cinética de hormônios endógenos causando desordens metabólicas lipídicas (COMBARNOUS e NGUYEN, 2019; COSTER e LAREBEK, 2012; HAMPL et al., 2016). Nesse sentido, compostos organoclorados, retardantes de chama polibromados, substâncias perfluoradas, alquilfenóis, ftalatos, pesticidas, aromáticos policíclicos hidrocarbonetos, solventes e alguns metais demonstraram possuírem efeitos desreguladores endócrinos (COSTER e LAREBEK, 2012).

Alguns eventos-chave podem caracterizar, por exemplo, contaminantes ambientais que atuam no metabolismo lipídico como obsegênicos, já que podem promover o aumento do acúmulo de lipídios e o aumento do peso em larvas de zebrafish (RIU et al., 2014). Como mecanismo de ação, a maioria desses compostos atua por meio da ativação dos receptores ativados por proliferadores de peroxissomos nucleares (PPARs) (YANG et al., 2015), que são reguladores essenciais em diversos processos relacionados ao metabolismo lipídico (LEMPRADL et al., 2015) e são ativados naturalmente por hormônios lipofílicos, ácidos graxos e seus metabólitos (YANG et al., 2015). Independentemente de seu envolvimento em diferentes aspectos do metabolismo lipídico, as diferentes isoformas do PPAR (PPAR-α/β/Y) formam heterodímeros com o receptor de ácido retinoico 9-cis (RXR) para se tornarem funcionalmente ativas (DEN BROEDER et al., 2015; IBABE et al., 2005; KERSTEN, 2008; WALCZAK e TONTONOZ, 2002). A ativação do complexo PPAR-Y/RXR parece ser o regulador principal da adipogênese (YANG et al., 2015), pois se liga aos elementos de resposta a proliferadores de peroxissomos (PPREs) em vários genes-alvo (incluindo apolipoproteínas) para regular sua transcrição (YANG et al., 2015; BERGER e MOLLER, 2002; CHIARELLI e MARZIO, 2008). A ativação do complexo PPAR-Y/RXR leva ao aumento dos transcritos que codificam várias apolipoproteínas, aumentando assim os níveis de lipoproteínas após a ligação a diferentes fosfolipídios (CINDOROVA-DAVIES et al., 2017), como foi demonstrado em diferentes organismos modelo (KERSTEN, 2008; DAHABREH e MEDH, 2012; HAI et al., 2015). Além disso, um outro tipo de receptor nuclear, lx (liver x factor), também pode atuar em conjunto com os receptores descritos anteriormente para regular processos do metabolismo lipídico (LI e GLASS, 2004; ULVEN et al., 2005).

## 1.5) Ferramentas utilizadas para avaliação do potencial tóxico em peixes

Diversos biomarcadores são empregados para investigar a qualidade de ambientes potencialmente impactados pelo uso de pesticidas ou efluentes urbanos/industriais, como rios cercados por áreas de atividade agrícola (VIANA et al., 2018; BARRETO et al., 2020). Entre eles, as alterações nucleares em eritrócitos e as análises bioquímicas e comportamentais são muito utilizadas, considerando que uma série de pesticidas é capaz de induzir estresse oxidativo, causar alterações comportamentais relacionadas com aspectos neurotóxicos e induzir a expressão de proteínas envolvidas com desregulação endócrina (MARINS et al., 2018; MULLER et al., 2018; PERSCH et al., 2018; SLANINOVA et al., 2009; VIANA et al., 2018; YAMAMOTO et al., 2017; YAMAMOTO et al., 2018). A capacidade de desregulação endócrina promovida por contaminantes ambientais é de grande preocupação, pois pode promover danos reprodutivos a seres humanos e diversas espécies animais, comprometendo o desenvolvimento embrionário e a manutenção da população. Alguns pesticidas organoclorados, bem como os hidrocarbonetos policíclicos aromáticos (HPAs) podem atuar como hormônios sintéticos e xenoestrógenos, causando efeitos estrogênicos em modelos *in vitro* e *in vivo* (FUDVOYE et al., 2014; MARTÍNEZ et al., 2018).

Os dados gerados através de experimentos biológico possibilitam a estruturação de Adverse Outcome Pathways (AOPs), as quais são uma abordagem para descrever e prever os efeitos adversos de substâncias tóxicas no organismo (TOLLEFSEN et al., 2014). Essas vias representam uma série de eventos biológicos interconectados que levam a um resultado adverso, como a doença ou lesão, a partir da exposição a uma substância. O uso de AOPs na toxicologia permite a integração de informações experimentais e biológicas, melhorando a compreensão dos mecanismos subjacentes aos efeitos adversos de uma substância e facilitando a previsão de seus efeitos em diferentes organismos (STINCKENS et al., 2018; TOLLEFSEN et al., 2014). Isso é útil para a identificação precoce de substâncias potencialmente tóxicas e para o desenvolvimento de estratégias de avaliação e gestão de risco.

Além disso, técnicas “ômicas” (transcriptômica, lipidômica e metabolômica) têm sido utilizadas recentemente na área da toxicologia ambiental para avaliar o efeito tóxico sistêmico do organismo frente à exposição a contaminantes ambientais,

utilizando embriões de *Danio rerio* (*zebrafish*) como modelo de estudo (MARTÍNEZ et al., 2018; MARTÍZNEZ et al., 2020a; MARTÍNEZ et al., 2020b; ORTIZ-VILLANUEVA et a., 2017). O uso de “ômicas” para análises ecotoxicológicas pode ser considerado um avanço e inovação tecnológica para a área, especialmente por serem técnicas elaboradas e com alto poder de análise, ao contrário da avaliação de *endpoints* específicos. Dessa forma, compreender as vias celulares e efeitos de contaminantes ambientais em diferentes modelos de estudo facilita a compreensão dos processos envolvidos com a toxicidade desses compostos, podendo servir como base para programas de mitigação de danos.

### 1.6) Uso de cultivo celular em toxicologia ambiental

A cultura de células de peixes tornou-se uma ferramenta cada vez mais importante na toxicologia ambiental, fornecendo um meio para estudar os efeitos de poluentes em espécies de peixes, eliminando a necessidade do sacrifício de animais. Este método provou ser uma ferramenta valiosa para prever a toxicidade de produtos químicos e seu potencial impacto nos ecossistemas aquáticos (BOLS et al., 2005). Além disso, tem a vantagem de serem relativamente fáceis de manter em condições de laboratório, proporcionando um ambiente controlado e padronizado para testar a toxicidade de produtos químicos (ZURITA et al., 2019).

Uma das principais vantagens da cultura de células de peixes em toxicologia ambiental é a capacidade de testar os efeitos dos poluentes em nível celular. Ao examinar o impacto de produtos químicos em células individuais, os pesquisadores podem obter uma melhor compreensão dos mecanismos pelos quais os poluentes causam danos aos peixes e outras espécies aquáticas (STADNICKA-MICHALAK et al., 2015). Além disso, as culturas de células podem ser usadas para estudar os efeitos de poluentes em tecidos ou sistemas de órgãos específicos, como brânquias ou fígado, que são críticos para a saúde geral e sobrevivência dos peixes (BOLS et al., 2005). Nesse sentido, o uso de células imortalizadas possibilita a avaliação do potencial tóxico em diferentes vertentes de compostos amplamente presentes no ambiente sem a necessidade do uso de animais (ZURITA et al., 2019).

O uso de culturas de células de peixes em toxicologia ambiental também demonstrou fornecer dados mais confiáveis em comparação com estudos in vivo tradicionais. Isso se deve à capacidade de controlar uma variedade de variáveis, como temperatura, luz e pH, que podem afetar os resultados de estudos *in vivo*

(BOLS et al., 2005). Além disso, culturas de células podem ser usadas para estudar os efeitos de poluentes em diferentes estágios do ciclo de vida dos peixes, fornecendo informações sobre o impacto de poluentes em peixes juvenis e adultos (STADNICKA-MICHALAK et al., 2015). Apesar das muitas vantagens da cultura de células de peixes em toxicologia ambiental, também existem algumas limitações a serem consideradas. Uma das principais limitações é a dificuldade em replicar com precisão o ambiente complexo dos ecossistemas aquáticos em laboratório (ZURITA et al., 2019; BOLS et al., 2005). Além disso, o uso de culturas celulares pode ser limitado pela disponibilidade de linhagens celulares apropriadas, bem como pelos custos associados à manutenção e condução de experimentos com culturas celulares (BOLS et al., 2005). Entretanto, apesar dessas desvantagens ainda assim é possível obter resultados confiáveis e avaliar mecanismos celulares importantes para compreender o potencial tóxico de substâncias presentes no ambiente, bem como o seu potencial efeito no organismo.

### 1.7) Toxicologia computacional

A compreensão das interações entre moléculas é fundamental para a investigação de processos bioquímicos e para o desenvolvimento de novas moléculas (PRIETO-MARTÍNEZ et al., 2018). Técnicas de toxicologia computacional, como o docking molecular e a dinâmica molecular, têm desempenhado um papel crucial na modelagem e análise dessas interações, permitindo insights valiosos sobre os mecanismos moleculares de ação de desreguladores endócrinos e agrotóxicos em organismos aquáticos (YANG et al., 2019; VUTUKURU et al., 2016). O docking molecular é uma técnica computacional amplamente utilizada para prever a conformação preferencial de ligantes em relação a uma proteína-alvo (ALONSO et al., 2006). Essa abordagem combina algoritmos de busca com funções de energia (kcal/mol) que avaliam a complementaridade entre as superfícies moleculares na tentativa de estimar a afinidade de interação entre essas moléculas (PRIETO-MARTÍNEZ et al., 2018; ALONSO et al., 2006).

Por outro lado, a dinâmica molecular (DM) é uma técnica que também utiliza as estruturas 3D como o docking, porém simula o movimento dos átomos em uma escala de tempo por meio da resolução das equações de movimento de Newton e não como uma interação pontual (PRIETO-MARTÍNEZ et al., 2018; ALONSO et al., 2006). Essas simulações são úteis para estudar a flexibilidade das proteínas, as

mudanças conformacionais e as interações dinâmicas entre ligantes e alvos, bem como a estabilidade do complexo ligante-proteína (ALONSO et al., 2006). O aumento dessa capacidade de análise computacional permitiu simulações de DM mais longas e realistas, proporcionando insights sobre eventos biologicamente relevantes sobre a ação de contaminantes em processos como câncer (MONTES-GRAJALES e OLIVERO-VERBEL, 2020). A importância da integração dessas duas técnicas é que o docking molecular pode fornecer uma primeira estimativa da conformação de ligantes ligados à proteína, que pode ser usada como ponto de partida para simulações de DM. Essas simulações podem revelar interações dinâmicas, efeitos de solvatação e mudanças conformacionais que não seriam observados apenas com o docking estático. A abordagem conjunta tem sido empregada com sucesso em estudos de afinidade de ligantes, compreensão de mecanismos de ligação e otimização de compostos candidatos (MONTES-GRAJALES et al., 2018; MONTES-GRAJALES et al., 2013; MONTES-GRAJALES e OLIVERO-VERBEL, 2020).

A Biologia de Sistemas é uma abordagem interdisciplinar que visa compreender os sistemas biológicos como um todo, em vez de focar em componentes individuais. Uma parte fundamental dessa abordagem envolve a construção e análise de redes de interação molecular, além da realização de análises de enriquecimento funcional para extrair informações significativas das redes biológicas complexas de dados provenientes de técnicas ômicas (PINU et al., 2019; YUAN et al., 2008).

As redes de interação molecular representam as relações entre diferentes moléculas biológicas, como proteínas, genes, metabólitos e interações de proteína-proteína (PINU et al., 2019). Essas redes podem ser construídas a partir de dados experimentais, como interações físicas ou regulação gênica, e podem ser obtidas de bancos de dados públicos (PINU et al., 2019; NANDAGOPEL e ELOWITZ, 2011; YUAN et al., 2008). A análise de redes permite identificar proteínas centrais (hubs), módulos funcionais e padrões de conectividade que podem revelar insights sobre processos biológicos complexos (ROY, 2012; ESCHRICH et al., 2009).

As análises de enriquecimento funcional são uma parte essencial da Biologia de Sistemas, permitindo atribuir funções biológicas às moléculas presentes nas redes de interação. Essas análises comparam as moléculas da rede com conjuntos de genes ou proteínas previamente caracterizados, como aqueles associados a vias metabólicas, processos celulares ou doenças (TIPNEY e HUNTER, 2010). Métodos

como Gene Ontology (GO) e análises de vias metabólicas ajudam a identificar termos enriquecidos, fornecendo informações sobre as funções biológicas predominantes na rede (LOPEZ-IBAÑEZ et al., 2023). Nesse sentido, o uso dessas ferramentas computacionais tem sido cada vez mais utilizadas para estudos de toxicologia ambiental, na tentativa de estimar de maneira holística como diferentes tipos de desreguladores endócrinos podem afetar o desenvolvimento e fisiologia de organismos aquáticos (SCHNEIDER et al., 2019; ORTIZ-VILLANUEVA et al., 2018; MARTÍNEZ et al., 2018; MARTÍNEZ et al., 2020).

## 2) OBJETIVOS

### 2.1) OBJETIVO GERAL

Avaliar o potencial desregulador endócrino de HPAs em peixes, utilizando uma abordagem multidisciplinar através de metodologias *in silico*, *in vitro* e *in vivo*.

### 2.2) OBJETIVOS ESPECÍFICOS

Capítulo I: Physicochemical and bioinformatic characterization of Oreochromis niloticus vitellogenin as an endocrine disruption biomarker

- Purificar vitelogenina do plasma de exemplares de *Oreochromis niloticus* machos induzidos com 17α-etinilestradiol (EE2) através de cromatografia de troca aniônica;
- Avaliar as características físico-químicas da vitelogenina em diferentes condições de pH, salinidade, temperatura e estabilidade;
- Caracterizar diferentes aspectos estruturais como composição de aminoácidos, predição da estrutura 3D e sítios de N e O-glicosilação da vitelogenina de *Oreochromis niloticus*.

Capítulo II*:* Exploring the toxicity and endocrine disrupting potential of a complex mixture of PAHs in the estrogen pathway in Oreochromis niloticus hepatocytes

- Validar o cultivo primário de hepatócitos de *O. niloticus* como modelo de responsivo a agentes desreguladores endócrinos, utilizando EE2 e tamoxifeno como agonista e antagonista, respectivamente;

- Realizar um *screening* com concentrações ambientalmente relevantes de uma misuta complexa de HPAs utilizando hepatócitos primários como modelo de estudo;

- Avaliar a modulação da expressão de genes envolvidos com resposta estrogênica, através da via genômica e não-genômica, em hepatócitos após a exposição a mistura de HPAs;

- Utilizar ferramentas bioinformáticas para explorar os resultados encontrados em um contexto sistêmico de biologias de processos.

Capítulo III: Molecular and lipid profile of zebrafish larvae exposed to PAHs: implications on endocrine disruption

- Realizar um screening de concentrações ambientalmente relevantes de mistura de HPAs;

- Analisar o perfil lipidômico de larvas expostas a concentrações sub-letais da mistura de HPA;

- Avaliar a modulação da expressão de genes envolvidos com a resposta de HPAs, bem como genes relacionados com o metabolismo lipídico;

Capítulo IV: Structure-based modeling to assess binding and endocrine disrupting potential of polycyclic aromatic hydrocarbons in *Danio rerio*

- Utilizar ferramentas bioinformáticas e de biologia estrutural como método de avaliação do potencial de desregulação endócrina de múltiplos HPAs;

- Elencar quais HPAs estão mais propensos a apresentar maior potencial de afinidade com proteínas que atuam em diferentes etapas da regulação endócrina (receptores nucleares e transportadores);

- Caracterizar a interação entre os HPAs afinidade de ligação com as proteínas-alvo e validar os resultados com dinâmica molecular e *training set*;

## 3) RESULTADOS

Os resultados obtidos nessa tese serão apresentados no formato de capítulos, onde cada um será publicado como um artigo original em revistas científicas internacionais.

*Capitulo I*

# Physicochemical and bioinformatic characterization of *Oreochromis niloticus* vitellogenin as an endocrine disruption biomarker

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**Abstract**

Aquatic biota is increasingly being exposed to chemical pollutants due to human activities and the relationship between the level of environmental pollution and fish reproduction is a continuously ongoing issue. The vitellogenin (Vtg) protein synthesis can be induced in the liver of juvenile and male fish after stimulation of the estrogen receptor and therefore, Vtg has been used as a biomarker of xenoestrogen exposure in several fish species. The current study reported the first physicochemical characterization of Vtg from *Oreochromis niloticus*. Adult male fish were exposed to 17α-ethinylestradiol for Vtg induction. Purified vitellogenin from plasma showed low stability at 25 ºC and 4ºC in saline conditions, and good stability in acidic (low pH) or in heated conditions. The 3D modeling provided useful information on the structure of *O. niloticus* Vtg showing conserved structural features. According to bioinformatics and experimental results, there are important structural differences between the two chemical forms of Vtg (VtgAb and VtgC) in a phylogenetic context. The present results add information about the development of ecotoxicological immunoassays to study the endocrine disruption in *O. niloticus* improving the Vtg performance as a biomarker of reproduction in fish.

**Keywords:** Nile Tilapia, Ecotoxicology, Immunoassay, Bioassay, 3D model, Phylogenetic.

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## Introduction

Vitellogenins (Vtgs) are large serum glycolipophosphoproteins (300–640 kDa) and yolk protein precursors synthesized by the liver of female organisms during vitellogenesis in response to 17β-estradiol [1, 2]. This protein was first described in insects by Pan [3] et al. and since then it has been isolated and characterized from diverse fish species [4, 5, 6). Although fish Vtg possess similar biochemical properties, they differ considerably in their molecular weights, polypeptide patterns and post-translational modifications [7 8]. Vtgs are studied for their role in reproduction and as a biomarker for endocrine disruption. After the expression in liver and secretion into the bloodstream, Vtg is taken up by developing oocytes via Vtg receptor [9], where is cleaved into smaller yolk proteins like lipovitellin (Lv), phosvitin (Pv), β′ component (β′) and C-terminal peptide (c) [2, 10] Vtgs are present in almost all oviparous organisms providing an important source of nutrients for the embryo development [11, 12, 13]. In normal physiological conditions, male organisms do not synthesize Vtg due to low or undetectable levels of estrogenic hormones in the plasma. However, these organisms can potentially synthesize the protein like females as a consequence of exposure to xenoestrogens [14]. Thus, the detection of Vtg occurrence in blood plasma of male is a consolidated biomarker used to investigate the bioavailability of estrogenic disruptors in fish [5, 15, 16]. Among the negative effects of exposure to endocrine disruptors, male fish can present kidney damage, increased susceptibility to infection, decreased testicular growth and spermatogenesis, and changes in sexual maturity [16, 17, 18, 19], leading to negative consequences for fish reproduction, a relevant endpoint for ecological studies. Considering that Vtg is a female-specific protein, the plasma of sexually mature male fish has been used to assess the presence of endocrine disruptors and levels of environmental pollution in ecotoxicology studies [15, 20, 21].

Bioinformatics is one of the ongoing trends of biological research integrating gene-based information and computational technology [22]. It synthesizes complex biological information from multiomics data by employing several bioinformatics tools [23], that analyze a vast biological dataset using the available databases [24]. The bioinformatics can be used for genomic data manipulation, genome annotation, expression profiling, molecular folding, protein characterization, modeling, biological network, and systems biology [25, 26]. Therefore, this area of science can contribute

to specified fields of ecotoxicology, such as disease diagnosis, aquatic health management, fish nutritional aspects, culture-able strain development and characterization of macromolecules of interest.

*Oreochromis niloticus* (Nile tilapia) is a popular freshwater fish in aquaculture due to its high growth rate, easy adaptation to commercial diets, and high tolerance against diseases and environmental stress [27]. This species has been recently used as a tropical fish model for studying the effects of different pollutants [16, 28, 29, 30, 31, 32]. The metabolism studies involving *O. niloticus* reproduction is of great interest as this species is largely used as protein source in human died and ecotoxicological studies.

The present investigation, aimed to characterize the *O. niloticus* vitellogenin through bioinformatics tools and analysis of Vtg stability to different physicochemical conditions, to provide useful information for future studies in ecotoxicology and fish reproduction.

## 2) Material and methods

Six male fish weighing ~350 g (300-400 g) were obtained from Panama fish-farming (Santa Catarina State, Brazil) and transported to the laboratory at Federal University of Paraná (UFPR). Prior to the experiment, the animals were distributed into tanks with 150 L capacity of a flow-through system containing submerged water circulation pumps with controlled water conditions, including dissolved oxygen, temperature, pH, nitrite and ammonia. The tanks also received a filter system composed of quartzite for biological filtration, activated charcoal and zeolite for nitrite and ammonia removal and Perlon™ type blanket for mechanical filtration of residues such as feces and food. The experiment was approved by the Ethics Committee for the Use of Animals (CEUA) of the UFPR (certificate 1323).

### 2.2) Induction of vitellogenin synthesis and sampling

To induce maximal Vtg levels in the plasma of *O. niloticus* 20 $mg.kg^{-1}$ of 17α-ethinylestradiol (EE2) was injected by a single intraperitoneal injection containing EE2 diluted in canola oil [15, 33]. After the 15th day of exposure, the fish were anesthetized with MS222 (0.12% ethil-ester-3-aminobenzoic acid in water, Sigma/Aldrich®) and the blood was sampled as described by [34] et al. Blood samples were mixed with the anti-proteolytic PMSF (phenyl-methyl-

sulfonylfluoride−10 mM and centrifuged at 4,000 *g* for 2 min at room temperature to obtain plasma samples (stored at −80 °C). After blood collection, the fishes were sacrificed with a spinal section (Figure 1).

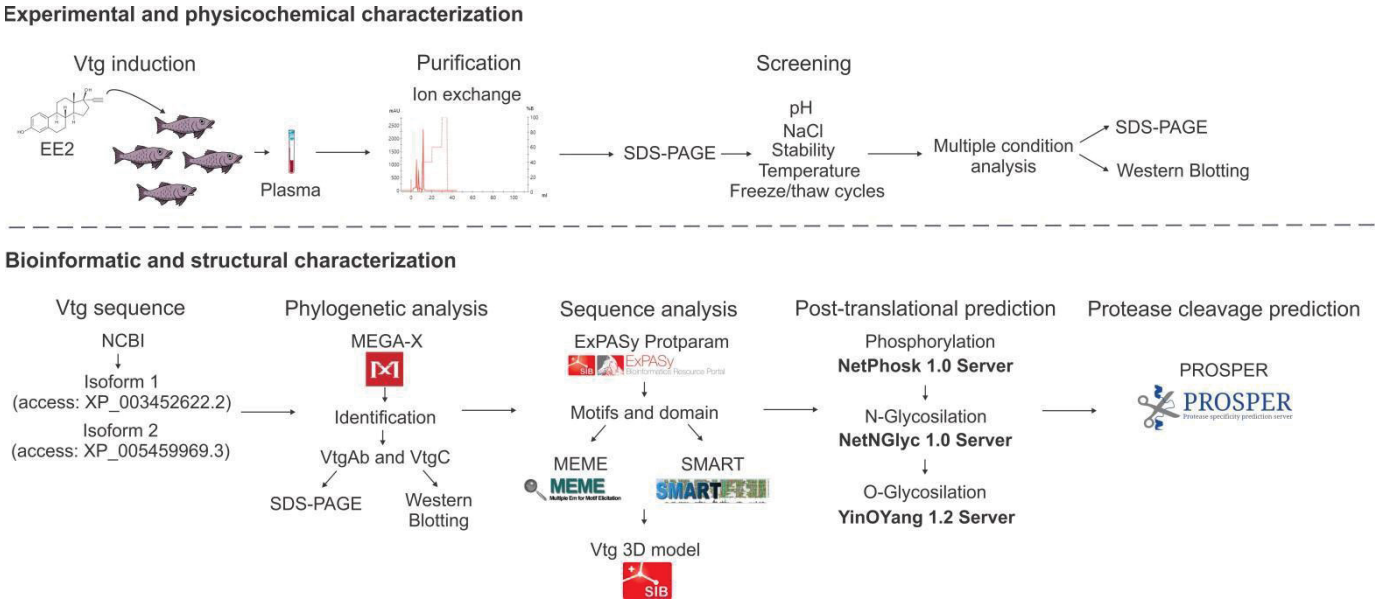


Figure 1 - Experimental design of vitellogenin induction in Oreochromis niloticus. Vtg was induced in *Oreochromis niloticus* by 17α-ethinylestradiol intraperitoneal injection and purified by ion exchange chromatography. Vtg stability was tested for different physicochemical conditions and analyzed by SDS-PAGE and western blotting. Vtg amino acid sequences from NCBI was accessed and used for phylogenetic and sequence analyses, post-translation and protease cleavage predictions.

### 2.3) Purification methods

For Vtg purification, the plasma was diluted (1:5) in buffer A (Tris-HCl 20 mM, pH 7.4) and applied in the ÄKTA go protein purification system (Cytiva©). For comparison purposes, two ionic exchange chromatographic methods were tested: the HiTrap Q HP 1 mL and the HiTrap DEAE capto 1 mL, both anion exchange columns (Cytiva©), with a 40 and 70 mL of total sample volume, respectively. The protein elution was performed by buffer B gradient (0-100% of Tris-HCl 20 mM, pH 7.4, and 1 M NaCl). A 0.5 mL volume from the fractions pool with the best yield and purity was loaded into the Superdex 200 Increase 10/300 GL column (Cytiva©) for size exclusion chromatography (SEC). The fractions, obtained according to the absorbance peaks at 280 nm, were evaluated by SDS-PAGE.

## 2.4) Physicochemical characterization

### 2.4.1) pH

The buffer of the Vtg samples (Tris-HCl, pH 7.4) was replaced using a disposable PD-10 Desalting Columns (17-0851-01, Cytiva©) for conditioning the sample in the desired pH buffers. To this end, Vtg stability tests were performed in: i) 20 mM MES (2-(N-morpholino) ethanesulfonic acid monohydrate) buffer, pH 4.0; ii) 20 mM Tris-HCl buffer, pH 7.4; iii) 50 mM Carbonate/Bicarbonate sodium buffer, pH 10.0.

### 2.4.2) Salinity

Different concentrations of sodium chloride (0, 0.5, 1.0 e 2.0 M NaCl) was added to the Vtg samples in order to characterize their stability regarding the aggregation.

### 2.4.3) Thermal stability

Two thermal stability tests were carried out. In the first test, the samples were kept at 4ºC and at room temperature (25ºC) for 120 h, with sampling at 24, 72 and 120h. In the second test, the samples were kept at 30, 60 and 90 ºC for 5 and 10 min.

### 2.4.4) Freezing and thawing

The protein was stored in a 20 mM Tris-HCl buffer (pH 7.4) containing a cryoprotective agent (0.1% tween, 25% glycerol or 50% glycerol). In total, 5 thawing cycles of the same sample were performed every 24 h for SDS-PAGE evaluation of Vtg fragmentation.

### 2.4.5) Multiple condition analysis

After testing the physicochemical stability of Vtg in isolation, some conditions were combined, as follow: pH 4.0, sample preparation (heated/unheated), thermal stability (5 thawing cycles), storage temperature ( -20, 4 and 25 ºC), addition of glycerol (25%) and NaCl (0 and 2 M). The tests were performed as described above. After the stability tests, all samples were centrifuged at 12,000 *g* and 4 ºC and stored performed in the absence of protease inhibitors.

### 2.5) SDS-PAGE

The SDS-PAGE was performed to obtain the electrophoretic profile of the protein content and evaluation of the tested conditions. Samples were denatured under reducing conditions in sample buffer (50 mM Tris-HCl, pH 6.5, 10% of Glycerol, 75 mM Dithiothreitol, 2 mM EDTA, 2 % of SDS and bromophenol blue) and heated to 95 ºC for 5 min. The run was performed in a vertical electric field using Mini-PROTEAN® Tetra Vertical Electrophoresis systems (Bio-Rad) in 0.75 mm polyacrylamide gels (9%). The gels were stained with Coomassie Brilliant blue R-250 and scanned for band identification. All reagents were purchased from Sigma-Aldrich®.

### 2.6) Western blotting

Samples previously separated by SDS-PAGE were transferred to 0.45 µm nitrocellulose membrane (Amersham™ Protran® Western blotting membranes, Merck) in a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) for 60 min at 25V. Non-specific sites were blocked with 1% (w/v) of Bovine Serum Albumin for 60 min. The membrane was washed in phosphate buffer saline containing 0.05 % Tween-20 (PBS-T) and incubated with 1.0 µg/mL of anti-vitellogenin primary antibody for 16 h at 4 ºC. Then, the membrane was washed three times for 5 min and incubated with anti-Rabbit secondary antibodies alkaline phosphatase (AP) conjugate (1:10,000, Catalog #31341, ThermoFisher Scientific) for 60 min and washed as described above. The reaction was revealed by NBT/BCIP (nitro blue tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate) color development substrate (NBT/BCIP Ready-to-Use Tablets, Catalog 11697471001, Roche). The antibodies used in this study were produced in rabbits immunized with a recombinant fragment of vitellogenin (VTG1) from *Oreochromis aureus* obtained from inclusion bodies of *Escherichia coli*. Both the antibodies and the recombinant protein were obtained at the Cell Toxicology Laboratory at UFPR by Folle [35].

### 2.7) Bioinformatic analysis

#### 2.7.1) Sequence analysis

Bioinformatics analyses were performed using the amino acid sequence of two Vtg isoforms of *O. niloticus* (Nile Tilapia) available on NCBI (*National Center for Biotechnology Information*), access code XP_003452622.2 (1789 amino acids,

predicted mass: 195 kDa) and XP_005459969.3 (1271 amino acids, predicted mass: 141 kDa). Multiple sequence alignment with the Vtg of *O. niloticus* and other fish species to build a phylogenetic tree (*Neighbour-Joining*) and identification of the Vtg type were performed using the software MEGA-X (Informer Techonologies, Inc., New York, USA). The species and sequences used as input for comparison and identification were obtained from the study published by [6].

The composition of the amino acid sequence and general characteristics of the protein were obtained by the ExPASy ProtParam server of the Swiss Institute of Bioinformatics [36]. In addition, prediction of cleavage sites in Vtg isoforms were performed on the PROSPER (protease specificity prediction server - https://prosper.erc.monash.edu.au/) [37]. Post-translational structural modification sites, such as phosphorylation, N- and O-glycosylation were generated through predictions by the servers NetPhosK 1.0 [38], NetNGlyc 1.0 [39] e YinOYang 1.2 [39], respectively.

Motif search in the selected sequences was conducted by MEME (Multiple Em for Motif Elicitation – http://meme.nbcr.net) [40]. The selected width parameter of motifs was 50. Simultaneously, to identify the functionally characterized protein domains, the sequences were scanned against SMART [41].

### 2.7.2) 3D modeling and structure

The 3D model of Vtg was built by homology from the sequences found in the NCBI by the server Swiss Model [42, 43, 44] and distance/contact maps of the structure were generated on the server nanoHUB (https://nanohub.org/resources/).

## 3) Results

### 3.1) Purification methods

For Vtg purification, strong and weak anion exchange chromatography columns were tested, using HiTrap Q and DEAE column, respectively. The chromatographic fractions were analyzed by SDS-PAGE to obtain the electrophoretic profile and the identification of the fraction of interest. The Vtg from the plasma of EE2-induced fish was successfully purified, and relatively pure fractions were obtained. The chromatography performed with the strong anion exchange column gives the best purity and yield (Figure 2A-B). The method using weak anion exchange column showed the highest presence of contaminants in the fractions

obtained and poorly defined peaks in the chromatogram (Figure 2C-D). Thus, it was possible to verify that the highest peak observed in the chromatogram corresponds to Vtg and fractions contained the purified protein in its ~250 kDa form (Figure 2A-B).

It is important to emphasize that Vtg has two main forms in denaturing condition, 200~250 kDa and 140~150 kDa, as predicted by the sequences found in NCBI. For comparison purposes, the results described were evaluated according to the SDS-PAGE profile and the 220~250 kDa band was considered in our analysis.

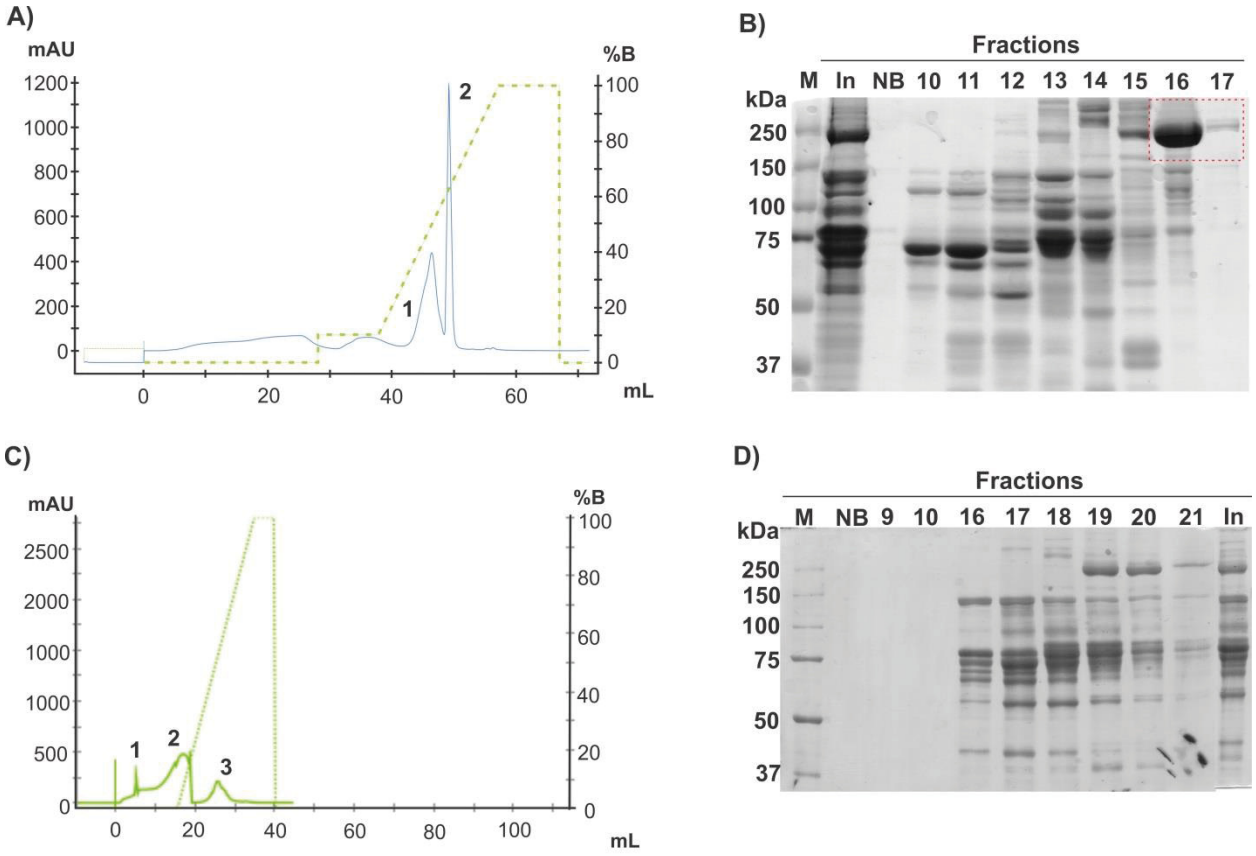


Figure 2 - Vitellogenin purification profile Vitellogenin purification profile. A) Chromatogram: column HiTrap Q. B) SDS-PAGE of fractions. Peak 1: lanes 10-14. Peak 2: lanes 15-17. C) Chromatogram: column DEAE. D) SDS-PAGE of fractions. Peak 1: lanes 9-10. Peak 2: lanes 16-18. Peak 3: lanes 19-21. B) and D) 9% polyacrylamide gel stained with Coomassie R-250 blue. M: BenchMark Protein Ladder. NB: unbound fraction. In: sample input. Reference: 250 kDa.

### 3.2) Physicochemical stability

Among the stability tests, there was an increase in the loss of Vtg with increasing temperature at the boil of the sample (95 ºC), where the conditions of 5 min is better than those of 10 min. However, in both situations, the temperature of 30 °C presented the best result for protein stability (Figure 3A). Vtg showed a greater thermal stability at 4 °C than at room temperature, although there is protein

degradation over time under any of the conditions (Figure 3B). Under different pH conditions, samples were better preserved at low and neutral pH (4.0 and 7.4), while there is loss of protein content at high pH (10) (Figure 3C). As for salt stability, at concentrations above 1M of NaCl there is protein precipitation and the condition without NaCl or with 0.5 M of NaCl were the most suitable for Vtg preservation (Figure 3D). Furthermore, no significant difference in the type of cryoprotectant (tween or glycerol) used to preserve the protein was observed, but there was a slight loss of content in the fifth thawing cycle (Figure 3E).

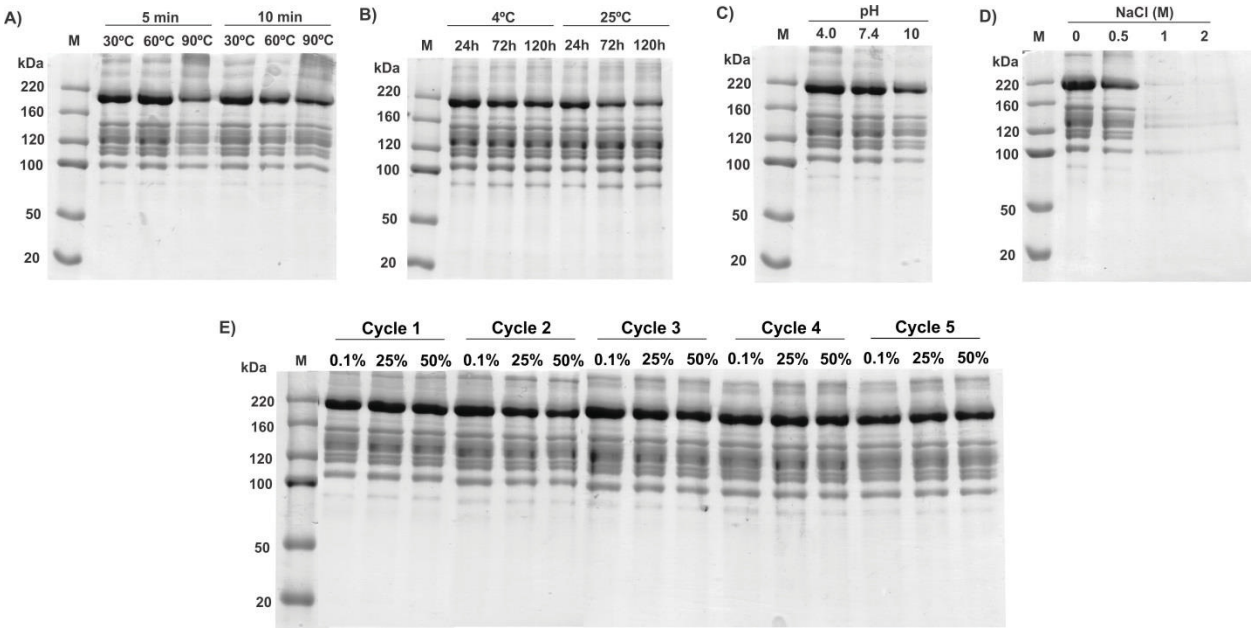


Figure 3 - Vitellogenin physicochemical stability. SDS-PAGE of purified Vtg from *O. niloticus*. Polyacrylamide gel stained with Coomassie blue R-250. A-B) Thermal stability. C) pH conditions. D) Salinity conditions. E) Freeze and thaw cycles. M: BenchMark Protein Ladder. Lanes: 0.1% (0.1% Tween), 25% (25% glycerol), 50% (50% glycerol). Reference: 220 kDa.

### 3.3) Characterization under multiple conditions

After the initial characterization, tests combining favorable and unfavorable conditions for the Vtg preservation were carried out divided into two main conditions at the time of sample preparation: heated to 95°C (Figure 4A) and unheated (Figure 4B). Most of the patterns agree with the tests of the individual conditions, demonstrating that the higher temperature storage is related with the protein degradation. However, it is noted that there is a greater amount of protein content in not heated samples stored at 25 ºC (with or without glycerol) and 4 ºC. The opposite situation was observed for storage at -20 °C. In addition, it is important to highlight that the reference band showed greater intensity compared to the absence of

glycerol at all analyzed temperatures, especially in -20 and 4 ºC, regardless the thermal denaturation treatment.

Interestingly, the main difference of the evaluated parameters in Vtg preservation was the presence of 2 M NaCl when heated, while in unheated samples a loss of almost all the protein content was found. Furthermore, the results obtained for the western blotting of the heated (Figure 4C) and unheated (Figure 4D) samples demonstrate the same pattern found in the SDS-PAGE.

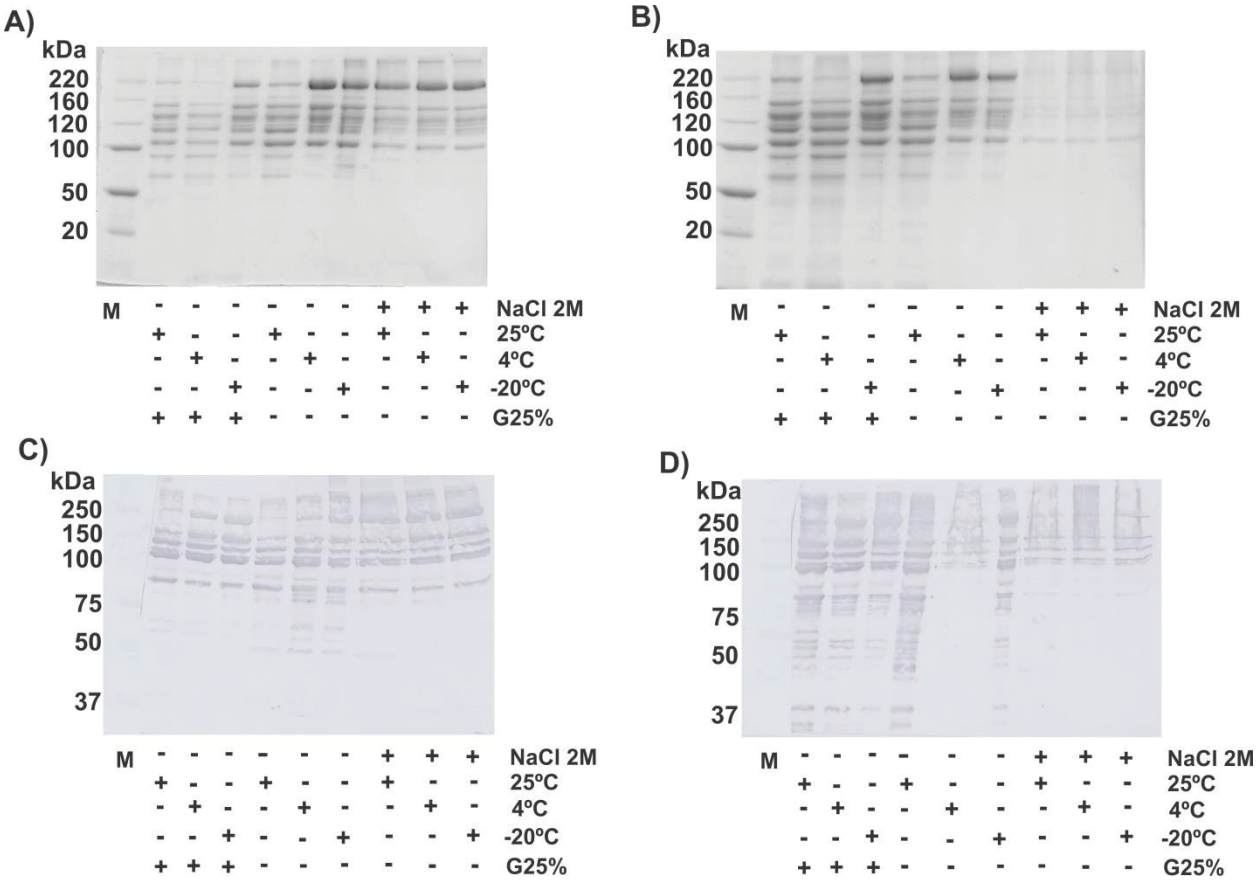


Figure 4 - Vitellogenin physicochemical stability in multiple condition test. A) SDS-PAGE of unheated samples. B) SDS-PAGE of heated samples. C) Western blotting of unheated samples. D) Western blotting of heated samples. The samples stored at -20 ºC were subjected to five thawing cycles, while the others were evaluated within 120 h of storage at the respective temperatures. For SDS-PAGE: polyacrylamide gel was stained with Coomassie blue R-250. M: BenchMark Protein Ladder. For western blotting: nitrocellulose membrane. M: Precision Plus Protein™ Molecular Weight Marker Kaleidoscope™ Pre-stained Protein Standards. G25 %: Glycerol 25 %. “+”: presence. “-“: absence. Reference: 220 kDa

### 3.4) Bioinformatic characterization

The amino acid sequence of *O. niloticus* vitellogenin found in the NCBI was used for phylogenetic and structural analysis, leading the identification of the Vtg type

corresponding to the accessions found based on sequence homologies, as follows: VtgAb - complete (XP_003452622.2, 1789 amino acids, predicted mass: 195 kDa) and VtgC (XP_005459969.3, 1271 amino acids, predicted mass: 141 kDa) – incomplete, phosvitin less (Figure 5A-D). After phylogenetic comparisons of Vtg types in multiple species, it was observed that there are four structural motifs conserved in the complete Vtgs, while in the incomplete form only two motifs are found (Figure 5B). Additionally, in the two isoforms of *O. niloticus* the following domains were evidenced: LPD_N (lipoprotein N-terminal domain), DUF1943 (domain of unknow function 1943, several large open beta-sheets), Pv (phosvitin), DUF1944 (domain of unknow function 1944, several large open beta-sheets) and VWD (von Willebrand factor type D domain). However, the Pv and VWD domains are not present in the incomplete form of the Vtg (Figure 5C).

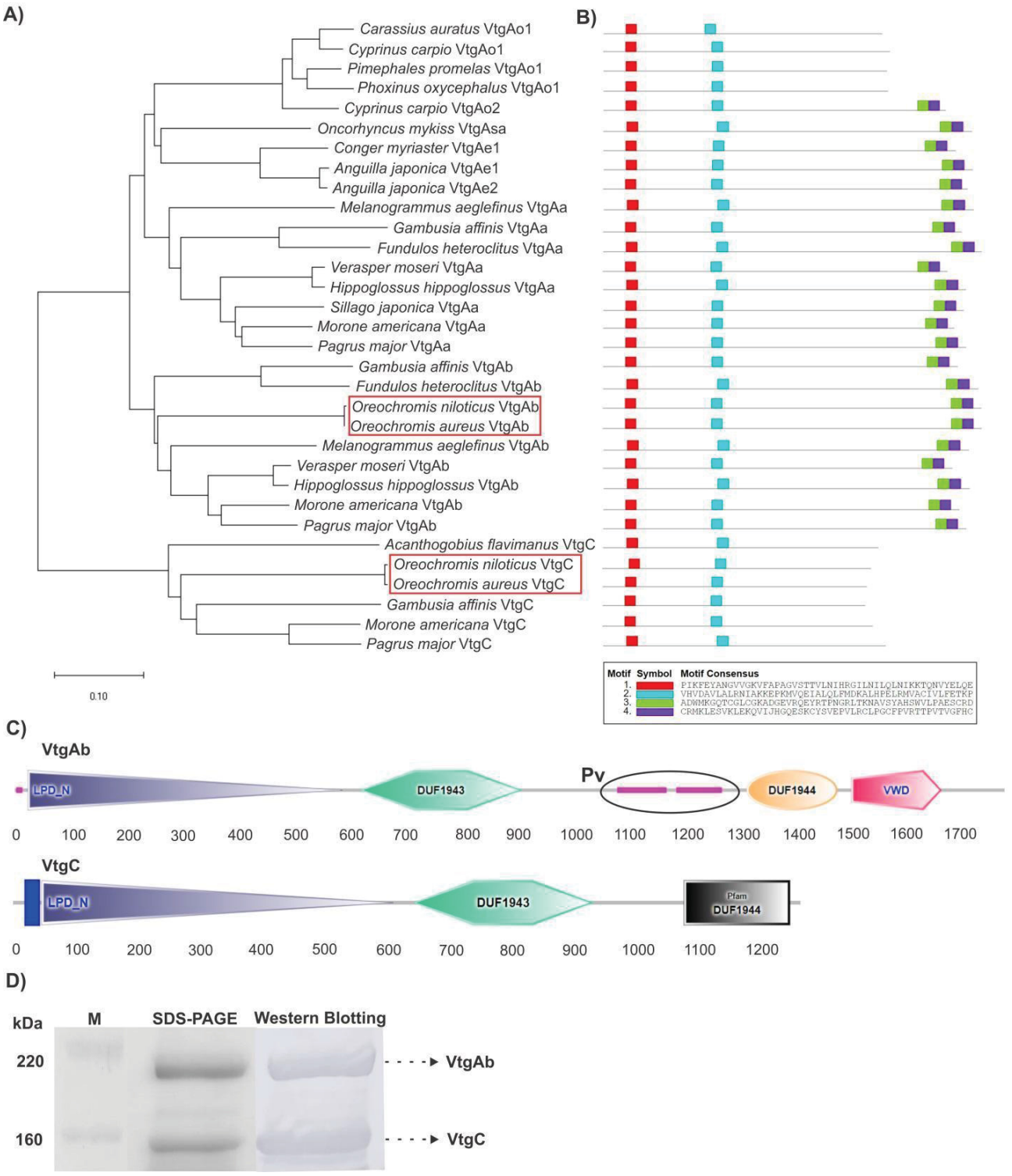


Figure 5 - Phylogenetic and structural characterization of O. niloticus Vtg. A) Phylogenetic tree constructed through the peptide sequence of Vtgs from different fish species. B) Analysis of motifs conserved in different fish species and isoforms. C) Domains present in the sequence of VtgAb and VtgC. D) Representative electrophoretic profile and immunobloting of VtgAb and VtgC isoforms according to predicted mass (kDa) from amino acid sequences.

The 3D model of Vtg was constructed based on the sequence homology of an already crystallized template protein (template - Protein Data Bank: 1lsh.1.A) and validated by the Swiss Model server through the QMEAN value (-2.95). The 3D model allows to elucidate the structure of Vtg, as well as demonstrate evolutionary conserved structural features, such as lipid cavity (Figure 6A). In addition, some features of the 3D model, such as energetically favored regions and model validation, evaluated by the Ramachandran graph, and distance/contact regions are shown in the figure 6B-D.

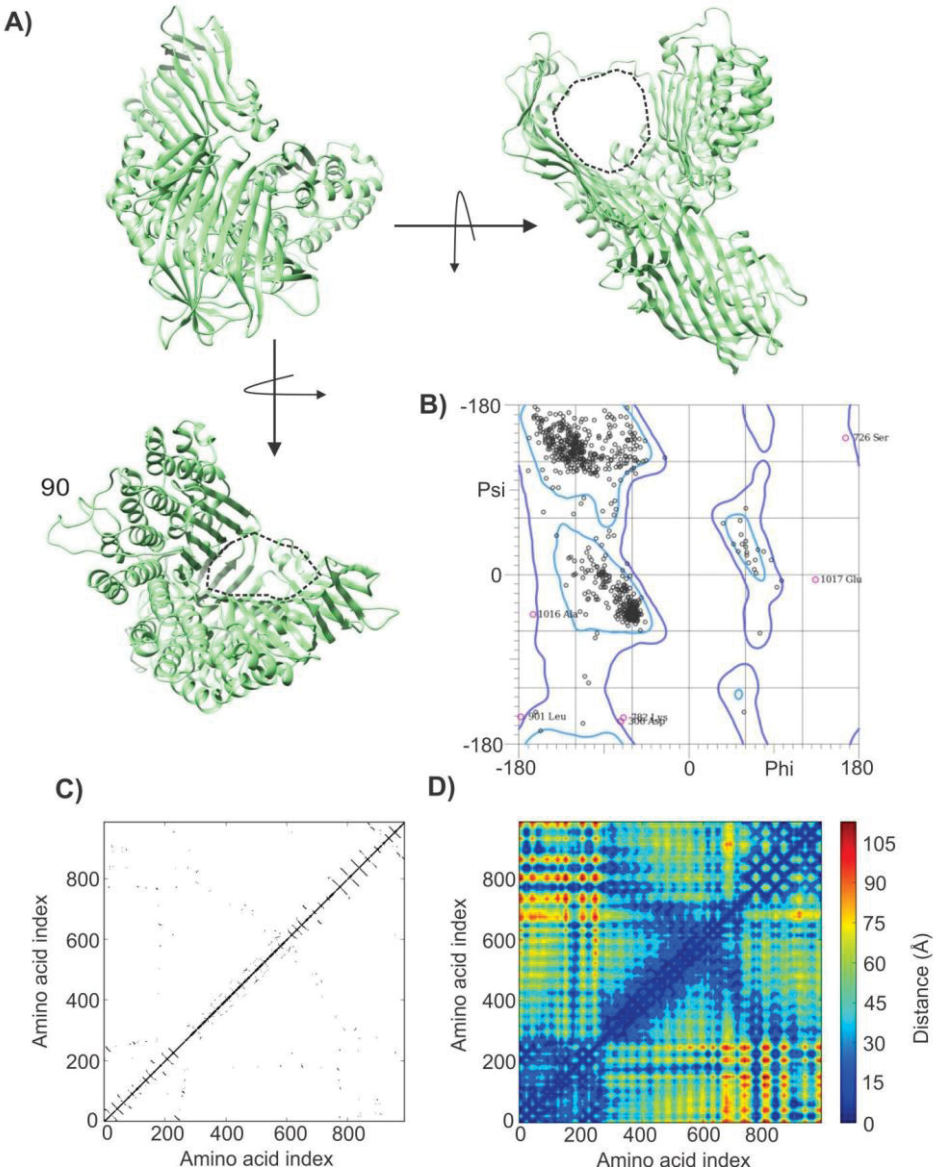


Figure 6 - 3D model and structural characterization of O. niloticus Vtg. A) 3D model of the Vtg at different angles, showing the lipid cavity in the dashed area. B) Ramachandran graph. D) Structural map of contact between protein regions. E) Structural map of distance between protein regions.

Characterization and prediction of the amino acid sequence between the two types of Vtg identified demonstrates a high serine content of VtgAb compared to VtgC (Figure 7A). Predictions of post-translational modifications, such as N- and O-glycosylation and phosphorylation demonstrated important structural differences between the two types of Vtg, indicating the presence of phosvitin – highly glycosylated and phosphorylated region – in VtgAb and absence in VtgC (Figure 7B-G). However, there are also some other glycosylation sites, and the difference in the low number of glycosylated regions between the two Vtg isoforms was evident.

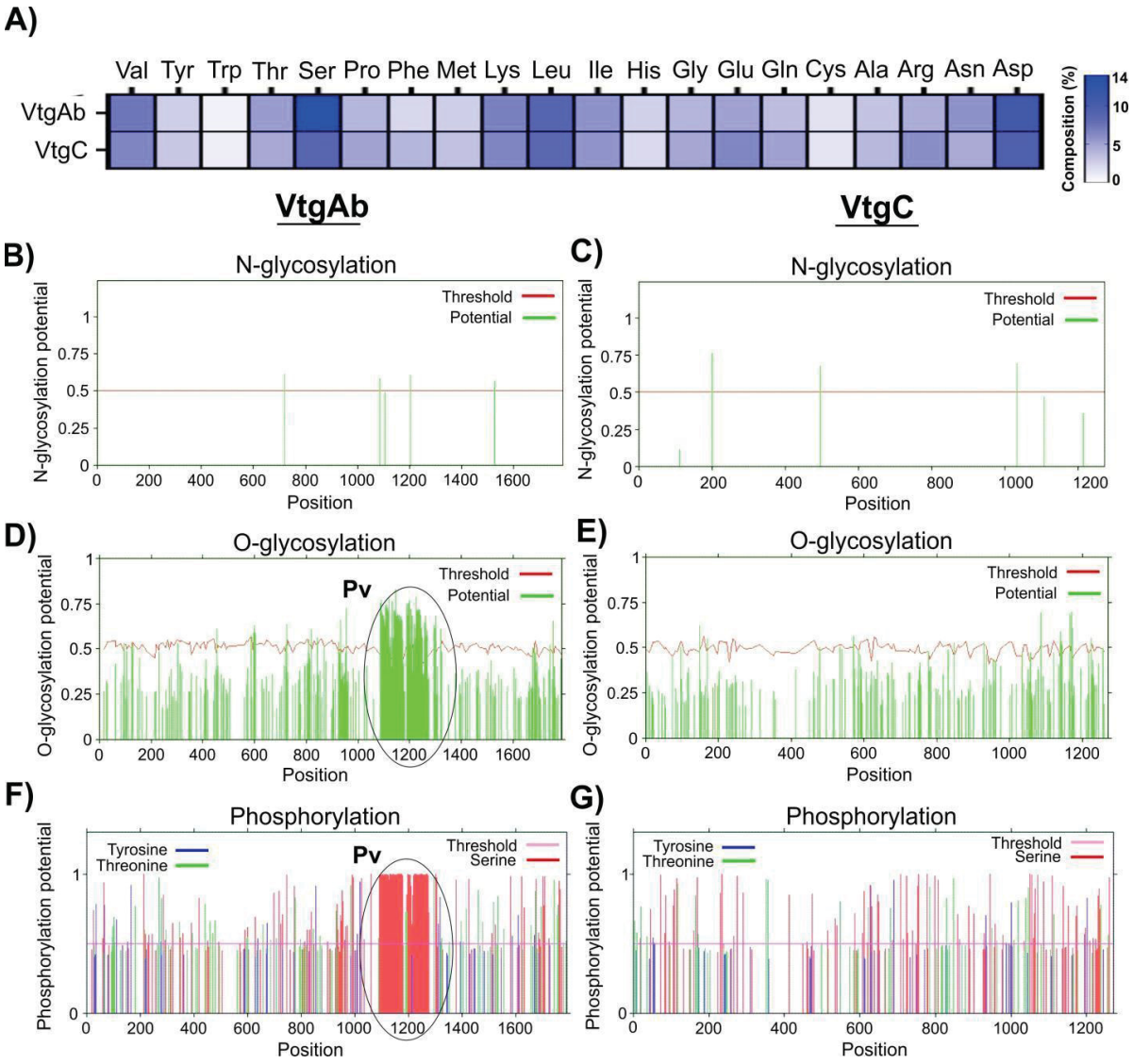


Figure 7 - Sequence characterization and prediction of post-translational modifications of O. niloticus Vtg. A) Amino acid composition of VtgAb and VtgC. B-C) N-glycosylation. D-E) O-glycosylation. F-G) Phosphorylation sites. Vertical lines correspond to amino acids and those above the threshold (horizontal red line) correspond to residues that are modified according to predictions.

From the sequences obtained from the database, a prediction map of cleavage sites by multiple classes of proteases was generated using the PROSPER server (Figure 8A-B). Furthermore, the table with the variety of proteases, characterization of the cleavage site and prediction of the mass (kDa) of the N and C-terminal fragments of each class and Vtg isoform were determined.

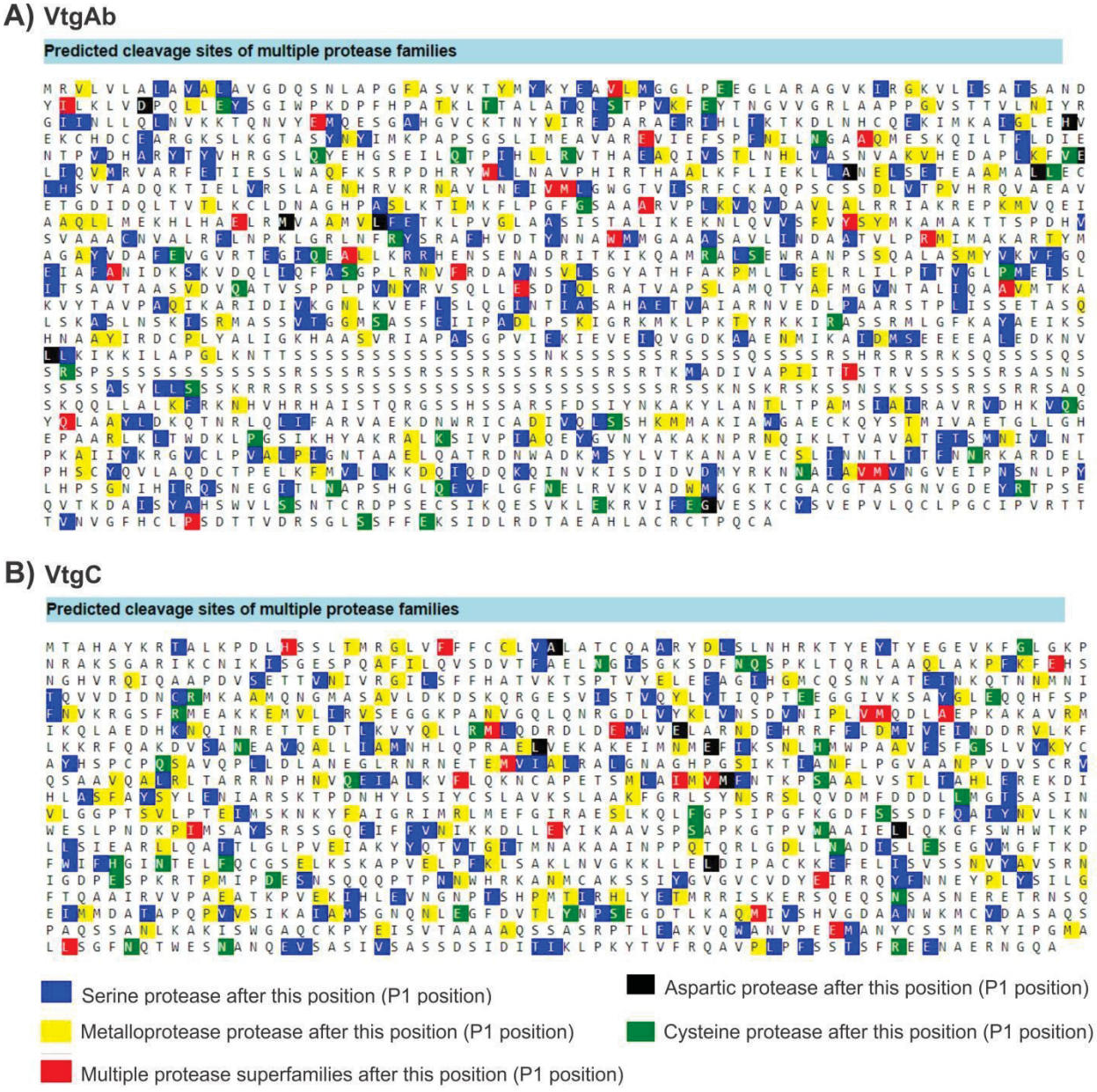


Figure 8 - Profile of cleavage sites by different proteases of O. niloticus VtgAb and VtgC, according to PROSPER server.

## 4) Discussion

It is well established that the Vtg synthesis can be artificially stimulated in teleosts by estradiol treatment, both in male and female fish. Estrogenic hormones are considered important chemicals capable to induce hepatic Vtg synthesis and

subsequent accumulation in the blood stream [45]. The results showed a significant Vtg increase in total plasma protein after the administration of one dose of 20 $mg.kg^{-1}$ of EE2.

Extraction, purification, and storage can directly affect the stability of proteins in solution and so, these procedures must be carefully selected and standardized. In the present study, some parameters that can impact the stability of Vtg were tested. Vtg is known as a high molecular mass protein precursor, but the cleavage sites for Vtg processing can make the molecule unstable, resulting in degradation in different number and size of fragments [5, 12, 46, 47]. On this way, the determination of Vtg physicochemical stability is therefore necessary before use Vtg performance as a biomarker.

During the *O. niloticus* Vtg purification, the presence of several low molecular weight bands in the elution fractions was observed both in the SDS-PAGE and Western blotting assays under reducing conditions, even performing the procedures immediately after sampling. Similar results are described by [47, 48 and 49]. To assess *O. niloticus* Vtg stability, the effects of temperature, ionic strength and pH on the cleavage and aggregation were analyzed by SDS-PAGE assay using the 220 kDa band as a reference, a sensitive and low-cost technique for effective separation of proteins based on molecular weight and assess of protein fragmentation profile.

Protein stability are of great interest in biochemistry because is directly related with its structure dynamics in each environment that can be affected by biological, chemical and physical factors, such as protein complexes, pH, temperature, and mechanical stress [50]. According to [52], the proteins may be affected by chemical and physical instability. The chemical reaction involves the hydrolysis degradation pathway in the primary structure of the protein mainly by deamidation and oxidation reactions. Deamidation and oxidation process can lead to conformational modifications in proteins structure and aggregation [52, 53]. The physical instability is the process whereby the protein changes its physical state without any change in the chemical composition and includes denaturation, aggregation, precipitation and surface adsorption. These two forms of instability are directly related, leaving one process more susceptible to the other with impact in stability of all structure levels of proteins [53].

In the deamidation reaction, the side chain amide linkage of the glutamine or asparagine amino acid residues is hydrolyzed to form a free carboxylic acid,

generating impurities and degradation products [51, 52, 53]. The proteins hydrolysis due to oxidation, including disulfide bond formation, is one of the most frequent chemical degradation. The side chains of histidine, methionine, cysteine, tryptophan and tyrosine residues are potential oxidation sites and can occur during any stage of protein production, purification, formulation and storage [51, 52, 53]. According to the ExPASy ProtParam tool *O. niloticus* Vtg (VtgAb – complete form) is composed of 65 glutamine, 78 asparagine, 45 histidine, 47 methionine, 27 cysteine, 12 tryptophan, 47 tyrosine amino acid residues that may be susceptible to chemical hydrolysis and consequently, to physical instability.

According to Basle [53], protein aggregation is the main physical instability and can result from weak nonspecific interactions (Van der Waals interactions, hydrogen bonding, hydrophobic and electrostatic interactions) or covalent bonds, including disulfide bonds. Aggregation can occur due to several factors, such as incorrect or partial protein folding, disturbance of native protein conformation due to purification process, buffer exchange for formulation, freeze-thaw cycles, ultra-filtration, dehydration, extreme pH, high or low temperatures and storage. Depending on the degree of disturbance, the process can be reversible or irreversible and can lead to precipitation and denaturation of the protein [52, 53]. Denaturation is a more drastic process because denotes the loss of native secondary and tertiary structures, while the preserving the protein chemical composition. This unfolded structure may undergo precipitation [51, 52, 53). The most common stress that causes proteins denaturation is extreme temperatures and pH [53].

On this sense, *O. niloticus* Vtg showed a reasonable resistance to degradation and precipitation at high temperatures and pH, and an excellent stability at lower temperature, pH, ionic strength and freeze. Vtg stability was also improved by glycerol. We noticed the presence of the 220 kDa band even after keeping Vtg at 25°C for 120 h and heating the protein at 90°C for 10 min, in the absence of protease inhibitors and also after the 5 freeze/thaw cycles with nonionic detergent or glycerol. The high stability for *O. niloticus* Vtg under these conditions may be related to: i) the presence of highly conserved motif of repeated cysteine residues that protect Vtg from premature or inappropriate proteolysis [12]. This explains the fragmented pattern observed in the denaturing and reducing SDS-PAGE, which can be confused with degradation or contamination of the chromatographic process. ii) the presence of amino acids that increase proteins stability. Panja [54] noticed that proteins of

extremophile organisms present higher amount of the non-polar amino acids Gly, Ala and Val for extreme temperature, and glutamic acid and aspartic acid for low pH environment. The authors relate this plasticity and molecular stability against stress as a process induced by natural selection. In fact, the *O. niloticus* Vtg (VtgAb – complete form) has 72 glycine, 191 alanine, 131 valine, 90 glutamic acid and 58 aspartic acid residues. iii) the adjustments of the physicochemical conditions, since a number of conditions can slow chemical and physical protein instability [52]. The deamidation and oxidation are favored by increases of pH, temperature and ionic strength, thus increasing the aggregation potential [51, 53]. Basle [53] reported that pH 5 to 6 are generally more protective against fragmentation than higher pH ranges (7-8) and that cold caused protein unfolding and aggregation, which is usually reversible. Moreover, non-ionic detergents and glycerol have been often employed as additives for the stabilization of proteins due their ability to prevent surfaces adsorption, aggregation, precipitation, and to hinder denaturation [51, 53, 55, 56, 57]. All these factors may provide high stability for Vtg.

Interestingly, the results showed that *O niloticus* Vtg precipitation occurs in high ionic strength (2 M NaCl) at neutral pH under thermal denaturation (heating), while at pH 4.0 under unheated condition its stability at room and low temperature was superior to that found in presence of glycerol. The thermally induced denaturation is irreversible, as the unfolded protein molecules rapidly associate to form aggregates [52, 53] and NaCl cannot prevent it. At lower temperatures and pH, both in presence of glycerol, the NaCl act as a stability protective agent, probably due ionic charge. According to Manning [51, 52], salts establish specific ionic interactions with the protein, increasing its stability. Furthermore, Farnum and Zukoski [57] verified that at low pH the presence of glycerol increases the solubility of the Bovine pancreatic trypsin inhibitor protein in high ionic strength (1 M NaCl).

Structurally, vitellogenin present a quite high variety of isoforms depending on the animal species referred as VtgAa or VtgAb. These forms have five main structural domains: heavy-chain lipovitelin (LvH); fosvitine (Pv); light chain lipovitelin (LvL); β' component; and C-terminal peptide (CT) [6]. However, there is still the incomplete form, called VtgC, which does not have the Pv domain and may or may not have the β' and CT domains [6, 46]. In the present study, it was possible to characterize two Vtg isoforms (VtgAb and VtgC) in *O. niloticus*. Phylogenetically, there are four main conserved motifs in the vtg of different teleost fish, which are comparable to the

identified domains. Comparatively, the LPD_N and DUF1943 domains correspond to LvH, while DUF1944 and VWD (Von Willebrand factor type D domain) correspond to LvL and β'-CT, respectively [10, 46]. Therefore, the difference between the structural components of VtgAb NH2-(LvH-Pv-LvL-β'-CT)-COO- and VtgC NH2-(LvH-LvL)-COO- is clear, especially considering that these structures are cleaved and incorporated into the oocyte. Furthermore, these different types of Vtg have already been identified in the genus *Oreochromis* and other species [4, 5, 58].

Thus, these data can be correlated with those obtained experimentally: the Vtg band at ~200-220 kDa possibly corresponds to the VtgAb (complete), while the bands at t~140-150 kDa correspond to the VtgC (incomplete). According to the identified and conserved domains of the *O. niloticus* Vtg, the following masses were predicted for these regions: LvH (94.79 kDa); Pv (17.45 kDa); LvL (18.43 kDa) and VDW (18.26 kDa). However, it is necessary to consider that the total mass of each isoform varies, as there are regions of the protein that were not considered in the prediction of domain mass. Furthermore, post-translational modifications were not considered, and it is important to emphasize that these alterations promote an increase of the protein mass, enabling the two isoforms described in this study to be differentiated. Thus, considering the electrophoretic profile of *O. niloticus* Vtg, associated with the low structural stability under reducing conditions and its role in the oocyte, it is possible to observe the large number of cleavage sites by different types of proteases, reflecting the electrophoretic pattern observed in the present study. Regarding the results obtained in the structural analyses, the largest number of possible glycosylation sites of the identified Vtgs are towards the C-terminal portion of the protein. This result agrees with that found for other animal species, considering that this region corresponds to the LvL domains, terminal components β' and CT [10] Glycosylation in these terminal regions is important to impart aqueous solubility to vitellogenin, as well as aiding in lipid transport [46]. The greater phosphorylation potential in the region corresponding to the Pv domain in VtgAb indicates a significant structural difference between the two types of Vtgs. This post-translational modification in this Serine-rich region may be important for structural stabilization of the protein and increased solubility in the plasma [10]. In addition, this phosphate-rich region allows the binding of several ions, especially calcium, being essential for the transport of important molecules for embryonic osteogenesis [10, 59]. The terminal β'-CT domains (VWD) have no clearly defined function but may be

associated with the stabilization of Vtg dimers, as well as facilitating recognition by extracellular proteins, including the Vtg receptor [10, 59].

Another important structural feature found in the 3D model of *O. niloticus* vitellogenin is the presence of the lipid cavity, which is a secondary and amphipathic structure that help to transport lipids to oocytes [46]. This structural framework is formed by the conformational interaction of the large number of β-sheets present in the LvL and LvH domains, as described by [60]. Furthermore, as shown in the contact map of the protein's 3D structure, there are regions where there is proximity between the side chains of different regions, which possibly facilitate the structural maintenance and conformation of the Vtg.

## 5) Conclusion

*O. niloticus* Vtg showed in the present study a high physicochemical stability that can be improved by addition of glycerol (25%), Tween-20 (0.1%) and 2 M NaCl at pH 4.0. The Vtg integrity must be assessed by electrophoresis under non-reducing and non-denaturing conditions. In addition, it was elucidated important structural characteristics of Vtg through bioinformatics tools, as well as the differentiation of its complete (VtgAb) and incomplete (VtgC) forms, increasing the knowledge about the protein stability and function.

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## 7) Declaration of interests

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

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Capítulo II

# Exploring the toxicity and endocrine disrupting potential of a complex mixture of PAHs in the estrogen pathway in *Oreochromis niloticus* hepatocytes

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**Abstract**

This study aimed to investigate the toxicity and endocrine disrupting potential of a complex mixture of polycyclic aromatic hydrocarbons (PAHs) in the estrogen pathway using hepatocytes of Oreochromis niloticus. The hepatocytes were exposed to various concentrations of the PAH mixture, and multiple endpoints were evaluated to assess their effects on cell viability, gene expression, oxidative stress markers, and efflux activity. The results revealed that the PAH mixture had limited effects on hepatocyte metabolism and cell adhesion, as indicated by the non-significant changes observed in MTT metabolism, neutral red retention, and crystal violet staining. However, significant alterations were observed in the expression of genes related to the estrogen pathway. Specifically, the biomarker vitellogenin (vtg) exhibited a substantial increase of approximately 120% compared to the control group. Similarly, estrogen receptor 2 (esr2) showed a significant upregulation of approximately 90%. In contrast, no significant differences were observed in the expression of estrogen receptor 1 (esr1) and the membrane receptor gper1. Furthermore, the PAH mixture elicited complex responses in oxidative stress markers. While reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels remained unchanged, the activity of catalase (CAT) was significantly reduced, whereas superoxide dismutase (SOD) activity, glutathione S-transferase (GST) activity, and non-protein thiols (NPT) levels were significantly elevated. In addition, the PAH mixture significantly influenced efflux activity, as evidenced by the increased efflux of rhodamine and calcein, indicating alterations in multidrug resistance-associated proteins (MXR). Overall, these findings, associated with bioinformatic analysis, highlight the potential of the PAH mixture to modulate the estrogen pathway and induce oxidative stress in O. niloticus hepatocytes. Understanding the mechanisms underlying these effects is crucial for assessing the ecological risks of PAH exposure and developing appropriate strategies to mitigate their adverse impacts on aquatic organisms.

Keywords: primary hepatocyte, Nile tilapia, vitellogenin, bioinformatics, oxidative stress

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds that are widely distributed in various environmental matrices as a result of anthropogenic activities and natural processes, such as fossil fuel combustion, biomass burning, and organic material degradation (Mojiri et al., 2019; Lawal, 2017). PAHs have been extensively studied in ecotoxicology due to their widespread occurrence and potential to cause environmental and human health hazards (Valderrama et al., 2022; Peng et al., 2019). These compounds pose significant concerns due to their toxicity and potential to interfere with the endocrine systems of aquatic organisms, including fish (Valderrama et al., 2022; Peng et al., 2019; Vondráček et al., 2018).

*Oreochromis niloticus*, known as Nile tilapia, is a freshwater fish widely distributed worldwide and is often used as a model organism in toxicological studies (Souza et al., 2022; Leão-Buchir et al., 2022). The exposure of fish to complex mixtures of PAHs is a growing concern due to their potential to cause adverse effects on the health and reproduction of these organisms (Perrichon et al., 2015; Vignet et al., 2014). Particulary, endocrine disruption can lead to alterations in embryonic development, hormonal function, and negative effects on the reproductive system through the modulation of nuclear receptor activity (Fang et al., 2022; Hawliczek et al., 2012).

One of the biomarkers of estrogenic endocrine disruption in fish is vitellogenin (Vtg). Vtg is a high molecular weight protein, ranging from 300 to 600 kDa, and is formed by two polypeptide homodimers that contain phosphate, carbohydrates, and lipids, characterizing the structure of a lipophosphoglycoprotein (Souza et al., 2023; Carducci et al., 2019). Vtg expression is regulated by hormones and occurs through the induction of transcription via estrogen-responsive elements (EREs) mediated by estrogen receptors in hepatocytes (Tokarz et al., 2015; Kiyama and Wada-Kiyama et al., 2015). However, estrogenic response can occur either through genomic pathways, mediated by nuclear receptors, or through non-genomic pathways involving intracellular signaling cascades and membrane proteins, such as GPER1 (Kiyama and Wada-Kiyama et al., 2015).

Thus, evaluating the mechanism of action of mixtures of PAHs in hepatocytes is of utmost importance to understand the adverse effects of these compounds on the estrogenic pathway and their toxic potential in fish. In this regard, the liver is an

important target organ for PAH toxicity, since many PAHs are bioactivated into more toxic forms by hepatic enzymes. Therefore, the main aim of this study was to evaluate the toxicity and endocrine-disrupting potential of a mixture of PAHs in primary culture of hepatocytes of *O. niloticus*. Primary culture hepatocytes can provide a more relevant approach compared to established cell lines, as they preserve morphophysiological functions closer to the in vivo environment and have a higher sensitivity for vtg expression and synthesis (Navas and Segner, 2006). These cells were isolated from the livers juvenile fish and cultured in an appropriate culture medium, providing a suitable model for assessing the effects of PAHs.

## 2. Material and methods

### 2.1. Fish

Juvenile *O. niloticus* specimens were obtained from a fish farm, maintained in water tanks with aeration and filtration systems for acclimation over a period of 7 days. Prior to euthanasia for cell extraction, the individuals were subjected to a fasting period of 2-3 days to reduce stomach content and the risk of bacterial contamination during the procedures. All procedures using animals were approved by the institutional ethics committee CEUA/BIO (certificate 1286).

### 2.2. Primary culture of *O. niloticus* hepatocytes

The isolation and primary culture of hepatocytes were performed following the method proposed by Kim and Takemura (2003) with modifications. The fish were anesthetized using MS-222 (ethyl 3-aminobenzoate methanesulfonate), euthanized by spinal cord transection, and dissected. The liver was removed from the abdominal cavity, transferred to a Petri dish, and perfused with calcium-free hepatocyte buffer (HB) solution (HB: 136.9 mM NaCl; 5.4 mM KCl; 0.81 mM $MgSO_4$; 0.44 mM $KH_2PO_4$; 0.33 mM $Na_2HPO_4$; 5 mM $NaHCO_3$ – pH 7.6) at room temperature in a laminar flow hood. After perfusion, the liver was cut into small fragments using a surgical blade, and the hepatic tissue was digested for 40 min at room temperature with HB containing 0.3 $mg.mL^{-1}$ collagenase IV.

The cells were filtered in a mesh, transferred to a 50 mL tube, and centrifuged once at 50 g for 3 min in HB and twice in culture medium (L15: 85% + F12: 15%) under the same conditions. The cell pellet was re suspended for cell viability assessment using the Trypan Blue exclusion method. The isolated hepatocytes were

cultured at a density of $2.1\times10^5$ cells/$cm^2$ in culture medium supplemented with L15 (85%), F12 (15%), fetal bovine serum (5%), insulin (0.01 $mg.mL^{-1}$), $NaHCO_3$ (0.15 $g.L^{-1}$), HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, 15 mM), penicillin (100 $U.mL^{-1}$), streptomycin (0.1 $mg.mL^{-1}$), and amphotericin B (0.25 $\mu g.mL^{-1}$) at 25 °C in an incubator without $CO_2$ injection. The cells were cultured for 48 h for cell attachment and establishment of cells clusters, and then exposed to different conditions (treatments) by culture medium replacement with fresh medium.

### 2.3. Experiment I: validation of cell responsiveness

To validate the responsiveness of hepatocytes to estrogenic pathway modulation, the cells were exposed to 1) ethinylestradiol (EE2), an endocrine disruptor and agonist of the estrogen receptor (ER), and 2) tamoxifen, an antagonist of the ER and a selective estrogen receptor modulator (SERM), both at concentrations of 0 (control), 0.1, 1 and 10 µM for 72 h. The highest concentration of EE2 was based on the vitellogenin synthesis-inducing concentration described in the study conducted by Kim and Takamura (2003). The same concentration of tamoxifen was used, and dimethyl sufoxide (DMSO) at 0.01 % was used as a solvent and in the control group.

### 2.4. Experiment II: exposure to a complex mixture of PAHs

After validating the responsiveness of primary cultured hepatocytes, the toxic and endocrine disrupting potential of a mixture of PAHs (Table 1) was assessed. Hepatocytes were exposed for 72 h to seven logarithmically scaled (log10) nominal concentrations, with the intermediate concentration of 0.02 $\mu g.L^{-1}$. Dimethyl sufoxide (DMSO) at 0.01 % was used as a solvent and in the control group. The range of concentrations used encompasses those found in water bodies, which are environmentally relevant for exposure to aquatic organisms (Barreto et al., 2020).

Table 1 - Components of the complex mixture of PAHs.

| **Total PAHs (Σ=18) stock concentration: 2000 µg.mL$^{-1}$** | |
|---|---|
| Acenaphthene | Chrysene |
| Acenaphthylene | Dibenz[a,h]anthracene |
| Anthracene | Fluoranthene |
| Benz[a]anthracene | Fluorene |
| Benzo[b]fluoranthene | Indeno[1,2,3-cd]pyrene |
| Benzo[k]fluoranthene | 1-Methylnaphthalene |
| Benzo[ghi]perylene | 2-Methylnaphthalene |
| Benzo[a]pyrene | Naphthalene |
| Pyrene | Phenanthrene |

## 2.5. Cytotoxicity assays

For the viability assays, liver cells were cultured in 96-well microplates at a density of 2.1x10$^5$ cells per cm$^2$ for 48 h, and then exposed to PAH mix for 72 h.

MTT Assay: To evaluate the cell dehydrogenases activity, 25 µL of a solution containing 5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) were added to each well. After 2 h of incubation at 25°C, the wells were washed with 200 µL of PBS (phosphate buffer saline), the formazan crystals were solubilized in 100 µL of DMSO, and the absorbance measured at 540 nm (Reilly et al., 1998; Mosmann et al., 1983).

Neutral Red Assay: To assess endolysosomal system integrity, 25 µL of a stock solution containing 1 mg.mL$^{-1}$ of neutral red was added to each well (final concentration of 0.1 mg.mL$^{-1}$). After a 2 h of incubation at 25°C, the wells were washed with 200 µL of PBS, the dye was extracted with 100 µL of 1% acetic acid and 50% ethanol solution, and the absorbance measured at 550 nm (Repetto and Del Peso, 2008).

Crystal Violet Assay: To evaluate cell adhesion, the cells were dyed with 0.25 mg.mL$^{-1}$ of crystal violet for 10 min and washed with 200 µL of PBS. Then, the dye was extracted with 100 µL of 33% acetic acid for 10 min, and the absorbance was measured at 570 nm (Feoktistova et al., 2016).

## 2.6. Biochemical biomarkers assays

The cells were cultured in 6-well plates at a density of 3.1x10$^5$ cells per cm$^2$ for 48 h, and then exposed to PAH mix for 72 h. Then, the cells were detached using a cell scraper, centrifuged at 50 g for 3 min, and the pellet was stored at -80 ºC. For the

assays, the cells were on ice, suspended in Tris-EDTA buffer (20 mM Tris−HCl, 1 mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 12,000 g for 20 min at 4 °C. The protein content of the supernatants was measured at 595 nm by mixing 10 µL of the supernatants with 250 µL of Bradford reagent in a 96-well microplate (Bradford, 1976), and comparison to a standard BSA (bovine serum albumin) curve, and then adjusted to a final concentration of 1.0 mg.mL$^{-1}$ for the determination of enzyme activities. For the reactive oxygen and nitrogen species and lipid peroxidation assays, the cells were cultured in 96-well cell culture plates at a density of 2.1x10$^5$ cells per cm$^2$. After exposure to a PAH mix, the assays were conducted.

### 2.6.1. Antioxidant enzymes

Superoxide dismutase (SOD) activity was determined as follows: in a 96-well microplate, 30 µL of supernatant (1.0 mg protein.mL$^{-1}$) was mixed with 70 µL of 572 µM nitrotetrazolium blue chloride (NBT) in 0.1 mM EDTA. For the reference curve, the sample supernatant was replaced with Tris-EDTA buffer (20 mM Tris−HCl, 1 mM EDTA, pH 7.4). The reaction was initiated by adding 100 µL of 51 mM hydroxylamine chloride in 0.5 M sodium carbonate (pH 10.2). The reduction of NBT by superoxide anion to blue formazan was measured as a constant increase in absorbance at 560 nm for 30 min. One unit of SOD activity was defined as the enzymatic activity necessary to inhibit the reduction of NBT to 50% of the reference rate (Crouch et al., 1981; Kono, 1978).

Glutathione S-transferase (GST) activity was measured as follows: in a 96-well microplate, 20 µL of supernatant (1.0 mg protein.mL$^{-1}$) was added to 180 µL of reaction medium (1.5 mM glutathione (GSH) and 2.0 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 0.1 M potassium phosphate buffer, pH 6.5). The increase in absorbance at 340 nm was measured for 5 min, and the GST activity was calculated using the molar extinction coefficient for CDNB (9.6 mM$^{-1}$cm$^{-1}$) (Keen et al., 1976).

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined as follows: in a 96-well microplate, 30 µL of supernatant (1.0 mg protein.mL$^{-1}$) was added to 170 µL of reaction medium (1 mM $NADP^+$, 2 mM D-glucose-6-phosphate, and 10 mM $MgCl_2$ in 0.1 M Tris-HCl buffer, pH 8.0). The increase in absorbance, reflecting the conversion of $NADP^+$ to NADPH, was measured at 340 nm for 6 min. One unit of G6PDH activity was defined as the amount of enzyme that catalyzes the

formation of 1 µmol NADPH per minute per milligram of protein (Glock and McLean, 1953).

Catalase (CAT) activity was measured as follows: in a 96-well UV-star® microplate (Greiner), 20 µL of supernatant (1.0 mg protein $mL^{-1}$) was mixed with 180 µL of the reaction medium (20 mM $H_2O_2$ in 50 mM Tris-base, 0.25 mM EDTA, pH 8.0). The decrease in absorbance was immediately measured at 240 nm for 5 min. The molar extinction coefficient for hydrogen peroxide (40 $M^{-1}cm^{-1}$) was used to calculate catalase activity (Aebi, 1984).

### 2.6.2. Non-protein thiols (NPT) levels

To measure NPT, the supernatant was collected after protein precipitation with 10% trichloroacetic acid and centrifugation at 1,000 g for 15 min at 4 °C, and transferred (50 µL) into a 96-well microplate. Then, 230 µL of 0.4 M Tris-base buffer (pH 8.9) and 20 µL of 2.5 mM DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)) were added to the microplate. The absorbance of was measured at 415 nm, and the concentration of NPT was determined by comparison to a standard curve of GSH (Sedlak and Lindsay, 1968).

### 2.6.3. Lipid peroxidation (LPO)

Following exposure to the PAH mix, the culture medium was replaced by fresh culture medium containing 100 µM of diphenyl-1-pyrenylphosphine (DPPP). The cells were incubated at 25 ºC for 15 min (protected from light), the culture medium was remoevd, and 100 µL of DMSO was added. The formation of DPPP oxide, resulting from the reaction between DPPP and lipid hydroperoxides, was measured in the Varioskan Lux (Thermo Scientific) at ex/em wavelenghts of 351/380 nm (Okimoto et al., 2000).

### 2.6.4. Reactive oxygen and nitrogen species levels

Reactive oxygen species (ROS): after exposure, the cells were incubated with fresh medium containing 10 µM of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) at 25 ºC for 15 min (protected from light), the culture medium was removed, and 200 µL of PBS was added. The formation of 2',7'-dichlorofluorescein (DCF), resulting from the cleavage of acetate groups by intracellular esterases and

subsequent oxidation, was quantified in the Varioskan Lux (Thermo Scientific) at ex/em wavelengths of 488/530 nm (Wu and Yotnda, 2011).

Reactive nitrogen species (RNS): after exposure, 150 µL of the culture medium was transferred to a black microplate, and 10 µL of 0.5 $mg.mL^{-1}$ of 2,3-diaminonaphthalene (DAN) in 0.62 M HCl were added. The microplates were kept at 25°C under gently shaken and protected from light. After 30 min, 5 µL of 2.8 M NaOH were added to each well. The formation of fluorescent 1-naphthotriazole, resulting from the reaction between nitrite and DAN, was determined in the Varioskan Lux (Thermo Scientific) at ex/em wavelengths of 365/450 nm (Misko et al. 1993 and Glinski et al. 2016).

### 2.7. Multixenobiotic resistance (MXR) transporters activity

The cells were cultured in 96-well microplates at a density of $2.1x10^5$ cells per $cm^2$ for 48 h, and then exposed to PAH mix for 72 h.

Rhodamine B assay: after exposure, the cells were incubated with 1 µM rhodamine B in PBS at 25°C for 30 min (protected from light), washed twice with PBS, and incubated with 200 µL of PBS at 25 ºC for 10 min. The supernatants were transferred to a black microplate, and the fluorescence intensity was measured in the Varioskan Lux (Thermo Scientific) at ex/em wavelenghts of 540/580 nm (Liebel et al., 2015 with modifications).

Calcein-AM assay: after exposure, the cells were incubated with 0.5 µM calcein-AM in FBS-free culture medium for 1 h, washed twice with PBS and incubated with 100 µL of PBS at 25 ºC for 10 min. The supernatants were transferred to a black microplate, and the fluorescence intensity was measured in the Varioskan Lux (Thermo Scientific) at ex/em wavelengths of 488/530 nm (Roque et al., 2023).

### 2.8. qPCR

The cells were detached using a cell scraper, centrifuged at 50 g for 3 min at room temperature, and suspended in 100 µL of RNAlater™. RNA was extracted with PureLink™ RNA Mini Kit (Invitrogen) and Trizol reagent (Invitrogen), and any potential genomic DNA contamination was eliminated with TURBO DNAse RNAse-free™ (Invitrogen). RNA was quantified using Quantifluor® (Promega), RNA purity was determined by absorbance ratios (260/280 for protein and 260/230 for phenol and other contaminantion), and RNA integrity was evaluated by agarose gel

electrophoresis. Then, the RNA was converted into cDNA using the SuperScript™ IV First-Strand Synthesis System (Invitrogen) and a Veriti™ Thermal Cycler. Each reaction was performed with 10 ng of cDNA and 500 µM of primers in a total volume of 10 µL. Real-time amplification was performed using the PowerUp™ SYBR™ Green Master Mix (Invitrogen) and the StepOnePlus™ Real-Time PCR System. Data analysis was performed in the Web-based LinRegPCR server (Untergasser et al., 2021). Ubiquitin-conjugating enzyme (ube2z), tubulin alpha chain-like (tuba), and beta-actin (actb) were used as reference genes. The target genes under investigation were esr1 (estrogen receptor α), esr2 (estrogen receptor β), gper1 (G protein-coupled estrogen receptor 1) and vtg (vitellogenin) (Table 2).

Table 2 – Gene and primer sequences

| Gene | GenBank | Forward | Reverse |
|---|---|---|---|
| *ube2z* | XM_003460024 | CTCTCAAATCAATGCCACTTCC | CCCTGGTGGAGGTTCCTTGT |
| *tuba* | XM_003445344 | AGCCAGACGGACAGATGCC | TTCCTGCACGCACCTCATC |
| *actb* | XM_003455949 | GTACCCCATTGAGCACGGTA | GAGCCTCTGTGAGCAGGACT |
| *esr1* | NM_001279770.1 | AGCCAAGCTGACTCCAGAAA | TTTGAGCTGGCAGTGTTTTG |
| *esr2* | NM_001279477.1 | ACAAGCTGCTGGGAAAGAAA | AGGACCACACACCGTAGTGA |
| *gper1* | XM_005468788.4 | TCTCACGTGGATGAGCTTCG | GCACAATGGTGAAGGGGAGA |
| *vtg* | FJ709597.1 | GCCCAAAGATCCTTTCCATC | TCAGCACTGTTGTGGAGACC |

### 2.9. Computational toxicology

The endocrine-disrupting effects of the 18 PAHs present in the mixture were assessed using the Comparative Toxicogenomics Database (https://ctdbase.org/; Davis et al., 2023). The "gene-chemical interactions" search module was employed with default settings, focusing on the interaction of the PAHs with genes involved in the estrogen signaling pathway (KEGG: hsa04915). This approach generated a list of genes affected by individual PAHs, and after removing duplicates, the final list was used as input for network analysis with medium confidence (0.400) on the STRING server (https://string-db.org/) (Szklarczyk et al., 2023).

The resulting network was exported and analyzed using Cytoscape software. The MCODE v. 2.0.2 plugin (Molecular Complex Detection) was applied to detect functional clusters in the network (Shannon et al., 2003; Bader and Hogue, 2003).

Furthermore, to identify proteins that may serve as potential key regulators of the biological processes within the network (including the cluster generated by MCODE), centrality analysis was performed using the CentiScaPe v. 2.2 plugin for Cytoscape (Scardoni et al., 2009). This analysis identifies nodes with central positions in the network based on their betweenness and degree measures. Nodes with high betweenness and degree levels, known as "bottlenecks" are more likely to act as central hubs for information flow or interactions with other nodes in the network (Yu et al., 2007).

A terminology enrichment analysis of the selected genes was performed using zebrafish (*Danio rerio*) databases in the server ShinyGO v.0.77 (http://bioinformatics.sdstate.edu/go/) to show the association of the selected genes involved in biological processes and KEGG pathways, using a False Discovery Rate (FDR) of 0.05 (Ge et al., 2020).

### 2.10. Statistics

The data of four to five independent experiments were submitted to ANOVA followed by Dunnett's post hoc test or t-student test (comparison: control x treated). $p < 0.05$ was considered statistically significant in all conditions.

## 3. Results and discussion

### 3.1. Hepatocytes responsiveness to estrogenic agonist/antagonist chemicals

Hepatocytes were exposed to EE2 and tamoxifen at concentrations of 0.1, 1, and 10 µM for 72 h, and none of the concentrations were cytotoxic, as measured by MTT (Figure 1A-B), neutral red (Figure 1D-E), and crystal violet (Figure 1G-H) assays. Based on this, the highest concentration was chosen to perform the EE2 + tamoxifen mixture exposure. This mixture caused 30% increase in MTT metabolism (Figure 1C), but no effect was observed for neutral red (Figure 1F) and crystal violet (Figure 1I) assays. The absence of cytotoxic effects was considered a positive outcome, as it would affect the induction of vitellogenin expression in cells, and is in accordance with the data reported by Liu et al. (2007) for *O. niloticus* hepatocytes exposed to E2 for 48 h.

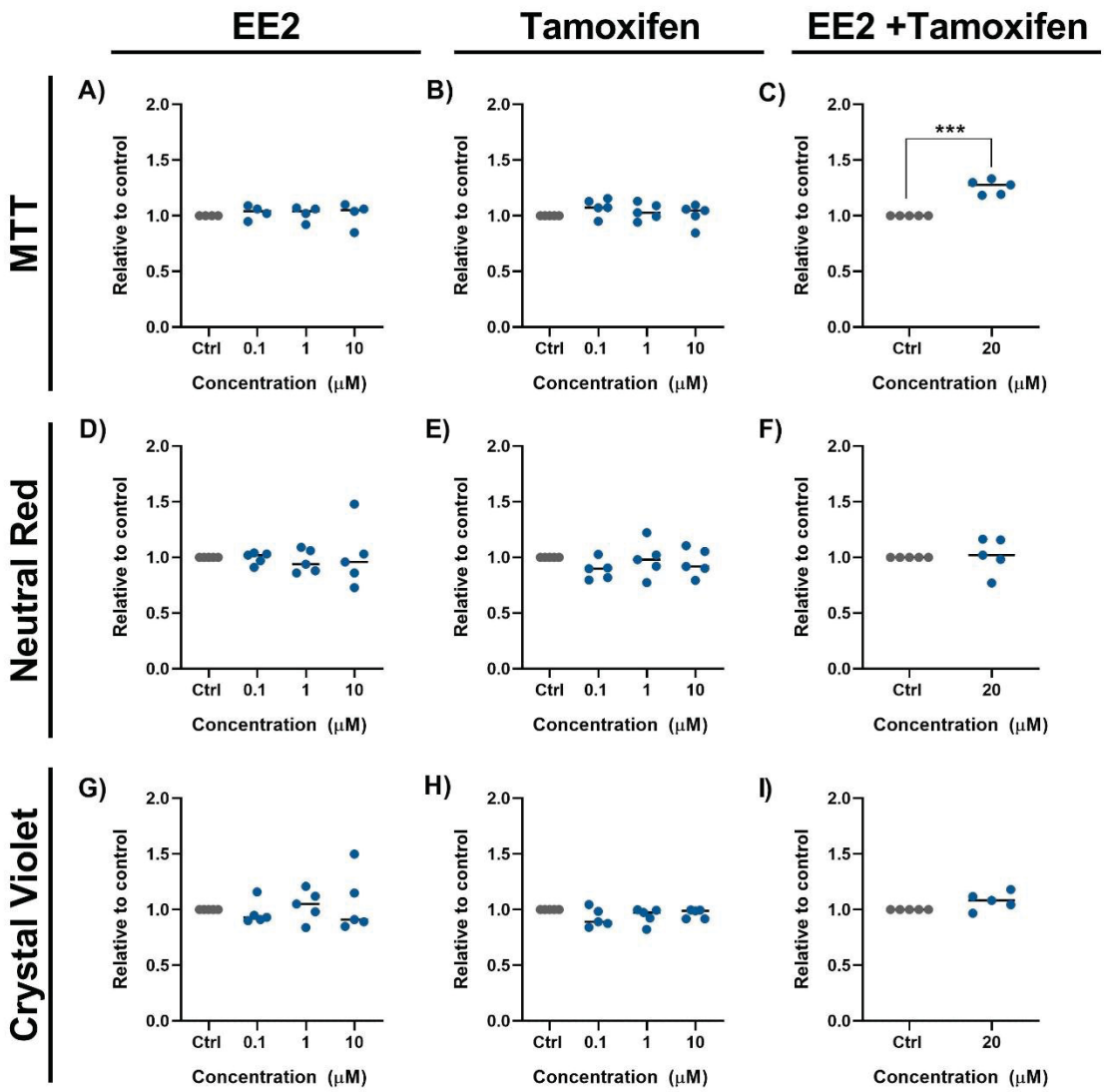


Figure 1. Cytotoxicity assays in *O. niloticus* hepatocytes after exposure for 72 h. EE2 (agonist), tamoxifen (antagonist), and the mix (EE2 + tamoxifen at 10 µM). Dots: independent experiments (n=5); Bar: mean. ANOVA followed by Dunnett post hoc test or t-student test (EE2 + tamoxifen). ***p < 0.001.

Exposure to EE2 modulated the expression of all the evaluated genes compared to the control group (Figures 2A-D). There was a pronounced induction (~2000%) of the vtg (Figure 2A), the nuclear receptor esr1 (~350%, Figure 2B) and the membrane receptor gper1 (~100%, Figure 2D), and decrease (~55%) of the esr2 isoform (Figure 2C). When the cells were exposed to the equimolar mixture (10 µM) of EE2 + tamoxifen (agonist + antagonist), a drastic modulation of the expression of genes related to the estrogenic pathway was observed. In all analyzed genes, there was an inhibitory modulation of expression, resulting in a reduction ranging from ~70% to ~97% (Figures 2E-H).

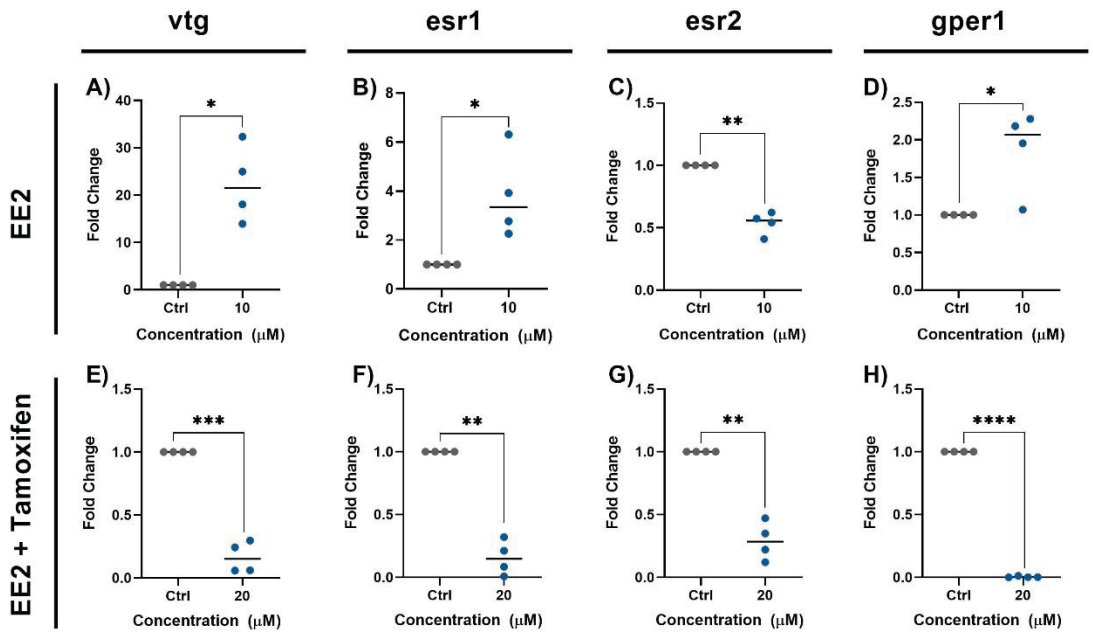


Figure 2. Expression of genes related to estrogen pathway in *O. niloticus* hepatocytes after 72 h exposure. 10 μM of EE2 (agonist), tamoxifen (antagonist), and the mix (EE2 + tamoxifen). Dots: independent experiments (n=4). Bar: mean. T-student test. *p<0.05, **p<0.01,***p<0.001, ****p<0.00001.

These results are in accordance with the use of a potent inducer and combination of inducer/inhibitor for estrogenic pathway regulation, which demonstrates that the cells properly respond to the treatments. In comparison to immortalized cell lines, the use of primary cultured hepatocytes has been widely employed for the detection of vitellogenin production, an important biomarker of endocrine disruption, due to their high responsiveness to environmental contaminants (Navas and Segner, 2006). In this regard, there is considerable variation among species and their ability to respond. In the present study, the concentration of 10 μM of EE2 caused a ~25-fold change in vtg expression in *O. niloticus* hepatocytes. However, exposure to the same concentration of E2 caused a ~100,000-fold increase in vtg expression in *Cyprinus carpio* (common carp) hepatocytes (Bickley et al., 2009). Similar studies have also been able to detect high vtg synthesis in the culture medium of *O. niloticus* hepatocytes after using agonists of estrogen receptors (ER) as inducers (Kim et al., 2003; Kim and Takemura, 2002, 2003). The induction of vtg expression occurs through the activation of the estrogenic pathway, which was modulated after exposure to the mixture of PAHs in hepatocytes by ER activity. For esr isoforms, the increase of esr1 and the decrease of esr2 expressions highlight the intricate and complex regulation of the estrogenic pathway

by both esr. Moreover, the increase of gper1 along with these results for esr indicates an intrinsic regulatory process involving crosstalk between responses from genomic and non-genomic pathways after EE2 exposure (Kiyama and Wada-Kiyama, 2015). Conversely, the treatment with EE2 + tamoxifen was effective in promoting drastic downregulation of all evaluated pathway elements, as observed by other authors for vtg production by *O. niloticus* hepatocytes under similar exposure conditions (Kim et al., 2003). However, it was an expected result, given that tamoxifen acts as a strong selective estrogen-receptor modulator (SERM), inhibiting several estrogen-responsive components (Kiyama and Wada-Kiyama, 2015). Overall, these results demonstrate the profound impact of both individual compounds and their combination on the regulation of genes associated with the estrogenic pathway in hepatocytes.

### 3.2. Responsiveness of hepatocytes to the PAH mixture

The tested PAH concentrations were not toxic to hepatocytes, regarding the cytotoxicity tests performed here, since only slight increases in MTT metabolism (~20-25%) and neutral red retention (~20-35%) were observed at concentrations of 0.002 and 0.2 $\mu g.L^{-1}$ (Figure 3A-B), and cell adhesion/cell number, measured by crystal violet assay, was not affected by exposure (Figure 3C). Furthermore, there were no observable morphological differences between PAHs and the control groups (Figure 3D).

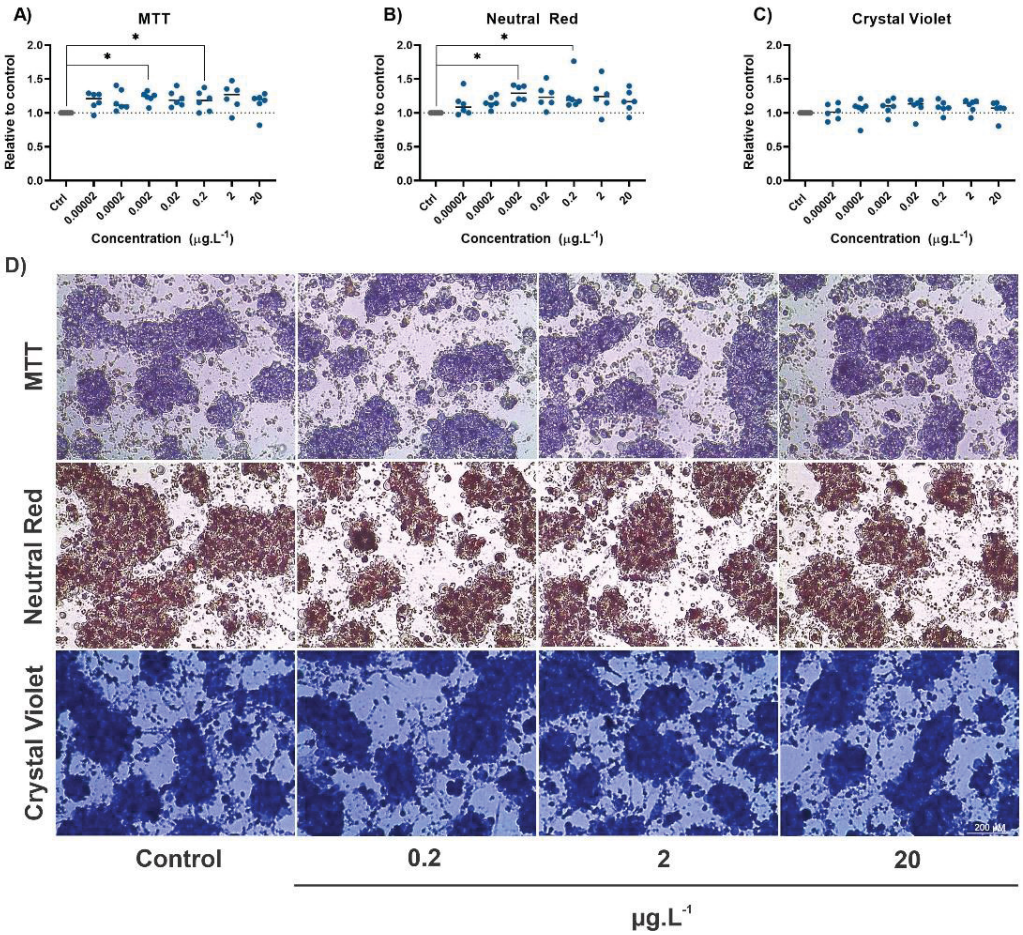


Figure 3. Cytotoxicity assays for PAH mix screening in *O. niloticus* hepatocytes after exposure for 72 h to 20 µg.L$^{-1}$. A) MTT. B) Neutral red. C) Crystal violet. D) Representative images from the hepatocytes exposed to the three highest concentrations of PAH mix. Dotted line: control baseline; dots: independent experiments (n=6). Bar: mean. ANOVA followed by Dunnett post hoc test. *p<0.05.

Considering these results, the toxic effects of the mixture of PAHs at environmentally relevant concentrations (0.002 and 0.2 µg.L$^{-1}$) can be considered mild concerning cellular dehydrogenases and lysosomal function. Isolated PAHs, such as naphthalene and pyrene, can reduce membrane integrity in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 24 h at concentrations of 0.01-10 µM (1.28-2.02 µg.L$^{-1}$; Yazdani, 2020). On the other hand, according to Bramatti et al. (2023), the PAHs benzo[a]pyrene, phenanthrene, and benzo[b]fluoranthene individually do not promote significant alterations in cellular metabolism. However, when binary mixtures of these compounds are made at different proportions, there is a significant increase in MTS metabolism (referred as 'one-step' MTT assay) in primary gilthead seabream (*Sparus aurata*) hepatocytes, demonstrating the effects of PAHs. Despite the mixture used in the present study being more complex than the

one previously described, a similar effect (increase) on MTT assay was observed, but we considered it as a cell modulation (e.g., of dehydrogenases activity) rather than proper toxicity. Furthermore, it is important to highlight that the absence of cytotoxic effects of environmentally relevant concentrations of PAHs does not mean that these compounds have no 'toxic effects' to other (not evaluated) cell activities and endpoints.

The levels of ROS and RNS were not altered after exposure to the PAH mixture (Figures 4A-B). However, antioxidants were significantly affected: catalase (CAT) activity decreased (~30%, Figure 4C), and increases occurred for superoxide dismutase (SOD: ~410%) and glutathione *S*-transferase (GST: ~45%) activities, as well as for non-protein thiols (NPT: ~45%) levels (Figures 4D-F). Likewise, a significant increase of ABC transporters efflux activity (~75-95%) was observed, as analyzed by rhodamine and calcein efflux assays (Figures 4I-J). No effects were observed for glucose 6-phosphate (G6PDH) activity and lipid peroxidation (DPP assay, Figures 4G-H).

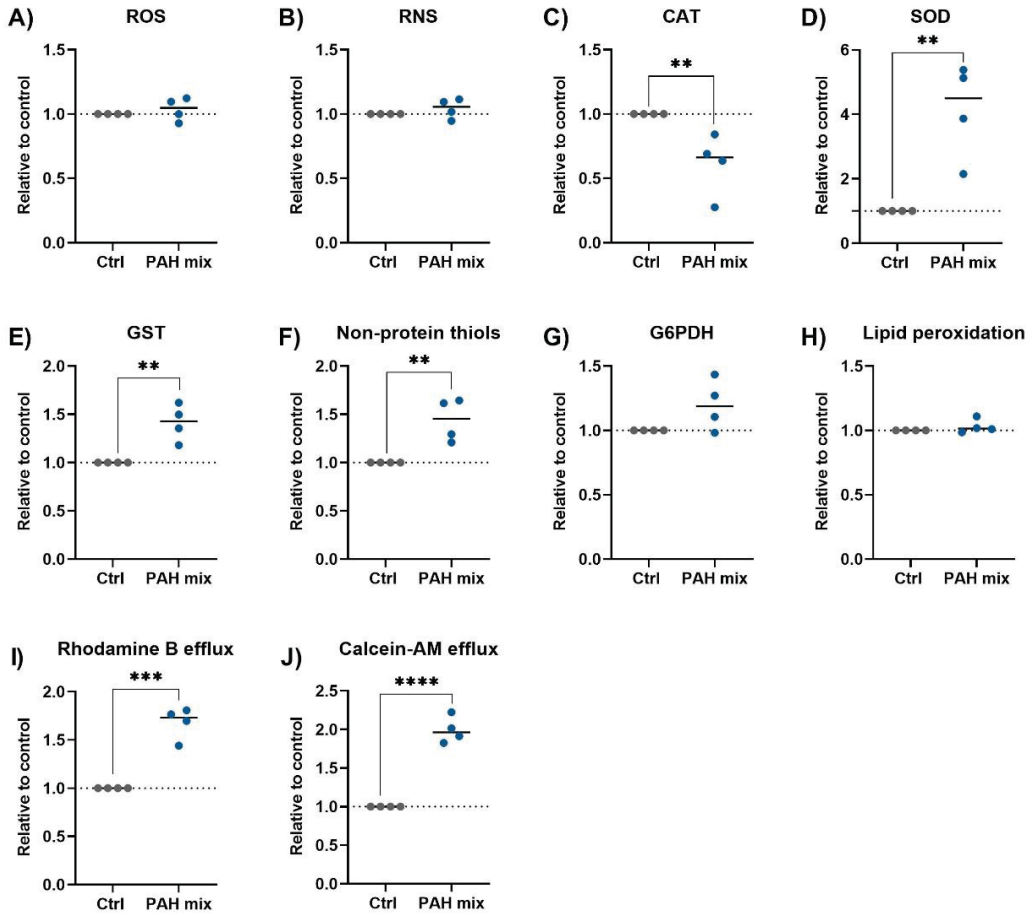


Figure 4. Oxidants, antioxidants and ABC efflux transporters in *O. niloticus* hepatocytes after exposure for 72 h to 20 µg.L$^{-1}$ of PAH mix. Dotted line: control baseline; dots: independent experiments (n=4). Bar: mean. T-student test. *p<0.05, **p<0.01, ***p<0.001, ****p< 0.00001.

Although PAHs are extensively metabolized by cytochrome P450 monooxygenases, such as CYP1A isoform, which usually produces high levels of ROS as by-products (Bramatti et al., 2023; Stading et al., 2020; Franco and Lavado, 2019), the PAH mixture did not cause mixture does not contribute to a significant increase in ROS/RNS levels in the hepatocytes. This indicates the antioxidant and detoxification defense systems (SOD, NPT and GST) as well as efflux mechanisms (PgP and MRP), were able to deal with prooxidant and toxicant stress, maintaining the cell redox milieu and cell integrity. Here, there was a significant increase in the activity of SOD, GST, and the levels of NPT in the PAH exposed group. SOD is responsible for neutralizing superoxide radicals by converting them in hydrogen peroxide, while GST plays a crucial role in the detoxification of electrophilic compounds through conjugation of glutathione (the most abundant NPT in the cells) with them (Agarwal et al., 2014). The increased activity of SOD and GST suggests an

adaptive response of the hepatocytes to counteract potential oxidative stress induced by the PAH mixture. The increased levels of NPT, which are important cellular antioxidant and detoxifying molecule, further support the idea of a compensatory response to oxidative insult. In this regard, HepG2 cells exposed to isolated PAHs and mixtures show enzymatic alterations and increased expression of Nrf2 (Nuclear factor erythroid 2-related factor 2), an important nuclear receptor and transcription factor for antioxidant response (Branco et al., 2021).

Furthermore, considering the adaptive response of hepatocytes to the PAH exposure, oxidative damage may have been minimized, as observed by control-like lipid peroxidation levels (measured by DPP assay) in the PAH-exposed cell groups. Interestingly, CAT activity decreased in the PAH-exposed cells. Although it can means a potential disruption in the antioxidant defense system (Agarwal et al., 2014), CAT and SOD work together as the first enzymatic antioxidant defense line and can have opposite modulation by superoxide anion/hydrogen peroxide levels. In addition, CAT decrease may be compensated by the large number of systems that deal with hydrogen peroxide (CAT, GST, glutathione peroxidise and peroxirredoxin enzymes, and glutathione, NADPH etc) and differences on enzymatic kinetics. Nevertheless, the antioxidant response to PAHs can also vary depending on the species and the duration of exposure, as observed in primary hepatocytes of Orange-spotted (*Epinephelus coioides*) exposed to benzo[a]pyrene, which had an increase in CAT activity (Derakhshesh et al. 2019).

In line with induction of defense mechanisms, PAH exposure caused an increase in hepatocyte efflux activity, as indicated by the increased efflux of rhodamine and calcein mediated by xenobiotic-efflux transporters. This increased may have an important role on intracellular concentrations of xenobiotics and some endogenous compounds, thus affecting their toxicity. In mammals, these ABC transporters exhibit functional similarities (Leslie et al., 2005) and have a wide range of substrates, encompassing GSH conjugates, as well as various glucuronide and sulfate conjugates of both xenobiotics and endobiotics (Leslie et al., 2005, Deeley et al., 2006). These characteristics render them highly suitable candidates for participation in phase III detoxification pathways in organisms, as indicated in a study involving Nile tilapia (Costa et al., 2012). Additionally, as shown in the present study, primary hepatocytes of European seabass (*Dicentrarchus labrax*), for example, exhibit

responsiveness in rhodamine efflux after exposure to the PAH benzo[a]pyrene (Ferreira et al., 2014).

### 3.3. Estrogen pathway modulation by the PAH mixture

The PAH mixture was able to significantly modulate the estrogenic pathway in *O. niloticus* hepatocytes. The expression of the biomarker vitellogenin (vtg) increased approximately 120% (Figure 5A). For the nuclear receptors, there was no significant difference for esr1 (Figure 5B), but esr2 expression was about 90% higher in cells exposed to the PAHs (Figure 5C). For the membrane receptor gper1, expression remained unchanged after PAH exposure (Figure 5D).

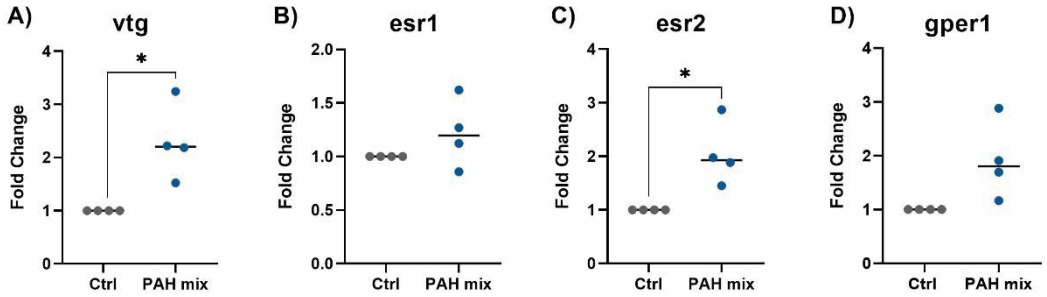


Figure 5. Expression of genes related to estrogenic pathway in *O. niloticus* hepatocytes after exposure for 72 h to 20 $\mu g.L^{-1}$ of PAH mix. A) vtg. B) esr1. C) esr2. D) gper1. Dots: independent experiments (n=4). Bar: mean. T-student test. *p<0.05.

The endocrine disrupting potential and estrogenic activity of PAHs are still controversial in the literature, as these compounds can act as inducers or repressors of estrogenic pathway (Meng et al., 2021; Zhang et al., 2016; Gozgit et al., 2004). Vtg is a well-established biomarker for estrogenic activity, and its upregulation suggests an estrogenic activity of the PAH mixture. Supporting it, vtg gene expression increased in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to environmental samples rich in PAH compounds, characterizing the effect of a complex mixture on one of the estrogen-responsive elements (EREs) of estrogenic pathway activation (Gagné et al., 2012). Furthermore, the plasma vitellogenin protein production, ER-mediated, is also used in fish to measure the toxic potential of PAHs in oil production discharges (Zhang et al., 2016).

Interestingly, the expression of the nuclear receptor esr1 (ERα) was similar between PAH-exposed and control groups so that PAH mixture may not directly affect the expression of esr1 or other factors, not evaluated in this study, may influence the modulation of esr1. Indeed, some methylated 4 and 5 ring-PAHs (by

CYP1A) can act as agonists (ER-α agonist CALUX assay) and promote an estrogenic response (Fang et al., 2022; van Lipzig et al., 2005), demonstrating a crosstalk between the AhR/ER pathways (Sondermann et al., 2023). Concerning esr2, the specific activation of esr2 (ERβ) is consistent with the estrogenic modulation observed in vtg expression. The differential response of esr1 and esr2 suggests distinct regulatory mechanisms and their potential roles in mediating the genomic pathway of the estrogenic response effects of the PAH mixture. In this regard, some compounds present in the mixture (anthracene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, and benzo[a]pyrene) used for hepatocyte exposure have been described as capable of activating ERs expression in MCF-7 cells (Gozgit et al., 2004).

In contrast, since the expression of the membrane receptor gper1 was not affected in hepatocytes exposed to the PAH mixture, the modulation of the estrogenic pathway may predominantly occur through the genomic pathway. Gper1 plays a crucial role in non-genomic pathway in aquatic organisms, especially for environmental contaminants belonging to multiple classes (Chen and Chan, 2021; Liu et al., 2021; Meng et al., 2021; Gonzáles-Rojo et al., 2019). However, it is worth noting that gper1 may still play a role in other cellular processes and respond to different stimuli promoted by PAHs that were not evaluated in this study. These findings provide valuable insights into the molecular mechanisms underlying the estrogenic modulation induced by the PAH mixture in *O. niloticus* hepatocytes, considering that the upregulation of vtg and esr2 expression suggests a potential estrogenic activity of the PAHs present in the mixture.

### 3.4. Bioinformatics analysis

A list of 94 genes affected by the 14 PAHs present in the mixture was obtained through the search in the CTD database and analyzed using the String database. From the 18 PAHs, no results were found only for acenaphthene, acenaphthylene, 1-methylnaphthalene, and 2-methylnaphthalene.

Using the MCODE app in the Cytoscape software, a sub-cluster with a high score (24) in this complex interaction network was obtained (Figure 6A). Furthermore, the CentiScape app analysis, using the betweenness and degree thresholds, determined which genes are highly connected in the network and may act as potential bottlenecks in the regulation of pathway interactions. In the complete network,

several genes exceeded the threshold values (betweenness: 19.23; degree: 23.07), with the top 5 being creb1a, mapk3, esr1, kras, and hrasa (Figure 6A). On the other hand, for the sub-cluster, only the esr1 gene had values above the threshold (betweenness: 1.45; degree: 22.7), being the main regulator of this network (Figure 6A).

Furthermore, based on the gene list, a functional enrichment analysis was performed using the ShinyGO server, allowing for the significant association (FDR-value < 0.05) of these genes with biological processes and pathways. Most biological processes are related to cellular signaling and response, involving organic compounds, as well as several metabolic processes associated with lipid metabolism. However, in terms of pathways, there was a greater association with reproductive processes and gonadal maturation, as well as calcium or MAPK-mediated cellular signaling (Figure 6B).

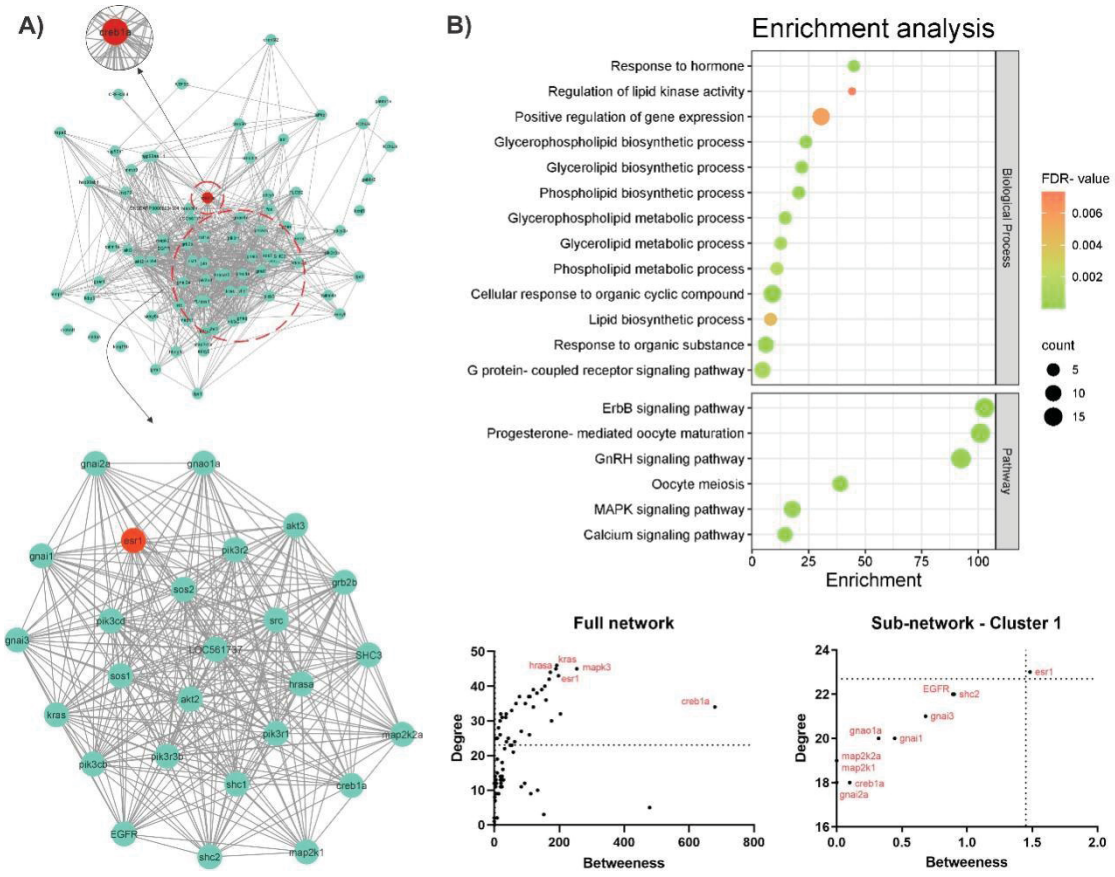


Figure 6. Network interaction and enrichment analysis of PAHs present in the mixture with genes associated with the estrogen pathway signaling (KEGG: hsa04915). A) General interaction network of genes found in the CTD database related to PAHs, highlighting the cluster with the highest score identified by the MCODE application, and graphical representation of the results obtained in the identification of bottlenecks for the general

network (in red: creb1a, gene with highest score) and the cluster (in red: esr1, the only gene above the thresholds). B) Results of the enrichment analysis for biological processes and pathways associated with the identified genes.

Despite the controversial literature regarding the estrogenic activity and endocrine-disrupting potential of PAHs, the bioinformatics analyses characterized and identified genes related to these processes based on the individual compounds present in the mixture. The interaction network analyses highlighted the top 5 genes with the highest potential to act as regulators in the network (hub/bottleneck). Among these, creb1a (CAMP responsive element-binding protein 1) is significantly regulated in benz(a)anthracene-exposed zebrafish embryos (48 hours post-fertilization) (Goodale et al., 2013). In this regard, other genes involved in signaling pathway regulation, such as mapk, kras, and hrasa, may be altered after PAH exposure and modulate cellular responses related to detoxification, cell migration, oxidative DNA damage, and carcinogenesis (Stading et al., 2021; Sun et al., 2021; Wang et al., 2020; Song et al., 2011). The esr1 gene was also identified as a bottleneck/hub in both the overall network and the sub-cluster, and although its expression was not altered in the present study, esr2 gene was. The differential expression of these genes after exposure to PAHs suggests that individual compounds, when present in a complex mixture, can modulate the activity of proteins involved in the estrogenic pathway in *O. niloticus* hepatocytes. On this regard, it is important to emphasize that during the estrogenic response process, nuclear receptors can form homodimers or heterodimers (e.g., ERα/ERα or ERα/ERβ), and alterations in the interaction dynamics of different isoforms can lead to changes in physiological responses (Zadja and Gregoraszczuk, 2019; Chakraborty et al., 2012).

Furthermore, through the functional enrichment analysis, it was identified that most of the genes involved in the estrogenic response to PAHs are associated with biological processes involving lipid metabolism. There are not many studies in the literature highlighting the relationship between PAHs and the affected processes. However, some studies have shown that multiple lipid subclasses, such as glycerophospholipids, glycerolipids, and fatty acids metabolism in mice, are affected by benzo[a]pyrene (Li et al., 2020; Li et al., 2019). Moreover, PAHs can also alter lipid metabolism by increasing the expression of peroxisome proliferator-activated receptor γ (pparγ) and sterol regulatory element-binding protein-1c (srebp1c), resulting in disruption of steroidogenic production and processes (Vondráček et al.,

2018; Jin et al., 2014). As for the pathways involved, all of them are related to reproductive processes, which are negatively affected by exposure to different types of PAHs in *in vivo* studies (Peng et al., 2019; Vondráček et al., 2018; Perrichon et al., 2015).

## 4. Conclusion

The current study demonstrated that a complex mixture of PAHs can induce significant alterations in the estrogen pathway and oxidative stress response in primary cultured hepatocytes of *O. niloticus*. The exposure to the PAH mixture resulted in an upregulation of the biomarker vitellogenin (vtg) and estrogen receptor 2 (esr2), suggesting the activation of the estrogen pathway. In addition, the hepatocytes had an effective response to mitigate the oxidative damage promoted by the exposure of PAHs. These results emphasize the importance of understanding the toxicological effects of complex mixtures of PAHs on aquatic organisms. The modulation of the estrogen pathway and oxidative stress response highlights the potential endocrine disrupting and toxic effects of these environmental contaminants. In this sense, the bioinformatics analysis enabled to evaluate which biological processes the genes related to the compounds present in the PAH mixture are involved, resulting in a better comprehension about the toxic mechanisms. Overall, this study provides useful data for further investigations aiming to elucidate the underlying molecular mechanisms and assess the long-term ecological consequences of PAH exposure.

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Capítulo III

# Molecular and lipid profile of zebrafish exposed to PAHs: implications on endocrine disruption

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**Abstract**

Understanding the environmental effects on lipid metabolism is crucial to assess the impacts of contaminant exposure on aquatic organisms. In this study, the effects of a complex mixture of Polycyclic Aromatic Hydrocarbons (PAHs) on lipid metabolism and related gene expression were investigated in zebrafish (*Danio rerio*) larvae. The larvae were exposed to environmentally relevant concentrations (0.00001 – 0.1 µg/L of water) of a complex mixture composed of 18 PAHs. Biological responses were evaluated at 5 different concentrations through qPCR and lipidomic analyses. Genes traditionally responsive to PAHs, ahr2 and cyp1a, were significantly upregulated, and the expression of nuclear receptor genes lxr and rxraa, responsible for regulating lipid metabolism, were altered. A pronounced downregulation in the classes of lipids such as glycerolipids, glycerophospholipids, sphingolipids, and fatty acyl was observed through lipidomic analysis, suggesting that exposure to PAHs significantly impacted larval lipid metabolism. However, this response was not uniform across all concentrations, with lipid levels preserved in these classes at the highest concentration (0.1 µg/L), possibly indicating an altered cellular response at higher exposure levels. Glycerolipids, sphingolipids, fatty acyls, and glycerophospholipids were the most relevant classes of lipids to separate experimental groups in the PLS-DA analysis. This variability in class importance suggests a distinct response to different PAH concentrations, highlighting the complex interaction between contaminants and lipid metabolism. These findings contribute to our understanding of the impacts of PAHs on aquatic organisms, emphasizing the importance of studying the intricate relationship between pollutants and lipid metabolism.

**Keywords**: lipidomics; developmental toxicity; FET; PAH mixture.

## 1) Introduction

Industrial expansion and urban growth increased the introduction of chemical pollutants into aquatic ecosystems, resulting in a complex amalgamation of substances that jeopardize the health and stability of these environments. Among the prominent pollutants, Polycyclic Aromatic Hydrocarbons (PAHs), stemming from diverse sources of organic matter, have emerged as a significant environmental concern (McGrath et al., 2019; Jacob et al., 2013). PAHs represent a class of chemical compounds containing condensed benzene rings and are widely distributed in aquatic environments due to both natural and anthropogenic factors, such as atmospheric deposition, oil spills and industrial activities (Gaurav et al., 2021; Mojiri et al., 2019; Weber et al., 2011). Owing to their lipophilic nature and persistence, bioaccumulation on adipose tissue and transfer through the food chain (biomagnification), the continuous and chronic exposure to PAHs potentially triggers adverse effects on the health of fish (Li et al., 2023; Mojiri et al., 2019; Wang et al., 2019; Froehner et al., 2018). Furthermore, other types of environmental contaminants, such as microplastics, can act as carriers of PAHs, enhancing their toxic potential to animal health (Li et al., 2023).

Particularly, endocrine disruption has emerged as a health concern since PAHs can act as endocrine disruptors capable of interfering with the hormonal systems of fish (Aguilar et al., 2021; Gonçalves et al., 2014). The ability of PAHs to bind to nuclear receptors can lead to alterations in hormone-regulated physiological processes (Aguilar et al., 2021; Gonçalves et al., 2014). In this regard, the upregulation of estrogen receptors (ERs), estrogen-responsive elements (EREs) and vitellogenin (vtg) biomarker has been observed in fish (Aguilar et al., 2021; Gonçalves et al., 2014). Consequently, the normal functioning of the endocrine system in these organisms may be impaired, impacting their reproduction, growth, development, and behavior.

Another critical impact of PAHs on fish resides in their influence on lipid metabolism. While methods to assess the endocrine-disrupting potential of a substance usually focus on EATS (estrogen, androgen, thyroid, and steroidogenesis), there is a need to develop non-EATS methods, of great concern for nuclear receptors involved in lipid and xenobiotic metabolism, such as RXR - retinoic

acid, AhR - aryl hydrocarbon receptor, and PPAR - peroxisome proliferator-activated receptor (Martyniuk et al., 2022). Moreover, the alteration of proteins involved in lipid metabolism can result in the abnormal accumulation of lipids in tissues, leading to dysfunction of cell membrane and energy homeostasis (Martyniuk et al., 2022; Ning et al., 2020). This influence is not limited to metabolic disruption but can also generate interconnected effects on fish endocrine systems through ERα receptor activation and modulation of genes associated with steroid hormone synthesis (Ning et al., 2020). Additionally, PAHs are considered potent inducers of responses mediated by the AhR pathway, through the induction of cytochrome P450 1A (CYP1A) expression, and this pathway may correlate with endocrine regulation through crosstalk (Gräns et al., 2010). This involves acknowledging the interaction between the EATS and non-EATS pathways, intricate combinations of diverse endocrine disrupting chemicals within mixtures, pathways leading to negative effects due to substances influencing non-EATS processes, and innovative approaches to evaluate chemical substances (Martyniuk et al., 2022).

Amidst the complex mixture of pollutants present in aquatic ecosystems, PAHs emerge as a pivotal point of interest due to their implications on fish health. Endocrine disruption and the disruption of lipid metabolism can be integral parts of the adverse effects caused by PAH exposure, especially in sensitive developmental stages. Thus, comprehending the underlying mechanisms of these effects is essential for formulating strategies to mitigate the risks associated with PAHs, preserving the health of aquatic ecosystems.

## 2) Material and methods

### 2.1) Zebrafish breeding

Zebrafish eggs were produced through natural mating of adult individuals (wild type *Danio rerio*) fed with dry flakes twice a day (TetraMin, Tetra, Germany), and in 4 L breeding tanks with 2 male and 3: female fish. To collect the eggs, a mesh was placed to prevent contact and predation by adult individuals. Adults, eggs, and embryos were maintained in in reverse osmosis purified water with the commercial salt mixture Instant Ocean (90 g/mL, Aquarium Systems, Sarrebourg, France) and $CaSO_4 \cdot 2H_2O$ (100 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) under standard conditions, at 28.0±1.0°C and 12 h light:12 h dark photoperiod. Two hours after

fertilization (hpf), the eggs were collected, rinsed with methylene blue (0.001%), and examined for fertilization. A total of 10 embryos were placed in each well of 6-well plates containing clean water (3 mL) prior to exposure to PAHs. All experimental procedures were conducted in accordance with institutional policies and the Institutional Animal Care and Use Committees of the Research and Development Center (CID) of the Spanish National Research Council (CSIC).

### 2.2) Exposure and toxicological screening

Zebrafish larvae were exposed to a complex mixture of PAHs (Table 1) for 24 h, from the 4th day post-fertilization (dpf) to the 5th dpf. This exposure period was chosen to ensure that the treatments would not affect critical stages of embryonic development, in order to maintain the status of an "animal replacement test" (Scholz et al., 2008; Raldua and Piña, 2014).

Table 1 - Components of the Complex Mixture of PAHs

| **HPAs (Σ=18) – stock: 2000 µg/mL** | |
|---|---|
| Acenaphthene | Chrysene |
| Acenaphthylene | Dibenz[a,h]anthracene |
| Anthracene | Fluoranthene |
| Benz[a]anthracene | Fluorene |
| Benzo[b]fluoranthene | Indeno[1,2,3-cd]pyrene |
| Benzo[k]fluoranthene | 1-Methylnaphthalene |
| Benzo[ghi]perylene | 2-Methylnaphthalene |
| Benzo[a]pyrene | Naphthalene |
| Pyrene | Phenanthrene |

First, a screening was conducted to assess the toxic potential of the PAH mixture and select sub-lethal concentrations for the analyses. To obtain a dose-response curve, concentrations of 0.6125, 1.25, 2.5, 5 and 10 µg/mL of the mixture were utilized. Endpoints such as larvae survival and swim bladder inflation before and after exposure were assessed to determine the toxic potential of the mixture. Then, new test concentrations were selected based on the screening, and the larvae were exposed to the mixture on a log10 basis: 0.00001, 0.0001, 0.001, 0.01 and 0.1 µg/mL. In all experiments, DMSO (0.02% v/v) was used as a negative control. The range of tested concentrations encompasses those of environmental relevance, found in water bodies (Barreto et al., 2020).

**2.3) qPCR**

After the exposure period, total RNA was extracted using the TRIzol Reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration and purity were measured using the NanoDrop™ ND-8000 spectrophotometer (Fisher Scientific). The samples were treated with DNase I, and cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) following the manufacturer's instructions. For RT-qPCR, each reaction was prepared with the addition of 2 µL of cDNA, 6.8 µL of nuclease-free ultrapure water, 1.2 µL of primer mix (forward and reverse), and 10 µL of SYBR® Green Master (Roche Diagnostics).

The qPCR reactions were performed using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics). The amplification program consisted of 10 min at 95ºC, followed by 45 cycles of 10 s at 95ºC and 30 s at 60ºC. After amplification, a dissociation curve was generated to evaluate reaction specificity. Non-template and RT-minus controls were used as negative controls for the reactions to assess primer dimer formation and genomic DNA contamination. The ppia2 gene was used as a reference gene for comparison with the target genes (ga) (Table 2). The relative mRNA abundance was calculated according to the second maximum derivative of their respective amplification curve (cp, calculated in technical duplicates), using the equation $\Delta Cpga = Cp\_reference\_gene - Cp\_ga$. Differences in mRNA abundance between samples from different treatments were calculated according to the ΔΔCp method (Pfaffl, 2001). In this regard, fold change values were obtained relative to the control group.

Table 2 - Genes and primer sequences.

| Gene | NCBI ID | Forward | Reverse |
|---|---|---|---|
| *cyp1a* | 140634 | GGTTAAAGTTCACCGGGATGC | CTGTGGTGTGACCCGAAGAAG |
| *ahr2* | 30517 | CTACTTGGGCTTCCATCAGTCG | GTCACTTGAGGGATTGAGAGCG |
| *ugt1a1* | 641488 | CGTTTGATTCCTTGTCGTCTTG | AACCACTGGAGTAGCGAGGC |
| *abcc1* | 100002010 | CAGTCCACCATCCGAACTCA | ATCGAGAACCAGCACCCTTG |
| *cyp19a1b* | 60640 | GCAAAGGGGACAAACCTAATC | CCTTCGTCATCACCATAGCA |
| *ppara* | 557714 | ATCCAAAGCACGAACCATCC | TGGCCACAAATGTCTGTTCTG |
| *rxraa* | 555578 | GAAGACATGCCCGTGGAGAA | GGTGAATTTGATGGCATGGG |
| *lxr* | 548341 | GACTAGGCAAATGAGAGCAATTTG | AACCTGAATTGTGCATGCTCAC |
| *cyp26b1* | 324188 | GCTGTCAACCAGAACATTCCC | GGTTCTGATTGGAGTCGAGGC |
| *cyp3a65* | 553969 | CGGAGAGCTTCAAACCCGA | CCAGACCGAACGGCATGTAC |
| *ppia2* | 336612 | GGGTGGTAATGGAGCTGAGA | AATGGACTTGCCACCAGTTC |

*cyp1a*: cytochrome P450, family 1, subfamily A; *ahr2*: aryl hydrocarbon receptor 2; *ugt1a1*: UDP glucuronosyltransferase 1 family, polypeptide A1; *abcc1*: ATP-binding cassette, sub-family C (CFTR/MRP), member 1; *cyp19a1b*: cytochrome P450, family 19, subfamily A, polypeptide 1b; *ppara*: peroxisome proliferator-activated receptor alpha b; *rxraa*: retinoid X receptor, alpha a; *lxr*: liver x factor; *cyp26b1*: cytochrome P450, family 26, subfamily b, polypeptide 1; *cyp3a65*: cytochrome P450, family 3, subfamily A, polypeptide 65; *ppia2*: peptidylprolyl isomerase Aa (cyclophilin A).

### 2.4) Lipid extraction

Lipids were extracted from 5 samples per group, each consisting of a pool of 10 larvae for lipidomic analysis. Metabolites from the samples were extracted by adding 900 µL of chilled methanol (Fisher), vortexing for 15 s and homogenization in a Tissue Lyzer LT (Qiagen) for 4 min (40 Hz). The samples were supplemented with 10 µL of 10 ppm methionine sulfone. To enhance the separation of metabolites and lipids, an additional hydrophobic phase was created by adding 500 µL of water and 400 µL of chloroform. The samples were shaken for 15 s and sonicated for 10 min at ice bath before centrifugation at 20,000 g for 10 min at 4°C. The aqueous fraction containing metabolites (upper phase) was separated from the lower phase (containing lipids) and evaporated under a nitrogen gas stream. The samples were reconstituted in 450 µL of 1:1 acetonitrile:water. To eliminate potential residues from the extracted samples, the samples were centrifuged again at 20,000 g for 5 min and stored in vials at -80°C until analysis. Additionally, a Quality Control (QC) sample was prepared by pooling 10 µL from each sample of all treatments and controls.

### 2.5) Liquid Chromatograph – High Resolution Mass Spectometry (LC-HRMS)

Chromatographic separations of lipids were performed on an ultra-high performance liquid chromatography (UHPLC - Bruker) system using a hydrophilic interaction liquid chromatography (HILIC) column (ACQUITY UPLC BEH, 100 mm length, 2.1 mm diameter, 1.7 µm particle size) from Waters (Ireland) at 50ºC. The protocol followed the manufacturer's instructions. To enhance chromatographic separations, only 2 µL of sample were injected into the UHPLC system at a flow rate of 0.4 mL/min. The 10-min separations started with an elution gradient of 99.9% mobile phase B1 (90:10 Acetonitrile/Water, 10 mM ammonium formate + 0.2% formic acid), increasing to 75% at 4 min and 50% at 6 min. The concentrations of mobile phase A1 (50:50 acetonitrile/water, 10 mM ammonium formate + 0.2% formic acid) reached 90% at 7 min and returned to 0.1% after 8 min.

Full scan mass spectra (broadband collision-induced dissociation, bbCID) ranging from 60 to 1000 m/z were acquired using a high-resolution QTOF mass spectrometer (Bruker Impact II) with a resolution of 50,000 FWHM. The ionization source operated solely in positive mode, with a normalized collision energy of 25 eV and ion energy of 4 eV. Additionally, the capillary voltage was set to 2500 V, nebulizer pressure at 2.0 Bar, dry gas flow at 8 $L.min^{-1}$, and dry temperature at 200ºC.

### 2.6) Data analysis

The analysis was conducted following Villasclaras et al. (2022). Omics data were converted from raw to abf format for analysis using the MSDIAL software (version 4.9). The file conversion was performed using the ibfConverter tool within the MSDIAL package. In addition to variable selection and feature alignment across all samples, this software package combines experimental MS2 AIF-DIA (All Ion Fragmentation Data Independent Acquisition) data with those stored in public libraries of chemical compounds. This step is essential for the provisional assignment and identification of lipids present in the samples. The peak area matrix of the compounds detected in all samples was then subjected to inter-group alignment, enabling the attribution of fold change differences between treatments. Furthermore, data distribution was evaluated using Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) to determine Variable

Importance in Projection (VIP) values. VIP is utilized to identify genes or variables with the greatest influence on distinguishing between the groups of interest, aiding in the selection of relevant biomarkers and interpretation of PLS-DA model results. Consequently, only VIP values >1 were considered in the outcomes. For other inter-group comparison parameters, a one-way ANOVA with Tukey's post hoc test was employed, with p-values <0.05 considered significant.

## 3) Results and Discussion

### 3.1) General toxicity

The initial screening of the toxicity of the complex mixture of PAHs demonstrated a dose-response effect on swim bladder inflation and individual survival (Figure 1A-C). Based on the screening, sub-lethal concentrations were selected and absence of severe toxicity was confirmed (Figure 1D-F). Most studies in the literature merely describe the toxicological effect of individual PAHs, rather than of mixtures. Nevertheless, severe morphological alterations and low survival of zebrafish embryos exposed to mixture of O-bounded PAHs, complex mixture of PAHs present in crude oil, and isolated benzo[a]pyrene have been previously reported (Cunha et al., 2020; Gonzáles-Penagos et al., 2022).

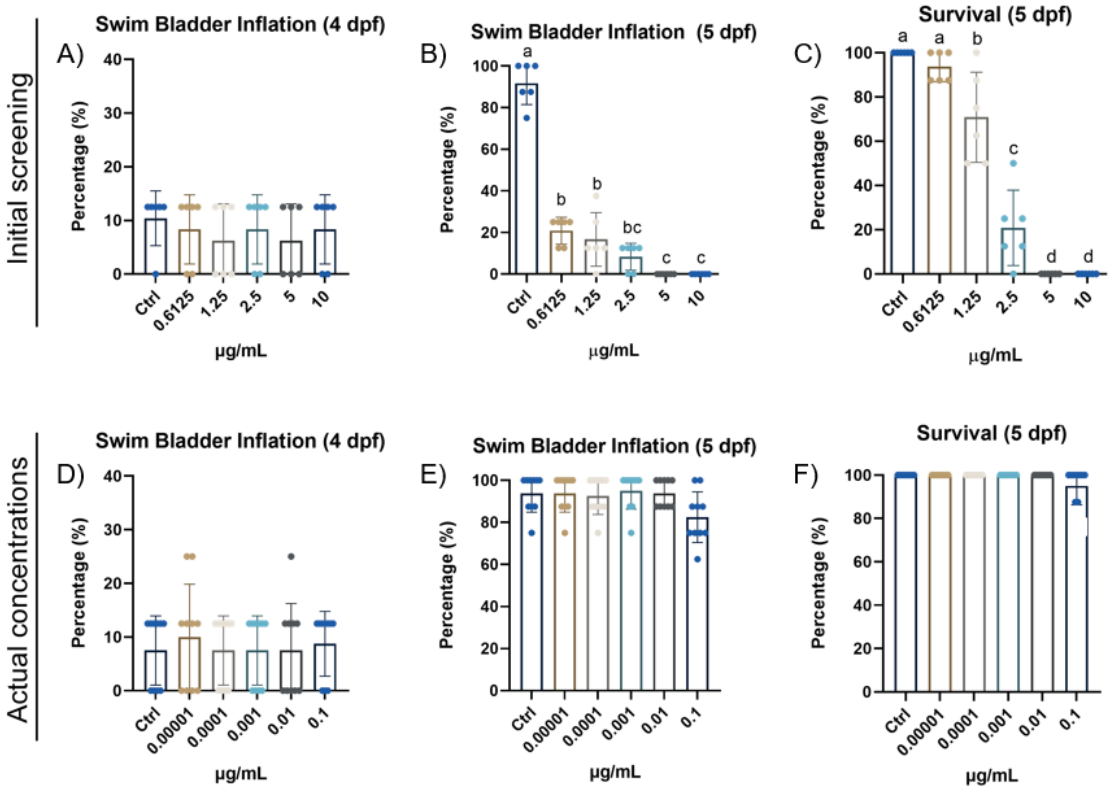


Figure 1. Toxicity screening of the PAH mixture. A) Swim bladder inflation at 4 dpf (before exposure). B) Swim bladder inflation after exposure to the PAH mixture at 5 dpf (after exposure). C) Survival following PAH exposure. D) Swim bladder inflation at 4 dpf (before exposure). E) Swim bladder inflation after exposure to the PAH mixture at 5 dpf (after exposure). F) Survival following PAH exposure. ANOVA followed by Tukey's post hoc test (n=6-8). Different letters indicate statistical differences among the groups ($p<0.05$).

### 3.2) Gene expression

*PAH metabolism*: increased expression of the nuclear receptor ahr2 was observed at the two highest concentrations of PAHs (0.01 and 0.1 µg/mL), as well as of cyp1a and ugt1a1 (phase 1 and 2 of xenobiotic metabolism), and the efflux transporter abcc1 (Figure 2A-D). However, there was no estrogenic effect observed for the PAH mixture, as the expression of cyp19a1b remained unchanged (Figure 2E). Similar effects have also been reported by Cunha et al. (2020) when evaluating the impact of a PAH mixture on zebrafish embryos, particularly for genes ahr2 (~1.5-2 fold change) and cyp1a (~100-300 fold change). Additionally, PAHs are recognized as potent inducers of AhR-mediated responses, leading to CYP1A expression, which can induce inflammatory, genotoxic, and oxidative stress effects (Endirlik et al., 2023; Gonçalvez et al., 2014). In this context, the interplay between the AhR/ERα cross-talk (Gräns et al., 2010), along with their concurrent action with other nuclear receptors, might be a pivotal event linking lipid metabolism and endocrine disruption caused by

PAHs. Although an increase of cyp19a1b expression was not observed, it is possible that other estrogenic mechanisms are activated. On the other hand, exposure to the PAH mixture altered the expression of an ABC transporter in zebrafish larvae involved in the efflux of xenobiotics and some endogenous compounds. Assessing the expression of the transporter abcc1 (~2 fold change) and the enzyme ugt1a (~5 fold change) in cellular lineages and primary cultures is crucial for understanding cellular responses, phase 2 detoxification and phase 3 efflux mechanisms, and cellular adaptability to environmental pollutants (Ferreira et al., 2014; Zaja et al., 2007). In this regard, efflux transporters exhibit functional similarities (Leslie et al., 2005), encompassing a wide range of substrates, including conjugates such as glucuronide and glutathione conjugates from ugt1a and gst metabolism of xenobiotics (Leslie et al., 2005; Deeley et al., 2006). Not only transporters but various proteins involved in xenobiotic metabolism in zebrafish embryos are affected following exposure to complex mixtures of PAHs, as demonstrated by González-Penagos et al. (2022).

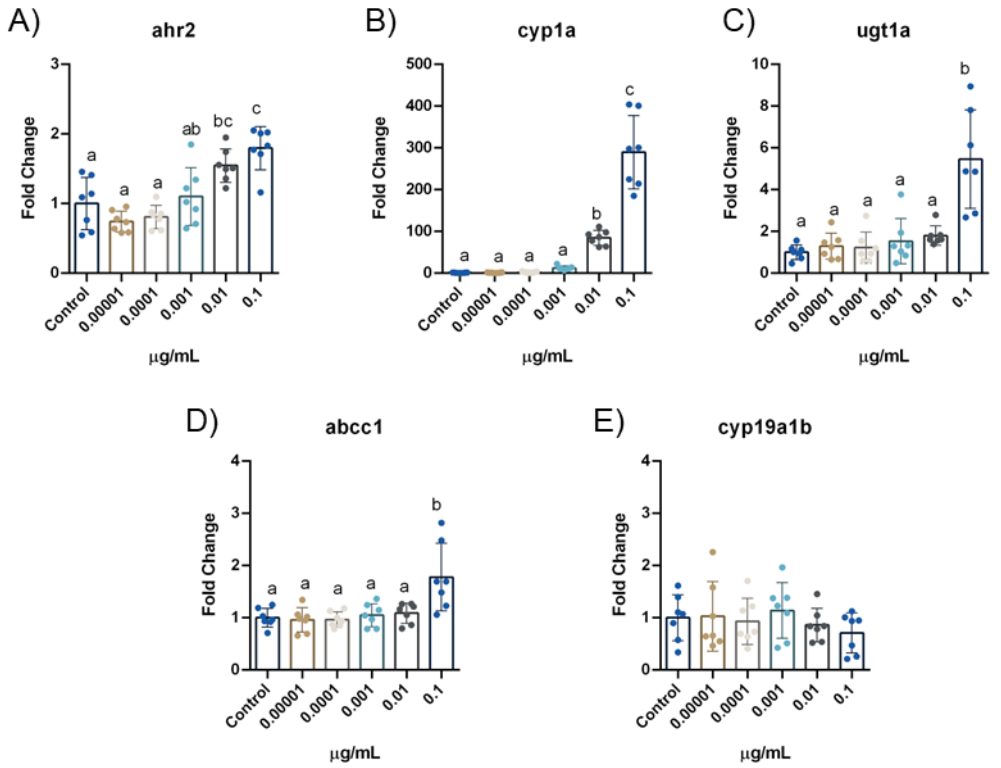


Figure 2. Expression of genes involved in PAH metabolism. A) ahr2. B) cyp1a. C) ugt1a1. D) abcc1. E) cyp19a1b. ANOVA followed by Tukey's post hoc test (n=7). Different letters indicate statistical differences among the groups ($p<0.05$).

*Lipid metabolism*: for the 3 nuclear receptors (ppara, rxraa, and lxr), gene expression was downregulated for rxraa and upregulated for lxr (Figure 3A-C), whereas for enzymes related to the lipid metabolism, there was no significant difference for cyp26b1, while the highest concentration (0.1 µg/mL) of PAH caused an increase in the expression of cyp3a65 (Figure 3D-E). Particularly, the distinct regulations of rxraa (down: ~-0.5 folg change) and lxr (up: ~1.5 fold change) may indicate a complex cross-regulation between these two nuclear receptors, possibly related to lipid metabolism modulation. The downregulation of rxraa could be associated with cellular adaptations in response to environmental changes, while the upregulation of lxr might represent an attempt to compensate for or reverse the adverse effects of exposure. This response following PAH exposure could have negative implications for lipid metabolism modulation, given that nuclear receptors like lxr, rxr, and ppara can form heterodimers to modulate gene expression (Martyniuk et al., 2022; Cocci et al., 2015).

The cyp26b1, responsible for metabolizing retinoid compounds and responsive to rxraa, did not show significant differences in gene expression in response to PAH

exposure. This suggests that the CYP coded by the gene is not sensitive to the compound concentration used in this study or may not play a central role in the pathways of lipid metabolism affected in the current study. Conversely, the expression of cyp3a65 (up regulated: ~2.5 fold change), which codes for an enzyme related to the metabolism of xenobiotics and steroid lipids, increased in response to the highest PAH concentration (0.1 µg/mL), and may be an adaptive response to stress caused by exposure to such high concentration. Thus, this enzyme could be involved in the cell's attempt to metabolize or neutralize the compounds, acting as a cellular defense against substances potentially harmful to the organism. Modulation of this CYP in zebrafish larvae exposed to environmental samples containing complex mixtures of PAHs or isolated compounds has been reported, demonstrating the lipid-disrupting potential of these contaminants (Lu et al., 2021; Bui et al., 2012; Weigt et al., 2011).

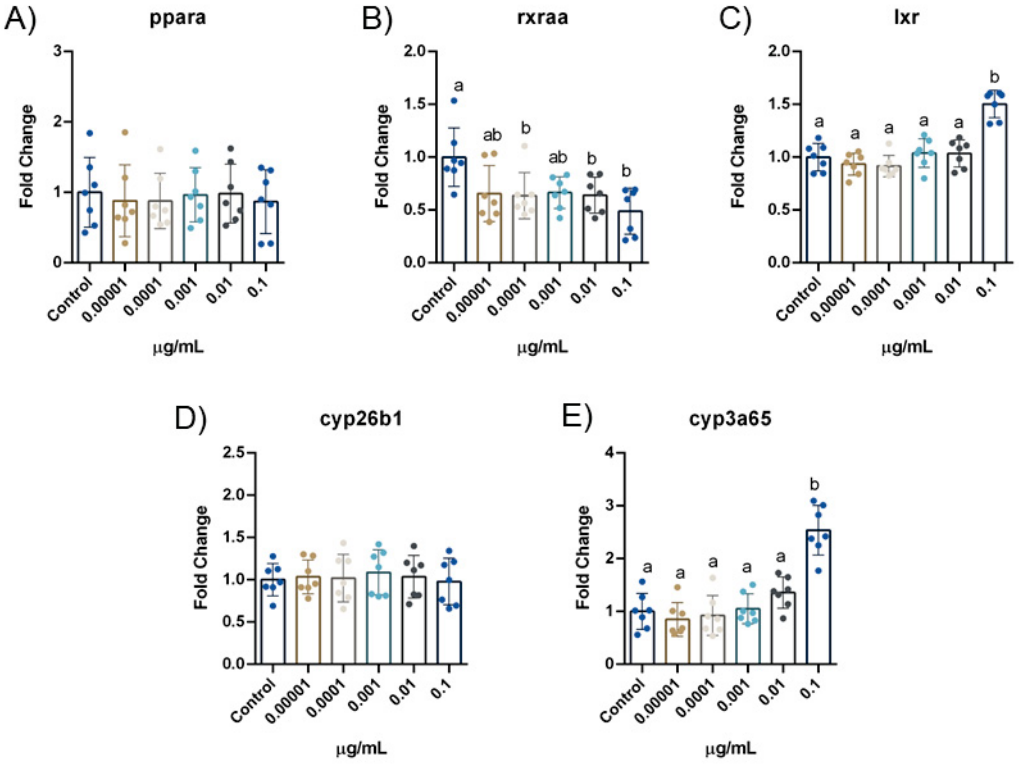


Figure 3. Expression of genes involved in lipid metabolism. A) ppara. B) rxraa. C) lxr. D) cyp26b1. E) cyp3a65. ANOVA followed by Tukey's post hoc test (n=7). Different letters indicate statistical significance among the groups ($p<0.05$).

### 3.3) Lipidomic analysis

The Principal Component Analysis (PCA) allowed to discriminate the 5 concentrations of the PAH mixture, as well as the control, blank, and Quality Control (QC) samples (Figure 4A). Furthermore, upon evaluating the dataset structure, the outcome is highly comparable with the exploratory multivariate analysis of PLS-DA (Figure 4B). These findings suggest a clear differentiation of altered parameters within each treatment based on the administered dose, enabling effective discrimination within the dataset.

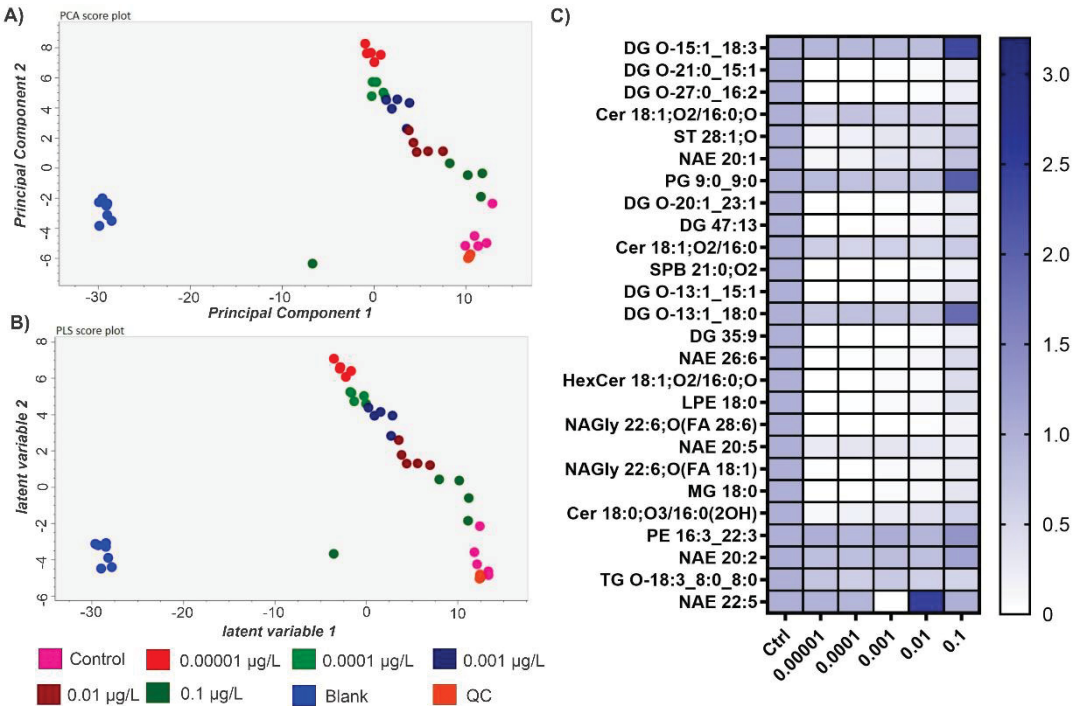


Figure 4. Multivariate analysis of lipidomic data. A) PCA analysis. B) PLS-DA analysis. C) Heatmap of lipid species with VIP values >1, depicting the fold change of the PAH mixture concentrations in relation to the control group. The values shown in the heatmap represent the fold change relative to the control group (n=5). DG: diacylglycerol; Cer: Ceramide; ST: Sterol; NAE: N-Acylethanolamine; DG-O: Diacylglycerol ethers; SPB: Sphingosine-1-phosphate; HexCer: Hexosylceramide; NAGly: N-Acylglycine; LPE: Lysophosphatidylethanolamine; PE: Phosphatidylethanolamine; MG: Monoglyceride; TG-O: Triglyceride ethers.

Furthermore, for the PLS-DA analysis, 42 lipid species exhibited VIP values greater than 1 and are depicted in Figure 4C. It is noticeable that there is a certain pattern of fold change among the tested concentrations of the PAH mixture, predominantly indicating a down-regulation pattern. The lipid classes of greatest importance for group separation, as indicated by PLS-DA results and the number of lipid subclasses, were as follows: glycerolipids (n=16) > sphingolipids (n=8) – fatty acyls (n=8) > glycerophospholipids (n=7) > sterol lipids (n=2) > prenol lipids (n=1).

Among these findings, the substantial quantity of glycerolipid subclasses involved stands out, suggesting that exposure to PAHs may influence various aspects of lipid metabolism related to these lipids, as already observed in mammals and invertebrates exposed to PAHs (Masutin et al., 2022; Hansen et al., 2020; Li et al., 2019). Within the glycerolipid class, Li et al. (2020) indicate that triglycerides (TG) are the most sensitive subclass to exposure within the class after exposure to benzo[a]pyrene. However, in the present study, diacylglycerols (DG) demonstrated greater sensitivity.

The drastic downregulation of glycerolipids, glycerophospholipids, sphingolipids, and fatty acyls levels suggests a potential coordinated response of lipid metabolic pathways to PAHs exposure (Figure 5A-D). Firstly, the downregulation of glycerolipids and glycerophospholipids implies a general suppression of triglyceride and phospholipid synthesis and storage pathways (Van Meer et al., 2008; Aguilera-Méndez et al., 2013). This might indicate that zebrafish larvae are reducing the production of lipid molecules involved in energy storage and construction of cell membranes, possibly as part of an adaptive response to PAHs. The downregulation of sphingolipids is also remarkable, as sphingolipids are involved in diverse cellular functions, including formation of lipid raft domains in cell membranes, and intracellular signaling (Alonso et al., 2001). The decrease of these lipids could indicate an alteration in the composition of plasma membrane domains and cellular signaling, impacting cell integrity, function, and organism development.

The downregulation of fatty acyls, except at the highest concentration of PAH mixture (Figure 5A), points to a possible concentration-dependent effect of PAHs on fatty acid composition. This lipid class is important for energy conversion (through oxidation) and lipid synthesis (via esterification) and serves as key components in signaling pathways (De Carvalho and Caramujo, 2018; Itoh et al., 2003). Therefore, the maintenance of fatty acyl levels at the highest concentration of PAHs might result from an inflection point where cellular response changes due to the high concentration of PAHs, possibly activating different regulatory pathways.

On the other hand, the lack of significant differences in sterols and prenols levels (Figure 5E-F) suggests that these lipid classes may not be directly affected by the exposure to PAHs at the tested concentrations. Given that sterols act as precursors for steroids, including estrogenic and androgenic hormones (Wollam and Antebi, 2011), the data are not robust enough to infer a link between exposure to PAHs in a

complex mixture and the endocrine disruption process in the present study. However, these results might still indicate that some classes of lipids are more susceptible to changes induced by PAHs (Figure 5). Based on these results, we propose a mechanism of action for acute exposure (24 hr) in zebrafish larvae from 4 dpf to 5 dpf, where the decrease of fatty acyl levels impacted the synthesis of glycerolipids, glycerophospholipids, and sphingolipids (Figure 5G). Interestingly, the recovery of metabolic homeostasis of fatty acyls observed at the highest concentration of the PAH mixture (0.1 µg/L) also reflects on the other lipid classes that are dependent on these compounds for their synthesis.

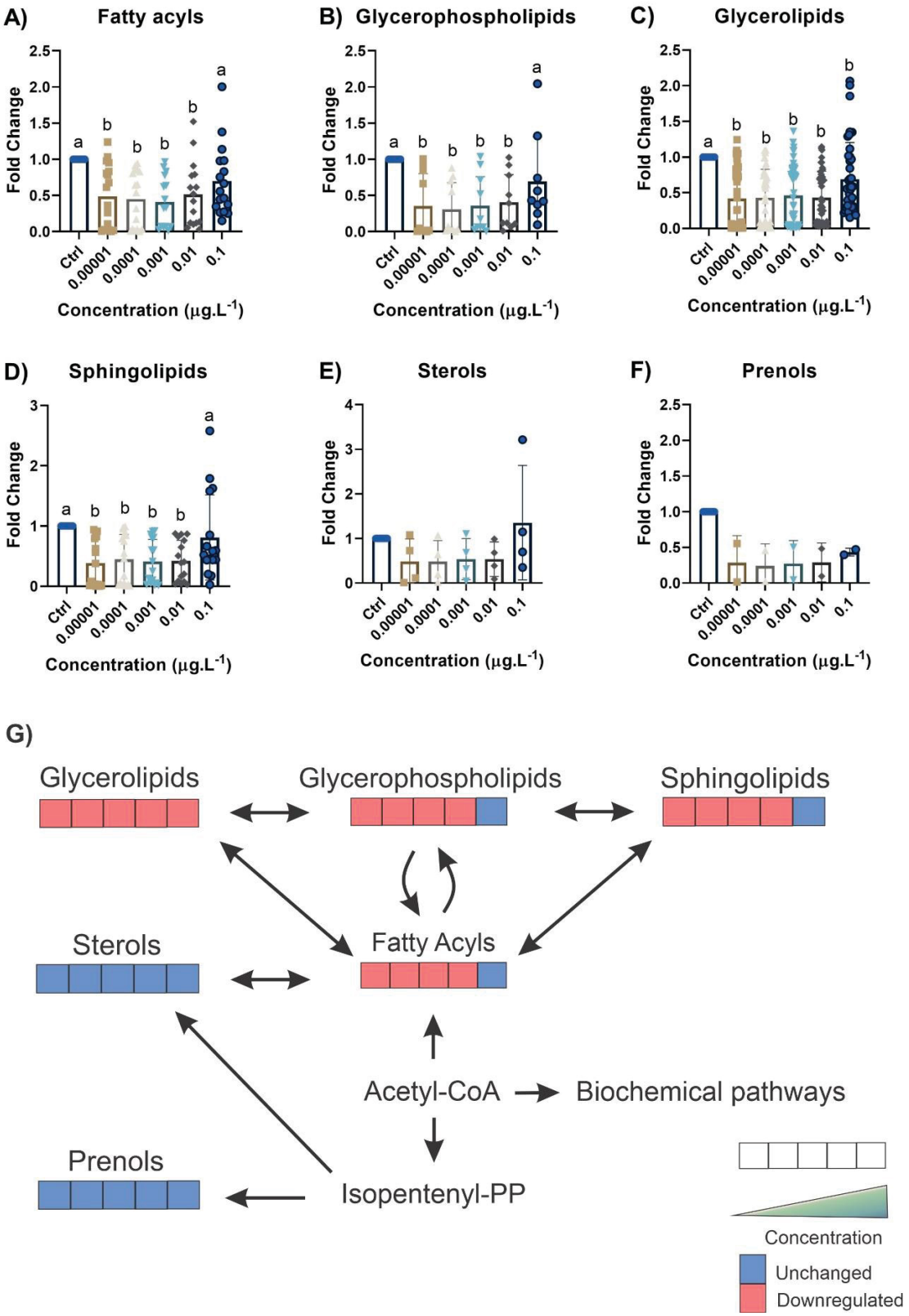


Figure 5. Lipid classes identified by LC-HRMS. A) Fatty acyls. B) Glycerophospholipids. C) Glycerolipids. D) Sphingolipids. E) Sterols. F) Prenols. One-way ANOVA followed by Tukey's post hoc test (n=7). Different letters indicate statistical differences among the groups ($p<0.05$).

## 4) Conclusion

The toxic potential and sub-lethal effects of a complex mixture of PAHs were demonstrated in zebrafish larvae. The expression of genes commonly responsive to PAHs (ahr2, cyp1a, ugt1a and abcc1) and genes related to lipid metabolism (rxraa, lxr, cyp3a65) was altered. The effects on the genes that code for the nuclear receptors lxr and rxraa suggest a potential metabolic modulation of their responsive elements, with effects on lipid levels, i.e., the downregulation of fatty acyls, sphingolipids, glycerolipids, and glycerophospholipids observed in the current study. However, the levels of sterols and prenols were not affected by the PAH exposure, so that we could not establish a clear correlation between endocrine disruption (estrogenic/androgenic) and lipid metabolism.

## 5) Acknowledgement

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Capítulo IV

# Structure-based modeling to assess binding and endocrine disrupting potential of polycyclic aromatic hydrocarbons in *Danio rerio*

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**Abstract**

Environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), have raised concerns regarding their potential endocrine-disrupting effects on aquatic organisms, including fish. In this study, molecular docking and molecular dynamics techniques were employed to evaluate the endocrine-disrupting potential of PAHs in zebrafish, as a model organism. A virtual screening with 72 PAHs revealed a correlation between the number of PAH aromatic rings and their binding affinity to proteins involved in endocrine regulation. Furthermore, PAHs with the highest binding affinities for each protein were identified: cyclopenta[cd]pyrene for AR (-9.7 kcal/mol), benzo(g)chrysene for ERα (-11.5 kcal/mol), dibenzo(a,e)pyrene for SHBG (-8.7 kcal/mol), dibenz(a,h)anthracene for StAR (-11.2 kcal/mol), and 2,3-benzofluorene for TRα (-9.8 kcal/mol). Molecular dynamics simulations confirmed the stability of the protein-ligand complexes formed by the PAHs with the highest binding affinities throughout the simulations. Additionally, the effectiveness of the protocol used in this study was demonstrated by the receiver operating characteristic curve (ROC) analysis, which effectively distinguished decoys from true ligands. Therefore, this research provides valuable insights into the endocrine-disrupting potential of PAHs in fish, highlighting the importance of assessing their impact on aquatic ecosystems.

**Keywords**: molecular docking, molecular dynamics, computational toxicology, reproduction, endocrine disruptors

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds consisting of fused aromatic rings. They are primarily formed during incomplete combustion of organic materials, such as fossil fuels, tobacco, and biomass burning, as well as through industrial processes, such as coal gasification, petrochemical production, and waste incineration (Mojiri et al., 2019; Lawal, 2017). Due to their persistent nature and widespread distribution in the environment, PAHs have become a matter of great concern in terms of biota exposure and environmental contamination (Lawal, 2017).

Several studies have documented the toxic effects of PAHs, including their mutagenic and carcinogenic potential, and effects on animal development (Wilson et al., 2023; Fang et al., 2022; McCarrick et al., 2019, Lawal, 2017). However, emerging evidence suggests that PAHs can also act as endocrine disruptors, exerting adverse effects on the endocrine system, which plays a critical role in regulating physiological processes, growth, development, and reproduction (Peng et al., 2019; Perrichon et al., 2015). Concerning the endocrine-disrupting properties of PAHs, they can interfere with hormonal signaling pathways, leading to disruptions in hormone synthesis, transport, receptor binding, and gene expression (Chen et al., 2021; Vondráček et al., 2018; Zhang et al., 2016; Vignet et al., 2014).

Understanding the molecular interactions between PAHs and endocrine receptors is fundamental in elucidating their potential endocrine-disrupting mechanisms. Experimental methods, such as *in vitro* assays and animal studies, have traditionally been employed to investigate the effects of PAHs on endocrine function (Zhou et al., 2022; Valderrama et al., 2022; Peng et al., 2019). However, these approaches are often time-consuming, expensive, and require large amounts of chemicals and resources.

Molecular docking, a computational technique widely used in drug discovery, provides a valuable alternative for studying the interactions between small molecules and protein receptors (Guedes et al., 2014). It predicts the binding affinity and preferred binding modes of a ligand within the binding site of a target receptor by exploring the conformational space and energy of the system (Sakkiah et al., 2018; Guedes et al., 2014). Molecular docking and other types of computational toxicology approaches have already been used to identify the mode of action of chemicals in

several contexts, such as chemical mixtures, breast cancer, and endocrine disruption in fish (Montes-Grajales and Olivero-Verbel, 2020; Yang et al., 2019; Vutukuru et al., 2016; Saxena et al., 2014; Yao et al., 2013). In this sense, molecular docking can be used to evaluate the potential of PAHs to bind to key endocrine receptors, such as estrogen receptors (ERs), androgen receptors (ARs), thyroid hormone receptors (TRs), and transport proteins, such as Sex Binding Hormone Protein (SHBG) and Steroidogenic Acute Regulatory protein (StAR) involved in endocrine regulation, thereby shedding light on their endocrine-disrupting capabilities.

Therefore, the present study aims to apply molecular docking as a computational tool to investigate the potential endocrine-disrupting properties of PAHs on *Danio rerio* (zebrafish), as a fish model. By employing established docking protocols and utilizing available crystal structures of endocrine receptors, this study will explore the binding interactions between PAHs and the active sites of these receptors. This approach provides valuable insights into the molecular mechanisms underlying PAH-induced endocrine disruption, allowing a better understanding of the potential risks associated with PAH exposure.

Overall, the application of molecular docking in the analysis of PAHs as potential endocrine disruptors represents a powerful tool for predictive toxicology and risk assessment. The findings of this study will contribute to the development of strategies for identifying and prioritizing the most hazardous PAHs, as well as designing more targeted and effective mitigation measures to reduce their impact on the biota and the environment.

## 2. Material and methods

### 2.1. Receptor Selection and Protein Modeling

To evaluate the endocrine-disrupting potential of PAHs, five proteins involved in different endocrine regulatory processes were chosen: Estrogen Receptor α (ERα), Androgen Receptor (AR), Thyroid Receptor α (TRα), Steroid Acute Regulatory Protein (StAR), and Sex Hormone Binding Globulin (SHBG). *D. rerio* was selected as the model organism to assess the toxic potential of these contaminants in the interaction with aquatic organism proteins. Amino acid sequences for the proteins were retrieved from the UniProt server (https://www.uniprot.org/), and 3D models (pdb format) of the proteins were built based on sequence homology using a

template available in the Protein Data Bank (PDB: https://www.rcsb.org/) through the Swiss-Model server (https://swissmodel.expasy.org/). From the 3D structures, data such as identity, similarity, range, coverage, and resolution (Å) were obtained and compared to the input sequence and the homology-generated model. Additionally, all constructed models were evaluated and validated for their structure using the MolProbity score (Chen et al., 2010), QMEAN (Qualitative Model Energy ANalysis, Benkert et al., 2008), and Ramachandran plot (% favored regions). The BIOVIA Discovery Studio 2019 software (San Diego, CA, USA) was used for visualization, file correction (if necessary), and removal of contaminants in the structure (Figure 1).

### 2.2. Ligand Preparation

The PAHs for the modeling tests were selected according to the "PAHLIST" list obtained from the EPA CompTox Chemicals server (https://comptox.epa.gov/dashboard). In total, the chemical information and 3D models (sdf format) of the 72 PAHs in the list were obtained from the PubChem server (https://pubchem.ncbi.nlm.nih.gov/). These files were converted to pdb format for analysis using the Discovery Studio 2019 software (Figure 1).

### 2.3. Virtual High-Throughput Screening (vHTS)

The molecular vHTS followed the method described by Montes-Grajales and Olivero-Verbel (2020) with modifications. The AutoDock Tools 1.5.6 software (Morris et al., 2009) was used. First, polar hydrogens and Kollman charges were added to the protein structure, and the number of ligand torsions was set. At this stage, the files were saved in pdbqt format. Second, the grid parameter was obtained, considering that the strategy employed in this study was based on blind docking, with dimensions of x: 120, y: 120, z: 120 points (spacing: 0.375 Å). Finally, the vHTS was performed using the AutoDock Vina software (Trott and Olson, 2010) based on the files generated during the system preparation. The molecular modeling was performed using the Cygwin 64 terminal emulator, and the docking parameters used were: energy range (5), exhaustiveness (10), and number of modes (20). Three independent vHTS runs were completed for each ligand/protein complex (Figure 1).

### 2.4. Conformation Analysis

Based on the results, only the ligand-protein complex with the best affinity score (kcal/mol) was represented by the analysis performed in the Discovery Studio 2019 software, following the protocol described by Yang et al. (2019). Using the toolbar, the types of polar and hydrophobic interactions between the ligand/amino acids present in the binding site were evaluated, such as hydrogen bonds, carbon hydrogen bonds, Pi-Sulfur, Pi-Sigma, Alkyl, among others. Additionally, the complexes were also evaluated using the UCSF Chimera software (Pettersen et al., 2004). Thus, 3D and 2D interaction images between the chemical compounds and the binding sites were generated (Figure 1).

### 2.5. Molecular Dynamics

Molecular dynamics simulations were performed for the protein-ligand complexes with the highest affinity score (kcal/mol) using the different modules of the PlayMolecule server, the first platform to offer molecular dynamics and machine learning applications for drug discovery with a focus on drug design (https://www.playmolecule.org/). The following modules were used:

1) Protein structure preparation using ProteinPrepare. The process includes protonation state titration (pH 7.4) using PROPKA 3.1 and the addition of missing atoms and overall optimization of the H-network using PDB2PQR 2.1 (Martinez-Rosell et al., 2017).

2) Ligand preparation using Parametrize: A fast molecular force field parameterization tool based on quantum-level machine learning. In this step, the force field is constructed using GAFF2 (Generalized Amber Force Field 2) parameters and AM1-BCC atomic charges for structure and dihedral angle adjustment (Bannwarth et al., 2021; Devereux et al., 2020; Galvelis et al., 2019).

3) The properly parameterized files were submitted to SystemBuilder, an application for system preparation to run molecular dynamics simulations in water, generating a system with the Amber force field (Doerr et al., 2016). The dimensions of each system were defined according to the server's default, following the characteristics of each file generated during the parameterization.

4) After the protein-ligand system preparation, molecular dynamics simulation was performed using SimpleRun (Doerr et al., 2016). The simulation had a total time

of 25 ns, with 10 ns for the equilibration phase and 15 ns for the production phase. The stability of the protein-ligand complexes was measured by RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) (Figure 1).

### 2.6. 2D and 3D Structure Comparison

The evaluation of structural similarity between the PAHs and endogenous molecules (natural ligands of the proteins) was performed using the PubChem Score Matrix Service cheminformatics tool (https://pubchem.ncbi.nlm.nih.gov/score_matrix). The 2D comparison (score: 0-1) was based on Tanimoto similarity, while shape and chemical characteristics were also assessed for the 3D comparison (score: 0-1) (Bolton et al., 2011). The five PAHs with the highest binding affinity (kcal/mol) for each protein were evaluated. The Compound ID (CID) was used as input for structural comparison with endogenous ligands, namely: ERα (Estradiol and Ethinylestradiol); AR (Testosterone and Dihydrotestosterone); TRα (triiodothyronine and thyroxine); SHBG (Estradiol and Testosterone); and StAR (Cholesterol) (Figure 1).

### 2.7. Validation

The validation of the methods used was based on 1) validation of the protocol used and the ability of AutoDock Vina to differentiate active ligands from decoys, and 2) correlation of affinity (kcal/mol) with experimental data.

The first step of validation involves evaluating the predictive power of AutoDock Vina in identifying the protein binding site and distinguishing ligands from decoys, following the approach proposed by Montes-Grajales et al. (2018) with modifications. Tests were performed with 100 compounds (50 active ligands and 50 decoys) for ERα, AR, and TR to assess the efficiency and sensitivity of the protocol used here. The files were obtained from the DUD-E server (Database of Useful Decoys - Enhanced). After analysis by AutoDock Vina, the binding affinity values (kcal/mol) were plotted in GraphPad Prism 6, and a Receiver Operating Characteristic (ROC) curve was generated to evaluate the potential for distinguishing ligands, as well as the sensitivity and specificity of the methodology.

The second validation step corresponds to correlating experimental data with binding affinity (kcal/mol), according to Montes-Grajales et al., 2013. Available experimental data (ERα, n=20; AR, n=17; TRα, n=12) obtained from *in vitro* assays

of the effects of different PAHs present in the "PAHLIST" with some of the proteins evaluated in this study (ERα, AR, and TRα) were used. All data were obtained from the Environmental Protection Agency, United States server (https://comptox.epa.gov/dashboard). Considering the available data, Pearson correlation between the AC50 value (concentration where half maximal activity occurs) and binding affinity was calculated (Figure 1).

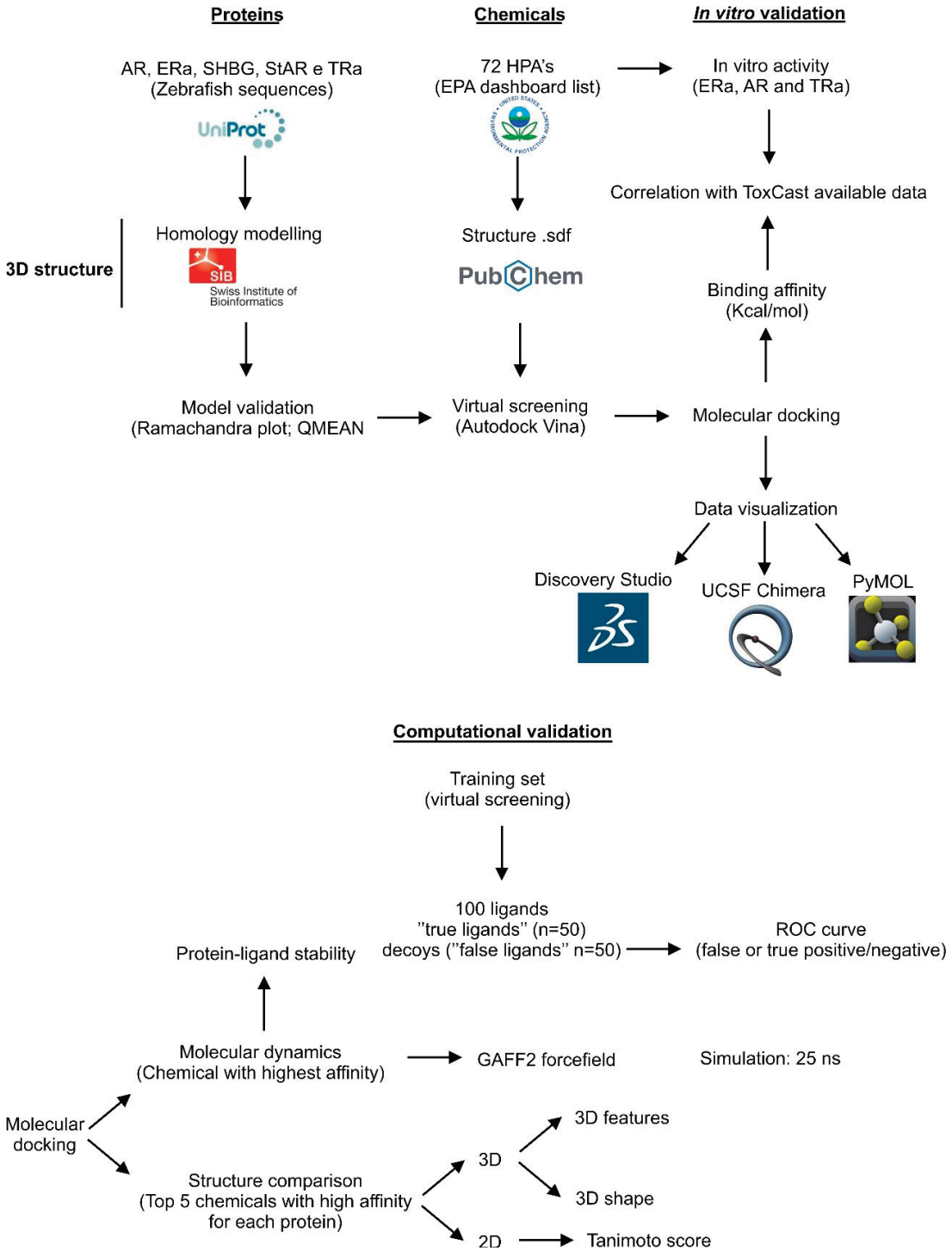


Figura 1. Experimental design.

## 3. Results

### 3.1. Protein models and virtual screening

The 3D structure of the proteins was duly validated, and the data regarding the construction of the homology-based models are shown in Supplementary Material 1. Virtual screening was performed with 72 PAHs, and the greater the number of aromatic rings in their structure, the higher the binding affinity with the proteins involved in endocrine regulation processes (Figure 2A-E). In this regard, the PAHs with the highest binding affinity for each protein were highlighted (Figure 2F-J): cyclopenta[cd]pyrene for AR (-9.7 kcal/mol), benzo(g)chrysene for ERα (-11.5 kcal/mol), dibenzo(a,e)pyrene for SHBG (-8.7 kcal/mol), dibenz(a,h)anthracene for StAR (-11.2 kcal/mol), and 2,3-benzofluorene for TRα (-9.8 kcal/mol).

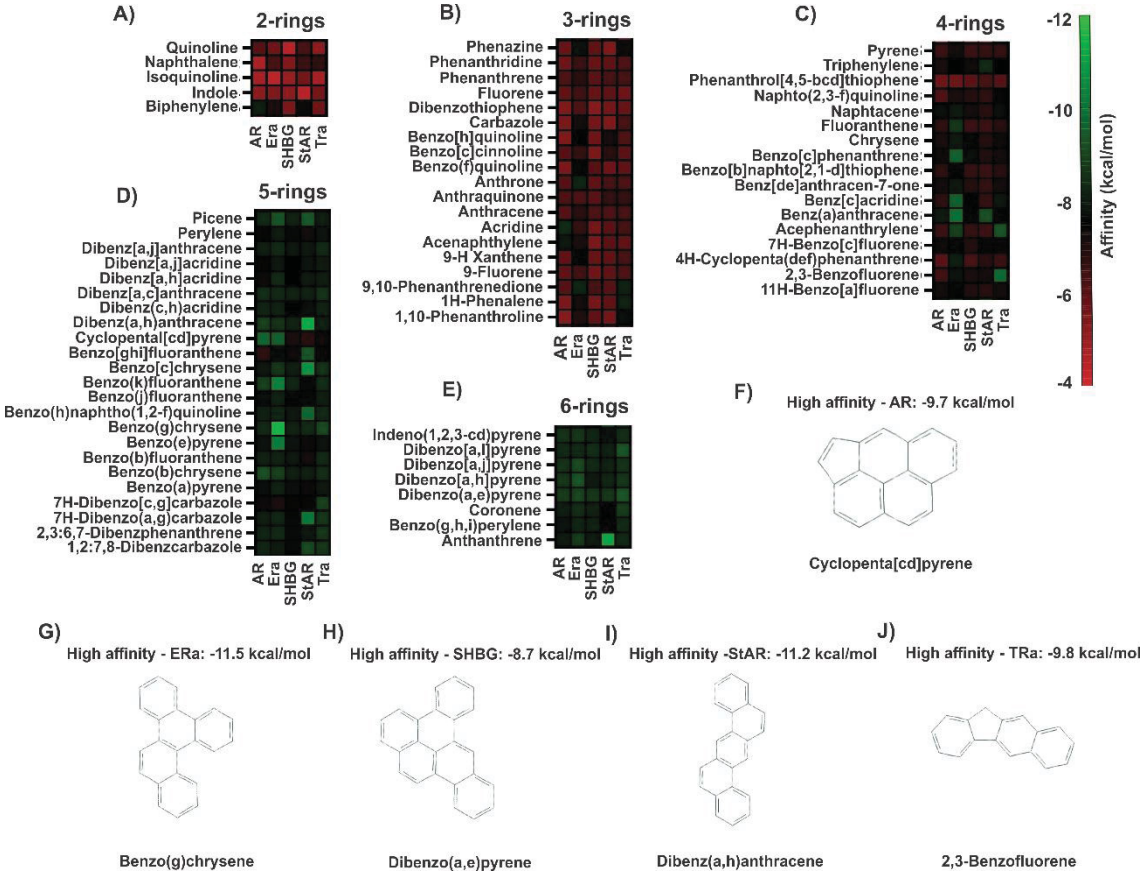


Figure 2. Virtual screening. The heatmaps show the binding affinity values for 2-6 ring PAHs. A) 2 rings. B) 3 rings. C) 4 rings. D) 5 rings. E) 6 rings. The PAHs with the highest binding affinity for each protein are shown in figures F-J. F) AR. G) ERα. H) SHBG. I) StAR. J) TRα.

### 3.2. Molecular docking

The PAH-nuclear receptor partners with the highest biding affinities (fig. 2F-J) were further analyzed. The interactions between PAHs and the proteins were

generally classified as Pi-Sigma, Pi-Anion, Pi-Pi T-Shaped, and Pi-Alkyl bonds (Figure 3). Cyclopenta[cd]pyrene interacts with the AR receptor through 8 amino acids: LEU-657, LEU-699, PHE-714, MET-692, LEU-821, LEU-654, MET-695, and VAL-696 (Figure 3A). Likewise, for benzo(g)chrysene, the interaction with ERα occurs through 8 amino acids: LEU-346, ALA-350, LEU-525, ILE-424, LEU-384, MET-388, LEU-391, and PHE-404 (Figure 3B). For 2,3-benzofluorene the interaction with TRα involves 7 amino acids: MET-259, LEU-279, ILE-225, PHE-404, LEU-295, MET-391, and ALA-228 (Figure 3C).

For the transporter proteins, dibenzo(a,e)pyrene binds to SHBG through interaction with 4 amino acids: TYR-361, VAL-224, VAL-222, and PRO-328 (Figure 3D). Additionally, the predicted interaction site of dibenz(a,h)anthracene with the StAR protein occurs in 5 amino acids: ARG-180, LEU-245, THR-261, SER-169, and GLU-167 (Figure 3E). Like the nuclear receptors, the interactions with the transporter proteins were also classified as Pi-Sigma, Pi-Anion, Pi-Pi T-Shaped, and Pi-Alkyl bonds. However, for StAR, there is a Pi donor hydrogen bond with SER-169.

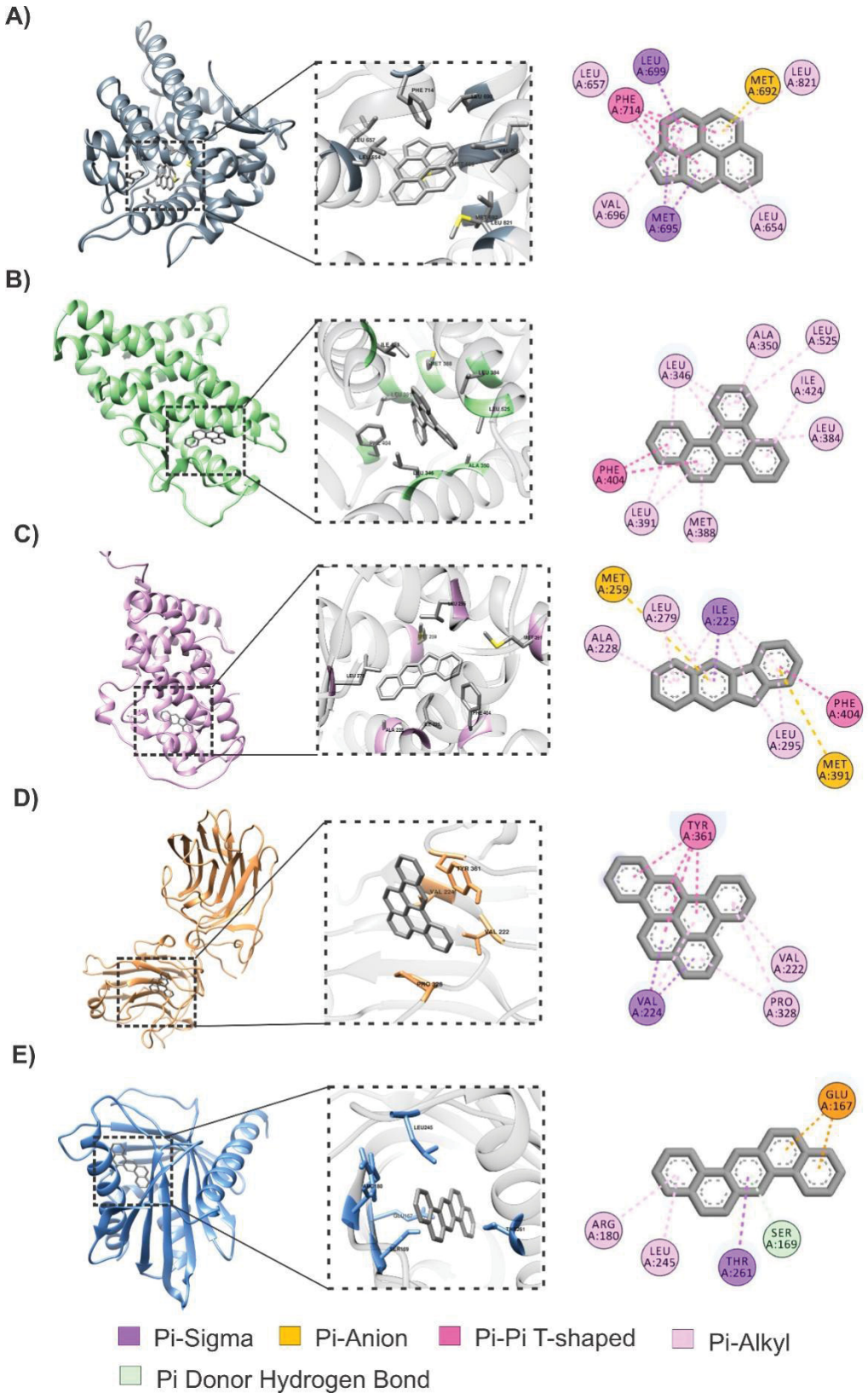


Figure 3. Conformational analysis and types of interaction of PAHs with target proteins. A) AR and Cyclopenta[cd]pyrene. B) ERα and benzo(g)chrysene. C) TRα and 2,3-benzofluorene. D) SHBG and dibenzo(a,e)pyrene. E) StAR and dibenz(a,h)anthracene.

### 3.3. Molecular dynamics

The average root-mean-square deviation (RMSD) over the simulation time (25 ns) for the AR-cyclopenta[cd]pyrene complex revealed a considerable variation in the ligand position, particularly during the equilibration phase, with a value of 4.84 Å. For the protein structure, both the side chain (2.62 Å) and the Cα (1.64 Å) of the protein exhibited relatively high structural stability, indicating a consistent conformation over time (Figure 4A). In the case of the ERα-benzo(g)chrysene complex, the ligand displayed an RMSD of 2.87 Å, indicating lower fluctuation in its position during the simulation. Both the side chain (2.5 Å) and the Cα (1.63 Å) of the protein also showed low RMSD values, suggesting a stable and consistent structural conformation (Figure 4B).

Conversely, the TRα-2,3-benzofluorene complex showed an increase in RMSD for both the ligand and the protein. The ligand had a value of 3.28 Å, indicating greater variation in its position. The side chain (3.18 Å) and the Cα (2.42 Å) of the receptor also had higher RMSD values (Figure 4C). Thus, the protein-ligand complex may have undergone more significant conformational changes during the simulation, affecting both the ligand and the protein structure in a correlated manner.

Analyzing the SHBG-dibenzo(a,e)pyrene complex, a large variation in RMSD values was observed for all categories. The ligand had a value of 5.29 Å, while the side chain and the Cα of the protein showed values of 5.21 Å and 4.25 Å, respectively (Figure 4D). These results indicate greater flexibility and structural instability for the SHBG complex compared to the other studied complexes. Finally, the StAR-dibenz(a,h)anthracene complex exhibited a stable and consistent structural conformation throughout the simulation (Figure 4E). The ligand had an RMSD value of 2.3 Å, while the side chain and the Cα of the protein showed values of 3 Å and 1.71 Å, respectively, suggesting high structural stability.

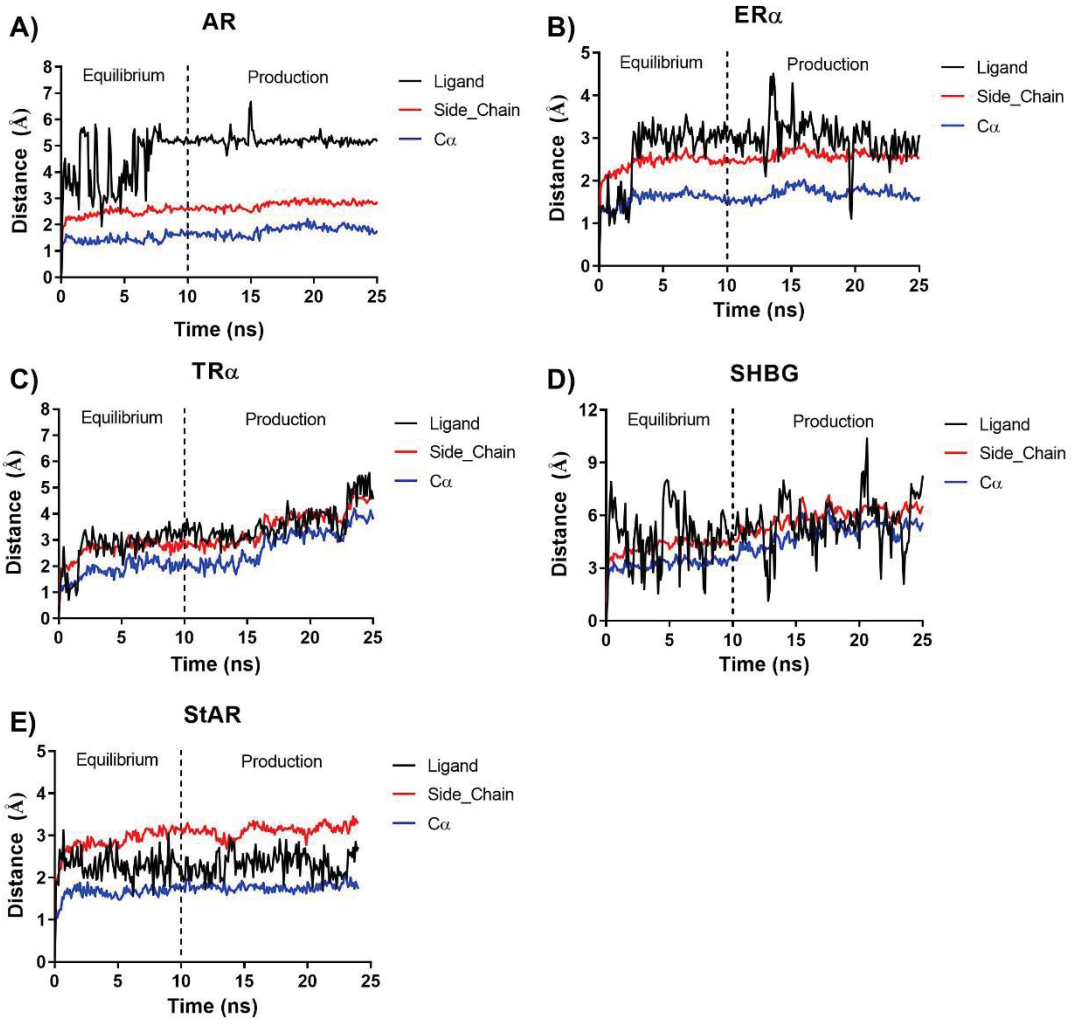


Figure 4. Molecular dynamics analysis of protein-ligand complexes using RMSD. A) AR + cyclopenta[cd]pyrene. B) ERα + benzo(g)chrysene. C) TRα + 2,3-benzofluorene. D) SHBG + dibenzo(a,e)pyrene. E) StAR + dibenz(a,h)anthracene.

When analyzing the root-mean-square fluctuation (RMSF), individual fluctuation values of the residues comprising the proteins and the atoms in the ligands were observed. For the AR receptor, the average value for Cα residues was 1.47 Å, while for the side chain it was 2.28 Å (Figure 5A). These values suggest a certain structural flexibility of the AR protein throughout the simulation. However, there are some regions near the N-terminal portion of the protein (residues ~200-250) where there was a higher variation in RMSF, indicating structural flexibility in that region. Regarding the ligand, cyclopenta[cd]pyrene showed an average RMSF of 5.20 Å for its atoms, indicating considerable fluctuation in the atom positions over time (Figure 5B).

For the ERα receptor, Cα residues showed an average RMSF of 1.45 Å, while the side chain had a value of 2.20 Å, indicating a relatively stable conformation (Figure 5C). Although the protein complex, in general, showed stability in the simulation, there are distinct regions (residues ~100-150) that exhibit flexibility in the protein structure. Concerning the ligand benzo(g)chrysene, the average RMSF for its atoms was 2.96 Å, suggesting moderate fluctuation in the atom positions during the simulation (Figure 5D).

For the TRα receptor, Cα residues had an average RMSF of 1.80 Å, while the side chain showed a value of 2.47 Å, indicating a slightly more flexible structural conformation (Figure 5E). Regarding the ligand 2,3-benzofluorene, the average RMSF for its atoms was 3.65 Å, suggesting significant fluctuation in the atom positions over time (Figure 5F).

When analyzing the SHBG protein, Cα residues showed an average RMSF of 4.33 Å, while the side chain had a value of 5.09 Å, indicating greater structural flexibility (Figure 5G). Additionally, there is noticeable flexibility in the region of residues ~250-300 compared to the others. Regarding the ligand dibenzo(a,e)pyrene, the average RMSF for its atoms was 5.61 Å, suggesting substantial fluctuation in the atom positions during the simulation (Figure 5H).

The StAR protein exhibited an average RMSF of 1.44 Å for Cα residues and 2.41 Å for the side chain, indicating a relatively stable conformation (Figure 5I). The ligand dibenz(a,h)anthracene had an average RMSF of 2.35 Å for its atoms, suggesting moderate fluctuation in the atom positions over time (Figure 5J).

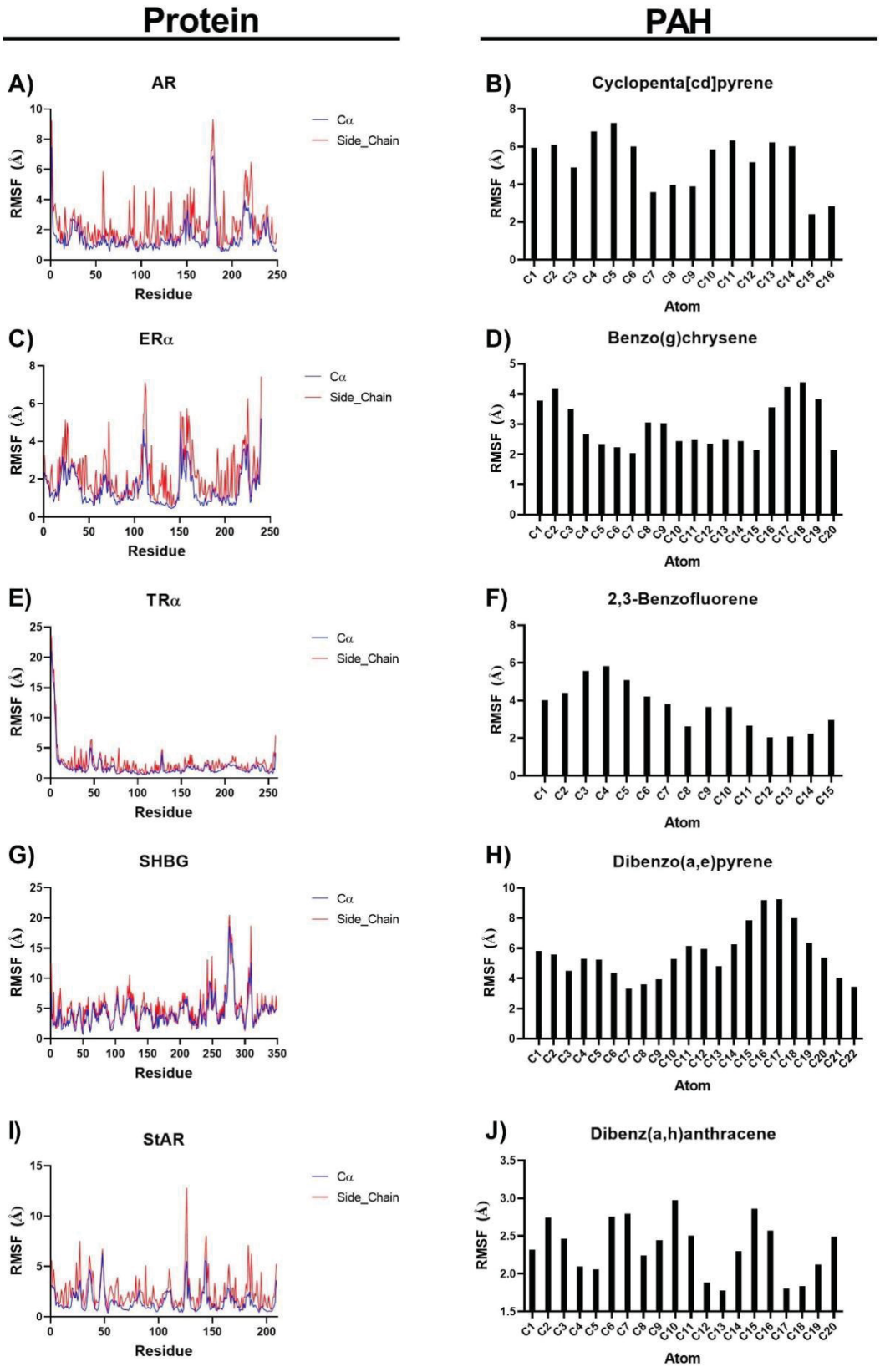


Figure 5. Molecular dynamics analysis of protein-ligand complexes using RMSF. A) AR. B) cyclopenta[cd]pyrene. C) ERα. D) benzo(g)chrysene. E) TRα. F) 2,3-benzofluorene. G) SHBG. H) dibenzo(a,e)pyrene. I) StAR. J) dibenz(a,h)anthracene.

### 3.4. Structural comparison

The structural comparison between the top 5 PAHs with the highest binding affinities for endogenous ligands of each protein was performed by evaluating the 2D and 3D parameters of these chemical compounds. For AR, testosterone and dihydrotestosterone (DHT) were used as endogenous ligands, and the comparison was made with the following PAHs: cyclopenta[cd]pyrene (-9.7 kcal/mol), benzo(b)chrysene (-9.2 kcal/mol), dibenz(a,h)anthracene (-8.9 kcal/mol), benzo(k)fluoranthene (-8.9 kcal/mol), and dibenzo(a,e)pyrene (-8.8 kcal/mol). Overall, these PAHs showed higher 2D similarities to testosterone than to DHT, while for the 3D shapes and overlap of these molecules, the compounds exhibited high similarity (>0.70). However, for the 3D characteristics, the highest similarity score (0.40) was observed for dibenzo(a,e)pyrene compared to DHT (Figure 6A).

For ERα, E2 (estradiol) and EE2 (17α-ethynylestradiol) were used as true ligands. Comparisons were made using the PAHs: benzo(g)chrysene (-11.5 kcal/mol), benzo(k)fluoranthene (-10.3 kcal/mol), benzo(e)pyrene (-10.2 kcal/mol), benz(a)anthracene (-9.9 kcal/mol), and benzo(c)phenanthrene (-9.6 kcal/mol). Structurally, all the aforementioned PAHs showed 2D similarities >0.5 and also exhibited a 3D similarity score >0.70 regarding to shape. Furthermore, benzo(g)chrysene had high 3D similarity with EE2, demonstrating an almost perfect overlap (Figure 6B).

For the nuclear receptor TRα, the endogenous hormones T3 (triiodothyronine) and T4 (thyroxine) were used as ligands. For this protein, the following PAHs were used for structural comparisons: 2,3-benzofluorene (-9.8 kcal/mol), dibenzo(a,l)pyrene (-9.3 kcal/mol), dibenzo(a,e)pyrene (-9.3 kcal/mol), acephenanthrylene (-9.3 kcal/mol), and benzo(g)chrysene (-9.1 kcal/mol). The 2D similarity between these compounds varied slightly (~0.32-0.38); however, the scores for the shape and characteristics of the 3D structures were lower than those obtained for the other nuclear receptors.

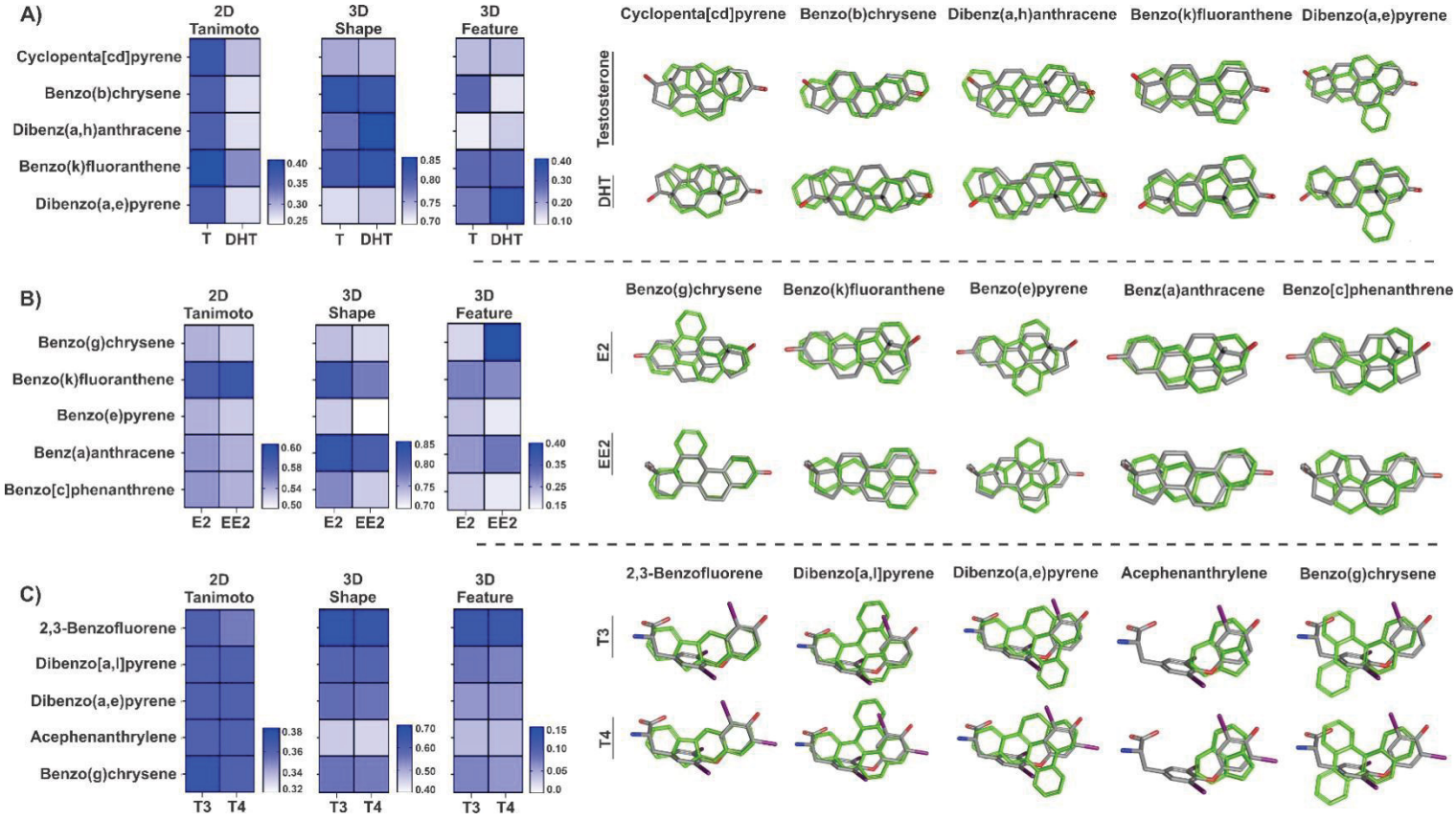


Figure 6. 2D and 3D structural comparison of the top 5 PAHs with endogenous ligands of the nuclear receptors. A) AR. B) ERα. C) TRα.

For the transporter proteins, E2 and testosterone were used as endogenous ligands for SHBG. Their structure was compared with the PAHs: dibenzo(a,e)pyrene (-8.7 kcal/mol), dibenzo(a,l)pyrene (-8.5 kcal/mol), dibenzo(a,i)pyrene (-8.4 kcal/mol), benzo(k)fluoranthene (-8.3 kcal/mol), and indeno(1,2,3-cd)pyrene (-8.3 kcal/mol). The 2D similarity of these compounds was higher with E2 (>0.50) than with testosterone, but when comparing the shape, characteristics, and 3D overlap, the scores demonstrated high similarity among these compounds (Figure 7A).

For StAR, only one endogenous ligand, cholesterol, was used. Comparisons were made with the PAHs: dibenzo(a,h)anthracene (-11.2 kcal/mol), anthanthrene (-11.1 kcal/mol), benzo(c)chrysene (-10.7 kcal/mol), 7H-dibenzo(a,g)carbazole (-10.1 kcal/mol), and benzo(h)naphtho(1,2-f)quinoline (-9.7 kcal/mol). The structural comparison revealed that most of the compounds had a 2D similarity of ~0.40. Additionally, for the shape in the 3D comparison, the scores were higher than 0.60, while for the 3D characteristics, benzo(h)naphtho(1,2-f)quinoline showed the highest similarity score of ~0.40 (Figure 7B).

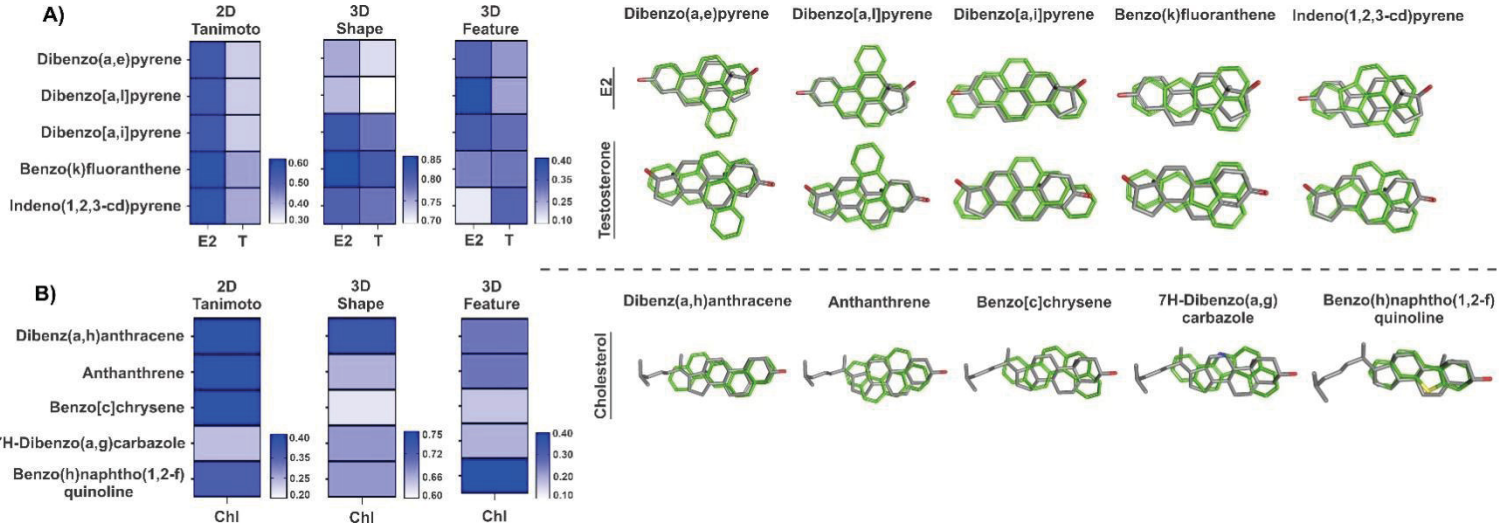


Figure 7. 2D and 3D structural comparison of the top 5 PAHs with endogenous ligands of the transporter proteins. A) SHBG. B) StAR.

### 3.6. Validation

A ROC (receiver operating characteristic) curve analysis was performed to evaluate the discrimination potential of the algorithm used for the three nuclear receptors (AR, ERα, and TRα) in relation to true ligands and decoys, with the aim of validating the virtual screening. Sensitivity and specificity of binding affinity (expressed in kcal/mol) were used as measures for this validation.

The results obtained for the nuclear receptors were as follows: the AR receptor showed an ROC curve with an area under the curve (AUC) of 0.81 (Figure 8A). The ERα receptor had an AUC value of 0.83, indicating a high potential for discrimination between true ligands and decoys (Figure 8B). On the other hand, the TRα receptor exhibited an AUC value of 0.68, suggesting slightly lower performance compared to the other two proteins (Figure 8C). However, it is important to note that all values were statistically significant, with a p-value < 0.05.

Furthermore, a correlation analysis was conducted between the binding affinity values (expressed in kcal/mol) obtained through molecular docking and the experimental Log[AC50] values from cell-based assays provided by the ToxCast database. The results obtained for Pearson correlation analysis were as follows: for the AR receptor, a Pearson correlation (r) of 0.33, $R^2$ = 0.11, and a p-value of 0.19 were observed (Figure 8D). These values indicate a weak and not statistically significant correlation between the binding affinity values and the experimental Log[AC50] results for the AR receptor.

However, for the ERα receptor, a Pearson correlation (r) of 0.80, $R^2$ = 0.65, and a p-value < 0.0001 were observed. These results indicate a strong and statistically significant correlation between the binding affinity values and the experimental Log[AC50] results for the ERα receptor (Figure 8E). As for the TRα receptor, the analysis revealed a Pearson correlation (r) of 0.51, $R^2$ = 0.27, and a p-value of 0.083 (Figure 8F). Although the correlation is moderate, the p-value indicates that this correlation is not statistically significant. These results suggest a possible trend of positive correlation between the binding affinity values and the experimental Log[AC50] results for the TRα receptor.

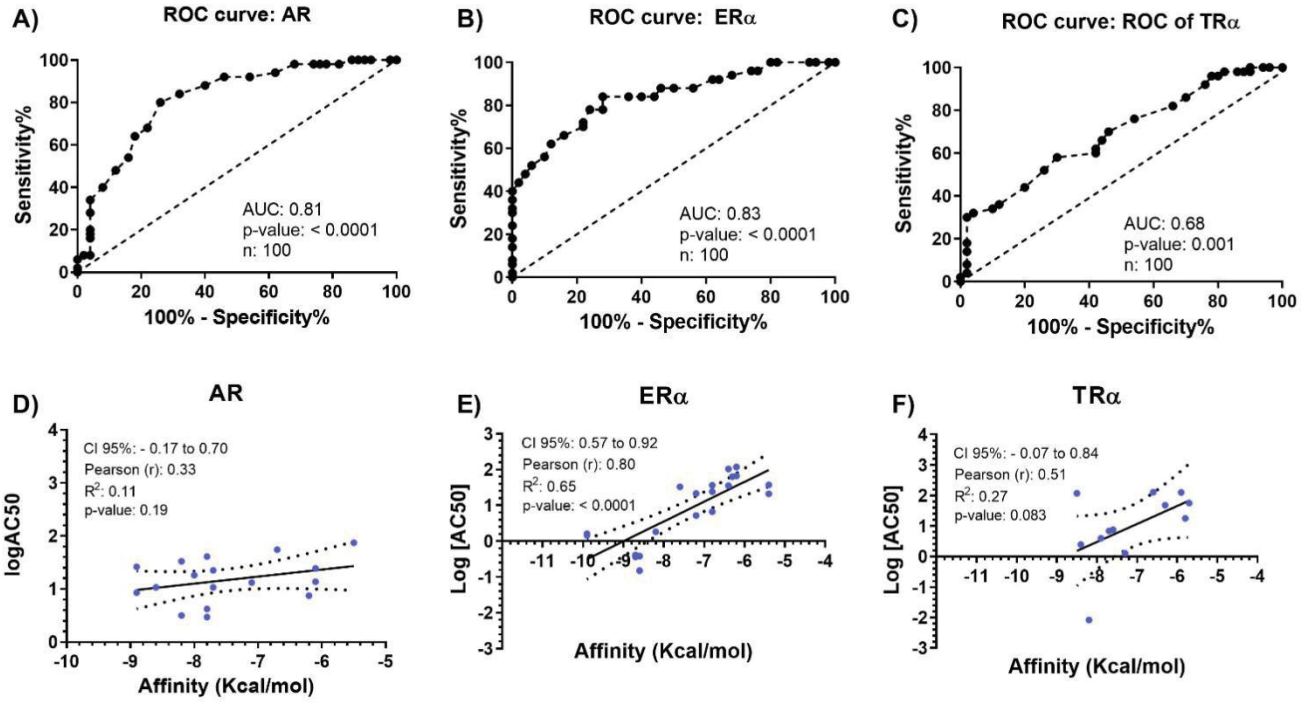


Figure 8. Validation tests and comparison with *in silico* results. A) ROC curve: AR. B) ROC curve: ERα. C) ROC curve: TRα. D) *In vitro* and *in silico* data correlation for the AR receptor. E) *In vitro* and *in silico* data correlation for the ERα receptor. F) *In vitro* and *in silico* data correlation for the TRα receptor.

## 4. Discussion

The growing concern about the effects of environmental contaminants on the endocrine system has driven scientific research in the field of environmental toxicology. In this context, the application of computational techniques such as molecular docking, molecular dynamics, and structural comparison has proven to be crucial for evaluating the endocrine-disrupting potential of chemical compounds present in the environment and inferring possible effects on organisms. In the

present study, these approaches to investigate different types of polycyclic aromatic hydrocarbons (PAHs) in relation to nuclear receptors (AR, ERα, and TRα) and transporter proteins (SHBG and StAR) were used. The main aim was to understand the molecular interactions and potential effects of these contaminants on the endocrine system, providing valuable information about the risks of exposure to PAHs.

### 4.1. PAHs and nuclear receptors

Endocrine disruptors usually act through nuclear receptors, which modulates the expression of a series of genes that regulate important processes for organism development, as well as reproductive aspects and hormonal regulation (Toporova and Balaguer, 2020). The ERα receptor, responsive to estrogenic compounds, acts as a key regulator of the development of some organs related to the female reproductive system in mammals (Toporova and Balaguer, 2020). In fish, this receptor is mainly involved in the regulation of gonadotropins and vitellogenesis, and in this sense, some xenobiotics can induce the production of vitellogenin (vtg) – a widely used biomarker to detect the activation of ERs in environmental toxicology (Peng et al., 2019; Nelson and Habibi, 2013).

Structurally, this receptor has three main domains: AF-1 (N-terminal domain, transcriptional activation function), DNA-binding domain (DBD), and AF-2 (C-terminal domain, ligand binding domain – LBD), which can interact in different ways with environmental contaminants, especially in the LBD domain that is involved in most receptor functions (Delfosse et al., 2014; Nelson and Habibi, 2013). Among the 72 PAHs used in virtual screening, benzo(g)chrysene showed the highest binding affinity (-11.5 kcal/mol) with ERα. The binding site of the ERα-benzo(g)chrysene complex corresponded to the amino acid residues LEU-346, ALA-350, LEU-525, ILE-424, LEU-384, MET-388, LEU-391, and PHE-404. According to the UniProt database (protein code: P57717), in *D. rerio*, the LBD domain of this protein comprises amino acids 279-515, encompassing the modeled interaction region for benzo(g)chrysene. It is important to note that there are differences in the specific residues that contribute to the generation of docking scores, and there is variation in how chemical compounds interact with receptors from different species (Asnake et al., 2019). However, the effectiveness of the response through the binding of a compound can

be similar, considering that the interaction occurred at the binding site of the receptor protein.

From this information, it is possible to infer that benzo(g)chrysene may have the potential to interact and modulate the activity of ERα, potentially causing endocrine disruption. Similar results obtained from computational models have been previously described for the interaction of ERα with other types of contaminants, such as pesticides (Montes-Grajales and Olivero-Verbel, 2020). Experimentally, PAHs such as benzo[a]pyrene, chrysene, anthracene, benzo(a)anthracene, and benzo(b)fluoranthene, as well as their metabolites, have already demonstrated potential estrogenic activity in *in vitro* assays (van Lipzig et al., 2005; Gozgit et al., 2004). Furthermore, in *D. rerio*, it has been described that different types of PAHs can promote alterations in the activity of estrogen receptors, causing effects possibly through crosstalk between ER-AhR activation pathways (Fang et al., 2022; Hawliczek et al., 2012).

Another type of steroid receptor, the AR, is also involved in maintaining the regulation of genes related to the development of reproductive structures and responsive to androgenic molecules, such as testosterone (in mammals) or 11-ketotestosterone (in fish) and 5α-dihydrotestosterone (DHT) (Toporova and Balaguer, 2020; Golshan and Alavi, 2019). *D. rerio* AR has three main domains: TAD (transactivation domain), DBD, and LBD (Hossain et al., 2007). Cyclopenta[cd]pyrene exhibited the highest binding affinity (-9.7 kcal/mol) with the AR receptor, through 8 amino acid residues: LEU-657, LEU-699, PHE-714, MET-692, LEU-821, LEU-654, MET-695, and VAL-696. According to the information obtained from the UniProt database (protein code: A4GT83), the interaction region between the PAH and the AR corresponds to the LBD domain, which is the site where receptor activity modulation occurs. Although cyclopenta[cd]pyrene is not a widely used PAH, it is known to be involved in modulating the apoptotic pathway in Hepa1c1c7 cells (Solhaug et al., 2004). However, based on the results obtained from the modeling, it is possible that cyclopenta[cd]pyrene can modulate the activity of AR.

In this regard, similar results have been reported for molecular docking models of the AR receptor and perfluoroalkyls and polyfluoroalkyls (Singam et al., 2020). Certain PAHs such as fluoranthene, benz[a]anthracene, benzo[a]pyrene, and benzo[k]fluoranthene can suppress the AR receptor *in vitro* (Šimečková et al., 2022). Furthermore, PAHs may possess a bidirectional inhibitory control pathway through

cross-talk between the AR/AhR pathways, similar to what has been observed for ER/AhR, although the mechanism is still unclear (Vondráček et al., 2018). Despite structural and sequence differences in AR among different species, the protein domains are highly conserved in mammals and fish (Golshan and Alavi, 2019), allowing for interspecies extrapolation. Many of the antiandrogenic effects caused by environmental contaminants are associated with reproductive dysfunctions that can impact in population size, such as reduced sperm quality in fish (Golshan and Alavi, 2019).

The TRα receptor is also directly related to reproduction, as it regulates the maintenance of homeostasis, differentiation, and embryonic development in fish through the transcription of thyroid hormone-responsive elements (Darras et al., 2011). Like ERα and AR, *D. rerio* TRα also consists of three domains: A/B, DBD, and LBD. The interaction of the TRα-2,3-benzofluorene complex (-9.8 kcal/mol) involves 7 amino acid residues: MET-259, LEU-279, ILE-225, PHE-404, LEU-295, MET-391, and ALA-228. According to the UniProt database (protein code: Q98867), this region corresponds to the LBD domain, and due to the high binding affinity potential, this compound is likely capable of modulating the activity of the thyroid receptor. Li et al. (2012) successfully demonstrated that the use of computational models applied to toxicology is a powerful tool for assessing the activity of polybrominated diphenyl ethers on thyroid receptors, thus supporting the results presented in this study.

To date, there are no studies investigating the endocrine-disrupting potential of 2,3-benzofluorene in the thyroid response system. However, it has been reported that PAHs with varying numbers of rings can significantly enhance the activity of the TRα receptor *in vitro* (Šimečková et al., 2022). Moreover, *D. rerio* exhibits sex-dependent dysregulation of the hypothalamic-pituitary-thyroid (HPT) axis after exposure to anthracene, leading to alterations in T3 and T4 levels, which are regulated by TR receptors (Zhong et al., 2023).

### 4.2. PAHs and transporter proteins

SHBG is a carrier protein involved in the transport of estrogens and androgens in the blood to target tissues. Despite structural differences among species, in *D. rerio*, some amino acid residues in the LG domain (laminin G-like), responsible for protein-ligand interaction, are conserved (Miguel-Queralt et al., 2004). When evaluating the dibenzo(a,e)pyrene-SHBG complex, the interaction occurs at 4 amino acid residues:

TYR-361, VAL-224, VAL-222, and PRO-328. According to the UniProt database (Uniprot: Q64HD0), this domain corresponds to amino acids 40-371, demonstrating that the molecular docking model effectively predicted the interaction of dibenzo(a,e)pyrene with SBHG and its binding site with a binding affinity of -8.7 kcal/mol.

*In silico* studies have been used to identify the ability of environmental contaminants and endogenous ligands to interact with SHBG, including in *D. rerio* (Abdi et al., 2021; Saxena et al., 2014; Thorsteinson et al., 2009). Saxena et al. (2014) reported that the PAH benzoanthracene is weakly active and has low interaction with SHBG. Similar results were observed by Thorsteinson et al. (2009) when evaluating the interaction of 9,10-dihydrobenzo(a)pyren-7(8H)-one (DBP) with *D. rerio* SHBG. However, for the pesticide tebuconazole, Abdi et al. (2021) demonstrated strong interactions with the active site of SHBG, suggesting potential endocrine-disrupting effects. Despite this, few studies have characterized the potential of PAHs to interfere with the activity of SHBG. In this regard, experimental studies linking PAH exposure to hormonal changes in humans, particularly during pregnancy, have indicated correlations between PAHs and SHBG (Cathey et al., 2020).

Finally, the StAR protein, which is involved in initiating the steroidogenic pathway by transporting cholesterol to the inner mitochondrial membrane, exhibited a higher binding affinity with dibenz(a,h)anthracene (-11.2 kcal/mol) at the amino acid residues ARG-180, LEU-245, THR-261, SER-169, and GLU-167. The same protein domain (cholesterol binding site) was reported by Kumar et al. (2022) as having *in silico* high binding affinity for PAHs from tobacco. Furthermore, Kumar et al. (2018) also described high affinity of benzo(a)pyrene and its metabolites for domains related to lipid transport in StAR protein isoforms using molecular docking models.

Experimental evidence suggests that PAH exposure negatively modulates the expression of the StAR gene in *D. rerio* and that certain PAH types, such as benzo(a)pyrene, can cause disturbances in testosterone levels in the organism (Zhang et al., 2016; Arukwe et al., 2008). Therefore, both computationally predicted and experimental results indicate that different types of PAHs can negatively impact aquatic organisms, causing functional alterations in cholesterol transport and affecting the steroidogenic pathway.

### 4.3. Chemical Interactions

Multiple types of forces are responsible for the folding and stabilization of the protein-ligand complex, predominantly of a non-covalent nature. These non-covalent interactions include hydrogen bonds (H-bonds) and other electrostatic interactions, as well as the hydrophobic effect (Brandl et al., 2001). The hydrophobic nature of PAHs, combined with the large number of Pi-type bonds of the aromatic rings with the active site of the studied proteins, plays an important role in the strong interaction and maintenance of complex stability, as observed for other types of molecules (Brena et al., 1992). Furthermore, the predominantly hydrophobic characteristics, especially of the LBD domains of nuclear receptors, may favor the interaction and response to multiple small molecules at low concentrations (Toporova and Balaguer, 2020).

The data obtained from the interaction simulation performed by molecular docking were validated by molecular dynamics analysis. These results demonstrated that both the protein backbone (Cα and side chain) and the ligand remained stably bound in a context where temperature, pressure, and solvent interaction forces are at play. The results obtained from RMSD demonstrate the strength of the interactions between PAHs and the proteins, as well as suggest their potential to act as possible endocrine disruptors in fish. Additionally, through RMSF, it was possible to identify protein and ligand regions that exhibit certain structural flexibility, enabling dynamic interaction within the protein-ligand complex. In this regard, various *in silico* techniques, such as molecular dynamics, have been used to assess and validate the endocrine-disrupting potential of multiple classes of environmental contaminants using nuclear receptors and transport proteins (Kumar et al., 2022; Abdi et al., 2021; Montes-Grajales and Olivero-Verbel, 2020; Singam et al., 2020; Kumar et al., 2018; Wahl and Smieško, 2020; Saxena et al., 2014; Li et al., 2012).

Another important factor to highlight is the structural comparison between the PAHs and the endogenous ligands of each protein. Most of them showed high 2D similarity based on the Tanimoto index and, especially, 3D shape similarities. These characteristics are essential for enabling protein interaction and maintaining the stability of the protein-ligand complex, further supporting the data obtained from the simulations. Some structural modifications can occur in PAHs, such as the addition of hydroxyl groups, which potentially increase the compound's affinity for nuclear

receptors and favor endocrine-disrupting effects in *D. rerio*, such as increased expression of vtg gene – an estrogen-responsive gene (Diamante et al., 2017).

Furthermore, in the present study, it was possible to correlate nuclear receptor activation data from *in vitro* tests with the binding affinity values obtained from molecular docking simulations. Few data are available in the scientific literature and open databases to enable a robust correlation analysis, due to the low number of samples. However, it was possible to identify a correlation for the ERα receptor and the *in vitro* data. In addition, through ROC curves, it was possible to confirm that the algorithm has a good discrimination capacity for true ligands versus decoys for the nuclear receptors, representing an important validation step for the current study.

## 5. Conclusion

The current virtual screening identified PAHs with potential endocrine-disrupting effects. The simulations performed here provide data useful to reduce the number of compounds and animals to be used in biological validations, and aid to direct efforts for specific pathways. Unfortunately, as a limitation of the method, these models are not capable of predicting whether the tested compounds can bind to allosteric sites and the type of response they may induce (i.e., agonist, antagonist, selective modulator). Furthermore, these models neither consider the compound metabolism, which may affect the binding affinity of the compounds to the target receptor, nor assess the subsequent impacts of these compounds. However, the present study and the methodologies used provide insights into the toxic potential of PAHs and can guide further *in vitro* and *in vivo* studies.

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## 6) DISCUSSÃO GERAL

A exposição de biota aquática a poluentes químicos devido às atividades humanas é uma preocupação crescente, e a relação entre a poluição ambiental e os mecanismos de ação desses compostos sob a fisiologia de organismos aquáticos é um problema contínuo em estudo. O processo de exposição a desreguladores endócrinos tem sido foco, e a proteína vitelogenina (Vtg) tem sido usada como um importante biomarcador desse processo. No primeiro capítulo foi possível caracterizar a Vtg de *Oreochromis niloticus*, através da sua indução em peixes para a padronização de procedimentos de purificação dessa proteína. Além disso, a estabilidade da Vtg foi avaliada, evidenciando boa estabilidade em condições ácidas e de aquecimento. A modelagem 3D da Vtg revelou características estruturais conservadas, onde notou-se que há diferenças estruturais entre as formas químicas da Vtg (VtgAb e VtgC) por meio de análises filogenéticas, o que pode ter implicações funcionais. Esses resultados foram importantes para contribuição das principais características de manutenção desse tipo de proteína em laboratório, uma vez que é importante conhecer esses processos para o desenvolvimento de ensaios ecotoxicológicos e a compreensão do mecanismo de desregulação endócrina em *O. niloticus* frente a exposição a contaminantes ambientais.

Após caracterizar um dos biomarcadores mais importantes para o processo de desregulação endócrina estrogênica em peixes, no segundo capítulo foi explorado a toxicidade de uma mistura complexa de HPAs e o seu impacto na modulação da via estrogênica *in vitro*, usando hepatócitos primários de *O. niloticus*. As concentrações ambientais da mistura de HPAs testada não afetou parâmetros de viabilidade celular, porém observaram-se alterações significativas na expressão de genes da via estrogênica, como a vtg e o receptor de estrogênio 2 (esr2). Além disso, a mistura de HPAs levou a respostas complexas nos marcadores de estresse oxidativo, indicando uma alteração no equilíbrio antioxidante e demonstrando o potencial tóxico do tratamento mesmo em condições sub-letais. A atividade de transporte de substâncias também foi influenciada, sugerindo uma interferência nas proteínas relacionadas à resistência a múltiplas drogas, bem como a maior metabolização desses compostos, através dos resultados obtidos pela expressão gênica de abcc1, cyp1a e ugt1a1. Esses resultados ressaltam o potencial dos HPAs em modular a via estrogênica e induzir adaptações fisiológicas em hepatócitos de *O.*

*niloticus*, destacando a importância de entender esses mecanismos para avaliar os riscos desses compostos mesmo em concentrações ambientais e sub-letais.

Em um contexto *in vivo*, a compreensão dos efeitos ambientais no metabolismo lipídico é fundamental para avaliar o impacto da exposição a contaminantes em organismos aquáticos. No terceiro capítulo, larvas de zebrafish (*Danio rerio*) foram expostas a mesma mistura utilizada no capítulo II, onde por meio de técnicas de lipidômica e expressão gênica foram avaliadas as alterações no metabolismo lipídico. A análise lipidômica revelou uma diminuição acentuada em várias classes de lipídios, especialmente esfingolipídios, glicerolipídios e fosfoglicerolipídios. Entretanto, para as respostas de expressão gênica as diferentes concentrações da mistura de HPAs foi heterogênea, sugerindo uma interação complexa entre a exposição e o metabolismo lipídico. Esses resultados podem indicar impactos no balanço energético, crescimento e saúde geral dos organismos. Além disso, as alterações nos perfis lipídicos podem influenciar processos fisiológicos diversos, como resposta ao estresse e desenvolvimento embrio-larval.

No contexto desses estudos, uma hipótese emergente é que a exposição crônica a HPAs em mistura pode ter efeitos significativos em níveis populacionais. Os efeitos de desregulação endócrina e o impacto no metabolismo lipídico observados nos sistemas *in vitro* e *in vivo* podem afetar a reprodução, o desenvolvimento e a sobrevivência de peixes e outros organismos aquáticos. Nesse sentido, uma abordagem integrada com o uso de ferramentas de bioinformática, como realizadas no capítulo IV são fundamentais para compreender plenamente os efeitos dessa classe de contaminantes em diferentes vias de regulação endócrina. A combinação de estudos moleculares com análises em nível de organismo e a consideração de fatores como a duração da exposição e as interações com outros poluentes são cruciais para uma avaliação precisa dos riscos e para o desenvolvimento de estratégias eficazes de mitigação.

Em resumo, os estudos apresentados contribuem para nossa compreensão dos efeitos complexos da exposição a HPAs em organismos aquáticos, especialmente peixes. Eles destacam a possibilidade de desregulação endócrina, alterações no metabolismo lipídico e implicações em múltiplos níveis fisiológicos. O uso de ferramentas interdisciplinares e integradas é essencial para elucidar os mecanismos subjacentes a esses efeitos, permitindo uma avaliação de risco eficaz.

## 7) CONCLUSÕES

- Foi possível avaliar o potencial desreuglador endócrino de HPAs em diferentes vertentes analíticas, demonstrando que esses compostos quando presentes em mistura complexa apresentam potencial desregulador endócrino;
- Através de análises de biologia molecular e bioinformática a vtg de O. niloticus foi caracterizada sob múltiplas condições, sendo possível utilizar otimizar físico-químicas (pH baixo e aquecimento) ideais para armazenamento desse biomarcador em laboratório para análises ecotoxicológicas;
- O modelo de cultivo primário de hepatócitos de O. niloticus foi validado utilizando o agonista (EE2) e antagonista (tamoxifen) para estudos de desregulação endócrina estrogênica;
- A mistura de HPAs causou respostas de desregulação endócrina, aumentando a expressão do gene vtg e esr2, sendo a modulação via genômica;
- Através de análises bioinformáticas foi possível elucidar um potencial mecanismo de ação dos múltiplos HPAs, identificando o receptor de estrógeno como molécula chave na regulação de processos;
- A mistura de HPAs promoveu alterações no metabolismo lipídico de larvas de zebrafish, através da modulação de glicerolipideos, fosfoglicerolipideos e esfingolipídios;
- Os genes dos receptores rxraa e lxr foram down e up regulados, respectivamente, caracterizando uma via de ação desses compostos quanto ao seu potencial de modulação do metabolismo lipídico;
- Através de ferramentas de biologia estrutural foi possível realizar simulações moleculares para avaliar a interação de diferentes tipos de HPAs com proteínas importantes para regulação de mecanismos endócrinos;
- Por meio do screening virtual e as validações realizadas, HPAs com maior potencial de ligação com receptores nucleares e transportadores foram identificados, sendo um indicativo de moléculas alvo para investigações em estudos in vivo/vitro.

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