Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais

Identificação do transcrito *CYP1A* no peixe *Phalloceros caudimaculatus* e resposta para a exposição a beta-naftoflavona e amostras ambientais de sedimento.

Roger Stacke Ferreira

Orientador: Juliano Zanette

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RESUMO

Contaminantes orgânicos, como os hidrocarbonetos policíclicos aromáticos (HPAs), podem atingir corpos da água e possuem potencial para causar efeitos tóxicos em organismos. A exposição aos HPAs causa indução nos níveis de citocromo P450 1A (CYP1A) em peixes, e portanto, é utilizado como um biomarcador de contaminação ambiental. O guarú *Phalloceros caudimaculatus* ocorre naturalmente em ambientes aquáticos dulcícolas e mixohalinos na América do Sul. O presente estudo identificou a sequência nucleotídica do transcrito *CYP1A* de *P. caudimaculatus*, que codifica uma proteína com 521 aminoácidos, e que apresenta 91% e 70% de identidade com CYP1A de *killifish* e paulistinha, respectivamente. A partir desta sequência foi possível realizar a avaliação dos níveis de mRNA de *CYP1A* deste peixe por RTq-PCR. Foi realizada uma caracterização de sua indução órgão- e tempo-dependente frente a exposição ao HPA beta-naftoflavona (BNF) e ao elutriato preparado a partir de sedimento de dois corpos da água possivelmente contaminados com HPAs. Foi constatado um aumento significativo nos níveis de mRNA de *CYP1A* em fígado, brânquia, intestino, cérebro, nadadeira anal de macho adultos e em alevinos na primeira hora de exposição a 1 μM de BNF, em relação ao grupo controle. O rim e as nadadeiras caudal e dorsal apresentaram indução de *CYP1A* após duas horas de exposição ao BNF. As maiores induções nos peixes dos grupos expostos ao BNF em relação ao controle foram de 176 no rim e 122 vezes no cérebro, observadas respectivamente após 8 e 48 horas de exposição. Os níveis de mRNA de *CYP1A* nos órgãos e tecidos de alevino, mantiveram-se induzidos pela exposição ao BNF até o final das 96 horas de exposição. A exposição dos peixes ao elutriato produzido a partir dos sedimentos coletados em dois locais potencialmente contaminados causou indução do *CYP1A* no fígado de 22 e 122 vezes em relação ao controle. Os resultados demonstram que a indução de *CYP1A* em *Phalloceros caudimaculatus* ocorre em tempos curtos de exposição, além da variação de acordo com o tempo de exposição e com o órgão analisado. Além disso, foi demonstrado que tecidos externos também podem ser utilizados para tais análises e que o elutriato feito a partir de sedimento de locais que recebem descargas de contaminantes podem causar indução de *CYP1A* nos organismos.

Palavras-chave: Ciprinodontiformes, citocromo P450, contaminação, guarú, elutriato.

ABSTRACT

Organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs), can reach water bodies and have the potential to cause toxic effects in organisms. Exposure to PAHs cause induction in the levels of cytochrome P450 1A (CYP1A) in fish and therefore is used as a biomarker of environmental contamination. The guppy *Phalloceros caudimaculatus* occurs naturally in freshwater and mixohaline aquatic environments in South America. This study identified the nucleotide sequence of *P. caudimaculatus CYP1A* transcript, which encodes a protein with 521 amino acids, and shows 91% and 70% identity with CYP1A of killifish and zebrafish, respectively. Based on this sequence it was possible to evaluate the *CYP1A* mRNA levels of this fish by RTq-PCR. A characterization of its organ- and time-dependent induction by exposure to PAH beta-naphthoflavone (BNF) and elutriate prepared from sediments of two bodies of water possibly contaminated with PAHs was carried out. A significant increase was seen in the *CYP1A* mRNA levels in liver, gill, gut, brain, anal fin of male adults and fingerlings in the first hour of exposure to 1 μM of BNF in compared to control group. Kidney, caudal fin and dorsal fin presented *CYP1A* induction after two hours of exposure to BNF. The highest induction in fish exposed to BNF groups compared to the control were 176 times in the kidney and 122 times in the brain, observed respectively after 8 and 48 hours of exposure. The *CYP1A* mRNA levels in organs and fingerling, remained induced by exposure to BNF until the end of 96 hours of exposure. The fish exposure to elutriate produced from sediments collected in two potential contaminated sites caused *CYP1A* induction in liver of 22- and 122- fold control. The results demonstrate that induction of *CYP1A* in *Phalloceros caudimaculatus* occurs in short exposure time and there is variation according to the time of exposure and analyzed organ. Furthermore, it was demonstrated that external tissues can also be used for such analysis and elutriate made from sediment of sites that recieve contaminant discharges induces *CYP1A* in those organisms.

Key-words: Cyprinodontiformes, cytochrome P450, contamination, guppy, elutriate.

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INTRODUÇÃO GERAL

Os Ambientes Aquáticos Continentais

Ambientes aquáticos continentais englobam vários ecossistemas, como lagoas de água doce e salobra; savanas, campos e florestas de inundação temporária ou permanentee os banhados (Maltchik et al., 2004). O estado do Rio Grande do Sul possui uma grande variedade de ambientes aquáticos, incluindo 3441 banhados que representam uma área total inundada de aproximadamente 30332 km² e ocupam aproximadamente 10,7% da área total do estado. Destes banhados, 72 % possuem uma área menor do que 1 km² (Maltchik et al, 2003; Rolon e Maltchik, 2006). Além dos banhados, o estado abriga o sistema da Lagoa dos Patos, considerada a maior lagoa costeira do mundo (Kjerfve, 1986). O sistema tem mais de 250 km de nordeste para sudoeste, com uma largura média de 40 km e profundidade média de 5 metros, com área de superfície de 10.360 km² e área estuarina de 900 km². A lagoa faz contato com o Oceano Atlântico através de um canal de entrada com aproximadamente 20 km de comprimento e 1 km de largura (Fernandes et al., 2004, Janeiro et al., 2008).

Banhados são áreas alagadas de forma permanente ou temporária, alimentados pela água da chuva e pelo escoamento de águas superficiais ou subterrâneas. São considerados ilhas de biodiversidade, pois têm cadeias alimentares extensivas e uma rica biodiversidade. Logo, estes ecossistemas são locais importantes para a conservação (Getzner, 2002; Rolon e Maltchik, 2006). Porém, devido à ocupação humana os banhados estão desaparecendo e ocupando áreas cada vez menores (Gibbs, 2000).

Os estuários, como o do sistema da Lagoa dos Patos, estão entre os ambientes mais importantes da zona costeira, os quais constituem zonas de transição, onde a água doce da drenagem de rios se mistura com a água marinha, criando alguns dos habitats mais produtivos conhecidos (Kennish, 2002). Os estuários são importantes para o ciclo de vida de muitos organismos marinhos e suas áreas rasas são utilizadas como berçários para diversas espécies de peixes e camarões, pois oferece proteção de predadores. Além disso, os estuários apresentam importância ecológica e comercial pela sua alta produtividade e por possuir abundância de alimento para os animais que o habitam (Pearcy e Myers, 1974; Weinstein, 1979; Martins et al., 2007).

O estuário da Lagoa dos Patos é delimitado pelas cidades de São José do Norte e Rio Grande, sendo a última a principal cidade no estuário, e que nos últimos anos tem aumentado sua importância no contexto nacional e América do Sul devido ao Mercosul.

Rio Grande tem como suas principais atividades a pesca, o turismo, atividade portuária e instalações industriais, além de uma refinaria de petróleo (Janeiro et al., 2008).

Os Contaminantes Ambientais

Compostos orgânicos naturais ou artificiais são aportados nos ecossistemas aquáticos por diversas formas, como por escoamento superficial, liberação de efluentes industriais e urbanos, derrames de combustíveis fósseis, deposição de partículas do ar (Neff, 1985; O'Malley et al., 1994; Garcia et al., 2010), e dispersados por movimento abióticos e bióticos, e transferência pela cadeia alimentar (Livingstone, 1998).

De maneira geral, os contaminantes estão presentes no ambiente fazendo parte de uma mistura de substâncias tóxicas, e possuem diferentes propriedades químicas, modos de toxicidade e potencial para interação com o ambiente e organismos. Além disso, os efeitos tóxicos de misturas não são a simples adição de toxicidades dos contaminantes individuais, o que torna a interpretação dos efeitos tóxicos causados por misturas na biota bastante complexa (Norwood et al., 2003).A cidade de Rio Grande carece de uma estrutura de tratamento de esgotos, o que resulta em uma descarga de esgotos para o estuário sem qualquer tipo de tratamento. As águas de escoamento urbano também são drenadas diretamente para o estuário. Estudo feito por Medeiros e colaboradores (2005) nos efluentes da região indicou pontos de contaminação por HPAs nos sedimentos perto da refinaria de petróleo e nos efluentes urbanos. Além disso, zonas de intensa navegação no estuário também são afetadas, superando o nível de contaminação em áreas rasas adjacentes da cidade e impactadas por efluentes industriais (Garcia et al., 2010).

Logo, o estuário da Lagoa dos Patos tem um histórico de contaminação antropogênica em pontos no Porto de Rio Grande, no pólo industrial, como na saída de efluentes industriais da refinaria de petróleo da região, produtoras de fertilizantes e da indústria pesqueira; e em saídas de efluentes urbanos não-tratados (Baisch et al., 1989). Estudo feito neste estuário demonstra que a contaminação da água e sedimento por metais pesados e contaminantes orgânicos dos efluentes industriais, urbanos e das atividades portuárias têm causado uma série de impactos sobre os ecossistemas abrigados pelo estuário (Tagliani et al., 2003).

Os Hidrocarbonetos Policíclicos Aromáticos

Os hidrocarbonetos policíclicos aromáticos (HPAs) são compostos por dois ou mais anéis de benzeno fundidos, que podem ser alquilados ou não. De acordo com o

tamanho, estrutura e solubilidade, podem ser divididos em compostos carcinogênicos (ex. benzo[a]pireno), e não carcinogênicos (ex. beta-naftoflavona). Os HPAs são relativamente mais solúveis do que outros hidrocarbonetos que possuem o mesmo número de átomos de carbono (McAuliffe, 1987) e esta solubilidade dos HPAs é uma característica importante, considerando que muitos HPAs estão entre os componentes mais tóxicos do petróleo bruto (Ramachandran et al., 2006), representando um risco para a biota aquática (Kennish, 1992).

As fontes de entrada de HPAs de origem antropogênica no meio aquático são feitas por combustão de combustíveis fósseis, derrame de derivados do petróleo e emissão de resíduos domésticos, sendo estas entradas mais aparentes em locais associados com as atividades industriais, atividades de transporte de petróleo e efluentes de esgoto doméstico (Medeiros et al., 2005). Segundo a organização dos Estados Unidos que coleta, analisa e dissemina informações sobre fontes energéticas, a *U.S. Energy Information Administration*, foram extraídos mais de 90 milhões de barris de petróleo por dia no ano de 2013 e produzidos quase 8 milhões de toneladas de carvão em 2012. A exploração, o refino, o transporte e as operações de armazenamento do petróleo e de seus derivados podem ocasionar derramamentos acidentais levando a contaminação tanto de solos quanto de ambientes aquáticos (Andrade et al., 2010).

O petróleo e seus derivados são uma classe ampla de contaminantes ambientais que podem entrar no ambiente aquático através de descargas de efluentes industriais e urbanos, atividades de transporte, derrames de petróleo, queima de combustíveis fósseis e afloramentos naturais (Medeiros et al., 2005) e o desenvolvimento dos centros urbanos e industriais aumentou consideravelmente os níveis de produtos petroquímicos no ambiente, principalmente em estuários e zonas costeiras (Lima et al., 2007). A contaminação ambiental por HPAs tornou-se uma preocupação global e existem relatos de contaminação significativa não apenas em sistemas costeiros e marinhos, como em também em água doce (Curran et al, 2000; Donahue et al., 2006).

De forma geral, os HPAs são incorporados por organismos (ex.: plantas e peixes) por difusão passiva, isso ocorre devido à natureza apolar dos HPAs que lhe conferem a capacidade lipofílica para atravessar a membrana das células (Gauthier et al., 2014). Após a incorporação, o HPA e seus metabólitos podem aumentar a produção de espécies reativas de oxigênio por vários mecanismos, os quais podem conduzir a danos celulares por meio de oxidação de proteínas, peroxidação lipídica e danos de DNA (Altenburger et al., 2003). Para evitar essas lesões, sistemas antioxidantes são ativados pela célula para

eliminar espécies reativas de oxigênio estimuladas pelos contaminantes, permitindo que o organismo supere o estresse oxidativo em ambientes contaminados (Livingstone, 2001; Lima et al., 2007).

Para analisar a contaminação de ambientes aquáticos por HPAs são estudadas amostras da coluna d'água, de sedimentos e de organismos (Volkman et al., 1992). Porém, a contaminação do sedimento tem recebido mais atenção, pois os HPAs são compostos hidrofóbicos com baixa solubilidade em água, que se associam principalmente com o material particulado, como o sedimento (Readman et al, 1984; Neff, 1985), fazendo com que a concentração de HPAs encontrada no sedimento seja superior a da água no mesmo local (Li et al., 2010).

Na Lagoa dos Patos, o lodo coletado diretamento nos pontos de escoamento (industrial, esgoto urbano e misto) apresentou níveis altos de contaminação por HPAs, sendo quase 20 vezes maiores que as encontradas nas amostras de sedimentos mais afastadas dos locais de descargas de efluentes. As concentrações de HPAs encontradas nos pontos amostrados revelam que a Lagoa dos Patos assemelha-se àoutros estuários com um nível de contaminação por HPAs moderada (Garcia et al., 2010). Dentre estes HPAs, o benzo[a]pireno (BaP) é um dos HPAs frequentemente utilizado por guias governamentais para indicar contaminação por HPAs, sendo estudado devido à sua propriedade carcinogênica e muito usado em experimentos com peixes como modelo para estudos que visam a compreensão do efeito de HPAs em vertebrados (Beyer et al., 1997). O BaP foi encontrado quase 100 vezes mais concentrado no lodo na saída do efluente do que no sedimento, demonstrando que a concentração dos contaminantes provenientes dos efluentes diminui drasticamente com o aumento da distância da fonte de contaminação (Medeiros et al., 2005; Garcia et al., 2010).

Remobilização de Contaminantes

A remobilização de contaminantes provenientes dos sedimentos afeta a mobilidade dos contaminantes, pode aumentar a biodisponibilidade e os efeitos sobre os organismos no local. Logo, a remobilização de contaminantes deve ser monitorada adequadamente, especialmente quando atividades de dragagem são feitas, pois mesmo após o término da atividade a concentração dos contaminantes pode permanecer elevada na água (Bocchetti et al., 2008) e causar impactos tanto subletais quanto letais nos organismos (Kennish, 2002). A ressupensão pode ocorrer tanto de modo natural, pelas tempestades, por grande aporte de água pluvial ou correntes de fundo, quanto por

atividades antrópicas, por operações de dragagem ou movimentação de barcos e navios (Bergen et al., 2005).

Para reprodução em laboratório de uma situação de ressuspensão dos contaminantes contidos no sedimento depositado no fundo de ambientes aquáticos, pode ser utilizado o elutriato (Bergen et al., 2005). A elutriação é feita pela a adição de água a uma substância sólida ou material solto (ex.: sedimentos, resíduos, lamas, lodos), as quais são misturadas por agitação ou aeração. Posteriormente é feita uma centrifugação, filtragem ou decantação do sobrenadante, sendo esta solução aquosa sobrenadante denominada elutriato, a qual é utilizada para os testes toxicológicos (EPA, 2001). A remobilização dos contaminantes do sedimento para a água ocorre em função do aumento do teor de água no sedimento e/ou mudanças nas condições de redução e oxidação (Di Toro et al, 1991;. Ankley et al., 1996).

Além disso, a elutriação remobiliza para água qualquer contaminante que esteja na fase particulada, trazendo à tona não só hidrocarbonetos policíclicos aromáticos (HPAs), mas também outros contaminantes (EPA, 2001). Estes contaminantes remobilizados podem interagir ao mesmo tempo sobre o organismo, podendo influenciar no transporte, metabolismo e desintoxicação através de competição por sítios ou complexação dos mesmos. Assim o uso do elutriato para testes toxicológicos mostra não só os efeitos dos um contaminante contido no sedimento, mas sim a interação do conjunto total de contaminantes remobilizados (Gauthier et al., 2014).

Phalloceros caudimaculatus: um Potencial Biomonitor de Contaminação

Ambientes aquáticos abrigam diversas espécies de plantas e animais, as quais entram em contato com os contaminantes carreados para os corpos d'água. As populações de peixes desempenham um papel importante na avaliação da contaminação aquática, pois algumas espécies respondem com sensibilidade a mudanças no ambiente aquático (Siroka e Drastichova, 2004).

A ordem Ciprinodontiformes é composta de diversas espécies de peixes, como os *killifish*, barrigudinhos e guarús. Têm de pequeno à médio porte (8 mm à 34 cm), são encontrados em ambientes de água doce e salobra. É considerada uma ordem cosmopolita (Parenti, 1981) e seus membros são conhecidos por sua tolerância a baixa qualidade da água (Araújo et al., 2007). Esta ordem tem entre seus representantes o *killifish Fundulus heteroclitus*, o qual vem sendo utilizado nos Estados Unidos como um modelo para o estudo de respostas adaptativas de organismos para a contaminação

ambiental (Burnett et al., 2007). *Poecilia vivipara* e *Jenynsia multidentata* também são representantes desta ordem que vem sendo utilizados para estudos de contaminação ambiental na América do Sul (Fig. 1).

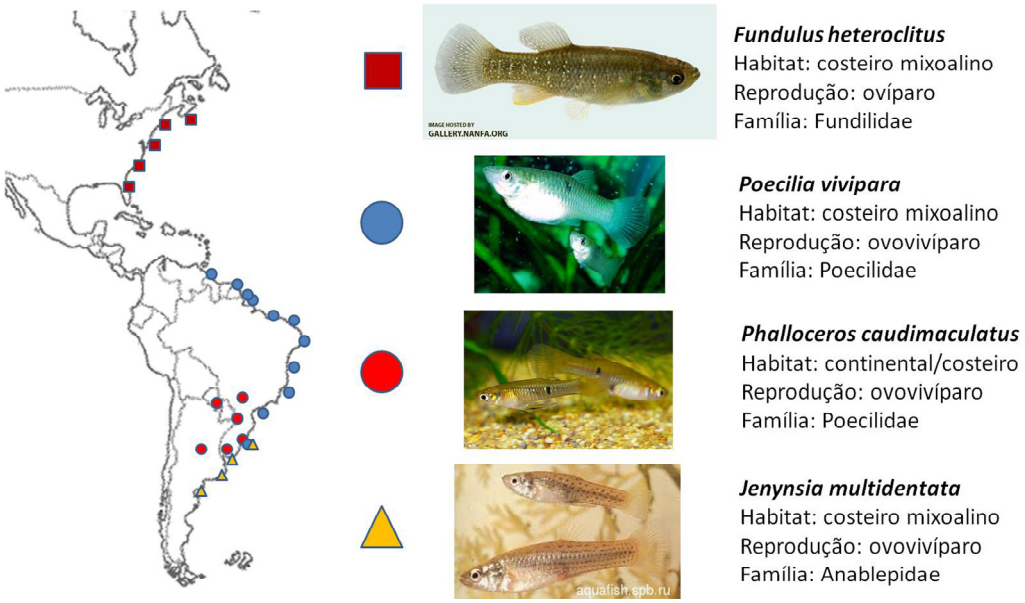

Figura 1. Distribuição, habitat e reprodução de espécies de Ciprinodontiformes estudados na América do Norte e América do Sul (Froese e Pauly, 2003).

A ordem Ciprinodontiformes é subdividida em dez famílias, Anablepidae, Aplocheilidae, Cyprinodontidae, Fundulidae, Goodeidae, Nothobranchiidae, Poeciliidae, Profundulidae, Rivulidae e Valenciidae. Sendo que no estado do Rio Grande do Sul foi observada uma alta riqueza e abundância de Ciprinodontiformes, principalmente a família Poeciliidae, em habitats de área reduzida, baixa profundidade, susceptibilidade ao estresse hídrico e com alta densidade de macrófitas (Maltchik et al., 2014).

Espécies de Poeciliidae ocorrem em ambientes de água doce e salobra (Nelson, 1994), são caracterizados pelo pequeno porte, onivoria, ovoviviparidade, fecundação interna e pelo dimorfismo sexual acentuado, no qual a fêmea é maior que o macho, e o macho possui a nadadeira anal modificada para transferência de esperma durante a cópula (Betito, 2006). Estudos relatam uma maior abundância de fêmeas do que de machos e a capacidade de reter esperma de uma cópula para fecundações sucessivas sem a necessidade de cópula. Essa família possui cerca de 200 espécies em 16 gêneros, entre as quais está o guarú *Phalloceros caudimaculatus* (Fig. 2) (Parenti, 1981; Araujo et al., 2007).

Como já descrito para a ordem, o guarú *P. caudimaculatus* é conhecido por ter capacidade de prosperar em condições ambientais adversas, podendo ser utilizado como bioindicador de más condições de água em estudos de comunidades. Esta capacidade faz com que colonize zonas impactadas por efluentes industriais e domésticos, caracterizando-a como uma espécie oportunista (Araújo et al., 2007). Esta espécie esta distribuída na área continental e costeira do sudeste do Brasil até o norte da Argentina (Froese e Pauly, 2003), sendo que estudos recentes a apontaram como uma das espécies mais abundantes e bem distribuídas nos banhados do estado do Rio Grande do Sul (Maltchik et al., 2014).

Figura 2. Guarú *Phalloceros caudimaculatus*, A) macho com 24,5 mm; B) fêmea com 36,8 mm (Lucinda, 2008)

Mudanças na faixa etária predominante e distribuição das espécies em uma população de peixes são bioindicadores gerais da contaminação da água, mas há também respostas específicas a produtos químicos estranhos ao organismo, conhecidos como xenobióticos (Siroka et al. 2004; Nebert e Dalton, 2006). Neste caso, os peixes podem ser utilizados como bioindicadores e biomonitores, pois os efeitos da exposição a xenobióticos podem ser medidos em respostas fisiológicas, bioquímicas ou moleculares (Mondon et al., 2001).

A análise química é capaz de medir quantitativamente uma ampla gama de contaminantes, porém somente estabelece a presença e concentraçãodo que é possível detectar, sendo incapaz de revelar alterações biológicas nos organismos. A utilização de biomarcadores, bioquímicos e moleculares, cumpre esse propósito através da mensuração das respostas bioquímicas e/ou moleculares em organismos, podendo

demonstrar tanto que organismos estão sendo expostos a certas classes de contaminantes, como demonstrar a ocorrência dos efeitos tóxicos causados (Siroka e Drastichova 2004). Biomarcadores celulares e moleculares têm o potencial de antecipar mudanças em níveis mais elevados de organização biológica, servindo como um alerta precoce, permitindo que sejam tomadas medidas de biorremediação antes de ocorrer mudanças irreversíveis no ecossistema (Cajaraville et al., 2000).

Biomarcador CYP1A

A biotransformação de xenobióticos e metabolização de substâncias endógenas podem ser divididas em três fases, a fase-I é conhecida pela introdução de um grupo funcional, como hidroxila (OH), carbonila (COOH) ou nitro (NO_2), ou alteração de algum grupo funcional existente por reações químicas, como oxidação, redução, hidratação ou hidrólise. A fase-II é realizada pelas transferases, as quais anexam uma unidade polar (ex.: glutationa, sulfato e aminoácidos) a um grupo funcional da molécula parental. A fase-III é o transporte e excreção do xenobiótico (Livingstone, 1998). O objetivo das fases-I e -II é aumentar a hidrofilicidade dos xenobióticos e das substâncias endógenas, ou seja, aumentar a afinidade destas moléculas com a água. A fase-I é realizada principalmente por enzimas da superfamília citocromo P450 (CYP) (Myers et al., 2003) e a fase-II por enzimas como sulfotransferases e glutationa S-transferases (Xu et al., 2005). Atividades de regulação do sistema de biotransformação/metabolização de fase-I e -II têm sido utilizados para quantificar os efeitos de xenobióticos em espécies aquáticas. No entanto, as enzimas de fase-II são geralmente menos responsíveis do que os sistemas de fase-I (Holth et al., 2008).

CYP é uma superfamília de hemoproteínas presente em praticamente todos os tecidos, e em todos os organismos vivos. A ligação heme-tiolato no sítio ativo da enzima é responsável por diversas propriedades catalíticas desta superfamília de enzimas. O CYP representa um dos sistemas enzimáticos mais importantes na biotransformação de xenobióticos e metabolização de lipídios endógenos (Estabrook e Werringloer, 1978).

Enzimas CYP podem ser classificadas de acordo com a sua função em três classes, designadas por FC-E, FC-S, e FC-X, baseado nas características dos seus substratos e produtos. Enzimas FC-E são essenciais para a síntese e degradação de compostos endógenos, tais como hormônios e vitaminas, enzimas FC-X são as envolvidas na biotransformação de xenobióticos, e as enzimas FC-S participam na síntese e catabolismo de metabólitos secundários. Porém esta classificação não é rígida,

pois são conhecidas enzimas CYPs das famílias CYP1, CYP2, CYP3 e CYP4 que são associadas tanto com a biotransformação de xenobióticos, quanto com o metabolismo dos esteróides e eicosanóides em mamíferos (Gotoh, 2012). Logo, uma alteração na indução de enzimas CYP por xenobióticos para biotransformação pode alterar a dinâmica nas vias de degradação e síntese de compostos endógenos.

Dentre os compostos que são metabolizados pela superfamília CYP estão drogas, solventes, anestésicos, pesticidas, petróleo e seus derivados, HPAs, antioxidantes, corantes, aromatizantes, esteróides, ácidos graxos, hidroperóxidos de lípidios, vitaminas lipossolúveis, aminoácidos, eicosanóides e retinóides (Coon, 2005).

O aumento do conteúdo de enzimas CYP é proposto como um biomarcador de exposição a xenobióticos, como os HPAs (Porte et al., 2001). Além disso, vários estudos de campo têm demonstrado relação direta entre a indução de enzimas da superfamília CYP e as concentrações de HPAs e bifenilas policloradas no ambiente (Petushok et al., 2002). Os efeitos de HPAs em organismos aquáticos têm sido avaliados através da atividade enzimática de fase-I e os subseqüentes efeitos provocados por estes compostos, tais como a formação de aduto de DNA e lesões histopatológicas (Myers et al., 2003). A toxicidade dos HPAs é atribuída principalmente a biotransformação dos compostos originais em metabólitos reativos, e, portanto, a toxicidade destes compostos é mediada por membros da superfamília CYP (Nebert e Dalton, 2006).

Devido ao grande número de enzimas na superfamília CYP, é necessário um sistema de classificação, no qual as enzimas são distribuídas em famílias e subfamílias, com base na porcentagem de identidade obtida pelo alinhamento destas sequências. A constatação de 40% ou mais de identidade entre sequências polipeptídicas de CYPs, leva ao agrupamento em uma determinada família designada por um algarismo arábico (ex.: CYP1). A constatação de 55% ou mais de identidade entre as sequencias de aminoácidos leva ao agrupamento em uma subfamília designada por uma letra (ex.: CYP1A), e caso haja mais de uma isoforma em uma dada subfamília, é adicionado mais um algarismo arábico (ex.: CYP1A1) (Nelson et al., 1996).

A biotransformação de HPAs tem recebido atenção devido a sua capacidade de alterar a via metabólica do CYP1, levando a indução de enzimas da família CYP1. Esta indução é mediada pelo receptor de hidrocarboneto aromático (AHR) (Hahn, 2002) que pode ser ativado pela ligação com HPAs, os quais podem ser agonistas do AHR, estimulando a biotransformação de HPAs pela indução da transcrição do gene *CYP1A* (Stegeman e Hahn, 1994; Hahn, 1998). Porém, existem HPAs que possuem pouca ou

nenhuma afinidade com o AHR, porém estes são considerados exceções (Van Tiem e Di Giulio, 2011; Wassenberg et al., 2005).

CYP é o principal complexo enzimático que atua na desintoxicação/bioativação de HPAs (Aas et al, 2000). Estudos feitos com paulistinha demonstraram que as diversas subfamílias de CYP1 diferem na magnitude da sua expressão basal e a expressão de CYPs varia entre órgãos e estágios de desenvolvimento (Jönsson et al., 2007; Goldstone et al., 2010), sugerindo que as diferentes enzimas CYP podem desempenhar diferentes papéis na susceptibilidade aos agonistas do AHR. Para mamíferos e peixes, quatro famílias de CYP são conhecidas por desempenhar um papel importante na biotransformação de xenobióticos, as famílias CYP1, CYP2, CYP3 e CYP4 (Stegeman e Hahn, 1994; Hahn, 1998; Nerbert e Dalton, 2006).

CYP1A é uma subfamília importante, em grande parte das classes de vertebrados, no processo de biotransformação de HPAs e outras substâncias agonistas dos AHR (Teramitsu et al., 2000). CYP1A é uma das formas de CYP muito estudada em peixes e vem sendo amplamente utilizada como biomarcador de contaminação aquática, pois é induzida por compostos tóxicos, como HPAs, bifenilas policloradas (PCB), dioxinas (TCDD) e furanos (TCDF) (Stegeman, 1993; Rattner et al, 1993; Bucheli e Fent, 1995).

De acordo com a revisão feita Kawajiri e Fujii-Kuriyama (2007) (Fig. 3), a ligação de compostos endógenos ou exógenos, tais como as dioxinas no AHR causa sua ativação, o que leva ao transporte nuclear do complexo AHR-agonista. Dentro do núcleo ocorre uma dimerização com o translocador nuclear do receptor de hidrocarbonetos (ARNT), formando um novo dímero, o AHR-ARNT, o qual reconhece regiões mediadoras conhecidas por elementos de resposta a xenobióticos (XREs). O dímero AHR-ARNT ligasse na região do XRE causando uma remodelação da cromatina, a qual aumenta a sensibilidade em um ponto próximo ao local de iniciação da transcrição do gene *CYP1A*. Assim, fatores de transcrição atuam no local sensibilizado fazendo a transcrição do gene em RNA mensageiro (RNAm), o qual é mobilizado para o citoplasma onde será traduzido na proteína CYP1A para biotransformação dos compostos.

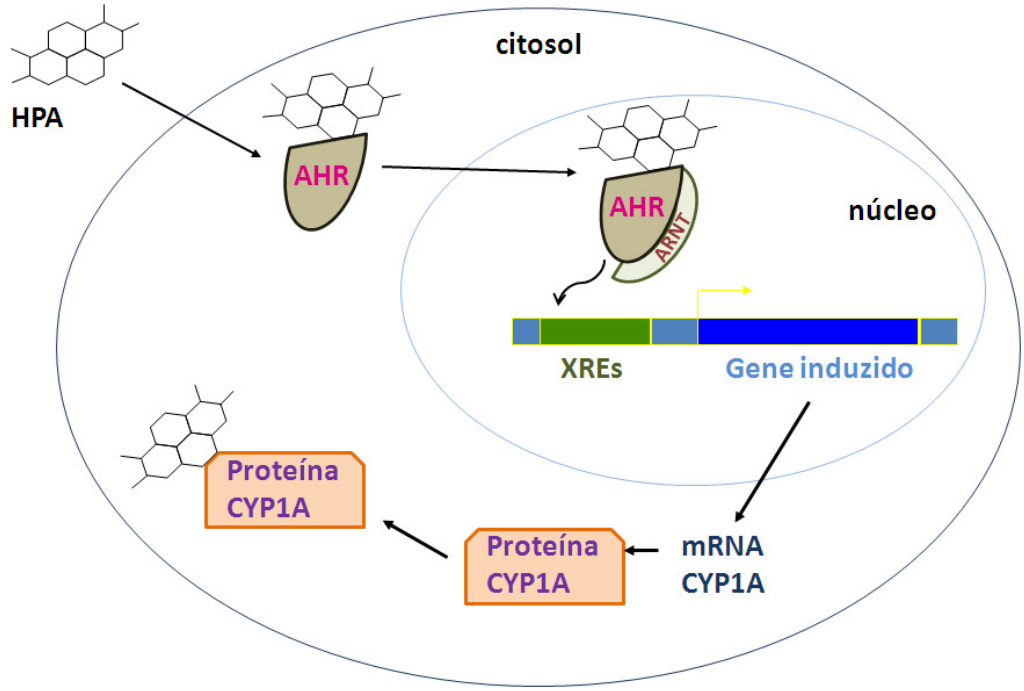

Figura 3. Desenho esquemático da indução do gene *CYP1A* na presença de um hidrocarboneto policíclico aromático (HPA) (Kawajiri e Fujii-Kuriyama, 2007).

A carcinogênese acionada pelos metabólitos das reações feitas pelo complexo enzimático CYP é o mecanismo mais reconhecido pelo qual os HPAs exercem efeitos tóxicos (Baird et al., 2005). Porém, estudos sobre a toxicidade do benzo[a]pireno (BaP), um forte agonista do AHR, demonstrou a função protetora da biotransformação feita pela enzima CYP1A durante a embriogênese de peixes (Van Tiem e Di Giulio, 2011; Wills et al, 2009), e que a inibição da CYP1A leva a um aumento da toxicidade (Gauthier et al., 2014)

O CYP1A é geralmente determinado em microssomas hepáticos, sendo que agonistas do AHR como os HPAs, podem ser facilmente biotransformados em outros órgãos, como as brânquias, fazendo com que a atividade hepática não reflita com precisão a exposição total de agonistas do AHR (Jönsson et al., 2006). A utilização de medições das alterações biológicas induzidas por contaminação química em ambientes aquáticos, como a atividade enzimática de CYP1A, tem sido amplamente empregada em ecossistemas aquáticos na América do Norte e Europa, em programas de biomonitoramento para detectar a exposição a esses produtos químicos (Stegeman, 1993; Bourgeot et al., 1996).

A informação de seqüência nucleotídica pode ser usada para produzir iniciadores e anticorpos para quantificar a transcrição/expressão gênica do gene/proteína de interesse no organismo e usá-los como uma medida do efeito contaminante sobre o mesmo (Greytak et al., 2005). Porém a baixa similaridade de seqüência entre genes *CYP1A* resulta em baixa reatividade cruzada entre diferentes espécies para anticorpos de CYP1A (Cousinou et al., 2000). Sendo assim, é necessário obter a seqüência de *CYP1A* do novo organismo de testes, para a produção de iniciadores e anticorpos específicos para analisar a transcrição/expressão gênica (Lee et al., 2005). A transcrição gênica de biomarcadores foca na etapa anterior, medindo a quantidade de RNAm, o qual codifica proteínas ou enzimas, sendo que na maioria dos casos, mas não todos, ambos os tipos de biomarcadores, transcrição gênica e ensaio enzimático/quantificação protéica estão mutuamente correlacionados (Piña et al., 2007). O estudo feito por Quirós e colaboradores (2007) demonstrou uma correlação positiva entre a atividade enzimática EROD, a qual o CYP1A é uma das principais enzimas associadas, com a transcrição gênica de *CYP1A*. Transcrição gênica de biomarcadores também pode ser destinada para avaliação de efeitos de contaminação observada nos organismos expostos ou para fornecer um aviso prévio nos casos de suspeita de impacto antropogênico no ambiente. A principal limitação do seu desenvolvimento é a falta de conhecimento sobre aspectos fisiológicos, genéticos, embriológicos, e, em geral, a biologia da biota impactada (Piña et al., 2007)

A mudança na transcrição gênica antecede as mudanças nos processos celulares, os quais podem afetar em nível de população, servindo de alerta anterior às mudanças em níveis mais complexos (Myers et al., 2003; Holth et al., 2008). Além disso, o custo de uma reação para análise de transcrição gênica é relativamente baixo, e um grande número de amostras pode ser analisada em um único ensaio. Além de ser um método relativamente rápido e preciso, após a otimização dos iniciadores, o qual não necessita de muito material biológico (Piña et al., 2007).

Neste estudo foi identificado o RNAm que codifica a enzima CYP1A em *Phalloceros caudimaculatus*. Foram avaliadas as respostas órgão-específicas e tempo-dependentes ocorridas nos níveis de RNAm de *CYP1A* frente a exposição ao HPA beta-naftoflavona (BNF) no fígado, intestino, cérebro, brânquia, rim, nadadeiras dorsal, caudal e anal e em alevinos. Avaliou-se também a resposta ocorrida nos níveis de RNAm de *CYP1A* de *P. caudimaculatus* expostos ao elutriato feito a partir dos sedimentos de locais possivelmente contaminados com HPAs, com a finalidade de demonstrar a

utilização potencial deste biomarcador no monitoramento ambiental. A hipóteses foide que o HPA BNF causaria aumento nos níveis de RNAm de *CYP1A* em alguns órgãos e em pelo menos algumas das janela temporais testadas. E de que a mistura de contaminantes contidos nos sedimentos dos locais potencialmente contaminados também causariam aumento nos níveis de RNAm de *CYP1A* no guarú *Phalloceros caudimaculatus*.

OBJETIVOS

Objetivo geral

Demonstrar o potencial do guarú *Phalloceros caudimaculatus* para utilização como bioindicador e biomonitor de ambientes contaminados por agonistas do receptor de hidrocarbonetos aromáticos (AHR) através da mensuração dos níveis de RNAm de *CYP1A* e a utilização das nadadeiras como um método não-invasivo para mensuração dos níveis de RNAm de *CYP1A*.

Objetivos específicos

- Identificar a sequência nucleotídica do transcrito *CYP1A* em *P. caudimaculatus*
- Avaliar os níveis de expressão de RNAm de *CYP1A* em fígado, intestino, cérebro, brânquia, rim, nadadeiras dorsal, caudal e anal e tecidos de alevinos de *P. caudimaculatus* expostos a 1 μM de BNF por 1, 2, 4, 8, 24, 48 e 96 horas.
- Avaliar os níveis de expressão de RNAm de *CYP1A* em fígado de *P. caudimaculatus* expostos por 8 horas aos elutriatos feitos a partir de sedimentos de dois locais possívelmente contaminados, e outro não contaminado.

BIBLIOGRAFIA DA INTRODUÇÃO GERAL

Aas, E., Baussant, T., Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquatic Toxicology. 51, 241-258.

Altenburger, R., Segner, H., Van dar Oost, R., 2003. Biomarkers and PAHs – prospects for the assessment of exposure and effects in aquatic systems. In: Douben, P.E.T. (Ed.), PAHs: An Ecotoxicological Perspective. Wiley, Chichester, England, 147–171.

Andrade, J.A., Augusto, F., Jardim, I.C.S.F., 2010. Biorremediação de solos contaminados por petróleo e seus derivados. Eclética Química. 35 – 3, 17-43.

Ankley, G.T., Di Toro, D.M., Hansen, D.J., Berry, W.J., 1996. Assessing the ecological risk of metals in sediments. Environmental Toxicology and Chemistry. 15, 2053–2055

Araújo, F.G., Peixoto, M.G., Pinto, B.C.T., Teixeira, T.P., 2007. Distribution of guppies *Poecilia reticulata* (Peters, 1860) and *Phalloceros caudimaculatus* (Hensel, 1868) along a polluted stretch of the Paraíba do Sul River, Brazil. Brazilian Journal of Biology. 69, 41-48.

Baird, W., Hooven, L., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environmental and Molecular Mutagenesis. 45, 106–114.

Baisch, P., Asmus, H., Jouanneau, J., 1989. Chemical composition of sediments from the Patos Lagoon (Brazil). Proceedings of International Symposium on Geochemical Exploration; p. 11 –20.

Bergen, B.J., Nelson, W.G., Mackay, J., Dickerson, D., Jayaraman, S., 2005. Environmental monitoring of remedial dredging at the New Bedford Harbor, MA, superfund site. Environmental Monitoring and Assessment. 111, 257–275.

Betito, R. 2006. Comparação da complexidade das adaptações bioecológicas de dois peixes (*Jenynsia multidentata* e *Poecilia vivipara*) Cyprinodontiformes no estuário da Lagoa dos Patos (RS - Brasil). Revista Didática Sistêmica. 3, 71-100.

Beyer, J., Sandvik, M., Skåre, J.U., Egaas, E., Hylland, K.,Waagbø, R., Goksøyr, A., 1997. Time- and dose-dependent biomarker responses in flounder (*Platichthys*

flesus L.) exposed to benzo[a]pyrene, 2,3,3',4,4',5-hexachlorobiphenyl (PCB-156) and cadmium. Biomarkers. 2 (1), 35–44.

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchiab, S., Olivierob, L., Pilatob, F., Pellegrini, D., Regoli, F., 2008. Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. Aquatic Toxicology. 89, 257–266

Bourgeot, T., Bocquéné, G., Porte, C., Dimeet, J., Santella, R.M., Garcia de la Parra, L.M., Pfhol-Leszkowicz, A., Raoux, C., Galgani, F., 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. Marine Ecology Progress Series. 131, 125–141.

Buchneli, T., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology. 25, 201–268.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Gomez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., Maclatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein,W.K., Whitehead, A., Winn, R.N., Crawford, D.L., 2007. *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. Comparative Biochemistry and Physiology, Part D. 2, 257-286.

Cajaraville, M.P., Bebianno, J.M., Blasco, J., Porte, C, Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. The Science of the Total Environment. 247, 295-311.

Coon, M.J., 2005. CYTOCHROME P450: Nature's Most Versatile Biological Catalyst. Annual Review of Pharmacology and Toxicology. 45, 1–25.

Cousinou, M., Nilsen, B., Lopez-Barea, J., Dorado, G., 2000. New methods to use fish cytochrome P4501A to assess marine organic pollutants. Science of the Total Environment. 247, 213–225.

Curran, K.J., Irvine, K.N., Droppo, I.G., Murphy, T.P., 2000. Suspended solids, trace metal and PAH concentrations and loadings from coal pile runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research. 281, 217–227.

Di Toro, D.M., Zarba, C.S., Hansen, D.J., Berry, W.J., Swartz, R.C., Cowan, C.E., Pavlou, S.P., Allen, H.E., Thomas, N.A., Paquin, P.R., 1991. Technical basis for establishing sediment quality criteria for nonionic organic chemicals using equilibrium partitioning. Environmental Toxicology and Chemistry. 10, 1541–1583.

Donahue, W.F., Allen, E.W., Schindler, D.W., 2006. Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada. Journal of Paleolimnology. 35, 111–128.

EPA, U.S., 2001. Methods for Collection, Storage and Manipulation of Sediments for Chemical and Toxicological Analyses: Technical Manual. EPA 823-B-01-002. U.S. Environmental Protection Agency, Office of Water, Washington, DC.

Estabrook, R.W., Werringloer, J., 1978. The measurement of difference spectra: Application to the Cytochromes of Microsomes. Methods in Enzymology. 52, 212-220.

Fernandes, E.H.L., Dyer, K.R., Moller, O.O., 2004. Spatial gradients in the flow of southern Patos Lagoon. Journal of Coastal Research. 21 (4), 759–769.

Froese, R., Pauly, D., 2003. Editors. FishBase. World Wide Web electronic 13 publication. Disponível em: http://www.fishbase.org. Acessado em: 31 de agosto de 2010.

Garcia, M.R., Mirlean, N., Baisch, P.R., Caramão, E.B., 2010. Assessment of polycyclic aromatic hydrocarbon influx and sediment contamination in an urbanized estuary. Environ Monit Assess. 168, 269-276.

Gauthier, P.T., Norwood, W.P., Prepas, E.E., Pyle, G.G., 2014. Metal–PAH mixtures in the aquatic environment: A review of co-toxic mechanisms leading to more-than-additive outcomes. Aquatic Toxicology. 154, 253–269.

Getzner, M., 2002. Investigating public decisions about pro- tecting wetlands. Journal of Environmental Management. 64, 237–246.

Gibbs, J.P., 2000. Wetland loss and biodiversity conservation. Conservation Biology. 14, 314–317.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jönsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics. 11:643.

Gotoh, O., 2012. Evolution of Cytochrome P450 Genes from the Viewpoint of Genome Informatics. Biological & Pharmaceutical Bulletin. 35 (6), 812–817.

Greytak, S.R., Champlin, D., Callard, G.V., 2005. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): Differential expression in fish from polluted and unpolluted environments. Aquatic Toxicology. 71, 371–389.

Hahn, M.E., 1998. The aryl hydrocarbon receptor: a comparative perspective. Comparative Biochemistry and Physiology C. 121, 23-53.

Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. Chemico-Biological Interactions. 141, 131–160.

Holth, T.F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G.I., Aleström, P., Hylland, K., 2008. Differential gene expression and biomarkers in zebrafish (*Danio rerio*) following exposure to produced water components. Aquatic Toxicology. 90, 277–291.

Janeiro, J., Fernandes, E., Martins, F., Fernandes, R., 2008. Wind and freshwater influence over hydrocarbon dispersal on Patos Lagoon, Brazil. Marine Pollution Bulletin. 56, 650–665.

Jönsson, E.M., Abrahamson, A., Brunström, B., Brandt, I., 2006. Cytochrome P4501A induction in rainbow trout gills and liver following exposure to waterborne indigo, benzo[a]pyrene and 3,3',4,4',5-pentachlorobiphenyl. Aquatic Toxicology. 79, 226-232.

Jönsson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007. Basal and 3,3”,4,4”,5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicology and Applied Pharmacology. 221, 29-41.

Kawajiri, K., Fujii-Kuriyama, Y., 2007. Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. Archives of Biochemistry and Biophysics. 464, 207–212.

Kennish, M.J., 1992. Ecology of estuaries: Anthropogenic effects. Boca Raton: CRC Press, Flórida. 2, 86–88.

Kennish, M.J., 2002. Environmental threats and environmental future of estuaries. Environmental Conservation. 29 (1), 78–107.

Kjerfve, B., 1986. Comparative oceanography of coastal lagoons. In: Wolfe, D.A. (Ed.), Estuarine Variability, 63–81.

Lee, Y., Williams, T.D., Jung, S., Lee, J., 2005. cDNA cloning and expression of a cytochrome P450 1A (CYP1A) gene from the hermaphroditic fish *Rivulus marmoratus*. Marine Pollution Bulletin. 51, 769–775.

Li, J., Shang, X., Zhao, Z., Tanguay, R.L., Dong, Q., Huang, C., 2010. Polycyclic aromatic hydrocarbons in water, sediment, soil, and plants of the Aojiang River waterway in Wenzhou, China. Journal of Hazardous Materials. 173, 75–81.

Lima, I., Moreira, S.M., Rendón-Von Osten, J., Soares, A.M.V.M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere. 66, 1230–1242.

Livingstone, D.R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comparative Biochemistry and Physiology Part A. 120, 43–49.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin. 42, 656–666.

Lucinda, P.H.F., 2008. Systematics and biogeography of the genus *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes: Poeciliidae: Poeciliinae), with the description of twenty-one new species. Neotropical Ichthyology. 6 (2), 113-158.

Maltchik, L., Costa, E.S., Becker, C.G., Oliveira, A.E., 2003. Inventory of wetlands of Rio Grande do Sul (Brazil). Pesquisas: Botanica. 53, 89–100.

Maltchik, L., Rolon, A.S., Guadagnin, D.L., Stenert, C., 2004. Wetlands of Rio Grande do Sul, Brazil: a classification with emphasis on plant communities. Acta Limnologica Brasiliensia. 16, 197-151.

Maltchik, L., Lanés, L.E.K, Keppeler, F.W., Rolon, A.S., Stenert, C., 2014. Landscape and habitat characteristics associated with fish occurrence and richness in southern Brazil palustrine wetland systems. Environmental Biology of Fishes. 97, 297–308.

Martins, I.M., Dias, J.M., Fernandes, E.H., Muelbert, J.H., 2007. Numerical modelling of fish eggs dispersion at the Patos Lagoon estuary - Brazil. Journal of Marine Systems. 68, 537–555.

McAuliffe, C.D., 1987. Organism exposure to volatile/soluble hydrocarbons from crude oil spills-a field and laboratory comparison. Oil Spill Conference. American Petroleum Industry, Washington, DC, 275-288.

Medeiros, P.M., Bícego, M.C., Castelão, R.M., Rosso, C.D., Fillmann, G., Zamboni, A.J., 2005. Natural and anthropogenic hydrocarbon inputs to sediments of Patos Lagoon Estuary, Brazil. Environment International. 31, 77-87.

Mondon, J.A., Duda, S., Nowak, B.F., 2001. Histological, growth and 7- ethoxyresorufin O-deethylase (EROD) activity responses of greenback flounder *Rhombosolea tapirina* to contaminated marine sediment and diet. Aquatic Toxicology. 54, 231-247.

Myers, M.S., Johnson, L.L., Collier, T.K., 2003. Establishing the causal relationship between polycyclic aromatic hydrocarbon (PAH) exposure and hepatic neoplasms and neoplasia-related liver lesions in English sole (*Pleuronectes vetulus*). Human and Ecological Risk Assessmen. 9 (1), 67–94.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nature Reviews Cancer. 6, 947–960.

Neff, J.M., 1985. Polycyclic aromatic hydrocarbons. In G. M. Rand, S. R. Petrocelli (Eds.), Fundamentals of aquatic toxicology. New York: Hemisphere. 416–454.

Nelson, J.S., 1994. Fishes on the world. New York: John Wiley and Sons, 600p.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R., Gunsalus, I.C., Nebert, D.W., 1996. P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics, 6: 1, 1-42.

Norwood, W.P., Borgmann, U., Dixon, D.G., Wallace, A., 2003. Effects of metal mixtures on aquatic biota: a review of observations and methods. Human and Ecological Risk Assessment. 9, 795–811.

O'Malley, V. P., Abrajano, T. A., Hellou, J., 1994. Determination of the 13C/ 12C ratios of individual PAH from environmental samples: Can PAH sources be apportioned?. Organic Geochemistry. 21, 809–822.

Parenti, L.R., 1981. A phylogenetic and biogeographic analysis of cyprinodontiform fishes (Teleostei, Atherinomorpha). Bulletim of the American Museum of Natural History. 168 (4), 557p.

Pearcy, W.G., Myers, S.S., 1974. Larval fishes of Yaquina Bay, Oregon: a nursery ground for marine fishes?. Fisheries Bulletin of United States. 72, 201–213.

Petushok, N., Gabryelak, T., Palecz, D., Zavodnik, L., Szollosi Varga, I., Deér K.A., 2002. Comparative study of the xenobiotics metabolising system in the digestive

gland of the bivalve molluscs in different aquatic ecosystems and in aquaria experiments. Aquatic Toxicology. 61, 65–72.

Piña, B., Casado, M., Quirós, L., 2007. Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. Trends in Analytical Chemistry. 26 (11), 1145-1154.

Porte, C., Biosca, M., Sole, M., Albaiges, J., 2001. The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Galician coast (NW Spain). Environmental Pollution.112, 26–268.

Quirós, L., Piña, B., Solé, M., Blasco, J., López, M.A., Riva, M.C., Barceló, D., Raldúa, D., 2007. Environmental monitoring by gene expression biomarkers in *Barbus graellsii*: Laboratory and field studies. Chemosphere. 67, 1144-1154.

Ramachandran, S.D., Sweezey, M.J., Hodson, P.V., Boudreau, M., Courtenay, S.C., Lee, K., King, T., Dixon, J.A., 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Marine Pollution Bulletin. 52, 1182–1189.

Rattner, B.A., Melancon, M.J., Custer, T.W., Hothem, R.L., King, K.A., LeCaptain, L.J., Spann, J.W., Woodin, B.R., Stegeman, J.J., 1993. Biomonitoring environmental con- tamination with pipping black-crowned night heron embryos: induction of cytochrome P450. Environmental Toxicology and Chemistry. 12, 1719–1732.

Readman, J.W., Mantoura, R.F.C., Rhead, M.M., 1984. The physico-chemical speciation of polycyclic aromatic hydrocarbons (PAH) in aquatic systems. Fresenius' Journal of Analytical Chemistry. 319, 126–131.

Rolon, A.S., Maltchik, L., 2006. Environmental factors as predictors of aquatic macrophyte richness and composition in wetlands of southern Brazil. Hydrobiologia. 556, 221–231.

Siroka, Z., Drastichova, J., 2004. Biochemical markers of aquatic environment contamination - cytochrome P450 in fish. Acta Veterinaria Czech. 73, 123-132.

Siroka, Z., Krijt, J., Randak, T., Svobodova, Z., 2004. Cytochrome P450 induction in fish as a biochemical marker of Labe river contamination. Toxicology and Applied Pharmacology. 197, 189-189.

Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and Molecular Biology of Monooxigenases: Current perspectives on forms, functions and regulation of cytochrome P450 in aquatic species, in:Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology: Molecular, Biochemical and Cellular Perspectives.Lewis Publishers, Boca Raton, pp. 87-206.

Stegeman, J.J., 1993. Cytochrome P450 forms in fish. In: Schenkman, J.B., Greim, H. (Eds.), Handbook of Experimental Pharmacology. Springer, Berlin. 105, 279-291.

Tagliani, P.R.A., Landazuri, H., Reis, E.G., Tagliani, C.R., Asmus, M.L., Sánchez-Arcilla, A., 2003. Integrated coastal zone management in the Patos Lagoon estuary: perspectives in context of developing country. Ocean & Coastal Management. 46, 807–822. Tagliani, 2003

Teramitsu, H., Yamamoto, Y., Chiba, I., Iwata, H., Tenace, S., Fujise, Y., Kazusaka, A., Akahori, F., Fujita, S., 2000. Identification of novel cytochrome P450 1A genes from five marine mammal species. Aquatic Toxicology. 51, 145–153.

U.S. Energy Information Administration. World Wide Web electronic publication. Disponível em: http://www.eia.gov/cfapps/ipdbproject/IEDIndex3.cfm. Acessado em: 13 de novembro de 2014.

Van Tiem, L., Di Giulio, R., 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology. 254, 280–287.

Volkman, J.K., Holdsworth, D.G., Neill, G.P., Bavor Jr, H.J., 1992. Identification of natural, anthropogenic and petroleum hydrocarbons in aquatic sediments. Science of the Total Environment. 112, 203–219.

Wassenberg, D., Nerlinger, A., Battle, L., Di Giulio, R., 2005. Effects of the poly- cyclic aromatic hydrocarbon heterocycles, carbazole and dibenzothiophene, on in vivo and in vitro CYP1A activity and polycyclic aromatic hydrocarbon-derived embryonic deformities. Environmental Toxicology and Chemistry. 24, 2526–2532.

Weinstein, M.P., 1979. Shallow marsh habitats as primary nurseries for fishes and shellfishes, Cape Fear River, North Carolina. Marine Biology (Berlin). 58, 227–243.

Wills, L.P., Zhu, S., Willett, K.L., Di Giulio, R.T., 2009. Effect of *CYP1A* inhibition on the biotransformation of benzo[a]pyrene in two populations of *Fundulus heteroclitus* with different exposure histories. Aquatic Toxicology. 92, 195-201.

Xu, C.J., Li, C.Y.T., Kong, A.N.T., 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Archives of Pharmacal Research. 28 (3), 249–268.

CAPÍTULO ÚNICO - MANUSCRITO

New cytochrome P450 1A transcript in the guppy *Phalloceros caudimaculatus* and *CYP1A* response to beta-naphthoflavone and environmental samples

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New cytochrome P450 1A transcript in the guppy *Phalloceros caudimaculatus* and *CYP1A* response to beta-naphthoflavone and environmental samples

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ABSTRACT

The cytochrome P450 1A (CYP1A) in fishes is induced in the transcriptional level by agonists of the aryl hydrocarbon receptor (AHR), such as the environmental contaminants polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs) and dioxins. Here we cloned the *CYP1A* gene from the guppy *Phalloceros caudimaculatus*, a potential model for toxicological studies in South America. The *P. caudimaculatus CYP1A* encodes a protein with 521 amino acids that shared 96%, 91% and 70% identity with CYP1A of the fishes *Poecilia vivipara*, *Fundulus heteroclitus* and *Danio rerio*, respectively. The characterization of organ- and time-dependent induction of *CYP1A* by waterborne exposure to the model CYP1A inducer beta-naphthoflavone (BNF) was carried out by RT-qPCR. The minimum exposure time that elicited significant *CYP1A* transcriptional induction was 1 h for liver, gill, gut, brain, anal fin and fingerlings; 2 h for dorsal fin; and 4 h for kidney and tail fin. In most of the organs *CYP1A* tends to reach the greatest induction in the first few hours (1 to 8 h) of experiment and *CYP1A* mRNA levels remained elevated until the end of the experiment (96 h; with water renewing every 24 h). This information could be important planning toxicological assays using fish. In addition, fins that has regeneration capacity, could also be used for such analyzes which could favor for the use of non-lethal biopsy for the use of *CYP1A* biomarker in guppies. Validation of *CYP1A* gene biomarker in fish was done by exposing *P. caudimaculatus* to elutriate made from sediment of three streams located in adjacent areas of the Patos Lagoon Estuary in Rio Grande city, RS, Brazil. There was a *CYP1A* induction by elutriates made from sediment from urban and industrial areas (22- and 122-fold control); and absence of induction by elutriate from an area located 22 Km away from the populated area of the city (reference site). The results suggest that two of the streams were potentially contaminated by organic contaminants that are AHR agonists. The responsiveness of *CYP1A* to BNF and elutriate from urban areas indicates that the guppy *P. caudimaculatus* could be used as model for environmental toxicology and monitoring of AHR agonist compounds.

Keywords: Cyprinodontiformes, guppy, contamination, cytochrome P450, PAH.

1. INTRODUCTION

The cytochrome P450 of family 1 and sub-family A (CYP1A) is a member of the CYP super family and play critical roles in the biotransformation of drugs, environmental contaminants and endogenous compounds (Livingstone, 1998; Schober et al., 2006). *CYP1A* is highly inducible by environment contaminants via aryl hydrocarbon receptor (AHR)-mediated gene transcription (Kawajari and Fujii-Kuriyama, 2007) and is used as a biomarker for the environmental exposure to contaminants in fish (Behnisch et al., 2001).

The environmental contaminants that induces *CYP1A* includes planar polychlorinated biphenyl (PCBs), dibenzo-p-dioxin (PCDD), dibenzofuran (PCDF), halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) (Hahn, 2002; Nebert and Russell, 2002). These compounds are highly associated with particulate matter, e.g. sediment and soil, due to the hydrophobic nature of these compounds (Readman et al, 1984). The resuspension of compounds associated with the sediment can be done in laboratory by elutriation, which simulates a real situation of dredging, storm or intake of large volumes of rainwater that can make a fraction of the compounds bioavailable (EPA, 2001).

The guppy *Phalloceros caudimaculatus* (Cyprinodontiformes; Poecillidae; Hensel, 1868) is widespread in contaminated and pristine freshwater and estuarine environments of South American countries, e.g. Brazil, Argentina, Paraguay and Uruguay, and has been introduced in countries from other continents too, e.g. Malawi and New Zealand (Froese and Pauly, 2003; Lucinda, 2008). Similar to other cyprinodontiforme fishes, including guppies and killifishes (e.g. *Poecilia reticulata* and *Fundulus heteroclitus*, respectively) *P. caudimaculatus* present characteristics

that favor the use as a model in environmental toxicology studies, to understand mechanisms of resistance to live in contaminated environments and to be employed as a biomonitor in environment monitoring (Aluru et al., 2011).

Enzymatic assay and/or gene expression in fishes have been used as biomarkers for biomonitoring different contaminants, such as PAHs, in the aquatic environment (Payne et al., 1996; Jönsson et al., 2010). The analysis of *CYP1A* mRNA levels by real time PCR has showed a positive correlation to traditional methods that evaluates the levels of CYP1A protein and activity (e.g., Western blot and EROD, respectively) (Piña et al., 2007). *CYP1A* transcript expression was demonstrated to reach a peak before the EROD activity, which could be due to lag between mRNA and protein synthesis (Gao et al., 2011).

The present study aimed to: (1) identify the full-length sequence of *P. caudimaculatus CYP1A* transcript; (2) analyze the expression in liver, gut, brain, gill, kidney, dorsal fin, tail fin, anal fin and fingerlings in a time course exposure to 1 µM of beta-naphthoflavone (BNF), a synthetic derivated of a natural flavonoid compound, which strongly induces *CYP1A1/2* in mammals and is a strong non-genotoxic inductor (Digiovanni, 1990); and (3) analyze the induction of *CYP1A* mRNA levels by exposure to elutriate made from sediments of reference and contaminated sites. The results aims to elucidate if the guppy *Phalloceros caudimaculatus* can be used for biomonitoring of AHR agonists in the aquatics environments. The investigation of responses in different exposure times can be useful for planning future toxicological tests with this specie and different organs

2. MATERIAL AND METHODS

2.1. Identification of *CYP1A* Transcript in *P. caudimaculatus*

Liver was excised from one wild male *Phalloceros caudimaculatus* (2 cm; 0.2 g) collected using fishing net in a watercourse at Cassino Beach (Rio Grande, RS, Brazil). The liver was placed in TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) and then stored at – 20 ºC. Total RNA was isolated using TRIzol Reagent and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random hexamer primer (provided in the reverse transcriptase kit) and RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen,Carlsbad, CA, USA). Degenerate primers were designed using highly conserved regions of previously known *CYP1A* sequences from *Fundulus heteroclitus* (Cyprinodontiformes), Japanese medaka *Oryzias latipes* (Beloniformes) and stickleback *Gasterosteus aculeatus* (Gasterosteiformes) (GenBank accession numbers AF026800, NM_001105087, and HQ202281, respectively) and avoiding conserved regions present in other CYP1 subfamilies, i.e. CYP1B, CYP1C and CYP1D. The forward and reverse degenerate primers used are presented in Table 1 and the PCR reactions were performed using the following PCR conditions: 95 °C for 3 minutes, 35 cycles of 95 °C for 30 seconds, 45 °C for 30 seconds and 72 °C for 1 minute and a final extension of 72 °C for 10 minutes. Primers were obtained from IDT Integrated DNA Technologies. PCR products were resolved on a 1% agarose gel and then isolated and purified using Illustra GFX PCR DNA and Gel Band PurificationKit (GE Healthcare, Buckinghamshire, UK). Partial sequences were obtained using an ABI Prism 3130xl Platform (Applied Biosystems). The sequence then was used to design specific primers with Primer3 (Rozen and Skaletsky, 2000) for Real-time qualitative PCR (RTq-PCR) and for the SMARTer^{TM} RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) (Table 1). The full-length sequence of *P. caudimaculatus CYP1A* was cloned using the SMARTer^{TM} RACE

cDNA Amplification Kit and sequenced using an ABI Prism 3130xl Platform. The full nucleotide sequence was translated to the predicted amino acid sequences and aligned with other CYP1 family members using Sequence Identity Matrix tool, Bioedit Sequence Alignment Editor Software (Hall, 1999). Calculation of identities among *P. caudimaculatus*, *Danio rerio* and *F. heteroclitus* nucleotide and amino acid predicted sequences was performed using the Multiple Sequence Alignment tool, Clustal Omega (Sievers et al., 2011). Substrate recognition sites were located based on those indicated by Gao et al. (2011) and Dorrington et al. (2012).

2.2. Fish Collection and Maintenance

Pregnant females and adult *P. caudimaculatus* were caught at the same place and collection process described in the item 2.1 in September 2012 and May 2013, respectively. The pregnant females (n = 10) were transferred to the laboratory and acclimated for two weeks at 20 ºC in 100 L aquaria with dechlorinated freshwater, constant aeration and fed twice a day with Alcon BASIC MEP 200 Complex *ad libitum* (Alcon, Camboriú, SC, Brazil), until the birth of the fingerlings. The born fingerlings were immediately separated from the females to a 20 L aquarium to prevent predation and were maintained under the same conditions as described above until the exposure experiment described in the item 2.3. Adult males of *P. caudimaculatus* (n = 140, length: ~2 cm) were transferred to the laboratory and acclimated for two weeks at the same conditions of water quality and feeding as described above and used for the beta-naphthoflavone (BNF) exposure experiment as described in the item 2.3.

Adult males of *P. caudimaculatus* (n = 56, length: ~2 cm) were caught at the same place and collection process described in the item 2.1 in August 2013,

transferred to the laboratory, acclimated for two weeks at the same conditions of water quality and feeding as described above and used for the elutriate exposure experiment as described in the item 2.5.

The collection of the wild fish to use in scientific activity have the authorization from SISBIO (process number: 24486-3) and the procedures used in these experiments were approved by the Animal Care and Use Committee (CEUA) at the Universidade Federal do Rio Grande (FURG).

2.3. BNF Exposure Experiment and Quantification of *CYP1A* mRNA levels

Adult *P. caudimaculatus* (n = 70, length: ~2 cm) were waterborne exposed to 1 uM of beta-naphthoflavone (BNF) (Sigma–Aldrich Co., St. Louis MO, USA) dissolved in DMSO (to reach 20 ppm in the final volume) in a glass aquarium. Adult *P. caudimaculatus* were also kept in a second glass aquarium with an equivalent concentration of DMSO in the water (control group; n=70; length: ~2 cm). The selection of the waterborne BNF concentration was based on Jönsson et al. (2007) and Kim et al. (2008) that showed strong induction of *CYP1A* mRNA levels. Fish were kept without food in the aquarium with aerated water and temperature 20 ºC for 24 h before the exposure experiment and no food was supplied during the exposure experiment. The proportion of five fish per liter of water in both aquariums (BNF and control) was maintained along the period of the experiment and the water was renewed every 24 h to maintain the BNF and DMSO concentration during the 96-h experiment. The same exposure procedures described using adult *P. caudimaculatus* was conducted for the fingerlings BNF exposure. Fingerlings with five to eight days of age were used and the exposure was performed with 10 fingerlings per 200 mL of water.

158 Adult fish and fingerlings, from BNF exposed or control group, were
159 randomly sampled at 1, 2, 4, 8, 24, 48 and 96 h of exposure, immersed in ice water
160 and euthanized by cervical transaction. Liver, gut, brain, gill, kidney, dorsal fin, tail
161 fin and anal fin were dissected from the adult fish and immediately placed in
162 RNAlater (Ambion, Austin, TX, USA). Each fingerling was collected as a whole
163 sample. The samples were held for 24 h at 4ºC, and then stored at −20 ºC according
164 to the RNAlater manufacturer's instructions.
165 Total RNA was extracted from adult fish organs and fingerlings. The cDNA
166 was synthesized (n = 6 per experimental group) as described in the item 2.1. *P.*
167 *caudimaculatus* specific primers (Table 1) were used to quantify *CYP1A* and *β-actin*
168 mRNA levels by real-time PCR (RTq-PCR). The RTq-PCR runs were performed in
169 duplicate using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) according
170 to the manufacturer's instructions and a 7300 Real-Time PCR System (Applied
171 Biosystems) using the program: 50 ºC for 2 minutes, 95 ºC for 2 minutes, and 40
172 cycles of 95 ºC for 15 seconds and 60 ºC for 30 seconds. Melting-curve analysis was
173 performed on all the PCR products at the end of each RTq-PCR run to verify the
174 amplification of a single product. The $2^{-\Delta Ct}$ method described by Schmittgen and
175 Livak (2008) was employed to calculate *CYP1A* relative mRNA levels of individual
176 samples, using *β-actin* as a housekeeping gene.
177
178 **2.4. Sediment Sampling**
179 Sediment samples used to produce the elutriate to the fish exposure
180 experiment (see item 2.5) were collected at no more than 1 m depth in three sites.
181 The site S1 (32º01'49.35"S, 52º06'31.15"W) was a watercourse in the middle of the
182 urban area that receive domestic and urban sewage discharges; the site S2

(32º02'56.48"S, 52º05'06.98"W) was nearby the petro distributor of the city in a watercourse close to the loading terminal of tank trucks; and the reference site (REF) (32º33'32.48"S, 52º23'54.59"W) was a watercourse 22 km south-west from the closer urban area at Cassino beach. The locations were selected to represent sites with different kinds and degrees of contamination. The sites (S1 and S2) were close to places that have historically received effluent of domestic and industrial activities, S2 site was close to a contaminated site with PAHs, being reported as moderately contaminated with PAH and S1 to a less PAH contaminated site but was more contaminated than the reference site for PAH levels (Medeiros et al., 2005; Garcia et al., 2010). One sediment sample of 500 ml was collected for each place. The sampling of surface sediment (0-10 cm) was performed using a stainless steel Ekman grab and the material was stored for less than 20 days at −20 ºC until the preparation of the elutriate solution.

2.5. Exposure to Elutriate and Quantification of *CYP1A* mRNA levels

Elutriate was obtained by mixing 300 ml of sediment and 1200 ml of dechlorinated water (ratio 1:4 v/v) inside a glass container. Detritus (i.g. small rocks and plant roots) were manually removed from sediment using forceps. The mix was maintained under vigorous agitation for 1 h, followed by a settling down process for at least 8 h (EPA, 2001). Afterwards, 1 L of the supernatant fraction was placed in glass aquariums for the fish exposure.

Adult *P. caudimaculatus* (n = 56, ~2 cm) were kept without food with aerated freshwater and temperature 20 ºC for 24 h before the exposure experiment and no food was supplied during the exposure. The exposure was held with 1 L of concentrated elutriates (100%). Elutriates made with sediments of REF site, S1 site

208 and S2 site were placed in aquarium with constant aeration. Groups of fourteen adult
209 fish were added to each of the experimental aquaria. A control group (n = 14) was set
210 in a separate aquarium containing the same freshwater that was used to prepare
211 elutriates. Fish from the four experimental aquaria were sampled after 8 hours of
212 exposure, anesthetized by immersion in a tricaine solution (150 $\mu g.ml^{-1}$) for ~2.5 min
213 (Sigma, St Louis, MO, USA) and euthanized by cervical transection. The use of
214 anesthetic procedure in this experiment was implemented, as this procedure
215 apparently did not alter significantly the *CYP1A* mRNA levels in guppy (Pinto et al.,
216 2015). The liver was dissected and immediately placed in RNAlater and stored. The
217 reverse transcription and quantification of the *CYP1A* mRNA levels was done as
218 described in the item 2.1.
219
220 **2.6. Statistical Analyses**
221 Statistical analyses for both experiments were performed using Past software
222 (version 2.17) (Hammer et al, 2001). No difference in the *CYP1A* mRNA levels in
223 different organs and fingerlings were found between the control groups for different
224 times of exposure and the data from control groups were pooled for the subsequent
225 analyses. All data was log-transformed and ANOVA assumptions were verified. For
226 the BNF exposure experiment a one-way ANOVA for repeated measures was
227 performed to test the differences among treatments. For the elutriate exposure
228 experiment a one-way ANOVA was performed to test for differences among the four
229 experimental groups. Tukey's *post hoc* test was performed for both analyses to
230 identify differences between groups. The significance level for all analyses was $p \leq$
231 0.05.
232

3. RESULTS

3.1. Identification of *CYP1A* mRNA Sequence in *P. caudimaculatus*

The first fragment achieved using the degenerate primers was 534 base pair long with 89% of identity with predicted *Fundulus heteroclitus* CYP1A amino acid sequence. Using SMARTer^{TM} RACE cDNA Amplification Kit, one sequence with 391 base pairs and another with 1516 base pairs were obtained, corresponding to the 3' and 5' cDNA ends of the *P. caudimaculatus CYP1A*, respectively. The contig of those three sequences revealed a 1750 bp nucleotide sequence containing the *P. caudimaculatus CYP1A* complete open reading frame with 1566 base pairs coding for a 521 amino acid protein. The obtained sequence also contained 130 bp in the 5' untranslated region (UTR) and 54 bp in the 3' UTR. The 3' UTR was apparently incomplete since no poly-A tail was found. The *CYP1A* nucleotide sequence of *P. caudimaculatus* and the predicted translated amino acid sequences were aligned with other CYP1A sequences from *Poecilia vivipara*, *F. heteroclitus* and *Danio rerio* (Fig. 1). The percent identities between those sequences and other CYP1 sequences of *F. heteroclitus* are shown in the Table 2. The *P. caudimaculatus* CYP1A amino acid sequence was 96%, 91% and 70% identical to the *P. vivipara*, *F. heteroclitus* and *D. rerio* CYP1A sequences, respectively. CYP1B1, CYP1C1 and CYP1D1 amino acid sequences showed less than 55% of identity with the *P. caudimaculatus* new sequence. The alignment of Fig. 1 showed that amino acid sequences were identical in *P. caudimaculatus* and *P. vivivapara* for the substrate recognition sites SRS 1, SRS 5, SRS 6 and the heme binding domain. Between *P. caudimaculatus* and *F. heteroclitus* sequences, SRS 3, SRS 4, SRS 5, SRS 6 and the heme binding domain were identical, and the SRS 6 was identical for all fishes in the alignment.

3.2. *CYP1A* mRNA levels in *P. caudimaculatus* Exposed to BNF

No mortality was observed for adult fish and fingerlings in the control or BNF exposed groups. The *CYP1A* mRNA level was induced in all organs of the adult fish and in fingerlings (Fig. 2) of *P. caudimaculatus* exposed to 1 μM of BNF in comparison to the respective controls. Liver, gill, gut, brain, anal fin and the fingerlings showed increased *CYP1A* mRNA level after one hour of BNF exposure; dorsal fin showed *CYP1A* induction after two hours, and kidney and tail fin showed *CYP1A* induction after four hours. In the first few hours (1 to 8 h), gill, gut, kidney of adult fish, and the fingerlings reached the highest mean value of *CYP1A* mRNA levels, while the remaining organs reached the highest mean value of *CYP1A* mRNA levels after 24 h.

The mean of the induction of *CYP1A*, compared to control group, ranged between 4- and 27-fold in the liver, 7- and 39- fold in gill, 21- and 97- fold in gut, 8- and 122- fold in brain, 19- and 176- fold in kidney, 14- and 45- fold in dorsal fin, 4- and 112- fold in tail fin, 8- and 64-fold in anal fin and 4- and 46- fold in fingerlings (data not shown). The highest mean inductions of *CYP1A* mRNA levels were observed in kidney at 8 h of BNF exposure (176 fold) and brain at 48 h of BNF exposure (122 fold). At 96 h after the beginning of BNF exposure the *CYP1A* level in all organs of the adult fish and in the fingerlings remained induced.

3.3. *CYP1A* mRNA levels in *P. caudimaculatus* Exposed to Elutriate

Mortality of three individuals was observed in response to the exposure to elutriate from the sediment of the site S1. The results of *CYP1A* mRNA levels for fish livers are presented in figure 3. Elutriate made of sediment from the REF site showed no difference comparing to the control water. The elutriate from S1 site was

283 different from the control water, but not from the REF site, inducing the *CYP1A*
284 mRNA levels 22 times in comparison to the control water. The elutriate from the S2
285 site was significantly different from all others sites and control water, with an
286 induction of 122 times in comparison to the control water.
287

288 **4. DISCUSSION**

289 The guppy *P. caudimaculatus* possess characteristics that are desired for use
290 as a model in environmental and mechanistic studies in toxicology, since it is
291 widespread in geographic areas of South America and potentially possess unique
292 adaptation to survive in habitats with adverse conditions, e.g. high levels of
293 contaminants, high turbidity and low oxygen (Froese and Pauly, 2003; Pinto and
294 Araújo, 2007; Araújo et al., 2009). In the present study, the nucleotide sequence of
295 CYP1A, a well established biomarker in fish, was identified in *P. caudimaculatus*,
296 permitting the characterization of *CYP1A* mRNA induction by BNF in different
297 exposure times and organs. We were able to show for the first time that *P.*
298 *caudimaculatus* has a fast transcriptional response (few hours) to AHR agonists in
299 many organs, including fins, and that *CYP1A* mRNA is strongly induced by exposure
300 to elutriate made from environmental samples. The results presented here could favor
301 for the use of this organism in ecotoxicology studies in the South American
302 subtropical environment.

303 The newly identified CYP1A predicted protein showed identity higher than
304 70 % with the CYP1A sequences of cyprinodontifornes *Poecilia vivipara* and
305 *Fundulus heteroclitus*; and the cypriniform *Danio rerio*. As the identity is greater
306 than 55% the new sequence should be classified in the family 1 and subfamily A
307 (CYP1A) according to the current classification criterion for CYP nomenclature

308 (Nelson et al., 1996). The higher similarity found to CYP1D1 than the other CYP1s
309 (CYP1B1, 1C1, 1C2) from *F. heteroclitus* was expected as the CYP1A and CYP1D1
310 have been show to group together in one clade in fishes, while CYP1B1 and CYP1Cs
311 are in another, according to previous molecular phylogenetic analysis (Zanette et al.,
312 2009). The high identity and high similarity among the substrate recognition sites
313 (SRSs) and the full-sequence between the CYP1A amino acid sequences of *P.*
314 *caudimaculatus*, *P. vivipara* and *F. heteroclitus* suggest these enzymes possibly
315 catalyzes similar compounds (Gotoh, 1992) as substantial similarity in the size and
316 shape of the crystal structure of the protein between isoforms and even different
317 subfamilies corresponds to the overlapping substrate profiles (Dong et al., 2012).
318 The *CYP1A* mRNA levels in *P. caudimaculatus* showed an increasing
319 response to BNF waterborne exposure. BNF treatment is known to enhance the
320 expression of *CYP1A* as the carcinogenics PAHs such as 3-methylcholanthrene and
321 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Nebert and Gonzalez, 1987).
322 Fingerlings and eight organs from adult fish were select for the expression of
323 *CYP1A*, five organs were removed with an invasive process: liver, gill, gut, brain and
324 kidney; and three organs removed with a non-invasive process: dorsal, tail and anal
325 fins; and the fingerlings. All adult fish organs and fingerlings showed *CYP1A*
326 induction to BNF exposure.
327 Previous studies demonstrated that *CYP1A* levels are induced in fish exposed
328 to PCBs and BNF, which are AhR agonists, i.g. *F. heteroclitus* (Elskus, 1999) and
329 *Jenynsia multidentata*, and in the same family (Poecillidae), i.g. *Poecilia vivipara*
330 (Ferreira et al., 2012). The induction of *CYP1A* mRNA levels by BNF was expected
331 in all organs, especially in the main detoxification organs, liver and intestine, which
332 both showed a fast induction (1 h). *CYP1A* expression was observed in the liver and

gill of several fishes, i.g. *F. heteroclitus*, *Danio rerio*, *P. vivipara*, *J. multidentata* exposed to AHR agonists, gill showed a higher increase than liver in *F. heteroclitus*, *D. rerio* and *P. vivapara*, and a higher increase in liver than gill was seen in *J. multidentata* (Jönsson et al., 2007; Zanette et al., 2009; Ferreira et al., 2012). The same pattern, higher induction of *CYP1A* mRNA level in gill over liver, can be seen for *P. caudimaculatus*. Since the liver is known to have a high basal level of *CYP1A* in some species of fish, e.g. *Danio rerio* and *Fundulus heteroclitus* (Jönsson et al., 2007; Zanette et al., 2009), it was possible that the expression could not increase much over the basal level (Gao et al., 2011).

Several studies were done to evaluate induction level of *CYP1A* over time course exposures and single time exposure to low concentrations of AHR agonists, e.g., PCB126, 3-methylcholanthrene, benzo[a]pyrene and BNF. Short time exposures ranged from 2 h to 24 h (Chung-Davidson et al., 2004; Kim et al., 2008; Gao et al., 2011; Ferreira et al., 2012), going to long time exposures that could take more than one week (Schlezinger et al., 2006; Gao et al., 2011; Holth et al., 2014) but time-course studies of *CYP1A* induction with lake trout and pufferfish show raise in the mRNA levels of *CYP1A* after 4 and 6 hours of exposure, respectively (Chung-Davidson et al., 2004; Kim et al., 2008).A recent study with *Jenynsia multidentata* (Pinto et al., 2015) analyzed gill, liver and anal fin demonstrating an increase in the mRNA levels of *CYP1A* after only one hour of exposure to BNF. *P. caudimaculatus* mRNA levels of *CYP1A* are different from the control group in the first hour of exposure. The raise of the mRNA levels of *CYP1A* in *P. caudimaculatus* and *J. multidentata* (Pinto et al., 2015) after only one hour of exposure to 1 µM of BNF in several organs and in the fingerlings suggests that the *CYP1A* induction, which is

357 mediated by the AHR, in this Cyprinodontiformes occurs rapidly and can provide an
358 early warning about AHR agonists in the environment.

359 *P. caudimaculatus CYP1A* mRNA levels reached a great induction in the first
360 hours (1 to 8 h) of experiment, this corroborates with Pinto et al. (2015), which
361 showed great inductions in the *CYP1A* mRNA levels of *Jenynsia multidentata*
362 between 1 to 8 h too. *P. caudimaculatus CYP1A* mRNA levels remained elevated
363 until the end of the experiment (96 h; with water renewing every 24 h) but Kim et al.
364 (2008) showed a decrease, even reaching a level similar to the control, in the *CYP1A*
365 mRNA levels, this could be due to the decay of the BNF in the water, as the BNF
366 was dissolved in the water once at the beginning of the experiment. This information
367 could be important to plan toxicological assays using fish.

368 Liver and gills have been used to analyze enzymatic and mRNA levels of
369 *CYP1A* (Jönsson et al., 2007; Curtis et al., 2011; Dorrington et al., 2012) but for this
370 methodology the death of the fish is required and actually there is a growing ethical
371 concern about the use of animals for research. The use of fins in this study
372 demonstrates that these organs that can be removed with a non-lethal procedure can
373 be used for analysis of mRNA levels of *CYP1A*. Besides this, the fins have a
374 regeneration capacity (Offen et al., 2008), which reinforces the utility for studies of
375 biomonitoring. Dorsal and tail fin showed an increase in the *CYP1A* mRNA levels
376 after two and four hours of exposure, respectively. Both fins increased and sustained
377 high mRNA levels of *CYP1A*, with the tail fin reaching 112 fold induction in 24
378 hours of exposure. While for the anal fin *CYP1A* mRNA levels was observed an
379 induction in the first hour of exposure like the one seen in liver, gill, gut, brain and
380 fingerlings. Different magnitudes and patterns of *CYP1A* mRNA levels during the
381 exposure among the fins could be associated with the histological composition of

each fin, as the dorsal and tail fins have more dermal skeleton, which is similar to scales, than the anal fin (Kuntz, 1914; Ogino et al., 2004; Zauner et al., 2003). Biopsied fins of *Jenynsia multidentata* exposed to BNF and showed induction of *CYP1A* mRNA levels (Pinto et al., 2015), this procedure does not expose the fish to the contaminants and could be used with *P. caudimaculatus.*

CYP1A have been showing induction after exposure to many different chemical in laboratory with the help of carriers (Gao et al., 2011; Ferreira et al., 2012; Dorrington et al., 2012) but this is not an approach that simulate a real condition. PAHs are hydrophobic compounds with low water solubility, which associate primarily with the particulate phase (Readman et al, 1984). The exposure to sediments and elutriates can be used to simulate real situations, e.g. dredging and intense storms, helping to understand the dynamic of the several contaminants that are remobilized to the water column (Bergen et al., 2005).

As expected, there was no statistical difference between the REF site and the control water, demonstrating that the elutriate method and exposure do not induced *CYP1A* mRNA levels in *P. caudimaculatus*. The higher expression of *CYP1A* mRNA levels, which are involved in biotransformation pathways, in *P. caudimaculatus* exposed to elutriate made from possibly contaminated S2 site sediment may be due to a higher concentration of contaminants present in the sediment that were resuspend by the elutriate method. Ribecco et al. (2012) performed a exposure of juvenile of the fish *Solea solea* to a sediment contaminated with pesticides, PCBs, HPAs and heavy metals, which was considered moderately contaminated, after 96 hours all the biomarkers analized, in which was *CYP1A* and *CYP3A*, were up regulated, being considered an early response to contamination..

406 In conclusion, this study showed that *Phalloceros caudimaculatus* respond to
407 AHR agonist inducing *CYP1A* mRNA levels in all organs analyzed and fingerlings.
408 This information can be used for toxicological porpoises, such as the use of *P.*
409 *caudimaculatus* for monitoring AhR agonist in environmental samples and the use of
410 fins as sample material for *CYP1A* mRNA analysis, as the excision of these organs
411 do not implies the death of the fish.
412
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419
420 **REFERENCES**
421 Aluru, N., Karchner, S.I., Hahn, M.E., 2011. Role of DNA methylation of AHR1 and
422 AHR2 promoters in differential sensitivity to PCBs in Atlantic Killifish,
423 *Fundulus heteroclitus*. Aquatic Toxicology. 101, 288–294.
424 Araújo, F.G., Peixoto, M.G., Pinto, B.C.T., Teixeira, T.P., 2009. Distribution of
425 guppies *Poecilia reticulata* (Peters, 1860) and *Phalloceros caudimaculatus*
426 (Hensel, 1868) along a polluted stretch of the Paraíba do Sul River, Brazil.
427 Brazilian Journal of Biology. 69 (1), 41-48.
428 Behnisch, P.A., Hosoe, K., Sakai, S., 2001. Bioanalytical screening methods for
429 dioxins and dioxin-like compounds a review of bioassay/biomarker
430 technology. Environment International. 27, 413–439.
431 Bergen, B.J., Nelson, W.G., Mackay, J., Dickerson, D., Jayaraman, S., 2005.
432 Environmental monitoring of remedial dredging at the New Bedford Harbor, MA,
433 superfund site. Environmental Monitoring and Assessment. 111, 257–275.

434 Curtis, L.R., Garzon, C.B., Arkoosh, M., Collier, T., Myers, M.S., Buzitis, J., Hahn,
435 M.E., 2011. Reduced cytochrome P4501A activity and recovery from
436 oxidative stress during subchronic benzo[a]pyrene and benzo[e]pyrene
437 treatment of rainbow trout. Toxicology and Applied Pharmacology. 254, 1–7.
438 Chung-Davidson, Y., Rees, C.B., Wu, H., Yun, S., Li, W., 2004. B-naphthoflavone
439 induction of CYP1A in brain of juvenile lake trout (*Salvelinus namaycush*
440 Walbaum). The Journal of Experimental Biology. 207, 1533-1542.
441 Digiovanni, J., 1990. Inhibition of chemical carcinogenesis. In: Chemical
442 Carcinogenesis and Mutagenesis, Copper CS, Grover PL (eds). Springer-
443 Verlag, Berlin, 159-253.
444 Dong, D., Wu, B., Chow, D., Hu, M., 2012. Substrate selectivity of drug-
445 metabolizing cytochrome P450s predicted from crystal structures and in silico
446 modeling. Drug Metabolism Reviews. 44 (2), 192–208.
447 Dorrington, T., Zanette, J., Zacchi, F.L., Stegeman, J.J., Bainy, A.C.D., 2012. Basal
448 and 3-methylcholanthrene-induced expression of cytochrome P450 1A, 1B
449 and 1C genes in the Brazilian guppy, *Poecilia vivipara*. Aquatic Toxicology.
450 124–125, 106–113.
451 Elskus, A.A., Monosson, E., McElroy, A.E., Stegeman, J.J., Woltering, D.S., 1999.
452 Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign
453 of pollutant resistance?. Aquatic Toxicology. 45, 99–113.
454 EPA, U.S., 2001. Methods for Collection, Storage and Manipulation of Sediments
455 for Chemical and Toxicological Analyses: Technical Manual. EPA 823-B-01-
456 002. U.S. Environmental Protection Agency, Office of Water, Washington,
457 DC.
458 Ferreira, R.S., Monserrat, J.M., Ferreira, J.L.R., Kalb, A.C., Stegeman, J., Bainy,
459 A.C.D., Zanette, J., 2012. Biomarkers of organic contamination in the South
460 American fish *Poecilia vivipara* and *Jenynsia multidentata*. Journal of
461 Toxicology and Environmental Health, Part A. 75, 1023–1034.
462 Froese, R., Pauly, D., 2003. Editors. FishBase. World Wide Web electronic 13
463 publication. Disponível em: http://www.fishbase.org. Acessado em: 31 de
464 agosto de 2010.
465 Gao, K., Brandt, I., Goldstone, J.V., Jönsson, M.E., 2011. Cytochrome P450 1A, 1B,
466 and 1C mRNA induction patterns in three-spined stickleback exposed to a

467 transient and a persistent inducer. Comparative Biochemistry and Physiology,
468 Part C. 154, 42–55.
469 Garcia, M.R., Mirlean, N., Baisch, P.R., Caramão, E.B., 2010. Assessment of
470 polycyclic aromatic hydrocarbon influx and sediment contamination in an
471 urbanized estuary. Environ Monit Assess. 168, 269-276.
472 Gotoh, O.,1992. Substrate Recognition Sites in Cytochrome P450 Family 2 (CYPB)
473 Proteins Inferred from Comparative Analyses of Amino Acid and Coding
474 Nucleotide Sequences. The Journal of Biological Chemistry. 267 (1), 83-90.
475 Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. Chemico-
476 Biological Interactions. 141, 131–160.
477 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and
478 analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series,
479 41, 95-98.
480 Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: Paleontological statistics
481 software package for education and data analysis. Palaeontologia Electronica
482 4(1): 9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm
483 Holth, T.F., Eidsvoll, D.P., Farmen, E., Sanders, M.B., Martínez-Gómez, C.,
484 Budzinski, H., Burgeot, T., Guilhermino, L., Hylland, K., 2014. Effects of
485 water accommodated fractions of crude oils and diesel on a suite of
486 biomarkers in Atlantic cod (*Gadus morhua*). Aquatic Toxicology. 154, 240–
487 252.
488 Jönsson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007. Basal
489 and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A,
490 1B and 1C genes in zebrafish. Toxicology and Applied Pharmacology. 221, 29-
491 41.
492 Jönsson, M.E., Gao, K., Olsson, J.A., Goldstone, J.V., Brandt, I., 2010. Induction
493 patterns of new CYP1 genes in environmentally exposed rainbow trout. Aquatic
494 Toxicology. 98, 311–321.
495 Kawajiri, K., Fujii-Kuriyama, Y., 2007. Cytochrome P450 gene regulation and
496 physiological functions mediated by the aryl hydrocarbon receptor. Archives
497 of Biochemistry and Biophysics. 464, 207–212.
498 Kim, J.H., Raisuddin, S., Ki, J.S., Lee, J.S., Han, K.N., 2008. Molecular cloning and
499 b-naphthoflavone-induced expression of a cytochrome P450 1A (CYP1A)

gene from an anadromous river pufferfish, *Takifugu obscurus*. Marine Pollution Bulletin. 57, 433–440.

Kuntz, A., 1914. Notes on the habits, morphology of the reproductive organs, and embryology of the viviparous fish *Gambusia affinis*. Bulletin of the U.S. Fish Commission. 33, 177–190.

Livingstone, D.R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comparative Biochemistry and Physiology Part A. 120, 43–49.

Lucinda, P.H.F., 2008. Systematics and biogeography of the genus *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes: Poeciliidae: Poeciliinae), with the description of twenty-one new species. Neotropical Ichthyology. 6 (2), 113-158.

Medeiros, P.M., Bícego, M.C., Castelão, R.M., Rosso, C.D., Fillmann, G., Zamboni, A.J., 2005. Natural and anthropogenic hydrocarbon inputs to sediments of Patos Lagoon Estuary, Brazil. Environment International. 31, 77-87.

Nebert, D.W., Gonzalez, F.J., 1987. P450 genes: structure, evolution and regulation. Annual Review of Biochemistry. 56, 945–993.

Nebert, D.W., Russell, D.W., 2002. Clinical importance of the cytochromes P450. The Lancet. 360, 1155-1162.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R., Gunsalus, I.C., Nebert, D.W., 1996. P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics, 6: 1, 1-42.

Offen, N., Blum, N., Meyer, A., Begemann, G., 2008. Fgfr1 signalling in the development of a sexually selected trait in vertebrates, the sword of swordtail fish. BMC Developmental Biology. 8, 98.

Ogino, Y., Katoh, H., Yamada, G., 2004. Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. FEBS Letters. 575, 119-126.

Payne, J.F., Mathieu, A., Melvin, W., Fancey, L.L., 1996. Acetylcholinesterase, an Old Biomarker with a New Future? Field Trials in Association with Two Urban Rivers and a Paper Mill in Newfoundland. Marine Pollution Bulletin. 32 (2), 225-231.

534 Piña, B., Casado, M., Quirós, L., 2007. Analysis of gene expression as a new tool in
535 ecotoxicology and environmental monitoring. Trends in Analytical
536 Chemistry. 26 (11), 1145-1154.
537 Pinto, B.C.T., Araújo, F.G., 2007. Assessing of biotic integrity of the fish community
538 in a heavily impacted segment of a tropical river in Brazil. Brazilian Archives
539 of Biology and Tecnology. 50 (3), 489-502.
540 Pinto, D.P., Chivittz, C.C., Ferreira, R.S., Sopezki, M.S., Zanette, J., 2015. Beta-
541 Naphthoflavone-Induced CYP1A Expression in the Guppy *Jenynsia*
542 *multidentata*: Time- Dependent Response, Anesthetic MS-222 Effect and Fin
543 Analysis. Ecotoxicology and Environmental Safety. 113, 38-44.
544 Readman, J.W., Mantoura, R.F.C., Rhead, M.M., 1984. The physico-chemical speciation
545 of polycyclic aromatic hydrocarbons (PAH) in aquatic systems. Fresenius'
546 Journal of Analytical Chemistry. 319, 126–131.
547 Ribecco, C., Hardiman, G., Šášik, R., Vittori, S., Carnevali, O., 2012. Teleost fish (*Solea*
548 *solea*): A novel model for ecotoxicological assay of contaminated sediments.
549 Aquatic Toxicology. 109, 133–142.
550 Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for
551 biologist programmers. In: S, KS, M. Bioinformatics Methods and Protocols:
552 Methods in Molecular Biology. Humana Press, Totowa, NJ. 365-386.
553 Schlezinger, J.J., Struntz, W.D.J., Goldstone, J.V., Stegeman, J.J., 2006. Uncoupling
554 of cytochrome P450 1A and stimulation of reactive oxygen species
555 production by co-planar polychlorinated biphenyl congeners. Aquatic
556 Toxicology. 77, 422-432.
557 Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the
558 comparative CT method. Nature Protocols. 3 (6), 1101-1108.
559 Schober, W., Luch, A., Soballa, V.J., Raab, G., Stegeman, J.J., Doehmer, J., Jacob,
560 J., Seidel, A., 2006. On the species-specific biotransformation of
561 dibenzo[a,l]pyrene. Chemico-Biological Interactions. 161, 37-48.
562 Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R.,
563 McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G.,
564 2011. Fast, scalable generation of high-quality protein multiple sequence
565 alignments using Clustal Omega. Molecular Systems Biology. 7, 539.
566 Zanette, J., Jenny, M.J., Goldstone, J.V., Woodin, B.R., Watka, L.A., Bainy, A.C.D.,
567 Stegeman, J.J., 2009. New cytochrome P450 1B1, 1C2 and 1D1 genes in the

killifish *Fundulus heteroclitus*: Basal expression and response of five killifish CYP1s to the AHR agonist PCB126. Aquatic Toxicology. 93, 234-243.

Zauner, H., Begemann, G., Mari-Beffa, M., Meyer, A., 2003. Differential regulation of msx genes in the development of the gonopodium, an intromittent organ, and of the "sword," a sexually selected trait of swordtail fishes (*Xiphophorus*). Evolution & Development. 5, 466-477.

FIGURES

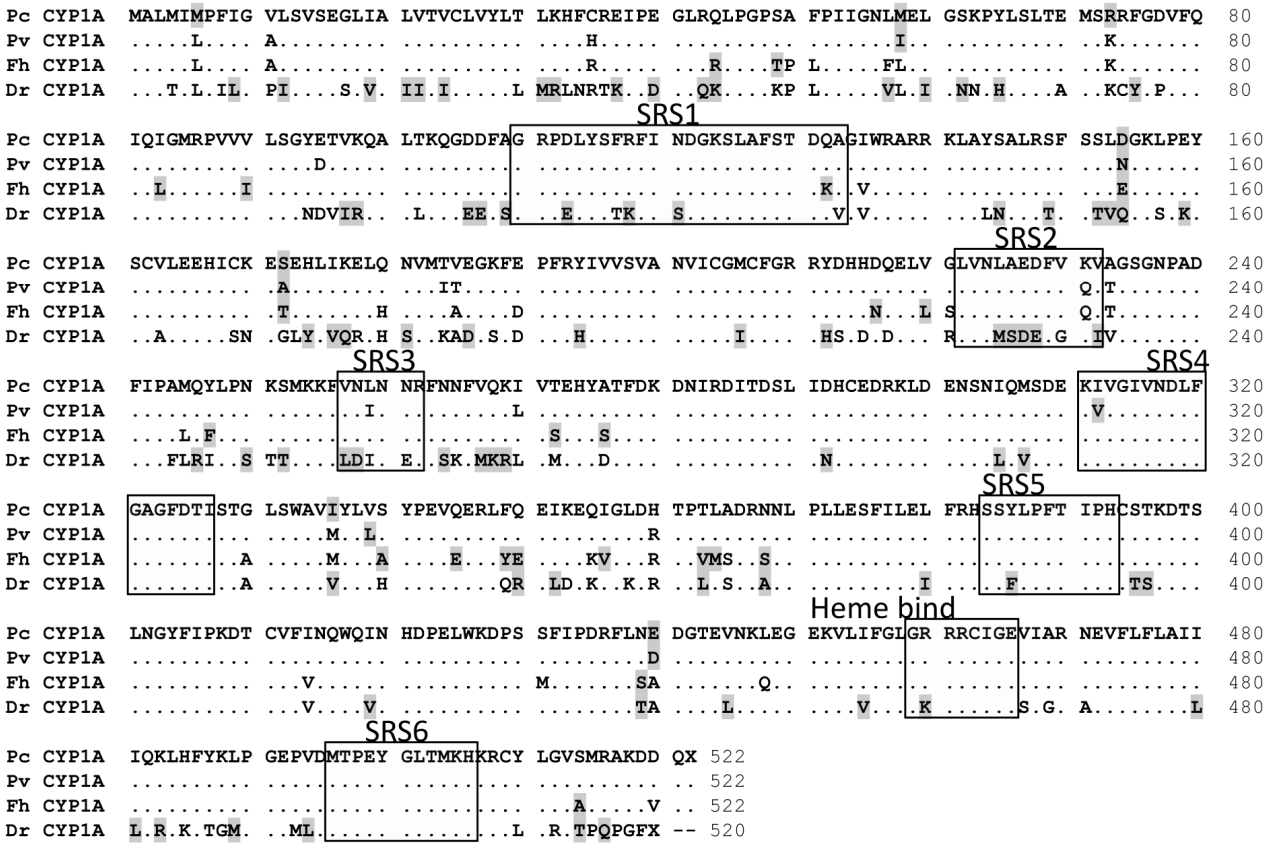

Fig. 1. Alignment of the CYP1A amino acid sequences of guppy *P. caudimaculatus* (Pc), guppy *Poecilia vivipara* (Pv), killifish *Fundulus heteroclitus* (Fh) and zebrafish *Danio rerio* (Dr). Identical and similar amino acids are represented by dot and gray background, respectively, comparing to Pc CYP1A sequence. The substrate recognition sites (SRS 1-6) and the heme binding site are indicated by boxes.

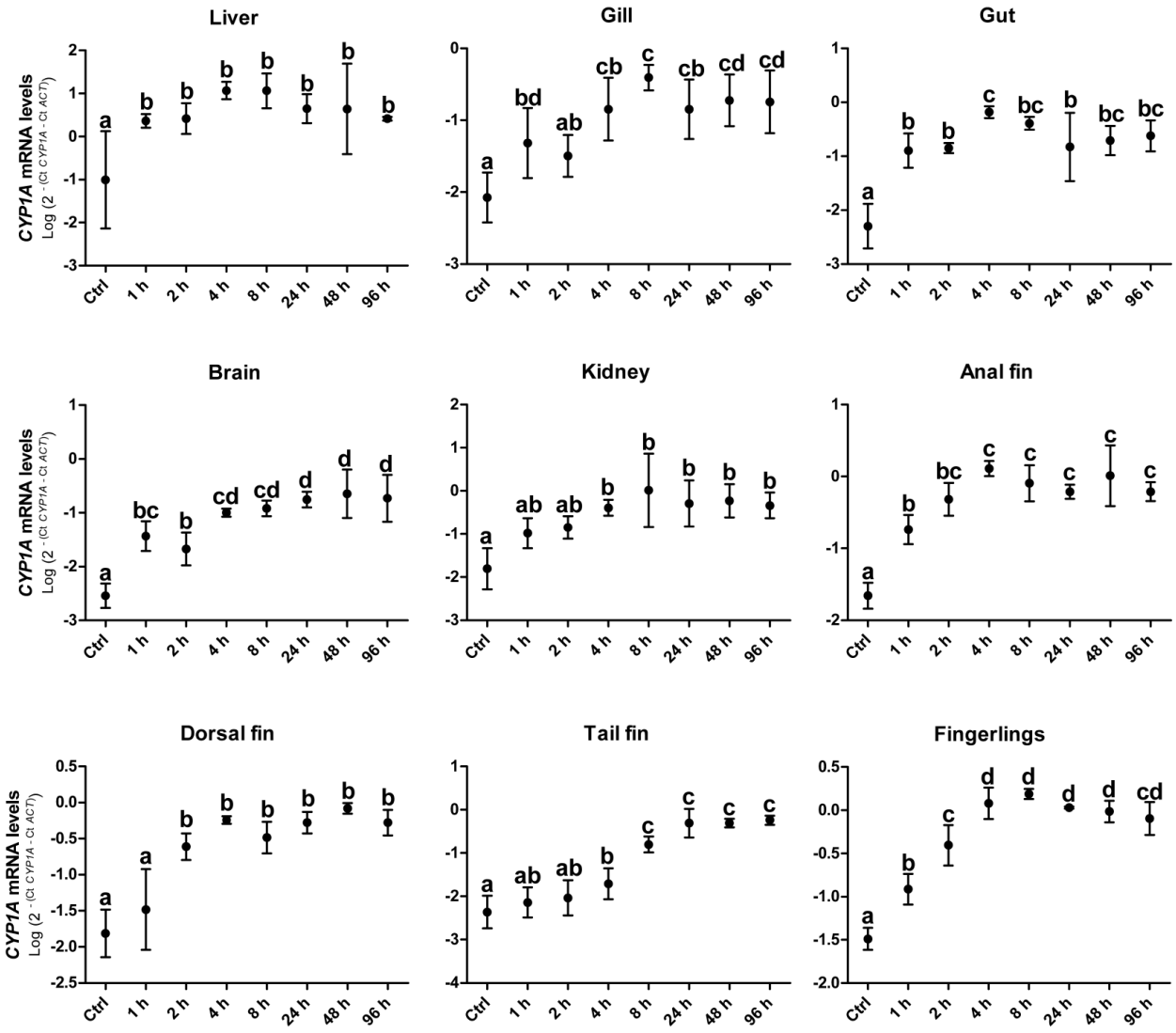

Fig. 2. *CYP1A* mRNA levels in liver, gill, gut, brain, kidney, anal fin, dorsal fin, tail fin and fingerling of *P. caudimaculatus* exposed to 1 µM BNF during 1, 2, 4, 8, 24, 48 and 96 h. Differences between groups were determined by one-way ANOVA for repeated measures followed by Tukey's *post hoc* test ($p < 0.05$, $n = 6$). Dots in the figures represent means ± SD.

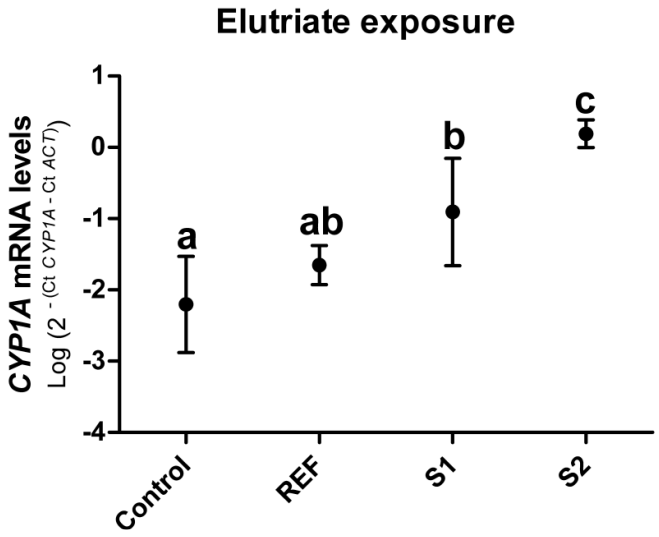

Fig. 3. *CYP1A* mRNA levels in adult *P. caudimaculatus* liver exposed for 8 hours to the control water, elutriate from the sediment reference site, domestic waste site and petrochemical pole site. Differences between groups were determined by one-way ANOVA followed by Tukey's *post hoc* test ($p < 0.05$, $n = 10$). Dots in the figures represent means ± SD.

TABLES

Table 1. Degenerate and specific primers employed in the *CYP1A* identification and in the *CYP1A* and *B-actin* RTq-PCR analysis in *P. caudimaculatus*.

Primer name	Sequence (5'-3')
CYP1A_degenerate_F	CAACCCWGCAGAYTTCATCCCTGC
CYP1A_degenerate_R	GGGTCRTGGTTTATYTGCCACTGG
CYP1A_RACE_F1	CCCTGCTATGCAGTATCTTCCCAAC
CYP1A_RACE_R1	GTCTGTGATGTCACGGATGTTGTCC
CYP1A_RACE_F2	GAGGACCGGAAGCTGGATGAGAA
CYP1A_RACE_R2	GCCCATGACAAACCAGTAGAGATGG
CYP1A_qPCR_F	GAGGACCGGAAGCTGGATGAGAA
CYP1A_qPCR_R	GCCCATGACAAACCAGTAGAGATGG
β-ACT_qPCR_F	ACCATCACCGGAGTCCATGACGA
β-ACT_qPCR_R	ATGTACGTTGCCATCCAGGCCGT

Table 2. Identities between nucleotide sequences (in the left-bottom) and amino acid sequences (right-top) among *P. caudimaculatus* (Pc), *Poecilia vivipara* (Pv) and zebrafish *Danio rerio* (Dr) CYP1As and *Fundulus heteroclitus* (Fh) CYP1A, 1B1, 1C1 and 1D1 sequences.

	Pc_CYP1A	Dr_CYP1A	Pv_CYP1A	Fh_CYP1A	Fh_CYP1B1	Fh_CYP1C1	Fh_CYP1D1
Pc_CYP1A		70	96	91	39	37	43
Dr_CYP1A	68		71	71	40	39	44
Pv_CYP1A	97	68		91	40	39	43
Fh_CYP1A	90	69	90		39	38	44
Fh_CYP1B1	49	49	49	49		49	37
Fh_CYP1C1	49	47	49	47	57		39
Fh_CYP1D1	55	54	54	54	50	48	

Acession numbers: NM_131879.1 (Dr_CYP1A); JX027071.1 (Pv_CYP1A); AF026800.1 (Fh_CYP1A); FJ786959.1 (Fh_CYP1B1); FJ786960.1 (Fh_CYP1C1); FJ786961.1 (Fh_CYP1D1)

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