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Efeitos fisiológicos e genotóxicos induzidos por contaminantes ambientais em peixes

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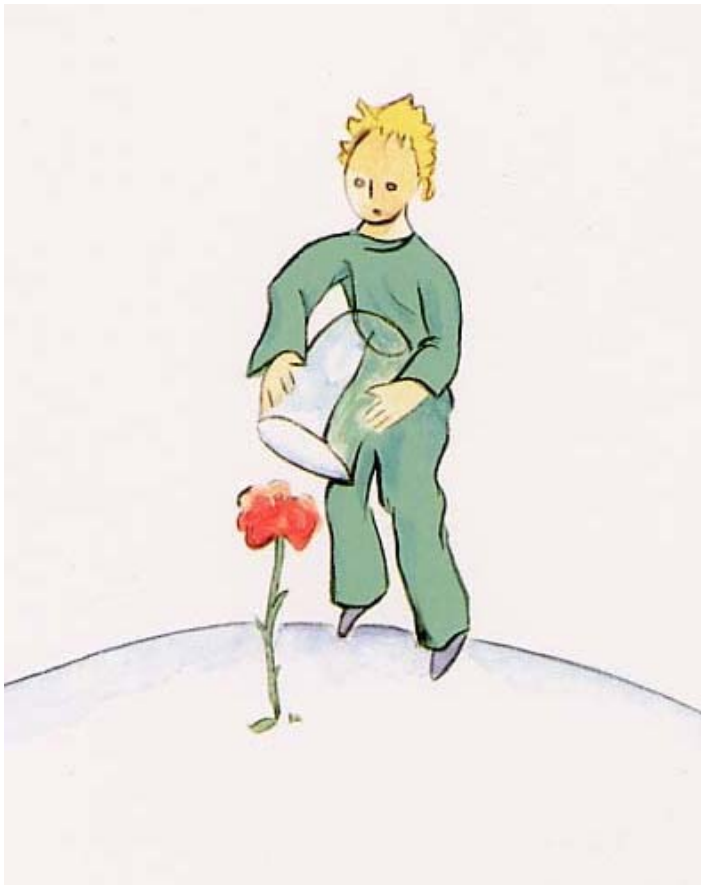

« C'est le temps que tu as perdu pour ta rose qui fait ta rose si importante. »
(Le Petit Prince, Saint-Exupéry)

À Blá, pelo seu apoio.

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palavras-chave

Peixes, contaminantes ambientais, efeitos fisiológicos, genotoxicidade.

resumo

No presente trabalho foi estudado um largo espectro de efeitos fisiológicos e genotóxicos induzidos pela exposição de três espécies de teleósteos - *Anguilla anguilla* L. (enguia Europeia), *Dicentrarchus labrax* L. (robalo) e *Sparus aurata* L. (dourada) a contaminantes aquáticos. Exposições de curta duração foram realizadas de acordo com três (1, 2, 3) perspectivas distintas:

(1) Exposições laboratoriais a contaminantes isolados (capítulos II a VI), nomeadamente aos ácidos abiético e desidroabiético, reteno, naftaleno, β-naftoflavona (BNF) e 17β-estradiol (E_2). Estas exposições causaram, maioritariamente, uma desregulação endócrina, expressa através da incapacidade dos peixes elevarem o cortisol no plasma face a situações de stresse, exceptuando a exposição a ácido abiético que induziu um aumento desta hormona e a BNF que não causou nenhuma alteração deste parâmetro. Os níveis plasmáticos de glucose e lactato foram determinados como respostas secundárias de stresse para todos os compostos anteriormente referidos, excluindo o naftaleno. Os peixes expostos a ácidos resínicos apresentaram um aumento dos dois parâmetros anteriores correspondente a um padrão de resposta típica de stresse. Contudo, o reteno e a BNF induziram aumentos unicamente na concentração de glucose plasmática. A exposição de *A. anguilla* a BNF também revelou um decréscimo da concentração de tiroxina (T4) no plasma. A capacidade do E_2 induzir a síntese de vitelogenina (Vtg) em *D. labrax* juvenis e a sua relação com os níveis de E_2 no plasma, foram avaliada no capítulo VI, adoptando duas vias de exposição diferentes: diluição na água ou injecção intraperitoneal (i.p.). A indução de Vtg foi verificada nos dois casos, tendo sido detectado um aumento concomitante de E_2 no plasma apenas para a exposição via água.

O naftaleno e a BNF foram capazes de induzir a biotransformação hepática, medida através do aumento da actividade da etoxiresorufina-*O*-desetilase (EROD) (fase I) (capítulo III). Apesar da capacidade destes compostos induzirem a actividade EROD, eles revelaram uma capacidade inibitória, especialmente para as concentrações mais elevadas e durante as primeiras horas de exposição. O naftaleno revelou ser também um indutor da actividade da glutationa-*S*-transferase (GST) (fase II).

A resposta genotóxica de *A. anguilla* variou claramente com o composto testado. Assim, as propriedades genotóxicas do naftaleno foram demonstradas através de um aumento da frequência de anomalias nucleares eritrocíticas (ANE), enquanto que a BNF não provocou qualquer alteração deste parâmetro (capítulo III). A exposição de *D. labrax* a E_2 resultou no aumento da frequência de ANE, demonstrando o seu potencial genotóxico (capítulo V).

Os compostos testados nos capítulos II a VI revelaram uma grande variedade de efeitos adversos, reafirmando o risco ecológico que representam.

(2) Exposições laboratoriais de *D. labrax*, *A. anguilla* e *S. aurata* a misturas simples ou exposições sequenciais a diferentes contaminantes (capítulos VII a IX). Os níveis plasmáticos de cortisol e glucose foram quantificados em todos estes capítulos. Um decréscimo significativo de cortisol foi observado em *S. aurata* exposta a E_2+nonilfenol (NP) (capítulo VII), sinalizando uma desregulação endócrina. A pré-exposição a BNF exerceu uma acção antagonista sobre a capacidade do cobre (Cu) aumentar o cortisol em *A. anguilla* (capítulo IX). Todas as respostas obtidas revelaram um aumento da glucose, tendo ainda sido verificada uma acção sinergística no caso da exposição de *D. labrax* a BNF+NP e de *A. anguilla* a BNF+100 μM crómio (Cr). Contudo, a BNF demonstrou diminuir a capacidade do E_2 elevar a glucose plasmática. O lactato plasmático (capítulos VIII e IX), de um modo geral, permaneceu inalterado, tendo-se observado um aumento apenas no caso de E_2+NP. No entanto, observou-se uma acção antagonista da BNF, impedindo o aumento de lactato no plasma de *A. anguilla* em resultado da exposição a Cu.

A frequência de ANE foi avaliada nos capítulos VII a IX. A exposição de *D. labrax* a BNF+E_2 e a BNF+NP induziu um aumento da frequência de ANE. A exposição de *A. anguilla* a BNF+Cu demonstrou igualmente o seu potencial genotóxico, revelando uma acção sinergística.

O conteúdo hepático em citocromo P450, as actividades EROD, GST e alanina aminotransferase (ALT) no fígado, bem como o índice hepato-somático (IHS) foram determinados nos capítulos VII e VIII. O E_2 e o NP reduziram a actividade de EROD induzida pela BNF em *D. labrax*. De forma semelhante, a mistura de E_2+NP teve a capacidade de afectar a expressão constitutiva do CYP1A, medida pela diminuição de EROD em *S. aurata*. A importância da mistura de compostos foi reforçada através da redução da actividade de GST observada após exposição a BNF+NP, uma vez que estes compostos isoladamente não demonstraram essa capacidade (capítulo VII). Contudo, no capítulo VIII, detectou-se um aumento de GST para a mistura E_2+NP, possivelmente devido à acção individual do E_2.

Um decréscimo de T4 plasmático foi observado em *A. anguilla* exposta a BNF+Cr e BNF+Cu, sugerindo uma acção sinergística entre BNF e Cu, em que a pré-exposição acentuou o efeito do Cu (capítulo IX). A importância da pré-exposição a BNF na acção do Cr e do Cu sobre os níveis T3 plasmático não foi detectada; embora, a interferência dessa pré-exposição não possa ser excluída no caso da exposição a 100 μM Cr.

(3) A terceira e última perspectiva disse respeito a exposições *in situ* em três ecossistemas aquáticos diferentes (capítulos X e XI). Estudou-se uma zona portuária, situada na Ria de Aveiro, tipicamente contaminada por hidrocarbonetos aromáticos policíclicos, metais pesados e compostos organoestânicos. A zona do rio Vouga situada a 13 Km da sua foz, receptora do efluente de uma unidade de produção de pasta de papel durante quase 5 décadas foi também estudada. Neste segundo local, foi avaliada a recuperação do ecossistema após o desvio do efluente, de acordo com os efeitos observados. Por fim, seleccionou-se um ecossistema de água doce – Pateira de Fermentelos – por ser uma bacia receptora de efluentes domésticos e industriais, bem como de lixiviados agrícolas. Em qualquer das áreas de

estudo foram avaliados indicadores de stresse como cortisol, glucose e lactato plasmáticos. Um conjunto de parâmetros relativos à biotransformação, genotoxicidade, hormonas do eixo hipotálamo-pituitária-tiróide (HPT) e E_2 no plasma foram também determinados após exposição na Pateira de Fermentelos (capítulo XI). Os indicadores de stresse mostraram alterações que se enquadram no perfil típico de resposta, ou seja aumento simultâneo de cortisol, glucose e lactato, nos três ecossistemas avaliados, com excepção do lactato que se manteve inalterado no caso da exposição na Pateira de Fermentelos. Os dados relativos ao rio Vouga revelaram alterações de stresse em *A. anguilla*, 2 anos após o desvio do efluente, indicando a persistência de contaminantes nos respectivos sedimentos. Na Pateira de Fermentelos observou-se uma tendência geral para a diminuição de T3, confirmada com o decréscimo significativo no local mais próximo da principal fonte de poluição, sem ser contudo acompanhada por alterações de TSH e T4. No que diz respeito ao E_2, observou-se um aumento significativo em 2 locais da Pateira de Fermentelos, indicando a presença desta hormona “natural” em níveis elevados na água. Os parâmetros relativos à biotransformação e genotoxicidade permaneceram inalterados. Os resultados obtidos nas exposições *in situ* na Pateira de Fermentelos forneceram fortes indicações da contaminação deste ecossistema.

Globalmente, os dados da presente tese permitem um melhor conhecimento do potencial tóxico de diferentes compostos de grande relevância ambiental, cuja importância surge acrescida pela escassez de informação disponível no que respeita aos parâmetros estudados. Os dados apresentados contribuíram também para um melhor conhecimento das respostas toxicológicas em peixes, particularmente como resultado do uso de uma bateria de biomarcadores que fornecem evidências das interferências de diferentes classes de xenobióticos, quer em misturas, quer em exposições sequenciais.

keywords Fish, environmental contaminants, physiological effects, genotoxicity.

abstract

In the present work a wide range of physiological and genotoxic effects due to aquatic contaminants exposure was studied, adopting three teleost species - *Anguilla anguilla* L. (European eel), *Dicentrarchus labrax* L. (sea bass) e *Sparus aurata* L. (sea bream). Short-term exposures were carried out, according to three (1, 2, 3) different approaches:

(1) Laboratorial exposures to individual compounds (chapters II to VI), namely to abietic and dehydroabietic acids, retene, naphthalene, β-nahthoflavone (BNF) and 17β-estradiol (E_2). The large majority of the previous exposures cause endocrine disruption, expressed through fish inability to elevate plasma cortisol under stress conditions, with the exception for abietic acid exposure, which induced an increase in this hormone and BNF that did not cause any alteration. Glucose and lactate plasmatic concentrations were determined, as secondary stress responses, for all the previous mentioned compounds except for naphthalene. Thus, fish exposed to resin acids presented a raise in both parameters corresponding to a typical stress pattern response. Though, retene and BNF only increased the plasma glucose concentration. *A. anguilla* BNF exposure also revealed a decrease in plasma thyroxine (T4) concentration. In chapter VI, the E_2 ability to induce vitellogenin (Vtg) synthesis in juvenile *D. labrax* was evaluated in relation with E_2 levels in plasma, adopting two different exposure routes: water diluted or intraperitoneally (i.p.) injected. A plasma Vtg increase was observed under both exposure conditions, though E_2 plasma increase was only significant for waterborne exposure.

Naphthalene and BNF demonstrated its ability to induce hepatic biotransformation, measured as an increased ethoxyresorufin-O-deethylase (EROD) activity (phase I) (chapter III). Despite these compounds EROD activity inducing capacity, it also revealed inhibition ability, especially for the highest concentrations and during the first exposure hours. Naphthalene also revealed its capacity as a glutathione *S*-transferase (GST) inducer (phase II).

A. anguilla genotoxicity response clearly varied with the tested compound. Thus, naphthalene has demonstrated its genotoxic properties by inducing erythrocytic nuclear abnormalities (ENA) increase, whereas BNF did not alter this parameter (chapter III). *D. labrax* E_2 exposure induced an increase in ENA frequency, demonstrating its genotoxic potential (chapter V).

Globally, the compounds tested in chapters II to VI revealed a wide variety of adverse effects, corroborating the ecological risk they represent.

(2) Laboratorial exposures to simple mixtures or sequential exposures to different contaminants in *D. labrax*, *A. anguilla* e *S. aurata* (chapters VII to IX). In any of the previous chapters, plasma cortisol and glucose levels were assessed. A significant plasma cortisol decrease was observed in *S. aurata* after E_2+nonylphenol (NP) exposure (chapter VII), signalling an endocrine disruption. BNF preexposure exerted an antagonistic action over copper (Cu) ability to induce cortisol increase in *A. anguilla* (chapter IX). Though all the plasma glucose responses observed revealed an increase, a synergistic action after *D. labrax* exposure to BNF+NP and *A. anguilla* exposure to BNF+100 μM chromium (Cr) was also observed. Nevertheless, BNF showed to limit the E_2 capability of elevating plasma glucose. In general, plasma lactate (chapters VIII and IX) remained unaltered, though its increase was observed only after E_2+NP exposure. A BNF antagonistic action preventing the plasma lactate increase in *A. anguilla* due to Cu exposure was detected.

ENA frequency was assessed in the chapters VII to IX. *D. labrax* exposure to BNF+E_2 and BNF+NP induced an ENA frequency increase. *A. anguilla* exposure to BNF+Cu also demonstrated its genotoxic potential, revealing a synergistic action.

In chapters VII and VIII liver cytochrome P450 content, liver EROD, GST and alanine transaminase (ALT) activities, as well as hepato-somatic index (HSI) were measured. E_2 and NP reduced the BNF EROD activity induction in *D. labrax*. The E_2+NP mixture had the ability to reduce the constitutive expression of CYP1A, measured as EROD activity in *S. aurata*. The importance of compound mixtures was supported by the GST activity reduction, observed after exposure to BNF+NP, since each of these compounds isolated does not induce the same effect (chapter VII). Though, in chapter VIII, a GST increase was detected for the mixture E_2+NP, probably due to E_2 individual action.

In chapter IX, an *A. anguilla* plasma T4 decrease was observed after BNF+Cr and BNF+Cu exposures, suggesting a synergistic action between BNF and Cu since the preexposure enhaced Cu effect. The BNF preexposure over Cr or Cu action on plasma T3 was not observed; though, its importance cannot be excluded considering the 100 μM Cr exposure.

(3) The third and last approach concerned *in situ* exposures in three different aquatic ecosystems (chapters X and XI). An offward fishing harbour located at the Ria de Aveiro, typically contaminated by polycyclic aromatic hydrocarbons, heavy metals and organometallics, was studied. An area situated 13 Km upstream from River Vouga mouth was also studied, considering its contamination for several decades as a receptor of a paper and pulp mill effluent. In this second area the contamination cleanup process after the effluent deviation was evaluated, according to the effects observed. Finally, a freshwater ecosystem was also selected – Pateira de Fermentelos – as a recipient of domestic and industrial effluents, agriculture runoff. Stress indicators such as plasma cortisol, glucose and lactate were determined in *A. anguilla* under exposure. A set of parameters concerning biotransformation, genotoxicity, HPT axis hormones and E_2 in plasma was also measured in *A. anguilla* after exposure at the Pateira de Fermentelos (chapter XI). The simultaneous increase in cortisol, glucose and lactate fit in the typical profile of response to stress indicators in the three evaluated ecosystems, with an exception for plasma lactate that remains unaltered after Pateira de Fermentelos exposure. The data concerning rio Vouga revealed that 2 years after the effluent deviation, contaminants were still able to induce stress alterations in *A. anguilla* indicating its persistence in the sediments. A general tendency for plasma T3 reduction was observed at the Pateira de Fermentelos and confirmed by its significant decrease at the closest site to the main pollution source. A significant E_2 increase was observed in 2 sites of Pateira de Fermentelos, indicating the presence of this natural “hormone” in elevated levels in water. The parameters related to biotransformation and genotoxicity remained unaltered. The results concerning *A. anguilla* Pateira de Fermentelos

in situ exposure provided strong indication of this ecosystem contamination.

Globally, the data concerning the present thesis allow a better knowledge of the toxic potential of different compounds with environmental relevancy. These results also contribute to a better understanding of fish toxicological responses particularly resulting from the use of a biomarker battery that give us the evidence of different classes of xenobiotics interference in mixtures as well as in sequential exposures.

ÍNDICE

Abreviaturas

Em Português:

ACTH – hormona adrenocorticotrófica
ADN – ácido desoxiribonucleico
AHH – aril hidrocarbono hidroxilase
ALT – alanina transaminase
ANE – anomalias nucleares eritrocíticas
AR – ácidos resínicos
BNF – β-naftoflavona
Cr – crómio
CRH – hormona libertadora da corticotrofina
Cu – cobre
DE – desreguladores endócrinos
E_2 – 17β-estradiol
ECOD – etoxicumarina-*O*-desetilase
EI – eritrócitos imaturos
EPP – efluentes da pasta de papel
EROD – etoxiresorufina-*O*-desetilase
GnRH – hormona libertadora da gonadotrofina
GST – glutationa *S*-transferase
GTH I e GTH II – gonadotrofinas I e II
HAP – hidrocarbonetos aromáticos policíclicos
HPG – hipotálamo-pituitária-gónadas
HPI – hipotálamo-pituitária-tecido interrenal
HPT – hipotálamo-pituitária-tiróide
IHS – índice hepatosomático
NP – 4-nonilfenol
PCB – bifenilo policlorados
PCDD – policloro dibenzo-*p*-dioxinas
PCDF – policloro dibenzofuranos
T3 – triiodotironina
T4 – tiroxina
TSH – hormona estimuladora da tiróide
TRH – hormona libertadora da tirotrofina
UDP – uridina difosfato
UDPGT – UDP-glucuronosil transferase
Vtg – vitelogenina

Em Inglês:

ACTH – adrenocorticotropic hormone
DNA – deoxyiribonucleic acid
AHH – aryl hydrocarbon hidroxilase
ALT – alanine transaminase
ENA – erythrocytic nuclear abnormalities
AR – resin acids
BNF – β-naphthoflavone
Cr – chromium
CRH – corticotropin releasing hormone
Cu – copper
ED – endocrine disrupters
E_2 – 17β-estradiol
ECOD – ethoxycoumarin-*O*-deethylase
IE – immature erythocytes
EPP – paper mill effluents
EROD – ethoxyresorufin-*O*-deethylase
GnRH – gonadotropin releasing hormone
GST – gluthatione *S*-transferase
GTH I e GTH II – gonadotropins I and II
PAH – polycyclic aromatic hydrocarbons
HPG – hypothalamus-pituitary-gonads
HPI – hypothalamus-pituitary-interrenal
HPT – hypothalamus-pituitary-thyroid
HSI – hepatosomatic index
NP – 4-nonylphenol
PCB – polychlorinated biphenyls
PCDD – polychlorinated dibenzo-*p*-dioxins
PCDF – polychlorinated dibenzofurans
T3 – triiodothyronine
T4 – thyroxine
TSH – thyroid stimulating hormone
TRH – thyrotrophin releasing hormone
UDP – uridine diphosphate
UDPGT – UDP-glucuronosyl transferase
Vtg – vitellogenin

A presente tese de Doutoramento está organizada em doze capítulos correspondendo a três partes distintas. Uma primeira parte representada pelo Capítulo I – Introdução Geral, em que se estabelece o contexto do trabalho e respectivos objectivos.

A segunda parte compreende a apresentação de dez artigos científicos publicados ou submetidos para publicação, e que correspondem sequencialmente aos Capítulos II a XI. A estrutura e conteúdo dos artigos científicos apresentados foram literalmente respeitados, tendo sido, contudo, adaptada a sua formatação de acordo com as normas da Universidade de Aveiro que regulamentam este aspecto.

A terceira e última parte (Capítulo XII) corresponde a uma Discussão Geral relativa aos dez capítulos precedentes, numa perspectiva global e integradora.

CAPÍTULO I

Introdução geral

1. MEIO AQUÁTICO E CONTAMINAÇÃO

O ambiente aquático tem vindo a ser progressivamente afectado pelas actividades antropogénicas resultando, fundamentalmente, da quantidade e variedade de poluentes que nele são regularmente introduzidos de forma intencional ou acidental. As actividades industriais e agrícolas, assim como os aglomerados urbanos são os principais contribuintes para o agravamento da saúde ambiental e consequentemente da qualidade da água. Recentemente, tem-se vindo a reconhecer também o risco que representam alguns compostos naturais, de que são exemplo as hormonas esteróides, quando presentes em elevadas concentrações no meio aquático. Os ecossistemas aquáticos constituem o destino final da maior parte dos resíduos provenientes dessas actividades, sendo as zonas costeiras particularmente afectadas uma vez que, de um modo geral, oferecem boas condições para a implantação de indústrias e centros urbanos importantes.

A gestão, protecção e conservação efectiva dos ecossistemas aquáticos obriga a uma monitorização regular, sustentada não só pela quantificação dos compostos químicos na água, sedimento e biota, mas também pela utilização de um leque adequado de respostas biológicas (biomarcadores). Estas respostas podem incluir alterações a nível bioquímico, fisiológico, celular, morfológico ou comportamental, permitindo assim um melhor conhecimento dos processos biológicos associados à acção dos contaminantes. Deste modo, o presente trabalho analisou os efeitos induzidos em peixes por contaminantes do meio aquático, em diferentes níveis biológicos, nomeadamente bioquímico, fisiológico e citogenético. As alterações a nível biomolecular podem ser interpretadas como um aviso prévio, oferecendo a possibilidade de antecipar uma intervenção relativamente à ocorrência de distúrbios importantes a nível do indivíduo ou a níveis organizacionais mais elevados.

Os xenobióticos após absorção, são submetidos a uma série de reacções que podem conduzir sequencialmente à sua activação, desintoxicação e excreção. Estes processos vão determinar a sua taxa de eliminação e consequentemente a sua toxicidade e potencial de bioacumulação. Os processos de desintoxicação têm

grande importância na toxicocinética dos xenobióticos, pelo que o presente trabalho abordou a modulação do metabolismo de biotransformação de fase I e fase II em resposta a exposições a contaminantes ambientais. Efeitos a nível do sistema endócrino foram também estudados, dada a sua importância na homeostasia. Tendo em consideração que a integridade do material genético é essencial ao funcionamento das células e dos organismos foram ainda investigados efeitos a nível citogenético.

A maioria dos compostos químicos testados no presente trabalho de investigação foram seleccionados tendo em consideração o facto de serem contaminantes ambientais e representarem risco potencial para os organismos aquáticos. Estes compostos integram as principais classes de contaminantes do meio aquático e apresentam uma grande relevância a nível regional, nomeadamente no que respeita à contaminação dos ecossistemas Ria de Aveiro e Pateira de Fermentelos. Adicionalmente, foram realizadas experiências *in situ* nos referidos ecossistemas, de forma a permitir o estudo de respostas perante situações reais de exposição, em particular no que respeita à acção de misturas complexas.

A Ria de Aveiro é uma laguna situada na região centro de Portugal (40º38'N, 8º45'W), com 45 km de comprimento e 10 km de largura, abrangendo uma área de 83 km^2 em preia-mar e 66 km^2 em baixa-mar (Dias e Lopes, 2006), representando um importante ecossistema do qual depende directamente uma considerável actividade piscatória e indirectamente actividades diversas como a indústria, turismo e tráfego naval. Assim, a contaminação da Ria de Aveiro resulta de uma enorme variedade de fontes antropogénicas em que se incluem efluentes industriais de diferente natureza, efluentes urbanos, resíduos da actividade portuária, bem como lixiviados agrícolas. Uma vez que a partir de 1999 se iniciou um processo de desvio destes efluentes através de um exutor submarino para o oceano Atlântico, é previsível que se venha a assistir a uma melhoria considerável na qualidade deste ecossistema. No entanto, a biomonitorização regular dos principais pontos críticos da Ria de Aveiro, constitui uma necessidade dada a ocorrência de descargas, assim como a persistência de alguns dos contaminantes.

O rio Vouga é o principal fonecedor de água doce da Ria de Aveiro, sendo o seu troço final caracterizado por uma considerável carga poluente resultante, principalmente, de descargas da indústria de pasta de papel de que foi receptor durante cinco décadas. No entanto, a zona que foi alvo de estudo, a juzante do rio Vouga, é condicionada pela acção das marés que aí se fazem sentir, transformando-se quase numa extensão da Ria de Aveiro.

A Pateira de Fermentelos é um ecossistema natural de água doce localizado na região centro de Portugal, com uma área total de 5 km^2. Esta massa de água é considerada uma expansão do rio Cértima e o principal afluente do rio Águeda. Durante as últimas décadas a qualidade da água na Pateira de Fermentelos tem vindo a decrescer devido ao aumento de descargas de efluentes domésticos, industriais e agrícolas. Apesar dos estudos realizados por Calado *et al.* (1991) e Almeida (1998) não existe referência a estudos relativos a efeitos de contaminantes sobre a ictiofauna.

Os peixes representam o grupo de Vertebrados mais antigo e diversificado, compreendendo a maior percentagem de espécies deste subfilo e ocupando um extraordinário leque de habitats tais como oceanos, mares, rios, lagos e fontes hidrotermais (Bolis *et al.*, 2001). Devido aos diferentes tipos de habitats que ocupam, os peixes têm desenvolvido diferentes estratégias de sobrevivência, tornando-os particularmente úteis em estudos de ecotoxicologia, permitindo avaliar os efeitos de uma vasta gama de contaminantes sob um largo espectro de condições de exposição. Os peixes têm sido eleitos como modelos biológicos preferenciais para a compreensão dos fundamentos de áreas tão diversas como a ecotoxicologia, genética, oncologia, biomedicina, neurobiologia, endocrinologia, aquacultura e de uma forma geral, como uma ferramenta que permite obter informação básica para outras ciências biológicas (Bolis *et al.*, 2001). Assim, no desenvolvimento do presente trabalho científico as espécies *Anguilla anguilla* L. (enguia Europeia), *Dicentrarchus labrax* L. (robalo) e *Sparus aurata* L. (dourada) foram seleccionadas como bioindicadores, por serem representativas da ictiofauna local, pelas suas características biológicas e elevado valor comercial, ocupando um lugar de destaque

na pesca e piscicultura. *A. anguilla* apesar da sua resistência, tem demonstrado grande sensibilidade no que respeita à detecção da presença de contaminantes na água e sedimentos, acrescida da possibilidade de poderem ser expostas em água salgada e/ou salobra o que é particularmente importante no âmbito de estudos de ecotoxicologia estuarina (Santos e Pacheco, 1995, 1996; Pacheco e Santos, 2001a, b; Maria *et al.*, 2004; Santos *et al.*, 2004; Ahmad *et al.*, 2005). *D. labrax* possui também grande sensibilidade à presença de xenobióticos (Gravato e Santos, 2002a, b, c; 2003) apesar da sua menor resistência. *S. aurata* foi também seleccionada devido ao facto de, apesar de fazer parte da ictiofauna da Ria de Aveiro, pouco ou nada se saber no que respeita aos efeitos induzidos pelos contaminantes xenobióticos presentes na respectiva água.

1.1. Etoxilados de alquilfenóis

Os etoxilados de alquilfenóis são surfactantes não iónicos largamente utilizados nas indústrias farmacêutica, cosmética, têxtil, de plásticos, detergentes e pasta de papel, estimando-se uma produção anual mundial de 665.000 toneladas (Hager, 1998). Neste contexto, os compostos mais relevantes são os etoxilados de octilfenol e nonilfenol, correspondendo os últimos a cerca de 80 % do consumo mundial (Renner, 1997). A degradação destes compostos conduz à formação de vários metabolitos, sendo o 4-nonilfenol (NP) identificado como o mais crítico devido à sua lipofilicidade, toxicidade e relativa resistência à biodegradação (Ying, 2005). O NP foi detectado em ecossistemas aquáticos poluídos em valores na ordem dos μg/L, sendo o nível mais elevado de 600 μg/L (Solé *et al.*, 2000). A degradação abiótica do NP é pouco significativa e a sua biodegradação moderada, sendo o seu tempo de meia-vida de aproximadamente 30 dias, em águas superficiais sob condições aeróbias (EU-RAR, 1998). O NP demonstrou ser bioacumulado em peixes, devido à sua lipofilicidade (Ying, 2005), e excretado predominantemente após conjugação com o ácido glucorónico (Arukwe *et al.*, 2000a). O NP demonstrou ser estrogénico em peixes, induzindo intersexualidade (Balch e Metcalfe, 2005),

produção de vitelogenina (Vtg) em machos e juvenis (Pait e Nelson, 2003; Soverchia *et al.*, 2005) e de proteínas da *zona radiata* em juvenis (Arukwe *et al.*, 2000b) e inibindo a espermatogénese (Sepúlveda *et al.*, 2003). Efeitos do NP em peixes a nível não reprodutivo incluem redução da capacidade de biotransformação (Arukwe *et al.*, 1997) e diminuição de crescimento (Arsenault *et al.*, 2004).

1.2. Hormonas sintéticas e "naturais"

As hormonas de síntese são substâncias produzidas com a intenção de modular o sistema endócrino, incluindo compostos com estrutura idêntica às hormonas "naturais", de que são exemplo os contraceptivos orais, fármacos aplicados em terapias hormonais de substituição e alguns aditivos alimentares usados em produção animal. Assim, estes compostos atingem o ambiente aquático através dos esgotos domésticos, bem como da indústria e pecuária. O etinilestradiol, em particular, é um estrogénio sintético usado nos contraceptivos orais, sendo considerado um potente desregulador endócrino em peixes (Van den Belt *et al.*, 2004). Este composto é excretado pelas mulheres na sua forma conjugada e portanto inactiva, podendo, no entanto, ser desconjugado no ambiente por acção bacteriana. Desta forma o etinilestradiol pode ser reactivado, pelo que é frequentemente encontrado em ambientes aquáticos poluídos (Van den Belt *et al.*, 2004).

Algumas hormonas "naturais", de que são exemplo o 17β-estradiol (E_2) e alguns fitoestrogénios, podem ser encontradas no ambiente aquático em concentrações anormalmente elevadas, o que aliado à sua capacidade de causar alterações endócrinas nos organismos, levou a que fossem consideradas como contaminantes ambientais. O E_2, em particular, chega ao meio aquático principalmente através de efluentes domésticos, tendo sido estimada uma excreção diária de 2,3 a 259 μg/mulher e 1,6 μg/homem (Johnson *et al.*, 2000). Outras duas importantes fontes de E_2 são os efluentes de produção animal (Ying *et al.*, 2002) e a lixiviação dos solos agrícolas (Céspedes *et al.*, 2004). Nichols *et al.* (1998) mediu em

lixiviados de um campo de pastorícia 3500 ng/L de E_2. Durante as últimas décadas, tem-se verificado um aumento significativo das concentrações de E_2 no meio aquático, principalmente em zonas urbanas, sendo os seus níveis na água na ordem das ng/L, podendo atingir 200 ng/L (Bowman *et al.*, 2000). O E_2 na água, sob condições ambientais aeróbias, é rapidamente biodegradado tendo sido determinado um tempo de meia-vida de 2 dias (Ying *et al.*, 2003). A biotransformação do E_2 em peixes é realizada pelas enzimas de fase I e de fase II (conjugação maioritariamente com ácido glucurónico), sendo a excreção através da bílis e da urina. Os conjugados resultantes da fase II da biotransformação do E_2 podem sofrer desconjugação no ambiente por acção bacteriana, levando ao seu reaparecimento como hormona livre (activa) e podendo desta forma ser absorvida pela fauna piscícola (Baronti *et al.*, 2000). O E_2 pode também ser excretado, em menor percentagem, na sua forma livre (Lange *et al.*, 2002). Os estudos relativos aos efeitos adversos do E_2 em peixes são, maioritariamente, direccionados para aspectos da biologia da reprodução. A exposição de peixes a E_2 induz a vitelogénese em machos e juvenis (Vaccaro *et al.*, 2005; Soverchia *et al.*, 2005), redução do índice gonado-somático (Bjerselius *et al.*, 2001), inibição do crescimento testicular (Panter *et al.*, 1998), decréscimo da produção de ovos (Imai *et al.*, 2005) e feminização de machos (Hirai *et al.*, 2005). Existe ainda uma ampla evidência de que esta hormona pode afectar aspectos não reprodutivos, alterando nomeadamente a biotransformação de xenobióticos (Vaccaro *et al.*, 2005) e os níveis plasmáticos de hormonas não sexuais (McCormick *et al.*, 2005).

1.3. Hidrocarbonetos aromáticos policíclicos

Os hidrocarbonetos aromáticos constituem um grupo de compostos químicos que apresentam uma configuração baseada na presença do anel aromático benzeno; assim, dois ou mais anéis aromáticos fundidos constituem os chamados hidrocarbonetos aromáticos policíclicos (HAP). As erupções vulcânicas e os incêndios florestais são as principais fontes naturais de HAP. No entanto, os valores

de HAP encontrados no ambiente aquático são marcadamente afectados por emissões antropogénicas, resultando nomeadamente de processos de incineração, derrames de combustíveis fósseis, assim como de efluentes industriais e domésticos. Os HAP são compostos ubíquos, estimando-se que sejam libertadas anualmente para o meio aquático cerca de 230.000 toneladas (Eisler, 1987). Os processos que mais contribuem para a sua degradação na água são a fotooxidação, oxidação química e biodegradação por microorganismos. Contudo, são compostos estáveis que pela sua natureza lipofílica podem atravessar facilmente as membranas biológicas, sendo na maioria dos casos rapidamente metabolizados e excretados pelos peixes. A relativa insolubilidade dos HAP na água e a sua forte capacidade de adsorção a partículas faz com que se acumulem facilmente nos sedimentos (Seruto *et al.*, 2005). As concentrações de HAP encontradas na água de locais contaminados atingem os 100 μg/L (Kennicutt *et al.*, 1991; Zhou e Maskaoui, 2003; González *et al.*, 2005). A contaminação por este tipo de compostos constitui uma preocupação generalizada devido à sua persistência no ambiente e potencial mutagénico e cancerígeno já demonstrado em peixes (Baumann, 1998; Gravato *et al.*, 2002a; Pacheco e Santos, 2002a; Maria *et al.*, 2002; Busetti *et al.*, 2006).

O naftaleno é o HAP mais simples, constituído apenas por dois anéis benzénicos, cuja introdução no ambiente aquático está predominantemente relacionada com a produção de repelentes anti-insectos e derrames de derivados de petróleo provenientes de refinarias e petroleiros. Desta forma, o naftaleno está amplamente distribuído no meio aquático onde pode atingir a concentração de 66 mg/L (ATSDR, 1995), tendo sido incluído na lista de poluentes prioritários elaborada pela U.S. Environmental Protection Agency (EPA, 1988). O naftaleno é considerado o HAP mais hidrosolúvel, ligando-se fracamente aos sedimentos, podendo sofrer acção bacteriana ou evaporação e desaparecer em 2 semanas. Os peixes absorvem o naftaleno contido na água o qual é sujeito à fase I da biotransformação e posterior conjugação com glutationa, ácido glucorónico ou sulfato activados, sendo excretado na urina e bílis. Este HAP, de uma forma geral, não se bioacumula, sendo encontrado em níveis muito baixos nos tecidos de peixes que habitam locais poluídos (ATSDR,

1995). Os estudos relativos aos efeitos tóxicos do naftaleno em peixes são escassos, apesar de já ter sido demonstrado que este composto é um indutor da biotransformação hepática (Pacheco e Santos, 2002a) e de stresse oxidativo (Ahmad *et al.*, 2003), apresentando potencial genotóxico (Gravato e Santos, 2002a).

1.4. Metais pesados

Os metais pesados que ocorrem no ambiente podem ter origem natural, resultando principalmente da erosão física, química e biológica das rochas, ou antropogénica como consequência da actividade agrícola, mineira, industrial, assim como da utilização de combustíveis fósseis. Vários metais pesados estão também incluídos na lista de poluentes prioritários da EPA (2004) devido à sua abundância, persistência e toxicidade. No meio aquático, os metais pesados podem encontrar-se dissolvidos na água sob a forma iónica, associados a uma série de ligandos ou adsorvidos a partículas em suspensão e sedimentos. O comportamento e distribuição dos metais pesados no ambiente aquático são influenciados por vários factores, tais como pH, oxigénio dissolvido, temperatura, salinidade, presença de agentes oxidantes e quelantes, bem como o conteúdo em matéria orgânica, afectando a sua biodisponibilidade.

A capacidade de excreção de metais pesados pelos peixes poderá aumentar sempre que estes absorvam quantidades que excedam as suas necessidades fisiológicas, de modo a manter a homeostase interna. A excreção desses metais pode ocorrer através das brânquias, bílis e urina. No entanto, acima de um determinado limite a capacidade de excreção esgota-se e os metais pesados podem ser sequestrados por proteínas intracelulares, tais como as metalotioninas (Langston *et al.*, 2002), ou incorporados em grânulos (Wallace *et al.*, 2003), limitando assim a sua biodisponibilidade.

A bioacumulação dos metais pesados nos peixes depende da via de absorção, encontrando-se níveis elevados nas brânquias no período inicial de exposição via água, enquanto que o sistema digestivo, fígado e rim acumulam

grandes quantidades de metais pesados em situações de exposição por via alimentar. Contudo, o fígado apresenta normalmente concentrações elevadas de metais pesados, independentemente da via de exposição. O músculo, comparativamente com outros tecidos, contém geralmente níveis baixos (Jezierska e Witeska, 2001).

Apesar de alguns metais pesados, tais como o cobre (Cu) e o crómio (Cr), serem essenciais para diversos processos fisiológicos, quando ocorrem no ambiente em concentrações elevadas podem tornar-se tóxicos para os organismos. O Cu pode atingir facilmente o ambiente aquático dada a sua extensa e variada utilização em inúmeros contextos tal como na indústria de galvanização, refinarias de petróleo, incorporação em canalizações e utensílios domésticos, assim como em fertilizantes, pesticidas e alimentos para animais. Assim, o Cu pode ser encontrado em águas superficiais em concentrações até 1 mg/L (ATSDR, 2002). A exposição de peixes a Cu pode causar imunosupressão (Shariff *et al.*, 2001), stresse oxidativo (Ahmad *et al.*, 2005), alterações hormonais (Flik *et al.*, 2002) e do metabolismo dos hidratos de carbono (Dhanapakiam e Ramasamy, 2001), alterações hematológicas (Carvalho e Fernandes, 2006) e morfológicas (De Boeck *et al.*, 2003), bem como redução do crescimento (Levesque *et al.*, 2002).

O Cr é um metal abundante na Crosta terrestre, podendo ser encontrado na forma trivalente [Cr (III)] ou hexavalente [Cr (VI)]. Esta última é considerada a forma mais tóxica do Cr, dado que atravessa facilmente as membranas biológicas. O Cr (VI) uma vez dentro da célula, é reduzido a Cr (III), o qual pode complexar-se com macromoléculas, incluindo o ácido desoxirribonucleico (ADN) (Farag *et al.*, 2005). A actividade industrial é a principal responsável pela emissão de importantes quantidades de Cr para o ambiente aquático, em especial a indústria têxtil, siderúrgica, metalúrgica, de cromagem, galvanização e produção de aço inoxidável. Os efluentes domésticos e de curtumes assumem ainda particular relevo, assim como lixiviados de solos agrícolas tratados com fertilizantes contendo Cr. Assim, este metal pode ser encontrado no ambiente aquático em concentrações até 110 μg/L (Pekey *et al.*, 2004). A exposição de peixes a Cr pode causar alterações no

metabolismo dos hidratos de carbono (Nath e Kumar, 1988), imunosupressão (Khangarot *et al.*, 1999), genotoxicidade (De Lemos *et al.*, 2001), stresse oxidativo, bem como alterações histopatológicas (Farag *et al.*, 2005).

1.5. Misturas de contaminantes

Os contaminantes raramente ocorrem individualmente no ambiente, estando os organismos aquáticos, de um modo geral, expostos a misturas mais ou menos complexas de poluentes. A ocorrência de interacções toxicológicas entre contaminantes introduz deste modo uma dificuldade adicional na previsão dos seus efeitos. Um exemplo típico de interacção entre compostos químicos é aquela que decorre da presença simultânea de indutores e inibidores da actividade do CYP1A, como são respectivamente os HAP e metais pesados. Estudos prévios demonstraram redução da actividade de etoxiresorufina-*O*-desetilase (EROD) em peixes expostos simultânea ou sequencialmente a estas duas classes de compostos (Viarengo *et al.*, 1997; Sorrentino *et al.*, 2005). Níveis anormalmente baixos de actividade EROD foram também observados em peixes colhidos em locais poluídos por hidrocarbonetos e que apresentavam elevada concentração de metais pesados nos tecidos (Romeo *et al.*, 1994). Uma interacção semelhante ocorre entre indutores da actividade de EROD e etoxilados de alquifenóis (Vaccaro *et al.*, 2005). Em ambos os exemplos referidos, os baixos níveis da actividade de EROD deverão ser interpretados não como resultado da ausência de compostos indutores, mas antes como uma impossibilidade de expressar a acção dos indutores pela presença de inibidores.

Uma interacção toxicológica ocorre quando a exposição a dois ou mais compostos químicos resulta numa resposta biológica quantitativa e qualitativamente diferente daquela que seria de esperar pela soma da acção individual de cada composto. Estas interacções podem configurar situações de sinergismo, potenciação ou antagonismo (Connell *et al.*, 1999). O sinergismo e a potenciação referem-se a situações em que o efeito tóxico da mistura é superior ao da soma dos efeitos dos

compostos químicos separadamente. Na potenciação, assume-se que um dos compostos, individualmente, tem pouca ou nenhuma toxicidade, enquanto que no sinergismo, ambos os compostos apresentam já toxicidade considerável individualmente. Por outro lado, o antagonismo refere-se à situação em que a exposição simultânea ou sequencial a dois ou mais compostos químicos, resulta num efeito menor que o esperado se a exposição fosse feita separadamente. Assim, predizer o impacto de misturas de compostos químicos com base no conhecimento dos efeitos individuais de cada composto pode não reflectir o que realmente se passa no organismo, tornando-se pois indispensável o conhecimento prático das respectivas interacções.

A ocorrência de misturas de contaminantes no ambiente pode ser o resultado quer da emissão de efluentes por si só complexos, quer de diferentes descargas com origem e composição diversas. Os sistemas lagunares, costeiros ou de águas interiores, pelas suas características hidrológicas, assim como pela concentração de aglomerados urbanos e unidades industriais na sua periferia, são ecossistemas que tipicamente exibem situações de multi-contaminação, resultantes dos dois cenários anteriormente descritos.

Um exemplo de efluente de enorme complexidade é o que resulta da indústria de pasta de papel. Os efluentes da pasta de papel (EPP) são constituídos por mais de 300 compostos distribuídos por diferentes classes, tais como os ácidos resínicos (AR), ácidos gordos, clorofenóis, dioxinas e furanos clorados, cloroguaicois e clorocatecois. Estes efluentes demonstraram ser responsáveis por efeitos adversos em peixes, nomeadamente alterações no metabolismo dos hidratos de carbono (Santos e Pacheco, 1996; Landman *et al.*, 2005), distúrbios hormonais (Santos e Pacheco, 1996; Sepúlveda *et al.*, 2001), indução de enzimas de biotransformação (Larsson *et al.*, 2002), stresse oxidativo (Santos *et al.*, 2004; Oakes *et al.*, 2004) e vitelogénese (Sepúlveda *et al.*, 2001), genotoxicidade (Ericson e Larsson, 2000; Maria *et al.*, 2004) e imunosupressão (Aaltonen *et al.*, 2000), bem como alterações histopatológicas (Couillard *et al.*, 1999; Pacheco e Santos, 2002b).

2. RESPOSTAS BIOLÓGICAS A CONTAMINANTES

No contexto da exposição a contaminantes, o termo "resposta biológica" refere-se ao conjunto de reacções de um organismo no sentido da manutenção da homeostase, assim como a alterações mais severas, não incluídas nos processos anteriormente referidos os quais representam desequilíbrios, que configuram situações de toxicidade. As respostas biológicas, a nível molecular e bioquímico, resultantes da exposição a contaminantes, quando não devidamente compensadas por homeostasia, poderão expressar-se a níveis superiores de um modo progressivo resultando, por exemplo, em alterações que afectam a reprodução, o crescimento e o comportamento. Estas alterações poderão afectar, em última análise, a sobrevivência dos organismos levando ao declínio de populações, comunidades e ecossistemas. As respostas biológicas a nível biomolecular e/ou bioquímico são indicações precoces de situações de stresse, podendo ser utilizadas como um sistema de alerta que permita prever e evitar efeitos tóxicos significativos. Assim, um vasto leque de respostas biológicas, a diversos tipos de contaminantes do meio aquático, tem sido estudado em peixes, pelo que se segue nos pontos seguintes uma síntese daquelas que foram alvo de estudo na presente tese.

2.1. Biotransformação

No meio aquático, o contacto permanente dos peixes com a água promove a absorção directa de contaminantes a partir desta ou do sedimento, através dos sistemas respiratório (via brânquias) e, em menor grau, através da pele. A absorção pode igualmente ter lugar através do sistema digestivo por via alimentar. A distribuição dos contaminantes pelos tecidos após absorção é influenciada por vários processos fisiológicos e pelas propriedades químicas dos mesmos, tais como a lipofilicidade e a sua capacidade de ligação a macromoléculas. Os compostos químicos, aquando da sua eliminação, podem encontrar-se na sua forma parental ou ter sofrido biotransformação. A eliminação desses pode ocorrer através das brânquias, pele, bílis e urina. A biotransformação de xenobióticos ocorre

maioritariamente no fígado e rim, e em menor grau nas guelras e mucosa intestinal. Os xenobióticos podem sofrer nestes órgãos, uma série de reacções sequenciais conducentes à sua activação, desintoxicação, acumulação e excreção. A biotransformação, em termos gerais, é entendida como a conversão de um composto noutro, através de um conjunto de processos catalisados biologicamente. A função principal da biotransformação é a conversão de xenobióticos lipofílicos em produtos mais hidrossolúveis, facilitando a sua conjugação e respectiva excreção, reduzindo deste modo a sua actividade biológica e tornando-os menos tóxicos. Contudo, convém salientar que a biotransformação conduz por vezes à bioactivação dos contaminantes, formando metabolitos toxicologicamente mais potentes do que os compostos parentais, como por exemplo alguns HAP e fungicidas.

Williams (1959) propôs a estruturação do processo de biotransformação em reacções de fase I e fase II, mantendo-se contudo presentes os conceitos de interdependência e continuidade entre estas duas etapas. Alguns autores consideram ainda a existência de uma fase III que consiste na metabolização adicional dos produtos resultantes das reacções de conjugação da fase II, catalisadas por enzimas também envolvidas nas reacções de fase I e/ou II (Vermeulen, 1996). Apesar da maioria dos xenobióticos serem submetidos consecutivamente às reacções de fase I e II, alguns podem ser submetidos apenas a uma dessas fases.

Os mecanismos de fase I incluem reacções de oxidação, redução ou hidrólise que podem activar ou inactivar a acção tóxica dos xenobióticos. As oxidações por monooxigenases associadas ao citocromo P450 ocorrem no retículo endoplasmático liso, através da inserção de um átomo de oxigénio no substrato (Nelson *et al.*, 1996). As monooxigenases possuem grande capacidade para metabolizar xenobióticos orgânicos e endobióticos, tais como esteróides e ácidos gordos, encontrando-se especialmente no fígado (Stegeman e Hahn, 1994), embora a sua actividade também tenha sido detectada no rim, tracto intestinal e tecido branquial.

A análise das inúmeras investigações realizadas nesta área, demonstra uma acentuada concentração de trabalhos no estudo da expressão do CYP1A, e em

particular das suas actividades enzimáticas de 7-etoxiresorufina *O*-desetilação (EROD), 7-etoxicumarina *O*-desetilação (ECOD) e aril hidrocarbono hidroxilação (AHH). Esta maior incidência de trabalhos está relacionada com o facto do CYP1A ser a principal forma de monooxigenase dependente do citocromo P450 (P450), em peixes, passível de ser induzida por xenobióticos orgânicos (Goksøyr *et al.*, 1991). Assim, a indução da actividade das monooxigenases microssomais hepáticas associadas ao P450 tem sido largamente utilizada em estudos realizados em peixes como uma resposta característica à presença de poluentes orgânicos, tais como HAP (Maria *et al.*, 2002; Sorrentino *et al.*, 2005), policlorodibenzo-*para*-dioxinas (PCDD) e policloro dibenzofuranos (PCDF) (Orrego *et al.*, 2005), bifenilo policlorados (PCB) (Vaccaro *et al.*, 2005) e terfenilos policlorados (Hale *et al.*, 1998). A indução destas monooxigenases tem sido também utilizada no âmbito da exposição a outros contaminantes, tais como pesticidas (Egaas *et al.*, 1999) ou ainda misturas de contaminantes, como os EPP (Pacheco e Santos, 1999; Larsson *et al.*, 2002). Contudo, alguns contaminantes ambientais tal como metais pesados (Sorrentino *et al.*, 2005) e compostos organoestânicos (Fent e Stegeman, 1993), podem actuar como inibidores da actividade do P450. Diversos indutores da síntese de CYP1A podem também inibir a sua actividade catalítica, tendo sido esta duplicidade de acções encontrada em peixes expostos a concentrações elevadas de PCB (Hahn *et al.*, 1993) e HAP (Haash *et al.*, 1993; Gravato e Santos, 2002c).

Os produtos da fase I e outros xenobióticos, contendo grupos funcionais como hidroxilo, amino, epóxido ou halogéneo, sofrem na fase II da biotransformação, reacções de conjugação com compostos polares endógenos, tais como o ácido glucorónico, sulfato, glutationa ou determinados aminoácidos como a glicina e a glutamina. Os produtos de conjugação, com raras excepções, são menos tóxicos, mais polares e mais facilmente excretáveis que os compostos químicos parentais. A actividade de enzimas de fase II, como a glutationa *S*-transferase (GST) e UDP-glucuronosil transferase (UDPGT), pode ser modulada em peixes pela exposição a uma variedade de compostos tais como as dioxinas (Guosheng *et al.*, 1998), HAP (Gravato e Santos, 2003), metais pesados (Ahmad *et al.*, 2005), compostos

organoestânicos (Wang *et al.*, 2005), pesticidas (Peixoto *et al.*, 2006) e E_2 (Vaccaro *et al.*, 2005).

2.2. Genotoxicidade

Alguns compostos químicos podem induzir alterações no material genético, sendo por isso designados de genotóxicos. A exposição a este tipo de compostos, mesmo em pequenas quantidades, podem originar disfunções celulares e tecidulares através das alterações induzidas no genoma, por exemplo como resultado da sua ligação covalente com as bases do ADN formando aductos (Dolcetti *et al.*, 2002; Hellou *et al.*, 2005). Esses aductos do ADN podem ser responsáveis por mutações impedindo a replicação fidedigna do ADN ou desencadeando processos de reparação do ADN, nem sempre efectivos (Maccubbin, 1994), os quais poderão levar a uma gradual acumulação de erros no material genético.

A avaliação das diferentes expressões de genotoxicidade tem sido levada a cabo recorrendo a métodos bioquímicos, moleculares e citogenéticos. Os métodos bioquímicos, tais como o desenrolamento alcalino (Everaarts *et al*., 1998) e a eluição alcalina do ADN (Bihari *et al*., 1990) constituem as principais técnicas para avaliação das quebras do ADN. As técnicas citogenéticas assumem particular relevância no que respeita aos testes de troca entre cromátides irmãs ou técnica de arlequim (*sister chromatid exchange*, SCE) (Santos e Pacheco, 1995), aberrações cromossómicas (Jha *et al*., 2000), micronúcleos (Sanchez-Galan *et al.*, 2001) e anomalias nucleares eritrocíticas (ANE) (Pacheco e Santos, 1999; Ayllón e Garcia-Vazquez, 2000). As alterações citogenéticas que estão na base das metodologias anteriores são entendidas como expressões de toxicidade genética, pressupondo a existência de acontecimentos moleculares anteriores, como a formação de aductos do ADN. De entre os parâmetros anteriormente mencionados, recentemente têm assumido particular relevância o teste de ANE devido à sua facilidade e rapidez de execução, sensibilidade, baixo custo e fiabilidade demonstrada em diferentes espécies de peixes expostos a metais pesados (Ayllón e Garcia-Vazquez, 2000),

fenóis clorados (Farah *et al.*, 2003), HAP (Pacheco e Santos, 2002a), assim como EPP (Maria *et al.*, 2004) e efluentes de uma refinaria de petróleo (Çavaş e Ergene-Gözükara, 2005).

Diversos estudos demonstraram claramente uma relação próxima entre os níveis de contaminação ambiental, níveis de contaminantes nos tecidos, e incidência de cancro, mutações e malformações nos embriões em diversas espécies de peixes (Connell *et al*., 1999).

2.3. Alterações endócrinas

Uma preocupação crescente, relativa à presença no ambiente de determinados compostos que podem interferir de algum modo com o sistema endócrino dos organismos, emergiu recentemente. A repetida constatação de efeitos adversos sobre diversas populações da fauna selvagem e até no próprio Homem, foi o ponto de partida para a eclosão mundial de inúmeras pesquisas acerca dos designados desreguladores endócrinos (DE). Após a publicação do livro *Our Stolen Future* (Colborn *et al.*, 1996), que reúne e sistematiza os conhecimentos acumulados sobre as alterações endócrinas provocadas por vários compostos químicos existentes no ambiente, essa preocupação deixou de ser exclusiva da comunidade científica, estendo-se também ao público em geral.

O conceito de desregulador ou disruptor endócrino (*endocrine disruptor*) está associado a compostos ou misturas de compostos exógenos, capazes de assumir função idêntica à de uma hormona natural ou inibir o funcionamento normal da mesma. Assim, as funções do sistema endócrino são alteradas, afectando a saúde do organismo e/ou da sua descendência (EDSTAC, 1998; IPCS, 2002).

Os DE são derivados de compostos químicos de origem antropogénica, na sua maioria provenientes de actividades industriais e domésticas, relacionadas com a utilização/produção de plásticos e detergentes, bem como do uso de alguns pesticidas. Em geral, estes compostos são estáveis, lipofílicos e semi-voláteis, o que facilita a sua rápida e vasta dispersão no ambiente, tendo como principais veículos

os recursos hídricos (Mills e Chichester, 2005), pelo que poderão ser bioacumulados ao longo da cadeia trófica durante largos períodos de tempo. Algumas hormonas "naturais", tais como o E_2 e a estrona, também passaram a ser recentemente consideradas DE (Ying *et al.*, 2002; Imai *et al.*, 2005).

A acção do sistema endócrino envolve três elementos distintos, nomeadamente, um conjunto de glândulas especializadas, mensageiros químicos (hormonas) sintetizados nessas glândulas e células alvo. Os mecanismos de activação do sistema endócrino têm início na reacção das células nervosas a um determinado estímulo (ex. fotoperíodo, presença de xenobiótico) e consequente envio de um sinal às glândulas endócrinas. Estas, por sua vez, libertam as hormonas adequadas, que se ligam a receptores específicos e se traduzem em instruções para as células alvo. O receptor interpreta a mensagem hormonal fazendo a sua tradução mediante um de dois processos celulares distintos: (i) indução de genes para a produção de novas proteínas e (ii) alteração da actividade de proteínas existentes na célula causando uma resposta rápida.

As glândulas endócrinas são o alvo preferencial dos contaminantes devido à sua organização anatómica associada a uma elevada vascularização. Este facto acentua a relevância e o risco ecológico dos DE, cujo estudo dos mecanismos de acção tem incidido na sua interacção com os receptores hormonais. Alguns compostos químicos contaminantes do meio ambiente podem, por exemplo, mimificar a acção das hormonas endógenas, levando o organismo a desencadear de um modo exagerado ou em tempo inapropriado um estímulo falso, sendo o fenómeno designado por efeito agonista. Outros xenobióticos podem ligar-se aos receptores específicos bloqueando-os, reduzindo ou anulando a respectiva estímulação, sendo este fenómeno denominado de efeito antagonista. Contudo, considerando a complexidade da acção endócrina, é plausível a existência de outros mecanismos de desregulação, que vão desde a acção directa nos órgãos até à interacção das próprias hormonas com o genoma (Reys, 2001). A alteração ou desregulação da função endócrina pode também estar associada à interferência a diferentes níveis, como sejam a síntese, secreção, transporte no plasma, ligação e

acção das hormonas, assim como a sua eliminação, conduzindo a uma nova resposta hormonal. Os principais factores que influenciam o modo de actuação dos DE no organismo relacionam-se com as propriedades químicas dos compostos, a sua concentração, o tipo de interacção, tempo e momento de exposição, factores genéticos, especificidade dos tecidos, idade e sexo. A acção hormonal resulta de estímulos induzidos por concentrações muito baixas de hormonas, devendo salientar-se que essa é a razão pela qual teores vestigiais de DE podem ser tão perniciosos para os organismos.

2.3.1. Disfunção de processos reprodutivos

A grande maioria dos estudos realizados em peixes sobre os efeitos adversos dos DE tem sido dirigida para aspectos reprodutivos. O controlo neuroendócrino da reprodução é dependente da acção coordenada de várias hormonas associadas ao eixo hipotálamo-pituitária-gónadas (HPG). Os estímulos sensoriais externos e internos condicionam a secreção da hormona libertadora da gonadotrofina (GnRH) pelo hipotálamo, a qual, por sua vez, regula a libertação de gonadotrofinas (GTH-I e GTH-II) pela pituitária. A GTH-I é funcionalmente similar à hormona folículo-estimuladora (FSH) e a GTH-II é similar à hormona luteinizante (LH) dos mamíferos (Schulz *et al.*, 2001). As principais células-alvo das gonadotrofinas são as células da granulosa e da teca no ovário das fêmeas e as células de Sertoli e Leydig nos testículos dos machos (Janz, 2000). Estas células, após estimulação pelas gonadotrofinas, sintetizam esteróides sexuais (estrogénios e androgénios) e de maturação (progesteronas). O E_2 e a estrona são os estrogénios mais importantes nas fêmeas, desempenhando um papel fundamental na diferenciação sexual, desenvolvimento dos órgãos reprodutores e indução do comportamento sexual. Nos machos, a 11-cetotestosterona e a testosterona são os androgénios mais importantes, sendo responsáveis pelo seu desenvolvimento sexual (Borg, 1994). As progesteronas são importantes na maturação dos ovócitos. Nos teleósteos, a

produção de esteróides sexuais é controlada por um sistema de retrocontrolo ligado ao eixo HPG.

A quantificação de E_2 no plasma de peixes tem sido utilizada como indicador da presença de DE no ambiente aquático, em particular de xenoestrogénios (substâncias que modulam ou mimificam a acção dos esteróides sexuais, principalmente do E_2). Assim, diversos autores observaram alteração dos níveis de E_2 plasmático após exposição a etoxilados de alquifenóis (Soverchia *et al.*, 2005), pesticidas (Singh e Canário, 2004), HAPs (Seruto *et al.*, 2005), PCBs (Jung *et al.*, 2005) e EPPs (McMaster *et al.*, 1996), bem como a misturas complexas inespecíficas (Sepúlveda *et al.*, 2002). Deste modo, alterações nos níveis plasmáticos de E_2 induzidas por contaminantes ambientais podem pôr em causa o sucesso reprodutivo. Por outro lado, elevados níveis de E_2 na água podem causar efeitos adversos nos peixes, tais como indução de intersexualidade em machos (Metcalfe *et al.*, 2001), redução do índice gonado-somático (Bjerselius *et al.*, 2001) e da produção de ovos (Oshima *et al.*, 2003), assim como alteração do comportamento reprodutivo em machos (Bjerselius *et al.*, 2001).

Além dos efeitos anteriormente referidos, uma das respostas mais importantes à presença de xeno/estrogénios é a indução da transcrição e tradução de determinadas proteínas, nomeadamente de uma fosfolipoglicoproteína, a Vtg. A sua síntese é um processo estimulado pelo E_2 envolvendo o controlo coordenado entre o hipotálamo, pituitária, fígado e gónadas (Thomas, 1990). A Vtg é sintetizada no fígado de peixes ovíparos, transportada até aos ovários onde é sequestrada e incorporada nos ovócitos durante o seu desenvolvimento. A Vtg é clivada proteoliticamente, após sequestro, formando as proteínas do vitelo (lipovitelina e fosfovitina). Assim, a Vtg constitui a principal fonte de nutrição do embrião em desenvolvimento, podendo ser encontrada no plasma de fêmeas maduras em concentrações elevadas. Normalmente, a presença de níveis significativos de Vtg no plasma ocorre apenas em fêmeas maduras. No entanto, os peixes juvenis e machos maduros possuem receptores de estrogénios (Kishida *et al.*, 1992), assim como um gene silencioso da Vtg, geralmente presente mas não expresso (Moncaut *et al.*,

2003). Deste modo, os machos e juvenis podem responder à presença de xeno/estrogénios, produzindo Vtg, a qual tende a permanecer no plasma em níveis elevados devido à falta de mecanismos de eliminação (Funkenstein *et al.*, 2000). Vários autores observaram aumentos da concentração de Vtg no plasma de diferentes espécies de peixes após exposição a E_2 e xenoestrogénios (Arukwe *et al.*, 1998; Berg *et al.*, 2004; Andreassen *et al.*, 2005; Soverchia *et al.*, 2005), o que forneceu evidência da grande sensibilidade deste parâmetro como indicador da exposição a DE.

2.3.2. Disfunção de processos não reprodutivos

Atendendo à concentração de trabalhos científicos que incidem nos mecanismos de acção dos DE na reprodução, poder-se-ia inferir que a acção destes se limita a aspectos da biologia da reprodução. No entanto, à medida que a investigação foi sendo aprofundada, concluiu-se que o sistema endócrino não-reprodutor poderia também ser um alvo potencial de desregulação por acção destes xenobióticos. Neste contexto, os eixos hipotálamo-pituitária-tecido interrenal (HPI) e hipotálamo-pituitária-tiróide (HPT) têm particular relevância pelo seu papel essencial nos mecanismos homeostáticos em peixes (Hontela, 1997; Janz, 2000). O cortisol é o principal corticoesteróide em peixes, sendo também o mais abundante e activo, ocorrendo a sua síntese em células do tecido interrenal, situadas na porção cefálica do rim. A síntese e libertação de cortisol na circulação sanguínea envolvem um sistema de retrocontrolo associado ao eixo HPI. Quando o organismo é submetido a uma situação de stresse, o hipotálamo produz a hormona libertadora da corticotrofina (CRH), a qual controla a síntese da hormona adrenocorticotrófica (ACTH) pela pituitária anterior, que por sua vez, estimula a síntese e libertação de cortisol pelo tecido interrenal (Hontela, 1997). Uma parte do cortisol libertado, circula no plasma sanguíneo, ligado de modo reversível a proteínas, numa forma biologicamente inerte, sendo que apenas a sua forma livre apresenta actividade fisiológica (Hazon e Balmen, 1998).

Os principais órgãos-alvo do cortisol são as brânquias, fígado e intestino, sendo também importante a sua acção sobre o músculo esquelético (Hontela, 1997; Hazon e Balment, 1998). O cortisol apresenta diversas acções fisiológicas, das quais se destacam os efeitos sobre o metabolismo intermediário (Hontela, 1997). Esta acção é crucial na manutenção da homeostase em situações de stresse prolongado e tende, em termos gerais, a aumentar a libertação de móleculas-substrato para a produção de energia, a partir das reservas corporais. Desta forma, é estimulado o catabolismo dos lípidos e proteínas, concomitantemente com a indução das aminotransferases e da gluconeogénese (Quintana, 2002). Para além dos efeitos anteriormente referidos, o cortisol exerce também a sua acção a níveis como a osmorregulação (Sakamoto e McCormick, 2006) e a função imune (Harris e Bird, 2000).

O aumento da concentração de cortisol no plasma de peixes foi demonstrado após exposições de curto prazo a uma vasta gama de contaminantes, desde metais pesados (De Boeck *et al.*, 2003), AR (Kennedy *et al.*, 1995), HAP (Pacheco e Santos, 2001a) e pesticidas (Waring e Moore, 2004), até hormonas "naturais" como o E_2 (Pottinger *et al.*, 1996). Apesar das evidências anteriores, a resposta dos peixes em termos de dinâmica do cortisol revelou-se mais complexa, tendo sido demonstrado que peixes expostos cronicamente a contaminantes ambientais eram incapazes de elevar os seus níveis de cortisol plasmático como resposta ao stresse resultante da captura. Este facto foi observado em peixes de águas poluídas com metais pesados, HAP, PCB, bem como EPP (Hontela, 1997). Níveis baixos de cortisol no plasma foram também detectados em exposições de curto prazo a AR (Kennedy *et al.*, 1995), derivados de pesticidas organoclorados (Benguira *et al.*, 2002), EPP (Santos e Pacheco, 1996), assim como à fracção solúvel de combustíveis fósseis (Pacheco e Santos, 2001b).

O valor informativo das alterações da dinâmica do cortisol, no que respeita a diferentes formas de stresse deve ser entendido como parte de uma resposta mais generalizada e complexa. O aumento de cortisol no plasma, precedido de um aumento de catecolaminas, constitui apenas uma etapa na sequência de

acontecimentos adaptativos mobilizadores de energia. Assim, alterações no metabolismo intermediário são também reconhecidas como potenciais indicadores de stresse. Neste contexto, a elevação dos níveis plasmáticos de lactato e glucose tem demonstrado a sua eficiência no que respeita à detecção de exposições de peixes a pesticidas (Sancho *et al.*, 1997), metais pesados (Hontela *et al.*, 1996) e EPP (Santos *et al.*, 1990).

Na maioria dos teleósteos, a tiróide é composta por grupos de folículos distribuídos de uma forma difusa, principalmente, em torno da faringe ventral onde são sintetizadas as hormonas tiroxina (T4) e triiodotironina (T3). Os folículos da tiróide podem também desenvolver-se em locais secundários como os ovários, rim e pericárdio (Hazon e Balment, 1998; Janz, 2000). A síntese e libertação destas hormonas no sangue são controladas por um sistema de retrocontrolo associado ao eixo HPT. O hipotálamo produz a hormona libertadora da tirotrofina (TRH), a qual controla a síntese da hormona estimuladora da tiróide (TSH) pela pituitária anterior, que por sua vez estimula a síntese e libertação de T4 e T3 pelos folículos da tiróide. Nos peixes, tal como nos outros vertebrados, existe uma síntese muito maior de T4 do que de T3. Estas hormonas circulam no sangue associadas a proteínas transportadoras (Power *et al.*, 2001) e apenas a fracção livre se pode difundir para os tecidos periféricos (Eales e Brown, 1993). Quando a T4 entra em contacto com os tecidos-alvo é, na sua maioria, convertida em T3 via 5'-monodesiodase, existindo fortes provas de que esta desempenha um papel fisiológico mais relevante (Hazon e Balment, 1998).

As hormonas tiróideias contribuem para o controlo do metabolismo, osmorregulação, crescimento e desenvolvimento (Hazon e Balment, 1998; Yamano, 2005). Alterações nos níveis plasmáticos de hormonas da tiróide foram detectadas em peixes após exposição a PCB (Brown *et al.*, 2004), metais pesados (Carletta *et al.*, 2002) e pesticidas (Thangavel *et al.*, 2005).

As hormonas da tiróide e o cortisol podem interagir e influenciar conjuntamente o metabolismo dos hidratos de carbono (Hontela *et al.*, 1995); no entanto, pouco se sabe acerca destas interdependências em peixes.

2.4. Respostas Biológicas como Indicadores de Poluição Ambiental

Para se proceder à avaliação do estado de um determinado ecossistema, cada vez mais se tem recorrido à adopção de programas de biomonitorização ambiental, baseados na medição de respostas biológicas, as quais são genericamente designadas de biomarcadores. Um biomarcador é definido como uma resposta biológica induzida, geralmente, por xenobióticos, mensurável em sistemas biológicos, ou amostras destes, abrangendo diferentes níveis, desde molecular, celular ou fisiológico até comportamental (NRC, 1989; WHO, 1993). Num contexto ambiental, os biomarcadores são considerados ferramentas altamente informativas no que respeita à detecção da exposição a contaminantes ou dos respectivos efeitos (Van der Oost *et al.*, 2003). A utilização de biomarcadores oferece ainda a possibilidade de obter um entendimento mecanístico ou de causa-efeito dos processos biológicos. Adicionalmente, esta estratégia apresenta uma vantagem, relativamente à monitorização química, que resulta do facto dos resultados integrarem eventuais interacções toxicológicas que podem ocorrer na sequência da exposição a misturas de contaminantes (Van der Oost *et al.*, 2003).

De acordo com o National Research Council (NRC, 1989) e a World Health Organization (WHO, 1993), os biomarcadores podem ser divididos em três classes: (i) biomarcadores de exposição - abrangem a detecção e quantificação de um composto exógeno, dos seus metabolitos ou da interacção entre este e moléculas ou células alvo, sendo medidos num compartimento do organismo; (ii) biomarcadores de efeito - incluem alterações bioquímicas, fisiológicas e outras, sendo mensuráveis nos tecidos ou fluídos corporais e reconhecidamente associadas a possíveis desequilíbrios; (iii) biomarcadores de susceptibilidade - indicam a capacidade inerente ou adquirida de um organismo alterar a susceptibilidade a uma exposição, envolvendo nomeadamente factores genéticos. Apesar das respostas medidas a níveis organizacionais mais básicos poderem não ter reflexo directo ao nível, por exemplo, da população ou comunidade, podem funcionar como um instrumento de vigilância ambiental que permite identificar o risco a esses níveis. Adicionalmente,

possibilitam intervenções correctivas que impedem que os efeitos adversos sejam transpostos para níveis superiores.

Na sequência do anteriormente exposto, as respostas biológicas a contaminantes consideradas na presente tese e anteriormente apresentadas, podem ser encaradas como potenciais biomarcadores, não obstando contudo a que, dependendo dos objectivos em causa, o seu estudo possa ser mais ou menos dirigido para o entendimento dos mecanismos envolvidos nas respostas tóxicas.

3. ORIENTAÇÕES E OBJECTIVOS DA TESE

A complexidade dos ecossistemas aquáticos torna a sua caracterização ecotoxicológica difícil, uma vez que as respostas dos organismos à presença de contaminantes dependem de uma série de factores bióticos e abióticos. Nesta perspectiva, deve recorrer-se a diferentes metodologias e estratégias de monitorização que podem englobar exposições laboratoriais, *in situ*, bem como amostragens de campo de espécimes selvagens.

As exposições laboratoriais proporcionam um grau de variabilidade menor relativamente às exposições de campo, permitindo um controlo de variáveis relativas à qualidade da água, tais como a temperatura, salinidade, oxigénio dissolvido, assim como outros factores abióticos incluindo o fotoperíodo. A anterior forma de exposição permite ainda a selecção de uma determinada espécie num estado específico de desenvolvimento, o controlo sobre o número de indivíduos expostos, o número de réplicas, o tipo de composto a testar e sua concentração, bem como o tempo e vias de exposição (Lindström-Seppä e Oikari, 1990). Apesar das vantagens anteriormente referidas relativamente a exposições laboratoriais, estas não reflectem completamente a complexidade do tipo de exposição que ocorre nos sistemas naturais, o que dificulta a extrapolação dos dados laboratoriais para situações reais de campo (Maycock *et al.*, 2003). Assim, as observações laboratoriais podem estar sub ou sobrestimados, pelo que se torna vantajosa e recomendável a sua validação no campo. Os dados fornecidos por estudos de campo usando espécies indígenas reflectem melhor aquilo que acontece no "mundo real" (Sibley *et al.*, 1999). No entanto, aspectos como a mobilidade dos peixes, distribuição espacial dos contaminantes, imprecisão do tempo de exposição e desconhecimento do "historial" dos peixes são factores limitantes para a interpretação dos dados de campo.

Algumas das variáveis associadas aos estudos de campo podem ser eliminadas recorrendo ao transplante de organismos para o local cuja contaminação se quer avaliar – exposições *in situ*. Esta estratégia é considerada uma ferramenta fundamental para a toxicologia aquática, reduzindo a incerteza associada às extrapolações laboratório-campo (Burton *et al.*, 2000) e constituindo uma abordagem

intermédia em que se reduzem as limitações dos estudos de campo e se usufrui de parte das vantagens das exposições laboratoriais. Contudo, as exposições *in situ* apresentam ainda algumas limitações, tais como a dificuldade em encontrar locais de referência adequados, o risco de vandalismo, os efeitos do enclausuramento dos organismos, a acção predatória e a fraca aplicabilidade em sistemas profundos e de fortes correntes (Burton *et al.*, 2005). Apesar das referidas limitações, esta estratégia tem sido largamente utilizada para prever efeitos em comunidades indígenas e orientar estudos futuros mais definitivos relativamente à quantificação de químicos, testes laboratoriais de toxicidade, bem como à adopção de medidas preventivas (Pacheco e Santos, 1999; Schulz, 2003; Burton *et al.*; 2005). A presente tese baseou-se na utilização de duas estratégias – ensaios laboratoriais e exposições *in situ* – representando uma evolução do estudo no sentido da máxima aproximação à situação real de campo (captura), cuja ausência não deve ser encarada como uma minimização da sua importância.

Os ambientes aquáticos poluídos são tipicamente caracterizados pela presença de misturas de contaminantes e não pela presença de um ou de outro contaminante isolado. As estratégias do presente estudo tiveram subjacente a ideia de que a previsão do resultado da interacção entre diferentes contaminantes pode ser imprecisa se baseada apenas no estudo do impacto dos compostos individuais. Contudo, o recurso à experimentação laboratorial com contaminantes isolados foi também considerado como um ponto de partida incontornável em direcção ao estudo do impacto de misturas. Assim, a estratégia global evoluiu para a realização de ensaios laboratoriais com misturas simples e cuja complexidade poderá de futuro ser progressivamente aumentada.

Os peixes utilizados no presente trabalho de investigação encontravam-se em estados de imaturidade sexual. A razão desta escolha prendeu-se com o facto de se pretender diminuir a interferência de processos metabólicos associados à reprodução em outros processos chave sob estudo. Assim, o conhecimento da acção tóxica de xenobióticos presentes na água sobre juvenis, poderá informar e prevenir essa acção em estádios posteriores de desenvolvimento.

Os objectivos gerais da presente tese assentam na investigação de efeitos induzidos por contaminantes ambientais em três espécies de teleósteos (*D. labrax*, *A. anguilla* e *S. aurata*), abrangendo o nível citogenético e diferentes vertentes ao nível fisiológico.

A presente tese engloba três perspectivas distintas mas complementares, relativamente ao tipo de exposição a contaminantes e substâncias padrão a que os organismos teste foram submetidos. Assim, estabeleceu-se um plano de trabalho que incluiu os seguintes objectivos:

(i) Estudo dos efeitos de exposições laboratoriais a contaminantes isolados, nomeadamente ácidos abiético e desidroabiético, reteno, β-naftoflavona (BNF), naftaleno e E_2 (capítulos II a VI);

(ii) Estudo dos efeitos de exposições laboratoriais a diferentes contaminantes, quer em misturas simples (BNF+E_2, BNF+NP, E_2+NP), quer em exposições sequenciais (BNF+Cr, BNF+Cu) (capítulos VII a IX);

(iii) Estudo dos efeitos de exposições *in situ* em ambientes aquáticos poluídos (Ria de Aveiro, Rio Vouga e Pateira de Fermentelos) (capítulos X e XI).

Os compostos testados incluem-se em diferentes classes de contaminantes do meio aquático, tendo sido seleccionados pela sua importância a nível global e regional, assim como pelo relevo que o seu estudo assume, dada a relativa escassez de informação no que respeita aos seus efeitos sobre peixes.

Os parâmetros avaliados foram: conteúdo em citocromo P450, actividade de EROD, GST e alanina transaminase (ALT) no fígado, índice hepato-somático (IHS), frequência de ANE e eritrócitos imaturos (EI), níveis plasmáticos de cortisol, glucose, lactato, TSH, T3, T4, E_2 e Vtg.

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CAPÍTULO II

Alterações nos níveis plasmáticos de cortisol, glucose e lactato em *Anguilla anguilla* L. exposta a ácido abiético, ácido desidroabiético e reteno

***Anguilla anguilla* L. plasma cortisol, lactate and glucose responses to abietic acid, dehydroabietic acid and retene**

M. Teles, V.L. Maria, M. Pacheco e M.A. Santos (2003)
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ABSTRACT

Anguilla anguilla L. were exposed to 0, 0.1, 0.3, 0.9 and 2.7 μM abietic (AA), dehydroabietic (DHAA) acids and retene (Re) during 8, 16, 24 and 72 h. The eels plasma cortisol, glucose and lactate were measured. A significant decrease in plasma cortisol was observed at 72 h exposure to 0.9 μM and 2.7 μM Re. DHAA (0.1 μM) significantly decreased plasma cortisol in eels after 8 and 24 h exposure. However, a significant plasma cortisol increase was found after 16 h, 2.7 μM AA exposure and after 24 h exposure to 0.1 μM and 2.7 μM AA. Furthermore, 72 h exposure to.0.9 μM AA also induced a plasma cortisol increase. A general rise in plasma glucose was detected after all exposure periods to Re. The plasma lactate also increased after 72 h exposure to 2.7μM AA and after 8 h exposure to 0.1 μM DHAA.

Keywords: *A. anguilla*; Abietic acid; Dehydroabietic acid; Retene; Cortisol; Glucose; Lactate; Endocrine disruption.

INTRODUCTION

Bleached kraft pulp mill effluents (BKPME) are complex mixtures of environmentally active compounds (Nestmann *et al*., 1980). The most prevalent compounds in BKPMEs are resin acids (RAs) representing an important toxicant group to fish. Abietic (AA) and dehydroabietic (DHAA) acids are the most abundant among the seven RAs. Thermal degradation of abietic-type RAs as well as anaerobic microbial DHAA degradation (Makris and Banerjee, 2002) represents important retene (Re) sources. Thus, the effluent treatment can also originate active metabolites such as Re due to anaerobic zones within aeration ponds.

Farm or wild eel's 4 h exposure to the 50% secondary treated bleached kraft pulp mill effluent (STBKPME), after 188 h recovery from capture, anoxia and transport, induces plasma lactate increase and prevents interrenal cortisol release to the blood, decreasing its plasma concentration (Santos and Pacheco, 1996). Additionally, cortisol and lactate uptake from plasma are depressed in eels after 6 h

recovery from capture, anoxia and transport, at 25 ºC in 50% STBKPME. The use of plasma cortisol as an indicator of physiological status has been well documented in numerous studies (reviewed by Hontela, 1997). Recent *in vivo* studies demonstrated that some environmental pollutants could act as endocrine disrupters in fish (Van der Kraak *et al.*, 1992), and one of the endocrine targets of xenobiotics seems to be the hypothalamo-pituitary-interrenal (HPI) axis (Hontela, 1998).

Acute stress, such as handling and confinement (Iwama *et al.*, 1976), exposure to low pH, heavy metals (Hontela *et al.*, 1995), chlorinated resin acids (RAs) (Kennedy *et al.*, 1995) and polycyclic aromatic hydrocarbons (PAHs) (Thomas and Rice, 1987) induced a plasma cortisol increase in several fish species. Further studies also demonstrated that eels exposed either to diesel water-soluble fraction (Pacheco and Santos, 2003) or to naphthalene (Teles *et al.*, 2003) had their plasma cortisol levels decreased. According to Hontela *et al.* (1997) and McMaster *et al.* (1994), fish sampled at contaminated sites had an impaired capacity to increase their plasma cortisol levels in response to stress. Nevertheless, it remains difficult to determine if the toxicological effects of pollutants occur at the level of cortisol synthesis in interrenal steroidogenic cells, upstream in the pituitary and adrenocorticotropin (ACTH) synthesis, in the hypothalamic cells, or elsewhere in the organism.

Changes in carbohydrate metabolism measured as plasmatic glucose and lactate can be used as general stress indicators in fish, and their relation to cortisol function were also investigated (Santos and Pacheco, 1996; Pacheco and Santos, 2001).

The vast majority of BKPME toxicity studies on fish concerns the effluent as a whole. Thus, the effects of single effluent compounds need to be investigated. The present research work intends to study the AA, DHAA and Re individual effects on *Anguilla anguilla* L. plasma lactate, glucose and cortisol levels.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade obtained from Sigma Chemicals Company (USA), Boehringer-Mannheim GmbH (Germany), and E. Merck-Darmstadt (Germany). Abietic acid and retene (7-isopropopyl-1-methylphenanthrene) were obtained from Sigma. Dehydroabietic acid was obtained from Helix Biotech (Canada).

Test Animals

A. anguilla L. with an average weight of 50 g and measuring 30±2 cm were captured at the Aveiro lagoon, Murtosa, Portugal. The eels were transported in anoxia and acclimated to laboratory conditions for 1 week prior to experimentation. During recovery and experimental periods, eels were kept in 80-L aquaria at 20 °C temperature under a natural photoperiod, in recirculating, aerated (dissolved oxygen: 7.4±0.2 mg/L), filtered and dechlorinated tap water with a pH of 7.1±0.2. Fish were fed neither under laboratory adaptation nor during the experimental procedure.

Experimental Design

Three different lots of eels were exposed during 8, 16, 24 and 72 h to abietic acid, dehydroabietic acid or retene in the following concentration range - 0 (control), 0.1, 0.3, 0.9 and 2.7 μM. Experiments were carried out using test groups of five eels (*n*=5). The appropriate amount of each chemical was previously dissolved in 1 mL of DMSO and added to the experimental aquaria. The same volume of DMSO was added to the control aquarium. This DMSO concentration has no effects (Pacheco and Santos, 1998). During the exposure period the water was not replaced.

Fish were killed at each sampling point and their blood was collected for plasma isolation using an Eppendorf centrifuge (14,000 rpm).

Biochemical Analyses

Cortisol, Lactate and Glucose Measurement

The determination of cortisol was performed in plasma using a diagnostic ELISA direct immunoenzymatic kit (Diametra, Italy, code 10011). Plasma lactate was determined with a diagnostic kit (Boehringer Mannheim GmgH, nº149993). Plasma glucose was measured using a diagnostic kit (Granutest, E. Merck-Darmstadt nº1.12194).

Statistical Analyses

Statistica software (StatSoft, Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. Variance analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P<0.05$.

RESULTS

Abietic Acid Experiment

The general trend in plasma cortisol is an increase observed from 16 up to 72 h (Fig. 1A). Nevertheless, a significant increase could be only observed at 72 h exposure to 0.9 μM, at 24 h exposure to 0.1 μM and to 2.7 μM, and at 16 h exposure to 2.7 μM AA. A significant glucose increase was observed after 24 h exposure to 0.9 and 2.7 μM and after 72 h to 0.3, 0.9, and 2.7 μM AA (Fig. 1B). A plasma lactate increase was observed after 72 h exposure to 2.7 μM AA.

Dehydroabietic Acid Experiment

A significant decrease in plasma cortisol levels was observed after 8 and 24 h exposure to 0.1 μM (Fig. 2A). A significant plasma glucose rise was found after 24 h exposure to 0.3 μM DHAA (Fig. 2B). Plasma lactate levels significantly increased only after 8 h exposure to 0.1 μM DHAA (Fig. 2C).

Retene Experiment

A decreasing tendency in plasma cortisol levels was observed during the whole experiment. However, this tendency was confirmed at 72 h where a statistically significant decrease was observed for 0.9 and 2.7 μM Re exposure (Fig. 3A). A plasma glucose rise was commonly found at all the exposure periods. Thus, a significant increase was observed after 8 h exposure to 0.1, 0.9 and 2.7 μM Re. After 16 h exposure to 2.7 μM Re, a significant increase in plasma glucose levels was observed, whereas at 24 h exposure, this increase was significant for all tested concentrations. Furthermore, eels showed a significant plasma glucose increase after 72 h exposure to 0.3, 0.9 and 2.7 μM (Fig. 3B). Despite the absence of statistically significant differences, a clear plasma lactate decrease was detected after 8 and 16 h exposure following the increase in Re concentration. However, this tendency seems to disappear for the longest exposure periods (Fig. 3C).

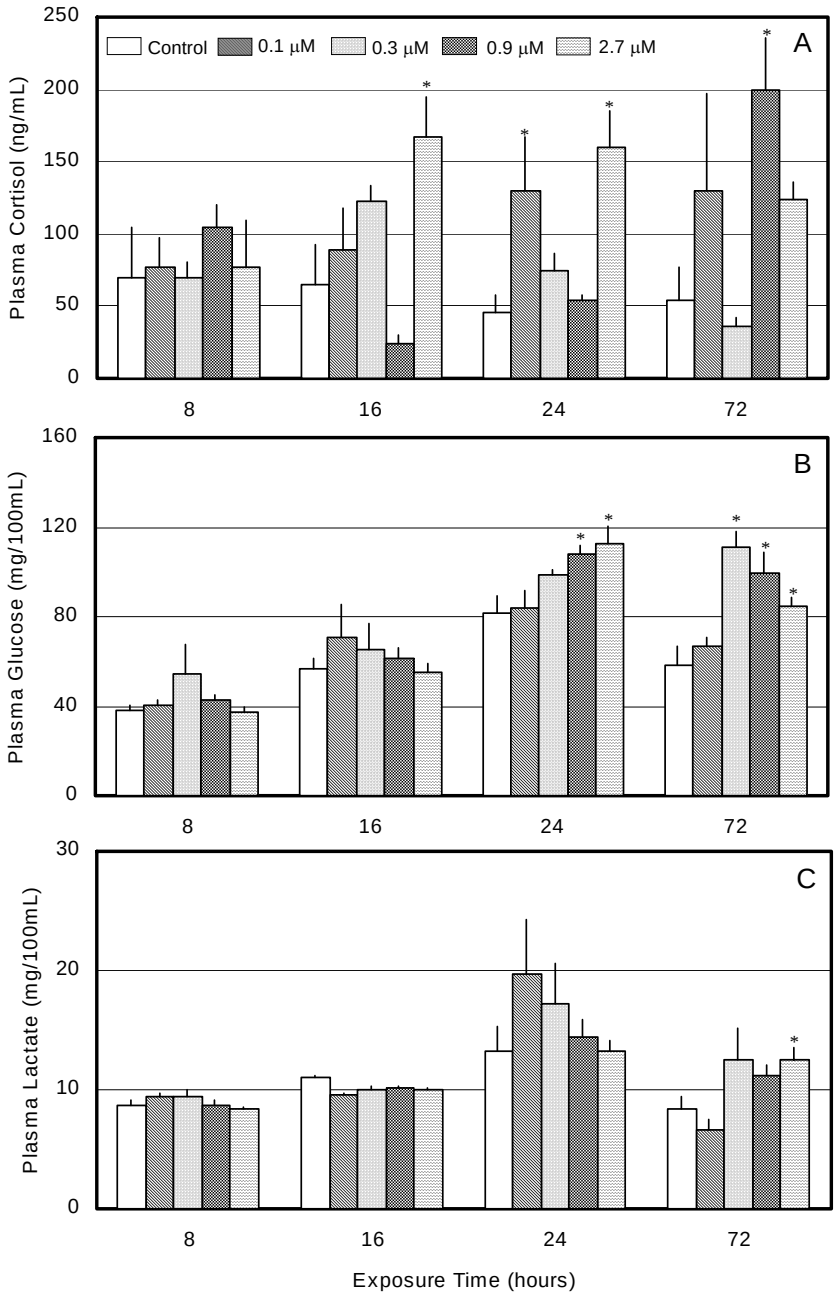

Fig. 1 – *A. anguilla* L. plasma cortisol levels (A), plasma glucose levels (B) and plasma lactate levels (C) after 8, 16, 24, and 72 h exposure to abietic acid (0, 0.1, 0.3, 0.9 and 2,7 μM). Values represent the means and S.D. (*n*=5/treatment). Differences from control: **P*<0.05.

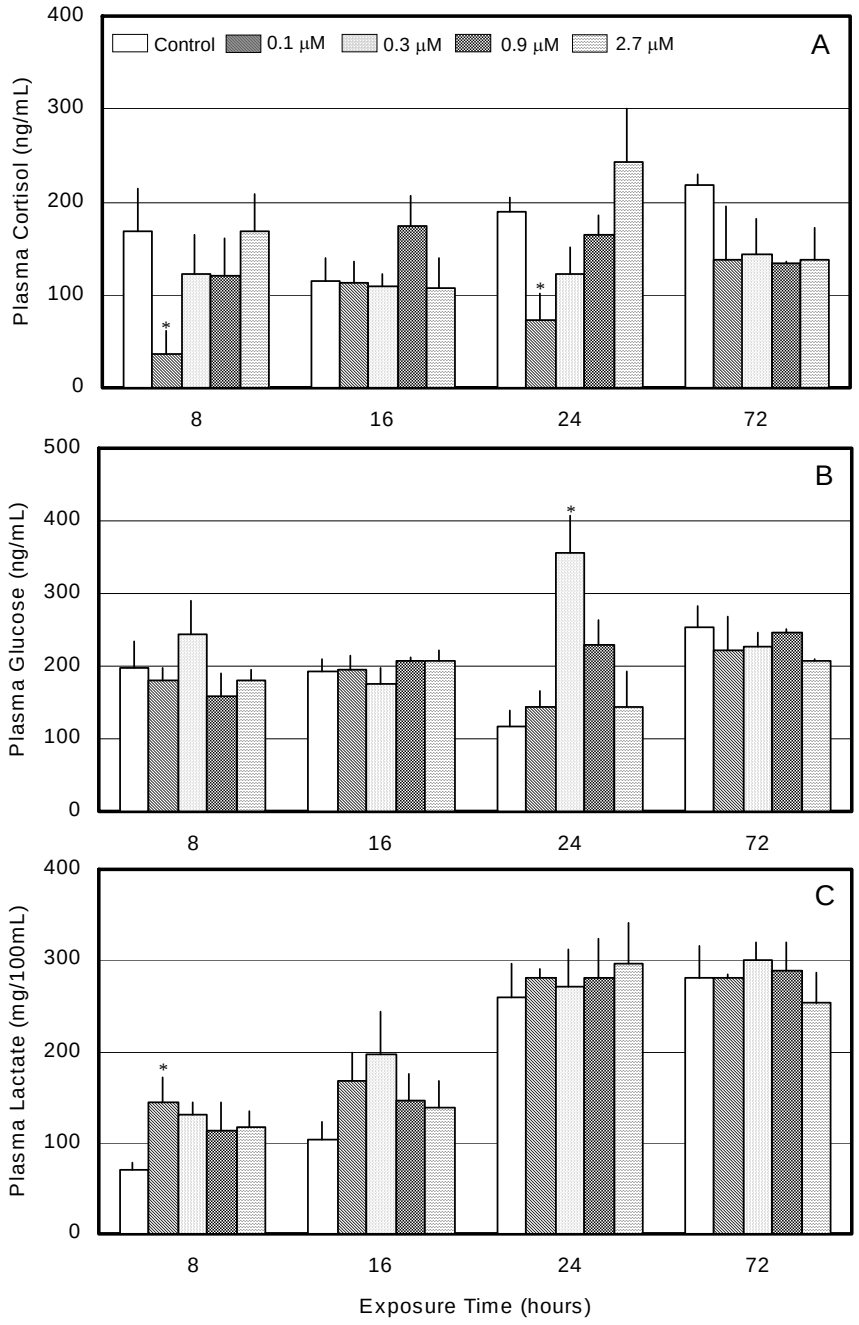

Fig. 2 – *A. anguilla* L. plasma cortisol levels (A), plasma glucose levels (B) and plasma lactate levels (C) after 8, 16, 24, and 72 h exposure to dehydroabietic acid (0, 0.1, 0.3, 0.9 and 2,7 μM). Values represent the means and S.D. (*n*=5/treatment). Differences from control: **P*<0.05.

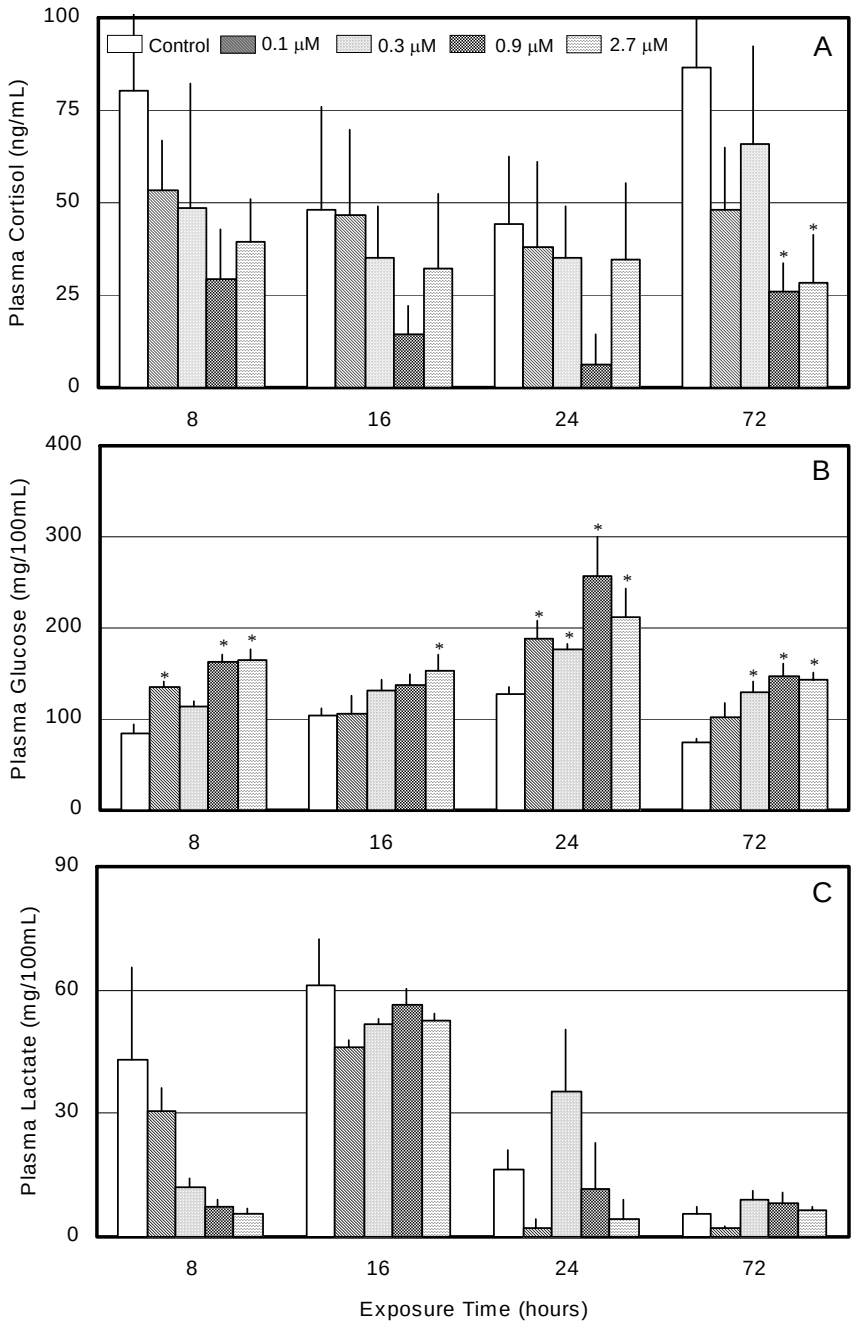

Fig. 3 – *A. anguilla* L. plasma cortisol levels (A), plasma glucose levels (B) and plasma lactate levels (C) after 8, 16, 24, and 72 h exposure to retene (0, 0.1, 0.3, 0.9 and 2,7 μM). Values represent the means and S.D. (*n*=5/treatment). Differences from control: **P*<0.05.

DISCUSSION

The assessment of the BKPME sublethal effects must also include the study of their individual components. Moreover, Oikari and Lindstöm-Seppä (1990) suggested that if some parameters are considered as important endpoints, it must be of primary interest to know which components of BKPMEs are responsible for those effects. In this perspective and considering the lack of data concerning AA, DHAA and Re effects on aquatic animals, namely fish; the present research work becomes ecotoxicologically relevant.

In this study, the eels exposed to the highest AA concentrations (0.9 and 2.7 μM) displayed a significant plasma cortisol increase. This response is in agreement with a well-established pattern for short-term exposures to chemical stressors, where fish exposed to chemical pollutants such as heavy metals, acid waters (Donaldson *et al.*, 1984; Thomas, 1990) or to chlorinated RAs (Kennedy *et al.*, 1995) exhibited elevated plasma cortisol. However, DHAA and Re exposures demonstrated that eels were unable to increase their plasma cortisol as a normal reaction to capture and handling, when compared to control levels, suggesting that an endocrine-impaired response has occurred. The eel's decrease in plasma cortisol levels found after DHAA and Re exposure seems to agree with previous results obtained after 4 h exposure to BKPME (Santos and Pacheco, 1996). Furthermore, *A. anguilla* exposed to diesel water-soluble fraction during 3 h up to 3 days (Pacheco and Santos, 2001), and to naphthalene during 24 h (Teles *et al.*, 2003) also exhibited a plasma cortisol decrease.

Hontela *et al.* (1992) stated that only fish chronically exposed to pollutants exhibited an impaired cortisol function. However, our results do not agree with the previous statement. Cortisol responses are complex, depending on fish species and tested xenobiotics rather than on short-term *vs.* long-term exposure. Furthermore, the fish plasma cortisol uptake by different body tissues after exposure to a xenobiotic compound mixture such as BKPME as previously demonstrated depends on the effluent temperature (20-25 ºC) (Santos and Pacheco, 1996).

Some authors explained the cortisol response impairment by an exhaustion of the cortisol-producing system and pituitary corticotrope atrophy, possibly as a result of its prolonged hyperactivity (Hontela *et al.*, 1992, 1995; Brodeur *et al.*, 1997). Considering short-term effects, Santos and Pacheco (1996) proposed that the interrenal cortisol release might be prevented by the contaminant (i.e. endocrine disruptor), since a high cortisol concentration in the interrenal tissue was observed in parallel with a low plasma cortisol concentration. This interpretation for short-term effects may complement Hontela's (1997) explanation, since an eventual long-term exposure may lead to a negative feedback, and consequent pituitary corticotrope atrophy. However, it is not possible to clarify the mechanism involved in this endocrine disruption, considering the present experimental design, and complementary parameters should be evaluated.

There is some evidence that mammals acute exposures to DDT and its metabolites exert direct adrenal cytotoxicity thus impairing the adrenal function and the ability to secrete cortisol (Lund *et al.*, 1988; Jönsson, 1994). Similarly, an eventual interrenal functional impairment produced by DHAA and Re may be due to a direct cytotoxic action and/or to sterodoigenesis interference.

Therefore, the cortisol impairment observed by Santos and Pacheco (1996) in *A. anguilla* after short-term exposure to BKPME may be associated with the presence of DHAA and Re in the effluent, suggesting their capacity to overcome the AA effects. A cortisol secretion dysfunction reduces the fish physiological competence, growth, and survivorship since this hormone is required for a wide range of important homeostatic mechanisms, including fuel reserves mobilization. Therefore, the current results demonstrate the ecotoxicological risks associated with fish populations exposed to pulp mill effluent discharges.

Fish carbohydrate metabolism may be altered through exposure to a variety of stressors. In this context, a typical stress response includes plasma glucose and lactate increase (Lowe-Linde and Niimi, 1984; Hontela *et al.*, 1996; Santos and Pacheco, 1996). These responses have an adaptive value since they augment the

availability of energy substrates, necessary for homeostasis maintenance (Donaldson *et al.*, 1984).

In the current study, eels exposed to AA and Re displayed a general increase in plasma glucose, whereas the response to DHAA was less comprehensible, revealing a significant increase only at 24 h. The plasma lactate response seems to be more complex, depending on the tested compound, since a significant plasma lactate increase was detected only for the shortest DHAA exposure and for longest AA exposure. Thus, a plasma lactate increase should be regarded as an expected response, considering previous results in the same species exposed to 50% BKPMEs during 4 h (Santos *et al.*, 1993).

The previously reported pattern, i.e., blood cortisol, glucose and lactate increase, was observed only after exposure to AA. However, DHAA and Re responses followed a different pattern, since a plasma cortisol decrease was accompanied by its glucose increase in both experiments and by a lactate increase in DHAA experiment. Therefore, the results concerning plasma glucose increase due to DHAA and Re exposure may be related to an increased cortisol uptake from blood by organs such as liver without its replacement in blood circulation by the interrenal tissue. The establishment of a consistent relation between plasma cortisol, glucose and lactate seems difficult. Further studies are required to elucidate the link between endocrine and metabolic dysfunctions in fish affected by pulp mill components.

CONCLUSIONS

A reduced capacity to elevate plasma cortisol after capture and handling was found in *A. anguilla* exposed to dehydroabietic acid and retene. However, this ability was not affected by abietic acid exposure, since an elevated plasma cortisol was observed. All the tested chemicals were able to induce a plasma glucose increase, while plasma lactate only responded to RAs.

The interaction between cortisol function and carbohydrate metabolism was not clear. Nevertheless, the measurement of plasma steroid levels such as cortisol,

complemented with plasma glucose and lactate measurements may constitute early warning signals of pulp mill effluent contamination.

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CAPÍTULO III

Alterações na actividade hepática de etoxiresorufina-*O*-desetilase e glutationa-*S*-transferase, indução de anomalias nucleares eritrocíticas e respostas endócrinas em *Anguilla anguilla* L. após exposição a naftaleno e β-naftoflavona

Anguilla anguilla L. liver ethoxyresorufin _O_-deethylation, glutathione _S_-transferase, erythrocytic nuclear abnormalities, and endocrine responses to naphthalene and β-naphthoflavone

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ABSTRACT

The effects of naphthalene (NAP) and β-naphthoflavone (BNF) on phase I biotransformation and genotoxicity in *Anguilla anguilla* L. were evaluated. Phase II biotransformation and cortisol levels were also assessed in NAP-treated fish. Two groups of eels were exposed to either a NAP or a BNF concentration range (0.1-2.7 μM) for different exposure periods (2-72 h). An early significant ethoxyresorufin *O*-deethylation (EROD) activity inhibition was observed, especially for the highest NAP concentrations at 2-6 h exposure and for BNF at 2 h exposure. However, a significant EROD activity increase was detected from 16 to 72 h exposure for NAP and from 4 to 72 h exposure for BNF. The cytochrome P450 (P450) content was not dose related. However, with regard to BNF exposure, P450 was the first biomarker to respond. Liver alanine transaminase (ALT) activity was measured as an indicator of hepatic health condition. ALT results demonstrated that the EROD activity decrease, previously described for NAP, was not related to tissue damage. Nevertheless, the highest BNF concentrations were demonstrated to induce liver damage and to impair the EROD activity response. An increased genotoxic response, measured as erythrocytic nuclear abnormalities (ENA), was observed during the first 8 h NAP exposure. However, for exposures longer than 8 h, ENA frequency returned to the control levels. This response profile may reflect a considerable DNA repair capacity and/or a metabolic adaptation providing an efficient NAP biotransformation and consequent detoxification. BNF revealed no ENA alterations for all concentrations and exposure lengths. In the NAP experiment a causal relationship between immature erythrocytes (IE) and ENA frequency disappearance was not found. BNF results with regard to IE frequency revealed an ability to alter the balance between erythropoiesis and removal of erythrocytes. Liver glutathione *S*-transferase (GST) activity was significantly induced after 2 and 48 h NAP exposure. A cortisol-impaired response seems to occur from 4 to 24 h NAP exposure, demonstrating an endocrine disruption. However, an adaptation process seems to occur after 48 hrs, since the plasma cortisol had a tendency to increase. The present findings confirm the

usefulness of the adopted biomarkers. The ecological risk associated with aquatic contamination by NAP was also confirmed by the present data.

INTRODUCTION

A large number of xenobiotic compounds have been produced in the 20th century as a result of petroleum exploitation, with the aquatic environment being the ultimate sink for most of them. Therefore, the vast majority of aquatic animals have become increasingly exposed to industrially derived xenobiotic pollutants, such as polycyclic aromatic hydrocarbons (PAHs) (George, 1994). These widespread environmental contaminants, originating mainly from combustion processes, oil spills, land runoff, and domestic and industrial wastes, are of international concern due to their persistence in the environment and mutagenic/carcinogenic potential. Naphthalene (NAP), the simplest PAH, is frequently encountered in soil and in aquatic environments (Irwin *et al.*, 1997).

Fish readily take up lipophilic organic contaminants such as NAP from the environment and possess a variety of cellular mechanisms for protection against the deleterious effects of such chemicals (Peters *et al.*, 1997). These mechanisms include biotransformation, which converts organic contaminants to water-soluble and excretable metabolites. However, biotransformation enzymes may also convert certain xenobiotics to intermediates, which are more toxic than the parent compounds. Biotransformation can generally be simply divided into phases I and II: phase I is the alteration of the original foreign molecule so as to add a functional group, which can be conjugated in phase II. Biotransformation studies in fish revealed that PAHs exposure induces cytochrome P450 (P450)-dependent enzymatic activities such as ethoxyresorufin *O*-deethylation (EROD) (phase I). Therefore, the EROD activity determination has been adopted as a valuable indicator allowing the detection of PAHs at presumably toxic levels.

A common mechanism for protection against biotransformed electrophilic xenobiotics is conjugation with glutathione (GSH) in phase II detoxification. This

reaction is catalyzed by glutathione *S*-transferase (GST) and accounts for one of the primary routes of NAP detoxification (Mitchell *et al.,* 2000). Since phase II detoxification enzymes are often inducible in aquatic animals, it has been suggested that its activity might also be a useful index of organic compounds exposure (Gallagher *et al.*, 2001).

Some authors have successfully employed aquatic organisms in cytogenetic studies, for chromosome aberrations, sister chromatid exchange, micronuclei (Pesch *et al.*, 1981; Kligerman, 1982) and erythrocytic nuclear abnormalities (ENA), which include micronuclei (supernumerary nucleus) and other nuclear abnormalities quantification (Pacheco and Santos, 1997, 2002; Ayllón and Garcia-Vasquez, 2001).

Fish endocrine system can be adversely affected by toxic chemicals. Therefore, biochemical alterations and consequent effects on specific hormonal functions may constitute important stress biomarkers (Hontela, 1997). In this context, changes in interrenal function, measured as plasma cortisol, are recognized as important fish stress biomarkers (Santos and Pacheco, 1996).

The toxic effects of NAP in fish have been poorly studied. The first reported studies concern lethality experiments (Boylan and Tripp, 1971; Anderson *et al.*, 1974). Levitan and Taylor (1979) found a general plasma cortisol increase in *Fundulus heteroclitus* exposed to NAP under different salinities. Both NAP genotoxic potential measured as ENA increase in *Dicentrarchus labrax* (Gravato and Santos, 2002) and an oxidative stress induction in *Anguilla anguilla* L. (Ahmad *et al.*, 2001) have been observed.

β-Naphthoflavone (BNF) has been considered the most potent P450 mixed-function oxydase (MFO) inducer among a number of synthetic flavonoid compounds (McKillop and Case, 1991). This compound is commonly used to evaluate fish biotransformation responses due to its MFOs responses, considered similar to those displayed by PAHs inducers, such as benzo(*a*)pyrene (BaP) (Okey, 1990; Novi *et al*., 1998). Thus, in a biotransformation context, BNF is regarded as a PAH-type inducer. Previous investigations with *A. anguilla* revealed that BNF is a strong EROD inducer both in adult eels intraperitoneally injected (Pacheco and Santos, 1998) and in glass

eels (leptocephalic stage) exposed to BNF-contaminated water (Pacheco and Santos, 1997). McKillop and Case (1991) reviewed the information available relating to BNF mutagenicity and carcinogenicity. Their literature search produced no primary reports on the mutagenicity/carcinogenicity of BNF, suggesting that no definitive study had been carried out specifically to assess these BNF effects. Furthermore, the majority of the studies were carried out in mammals; thus, doubt with regard to its potential in other animal groups remains. Recently, the BNF mutagenicity potential was demonstrated in fish by Pacheco and Santos (1997) who observed a significant ENA increase in glass eels. In this perspective, further fish studies are needed to clarify the BNF genotoxic potential in fish.

This research concerns the study of the effects of NAP on liver biotransformation, measured as P450 content, EROD (phase I), and GST activity (phase II), and on genotoxicity, measured as ENA. The immature erythrocytes (IE) frequency was determined to assess changes in the erythropoiesis/erythrocytic catabolism balance and its influence on ENA expression. Liver alanine transaminase (ALT) activity was measured as an indicator of hepatic health condition. The endocrine disruption was evaluated as plasma cortisol concentration.

Additionally, BNF was tested as a reference compound for biotransformation parameters (phase I) and to assess its genotoxic potential in fish, as ENA.

MATERIAL AND METHODS

Test Animals

A. anguilla L. (yellow eel), with an average weight of 30 g (yellow eel), were collected from the Aveiro lagoon, Murtosa. The eels were transported in anoxia and acclimated to laboratory conditions for 1 week prior to experimentation. During recovery and experimental periods, eels were kept in 80-L aquaria at 20°C under a natural photoperiod, in recirculating, aerated, filtered, and dechlorinated tap water. Fish were fed neither under laboratory adaptation nor during the experimental procedure.

Chemicals

NAP, 7-ethoxyresorufin, dithionite, 1-chloro-2, 4-dinitro-benzene (CDNB) and BNF were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). NADPH was purchased from Roche (Germany).

Experimental Design

Two different lots of eels were exposed either to a NAP or a BNF concentration range - 0 (control), 0.1, 0.3, 0.9 and 2.7 μM - for 2, 4, 6, 8, 16, 24, 48, and 72 h. The appropriate amount of NAP or BNF was previously dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the experimental aquaria. The same volume of DMSO was added to the control aquaria. During the exposure time the water was not replaced.

Fish were killed at each sampling point and their blood and liver were collected. Liver was immediately frozen in liquid nitrogen and stored at -20ºC until homogenization. Blood was used for smear preparation and for plasma isolation using an Eppendorf centrifuge (14,000 rpm). The following parameters were determined for NAP and BNF experiments with the exceptions of plasma cortisol and liver GST activity which were measured only for the NAP experiment.

Biochemical Analysis

Liver EROD Activity

Liver microsomal fraction was obtained according to the methods of Lange *et al.* (1992) and Monod and Vindimian (1991), as adapted by Pacheco and Santos (1998). Liver EROD activity was measured in microsome suspension as described by Burke and Mayer (1974).

Liver Cytochrome P450 Content

Cytochrome P450 content was determined using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm, as previously described by Hermens *et al.* (1990).

Liver GST Activity

GST activity was determined in the cytosolic fraction as described by Lemaire *et al.* (1996), using CDNB as substrate (Habig *et al.*, 1974). The assay, prepared in the cuvette, was carried out in a 2-mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB, and 0.2 mM GSH. The reaction was initiated by addition of 10 µl sample. The increase in absorbance at 340 nm was recorded at 25°C for 3 min.

Liver ALT Activity

The liver ALT activity was measured in the cytosolic fraction according to the method of Reitman and Frankel (1957).

Protein measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornall *et al.*, 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard.

Plasma Cortisol

Plasma cortisol quantification was performed using a diagnostic enzyme-immunological kit (Boehringer Mannheim GmbH No. 1288946).

ENA Frequency

Genotoxicity was tested using the ENA assay. The nuclear abnormalities were scored in 1000 mature erythrocytes (ME) per fish, according to the criteria of Schmid (1976), Carrasco *et al.* (1990), and Smith (1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei, lobed nuclei, dumbbell-shaped or segmented nuclei, and kidney-shaped nuclei. The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

IE Frequency

IE were scored for each of the 1000 erythrocytes (immature+mature) per fish. Results were presented as a frequency (‰) resulting from the expression:

$$\text{IE frequency (‰)} = [\text{No. IE}/(\text{No. IE}+\text{No. ME})]\text{x}1000.$$

The distinction between ME and IE was based on the criteria established by Hibiya (1982) and Smith (1990).

Statistical Analysis

Mean ± standard error (SE) was calculated for each experimental group, and data were analyzed for significance of differences between control and exposed groups according to the two-tailed Student *t* test (Bailey, 1959). Experiments were carried out using test groups of five eels (*n*=5).

RESULTS

NAP Experiment

Liver EROD Activity

A significant inhibition in liver EROD activity was observed during the first 6 h exposure (Fig. 1), especially for the highest NAP concentrations. However, a general significant liver EROD increase was detected from 16 to 72 h exposure. The maximum liver EROD activity level (7.06 pmol/min/mg protein) was reached at 48 h exposure to 2.7 μM.

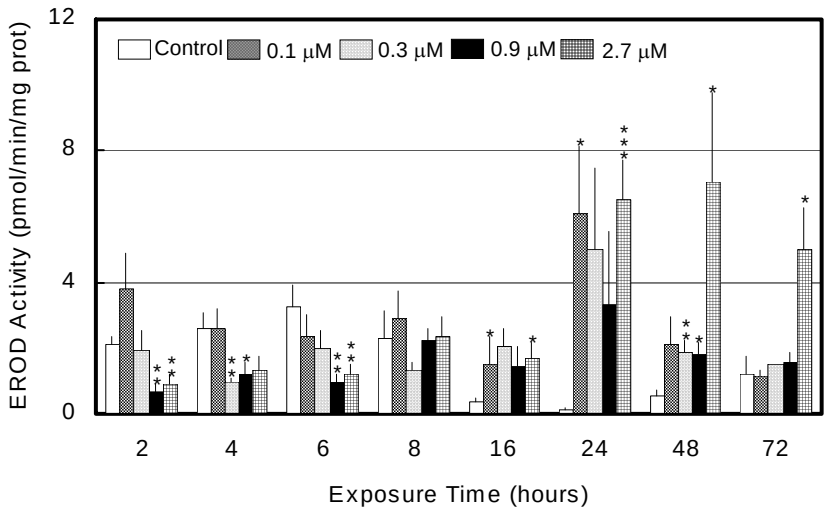

Fig. 1 - *A. anguilla* liver EROD activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2,7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

Liver P450 Content

A general decreasing tendency for liver cytochrome P450 content was observed for the first 6 h exposure to all NAP concentrations (Fig. 2), despite the absence of any statistical significance. A significant decrease was observed at 16 h exposure to 0.9 and 2.7 μM (*P*<0.05 and *P*<0.01, respectively).

Liver GST Activity

The GST activity demonstrated a significant induction only at 2 (*P*<0.01) and 48 h (*P*<0.001) exposure to 0.9 μM NAP (Fig. 3). However, this concentration induced a general GST increase during the whole experiment.

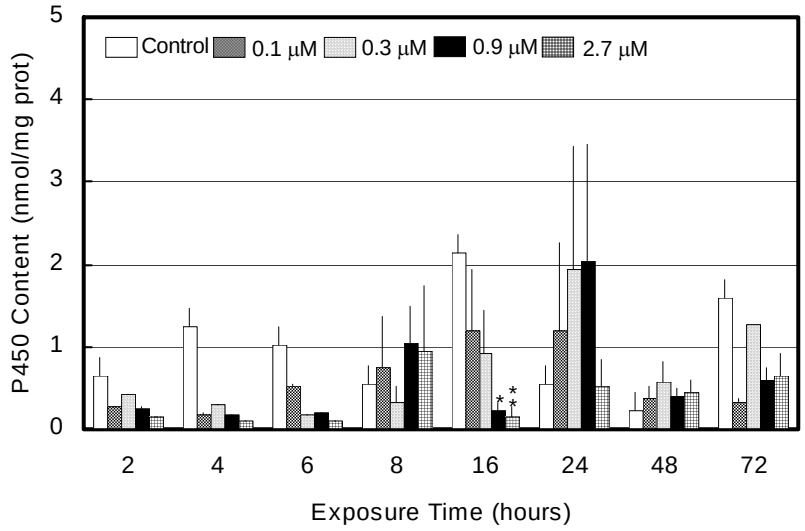

Fig. 2 - *A. anguilla* liver P450 content after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

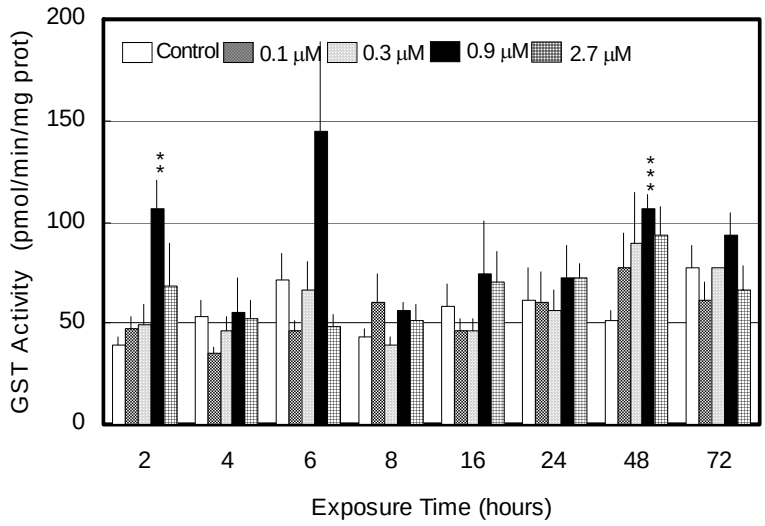

Fig. 3 - *A. anguilla* liver GST activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

Liver ALT Activity

In general, no significant alterations were observed in liver ALT activity during the whole NAP exposure period (Fig. 4), except for 0.3 μM at 8 h exposure where a significant increase was detected (*P*<0.05).

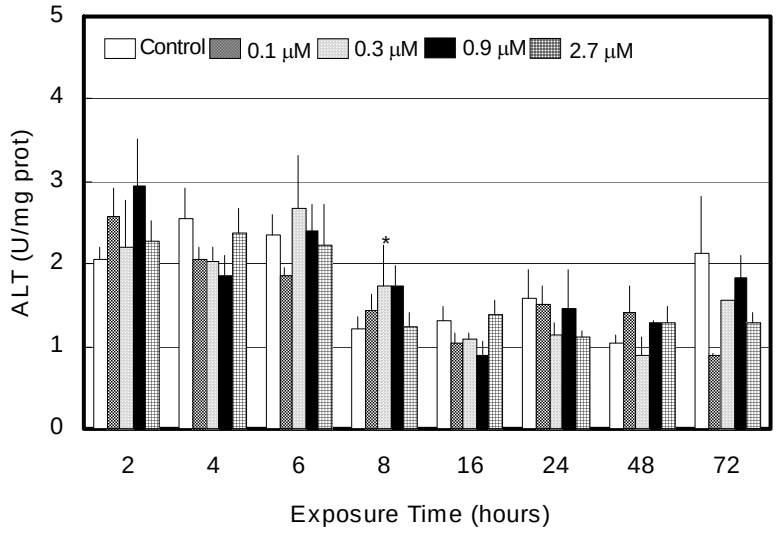

Fig. 4 - *A. anguilla* liver ALT activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

ENA Frequency

At 2 h exposure to NAP, significant ENA increases were observed for 0.3 and 2.7 μM (*P*<0.01); an increased ENA frequency was observed at 4 h exposure for 0.9 and 2.7 μM (*P*<0.05) and at 6 h for 0.3 μM (*P*<0.05), whereas at 8 h all the tested concentrations significantly increased ENA (0.1 μM, P<0.01; 0.3 μM, *P*<0.01; 0.9 μM, *P*<0.01; 2.7 μM, *P*<0.05) (Fig. 5). ENA frequency returned to control levels after exposures longer than 8 h.

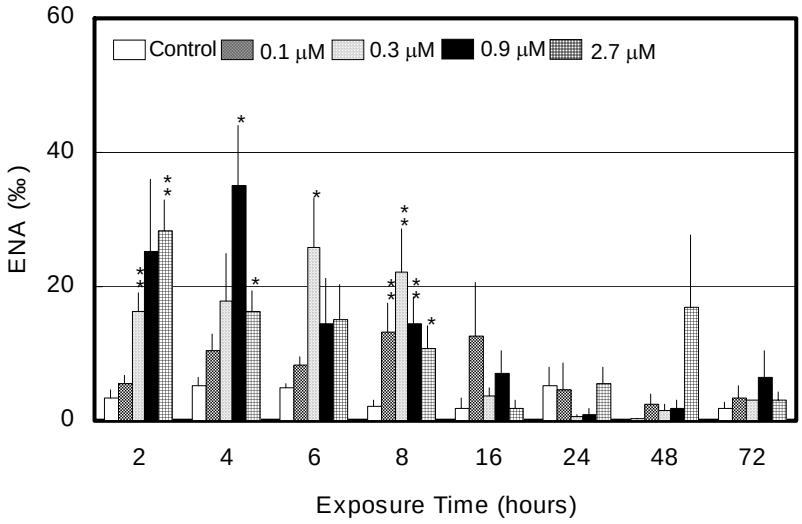

Fig. 5 - *A. anguilla* ENA frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

IE Frequency

At 4 h a significant increase was observed for 0.1 (*P*<0.05) and 0.3 μM (*P*<0.001), whereas at 24 h a significant decrease (*P*<0.05) was detected for 0.1 μM (Fig. 6).

Plasma Cortisol

A plasma cortisol level decreasing trend was observed from 4 to 24 h (Fig. 7). This tendency was confirmed at 6 h where a significant decrease was observed for 0.1 μM (*P*<0.05), 0.3 μM (*P*<0.01), and 0.9 μM (*P*<0.05). After 48 h the previous tendency disappeared and an opposite trend was observed.

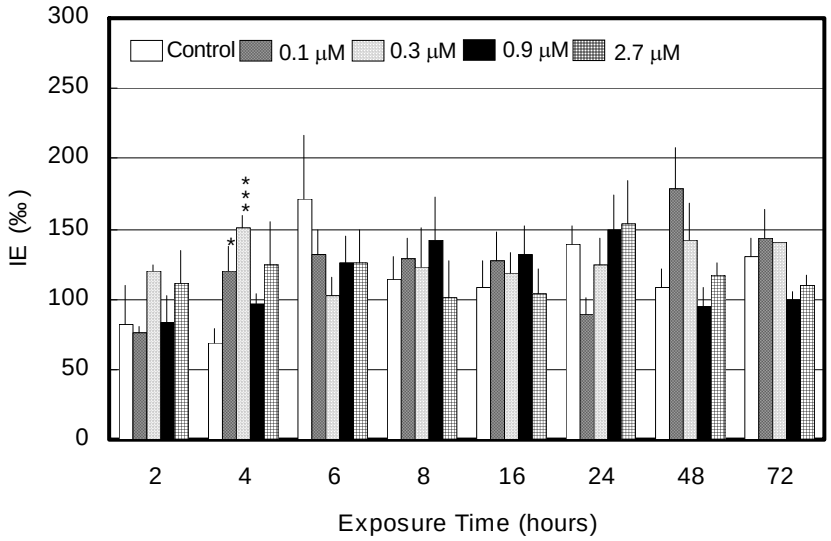

Fig. 6 - *A. anguilla* IE frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

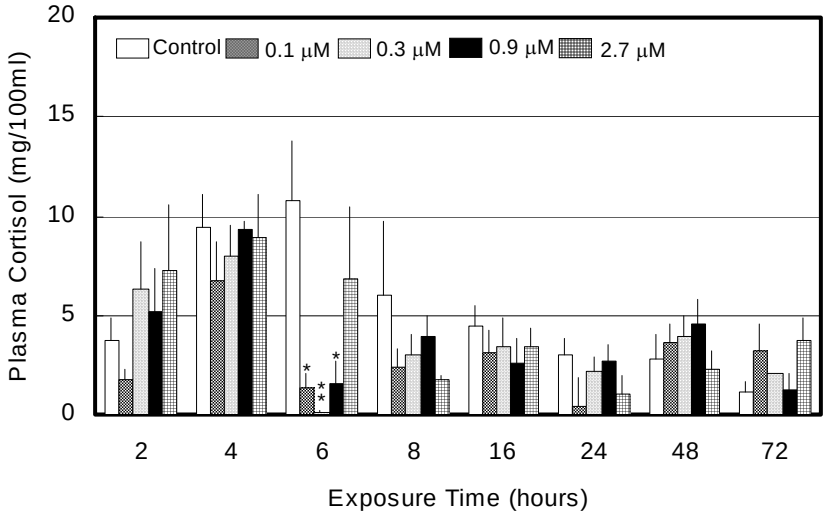

Fig. 7 - *A. anguilla* plasma cortisol activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to naphthalene (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

BNF Experiment

Liver EROD Activity

The *A. anguilla* response after 2 h exposure suggests an initial inhibition effect which became evident by the complete absence of EROD activity increase and mainly by the significant activity decrease observed for 0.9 μM BNF (*P*<0.001) (Fig. 8). The first significant EROD increase was observed after 4 h exposure (*P*<0.05) but just for the lowest BNF concentration (0.1 μM). During the exposure period between 6 and 72 h, all the concentrations were able to significantly increase EROD activity. For each exposure concentration, a global analysis of EROD results reveals a time-related increase tendency from 2 to 8 h exposure, where it reaches the maximum values.

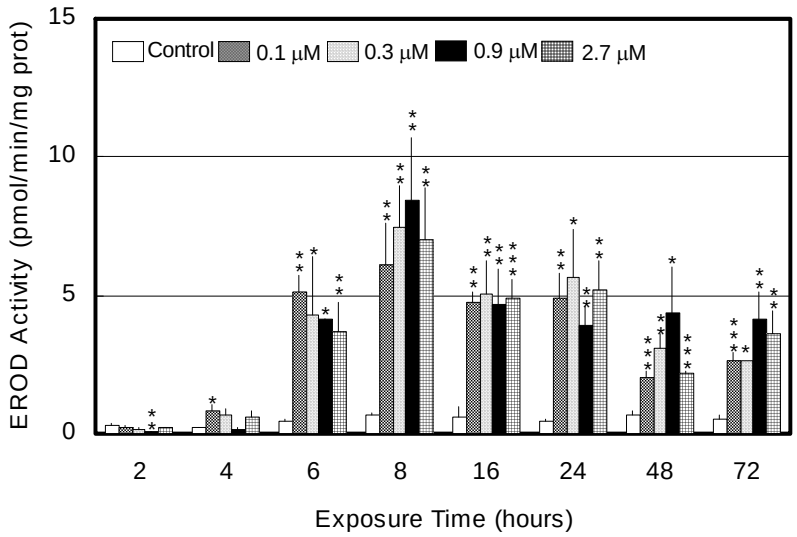

Fig. 8 - *A. anguilla* liver EROD activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to β-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

Liver P450 Content

This biomarker exhibited a fast significant increase at 2 h 0.1 μM BNF exposure (*P*<0.01) (Fig. 9). This parameter demonstrated a general tendency to increase at all BNF exposure lengths. However, significant responses were observed only at dispersed points and there was an unclear dose-response relationship. A response profile similar to that described for EROD activity was observed, i.e., the maximum values were obtained after 8 and 16 h exposure, followed by tendency to decline. Similar to the EROD response, an inhibition effect seems to occur in P450 content, particularly for short exposures, as confirmed by a significant decrease observed at 2 h for 0.3 μM, compared to 0.1 μM (*P*<0.05). Nevertheless, a decline of this inhibitory action was observed as result of an increased exposure length.

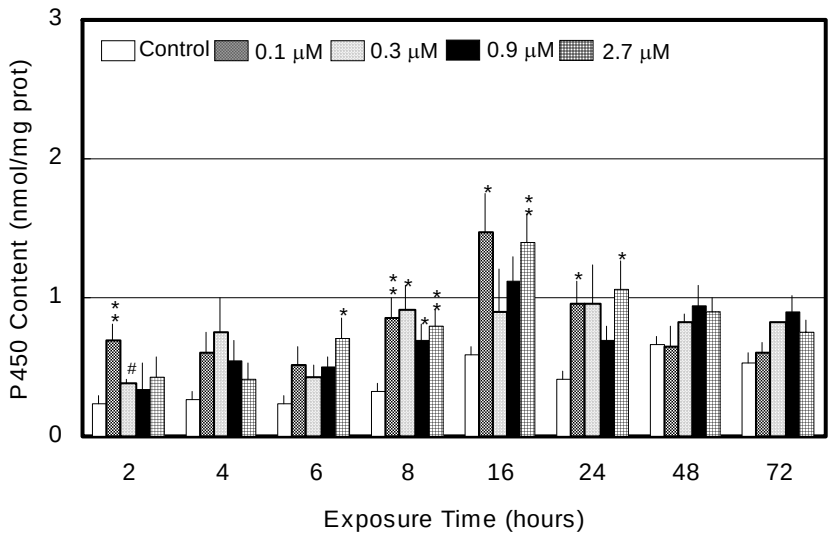

Fig. 9 - *A. anguilla* liver P450 content after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to β-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001. #*P*<0.05: 0.1 μM vs 0.3 μM.

Liver ALT Activity

The lowest BNF concentration (0.1 μM) was able to significantly increase ALT activity, as demonstrated at 2 and 24 h exposure (*P*<0.05) (Fig. 10). However, the highest BNF concentrations were able to induce liver damage sporadically, as it occurred for 0.9 μM at 8 (*P*<0.05) and 16 h exposure (*P*<0.01), and for 2.7 μM at 6 (*P*<0.05) and 16 h (*P*<0.01) exposure.

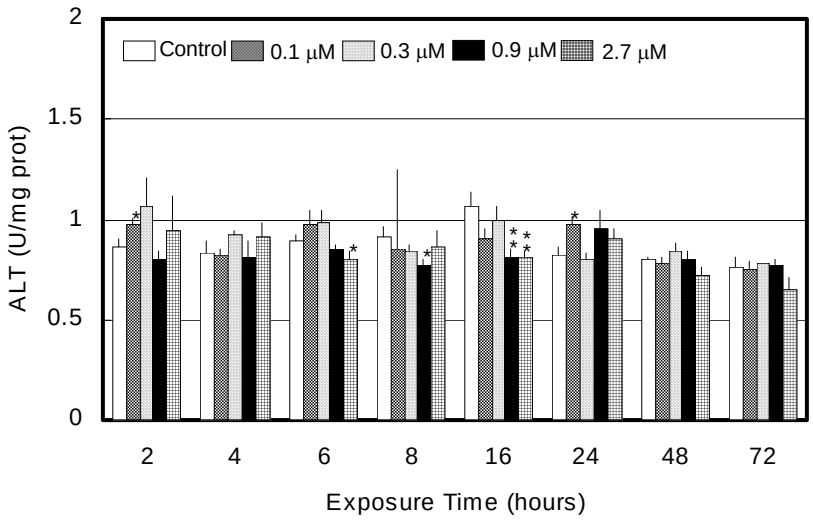

Fig. 10 - *A. anguilla* liver ALT activity after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to β-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

ENA Frequency

The genotoxic response revealed no alterations for all tested concentrations and exposure times (Fig. 11).

IE Frequency

The IE frequency was markedly affected by BNF exposure (Fig. 12). The results revealed significant decreases for 24 h and longer exposures. At some points in time, such as 24 h, a dose-response relationship seems to be evident, i. e., the IE frequency decreases as the BNF dose increases.

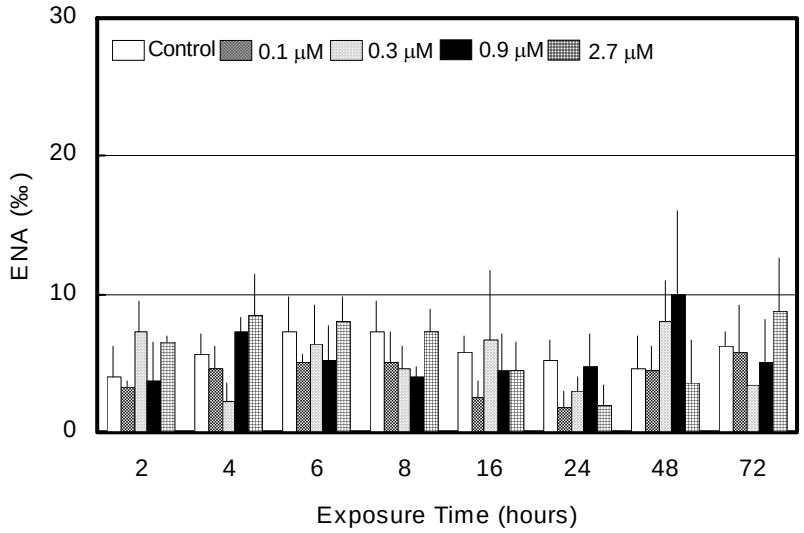

Fig. 11 - *A. anguilla* ENA frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to β-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

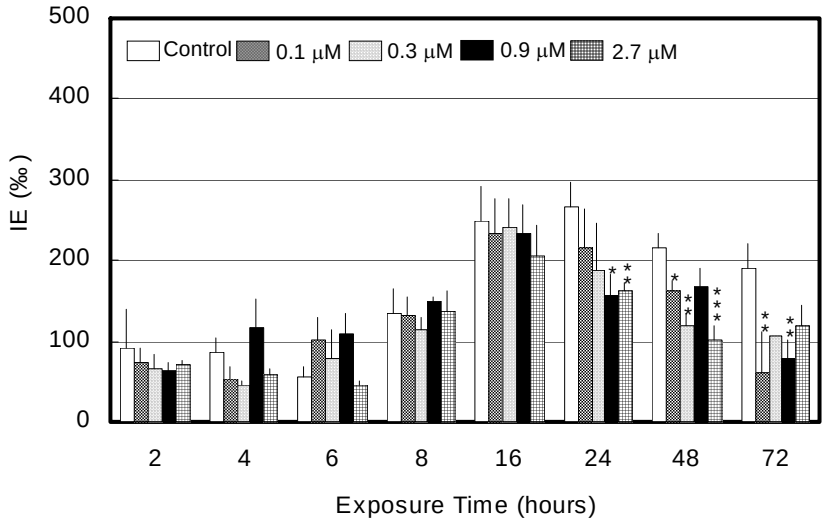

Fig. 12 - *A. anguilla* IE frequency after 2, 4, 6, 8, 16, 24, 48, and 72 h exposure to β-naphthoflavone (0, 0.1, 0.3, 0.9, and 2.7 μM). Values represent the means and SE (*n*=5/treatment). Differences from control: **P*<0.05; ***P*<0.01;****P*<0.001.

DISCUSSION

To our knowledge there are very few studies on the effects of NAP on biotransformation, genotoxicity, and endocrine parameters in fish.

It is well known that xenobiotics classified as PAH-type compounds induce hepatic microsomal cytochrome P450-dependent reactions in fish (Fouchécourt *et al.*, 1999). Measurements of aryl hydrocarbon hydroxylase (AHH) and EROD (phase I) activities in fish appear to be the most sensitive means for determining water contamination by PAHs. These phase I enzyme activities occur at low and sometimes undetectable levels in control or untreated fish (Förlin *et al.*, 1994). Several studies have demonstrated liver EROD induction in fish exposed to PAHs such as dibenzo[a]pyrene (Veter *et al.*, 1985) and BaP (Wolkers *et al.*, 1996). Nevertheless, Bols and coworkers (1999) failed to obtain EROD activity induction by NAP in a trout liver cell line.

The results of the present study indicated that NAP could modulate the biotransformation metabolism, expressed as liver EROD activity increase. However, compared to the positive control (BNF), NAP induced a later EROD rise (16-72 h).

Despite the EROD induction potential, the current NAP and BNF results also revealed an inhibitory capacity detected especially for the highest concentrations (0.9 and 2.7 μM) during the first 6 h NAP exposure and at 2 h BNF exposure (0.9 μM). This inhibitory effect may be explained by the high xenobiotic exposure concentrations. Previous studies demonstrated that several CYP1A1 inducers could also inhibit the MFO catalytic activity (EROD or AHH) at high concentrations (Stegeman and Hahn, 1994). Such double performance was found in fish exposed, either *in vivo* or *in vitro*, to high doses of polychlorinated biphenyl's (PCBs) congeners (Boon *et al.*, 1992; Hahn *et al.*, 1993), BaP (Goddard *et al.*, 1987), and BNF (Haasch *et al.*, 1993). The majority of cythocrome P450 enzyme inhibitors act either by competitive inhibition or by mechanism-based inactivation in which the inhibitor is metabolized by the cythocrome P450 into a product that covalently modifies the active site and thereby inactivates the enzyme (Stegeman and Hahn, 1994). In the present study, EROD activity and P450 content presented a concomitant inhibitory

tendency during the first hours of exposure, especially in NAP experiment. However, in this experiment a significant EROD activity increase corresponds to a significant decrease in cytochrome P450 content (16 h).

The liver EROD decrease observed during the first 6 h NAP exposure was not followed by a liver ALT decrease. Moreover, liver ALT activity at 8 h exposure to 0.3 μM NAP revealed a significant increase. Thus, the observed EROD activity decrease is not justified by liver lesions.

Liver EROD induction observed after 16-h NAP and 4-h BNF exposures might be explained by either a progressive fish acclimation and physiological adjustment, reducing the inhibition efficiency, or by a decline of the xenobiotic levels in the aquaria.

Globally, the current phase I results proved that liver EROD activity rather than total cytochrome P450 is a good biomarker for PAH and PAH-like compounds exposure, providing an early biological warning signal.

Increases in GSH, glutathione reductase and GST levels have been linked to organism resistance and adaptation to a variety of physical and chemical stresses found in the environment (Gallagher *et al.*, 2001). Considering the current NAP results, a liver GST activity tendency to increase was observed from 2 to 72 h, being significantly expressed at 2 and 48 h. GST induction could reflect a stress response to the chemical, resulting in a phase II conjugation process, where the metabolites formed in phase I are combined with endogenous molecules to form conjugates. Nevertheless, it is difficult to establish a direct correspondence between GST, EROD, and P450 content responses.

In agreement with our results, a hepatic GST activity increase was previously observed in NAP-treated mice (Mitchell *et al.*, 2000). Additionally, the same response was observed in BaP-treated fish (Lemaire *et al.*, 1992; George, 1994). Some authors suggested that the absence of GST activity variation could be followed by induction of other conjugases, such as UDP-glucoronyl transferase (Novi *et al.*, 1998).

The *A. anguilla* ENA response to both compounds was clearly different. A significant ENA frequency increase from 2 to 8 h exposure to NAP was observed,

demonstrating an early genotoxic effect. However, BNF did not reveal any ENA increase for all the exposure durations.

PAHs mutagenic characteristics are strongly related to their biotransformation, especially the initial oxidative metabolism carried out by cytochrome P450 monooxygenases. However, considering the early ENA induction observed in the NAP experiment concomitant to an EROD decrease, it might be suggested that NAP is genotoxic itself, without the need of bioactivation. Since NAP is the simplest PAH, having only two fused aromatic rings, its entrance in the cell is facilitated and a large number of these molecules might rapidly injure the DNA. The increased EROD activity observed from 16 to 72 h NAP exposure coincides with an ENA decline. Furthermore, a significant GST increase occurred at 48 hours exposure. These facts indicate that the phase I activation did not carry negative consequences for erythrocytic DNA and the whole detoxification process (phases I and II) seems to be effective, preventing ENA appearance. This explanation is supported by a previous human study (Tingle *et al.*, 1993) in which liver cytochrome P450 enzymes metabolized NAP to a cytotoxic and protein-reactive, but not genotoxic, metabolite (probably an epoxide). This is rapidly and efficiently detoxified by microsomal epoxide hydrolase forming stable metabolites.

The observed NAP genotoxic potential is in agreement with previous results of Gravato and Santos (2002) in *Dicentrarchus labrax* and Delgado-Rodriguez *et al.* (1995) in *Drosophila melanogaster.*

The hypothesis presented above for the NAP genotoxic mechanism may also be applicable to the current ENA/EROD results in the BNF experiment, since an EROD induction was observed from 4 to 72 h exposure without a simultaneous ENA response. This is in agreement with the results of Pacheco and Santos (1997) who observed in glass eels an ENA appearance at 6 and 9 days BNF exposure following a time-related tendency to decrease in EROD activity. Additionally, with respect to the previous findings, it must be suggested that longer BNF exposures should be used to assess its genotoxic potential.

Previous investigations suggested that the expression of genotoxic effects, such as micronuclei or other ENA, could be masked by a direct erythropoiesis inhibition (Das and Nanda, 1986; Dinnen *et al.*, 1988) and/or by an increased erythrocytic catabolism (Pacheco and Santos, 1997). Alterations in the balance of erythropoiesis/erythrocytic catabolism could be assessed by IE frequency changes. In the NAP experiment a causal relationship between IE frequency and ENA disappearance was not found. However, erythrocytic catabolism needs to be studied. In the BNF experiment IE frequency data indicated an ability to alter the balance between erythropoiesis and erythrocytes removal. Therefore, it was evident that ENA expression from 24 to 72 hours was affected by an IE frequency decrease, becoming more obvious at longer exposures to high BNF doses. Consequently, a causal relationship between the previous disruption observed at 24, 48 and 72 h and the absence of ENA increase may be suggested. If the erythrocytes are not produced or are catabolized more intensely, the ENA appearance can be masked despite the presence of genotoxic metabolites.

The endocrine system plays a central role in fish stress mechanisms. Cortisol, in particular, is a crucial hormone in the physiological response to stressors. Previous studies demonstrated that acute exposures to xenobiotics, namely PAHs (Thomas and Rice, 1987), in addition to capture and handling (Vijayan *et al.*, 1997), elevate plasma cortisol. Nevertheless, effects of chronic exposures to pollutants are less well understood (Hontela, 1997). Fish exposed to heavy metals exhibited an initial plasma cortisol rise followed by a decline to control levels (Pratap and Wendelaar, 1990). According to Hontela *et al.* (1997), fish chronically exposed to PAHs, PCBs, and heavy metals were unable to increase cortisol in response to a capture stress. The authors explained this fact by an exhaustion of the cortisol-producing endocrine system, possibly as a result of its prolonged hyperactivity. Other authors demonstrated that the interrenal function might also be affected by short-term exposures. Eels exposed either to bleached kraft pulp mill effluent for 4 h (Santos and Pacheco, 1996) or to diesel water-soluble fraction during 3 h to 3 days (Pacheco and

Santos, 2001) exhibited an impairment in the expected plasma cortisol increase, as a response to the stress of capture and handling.

In the current study, a cortisol-impaired response seems to occur from 4 to 24 h NAP exposure, demonstrating an interrenal disruption after short-term exposures. However, an adaptation process seems to occur after 48 h NAP exposure, since the plasma cortisol levels revealed a tendency increase in treated fish. These results are in agreement with the findings of Pacheco and Santos (2001) who detected a similar response profile. In opposition, Levitan and Taylor (1979) found a general plasma cortisol increase in *F. heteroclitus* exposed to 4 mg/L NAP for 2-12 h. This disagreement may be explained by the use of different fish species and/or by the concentration levels adopted (about 10-fold lower in the current work).

There is some evidence that acute exposures could exert adrenal cytotoxicity, impairing the adrenal function and the ability to secrete cortisol (Jönsson, 1994). Nevertheless, if this cytotoxic effect is related to the observed interrenal impairment in the present study, a recovery occurs after 24 h NAP exposure. Further investigations are needed to clarify the mechanisms involved in the cortisol responses, focusing mainly on the inhibitory processes in short-term exposures.

CONCLUSIONS

The current results led to the following conclusions:

(1) Naphthalene (NAP) is an aquatic contaminant, which induces early genotoxic damage in erythrocytes and a later liver ethoxyresorufin *O*- deethylation (EROD) induction. (2) NAP is a less potent EROD inducer than the positive control (BNF). (3) NAP is more genotoxic than BNF. (4) The increased ENA frequency induced by NAP is not directly related to high EROD activity. Furthermore, ENA seems to be expressed when the detoxification mechanisms are depressed. (5) The adoption of a multibiomarker battery focused on biotranformation, genotoxicity and endocrine levels should be a good biomonitoring strategy, facilitating the understanding of the mechanisms involved in fish toxic responses.

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CAPÍTULO IV

Alterações endócrinas e metabólicas em *Anguilla anguilla* L. após exposição a β-naftoflavona – um indutor das enzimas microssomais

Endocrine and metabolic changes in *Anguilla anguilla* L. following exposure to β-naphthoflavone - a microsomal enzyme inducer

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Environment International **31**, 99-104.

ABSTRACT

Anguilla anguilla L. were exposed during 24 and 48 h to 2.7 μM β-naphthoflavone (BNF), a known microsomal enzyme inducer. The BNF effects on thyroid-stimulating hormone (TSH), free triiodothyronine (T3), free thyroxine (T4) and cortisol plasma levels were investigated. Alterations on plasma glucose and lactate levels were also measured as an indication of energy-mobilizing hormones alterations. BNF showed to be able to decrease significantly *A. anguilla* plasma T4 levels, whereas TSH, T3 and cortisol plasma remained constant. However, plasma glucose levels were significantly increased, demonstrating that intermediary metabolism has been affected. These results demonstrate that BNF a PAH-like compound alters the normal functioning of the hypothalamo-pituitary-thyroid (HPT) axis in *A. anguill*a.

Keywords: β-Naphthoflavone; Plasma; Endocrine.

INTRODUCTION

In the vast majority of living organisms, the induction of phases I and II biotransformation enzymes is a well-known response to organic xenobiotic exposure. However, the knowledge of the linkage between these activations and other biological functions, namely endocrine regulation, is still a challenge to environmental toxicology. The majority of studies carried out on this subject concern effects on sexual hormones. For instance, β-naphthoflavone (BNF), an aryl-hydrocarbon (AhR) prototype ligand and cytochrome P4501A (CYP1A) inducer, demonstrated to impair the systemic hormonal control of reproductive processes by acting both at the hepatic level (vitellogenin production) and at the pituitary-gonad axis (Navas *et al.*, 2004). Nevertheless, the interference of this kind of xenobiotics with nonsexual endocrine responses in fish is still poorly understood.

Previous research work concerning mammals showed that typical microsomal enzyme inducers affected thyroid function. Thus, a decrease in plasma thyroxine (T4)

and triiodothyronine (T3) concentrations, after exposure to phenobarbital, 3-methylcholanthrene, polychlorinated biphenyls (PCBs), and BNF was observed (Vansell and Klaassen, 2002; Hood *et al.* 2003; Kato *et al.*, 2003). Nevertheless, to our knowledge, there are no studies on fish concerning the effects of this type of chemicals over the hypothalamo-pituitary-thyroid (HPT) axis. The information on the effects of microsomal enzyme inducers on corticosteroid hormones is also scarce. According to Wilson *et al.* (1998), BNF can affect fish pituitary-interrenal (HPI) axis since it was demonstrated that it abolishes interrenal sensitivity to adrenocorticotropic hormone (ACTH). Considering the previous statements, it seems relevant to investigate how chemicals that are not commonly regarded as endocrine disruptors can interfere at hormonal levels.

HPT and HPI axes play a central role on a wide range of important homeostatic mechanisms in fish. Thyroid hormones regulate growth, and hydromineral balance (Van Anholt *et al.*, 2003), while cortisol is involved in the regulation of energy metabolism, anti-inflammatory response as well as immune competence (Hontela, 1997; Wendelaar Bonga, 1997). Thyroid hormones and cortisol can both interact and influence carbohydrate metabolism (Hontela *et al.*, 1995). Alterations in these hormone plasma concentrations, as well as on glucose and lactate levels can reflect endocrine alterations, reducing fish physiological competence and possibly survivorship. Thus, the previously mentioned parameters can also be usefull tools on monitoring the impact of anthropogenic stressors in fish.

Previous studies have been performed with *Anguilla anguilla* concerning the effects of different xenobiotics on plasma cortisol, glucose and lactate levels (Santos *et al.*, 1990, 1992, 1993, 1996; Pacheco and Santos, 2001; Teles *et al.*, 2003a, b).

The choice of *A. anguilla* L. as test organism was based on its well-known resistance and sensitivity in the presence of adverse conditions making it a good option as an aquatic biological model for sublethal toxicological studies. A significant knowledge of eel's physiology was achieved by previous works namely those carried out in our laboratory. Moreover, the responses of *A. anguilla* to BNF in terms of biotransformation responses were extensively studied as an increase in microsomal

EROD activity, despite the lack of knowledge concerning its endocrine and intermediary metabolic effects. Thus, the purpose of the present study was to investigate the effects of BNF on the *A. anguilla* L. plasma thyroid-stimulating hormone (TSH), free triiodothyronine (T3), free thyroxine (T4), cortisol, as well as glucose and lactate levels.

MATERIAL AND METHODS

Chemicals

β-Naphthoflavone (BNF), β-nicotinamide adenine dinucleotide (β-NAD), L-lactic dehydrogenase, and glutamic-pyruvic transaminase were purchased from Sigma (USA). All the other chemicals were of analytical grade.

Biochemical Analysis

The determination of cortisol, TSH, T3 and T4 were performed in plasma, using diagnostic ELISA direct immunoenzymatic kits (Diametra, Italy). The absorbance in each well was measured at 450 nm in a microplate reader (ASYS Hitech).

The cortisol in the sample competes with horseradish peroxidase (HRP)-cortisol for binding onto the limited number of anti-cortisol sites in the microplate wells. The enzyme substrate (H_2O_2) and the TMB-substrate (TMB) are added, and after an appropriate time has elapsed for maximum color development, the enzyme reaction is stopped and the absorbances are determined. Cortisol concentration in the sample is calculated based on a series of standards and the color intensity is inversely proportional to the cortisol concentration in the sample.

The methods for free T3 and free T4 follow the same principles of the cortisol test, requiring immobilized T3 or T4 antibodies, as well as HRP-T3 or HRP-T4 conjugates.

Concerning TSH, an antibody specific to the β-chain of TSH molecule is immobilized on microwell plates and other antibodies to the TSH molecule are

conjugated with HRP. TSH from the sample is bound to the plates. The enzymatic reaction is proportional to the amount of TSH in the sample.

Plasma glucose was measured according to the method modified from Banauch *et al*. (1975). Plasma lactate was determined according to the method modified from Noll (1974).

Test Animals

The experiment was carried out using *A. anguilla* (European eel) collected from the Aveiro lagoon area – Murtosa, Portugal. The eels with a 25±3 cm (yellow eel) average length and weighing 30±5 g were acclimated to laboratory conditions in aerated (dissolved oxygen: 7.6±0.3 mg/L), filtered, dechlorinated tap water with pH 7.2±0.4, under a natural photoperiod at 20 °C, for 1 week prior to experimentation. Fish were neither fed under laboratory adaptation nor during the experimental procedure. The experiment was carried out in 20 L aquaria under the previous conditions.

Experimental Design

The eels were exposed to BNF 2.7 μM during 24 and 48 h. The appropriate amount of BNF was previously dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the experimental aquaria. The same volume of DMSO was added to the control aquaria. Fish blood was collected from the posterior cardinal vein using a heparinized Pasteur pipette and its plasma isolated using an Eppendorf centrifuge, 14,000 rpm during 5 min. Experiments were carried out using test groups of five eels (n=5). The BNF concentration and exposure time adopted were based on a previous study with same species, where a significant increase in liver EROD activity was observed (Teles *et al.*, 2003b).

Statistical Analysis

Mean±standard error (S.E.) was calculated for each experimental group, and data were analyzed for significance of differences between control and exposed groups according to the two-tailed Student's *t*-test (Bailey, 1959). Experiments were carried out using test groups of five eels (n=5). Differences between means were considered significant when $P<0.05$.

RESULTS

Hormonal Responses

A. anguilla plasma T4 was significantly decreased after 24- and 48-h exposure to BNF 2.7 μM when compared to the control. The plasma T4 decrease was time-related since a 2.5- and 5.1-fold decrease was, respectively, detected after 24 and 48 h. However, BNF did not induce any significant alteration on plasma cortisol, TSH and T3 concentrations (Fig. 1).

Intermediary Metabolism Responses

A. anguilla plasma glucose concentration significantly increased after 24- and 48-h BNF exposure. A 2.4-fold increase was observed after 24 h, while after 48-h exposure the increase was 4.3-fold. On the other hand, plasma lactate was not significantly altered (Fig. 2).

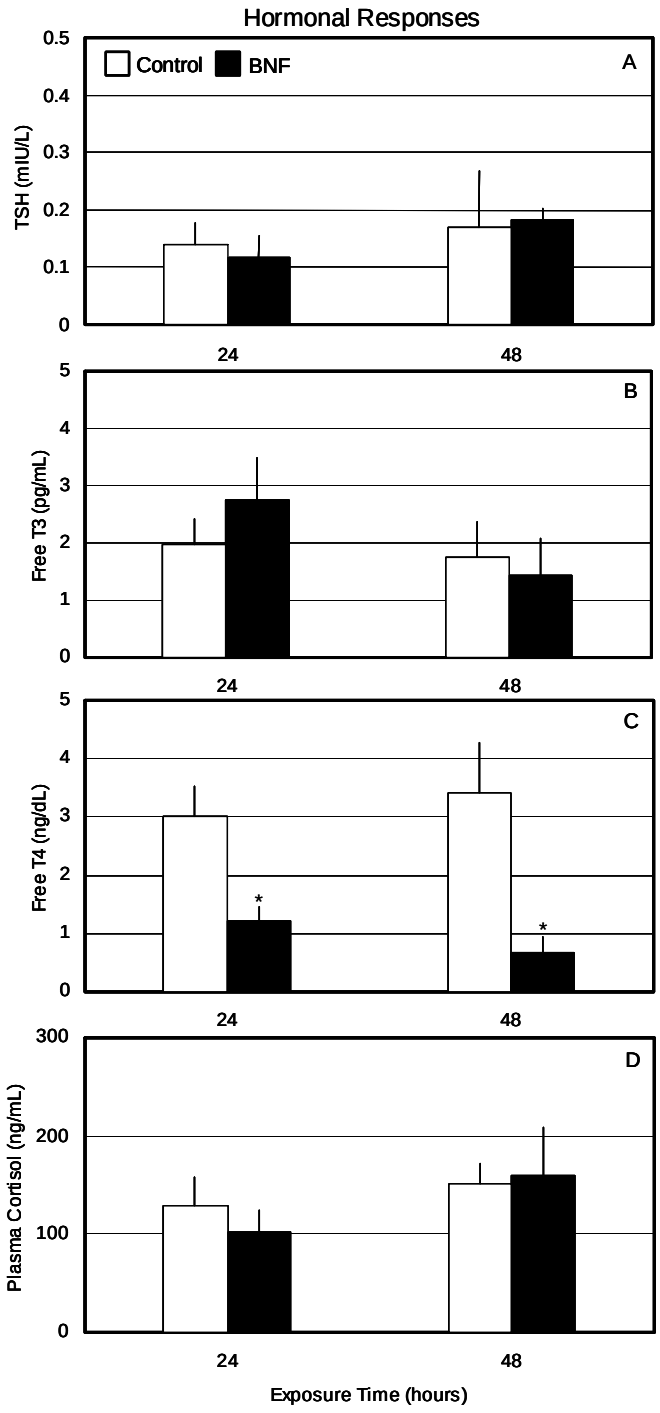

Fig. 1 - Plasmatic concentrations of TSH (A), free T3 (B), T4 (C) and cortisol (D) after 24 and 48 h of exposure to BNF 2.7 μM. Values represent the means and S.E. (*n*=5/treatment). Differences from control: * P<0.05.

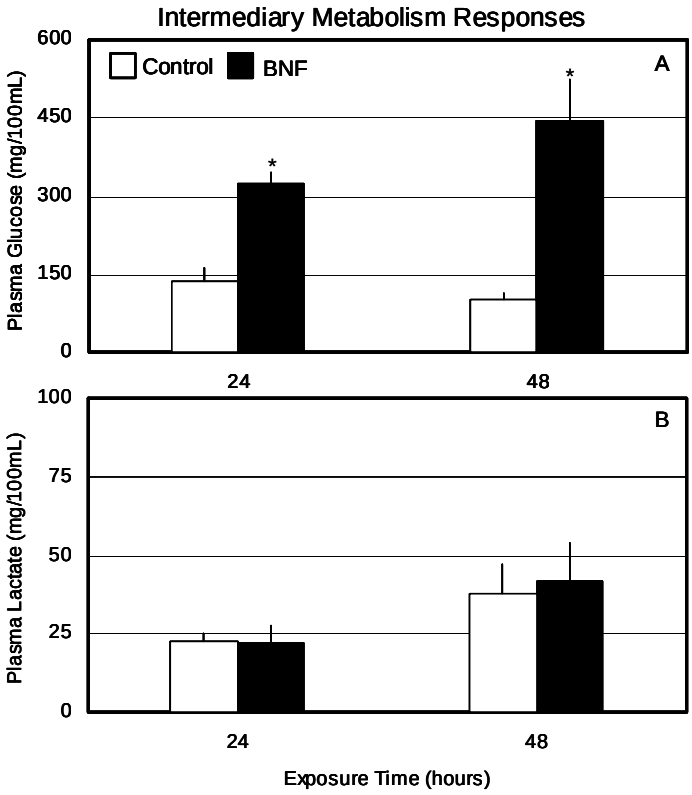

Fig. 2 - Plasmatic concentrations of glucose (A), and lactate (B) after 24 and 48 h of exposure to BNF 2.7 μM. Values represent the means and S.E. (*n*=5/treatment). Differences from control: * P<0.05.

DISCUSSION

The effects of different microsomal enzyme inducers (Liu *et al.*, 1995; Hood and Klassen, 2000), including BNF (Johnson *et al.*, 1993), on the thyroid hormone dynamics have been extensively studied in rats, unanimously revealing plasma T4 decrease. To our knowledge, no studies were performed on this issue concerning fish. Current results revealed decreased *A. anguilla* plasma T4 levels after exposure to BNF, confirming the data previously obtained with mammals. This similarity is not surprising considering that the morphofunctional organization of the thyroid gland is similar in fishes and in rats (Kornienko and Kozhin, 1997).

A wide range of mechanisms can be implicated in the xenobiotic-induced alterations of plasma thyroid hormones, corresponding to changes on thyroid status and/or alterations upstream or downstream the hormone production. These mechanisms include alterations on the: (i) hypothalamus and/or pituitary status (Alkindi *et al.*, 1996), (ii) biosynthesis and secretion steps of T3 and T4 (Capen, 1997), (iii) uptake by peripheral tissues, (iv) hepatic 5'-monodeiodinase activity (Waring *et al.*, 1996), or (v) hormone catabolism and clearance rates (Saito *et al.*, 1991; Hontela *et al.*, 1995). Among all the possible mechanisms involved in plasma T4 decrease following liver microsomal enzyme inducers exposure, it has been frequently proposed that UDP-glucuronyl-transferase (UDP-GT) inducers can increase T4 glucuronidation and biliary excretion (mechanism v), reducing plasma T4 concentration (McClain, 1992; Johnson *et al.*, 1993; Liu *et al.*, 1995). In fact, it was demonstrated that BNF induces liver UDP-GT activity in *Dicentrarchus labrax* (Novi *et al.*, 1998; Gravato and Santos, 2002). Thus, besides the similarity between mammals and fish responses, a mechanism resemblance can also be proposed, applying the previous explanation to the current responses observed in *A. anguilla*. Plasma T3 concentrations were unaffected by the current BNF fish treatment, which is consistent with the effects of other hepatic microsomal enzyme inducers on plasma T3 levels in rats (Hood *et al.*, 1999, 2003; Liu *et al.*, 1995). The ability to maintain plasma T3 concentrations is stronger than the ability to maintain T4 levels (Hood *et al.*, 2003), probably due to an activation of homeostatic mechanisms, such as increased synthesis of T3 by the thyroid or by extra-thyroidal tissues (5'monodeiodinase activity increase), recovery of T3 from T3-SO_4 by sulfatases, and increased enterohepatic circulation (Alkindi *et al.*, 1996; Hood *et al.*, 2003). According to Sapin and Schlienger (2003), plasma T3 is a less reliable reflection of thyroid hormone production than T4 since most of circulating T3 (around 80%) is produced extrathyroidally from T4 deiodination.

In response to reduced plasma T4, an increase in plasma TSH would be expected; however, in the present study plasma TSH levels remained unaltered. Previous studies concerning mammals exposed to microsomal enzyme inducers

revealed divergent responses, i.e., increased (Hood *et al.*, 2003) or unaltered (Liu *et al.*, 1995; Hood and Klassen, 2000) plasma TSH concomitantly with decreased plasma T4. According to Hood and Klassen (2000), these mechanisms are still poorly understood.

Previous fish studies (Anderson *et al.*, 1996; Navas *et al.*, 2004) suggested a disruptive action of BNF upon the hypothalamus-pituitary-gonad axis through the disappearance of the negative feedback control on pituitary luteinizing hormone (LH) release, explained through its absence of effect on estrogen receptors (ER) at the pituitary and hypothalamus level. Considering the current data, the occurrence of alterations on thyroid hormone receptors (TR) at the hypothalamus/pituitary level is not excluded; though it cannot be adopted as an explanation for the absence of any TSH response to plasma T4 depression. Additionally, an alteration on thyrotrophin releasing hormone (TRH) receptors at pituitary cells could also be suggested.

Thyroid hormones deiodination, conjugation as well as transport and their receptors interaction, besides measuring its plasma levels, should also be evaluated in order for a better understanding of the HPT axis dynamic, following xenobiotic exposure.

Fish respond to stress with characteristic acute increases in plasmatic levels of catecholamines, and slower but more sustained increases in plasmatic levels of the corticosteroid cortisol (Grutter and Pankhurst, 2000). Thus, alterations on the levels of plasma cortisol could provide valuable information on the fish stress condition. BNF is a potent cytochrome P450 1A inducer in fish tissues, including interrenal (Husøy *et al.*, 1994). CYP1A1 isozyme of fish does not catalyze oxidative metabolism of cortisol; however, CYP1A1 inducing compounds may interfere with steroid plasma levels through different mechanisms namely the increase of phase II conjugation of steroids, as suggested by Förlin and Haux (1985) for the effects of BNF on β-estradiol levels.

Furthermore, according to Wilson *et al.* (1998) a BNF interference on steroid biosynthetic pathways through a substrate competition or alterations on key mitochondrial enzymes involved in cortisol synthesis would be expectable, resulting in lower cortisol production. However, the present results did not confirm this hypothesis

since *A. anguilla* plasma cortisol levels were not altered by the BNF treatment. It is well known that the stress of capture and handling induces an acute rise in plasma cortisol levels (Hontela, 1997); therefore, when control and treated fish present similar plasmatic levels of cortisol it means that both groups were equally able to elevate cortisol under stress.

A previous study concerning *D. labrax* exposure to BNF 0.9 μM during 24 h revealed no plasma cortisol alteration (Teles *et al.*, 2004). In addition, no changes were found, after BNF injection, in *Oncorhynchus mykiss* plasma cortisol concentration, despite the ACTH interrenal sensitivity abolishment demonstration (Wilson *et al.*, 1998). The authors suggested that the ability of BNF-treated fish to normally increase plasma cortisol concentration could be due to other hormonal pathways, in addition to ACTH sensitivity abolishment, as well as alterations in the plasma cortisol clearance rate. Therefore, the absence of significant plasma cortisol alterations cannot ensure that the cortisol dynamics was unaffected, remaining the possibility alterations of HPI axis as well as plasma cortisol uptake by the target cells.

In the present study, plasma glucose increased after BNF exposure, despite the unaltered plasma cortisol and lactate levels. Vijayan *et al.* (1997) stated that besides interrenal cortisol release, other mechanisms might be controlling glucose availability in fish. Moreover, under acute stress catecholamines could be rapidly released resulting in increased glycogenolysis. However, this explanation cannot be applied to the current glucose response, since the persistence of catecholamine effects up to 48 h is not likely. Van der Boon *et al.* (1991) stated that the influence of plasma cortisol on fish carbohydrate metabolism is not very comprehensive and thus the establishment of a consistent relation between plasma cortisol, glucose and lactate seems difficult. However, the authors suggest that the current plasma glucose increase observed following BNF exposure may be related to an increased liver gluconeogenesis induced by the previously uptaken plasma cortisol.

Plasma T4 frequently follows a response pattern similar to that one of plasma cortisol, and T4 may also activate the interrenal function (Hontela *et al.*, 1995). On the

other hand, cortisol can induce T4 deiodination originating T3, as well as increase the clearance rate of T3 (Redding *et al.*, 1991) and T4 (Leatherland, 1987).

Finally, the present results confirm the studied parameters as important biomonitoring tools to assess the presence of stressors in aquatic environment contributing to a broader knowledge of fish responses as integration at different physiological levels. Moreover, these findings highlight the need of a careful interpretation of field data, since the occurrence of sequential or simultaneous exposures to different classes of chemicals is likely.

CONCLUSIONS

- *A. anguilla* displayed a significant decrease in plasma T4 levels after BNF exposure, revealing a similarity with the known mammal responses. These results demonstrate that typical microsomal enzyme inducers, namely BNF, can also interfere with neuroendocrine processes.

- BNF affected the intermediary metabolism since plasma glucose levels were significantly increased; however, it seems difficult to establish a correlation between cortisol, thyroid hormones and carbohydrate metabolism.

- In the future, it would be of interest assess thyroid hormones conjugation and deconjugation processes.

- The above results confirm the studied parameters as important biomonitoring tools to assess the presence of stressors in aquatic environment, providing significant information related to fish intermediary metabolism and endocrine responses to complex environmental mixtures.

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CAPÍTULO V

Efeitos do 17β-estradiol na biotransformação, stresse e genotoxicidade em robalos juvenis (*Dicentrarchus labrax* L.)

Biotransformation, stress and genotoxic effects of 17β-estradiol in juvenile sea bass (*Dicentrarchus labrax* L.)

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ABSTRACT

The effects of 17β-estradiol (E_2) on fish became a matter of concern, since significant levels of this hormone were detected in the aquatic environment released mainly by domestic sewage treatment plants. In this perspective, the current study was focused on E_2 effects upon biotransformation, stress and genotoxic responses of juvenile *Dicentrarchus labrax* L. (sea bass). Fish were exposed to E_2 during 10 days in two different ways: water diluted (200 ng/L or 2000 ng/L) and i.p. injected (0.5 mg/Kg or 5 mg/Kg). A battery of biological responses was evaluated: liver ethoxyresorufin-*O*-deethylase (EROD) and alanine transaminase (ALT) activities, liver somatic index (LSI), plasma cortisol, glucose and lactate concentrations, as well as erythrocytic nuclear abnormalities (ENA).

All the exposure conditions induced endocrine disruption, measured as plasma cortisol decrease, and genotoxicity, measured as ENA increase. Thus, no differences were detected either between different exposure routes or tested concentrations. Concerning liver EROD and ALT activities, as well as plasma glucose and lactate concentrations no differences were found between treated and control groups. LSI was the only parameter to respond differently in the two exposure routes, as only E_2 water diluted induced a significant increase in this hepatic indicator.

Keywords: 17β-Estradiol; *Dicentrarchus labrax* L.; Biotransformation; Stress Responses; Genotoxicity.

INTRODUCTION

Natural female estrogens are excreted via bile and urine mainly as water-soluble glucuronides, sulfates or, to a minor extent, in its free form (Fürhacker *et al.*, 1999). Before conjugation, estrogens undergo hydroxylation, reduction, methylation or oxidation predominantly in the liver. 17β-Estradiol (E_2), the most effective estrogen secreted by the ovaries, can be oxidized to estrone and further converted into estriol.

Polar metabolites like 16-hydroxyestrone, 4-hydroxyestradiol, 16-ketoestrone and/or 16-epiestriol are formed and can also be found in urine and faeces (Ying *et al.*, 2002).

Estrogens and E_2 in particular reach aquatic environments mainly through domestic effluents. Previous measurements of human estrogen excretion estimated that females were excreting 2.3-259 μg/person/day and males 1.6 μg/person/day of E_2 through urine (Johnson *et al.*, 2000). Two other important sources of E_2 are livestock waste (Ying *et al.*, 2002) and agriculture runoff (Céspedes *et al.*, 2004). For example, in poultry waste a concentration ranging from 14 to 533 ng/g dry waste for E_2 was reported by Shore *et al.* (1995). The E_2 concentration in urine of cattle was found to be 13 ng/L on average (Erb *et al.*, 1977). Recent studies have shown that the utilization of animal manure to agricultural land can lead to movement of E_2 into surface and ground water (Peterson *et al.*, 2001). E_2 has been found mobile and detected in runoff from a pastural land in an average concentration of 3500 ng/L (Nichols *et al.*, 1998). Therefore, in environmental waters E_2 concentration range is between the detection limit up to ng/L (Spengler *et al.*, 2001).

Although E_2 conjugates released into aquatic environments do not possess a direct biological activity, they can act as precursor hormone reservoirs able to be reconverted by bacteria to free E_2. Moreover, this cleavage is particular relevant in both raw and treated sewages (Baronti *et al.*, 2000).

Taking into consideration the scenario previously described, E_2 has been increasingly reported as an environmental contaminant (Ying *et al.*, 2002; Imai *et al.*, 2005), being also regarded by Dorabawila and Gupta (2005) as “the most potent of all xenoestrogens”, able to affect aquatic organisms, namely fish. Furthermore, E_2 is among the most potent endocrine disrupting chemicals having the potential to exert effects at extremely low concentrations (Bowman *et al.*, 2002).

Studies regarding E_2 effects on fish have been focused on endocrine aspects, mainly reproduction. It is known that it may alter gonadosomatic index in males, reduce egg production in females, induce vitellogenesis in males and juveniles as well as decrease fertility (Mills *et al.*, 2001; Kang *et al.*, 2002). However, there is now ample evidence that non-reproductive endocrine events can be disrupted by E_2,

namely the response to stress. Glucocorticoids, such as cortisol, are important for stress responses in fish, particularly in metabolic adjustments by the regulation of energy production, hydro-mineral balance, oxygen uptake and immune competence (Hontela, 1997). Nevertheless, only a few studies have been carried out on E_2 effects on plasma cortisol, leading to non-coincident results (Pottinger *et al.*, 1996; Teles *et al.*, 2005). Similarly, the effects of E_2 on fish secondary stress responses, such as alterations on plasma glucose and lactate concentrations, are still poorly understood (Petersen *et al.*, 1983; Teles *et al.*, 2005).

In the field of non-endocrine E_2 effects on fish, special attention has been paid on regulation of CYP1A expression; hence, several authors observed the suppression of CYP1A-associated 7-ethoxyresorufin-*O*-deethylase (EROD) activity, in both maturing females *Scophthalmus maximus* L. and juvenile *Sparus aurata* L. treated experimentally with E_2 (Arukwe and Goksøyr, 1997; Teles *et al.*, 2005). Moreover, several mammal studies indicated that E_2 is genotoxic (Liehr, 2000; Joosten *et al.*, 2004). Thus, the ecotoxicological relevance of E_2 genotoxicity on fish is worthwhile to study since little is known about this subject.

The present study was undertaken to assess E_2 effects on *Dicentrarchus labrax* L. (sea bass) at different levels: CYP1A expression, measured as liver EROD activity, genotoxicity, measured as erythrocytic nuclear abnormalities (ENA), as well as stress responses, namely plasma cortisol, glucose and lactate levels. Hepatic indicators, such as liver somatic index (LSI) and alanine transaminase (ALT) activity were also measured. Furthermore, the previous responses were evaluated under two different E_2 exposure routes, i.e., either water diluted or intraperitoneally (i.p.) injected, in order to clarify the influence of the exposure route.

MATERIAL AND METHODS

Chemicals

17β-Estradiol was purchased from Sigma-Aldrich (Germany), marine salt from Sera Premium (France). The kit for cortisol determination was obtained from

Diametra (Italy). All the other chemicals were of analytical grade obtained from Sigma-Aldrich (Germany) and E. Merck-Darmstadt (Germany).

Test animals

The experiment was carried out with juvenile *D. labrax* specimens purchased from a local fish farm, Materaqua – Ílhavo, Portugal. Fish weighing 21.80±1.32 g and measuring 12.4±3 cm were transported in aerated water and acclimated to laboratory conditions for 1 week prior to experimentation. During acclimatization and experimental periods fish were kept in 80 L aquaria, at 20ºC in aerated (dissolved oxygen: 7.3±0.4 mg/L) and filtered artificial seawater (34 g/L), with a pH of 8.4±0.3. Fish were neither fed under laboratory adaptation nor during the experimental period.

Experimental design

This study includes two experiments, corresponding to two different E_2 exposures routes – water diluted (WD) and i.p. injection (IP).

In WD experiment, fish were exposed to water diluted E_2 at the concentrations of 200 ng/L (0.734 nM) or 2000 ng/L (7.34 nM). The appropriate amount of E_2 was previously dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the experimental aquaria to reach the previous initial nominal concentrations. The same E_2 amount was daily added to the experimental aquaria, without water renewal. This procedure was adopted, since it was previously observed that a 4000 ng/L E_2 water concentration suffered a 99 % reduction 4 h after addition (Teles *et al.*, 2005). DMSO (1 mL) was also daily added to the control aquarium.

In the IP experiment, fish were initially treated with a single E_2 i.p. injection, using corn-oil as a carrier and two doses - 0.5 mg/Kg and 5 mg/Kg - were tested. Control fish were also i.p. injected with corn-oil alone.

Both experiments were performed with an exposure length of 10 days using test groups of five fish (n=5). Blood was collected from the posterior cardinal vein using a Pasteur pipette with heparin. Following blood sampling, fish were sacrificed by decapitation and its liver sampled. Liver was immediately frozen in liquid nitrogen

and stored at -80 ºC until homogenization. Blood was used for smear preparation and for plasma isolation using an Eppendorf centrifuge (14,000 rpm).

Biochemical analysis

Liver EROD activity

The liver EROD activity was measured in microsome suspension as described by Burke and Mayer (1974) and adapted by Pacheco and Santos (1998). The reaction was carried out, at 25ºC, in the fluorometer cuvette containing 1 mL 0.5 μM ethoxyresorufin (in 0.1 M Tris–HCl pH 7.4, containing 0.15 M KCl and 20% glycerol) and 25 μL of microsomal suspension. The reaction was initiated by adding 10 μL of NADPH (10 mM) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD-activity was expressed as picomoles per minute per milligram of microsomal protein.

Liver ALT activity

ALT activity was measured, in the supernatant resulting from microsomal isolation, according to a colorimetric method based on the measurement of the pyruvate produced by the transamination reaction (Reitman and Frankel, 1957).

Protein measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornal *et al.*, 1949) using bovine serum albumin as a standard.

Plasma cortisol, glucose and lactate measurement

The cortisol determination was performed using a Diametra (Italy) kit. Briefly, cortisol in the sample competes with horseradish peroxidase-cortisol for binding onto the limited number of anti-cortisol sites in the microplate. Cortisol concentration, measured spectrophotometrically (450 nm), is calculated based on a series of

standards and the color intensity is inversely proportional to the cortisol concentration in the sample.

Plasma glucose was measured spectrophotometrically (340 nm) according to the method modified from Banauch *et al.* (1975) based on the quantification of NADH after a glucose oxidation catalysed by the glucose-dehydrogenase. The quantity of NADH formed is proportional to the glucose concentration.

Plasma lactate levels were determined spectrophotometrically (340 nm) according to the method modified from Noll (1974) using lactate-dehydrogenase, ALT and NAD, measuring the NADH appearance.

Liver somatic index

LSI results were presented as a percentage, resulting from the following expression: [liver mass (g)/body mass (g)]×100.

Erythrocytic nuclear abnormalities (ENA) assay

The blood smears were fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. In order to evaluate genotoxicity, the erythrocytic nuclear abnormalities were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid (1976), Carrasco *et al.* (1990) and Smith (1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei (a supernumerary nuclear structure within the cytoplasm), lobed nuclei (nuclei with evaginations, ranging from single swellings of the nuclear surface to the presence of multiple lobes), dumbbell shaped or segmented nuclei (two lobes are present as a consequence of a central and bilateral constriction) and kidney shaped nuclei (nuclei with a central and unilateral constriction). The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

Micronuclei are expressed in dividing cells that either contain chromossome breaks lacking centromeres (acentric fragments) and/or whole chromossomes that

are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromossomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term “micronucleus”. Micronuclei, therefore, provide a convenient and reliable index of both chromossome breakage and chromossome loss (Fenech, 2000). It is assumed that the other nuclear abnormalities express DNA damaging events similar to those described for micronuclei formation.

Statistical Analysis

Statistica software (StatSoft, Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P< 0.05$.

RESULTS

D. labrax liver EROD (Fig. 1) and ALT (Fig. 2A) activities showed no statistically significant changes for both water diluted (WD) and intraperitoneal injection (IP) experiments, comparing to the respective control groups.

LSI (Fig. 2B) increased significantly in *D. labrax* exposed to water diluted E_2 in both tested concentrations, displaying a less pronounced rise for the highest concentration – 72 % increment for 200 ng/L *vs.* 25 % for 2000 ng/L. On the other hand, no significant alterations were observed in the LSI of i.p. injected fish.

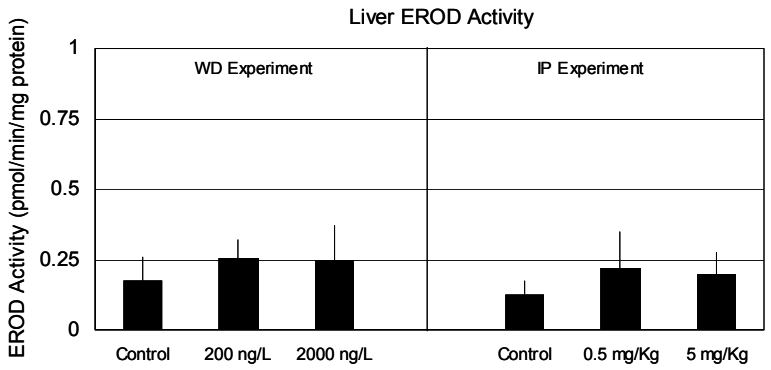

Fig. 1 - *D. labrax* liver EROD activity after 10 days of exposure to 17β-estradiol (E_2) water diluted (WD experiment, 200 ng/L or 2000 ng/L) and intraperitoneally injected (IP experiment, 0.5 mg/Kg or 5 mg/Kg). Values represent the means and S.E. (*n=5*/treatment).

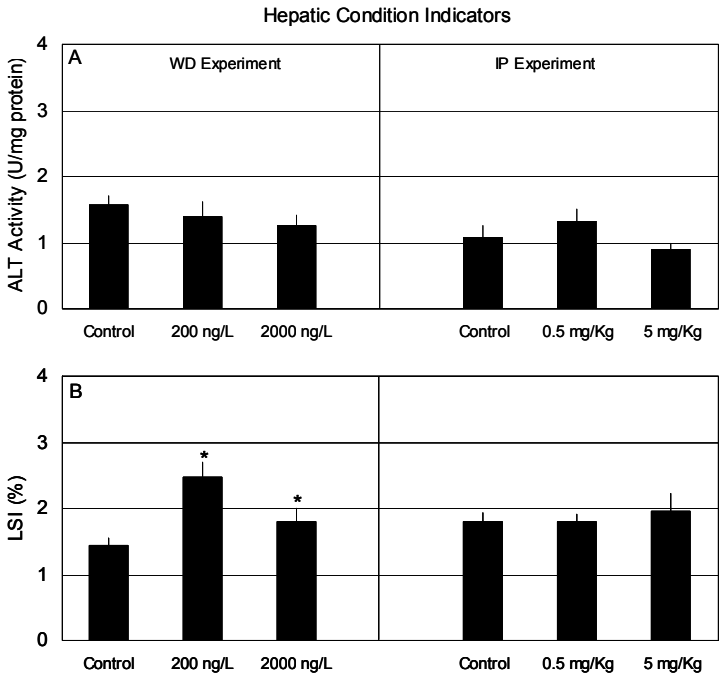

Fig. 2 - *D. labrax* liver ALT activity (A) and LSI (B) after 10 days of exposure to 17β-estradiol (E_2) water diluted (WD experiment, 200 ng/L or 2000 ng/L) and intraperitoneally injected (IP experiment, 0.5 mg/Kg or 5 mg/Kg). Values represent the means and S.E. (*n*=5/treatment). Differences from control: *P<0.05.

Concerning stress responses (Fig. 3), both WD and IP experiments induced a significant decrease in *D. labrax* plasma cortisol concentrations (Fig. 3A). Moreover, a dose dependency was perceptible in this response for both experiments – 38% decrease for 200 ng/L *vs.* 55% for 2000 ng/L and 37% decrease for 0.5 mg/Kg *vs.* 66% for 5 mg/Kg. Plasma glucose (Fig. 3B) and lactate (Fig. 3C) concentrations were unaltered in both E_2 exposure routes.

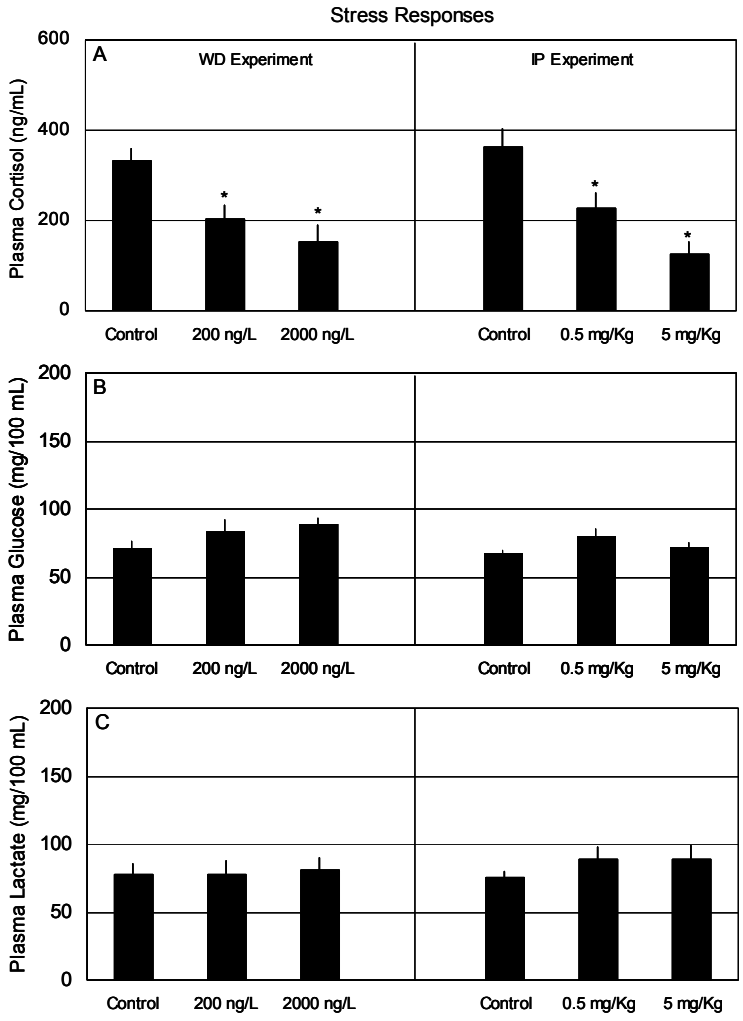

Fig. 3 - *D. labrax* plasma cortisol (A), plasma glucose (B), and plasma lactate (C) after 10 days of exposure to 17β-estradiol (E_2) water diluted (WD experiment, 200 ng/L or 2000 ng/L) and intraperitoneally injected (IP experiment, 0.5 mg/Kg or 5 mg/Kg). Values represent the means and S.E. (*n*=5/treatment). Differences from control: *P<0.05.

An E_2 genotoxic effect, measured as ENA frequency increase, was detected for all tested groups in comparison to their controls (Fig. 4); however, the ENA increase showed no relation to the dose. Comparing the experiments, a higher increase in WD experiment (around 7 times increase for 200 ng/L and 6 times increase for 2000 ng/L) than in IP experiment (around 5.5 times increase for 0.5 mg/Kg and 5 times increase for 5 mg/Kg) is perceptible.

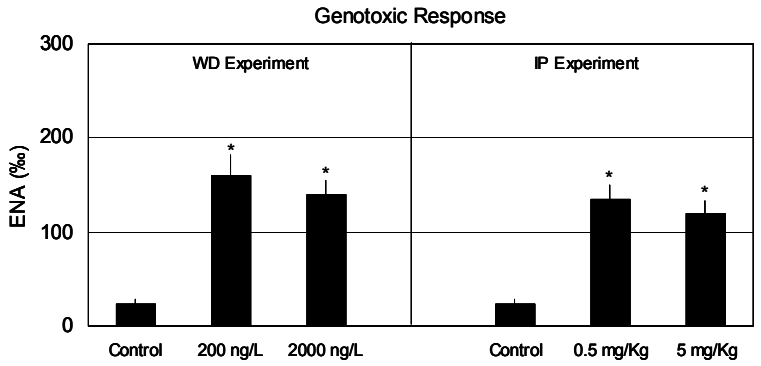

Fig. 4 - *D. labrax* ENA frequency after 10 days of exposure to 17β-estradiol (E_2) water diluted (WD experiment, 200 ng/L or 2000 ng/L) and intraperitoneally injected (IP experiment, 0.5 mg/Kg or 5 mg/Kg). Values represent the means and S.E. (*n*=5/treatment). Differences from control: **P*<0.05.

DISCUSSION

Hormonal steroids have always been present in the aquatic environment. However, their occurrence in natural waters strongly increased in the last decades due to its growing use in human medicine and livestock farming release, becoming a matter of concern and an interesting research topic for ecotoxicology.

The highest concentration (2000 ng/L) employed in the current WD experiment was chosen to mimic endogenous levels of circulating estrogen in fish spawning females, whereas the lowest concentration (200 ng/L) was chosen according to the highest concentrations found in the aquatic environment, as previously reported by Bowman *et al.* (2000). On the other hand, the doses adopted in the IP experiment

took into consideration several studies where 0.5 and 5 mg E_2/Kg induced estrogenic effects in fish (Anderson *et al.*, 1996; Pait and Nelson, 2003).

D. labrax was adopted as a bioindicator, since it represents a keystone species with economic and ecological importance in the Eastern Atlantic Ocean, Mediterranean and Black Sea.

Biotransformation Response

The CYP1A subfamily normally is expressed in fish at low levels in the absence of aryl hydrocarbon receptor (AhR) agonists. These basal levels can be depressed by non-AhR agonists as it was demonstrated for E_2 in *Oncorhynchus mykiss* (Navas and Segner, 2000; Elskus, 2004) and *S. aurata* (Teles *et al.*, 2005). In general, an enzyme activity inhibition can be achieved either by an interaction at the gene transcription level or by a direct competitive or non-competitive interaction of the tested chemicals with the enzyme molecule (Navas and Segner, 2000). The ability of steroids to bind to the CYP1A molecule acting as EROD activity competitive inhibitors was well established (Chan and Hollebone, 1995). Furthermore, Navas and Segner (2001) found that E_2 mediated suppression of CYP1A is at least not restricted to the enzyme catalytic level but occurs also at the pretranslational level.

The results of the present study indicated that E_2 was not able to suppress the constitutive CYP1A-associated EROD activity in *D. labrax*. Considering the findings of Navas and Segner (2000), Elskus (2004) and Teles *et al.* (2005), the current unaltered EROD levels can be surprising; though, they agree with our previous findings concerning the same species, where it was also found unaltered EROD activity in fish exposed to 36,000 ng/L of E_2 during 24 h (Teles *et al.*, 2004).

Considering the existing literature and the present results, a dependence on the tested species seems to exist, justifying further comparative studies in order to better understand the E_2 effects on CYP1A expression.

Hepatic Condition Indicators

The LSI increase under WD exposure suggests an altered metabolic state. Though, in a previous study with the same species no LSI alterations were found after 24 h exposure to 36,000 ng/L of E_2 (Teles *et al.*, 2004). This difference might be related mainly with the exposure length. On the other hand, an LSI increase was observed in juvenile *S. aurata* exposed during 16 h to 4000 ng E_2/L (Teles *et al.*, 2005) and in *Oryzias javanicus* exposed to 243 ng E_2/L during 6 months (Imai *et al.*, 2005). Verslycke *et al.* (2002) suggested that the liver weight increase observed after E_2 exposure may be related with a stimulation of hepatic vitellogenin synthesis.

D. labrax i.p. exposed to E_2 behaved differently from the WD E_2 exposed fish, since no LSI alterations were found for any of the tested doses. The results are different from a previous study where *Paralichthys dentatus* injected with 2 and 20 mg/Kg during 4 weeks exhibited an increased LSI, coincident with a plasma vitellogenin increase (Mills *et al.*, 2001).

Liver ALT activity was not altered in any of the performed experiments; these results are in agreement with a previous study where *S. aurata* exposed to E_2 had unaltered ALT activity (Teles *et al.*, 2005).

Stress Responses

Plasma cortisol increase is a typical fish response to short-term exposure to stressors. This hormonal increase is commonly followed by a rise in plasma glucose and lactate concentrations (Hontela, 1997). Nevertheless, fish responses to stressors demonstrated to be more complex, since different patterns of response have been observed either concerning the cortisol variation (Santos and Pacheco, 1996; Pacheco and Santos, 2001; Benguira *et al.*, 2002; Teles *et al.*, 2003) or its relation with the secondary stress responses (Teles *et al.*, 2003, 2005).

The present results revealed a plasma cortisol decrease suggesting a concentration-dependent response for both E_2 exposure routes. Despite the scarcity of fish studies on cortisol alterations after E_2 exposure, this kind of response is not

surprising as it was previously found in *S. aurata* after short-term exposure to 4000 ng E_2/L (Teles *et al.*, 2005).

A plasma cortisol decrease may indicate the occurrence of an endocrine impairment, preventing the animal to respond to the stress of capture and handling or alternatively, may represent a reduction in baseline levels of plasma cortisol. Different explanations were previously presented for the decrease of circulating cortisol. Santos and Pacheco (1996) proposed a chemical induced impairment on interrenal cortisol release, since an accumulation of cortisol was found in this tissue in parallel with a low plasma cortisol concentration. Alternatively, an *in vitro* incubation experiment using interrenal salmonid tissue demonstrated that E_2 suppressed the interrenal cells ability of using pregnenolone as a substrate for cortisol synthesis, and reduced interrenal response to ACTH through a post-receptor cyclic adenosine monophosphate (cAMP)-mediated steroidogenesis (McQuillan *et al.*, 2003). Moreover, it can be suggested that E_2 might affect the cortisol levels in plasma through its fast clearance as demonstrated in an early work with *O. nerka* for androgens (Donaldson and Fagerlund, 1970).

Other studies with fish short-term exposed to E_2 showed divergent results. Salmonids exposed to E_2 had elevated levels of plasma ACTH and cortisol (Pottinger *et al.*, 1996). Unaltered cortisol levels were observed in *D. labrax* water exposed (Teles *et al.*, 2004), as well as in i.p. injected *Oreochromis mossambicus* (Vijayan *et al.*, 2001) and *Salmo salar* (McCormick *et al.*, 2005).

Considering the number of studies where a plasma cortisol decrease was found, following short-term exposures either to E_2 or other classes of chemicals (Pacheco and Santos, 2001; Teles *et al.*, 2003, 2005), this response should be regarded as a new a pattern of response to short-term exposures to stressors.

The biological significance of the observed plasma cortisol decrease may be a reduced fish physiological competence as survivorship and growth since this hormone is required for a wide range of important homeostatic mechanisms.

Concerning the current intermediary metabolism data, plasma glucose and lactate levels remained unaltered after E_2 exposure, for both exposure routes. A

previous study with the same species exposed to 36,000 ng E_2/L during 24 h detected a significant glucose increase (Teles *et al.*, 2004); this discrepancy may be explained by the difference on tested concentrations and/or exposure length. Teles *et al.* (2005) also found unchanged levels of plasma glucose in *S. aurata* exposed to E_2, while lactate was significantly increased. On the other hand, Washburn *et al.* (1993) measured a decrease in plasma glucose concentration in *O. mykiss* E_2–exposed. Taking into account the available data on E_2 effects over plasma glucose and lactate concentrations, a considerable unpredictability of these fish responses, depending on the species and experimental protocol is evident. The absence of glucose and lactate changes in the present study can be expected, as a concomitant plasma cortisol decrease was observed. Moreover, Washburn *et al.* (1993) detected a gluconeogenesis depression in *O. mykiss* E_2 exposed.

Genotoxic Response

According to Joosten *et al.* (2004) among the hormonal steroids, estrogens take the head position with respect to incidence of positive outcome of genotoxicity in mammals. E_2, in particular, was classified as a carcinogen class I by the International Agency for Research on Cancer (IARC) (1999). This hormone caused various types of DNA damage including permanent genetic changes in mammals, that may have relevance for carcinogenesis (Liehr, 2000). Hence, an E_2-induced increment of sister chromatid exchanges (SCE), DNA adducts, polyploidy or aneuploidy was observed (Joosten *et al.*, 2004). Furthermore, it was demonstrated that E_2 itself can link to DNA and proteins inducing DNA single strand breaks (Han and Liehr, 1994) and micronuclei (Yared *et al.*, 2002). A different picture showing negative results was observed in Ames and chromosome aberration tests (Joosten *et al.*, 2004).

Mammal studies demonstrated that besides the parental hormone, E_2 metabolites may act at gene and/or chromosome levels (Roy and Liehr, 1999). Potentially carcinogenic metabolites include catechol estrogens such as 16α-hydroxyestrone, 4-hydroxyestrone and 4-hydroxyestradiol. 4-Hydroxyestradiol, in particular, can undergo metabolic redox cycling generating mutagenic free radicals

and oxidative stress, which was associated with estrogen carcinogenicity in rodents (Li *et al.*, 2004).

Taking into account the above results in mammals and the E_2 concentrations detected in the aquatic environment, it arise an important question about the E_2 genotoxic effects on fish. However, only a few studies were carried out in this context. Experiments performed with *D. labrax* (Teles *et al.*, 2004) and *S. aurata* (Teles *et al.*, 2005) did not confirm the E_2 genotoxicity, measured as ENA frequency, after short-term exposure. However, considering the ENA increasing tendency observed for 24 h *D. labrax* exposure, the authors suggested the need to perform longer exposures to obtain conclusive results.

It was observed that the most prominent estrogen metabolites in fish are 2-hydroxyestradiol and estrone (Snowberger and Stegeman, 1987; Stein *et al.*, 1991; Butala *et al.*, 2004). Nevertheless, Butala *et al.* (2004) detected formation of both 2- and 4- hydroxyestradiol in fish liver. Considering the genotoxicity of the previous E_2 metabolites demonstrated in mammals, these studies corroborate the E_2 genotoxic potential in fish.

Present results showed an ENA increase induced by E_2 in *D. labrax* for both exposure routes and both concentrations. These results clearly demonstrate the E_2 genotoxicity, reinforcing the hypothesis previously presented by Teles *et al.* (2004).

The correlation between genotoxicity and CYP1A expression is generally based on the assumption that CYP1A induction can potentiate the genotoxic properties of the xenobiotics. Considering that the expectable effect of E_2 on CYP1A expression would be its down-regulation, the correlation between EROD activity and ENA increase is less relevant. Moreover, the hypothesis of CYP1A down-regulation by E_2 was not confirmed in the present study.

The direct or indirect effects of stress can render fish more vulnerable to genotoxins. However, considering present results, *i.e.*, plasma cortisol decrease and unaltered plasma glucose and lactate levels, no correlation could be established between a stress induction and the observed genotoxic effects.

CONCLUSIONS

- The comparison between the responses to the different E_2 exposure routes revealed no differences for all the assessed parameters, excluding LSI. Thus, it may be suggested that E_2 distribution and/or elimination processes seem not to be affected.

- E_2 decreased plasma cortisol levels, revealing its endocrine disruptive role affecting fish stress responsiveness. This alteration was confirmed as a possible response pattern to xenobiotics short-term exposures.

- E_2, considered an aquatic contaminant, induced genetic damage in *D. labrax* since an ENA frequency increase was observed for both exposure routes and all tested concentrations.

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CAPÍTULO VI

Indução de vitelogenina em juvenis de *Dicentrarchus labrax* L. tratados com 17β-estradiol (E_2) – relação entre vias de exposição e níveis plasmáticos de E_2

Vitellogenin induction in juvenile *Dicentrarchus labrax* L. treated with 17β-estradiol (E_2) – relation with exposure route and E_2 plasmatic levels

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ABSTRACT

17β-Estradiol (E_2) is a female hormone, which has been increasingly reported as a water pollutant, being one of the strongest estrogenic chemicals in the environment. The present study concerns the 10 days exposure of immature *Dicentrarchus labrax* L. (sea bass) either to water diluted (WD, 200 or 2,000 ng/L) or intraperitoneally injected (IP, 0.5 or 5 mg/kg) E_2 in order to investigate its uptake and resulting vitellogenesis induction, measured as E_2 and vitellogenin (Vtg) plasma levels, respectively. Thus, E_2 plasma levels were significantly increased only in WD exposure for both tested concentrations, whereas plasma Vtg increased for the highest concentrations in both experiments. These results demonstrated that both exposure routes were able to induce vitellogenesis in immature sea bass and that an E_2-induced increase in plasma Vtg could be found without a concomitant increase in plasma levels of E_2. Based on environmental realistic concentrations used, these results confirm that environmental exposures to E_2 may represent an important risk mainly to juvenile fish, namely due to the adverse consequences of an abnormal Vtg production.

Keywords: *Dicentrarchus labrax*; 17β-Estradiol; Vitellogenin; Endocrine disruption.

INTRODUCTION

During the last decades, a great concern has been raised regarding the possible harmful effects of certain chemicals, both synthetic and natural, upon wildlife modulating and/or disrupting the endocrine system - the so-called endocrine-disrupting compounds (EDCs).

Particular attention has been paid to persistent environmental contaminants that mimic the effects of the female steroid hormone 17β-estradiol (E_2) - xenoestrogens - namely organochlorine pesticides, polychlorinated biphenyls and their hydroxylated metabolites, alkyphenols and a few phthalates (Witters *et al.*, 2001). However, it is becoming clear that excessive inputs of natural estrogens into

surface waters can be responsible for endocrine disruption in wildlife, namely fish (Schultz *et al.*, 2001). For example, female hormones excreted by women and other animals are ubiquitous in aquatic environments receiving sewage inputs (Bowman *et al.*, 2002). Therefore, E_2 and estrone are included in a preliminary list of priority substances for which endocrine-disrupting characteristic have been reported (López de Alda and Barceló, 2001). These naturally-occurring estrogens are found in environmental waters at very low concentrations (low ng/L) compared to other EDCs arising mainly from industrial discharges that are often present at μg/L levels. In fact, despite the significantly lower concentrations of the natural estrogens they may have a more important ecotoxicological impact due to their high estrogenic potency and prevalence in the aquatic environment (López de Alda and Barceló, 2001).

Taking into account several studies conducted to assess the estrogenic activity of different EDCs, E_2 was considered one of the strongest estrogenic chemicals in the environment (Purdom *et al.*, 1994; Hansen *et al.*, 1998; Van den Belt *et al.*, 2004), reinforcing the need for E_2 reproductive toxicity assessment. The E_2 presence in the environment has received little attention until recently, although as a natural hormone excreted mainly in the urine of mammals, it has always been present. Through the years E_2 levels increased steadily in the water, as a result of the continuously growing global population and livestock-farming practices. Moreover, point-source higher levels of E_2 can be expected in large cities, as a consequence of the population concentration.

Some E_2 exposure effects include testis-ova induction in males (Metcalfe *et al.*, 2001), reduced gonadosomatic index and courting behaviour in males (Bjerselius *et al.*, 2001), male spawning (Mills *et al.*, 2003) and testicular growth inhibition (Panter *et al.*, 2004), as well as decreased egg production by females (Imai *et al.*, 2005). Besides the previous effects, the measurement of the estrogen-inducible phospholipoglycoprotein - vitellogenin (Vtg) - as a sensitive marker of exposure to estrogens, including E_2, has gained widespread endorsement by scientists, industry and regulatory agencies (EPA, 1999; Arukwe *et al.*, 2002; Craft *et al.*, 2004; Marin and Matozzo, 2004;). Vitellogenesis or egg yolk production represents an E_2 initiated

key process in oviparous vertebrates and is crucial for oocyte maturation. This induction requires neuroendocrine coordinated feedback loops involving the hypothalamus, pituitary, liver and gonads (Thomas, 1990). Vtg is synthesized in the liver, transported via vascular system to the ovaries being sequestered and incorporated in developing oocytes. After sequestration by oocytes, Vtg is protealytically cleaved forming the yolk proteins lipovitellin and phosvitin. Thus, Vtg constitutes a major source of nutrition for the developing oocytes, being found in the plasma of maturing females in concentrations up to 100 mg/mL, often constituting over 50 % of the blood protein.

Significant levels of Vtg are normally only present in sexually maturing female. However, male, immature and nonvitellogenic females had estrogen receptors (Kishida *et al.*, 1992), as well as a silent Vtg gene, generally present but not expressed (Moncaut *et al.*, 2003). Thus, male and immature fish can respond to exogenous estrogens, such as E_2, producing Vtg which tends to remain elevated because of lack of mechanisms to clear the protein (Funkenstein *et al.*, 2000). A Vtg increase was observed in different fish species namely *Dicentrarchus labrax* L. (Mañanos *et al.*, 1990), S*almo salar* L. (Arukwe *et al.*, 1999), *Sparus aurata* L. (Funkenstein *et al.*, 2000), *Fundulus heteroclitus* L. (Pait and Nelson, 2003) and *Salvelinus alpinus* L. (Berg *et al.*, 2004) after laboratory exposure to E_2.

In the present research work *D. labrax* was adopted as a bioindicator, since it represents a keystone species with economic and ecological importance in the Eastern Atlantic Ocean, Mediterranean and Black Sea.

The objectives of this research were:

- The assessment of Vtg induction on immature fish following an E_2 waterborne or intraperitoneal (i.p.) exposure. Moreover, it was intended to compare the relative importance of the exposure route on the estrogenic activity of E_2.

- The evaluation of E_2 uptake following a 10 days exposure period, measured as its levels in plasma.

- The study of the relationship between levels of E_2 and Vtg in plasma, in order to a better understanding of vitellogenesis process in this fish species.

MATERIAL AND METHODS

Chemicals

17β-Estradiol (E_2) was purchased from Sigma-Aldrich (Germany). Marine salt was obtained from Sera Premium (France). All the other chemicals were of analytical grade obtained from Sigma-Aldrich (Germany) and E. Merck-Darmstadt (Germany).

Test animals

The experiments were carried out with juvenile *D. labrax* specimens purchased from a local fish farm, Materaqua – Ílhavo, Portugal. Fish weighing 21.80±1.32 g and measuring 12.4±3 cm were transported in aerated water and acclimated to laboratory conditions for one week prior to experimentation. During acclimatization and experimental periods fish were kept in 80 L aquaria, at 20ºC in aerated (dissolved oxygen: 7.3±0.4 mg/L) and filtered artificial seawater (34 g/L salinity), with a pH of 8.4±0.3. Fish were neither fed under laboratory adaptation nor during the experimental period.

Experimental design

This study includes two experiments, corresponding to two different E_2 exposure routes – water diluted (WD) and i.p. injected (IP).

In WD experiment, fish were exposed to water diluted E_2 at the concentrations of 200 ng/L (0.734 nM) or 2000 ng/L (7.34 nM). The appropriate amount of E_2 was previously dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the experimental aquaria to reach the previous initial nominal concentrations. The same E_2 amount was daily added to the experimental aquaria, without water renewal. This procedure was adopted, since it was previously observed that a 4000 ng/L E_2 water concentration suffered a 99 % reduction 4 h after addition (Teles *et al.*, 2005). DMSO (1 mL) was also daily added to the control aquarium.

In the IP experiment, fish were initially treated with a single E_2 i.p. injection, using corn-oil as a carrier and two doses - 0.5 mg/Kg and 5 mg/Kg - were tested. Control fish were also i.p. injected with corn-oil alone.

Both experiments were performed with an exposure length of 10 days using test groups of five fish (*n*=5). Blood was collected from the posterior cardinal vein using a Pasteur pipette with heparin. Following blood sampling, fish were sacrificed by decapitation. Blood was used for plasma isolation using an Eppendorf centrifuge (14,000 rpm).

17β-Estradiol Measurement

Plasma E_2 determination was performed using a diagnostic ELISA direct immunoenzymatic kit (Diametra, Italy). Briefly, E_2 in the sample competes with horseradish-peroxidase E_2 for binding onto the limited number of anti-E_2 sites on the microplates. E_2 concentration in the sample is calculated based on a standard series; the color intensity is inversely proportional to the E_2 concentration in the sample. The method allows the determination of E_2 from 20 pg/mL to 4000 pg/mL.

Plasma vitellogenin determination

Vtg was measured in the sea bass plasma using the components of a semi-quantitative ELISA kit from Biosense laboratories (Norway). The primary monoclonal antibody for sea bass was developed in Instituto de Acuicultura de Torre de la Sal (Spain). The Biosense protocol was adapted according to the protocol developed by Mañanós *et al.* (1994).

Statistical Analysis

Statistica software (StatSoft, Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish

groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P<0.05$.

RESULTS

WD experiment revealed a significant increase in E_2 plasma levels for both exposed groups (Fig. 1A). A clear relation to the exposure concentrations was not observed, since the E_2 increment in the plasma of 200 ng/L exposed group was 1115%, while the 2,000 ng/L exposed group had an increment of 1340%. The methodology adopted for Vtg measurement provided a suitable standard curve, allowing a quantitative analysis approach. Thus, fish exposed to the highest E_2 concentration displayed a significant increase in plasma Vtg concentrations (28.8 mg/mL of Vtg), corresponding to a 6175 % increment when compared to the control (Fig. 1B). Moreover, levels of plasma Vtg were significantly different between exposed groups.

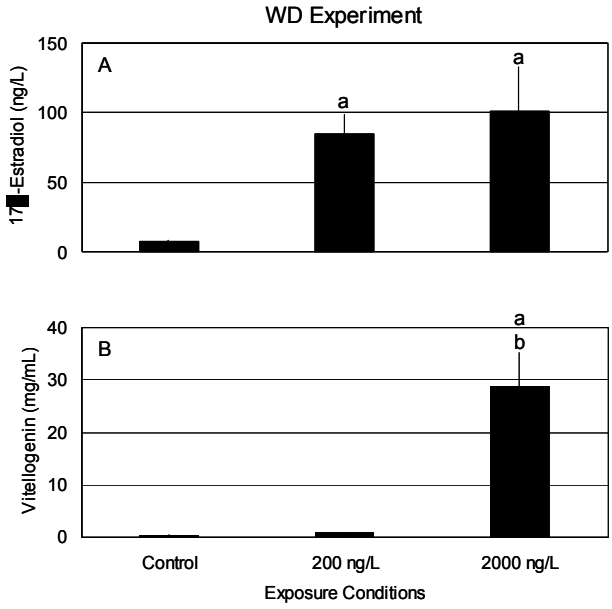

Fig. 1 - *Dicentrarchus labrax* (sea bass) 17β-estradiol (E_2) (A) and vitellogenin (B) plasma levels after 10 days of exposure to 200 ng/L or 2000 ng/L of E_2 via water-dilution (WD experiment). Values represent the means and S.E. (n=5/treatment). Significant differences are: a *vs.* control; b *vs.* 200 ng/L (P<0.05).

Concerning IP experiment, the E_2 plasma levels displayed a slight, but not statistically significant, increase in treated groups (Fig. 2A). Plasma Vtg increased significantly only in the group injected with 5 mg/kg when compared to the control (Fig. 2B). Moreover, this increase was also significant when compared to the 0.5 mg/kg treated group. Plasma Vtg concentration in 5 mg/kg treated group was 0.98 mg/mL, representing an increment of 110%.

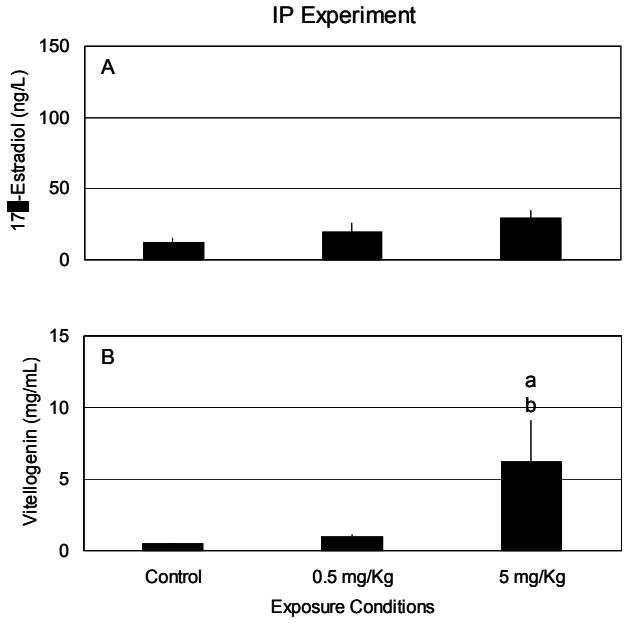

Fig. 2- *Dicentrarchus labrax* (sea bass) 17β-estradiol (E_2) (A) and vitellogenin (B) plasma levels after 10 days of treatment with 0.5 mg/Kg or 5 mg/Kg of E_2 via intraperitoneal injection (IP experiment). Values represent the means and S.E. (n=5/treatment). Significant differences are: a *vs*. control; b *vs*. 0.5 mg/Kg (P<0.05).

DISCUSSION

Few publications comparing relative toxicities of estrogens, namely E_2, administered via different routes into the fish body are available. In this perspective, two different exposure routes were chosen in the present study – waterborne and i.p. injection. The waterborne exposure represents a natural intoxication path, while, i.p. administration was chosen since it may provide a closer E_2 contact with the

gastointestinal tract, simulating an uptake via ingested food, and ensures its substantial uptake.

The highest E_2 concentration (2,000 ng/L) employed in the WD experiment was chosen to mimic endogenous levels of circulating estrogen spawning female fish, whereas the lowest concentration (200 ng/L) was adopted according to the highest levels found in the aquatic environment, as previously reported by Bowman *et al.* (2000). Otherwise, in the IP experiment fish were injected either with a low (0.5 mg/kg) or a high (5 mg/kg) dose of E_2, since it was demonstrated that these doses produces E_2 plasma levels similar to that of early and late vitellogenesis in fish (Anderson *et al.*, 1996; Asturiano *et al.*, 2000).

The 10 days exposure period adopted took into consideration that usually a period of days is necessary to induce Vtg production in fish following the occurrence of an E_2 peak in plasma (Kramer *et al.*, 1998; Hiramatsu *et al.*, 2002).

Current results of WD experiment are discussed assuming that E_2 uptake from the water was greater in fish exposed to the highest dose. This is supported by studies where E_2 in the water, ranging from 4,000 to 27,000 ng/L, was rapidly taken up by fish, reaching levels above the exposure concentration, within 0.5 to 4 h (Specker and Chandlee, 2003; Teles *et al.*, 2005). In the present study, E_2 plasma levels increased for both exposure concentrations compared to the control, revealing an unclear relation to the doses and exhibiting plasma levels below the initial water exposure concentrations. Even if these results appear to disagree with the previous statements, an augmented exposure length may justify the differences. Thus, considering that the current plasma E_2 measurements were carried out 24 h after the last E_2 addition, the possibility that E_2 peak in plasma above the exposure concentrations could have earlier occurred cannot be excluded. This idea is supported by Teles *et al.* (2005) previous data where 4,000 ng/l E_2 was almost completely removed within 16 h from *Sparus aurata* plasma. Nevertheless, in the current study E_2 was not completely removed from plasma since its levels in treated groups were still higher than those found in the control group. These results may be explained by a different E_2 uptake from plasma by target organs and/or its catabolism,

as a consequence of daily E_2 additions along the 10 days of exposure, probably leading to a less efficient E_2 removal from plasma.

The E_2 plasma levels were quite similar in both WD treated groups though the exposure concentrations were 10-fold different. This can also be explained by differences on E_2 plasma removal. Hence, it can be suggested that a higher E_2 removal rate occurred in fish exposed to the highest E_2 concentration, concomitantly with a greater uptake by target organs, namely liver. This explanation is corroborated by current plasma Vtg data, as a significant induction was observed only for the highest E_2 concentration, while fish exposed to the lowest dose displayed a Vtg concentration similar to the control level. These results are also in agreement with the premise that the E_2 uptake from the water was greater in the fish exposed to the highest E_2 dose, since Vtg levels were significantly different between exposed groups, though E_2 plasmatic levels were comparable. Therefore, only in fish exposed to 2,000 ng/L E_2, sufficient E_2 would be available to enter the hepatocytes, bind to estrogen-receptors (ER) and activate the transcription of the Vtg loci. This idea is supported by previous studies where it was demonstrated the ER recruitment (Schultz *et al.*, 2001) and E_2 binding affinity (Donohoe and Curtis, 1996) increase with higher doses of E_2, leading to more Vtg synthesis. The analysis of the available literature concerning the vitellogenesis induction in fish exposed to waterborne E_2 and the current data reveals comparable results (Kramer *et al.*, 1998; Denslow *et al.*, 2001; Imai *et al.*, 2005). This agreement is particularly obvious in relation to *Cyprinodon variegatus* where the same water diluted E_2 doses induce the same responses after 7 days exposure (Bowman *et al.*, 2000).

In IP experiment, plasma levels of E_2 did not differ significantly from the control, which is indicative of a complete E_2 disappearance from the plasma in the period of 10 days. These results agree with the findings of Korte *et al.* (2000) who observed E_2 plasma concentrations close to control levels in i.p. treated *Pimephales promelas* after 48 h of exposure. Additionally, Teles *et al.* (2005) previously mentioned results concerning E_2 plasma half-lifes also corroborate the current data.

Despite the similar E_2 levels found in plasma of both i.p. exposed groups, the Vtg increase may suggest the occurrence of a plasma E_2 peak before the sampling moment, which provided a considerable liver uptake only for the highest i.p. dose as Vtg production was induced only in this case. The previous statement is corroborated by Kramer *et al.* (1998) who observed in *P. promelas* that a peak in plasma E_2 typically precedes plasma Vtg increase.

Results of IP experiment are in agreement with previous studies using a single i.p. injection of 5 mg/kg of E_2 in males or juveniles fish. Thus, a plasma Vtg increase was observed *C. variegatus* (Denslow *et al.*, 2001), *Oncorhynchus mykiss* (Anderson *et al.*, 1996), *S. salar* (Arukwe *et al.*, 1999) and *F. heteroclitus* (Pait and Nelson, 2003), respectively at 4, 8, 14 and 21 days post-treatment. Despite the differences on the exposure time necessary for vitellogenesis induction, this particular physiological response is similar among different fish species.

A counter-current mechanism in fish gills provides a potentially efficient transport route for the uptake of chemicals from water to the bloodstream (Rand, 1995). In fish, the oxygenated blood travels from the gills directly to most organs. Therefore, E_2 entering the bloodstream in this way is distributed by all the target organs, including liver where it can also undergo biotransformation. Chemicals ingested through diet are absorbed in intestine and travel via the hepatic portal vein directly to the liver. Similarly, chemicals diluted in corn oil and i.p. injected also get in close contact with viscera being rapidly absorbed by the liver. In the present study, vitellogenesis was induced in both experiments, indicating that this hormone was efficiently taken up by juvenile sea bass, reaching liver, the target organ, when administered either via water or i.p. injection. However, only the highest concentrations were effective in both cases.

Present observations corroborate Bowman and coworkers proposition (2000) suggesting that a constant fish E_2 water exposure appears to stimulate more the liver estrogen receptor pathway than a single injection with a high dose. According to Korte *et al.* (2000), the injected E_2 is quickly metabolized by the liver and cleared from *P. promelas* plasma (70% in 6 h), suggesting a possible explanation for the diferent

vitellogenesis induction levels that were observed in current experiments. Moreover, the amount of injected E_2 that really reaches the estrogen receptor also depends on its binding to plasma proteins such as albumin or sex hormone-binding globulins (Nagel *et al.*, 1998; Bowman *et al.*, 2000).

In mature female fish Vtg is transported to the gonads, taken up into oocytes and cleaved to form the bulk of yolk protein. Nonetheless, in male and juvenile fish such pathway for the removal of Vtg from blood does not exist and it was demonstrated that Vtg elimination time from the blood could reach up to 5 months even after the source of the estrogenic stimulus had been removed. A number of studies have been focused on the correlation between the abnormal Vtg production and adverse reproductive effects and overall health (Kramer *et al.*, 1998; Panter *et al.*, 1998; Mills *et al.*, 2003). In this direction, the induction of Vtg has been associated with reduced testicular growth (Jobling *et al.*, 1996), intersexuality (Bortone and Davis, 1994), decreased egg production (Kramer *et al.*, 1998), kidney and liver damage (Herman *et al.*, 1988). Accordingly, current results demonstrated that both via of E_2 entrance into fish body could represent a risk for icthyc fauna living in contaminated areas, either through the direct effects of Vtg abnormal production or other overall deleterious effects.

CONCLUSIONS

Vtg production in juvenile *D. labrax* was induced by the E_2 highest doses either following both waterborne and intraperitoneal exposures. However, a repeated exposure to waterborne E_2 is more effective than a single injection.

E_2 plasma levels measured at the end of the experiment displayed an increment only for waterborne exposure. Thus, it was demonstrated that a Vtg plasma increase was measured without a concomitant increase in E_2 plasma levels.

Considering the deleterious effects of an abnormal Vtg production previously demonstrated, environmental exposures to E_2 can represent an important risk to fish.

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CAPÍTULO VII

Biotransformação, genotoxicidade e respostas endócrinas em robalos juvenis expostos a β-naftoflavona, 4-nonilfenol e 17β-estradiol, individualmente ou em combinação

Juvenile sea bass biotransformation, genotoxic and endocrine responses to β-naphthoflavone, 4-nonylphenol and 17β-estradiol individual and combined exposures

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ABSTRACT

Juvenile sea bass, *Dicentrarchus labrax* L., were exposed during 2, 4, 8, and 24 h to 0.9 μM β-naphthoflavone (BNF),131 nM 17β-estradiol (E_2), 4.05 μM 4-nonylphenol (NP), as well as to BNF combined either to E_2 or NP (maintaining the previous concentrations). Liver cytochrome P450 content (P450), ethoxyresorufin-O-deethylase (EROD), and glutathione *S*-transferase (GST) activities were measured in order to evaluate biotransformation responses. Genotoxicity was assessed as erythrocytic nuclear abnormalities (ENA) frequency. The effects on endocrine function were evaluated as plasma cortisol and glucose. Cortisol was not affected by xeno/estrogens tested, either in single exposure or mixed with BNF. Nevertheless, the intermediary metabolism was affected since glucose concentration increased after 4 h exposure to E_2, and after all BNF+NP exposure lengths. Moreover, a synergism between BNF and NP was thoroughly demonstrated, whereas a sporadic antagonistic interaction was found at 4 h BNF+E_2 exposure. Liver EROD and GST activities were not significantly altered by single E_2 or NP exposure. However, both compounds were able to induce EROD activity in the presence of BNF. NP single exposure was able to significantly increase liver P450 content, while its mixture with BNF displayed an antagonistic interference. Considering the xeno/estrogens single exposures, only NP induced an ENA increase; however, both mixtures (BNF+E_2 and BNF+NP) displayed genotoxic effects. Fish responses to mixtures of xenobiotics are complex and the type of interaction (synergism/potentiation or antagonism) in a particular mixture can vary with the evaluated biological response.

Keywords: P450 monooxygenases; GST; ENA; Cortisol; Glucose.

INTRODUCTION

A wide range of common anthropogenic chemicals, accidental or deliberately released into the aquatic environment, can affect aquatic organisms at several biological levels. Alkylphenol polyethoxylates (APEs) are typically regarded as

endocrine-disrupting chemicals (EDCs), being 4-nonylphenol (NP) identified as the most critical APEs metabolite, since it interacts with fish estrogen receptors inducing estrogenic responses (Arukwe *et al.*, 1998; Cheek *et al.*, 2001) and gametogenesis impairment (Tanaka and Grizzle, 2002). Moreover, the recent detection of significant 17β-estradiol (E_2) concentrations in municipal treated effluents revealed the need for the toxicity assessment of this natural estrogen itself as an aquatic contaminant (Kramer *et al.*, 1998). Besides the increasingly studied effects of the previous EDCs on reproduction-related processes, some authors demonstrated that NP suppressed the hepatic CYP1A in *Salmo salar* (Arukwe *et al.*, 1997) and *Oncorhynchus mykiss* (Navas and Segner, 2000). Furthermore, E_2 is known to affect CYP1A expression in fish, decreasing P450 content and EROD activity (Elskus *et al.*, 1991; Arukwe *et al.*, 1997). The P450 related MFOs metabolizes both endogenous and exogenous compounds; thus, xenobiotics may interact with the endogenous metabolic processes. According to some authors a cross-talk mechanism between P450 activities and steroid synthetic pathways may be suggested (Larsen *et al.*, 1992; Arukwe *et al.*, 1997). However, the CYP1A functions and its regulation by endogenous compounds or xenoestrogens have not been fully explored.

A large number of studies reported CYP1A induction, measured as ethoxyresorufin-*O*-deethylase (EROD) activity increased, after exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), in different fish species (Wolkers *et al.*; 1996; Pacheco and Santos, 1997; Vijayan *et al.*, 1997; Gravato and Santos, 2002a). Nevertheless, it was found that CYP1A induction may generate reactive intermediate compounds (Varanasi *et al*. 1985; Stegeman and Hahn, 1994; Cantrell *et al.*, 1998) and the consequent genotoxic and/or carcinogenic effects (Kantoniemi *et al.*, 1996; Tuvikene *et al.*, 1999). Several mammal studies indicated that estrogens could be genotoxic, inducing multiple types of genetic insults in cells (Liehr, 1990; Liehr and Roy, 1990). To our knowledge, minimal research has been conducted to assess the genotoxic potential of estrogens and xenoestrogens despite the growing concern over the potential effects of environmental endocrine disruptors

on wildlife. Thus, considering that aquatic ecosystems may be the final repository of those compounds, it is ecotoxicologically relevant to determine their effects in fish at the DNA level.

Following phase I metabolization, environmental chemicals detoxification is carried out through phase II biotransformation reactions, namely glutathione *S*-transferase (GST)-mediated conjugation. It was demonstrated that, besides xenobiotics (Sandbacka and Isomaa, 2000), GST might be modulated by endogenous steroids (Gallagher *et al.*, .2001).

Analyzing the available bibliography, it is evident that fish studies on E_2 and NP endocrine disruption have been focused mainly on reproductive impairments. However, the endocrine disruption by environmental pollutants is not confined to reproduction, since other crucial hormonal functions can be affected, namely those involved in the maintenance of homeostasis and physiological responses to chemical stressors. Thus, the hypothalamus-pituitary-interrenal (HPI) alterations induced by chemical exposures should be an important topic of research on endocrine toxicology. Cortisol is the major glucocorticosteroid secreted by the teleosts interrenal tissue in response to adrenocorticotropic hormone (ACTH) stimulation (Hontela, 1997). A considerable number of reports have indicated plasma cortisol alterations in fish after short-term exposure to pollutants, such as heavy metals (Bleau *et al.*, 1996; Hontela *et al.*, 1996), PAHs (Pacheco and Santos, 2001), and pulp mill effluent contaminants (Kennedy *et al.*, 1995; Santos and Pacheco, 1996; Teles *et al.*, 2003a). Despite previous research work reporting E_2 as responsible for the HPI axis hyperactivation in *O. mykiss* (Pottinger *et al.*, 1996; McQuillan *et al.*, 2003), the information concerning the effects of the so-called xenoestrogenic chemicals at this level is limited.

Common cytochrome P4501A (CYP1A) inducers, such as PAHs, are known to exert endocrine disruption in fish measured as lower plasma E_2 levels (Casillas *et al.*, 1991), precocious sexual maturation (Collier *et al.*, 1998), and reduced fertility (Cheek *et al.*, 2001). However, the effects at other endocrine levels, such as HPI axis, are poorly investigated. Wilson *et al.* (1998) showed that β-naphthoflavone (BNF) affects

the pituitary-interrenal axis in *O. mykiss*, abolishing the interrenal sensitivity to ACTH stimulation as a consequence of alterations on ACTH receptor dynamics and/or on the steroidogenic pathway.

Considering the occurrence of significant effects at different biological levels not included in the commonly described responses, the adoption of an integrative approach is desirable in environmental toxicology. Therefore, this point of view is particularly relevant when aquatic species are facing environmental mixtures of contaminants, containing xenobiotics that may behave differently when acting in isolated or in mixtures, raising the question about additive, synergistic or antagonistic interactions.

The aims of the present research work, using juvenile sea bass (*Dicentrarchus labrax* L.) as a biological model, were to investigate:

- How xeno/estrogenic chemicals, namely E_2 and NP, affect the biotransformation, measured as total cytochrome P450 content (P450), EROD (phase I) and GST activities (phase II).
- The liver somatic index (LSI) and alanine transaminase (ALT) activity as general hepatic condition indicators.
- NP and E_2 genotoxicity, measured as erythrocytic nuclear abnormalities (ENA).
- How the previous chemicals can affect HPI axis measured as plasma cortisol levels and intermediary metabolism, measured as plasma glucose.
- The interactions between a PAH-like compound (BNF) and E_2 or NP, as a binary mixture, on the above responses, trying to simulate a likely environmental contamination by PAHs and xeno/estrogens.

MATERIAL AND METHODS

Chemicals

β-Naphthoflavone, 4-nonylphenol, 17β-estradiol were purchased from Sigma-Aldrich Co. Ltd. (Germany) and marine salt from Sera Premium (Germany). All the

other chemicals were of analytical grade obtained from Sigma-Aldrich Co. Ltd. (Germany), Roche (Germany), and E. Merck-Darmstadt (Germany).

Test Animals

The experiment was carried out with juvenile *D. labrax* L. specimens purchased from a local fish farm, Materaqua - Ílhavo, Portugal. Sea bass weighing 14±0.30 g, measuring 8±2 cm, were transported in aerated water and acclimated to laboratory conditions for one week prior to experimentation. During acclimatization and experimental periods fish were kept in 80 L aquaria, at 20 ºC in aerated (dissolved oxygen: 7.4±0.2 mg/L) and filtered artificial seawater (34 g/L), with a pH of 8.386±0.02. Fish were neither fed under laboratory adaptation nor during the experimental procedure.

Experimental Design

Fish were divided in six lots: one as control and the others were exposed to 0.9 μM BNF, 4.05 μM NP, 131 nM E_2, and to the mixtures BNF+NP or BNF+E_2 (maintaining the previous concentrations), during 2, 4, 8, and 24 h. Experiments were carried out using test groups of five fish (*n*=5). The appropriate amount of each chemical was previously dissolved in 1 mL of DMSO and added to the experimental aquaria. The same volume of DMSO was added to the control aquarium. Previous studies have demonstrated that the 0.0125 ‰ DMSO concentration has neither detectable biotransformation or genotoxic effects in sea bass (Gravato and Santos, 2002a, b). First, the fish abdominal cavity was opened, and blood was collected from the posterior cardinal vein using a heparinised Pasteur pipette. After collecting the blood, fish were immediately killed by decapitation and its liver sampled. Liver was immediately frozen in liquid nitrogen, stored at -80ºC until homogenization. Blood was used for smear preparation and for plasma isolation using an Eppendorf centrifuge (14,000 rpm). The blood smears were fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min.

Biochemical Analysis

Liver Cytochrome P450 Content and EROD Activity

Liver microsomal fraction was obtained according to the methods of Lange *et al.* (1993) and Monod and Vindimian (1991), as adapted by Pacheco and Santos (1998). Cytochrome P450 content was determined using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm, as previously described by Hermens *et al.* (1990). The liver EROD activity was measured in microsome suspension as described by Burke and Mayer (1974).

Liver GST Activity

GST activity was determined in the cytosolic fraction as described by Lemaire *et al.* (1996), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig *et al.*, 1974). The assay, prepared in the cuvette, was carried out in a 2 mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM reduced glutathione (GSH). The reaction was initiated by addition of 10 µL sample. The increase in absorbance at 340 nm was recorded at 25°C for 3 min.

Liver ALT Activity

ALT activity was measured in the cytosolic fraction according to the method of Reitman and Frankel (1957).

Protein Measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornall *et al.*, 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard.

Plasma Cortisol and Glucose Measurement

The determination of cortisol was performed using a diagnostic ELISA direct immunoenzymatic kit (Diametra, Italy). Plasma glucose was measured using a diagnostic kit (Granutest, E. Merck-Darmstadt No. 1.12194).

Liver Somatic Index

LSI was expressed as a percentage (%) resulting from the following expression: [liver mass (g)/body mass (g)] x 100.

ENA Assay

Genotoxicity was tested using the ENA assay. The nuclear abnormalities were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid (1976), Carrasco *et al.* (1990) and Smith (1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei, lobed nuclei, dumbbell shaped or segmented nuclei and kidney shaped nuclei. The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

Statistical Analysis

Statistica software (StatSoft, Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P<0.05$.

RESULTS

Liver Biotransformation Responses

Cytochrome P450 Content (Fig. 1A)

Sea bass exposed during 2 and 8 h to NP exhibited a significant increase in P450 compared to the control group. E_2 and BNF+NP significantly decreased P450 at 2 and 8 h, compared to NP exposure.

EROD Activity (Fig. 1B)

Sea bass exposed to BNF exhibited a significant liver EROD activity increase for all the exposure lengths, reaching its maximum value (44.6-fold increase) after 8 h exposure that was maintained up to 24 h. During the whole experiment, sea bass exposed to NP or to E_2 revealed significantly lower EROD activity than BNF exposed fish. A significant increase in EROD activity was observed after 8 h exposure to BNF+E_2 as well as after 2 and 4 h exposure to BNF+NP compared to control. BNF+E_2 also revealed significantly lower EROD activity at 2, 4 and 24 h compared to BNF exposure. On the other hand, fish exposed to BNF+E_2 exhibited a significantly higher EROD activity than fish exposed to E_2 during 8 h. BNF+E_2 induced a significantly higher EROD activity than BNF+NP at 8 h exposure. Additionally, BNF+NP displayed a less pronounced EROD activity increase during the entire exposure length, though significantly lower after 8 and 24 h compared to BNF exposure.

GST Activity (Fig. 1C)

A significantly decrease in GST activity was observed at 8 h exposure to BNF+NP compared either to control or to BNF exposed fish.

Hepatic Health Indicators

Liver ALT Activity (Fig. 2A)

ALT activity significantly decreased at 2 h exposure to E_2 and BNF+E_2.

Liver Somatic Index (Fig. 2B)

The LSI did not present any significant alteration.

Genotoxic Response

ENA Assay (Fig. 3)

A significant ENA increase was observed in sea bass after 4, 8 and 24 h BNF exposure, when compared to control group. An increased ENA frequency was also

found at 24 h NP exposure, as well as after 4 and 8 h exposures either to BNF+NP or BNF+E_2 compared to the control. E_2 exposure induced at 24 h, a lower ENA frequency than NP. BNF+NP induced a significantly higher ENA frequency than NP at 4 h exposure; whereas at 24 h exposure BNF+NP induced an ENA frequency lower than NP. BNF+E_2 induced a significantly higher ENA frequencies than E_2 only at 4 h exposure.

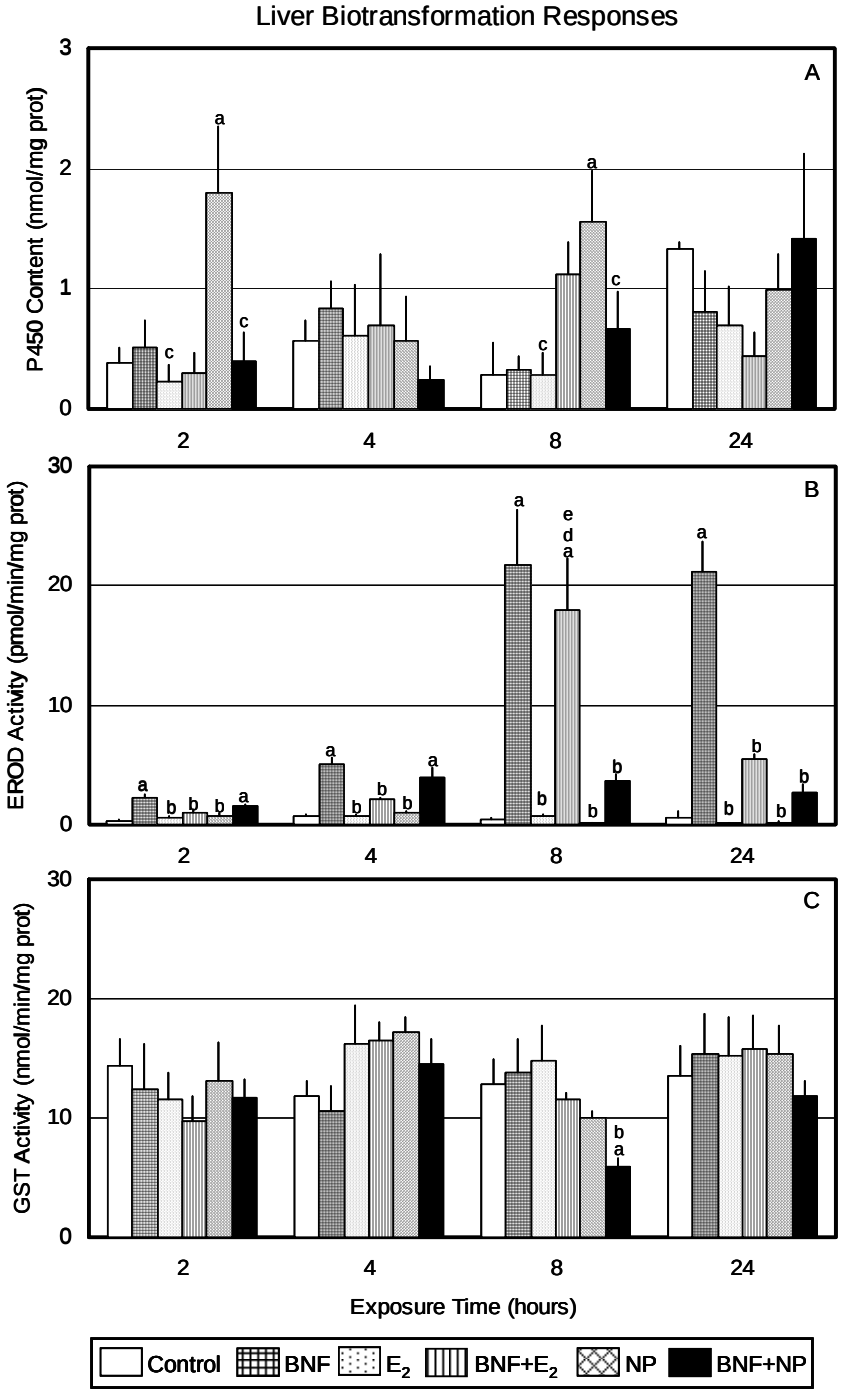

Fig. 1 – *D. labrax* liver P450 content (A), liver EROD activity (B), and liver GST activity (C) after 2, 4, 8 and 24 h exposure to 0.9 μM of β-naphthoflavone (BNF), 4.05 μM of 4-nonylphenol (NP), 131 nM of 17β-estradiol (E_2), and to a mixture of BNF+NP (0.9 and 4.05 μM, respectively) and BNF+E_2 (0.9 μM and 131 nM, respectively). Values represent the means and SE (*n*=5/treatment). Significant diferences are: **a** *vs.* control; **b** *vs.* BNF; **c** *vs.* NP; **d** *vs.* E_2; **e** *vs.* BNF+NP.

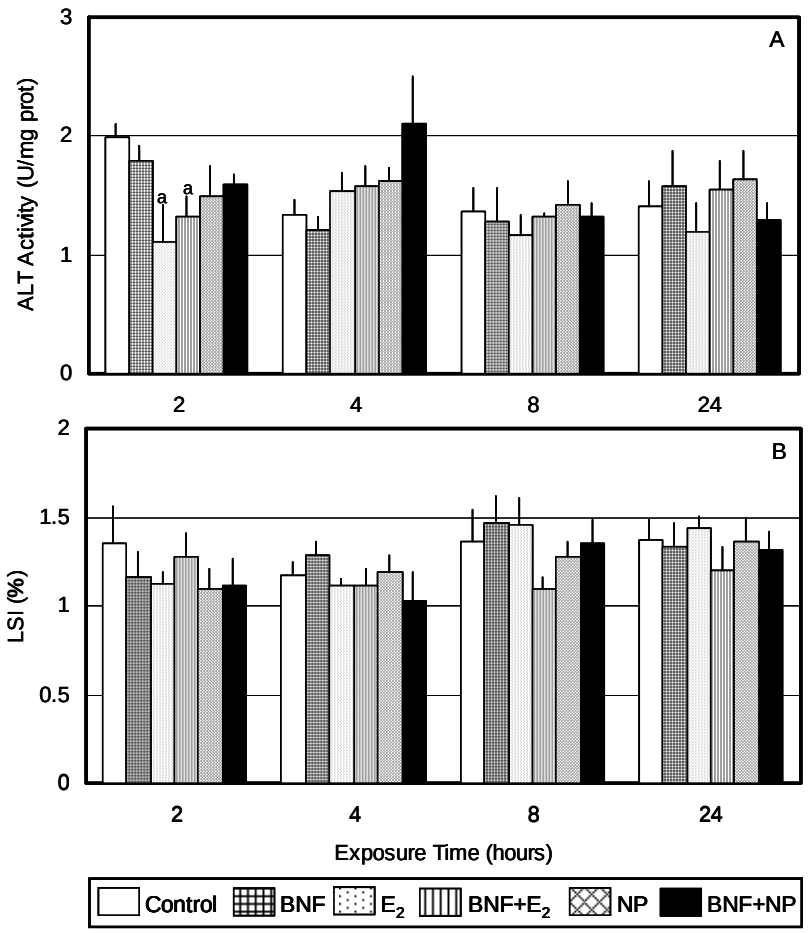

Fig. 2 - *D. labrax* liver ALT activity (A) and LSI (B) after 2, 4, 8 and 24 h exposure to 0.9 μM of naphthoflavone (BNF), 4.05 μM of 4-nonylphenol (NP), 131 nM of 17β-estradiol (E_2), and to a mixture of BNF+NP (0.9 and 4.05 μM, respectively) and BNF+E_2 (0.9 μM and 131 nM, respectively). Values represent the means and SE (*n*=5/treatment). Significant differences are: **a** *vs.* control.

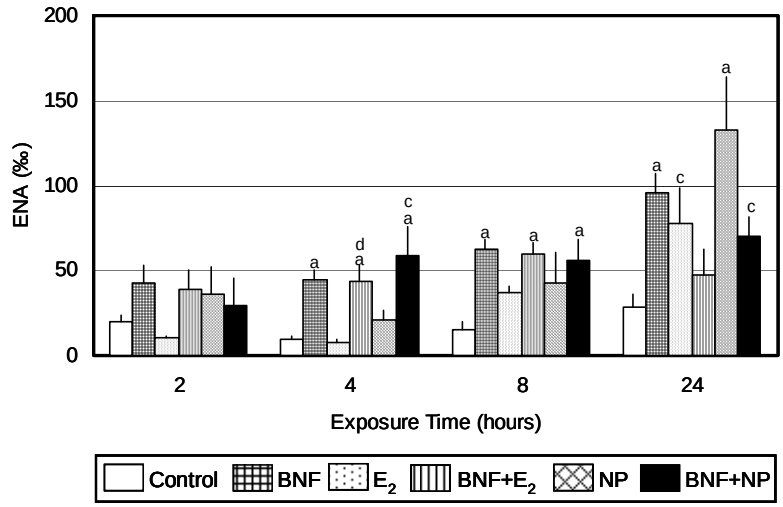

Fig. 3 - *D. labrax* ENA frequency after 2, 4, 8 and 24 h exposure to 0.9 μM of β-naphthoflavone (BNF), 4.05 μM of 4-nonylphenol (NP), 131 nM of 17β-estradiol (E_2), and to a mixture of BNF+NP (0.9 and 4.05 μM, respectively) and BNF+E_2 (0.9 μM and 131 nM, respectively). Values represent the means and SE (*n*=5/treatment). Significant differences are: a *vs.* control; **b** *vs.* BNF; **c** *vs.* NP; **d** *vs.* E_2.

Endocrine Responses

Plasma Cortisol Measurement (Fig. 4A)

Cortisol levels did not exhibit any significant alteration.

Plasma Glucose Measurement (Fig. 4B)

A significant increase in plasma glucose was detected after 4 h exposure to E_2 and to BNF+E_2 compared to control. However, sea bass exposed to BNF+E_2 revealed a glucose level significantly higher than E_2 at 4 h exposure. BNF+NP exposure significantly increased plasma glucose at all exposure times, when compared to the control group. The BNF+NP exposure displayed a continuous significant plasma glucose elevation when compared either to BNF or to NP exposed groups. BNF+NP exposed group exhibited plasma glucose concentrations higher than BNF+E_2 exposed group. Thus, significant differences between plasma glucose responses to the tested mixtures were observed during the whole experiment.

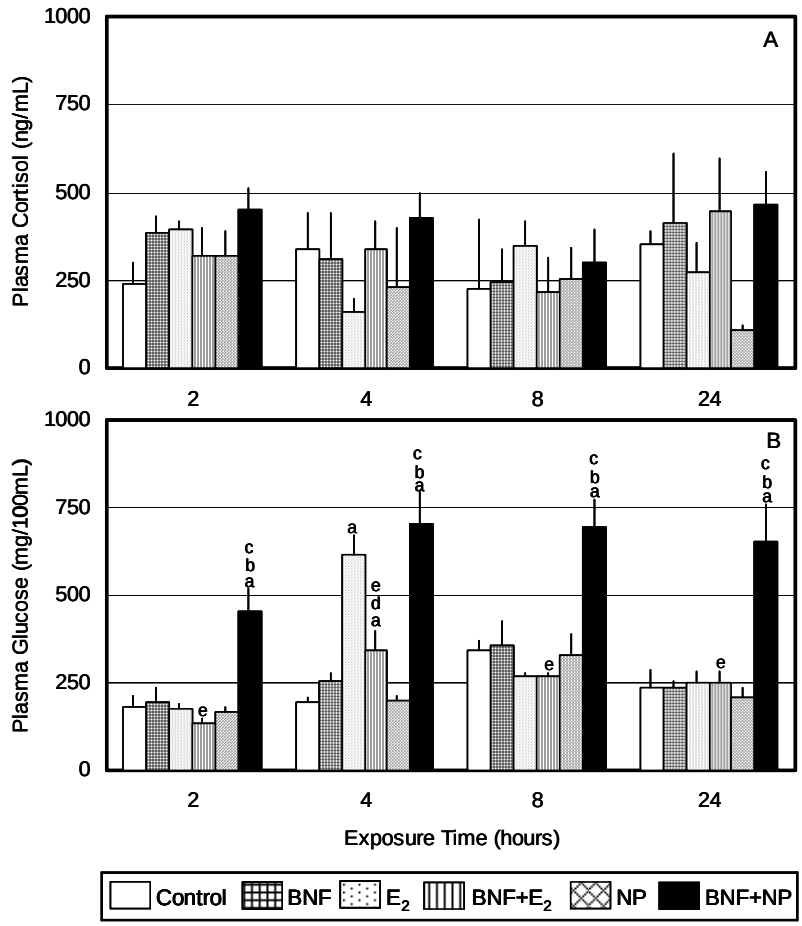

Fig. 4 - *D. labrax* plasma cortisol levels (A) and plasma glucose levels (B) after 2, 4, 8 and 24 h exposure to 0.9 μM of β-naphthoflavone (BNF), 4.05 μM of 4-nonylphenol (NP), 131 nM of 17β-estradiol (E_2), and to a mixture of BNF+NP (0.9 and 4.05 μM, respectively) and BNF+E_2 (0.9 μM and 131 nM, respectively). Values represent the means and SE (*n*=5/treatment). Significant differences are: **a** *vs.* Control; **b** *vs.* BNF; **c** *vs.* NP; **d** *vs.* E_2; **e** *vs.* BNF+NP.

DISCUSSION

Biotransformation Responses

Liver P450 results concerning BNF exposure did not reveal any significant alteration, which is in agreement with a previous sea bass study (Gravato and Santos, 2002a) where similar experimental conditions were adopted. Considering the NP potential to increase P450 content after 2 and 8 h in the present study, we observed that BNF is able to prevent that raise when mixed with NP. Despite some

time-related fluctuations, it is evident that after 2 and 8 h exposure to NP and E_2, their responses are different. The absence of P450 response to E_2 exposure, as well as its decreased tendency observed after 24 h NP exposure, agrees with Solé *et al.* (2000) results who also found an unaltered total P450 in *Cyprinus carpio* treated with the synthetic steroid 17α-ethynylestradiol. Considering the strong CYP1A induction potential previously demonstrated by BNF in different fish species such as *Perca fluviatilis*, *Anguilla anguilla* (Förlin and Celander, 1993; Pacheco and Santos, 2002; Teles *et al.*, 2003b), and *D. labrax* (Lemaire *et al.*, 1996; Jakšic *et al.*, 1998; Novi *et al.*, 1998; Gravato and Santos, 2002b), this synthetic flavonoid compound was adopted in the present study as a positive control for EROD activity induction. Thus, in the present study after any BNF exposure, EROD activity increase was as expected.

Steroid hormones are included in the endogenous factors that can regulate CYP1A expression, however their interaction mechanisms are not well established (Navas and Segner, 2000). It was suggested that the mechanism of E_2 effect on CYP1A is mediated by the 17β-estradiol receptor (ER) and putative estrogen-responsive elements in the CYP1A (Stegeman, 1993) altering gene transcription (Navas and Segner, 2000). Additionally, Arukwe *et al.* (1997) presented strong indications of cross-talk between CYP1A and estrogen-responsive genes and/or their receptor. Hence, the inhibition of CYP1A expression and activity in fish by E_2 has been demonstrated (Arukwe and Goksøyr, 1997). A significant reduction in EROD activity and CYP1A protein after NP administration was also reported in fish (Arukwe *et al.*, 1997) and mammals (Lee *et al.*, 1996a,b), demonstrating the possible role of CYP1A and 3A isozymes in NP metabolism. According to Lee *et al.* (1996a, b), NP down-regulates CYP1A activity and up-regulates CYP3A activity in rat liver. The competitive nature of inhibition by NP on hepatic microsomal EROD activity indirectly suggests that this compound is a possible substrate of the CYP1A enzyme.

The current liver EROD activity results concerning NP or E_2 single exposures did not allow the confirmation that these compounds down-regulate CYP1A activity when compared to their controls, probably due to a short-term exposure (up to 24 h).

However, analyzing BNF+NP or BNF+E_2 effects upon sea bass EROD activity, the results reveal that NP and E_2 reduced BNF inducing potential. Consequently, a less pronounced EROD activity increase was evident after BNF+NP exposure compared to the BNF exposed group, despite the observed general increase tendency compared to the control. Similarly, sea bass exposed to BNF+E_2 shows an EROD activity general increase tendency compared to control group, though lower than BNF exposed fish reaching its maximum difference at 24 h (4 times lower). Chan and Hollebone (1995) suggested that the steroids ability to bind to the CYP1A molecule may act as competitive inhibitors of EROD activity. Alternatively, Villalobos *et al.* (1996) and Anderson *et al.* (1996) presented a different perspective describing a more complex scenario. According to these authors, the CYP1A response to mixtures of CYP1A inducers and E_2 may be dependent upon the concentration of both agents. These responses are mediated through the AhR and ER, respectively, though the physiological implications for fish of inhibited and/or elevated P450 levels and activities have not been fully established. Therefore, the explanation for the NP and E_2 interference on BNF liver EROD induction ability is probably related to sea bass different uptake of individual or combined chemicals from the water.

Considering the current EROD results, it is predictable that, under environmental conditions, a reduced ability of fish to metabolize and excrete PAHs as well as other xenobiotics will depend on the eventual reduction on CYP1A inducibility due to the presence of xeno/ estrogens.

The relation between total P450 content and EROD activity is difficult to establish, since the EROD increase was not accompanied by a P450 elevation. Furthermore, NP induced the highest P450 level and the corresponding lowest EROD activity level. This result may indicate that a CYP3A induction may be mainly responsible for most of the liver P450 increase, suggesting a CYP3A up-regulation and simultaneous CYP1A down-regulation as stated by Lee *et al.* (1996b).

Despite sea bass liver GST activity decreased tendency at 8 h exposure to NP, one punctual significant decrease at 8 h exposure to BNF+NP was observed, compared either to control or to BNF exposure enhancing the difference between

individual or combined chemical exposure. This particular GST decrease caused by BNF+NP at 8 h exposure may be due either to an enzymatic inhibition or to the hepatocyte decline in GSH. However, sea bass GST decreased activity seems to be able to recover from BNF+NP at 24 h exposure, returning to control and BNF levels. Furthermore, Sandbacka and Isomaa (2000) reported an increase GST activity in the *O. mykiss* gill epithelial cells primary cultures at 48 h exposure to BNF. *C. carpio* exposure to 17α-ethynylestradiol revealed no significant GST activity alterations (Solé *et al.*, 2000). On the other hand, in mammals, GST activities increased significantly in direct correlation with increased bisphenol A concentrations (Nieminen *et al.*, 2002). However, Thibaut *et al.* (1999) reported that in *O. mykiss* the majority of NP metabolites were hydrolyzed after β-glucuronidase treatment, suggesting the formation of glucuronide compounds. Arukwe *et al.* (2000) confirmed those findings in a study with *S. salar* where NP biliary and urinary metabolic products were glucuronic acid conjugates.

Considering the previous findings and the absence of any significant GST increase during the current BNF, NP, E_2 or its combined exposure, the probable predominant involvement of other phase II conjugation enzymes such as UDP-glucoronosyltransferase (UDPGT) in sea bass, is suggested.

Hepatic Condition Indicators

E_2 was the only tested chemical that significantly decreased ALT activity, either individually or mixed with BNF, suggesting an initial metabolic alteration in the amino acid metabolism followed by an efficient adaptation process, demonstrated by its increased activity. The absence of any LSI alteration is probably due to the short exposure length.

Genotoxic Responses

Taking into account the BNF genotoxic potential previously observed in sea bass by Gravato and Santos (2002c), this compound can be regarded also as a

positive control to genotoxicity induction, measured as ENA. Thus, the present findings corroborate the above considerations, since an ENA frequency increase was observed after BNF exposure.

Estrogens administration to mammals resulted in various types of DNA damage and ultimately led to tumors (Roy and Liehr, 1999). Bolt (1979) found that E_2 links to DNA and proteins, inducing DNA single strand breaks (Han and Liehr, 1994) and micronuclei (Yared *et al.*, 2002). In contrast, other authors observed that E_2 was unable to form covalent bonds with nucleotide bases (Ashburn *et al.*, 1993). Besides the parental hormones, reactive estrogen metabolites, such as catecholestrogens, may also act at the gene and/or chromosome levels (Ashburn *et al.*, 1993; Roy and Liehr, 1999).

In the present study, E_2 exposure did not induce any significant ENA increase. Thus, in sea bass and under the present exposure conditions, the previous results concerning the E_2 genotoxic potential to mammals could not be demonstrated. Nevertheless, the current absence of ENA may reflect a tissue specific response to E_2 rather than a difference between fish and mammal metabolism, since the nuclear abnormalities were searched in blood, whereas the previously described DNA damage (Han and Liehr, 1994; Yared *et al.*, 2002) was detected in estrogen responsive tissues. Despite the previous statements, it is recommended to perform exposures longer than 24 h, since an ENA increased tendency was detected for this exposure length, despite the absence of any statistical significance.

The covalent addition of estrogens to DNA has been thoroughly investigated in mammals with synthetic estrogens (Roy and Liehr, 1999). Despite the relative abundance of results concerning the diethylstilbestrol genotoxicity, there are other xenoestrogens, namely NP, which interference with DNA is poorly studied. NP was reported as genomic DNA damaging agent to crustacean larvae (Atienzar *et al.*, 2002). To our knowledge, there are no previous data concerning NP genotoxicity in fish. The current experiment demonstrated that 24 h exposure to NP has a genotoxic effect on erythrocytes, expressed as an ENA increased frequency. Arukwe *et al.* (2000) investigated the NP metabolism and organ distribution in *S. salar*

demonstrating that the highest NP concentrations were reached in blood after 21 h exposure. Since the NP blood peak in *S. salar* was found after 21 h and the maximum ENA frequency was observed in sea bass after 24 h exposure to NP a cause-effect relation between both events may be suggested. NP exposed sea bass displayed a highly significant ENA frequency at 24 h compared to E_2 exposed fish.

The tested mixtures effects, compared with the individual chemical exposures, demonstrate that after 4 and 8 h exposure, the combination of xeno/estrogens with BNF, did not significantly affect the BNF genotoxic potential; moreover, the ENA frequency increases observed can be attributable only to BNF. In contrast, the ENA induction is prevented after 24 h exposure to BNF+E_2 and BNF+NP mixtures, demonstrating an antagonistic interaction. After the xenobiotic uptake in fish, its fate and effects are largely governed by CYP1A catalytic activities, playing a central role in their bioactivation producing genotoxic metabolites. The comparative analyses of the observed EROD activities and ENA frequencies were not always coincident in sea bass. Thus, at 24 h NP exposure, the highest ENA frequency coincides with the lowest EROD activity level. Changes at the DNA level may be the precursors of some effects reported at higher levels of biological organization, namely affecting the fertility (Atienzar *et al.*, 2002). Hence, in the field of endocrine disruption, the study of estrogens and/or xenoestrogens effects on DNA justifies further investigation.

Endocrine Responses

The cortisol and glucose plasmatic concentrations are commonly used, respectively, as indicators of primary and secondary acute stress responses in fish (Santos *et al.*, 1992, 1993; Santos and Pacheco, 1996; Hontela, 1997). In the present research work, exposure to individual or combined chemicals did not induce any significant alterations in plasma cortisol levels. Considering previous fish studies where plasma cortisol was increased (Thomas *et al.*, 1993; Miller *et al.*, 2002; Teles *et al.*, 2003b) or decreased (Santos and Pacheco, 1996; Pacheco and Santos, 2001; Teles *et al.*, 2003a, b) and the present absence of effects, suggested that the adrenal susceptibility may depend on the chemical nature of the water contaminants.

Analyzing the current plasma glucose data, after exposure to individual chemicals, it is clear that only E_2 was able to induce a significant early and punctual increase at 4 h exposure followed by recovery at 8 and 24 h. Sea bass BNF+NP exposure increased plasma glucose levels during the whole experiment, demonstrating the interaction between BNF and xeno/estrogens as none of these individual chemicals were able to induce those alterations. Furthermore, BNF+NP regularly displayed significantly higher glucose levels than BNF+E_2, BNF and NP individually. BNF+E_2 also induced a significant plasma glucose increase at 4 h exposure compared to control, though significantly lower than the observed E_2 exposure plasma glucose. Globally, plasma glucose levels demonstrated that fish stress responses to mixtures are clearly different from responses to individual chemicals. Furthermore, a BNF+NP consistent synergistic effect is observed in sea bass as increased plasma glucose levels during the entire experiment.

Generally, a fish stress response includes plasma cortisol and glucose increase (Vijayan *et al.*, 1997). However, different patterns of response have been also found in *A. anguilla*, i.e. glucose increase and plasma cortisol decrease (Santos and Pacheco, 1996; Teles *et al.* 2003a, 2004), indicating that the relation between both parameters is difficult to establish. In the present study, the glucose increase was not accompanied by any cortisol variation. These results corroborated Van Der Boon *et al.* (1991) suggestion that cortisol influence on fish carbohydrate metabolism is not very comprehensive, since glucose is not the most important fuel in fish energy metabolism. In fish, other mechanisms besides interrenal cortisol release may be controlling glucose availability; according to Vijayan *et al.* (1997), under acute stress, catecholamines could be rapidly released resulting in increased glycogenolysis.

Summarizing, the current results, using *D. labrax* as a biological model, demonstrate that:

- Plasma cortisol was not affected by xeno/estrogens tested, either in single exposure or mixed with BNF. Nevertheless, the intermediary metabolism was affected since plasma glucose concentration increased after 4 h exposure to E_2, and after all BNF+NP exposure lengths. Moreover, a synergism between BNF and NP was

thoroughly demonstrated, whereas a sporadic antagonistic interaction was found at 4 h BNF+E_2 exposure.

- Liver EROD and GST activities were not significantly altered by single E_2 or NP exposure. However, both compounds were able to induce EROD activity in the presence of BNF. NP single exposure was able to significantly increase liver P450 content, while its mixture with BNF displayed an antagonistic interference.

- Considering the xeno/estrogens single exposures, only NP induced an ENA increase; however, both mixtures (BNF+E_2 and BNF+NP) displayed genotoxic effects.

- Fish responses to mixtures of xenobiotics are complex and the type of interaction (synergism/potentiation or antagonism) in a particular mixture can vary with the evaluated biological response.

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CAPÍTULO VIII

Actividade hepática de EROD e GST, níveis plasmáticos de cortisol, lactato e glucose, e anomalias nucleares eritrocíticas em *Sparus aurata* L. após exposição a 17β-estradiol isoladamente ou em combinação com 4-nonilfenol

***Sparus aurata* L. liver EROD and GST activities, plasma cortisol, lactate, glucose and erythrocytic nuclear anomalies after short-term exposure either to 17β-estradiol (E_2) or E_2 combined with 4-nonylphenol**

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ABSTRACT

Immature *Sparus aurata* L. (gilthead seabream) were exposed to 17β-estradiol (E_2) 4000 ng/L and to the same E_2 concentration mixed with 50,000 ng/L 4-nonylphenol (E_2+NP) during 4, 8, 12 and 16 h. E_2 availability and E_2 plasma level variations were assessed. Liver biotransformation capacity was measured as ethoxyresorufin-*O*-deethylase (EROD) and glutathione-*S*-transferase (GST) activities. Plasma cortisol, lactate and glucose were also determined. Genotoxicity was assessed through erythrocytic nuclear anomalies (ENA) frequency. Liver EROD activity significantly decreased during the whole experiment for both treatments, with the exception of 16 h exposure to E_2. Liver GST activity was significantly increased after 8 and 12 h of exposure either to E_2 or E_2+NP. An endocrine disruption expressed as plasma cortisol decrease was observed after 16 h exposure under both tested conditions, concomitantly with a plasma lactate increase. No genotoxic responses, measured as ENA frequency, were detected. Analyzing the E_2 water concentration in aquaria without fish it was demonstrated an intense and fast E_2 loss, considerably reducing its availability to fish. In the presence of fish, E_2 water levels were drastically reduced after 4 h exposure, being this reduction more pronounced in E_2 aquarium when compared to E_2+NP aquarium. In addition, it was demonstrated a rapid E_2 uptake from the water since the highest E_2 plasma concentrations were observed after 4 h exposure, followed by a continuous decrease, which became more pronounced between 8 and 12 h of exposure. Furthermore, during the first 8 h exposure to E_2 and E_2+NP, seabream plasma E_2 concentrations were higher than the initial water exposure concentration. Comparing the E_2 plasma levels in both seabream-exposed groups, it was clear that its concentration is always higher in E_2+NP-treated fish. Despite the previous results, no significant differences were found in the measured responses between E_2 and E_2+NP.

Keywords: Seabream; Biotransformation; Genotoxicity; Endocrine disruption.

INTRODUCTION

The aquatic environment contamination by xenoestrogens - substances that modulate or mimic the action of sex steroid hormones, mainly 17β-estradiol (E_2) - has received considerable scientific, government and public attention (White *et al.*, 1994; Brighty, 1996). However, natural estrogens such as E_2 and estrone can also be regarded as aquatic contaminants. The discovery of significant E_2 concentrations in municipal sewage treatment plants effluents (Brighty, 1996) became a matter of concern, being the assessment of its effects on fish at different levels, strongly recommended.

The degradation products of nonylphenol polyethoxylates, such as 4-nonylphenol (NP), are able to activate fish estrogen-dependent gene expression (Ackermann *et al.*, 2002), revealing estrogenic properties either *in vitro* (White *et al.*, 1994) or *in vivo* (Jobling *et al.*, 1996). The polymer industry is the most important source of NP environment contamination, being the major part (95%) released to water. The majority of fish studies carried out on this subject concerned the effects on reproductive aspects. Either E_2 or NP elevate plasma vitellogenin and *zona radiata* proteins in both males and females (Ackermann *et al.*, 2002), induce intersexuality (Jobling *et al.*, 1996), inhibit spermatogenesis and alter gonadosomatic index (Sepúlveda *et al.*, 2003). Besides the known interference on reproduction, it is also important to study the interaction of synthetic and natural steroids with other biological responses. In this perspective, a substantial lack of information concerning the effects of this type of compounds on nonreproductive endocrine responses is evident. Considering the important role of cortisol on fish physiological responses to stressors through intermediary metabolism regulation, immune function and hydromineral homeostasis (Hontela, 1997), its alterations after exposure to xeno/estrogens must be investigated.

Different authors (Pajor *et al.*, 1990; Arukwe *et al.*, 1997) proposed a cross-talk mechanism between P450 activities and steroid synthetic pathways. Thus, cytochrome P450-based mixed-function oxygenase (MFO) system is involved in xenobiotics and endogenous steroids metabolism. Despite the lack of information

concerning CYP1A regulation by endogenous compounds, it was demonstrated that both EROD activity and CYP1A respond to estrogens (Solé *et al.*, 2000). A significant reduction in EROD activity and CYP1A protein was reported in several fish species, namely *Pseudopleuronectes americanus*, *Stenotomus chrysops* (Gray *et al.*, 1991) and *Salmo salar* (Arukwe and Goksøyr, 1997) after E_2 exposure. The same pattern of response was also observed in *S. salar* (Arukwe *et al.*, 1997) after NP exposure. Metabolites resulting from biotransformation processes (phase I) may be conjugated with endogenous molecules (phase II) making them more readily excretable (Goksøyr and Förlin, 1992). Gallagher *et al.* (2001) found that conjugase activities are also modulated by endogenous steroids. Additionally, effects of synthetic or natural steroids include genotoxicity (Liehr, 2000) as it was proposed that they could induce genetic damage and gene mutations in mammals (Roy and Liehr, 1999; Yared *et al.*, 2002). However, to our knowledge only a few studies are available concerning genotoxicity of these compounds in nonmammal species. NP was reported as genomic DNA damaging agent to crustacean larvae (Atienzar *et al.,* 2002), and genotoxic to sea bass, measured as erythrocytic nuclear abnormalities (ENA) (Teles et al., 2004a).

Gilthead seabream, *Sparus aurata* L., seawater teleost widespread in Atlantic and Mediterranean coastal waters, is one of the most commercially important species, and as protandrous hermaphrodite is particularly appropriate as a biological model to endocrine studies.

Environmental waters are frequently contaminated simultaneously by different natural and synthetic steroids, being therefore relevant to study xeno/estrogen mixtures effects, as well as their eventual interference on single compounds effects, in fish.

The aims of the present research work, using juvenile seabream, were to assess the waterborne E_2 or E_2 mixed with NP (E_2+NP) effects on biotransformation, measured as total P450 content, ethoxyresorufin-*O*-deethylase (EROD) activity (phase I) and glutathione-*S*-transferase (GST) activity (phase II). The variation on E_2 concentrations in aquaria water and in seabream plasma was determined during the

whole experiment. Liver somatic index (LSI) and alanine transaminase (ALT) activity were measured as general hepatic condition indicators. Additionally, the stress responses were evaluated as plasma cortisol, glucose and lactate, and genotoxicity was investigated using the ENA assay.

MATERIAL AND METHODS

Chemicals

NP and E_2 were purchased from Sigma-Aldrich (Germany). Marine salt was supplied by Sera Premium (Germany). All the other chemicals were of analytical grade obtained from Sigma-Aldrich (Germany), Roche (Germany) and E. Merck-Darmstadt (Germany).

Test Animals

The experiment was carried out with juvenile *S. aurata* specimens obtained from a local fish farm, Materaqua - Ílhavo, Portugal. Seabream weighing 34±0.3 g were transported in aerated saltwater and acclimated to laboratory conditions for 1 week prior to experimentation. During acclimatization and experimental periods, fish were kept in 80-L aquaria, at 20ºC in aerated (dissolved oxygen: 7.4±0.2 mg/L) and filtered artificial seawater (34 g/L salinity), with a pH of 8.4±0.2.

Experimental Design

Fish were divided in three lots: one as control and the others were exposed either to E_2 or E_2 mixed with NP (E_2+NP), during 4, 8, 12 and 16 h. The appropriate amount of each chemical was previously dissolved in 1 mL of DMSO and added to the experimental aquaria in order to prepare the following nominal concentrations: 14.68 nM (4000 ng/L) of E_2 and 183.5 nM (50,000 ng/L) of NP. The same volume of DMSO was added to the control aquarium, since this concentration has no damaging effects on fish (Pacheco and Santos, 1998). Experiments were carried out using test groups of five seabream (n=5). E_2 or E_2+NP (in the previous nominal concentrations)

were added to two additional aquaria (E_2nf and E_2+NPnf), kept without fish, in order to determine the E_2 availability to fish after the eventual adsorption and degradation occurring during the same exposure period.

Fish were killed at each sampling point and their blood and liver were collected. Liver was immediately frozen in liquid nitrogen, stored at -80ºC until homogenization. Blood smears were prepared. Blood plasma was isolated using an Eppendorf centrifuge (14,000 rpm).

Biochemical Analysis

Liver Cytochrome P450 Content and EROD Activity

Cytochrome P450 content was determined using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm, as previously described by Hermens *et al.* (1990).

The liver EROD activity was measured in microsome suspension as described by Burke and Mayer (1974) as adapted by Pacheco and Santos (1998). The reaction was carried out, at 25 ºC, in the fluorometer cuvette containing 1 mL 0.5 μM ethoxyresorufin (in 0.1 M Tris-HCl pH 7.4, containing 0.15 M KCl and 20% glycerol) and 100 μL of microsomal suspension. The reaction was initiated by adding 10 μL of NADPH (10 mM) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD-activity was expressed as picomoles per minute per milligram of microsomal protein.

Liver GST Activity

GST activity was determined in the cytosolic fraction as described by Lemaire *et al.* (1996), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig *et al.*, 1974). The assay was carried out in a quartz cuvette with a 2 mL mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM GSH. The reaction was initiated by the addition of 10 µL sample at 25°C. The increase in absorbance was recorded at 340 nm wavelength during 3 minutes.

Liver ALT Activity

ALT activity was measured, in the supernatant resulting from microsomal isolation, according to a colorimetric method based on the measurement of the pyruvate produced by the transamination reaction (Reitman and Frankel 1957).

Protein Measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornall *et al.*, 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard.

Cortisol, Glucose and Lactate Measurement

The determination of cortisol was performed in plasma using a diagnostic ELISA direct immunoenzymatic kit (Diametra, Italy). The cortisol in the sample competes with horseradish peroxidase-cortisol for binding onto the limited number of anti-cortisol sites in the microplate. Cortisol concentration in the sample is calculated based on a series of standards and the color intensity is inversely proportional to the cortisol concentration in the sample. The method allows the determination of cortisol from 10 ng/mL to 500 ng/mL.

Plasma glucose was measured spectrophotometrically (340 nm) according to the method modified from Banauch *et al.* (1975) based on the quantification of NADH after a glucose oxidation catalysed by the glucose-dehydrogenase. The quantity of NADH formed is proportional to the glucose concentration.

Plasma lactate levels were determined spectrophotometrically (340 nm) according to the method modified from Noll (1974) using lactate-dehydrogenase (LDH), ALT and NAD, measuring the NADH appearance.

17β-Estradiol Measurement

The E_2 determination was performed using a diagnostic ELISA direct immunoenzymatic kit (Diametra). E_2 in the sample competes with horseradish-peroxidase E_2 for binding onto the limited number of anti E_2 sites on the microplates.

E_2 concentration in the sample is calculated based on a series of standard; the color intensity is inversely proportional to the E_2 concentration in the sample. The method allows the determination of E_2 from 20 pg/mL to 4000 pg/mL.

Plasmatic and water E_2 measurements were carried out during the entire experiment. The E_2 quantification on water was performed in the experimental aquaria (E_2, E_2+NP) containing seabream, as well as in two additional aquaria kept without fish (E_2nf, E_2+NPnf).

Liver Somatic Index

LSI results were presented as a frequency (%) resulting from the following expression: [liver mass (g)/body mass (g)]x100%.

ENA Assay

The blood smears were fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. In order to evaluate genotoxicity the erythrocytic nuclear abnormalities were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid (1976), Carrasco *et al.* (1990) and Smith (1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei, lobed nuclei, dumbbell shaped or segmented nuclei and kidney shaped nuclei. The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

Statistical Analysis

Statistica software (StatSoft, Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P<0.05$.

RESULTS

Water and Plasma 17β-Estradiol Quantification (Table I)

A time-related reduction in 17β-estradiol concentrations was observed either in aquaria with fish (E_2 and E_2+NP) or without fish (E_{2nf} and E_2+NP_{nf}), during the entire experiment. However, this E_2 reduction was more pronounced in aquaria containing fish. On the other hand, the E_2+NP aquaria always presented clearly higher E_2 concentrations than the E_2 aquaria, either in the fish presence or absence. After 4 h exposure E_2 levels in the aquaria with fish were drastically reduced, i.e., a 99% reduction in E_2 aquarium and a 93.5% reduction in E_2+NP aquarium. Furthermore, the control level was reached after 4 h in E_2 aquarium, whereas in E_2+NP aquarium this reduction occurred after 12 h of exposure. The maximum E_2 plasma levels were observed after 4 h exposure in both treated groups, presenting thereafter a time-related decrease. Seabream exposed to E_2+NP exhibited E_2 plasma concentrations higher, at least two-fold, than E_2-treated group. In the period between 4 and 8 h, it was observed, for treatments, the lowest E_2 plasma reduction, 15% and 30% reduction for E_2- and E_2+NP-treated fish, respectively. The highest plasma E_2 reduction was observed after 12 h in E_2 (83% reduction) and at 16 h in E_2+NP-treated fish (67% reduction) Table I.

Taking into account the E_2 levels detected in the E_{2nf} and E_2+NP_{nf} aquaria, indicating the E_2 availability, it is noticeable that its uptake from the water in E_2 group was slightly lower than in E_2+NP group during the first 4 h.

Table I – 17β-Estradiol (E_2) measurement in the aquaria water 5 min, 4, 8,12 and 16 h and in the *S. aurata* plasma 4, 8, 12 and 16 h after the adition of E_2 (4000 ng/L) or E_2 mixed with 4-nonylphenol (NP) – E_2+NP - 4000 ng/L and 50,000 ng/L, respectively.

	Water Estradiol (ng/L)					Plasma Estradiol (ng/L)		
Time	Control	E_2	E_2+NP	E_{2nf}	E_2+NP_{nf}	Control	E_2	E_2+NP
5 min	33.05	666.01	1618.18	3426.21	3879.99	-	-	-
4 h	29.09	31.46	258.86	1605.71	2039.32	59.14	5997.43	14329.58
8 h	17.38	24.16	102.08	1238.95	1481.23	145.55	5111.98	10040.00
12 h	29.75	17.62	32.05	958.90	1203.81	101.31	882.13	4051.00
16 h	32.98	10.18	19.99	630.71	686.84	51.41	206.10	1355.04

The water quantification was performed in aquaria with fish (E_2 and E_2+NP) or without fish (E_2nf and E_2+NPnf).

Liver Biotransformation Responses (Fig. 1)

P450 content did not exhibit any statistically significant alteration (Fig. 1A). However, a significant EROD activity decrease was observed in seabream after 4, 8, and 12 h exposure to E_2 and E_2+NP compared to the control (Fig. 1B). Furthermore, EROD activity inhibition significantly persisted at 16 h of exposure to E_2+NP. Seabream exposed either to E_2 or to E_2+NP did not demonstrate any significant differences between them. A significant increase in GST activity was observed in seabream at 8 and 12 h exposure to E_2 or E_2+NP (Fig. 1C).

Hepatic Health Indicators (Fig. 2)

Liver ALT activity did not present any significant alteration (Fig. 2A). However, LSI significantly rises after 16 h exposure to E_2 and E_2+NP (Fig. 2B).

Genotoxic Response (Fig. 3)

The ENA frequency in treated fish was similar to that displayed by the control (Fig. 3).

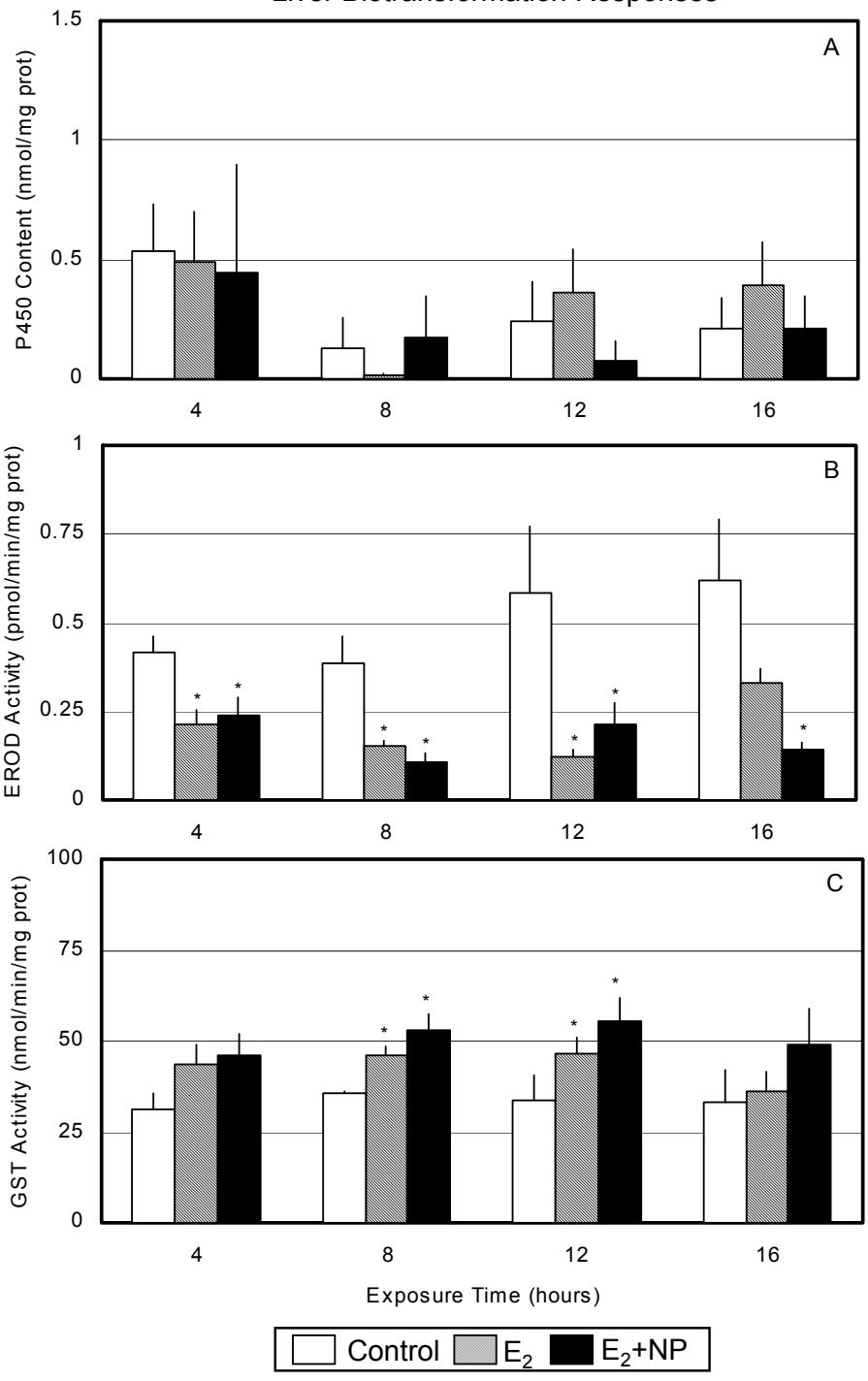

Fig. 1 – *S. aurata* liver P450 content (A), liver EROD activity (B) and liver GST activity (C) after 4, 8, 12 and 16 h exposure to (4000 ng/L) of 17β-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP) – E_2+NP – 4000 ng/L and 50,000 ng/L, respectively. Values represent the means and S.E. (*n*=5/treatment). Differences from control: **P*<0.05.

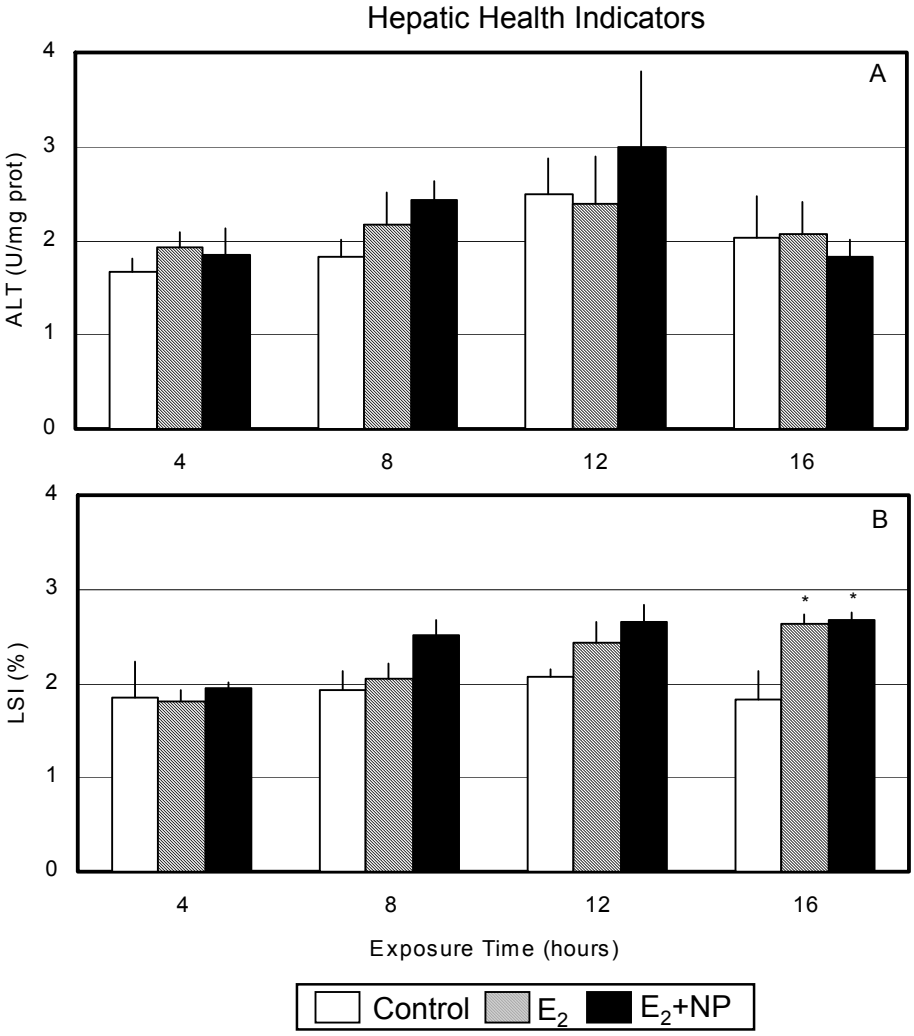

Fig. 2 – *S. aurata* liver ALT activity (A) and LSI (B) after 4, 8, 12 and 16 h exposure to (4000 ng/L) of 17β-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP) – E_2+NP – 4000 ng/L and 50,000 ng/L, respectively. Values represent the means and S.E. (*n*=5/treatment). Differences from control: **P*<0.05.

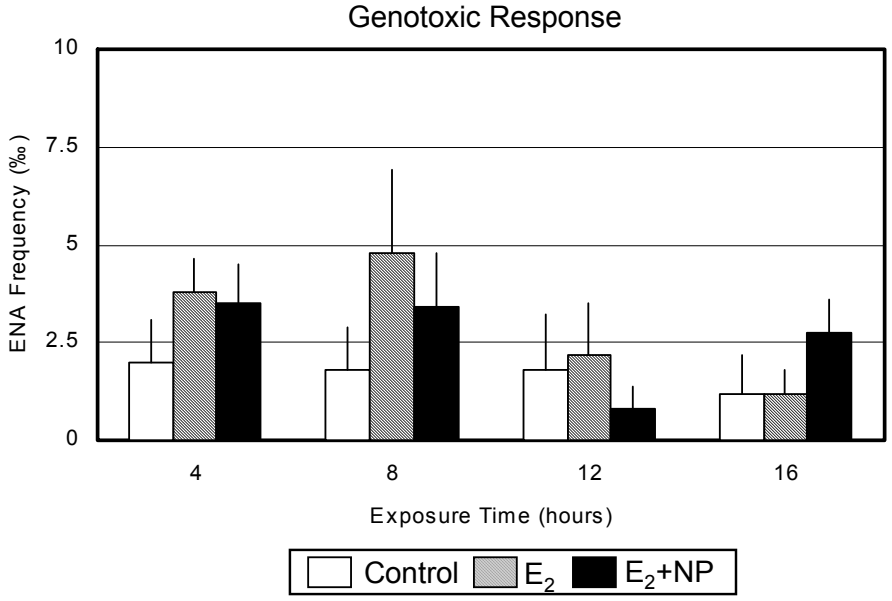

Fig. 3 – *S. aurata* ENA frequency after 4, 8, 12 and 16 h exposure to (4000 ng/L) of 17β-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP) – E_2+NP – 4000 ng/L and 50,000 ng/L, respectively. Values represent the means and S.E. (*n*=5/treatment). Differences from control: **P*<0.05.

Stress Responses (Fig. 4)

Seabream plasma cortisol levels significantly decreased after 16 h exposure to E_2 or E_2+NP despite its decreased tendency since the initial exposure (Fig. 4A). The plasma glucose concentration did not exhibit any significant alteration (Fig. 4B). Plasma lactate levels displayed a significant increase after 16 h exposure either to E_2 or E_2+NP, though this increasing tendency was noticed from 8 h exposure onwards (Fig. 4C).

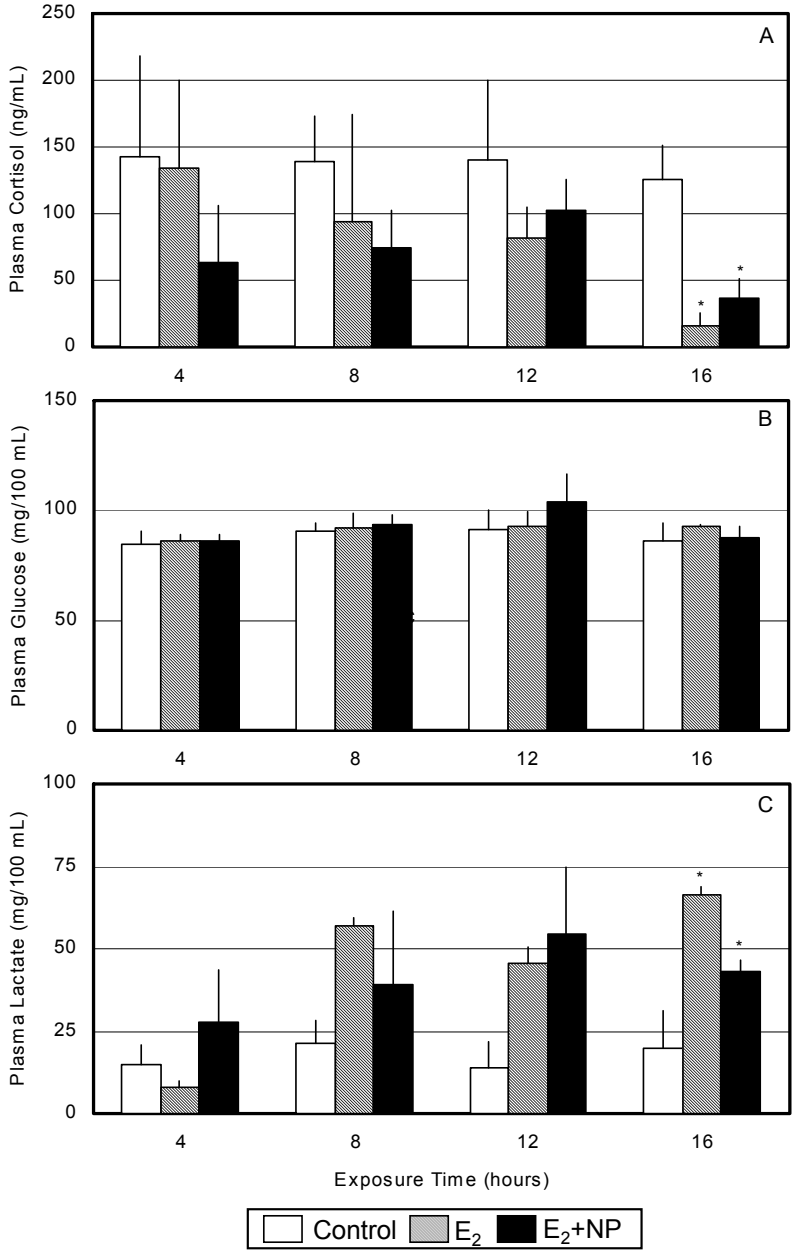

Fig. 4 – *S. aurata* plasma cortisol levels (A), plasma glucose levels (B) and plasma lactate levels (C) after 4, 8, 12 and 16 h exposure to (4000 ng/L) of 17β-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP) – E_2+NP – 4000 ng/L and 50,000 ng/L, respectively. Values represent the means and S.E. (*n*=5/treatment). Differences from control: **P*<0.05.

DISCUSSION

17β-Estradiol Measurements

E_2 has been detected in effluent sewage treatment plants in different countries such as USA, Japan, Germany, Italy and the Netherlands, at concentrations up to 64 ng/L (Spengler *et al.*, 2001). Considering the previous environmental data, a spiking concentration was adopted in the current study, to ensure a substantial uptake in a short period. Thus, this fact should be kept in mind in eventual extrapolations of the current findings to environmental situations.

The NP concentrations detected in environment ranged from near or below limits of detection up to 180 μg/L (Nielsen *et al.*, 2000).

The measurement of E_2 in aquaria without fish demonstrated a time-related reduction in E_2 concentration, which is less pronounced in the presence of NP. Some studies were previously carried out on E_2 sorption and degradation, which can help to explain the current results. A study testing an E_2 concentration range which includes the current E_2 concentration revealed that microorganisms were capable of transforming E_2 with half-lives of 0.2-9 days (Jurgen *et al.*, 2002). According to the same authors, E_2 was susceptible to photodegradation with half-lives in order of 10 days. Additionally, Holthaus *et al.* (2002) observed that 80-90% of E_2 bound to bed sediments within 1 day, whereas Ying *et al.* (2003) stated that E_2 had a modest sorption capability to sediment.

The E_2 decrease in water was more pronounced in aquaria with than without seabream, suggesting its rapid uptake from the water, which is corroborated by the plasma E_2 increase. Thus, E_2 on water was drastically reduced during the first 4 h of exposure, and concomitantly the highest E_2 plasmatic concentrations were detected after 4 h, followed by its continuous decrease, which became more pronounced between 8 and 12 h exposure. Moreover, plasma E_2 concentrations were higher than the initial water exposure concentration (4000 ng/L), during the first 8 and 12 h in fish, respectively exposed to E_2 and E_2+NP. The present results agree with Specker and Chandlee (2003) who found that E_2 uptake by *Paralichthys dentatus* reached an initial plateau within 30 min, being accumulated in the whole-body above the exposure

concentrations. Comparing the E_2 plasma levels in seabream exposed either to E_2 or E_2+NP, it is clear that its concentration was always higher in the last group. This fact may reflect alterations on E_2 water/plasma/tissue uptake, and excretion, as a consequence of the simultaneous NP exposure.

Despite the different E_2 plasma levels between seabream exposed to E_2 and E_2+NP, in general the measured responses were not different, as discussed below.

Biotransformation Responses

The CYP1A induction measured either by immunodetection or through its catalytic activity is probably the best-studied biomarker (Bucheli and Font, 1995). Hence, EROD activity has been widely used as a biomarker for fish exposure to substances that bind to the aryl hydrocarbon (Ah) receptor (Lemaire *et al.*, 1996; Pacheco and Santos, 1998, 2001).

In the present research work P450 content did not demonstrate any alteration after E_2 or E_2+NP exposures, which is in agreement with other authors who observed an unaltered total P450 content in *Cyprinus carpio* treated with the synthetic steroid 17α-ethynylestradiol (Solé *et al.*, 2000) and in *D. labrax* exposed to E_2 (Teles *et al.*, 2004a).

It is known that steroid hormones can regulate CYP1A expression. A decrease in the catalytic CYP1A activities was observed in sexually mature females with elevated levels of E_2, as well as in E_2-treated fish, namely *Oncorhynchus mykiss* (Andersson and Rafter, 1990), *P. americanus* and *S. chrysops* (Gray *et al.*, 1991). Thus, the current EROD activity decrease regularly found during the whole experiment in both exposed groups is not surprising. Though, the interaction mechanism between E_2 and CYP1A is not well understood and different possibilities were presented. It was suggested that the possible binding of E_2 to the estrogen receptor (ER) exhibits an anti-CYP1A influence on the CYP1A gene, indicating a probable receptor and/or gene cross-talk between CYP1A and estrogen-responsive genes (Arukwe *et al.*, 1997; Navas and Segner, 2000). However, the ability of steroids, including E_2 binding to the CYP1A molecule, acting as a competitive inhibitor

of its enzymatic activities, e.g. EROD, was established in fish (Chan and Hollebone, 1995).

Despite NP estrogen-like properties (White *et al.*, 1994) its interference with CYP1A function was demonstrated as an EROD activity reduction in juvenile *S. salar* (Arukwe *et al.*, 1997). Furthermore, BNF potential to induce EROD was also reduced either by NP or E_2 *D. labrax* exposure (Teles *et al.*, 2004a). Considering the previous findings a higher EROD activity reduction should be expected in E_2+NP than in E_2 seabream exposed groups; however, current results did not confirm this hypothesis, since there was no NP additional effect under co-exposure.

The less pronounced decrease in EROD activity was observed at 16 h exposure to E_2 coinciding with the lowest E_2 plasma concentration and thus suggesting an E_2 metabolic turnover in the hepatocytes. Furthermore, the E_2 inhibitory action was kept up to the end of the experiment in E_2+NP exposed fish, which coincided with a substantially higher E_2 blood concentration than that observed in E_2 exposed fish.

P450 enzymes are constitutively expressed being also modulated (induced/inhibited) by chemicals. Since no potential CYP1A inducers were used in the present study, it seems that E_2 affects the constitutive liver CYP1A expression, as previously demonstrated by Navas and Segner (2000) in monolayer cell cultures of *O. mykiss*.

The physiological implications of P450 MFO activities inhibition have not been fully established (Arukwe *et al.*, 1997). Nevertheless, this MFO inhibition by environmental estrogens may reduce fish ability to metabolize and excrete xenobiotics as well as endogenous estrogens, causing alterations at different biological levels and not only on reproduction. These metabolic changes may have important ecological consequences, as fish are frequently exposed in the environment to different classes of chemicals. Furthermore, the mechanisms involved in these interaction processes should be studied.

To our knowledge, no previous data concerning E_2 effects on fish GST activity are available. Concerning synthetic steroids, 17α-ethynylestradiol induced no

alterations on *C. carpio* GST activity (Solé *et al.*, 2000). On the other hand, a mammalian study demonstrated that bisphenol A increased GST activity (Nieminen *et al.*, 2002). The GST activity increase observed in the current study is suggestive of an increased hepatic steroid catabolism in fish. CYP induction and conjugation activities may occur simultaneously, depending on the enzyme involved and its regulation. Thus, the inhibition detected on CYP1A activity was not extended to other CYP families. Taking into account that steroid metabolism and elimination may be catalyzed by CYP3A and that previous investigations on synthetic steroids effects demonstrated a CYP1A down-regulation and a CYP3A up-regulation, further laboratory studies on E_2 biotransformation, namely CYP3A induction should be carried out (Lee *et al.*, 1996a, b).

Hepatic Condition Indicators

The LSI increase observed in both treatments at 16 h exposure indicates metabolic alterations. The ALT kept constant, indicating that no cell damage was induced under the experimental conditions. Concerning LSI and ALT no differences were detected between E_2 single exposure and the NP co-exposure.

Genotoxic Response

Recent works on genotoxicity of aquatic environment contaminants have demonstrated the suitability of the ENA assay, based on micronuclei and other nuclear anomalies detection in mature erythrocytes. This test, successfully adopted in different fish species (Pacheco and Santos, 1996, 1998, 2001, 2002; Ayllón and Garcia-Vasquez, 2001; Gravato and Santos, 2002), is expanding in application due to its simplicity, rapidity, sensitivity, and low cost. However, its utilization may present some limitations in the presence of intense contamination since it can be masked by an increased splenic erythrocytic catabolism and/or erythropoiesis rate reduction (Pacheco and Santos, 2002).

In the present study, E_2 or E_2+NP exposure did not induce any significant ENA increase. However, an ENA increasing trend was observed at 4 and 8 h that returned

to control levels after 12 h exposure to E_2 or E_2+NP. Comparing the ENA frequency and E_2 plasmatic levels the explanation previously presented for EROD and GST activities can be adopted, i.e., the ENA highest values were found concomitantly to the highest plasma E_2 concentrations. Thus, the occurrence of an ENA expression limitation in the presence of intense contamination, as previously suggested by Pacheco and Santos (2002), does not apply to this particular situation.

E_2 was classified as nonmutagenic and nongenotoxic, based on the failure to induce gene mutations in classical bacterial and mammalian mutation assays; however, this matter seems to be highly controversial (Roy and Liehr, 1999). In a recent review work on estrogens DNA damage and mutations Roy and Liehr (1999) stated that this classification was probably related to the lack of a sensitive method for detection of estrogen covalent binding to DNA bases, and because the classical mutation assays performed were designed to uncover mutations only at one specific locus and could not have detected other types of mutations or changes in other genes. According to International Agency for Cancer Research (IARC) (1999) E_2 genotoxicity was not demonstrated *in vivo*, neither in humans, nor in mice; nevertheless, E_2 was classified as a carcinogen, class I (IARC, 1999).

In the present study, the E_2 genotoxic potential was not demonstrated. However, the importance of further fish studies on estrogen genotoxicity is increased by the lack of data in nonmammal organisms, being recommended the adoption of longer exposures in realistic conditions.

Stress Responses

The studied stress responses and the respective methodologies proved their suitability and reproducibility in previous studies with different fish species and different classes of contaminants (Teles *et al.*, 2003a, b, 2004a, b), encouraging their utilization on toxicological endocrine research works.

The plasma cortisol decrease observed for both exposure conditions is an indication of endocrine impairment. Despite the lack of fish studies concerning cortisol alterations after E_2 exposure, plasma cortisol decrease was also found in various fish

studies after short-term exposure to different contaminants (Pacheco and Santos, 2001; Teles *et al.*, 2003a). The mechanism involved in this response is not completely clarified. Santos and Pacheco (1996) found that the interrenal cortisol release into the blood is prevented by the contaminant (i.e. endocrine disruptor) since an interrenal cortisol accumulation was observed in parallel with a reduced plasma cortisol concentration. The observed cortisol secretion dysfunction may reduce fish physiological competence, growth, and survivorship since this hormone is required for a wide range of important homeostatic mechanisms, including fuel reserves mobilization.

The E_2 potential to induce a plasma glucose increase was previously demonstrated in *D. labrax* (Teles *et al.*, 2004a); however, this effect was not confirmed for the current *S. aurata* study.

The plasma lactate increase, observed for E_2 and E_2+NP exposures can be regarded as an indication of stress. This response seems to be expectable since it was demonstrated for a wide range of xenobiotics (Santos and Pacheco, 1996; Pacheco and Santos, 2001; Teles *et al.*, 2003a), despite the lack of any publications concerning specifically the current compounds.

Cortisol effect includes the gluconeogenic activation, mobilizing substrates such as lipids, amino acids and lactate as fuel energy, besides carbohydrates. Analyzing simultaneously the plasma cortisol and lactate responses it is evident an opposite trend of variation; thus, it can be suggested that the observed plasma lactate increase could have been potentiated by a reduction of the cortisol induced gluconeogenesis.

CONCLUSIONS

The current data concerning seabream exposure either to E_2 or to its mixture with NP revealed:

- a liver EROD activity depression after both treatments, while GST activity was elevated;

- an endocrine disruption, expressed as plasma cortisol decrease after exposure either to E_2 or E_2+NP. Furthermore, a plasma lactate increase was measured for both conditions;

- that despite the higher E_2 levels measured in plasma of E_2+NP comparing to E_2-treated fish, no significant differences were detected on the biological responses due to NP co-exposure.

It was also demonstrated a high E_2 spontaneous loss in the aquaria, and a rapid E_2 uptake by fish (in the first 4 h).

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CAPÍTULO IX

Respostas fisiológicas e genéticas de enguia Europeia (*Anguilla anguilla* L.) a uma exposição de curta duração a crómio ou cobre – influência da pré-exposição a um composto do tipo HAP

Physiological and genetic responses of European eel (*Anguilla anguilla* L.) to short-term chromium or copper exposure – influence of preexposure to a PAH-like compound

M. Teles, Pacheco e M. A. Santos (2005)
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ABSTRACT

Anguilla anguilla L. (European eel) was exposed for 24 h to chromium (Cr - 100 μM and 1 mM) or copper (Cu - 1 and 2.5 μM), with or without a 24-h preexposure to β-naphthoflavone (BNF - 2.7 μM), a polycyclic aromatic hydrocarbon (PAH)-like compound, simulating sequential exposure to PAHs and heavy metals. Plasma cortisol, thyroid-stimulating hormone (TSH), free triiodothyronine (T3), and free thyroxine (T4) were determined in order to assess the effects on endocrine function. Plasma glucose and lactate also were measured. The frequency of erythrocytic nuclear abnormalities (ENA) was scored as a genotoxicity indicator. Plasma T4 decreased in eels when exposed to Cr only. The interference of BNF preexposure on Cr effects was observed as a significant plasma glucose increase. Single exposures to Cu elevated plasma cortisol and glucose (2.5 μM), as well as plasma lactate (1 μM), whereas a T4 decrease was found for both concentrations. BNF preexposure prevented plasma cortisol and lactate increases; however, a greater T4 decrease was observed in eels exposed to 2.5 μM Cu. Moreover, this pretreatment was crucial for genotoxicity expression because only BNF+2.5 μM Cu-exposed fish exhibited significant ENA induction. In general, plasma T4 was the most affected hormone, as it responded to all Cr and Cu exposure conditions.

Keywords: Heavy metals; β-Naphthoflavone; Endocrine disruption; Genotoxicity.

INTRODUCTION

Fish inhabiting polluted waters are exposed to several compounds that might have negative consequences to their health and reproductive success. Heavy metals, in particular, are widespread contaminants released into aquatic systems from numerous anthropogenic sources, constituting a serious threat to fish. Though some heavy metals, such as chromium (Cr) and copper (Cu), are essential for physiological processes, abnormally high environmental concentrations of these chemicals may become toxic. The effects of Cr and Cu have been a matter of numerous

investigations, establishing indubitably their wide spectrum of toxicity. Fish exposed to these metals revealed reduced immunity (Bennani *et al.*, 1996), as well as morphological (Nguyen and Janssen, 2002) and histopathologic (Krishnani *et al.*, 2003) effects.

Apart from investigations of the response levels mentioned above, very few studies have been carried out on Cr- and Cu-induced endocrine alterations. The endocrine system is particularly important because of its crucial function in maintaining internal fish homeostasis. In this context, cortisol plays a central role through gluco- and mineral-corticoid functions, as it is an end product of the hypothalamo-pituitary-interrenal (HPI) axis in response to different stressors. Thus, Cu has been shown to increase fish plasma cortisol (Dethloff *et al.*, 1999; De Boeck *et al.*, 2003); however, no data are available about the effects of Cr at this level.

Fish growth, reproduction, and osmoregulation can be affected by xenobiotic-induced alterations of the hypothalamo-pituitary-thyroid (HPT) axis (Bhattacharya *et al.*, 1989; Zhou *et al.*, 2000). Thyroid physiological alterations induced by heavy metals were detected following fish exposure to mercury (Bhattacharya *et al.*, 1989) and cadmium (Ricard *et al.*, 1998), as well as to a heavy metal-contaminated environment (Levesque *et al.*, 2003). Nevertheless, information concerning the effects of heavy metals on the HPT axis is scarce, and to our knowledge, Cr and Cu effects have not been studied.

Both cortisol and thyroid hormone can interact and influence carbohydrate metabolism (Hontela *et al.*, 1995). Exposure to Cr increased plasma glucose and lactate (Sastry and Tyagi, 1982; Nath and Kumar, 1988), whereas unclear responses were observed in Cu-exposed fish (Dethloff *et al.*, 1999). Changes in carbohydrate metabolism, measured as plasma glucose and lactate, can be regarded as secondary fish stress responses to xenobiotics (Teles *et al.*, 2003, 2004). Given this, understanding the interdependence of intermediary metabolism and the HPI and HPT axes becomes an important challenge to endocrine toxicology.

Heavy metals also have been reported as genotoxic agents in fish (De Lemos *et al.*, 2001; Sanchez-Galan *et al.*, 2001). Cr, in particular, increased the number of

erythrocytic micronuclei in *Carassius auratus* (Al-Sabti *et al.*, 1994) and in *Pimephales promelas* (De Lemos *et al.*, 2001). Furthermore, Cr has been classified as a carcinogenic compound by the International Agency for Research on Cancer (IARC, 1990). However, there have been contradictory indications about Cu genotoxicity. Thus, Cu was suggested as a possible cause of micronuclei induction in a field study with *Aldrichetta forsteri* and *Sillago schomburgkii* (Edwards *et al.*, 2001), whereas *A. anguilla* injected with Cu did not display significant induction of micronuclei (Sanchez-Galan *et al.*, 2001).

The majority of fish studies conducted on heavy-metal toxicity adopted an experimental design with single-metal exposures or, in a few cases, with exposures to metal combinations. However, aquatic systems represent a vast sink of pollutants in which interactions between heavy metals and organic pollutants frequently occur. Despite the knowledge that antagonistic or synergistic mechanisms can greatly modify toxic effects, the assessment of the interactions of heavy metals with other classes of contaminants is a poorly explored area of research. For instance, polycyclic aromatic hydrocarbons (PAHs) and heavy metals are known to interact at different levels, namely, in MFO biotransformation activities. It was previously reported that PAH-induced ethoxyresorufin-*O*-deethylase (EROD) activity could be inhibited by heavy metals, including Cr and Cu (Oliveira *et al.*, 2003).

The aims of the present study were to assess the effects of chromium [Cr(VI)] and copper (Cu^{2+}) on:

- Endocrine function, measured as cortisol, thyroid-stimulating hormone (TSH), free triiodothyronine (T3), and free thyroxine (T4) plasmatic levels;
- Intermediary metabolism, evaluated as plasma glucose and lactate levels; and
- Erythrocytic nuclear abnormality (ENA) frequency as a genotoxicity indicator.

Sequential exposures to β-naphthoflavone (BNF, a PAH-like compound) and heavy metals (Cr or Cu) were performed in order to determine if PAH preexposure can interfere with Cr and Cu effects.

MATERIAL AND METHODS

Chemicals

β-Naphthoflavone (BNF), β-nicotinamide adenine dinucleotide (β-NAD), L-lactic dehydrogenase, and glutamic-pyruvic transaminase were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Copper chloride ($CuCl_2$) and potassium dichromate ($K_2Cr_2O_7$) were from E. Merck-Darmstadt (Germany). All other chemicals were of analytical grade.

Test Animals

The experiment was carried out with *Anguilla anguilla* L. (European eel) whose average weight was 30 ± 5 g an average length was 25 ± 3 cm (yellow eel); they were collected from a clean site in the Aveiro Lagoon, Murtosa, Portugal. The eels were acclimated to laboratory conditions for 1 week prior to experimentation. During recovery eels were kept at a temperature of 20°C under a natural photoperiod in aerated (dissolved oxygen: 7.6 ± 0.3 mg/L), filtered, dechlorinated, and recirculating tap water with a pH of 7.2 ± 0.4. The experiment was carried out in 20-L aquariums in the same conditions except without a filtering and recirculating system. Fish were not fed either during laboratory adaptation or during the experimental procedure.

Experimental Design

The eels were divided into 10 groups according to the following experimental protocol (Fig. 1).

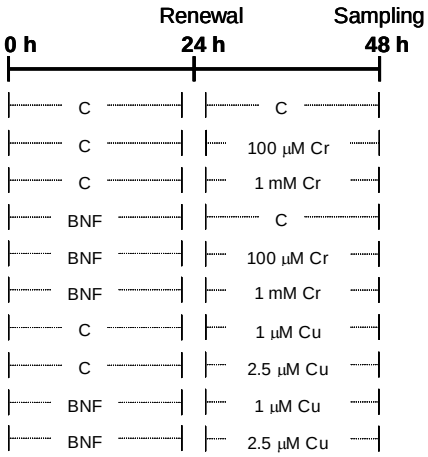

Fig. 1 – Schematic representation of the experimental design (C - clean tap water; BNF - β-naphthoflavone; Cr - chromium; Cu - copper).

The control group was kept in clean tap water (C) for 48 h, with water renewal after 24 h (control – C+C). Four groups were kept in C for the first 24 h and then exposed to Cr(VI) as potassium dichromate (100 μM or 1 mM) or Cu^{2+} as copper dichloride (1 or 2.5 μM) during the next 24 h. Another five groups were exposed for 24 h to 2.7 μM of BNF previously dissolved in 1 mL of DMSO. One milliliter of DMSO also was added to the control and to all the other aquariums in which there was heavy-metal exposure without BNF preexposure. After that, one group was transferred to C, and the remaining four groups were exposed for 24 h to either Cr or Cu in the concentrations previous stated. Fish were killed 48 h after the beginning of the experiment, and their blood was collected. Blood smears were prepared, and the blood plasma was isolated using an Eppendorf centrifuge (14.000 rpm). Experiments were carried out in test groups of five eels (*n*=5).

Biochemical Analyses

Plasma Cortisol, TSH, Free T3, and Free T4 Measurement

Hormonal determination was performed by ELISA direct immunoenzymatic methods using commercial kits from Diametra, Italy.

Plasma Glucose and Lactate Measurement

Plasma glucose was measured according to a method modified from Banauch *et al.* (1975). Lactate was determined according to a method modified from Noll (1974).

ENA Assay

To evaluate genotoxicity, erythrocytic nuclear abnormalities were scored in 1000 mature erythrocytes per fish, according to the criteria of Schmid (1976), Carrasco *et al.* (1990), and Smith (1990), as adapted by Pacheco and Santos (1996). In accordance with these criteria, nuclear lesions were scored as one of these categories: micronuclei, lobed nuclei, dumbbell-shaped or segmented nuclei, and kidney-shaped nuclei. The final result was expressed as the mean value (%) of the sum of all the individual lesions observed.

Statistical Analysis

Statistica software (StatSoft, Inc., Tulsa, OK, USA) was used for the statistical analyses. All data were first tested for normality and homogeneity of variance in order to meet statistical assumptions. ANOVA analysis was used to compare the results of the various fish groups, followed by the least significant differences test (Zar, 1996). Differences between means were considered significant at $P<0.05$.

RESULTS

Chromium Effects

Hormonal Responses

A. anguilla L. exposure to Cr, with or without BNF preexposure, did not reveal any significant alterations in cortisol or TSH plasma levels (Fig. 2). The plasma T3 was significantly lower in the BNF+100 μM Cr group than in the C+100 μM Cr group. A significant plasma T4 decrease compared to the controls was observed in all

experimental conditions. In addition, the T4 level was significantly decreased in BNF+100 μM Cr compared to that in BNF+C.

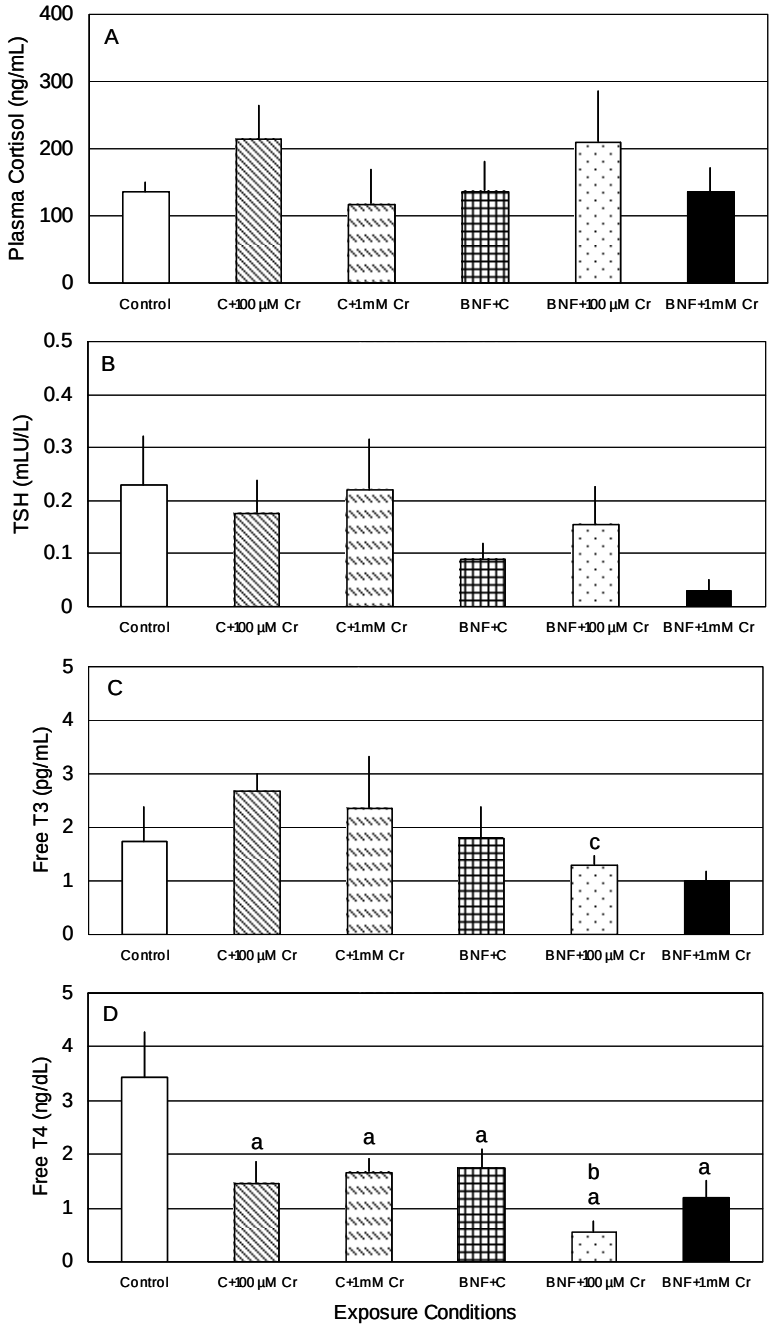

Fig. 2 – Plasma concentrations of (A) cortisol, (B) TSH, (C) free T3, and (D) T4 after Cr exposure (100 μM or 1 mM) with or without BNF preexposure. Values represent the means and SEs (*n*=5/treatment). Differences between groups are: **a**. *vs.* control; **b**. *vs.* BNF+C; **c**. *vs.* C+100 μM Cr.

Intermediary Metabolism Responses

The *A. anguilla* plasma glucose concentration significantly increased in all BNF-treated groups (BNF+C, BNF+100 μM Cr, and BNF+1 mM Cr) in comparison to the control group (Fig. 3). BNF+100 μM Cr exposure also induced a significant plasma glucose increase compared to that from C+100 μM Cr or BNF+C exposure. Furthermore, a plasma glucose increase was observed in BNF+1 mM Cr compared to that in C+1 mM Cr. Multiple comparisons of plasma lactate concentrations did not reveal any significant differences.

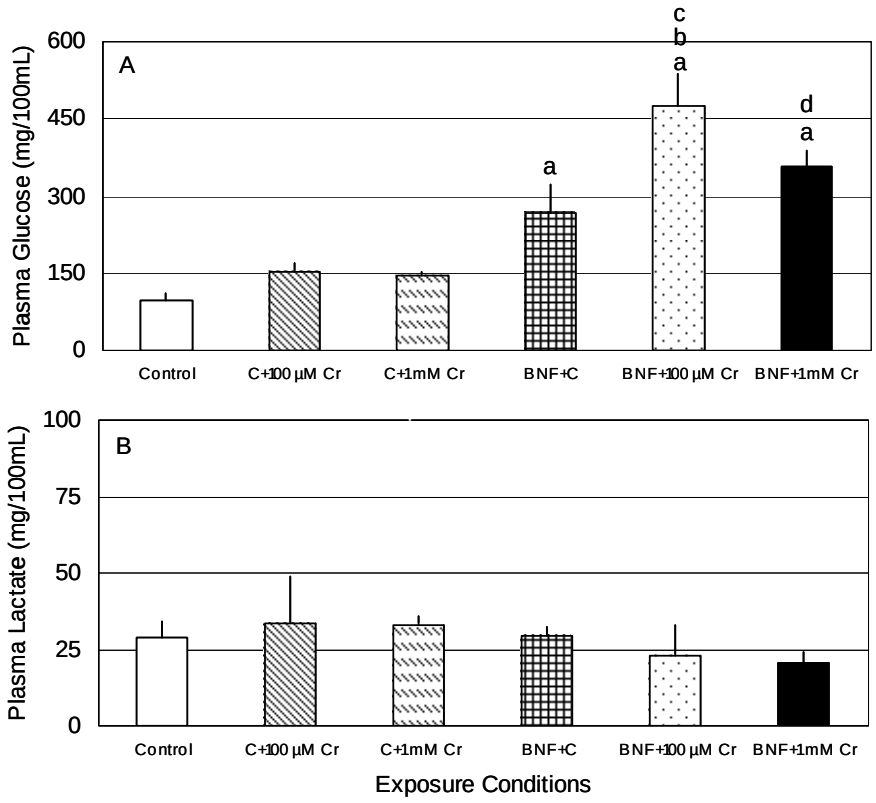

Fig. 3 – Plasma concentrations of (A) glucose and (B) lactate after Cr exposure (100 μM or 1 mM) with or without BNF preexposure. Values represent the means and SEs (*n*=5/treatment). Differences between groups are: **a**. *vs.* control; **b**. *vs.* BNF+C; **c**. *vs.* C+100 μM Cr; **d**. *vs.* C+1 mM Cr.

Genotoxic Response

No intergroup differences were detected in ENA frequency (Fig. 4).

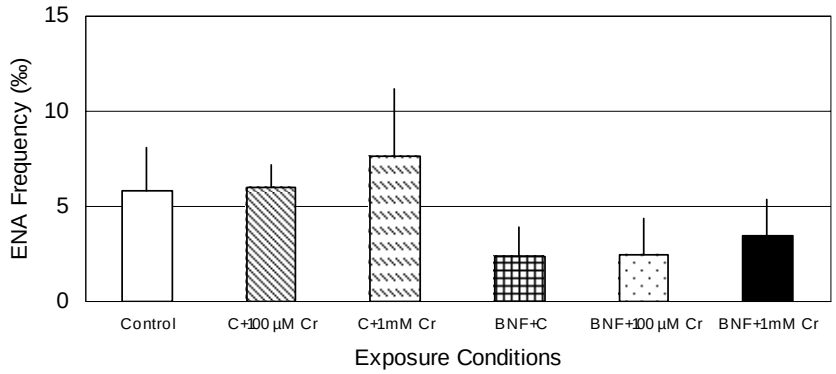

Fig. 4 – ENA frequency after Cr exposure (100 μM or 1 mM) with or without BNF preexposure. Values represent the means and SEs (*n*=5/treatment).

Copper Effects

Hormonal Responses

A. anguilla exposed to the highest Cu concentration (C+2.5 μM Cu) exhibited a significant increase in plasma cortisol compared to the control. No differences were observed in any of the other experimental conditions (Fig. 5). Exposure to C+1 μM Cu induced a significant decrease in TSH plasma levels compared to exposure to C+2.5 μM Cu. Plasma T3 did not show any significant alterations. However, T4 displayed a significant decrease compared to the control under all exposure conditions. The groups exposed to both Cu concentrations after BNF pretreatment revealed decreased T4 compared to BNF+C. Moreover, BNF+2.5 μM Cu caused a T4 reduction in comparison to C+2.5 μM Cu.

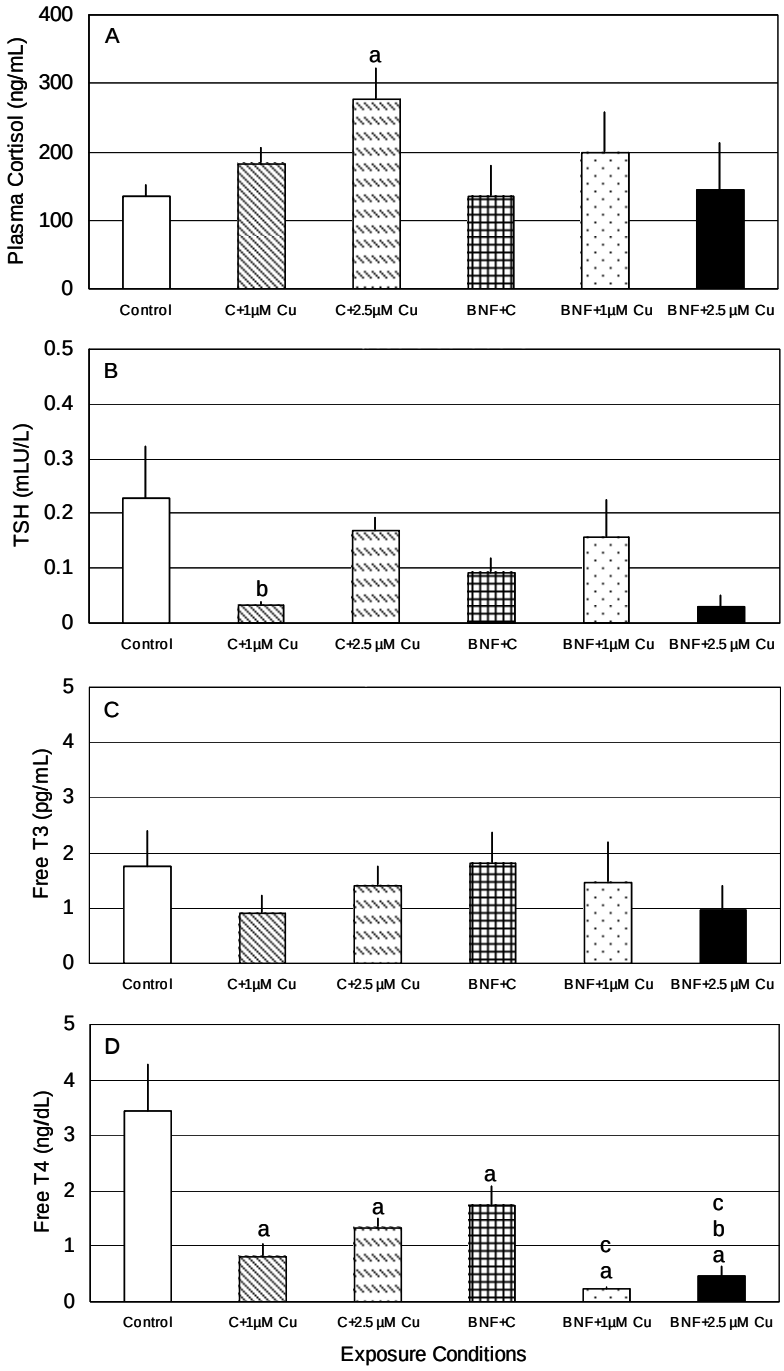

Fig. 5 – Plasma concentrations of (A) cortisol, (B) TSH, (C) free T3, and (D) T4 after Cu exposure (1 or 2.5 µM) with or without BNF preexposure. Values represent the means and SEs (*n* =5/treatment). Differences between groups are: **a**. *vs.* control; **b**. *vs.* C+2.5 µM Cu; **c**. *vs.* BNF+C.

Intermediary Metabolism Responses

Plasma glucose levels showed a significant increase in all treated groups compared to the control, except for C+1 μM Cu. The same glucose increase was detected in BNF+1 μM Cu when compared to C+1 μM Cu exposure (Fig. 6).

C+1 μM Cu induced a significant increase in plasma lactate compared either to the control or the BNF+1 μM Cu.

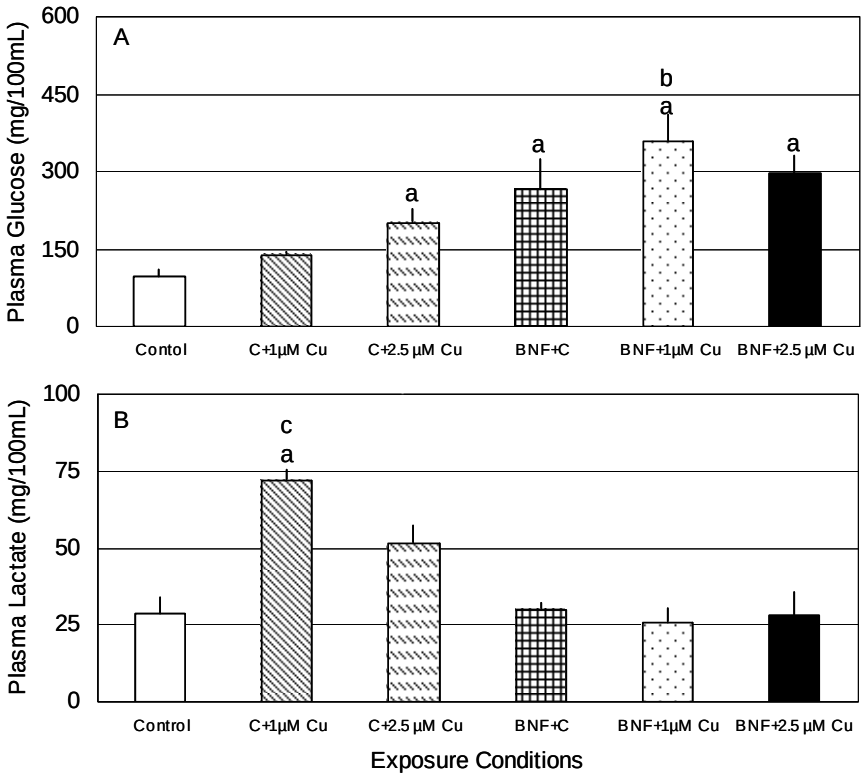

Fig. 6 – Plasma concentrations of (A) glucose and (B) lactate after Cu exposure (1 or 2.5 μM) with or without BNF preexposure. Values represent the means and SEs (*n*=5/treatment). Differences between groups are: **a**. *vs.* control; **b**. *vs.* C+1 μM Cu; **c**. *vs.* BNF+1 μM Cu.

Genotoxic Response

Exposure to BNF+2.5 μM Cu induced an increase in the frequency of ENA compared to either BNF+C or BNF+1 μM Cu (Fig. 7).

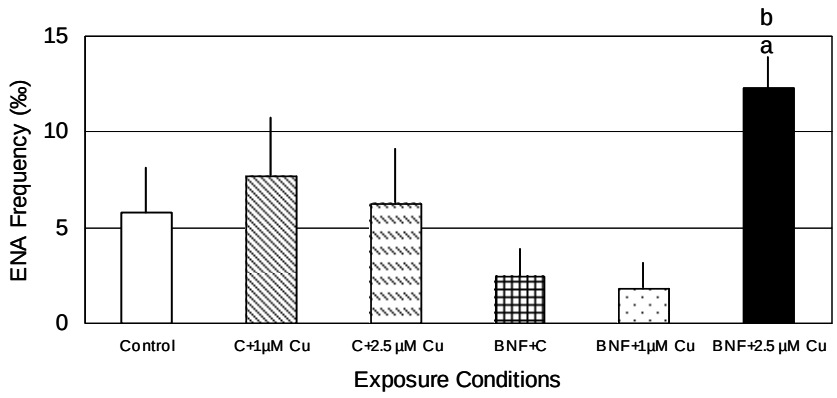

Fig. 7 – ENA frequency after Cu exposure (1 or 2.5 μM) with or without BNF preexposure. Values represent the means and SEs (*n*=5/treatment). Differences between groups are: **a**. *vs.* BNF+C; **b**. *vs.* BNF+1 μM Cu.

DISCUSSION

The choice of metal concentration and exposure time were made based on findings of previous works with *A. anguilla*. Thus, the Cr and Cu concentrations adopted in this study previously had been shown to inhibit liver microsomal EROD activity in eels (Oliveira *et al.*, 2004), and a liver organ culture study demonstrated a decrease in EROD activity after 24 h of exposure to Cr (Oliveira *et al.*, 2003). To ensure a substantial phase I induction following a BNF treatment, the adopted concentration and exposure length took into consideration the results obtained by Teles *et al.* (2003).

Hormonal Responses

Alterations in the HPI axis have been considered informative stress indicators and are widely used in environmental monitoring. In this context, alterations in plasma

cortisol levels have been detected following exposure to different types of stressors, namely, handling and xenobiotic exposures (Hontela *et al.*, 1995; Pacheco and Santos, 2001).

Concerning Cr, there has been a dearth of studies on fish interrenal physiology. In the present study, short-term Cr exposure did not induce any significant alteration in eel plasma cortisol with or without BNF preexposure. On the other hand, plasma cortisol was significantly increased by the highest Cu concentration (C+2.5 μM Cu). This finding on Cu is in agreement with those of De Boeck *et al.* (2003), who observed peak plasma cortisol in *Cyprinus carpio* after a 24-h exposure to 1.9 μM Cu. Analogous results were found in *Oncorhynchus mykiss* (Dethloff *et al.*, 1999) after longer exposures to Cu. Short-term exposure to cadmium (Brodeur *et al.*, 1997) resulted in increased plasma cortisol, which was related to an osmotic disturbance. The cortisol hypersecretion found in the present study may have been preceded by HPI axis stimulation upstream of the interrenal response, as observed by Norris *et al.* (1997) in *Salmo trutta* living in cadmium- and zinc-contaminated waters. Nonetheless, plasma cortisol concentrations greatly depend on the duration and concentration of the applied stressor; thus, it is recommended longer exposures and an extended concentration range be investigated in order to clarify effects of Cr at this level.

Previous *in vitro* studies using trout interrenal tissue reported that BNF abolishes interrenal sensitivity to adrenocorticotropic hormone (ACTH) stimulation as a consequence of alterations in ACTH receptor dynamics and/or in the steroidogenic pathway (Wilson *et al.*, 1998). Moreover, Teles *et al.* (2004) observed no cortisol alteration in *Dicentrarchus labrax* after a 24-h exposure to BNF. These findings may explain the current antagonistic action of BNF preexposure to the Cu cortisol induction.

The information on heavy metal effects in fish thyroid function is quite limited because most studies have investigated effects in mammals. Furthermore, to our knowledge, no studies have been conducted on the effects of Cr or Cu at this level. In the present study, eels revealed no changes in plasma TSH after Cr or Cu exposure

with or without BNF preexposure; however, the lowest Cu concentration (without BNF preexposure) induced a significant TSH reduction when compared with the highest Cu concentration. This result may suggest a fish adaptive capability at very high metal concentrations through alteration of its uptake and elimination, which can be expressed as an inverse relationship between metal exposure concentration and tissue burden, as suggested by Shulkin *et al.* (2003).

Regarding plasma T3, a significant reduction was observed for BNF+100 μM Cr when compared to C+100 μM Cr, enhancing the determinant role of BNF pretreatment. According to Sapin and Schlienger (2003), plasma T3 is a less reliable reflection of thyroid hormone production than is T4 because most circulating T3 (around 80%) is produced extrathyroidally from T4 deiodination. Hence, BNF pretreatment seems to affect circulating T3 levels through alteration of extrathyroidal processes occurring mainly in the liver and kidney.

Plasma T4 was revealed to be more responsive than T3 because it decreased after all exposures to Cr or Cu, which is in agreement with the finding of the majority of previous studies on heavy-metal effects. For instance, this alteration was found in *Channa punctatus* (Bhattacharya *et al.*, 1989) and in *O. mykiss* (Ricard *et al.*, 1998) exposed to cadmium, as well as in *C. punctatus* exposed to mercury (Bhattacharya *et al.*, 1989). In the current study, the T4 decrease induced by Cu was potentiated by BNF preexposure, suggesting a synergistic interaction. Levesque *et al.* (2003) observed decreases in T3 and T4 in *Perca flavescens* in a heavy-metal-contaminated lake. Additionally, *P. flavescens* from a PAH- and heavy-metal-polluted site revealed decreased plasma T4 levels (Hontela *et al.*, 1995), corroborating our results about BNF pretreatment followed by exposure to heavy metals.

Given the lack of TSH alterations found in this study, the general T4 decrease observed in the absence of a correspondent T3 reduction can be explained by the occurrence of some regulatory input, such as increased 5’monodeiodinase activity and/or decreased T3 clearance.

It is also known that thyroid hormones interact with cortisol. T4 plasma levels often follow a pattern similar to that of plasma cortisol, and T4 may activate the

interrenal function (Hontela *et al.*, 1995). On the other hand, cortisol can promote conversion of T4 to T3 and increase the clearance of T3 and T4. Correlating cortisol and thyroid hormones was difficult to accomplish from the current data because plasma cortisol displayed significant changes only for one exposure condition. However, the cortisol increase observed for C+2.5 μM Cu concomitantly with a plasma T4 decrease agrees with the findings of Redding *et al.* (1986) on the same fish species.

Intermediary Metabolism Responses

The increase in plasma glucose is an energy-mobilizing adaptive event to stressors. In the current study it was found that in the absence of BNF preexposure, only the highest Cu concentration induced hyperglycemia. This response agrees with that in a previous study carried out with *O. mykiss* exposed to Cu (Dethloff *et al.*, 1999). However, the plasma glucose increase previously detected in *C. punctatus* (Sastry and Tyagi, 1982) and *Colisa fasciatus* (Nath and Kumar, 1988) exposed to Cr was not corroborated by the present data. Moreover, the current results revealed an interaction between BNF and heavy metals, but that interference seems to depend on the metal and its concentration. Thus, BNF pretreatment seems to promote hyperglycemia for the lowest Cr and Cu concentrations, evidently being a synergistic effect for BNF+100 μM Cr. However, for the highest Cr and Cu concentrations, the BNF interference seemed to diminish.

The direct involvement of high plasma cortisol on the observed hyperglycemic responses seemed to be evident only for the highest Cu concentration. The plasma glucose increase could be related to other hormones such as T4 and glucagon rather than catecholamines because of the duration of exposure chosen.

An increase in plasma lactate is one of the earliest responses associated with “urgent” fuel consumption in tissues, that is, by anaerobic metabolism in white muscle (Trenzado *et al.*, 2003). In the present study, only the lowest Cu concentration induced a plasma lactate increase, which was prevented by the BNF preexposure, revealing an antagonistic interaction. The results obtained in the present work

concerning heavy-metal exposures without BNF pretreatment do not corroborate previous findings of a lactate increase in *C. punctatus* (Sastry and Tyagi, 1982) and *C. fasciatus* (Nath and Kumar, 1988) exposed to Cr, although no alterations were detected in Cu-exposed *O. mykiss* (Dethloff *et al.*, 1999). The divergence in plasma glucose and lactate findings between our results and previous studies is probably related to differences in the chosen species and protocols.

Genotoxic Responses

According to the literature, heavy-metal genotoxicity in fish depends on the heavy metal considered. Cr(VI) is readily taken up by cells, being reduced intracellularly to Cr(III), a stable form that binds DNA efficiently (De Flora, 2000), and is recognized as genotoxic by the IARC (1990). Thus, Cr(VI) exposure induced the development of micronuclei in the fish species *C. auratus* (Al-Sabti *et al.*, 1994) and *P. promelas* (De Lemos *et al.*, 2001). However, the current results do not corroborate the previous findings because none of the Cr concentrations used induced a significant increase in ENA frequency, probably because of the short duration of the exposure compared to the 7-day exposure carried out by Al-Sabti *et al.* (1994) and De Lemos *et al.* (2001). Sanchez-Galan *et al.* (2001) observed that *A. anguilla* injected with Cu did not display significant micronuclei induction, which agrees with the present data.

The absence of BNF genotoxicity effects observed after the eels were exposed for 24 h is in agreement with a previous study performed with the same species (Teles *et al.*, 2003). Nevertheless, an increase in ENA frequency was observed for BNF+2.5 μM Cu compared to BNF+C, suggesting a positive interaction between BNF and the highest Cu concentration. Edwards *et al.* (2001) observed micronuclei induction in *A. forsteri* and *S. schomburgkii* inhabiting heavy-metal-contaminated waters, suggesting that Cu was responsible for that effect. From the current results, it can be suggested that the genotoxic effects found in Cu-contaminated environmental waters can be attributed to a combined effect of Cu and other contaminants (e.g., PAHs), rather than merely to Cu.

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CAPÍTULO X

Respostas de enguia Europeia (*Anguilla anguilla* L.) a dois ambientes poluídos: experiências *in situ*

Responses of European eel (*Anguilla anguilla* L.) in two polluted environments: *in situ* experiments

M. Teles, M.A. Santos e M. Pacheco (2004)
Ecotoxicology and Environmental Safety **58**, 373-378.

ABSTRACT

European eels (*Anguilla anguilla* L.) were caged for 8 and 48 h in two polluted areas to assess the contamination cleanup process in the Vouga River 2 years after the official closing of a bleached kraft pulp mill effluent (BKPME; experiment 1), and to monitor the effects induced by contaminated offward fishing harbor waters (experiment 2). Plasma cortisol, glucose, and lactate levels were evaluated as stress responses. In experiment 1, plasma cortisol, glucose, and lactate increased after 8 h of exposure in site 3, which is located farthest from the deactivated sewage outlet. However, *A. anguilla* seemed to adapt after 48 h of exposure in site 3, because all three parameters returned to control levels. Plasma glucose also significantly increased after 8 h of exposure at sites 1, 2, and 3, returning to control levels after 48 h. Plasma lactate levels increased after 8 h of exposure at site 3 and after 48 h of exposure at site 1. In experiment 2, *A. anguilla* exposed to contaminated harbor waters increased their plasma lactate after 8 and 48 h of exposure, whereas their cortisol and glucose plasma were elevated only after 48 h of exposure. The results demonstrate that even 2 years after the official closing of the BKPME sewage outlet, the river Vouga water remains contaminated by the sediment associated chemicals. Because the fishing harbor induced in *A. anguilla* the same type of stress responses, it is also an area of concern. The adopted stress parameters allied to a caging strategy are recommended for future environmental monitoring assessments.

Keywords: Eels; BKPME; Harbor waters; Cortisol; Glucose; Lactate.

INTRODUCTION

A wide range of synthetic chemicals used for several industrial and household activities have been shown to cause endocrine disturbances in living organisms. The consequences of such perturbation can be important because of the crucial role played by the endocrine system in the coordination of physiological processes and homeostasis maintenance (Hontela, 1998). The role of cortisol as an intermediary in

metabolism is vital, since it is the major glucocorticosteroid secreted by the teleosts' interrenal tissue in response to adrenocorticotropic hormone stimulation (Pottinger *et al.*, 2000). Cortisol mobilizes fuels such as amino acids, converting them into glucose and lipids, thus exerting direct and indirect effects on intermediary metabolism. Cortisol plasma levels are the most commonly used stress indicator in fish because of the rapid elevation that occurs in response to various stressors such as handling, confinement, poor water quality, and a wide variety of toxicants (Wendelaar-Bonga, 1997). An elevation in plasma cortisol levels has been observed in fish after short-term exposure to pesticides (Bennet and Wolke, 1987), heavy metals (Bleau *et al.*, 1996), polycyclic aromatic hydrocarbons (PAHs) and crude oil (Thomas *et al.*, 1993), as well as pulp mill effluent (Kennedy *et al.*, 1995) and resin acids (RAs) (Teles *et al.*, 2003a). Nevertheless, short-term exposure of *Anguilla anguilla* L. to bleached kraft pulp mill effluent (BKPME) (Santos and Pacheco, 1996), diesel water soluble fraction (Pacheco and Santos, 2001), naphthalene (Teles *et al.*, 2003b), retene and dehydroabietic acid (DHAA) (Teles *et al.*, 2003a) led to a decrease in plasma cortisol levels.

Changes in carbohydrate metabolism measured as plasma glucose and lactate can also be used as general stress indicators in fish, and their relation to cortisol function has been investigated (Jardine *et al.*, 1996; Santos and Pacheco, 1996; Pacheco and Santos, 2001; Teles *et al.*, 2003a). Generally, stress responses include increases in plasma glucose and lactate (Hontela *et al.*, 1996; Santos and Pacheco, 1996). However, divergent results have been obtained (Teles *et al.*, 2003a), indicating the complexity of establishing a model response. Therefore, fish stress responses concerning the interdependence between interrenal function and carbohydrate metabolism due to various exposure conditions have become a subject of interest to study.

The Aveiro Lagoon, situated on the Portuguese coast at the mouth of the River Vouga, is an area polluted mainly by industrial and harbor activities as well as urban and agricultural wastes. The offward fishing harbor is contaminated, as usually occurs in these areas, by heavy metals (Abreu *et al.*, 2000), organometallics such as

tributyltin (TBT) (Barroso *et al.*, 2000), and PAHs (Pacheco and Santos, 2001). The pulp mill industry, on the Vouga River left bank, was responsible during the past five decades for continuous hazardous discharges. The toxicological risk to fish caused by these discharges has been assessed by several studies in *A. anguilla* (Santos *et al.*, 1993; Santos and Pacheco, 1995; Pacheco *et al.*, 1993; Pacheco and Santos, 1999) and *Gambusia holbrooki* (Pacheco *et al.*, 2002), with general stress, genotoxic effects, and biotransformation activation being observed.

In May 2000, the paper and pulp mill effluent was diverted to the Atlantic Ocean through a submarine pipe outlet. However, considering that some of the BKPME components are recognized as highly persistent in the environment, the risk for aquatic organisms may remain even after the discharge suspension. For instance, the half-life of RAs, one of the major toxicant groups in pulp mill effluents, was estimated to be ~30 years in the sediment (Stuthridge and Tavendale, 1996). The Cacia BKPME uses one-third *Pinus* spp. and two-thirds *Eucaliptus* spp. and according to Hall (1980) and Subtil *et al.* (1984) it enriches the effluent waters and sediments in RAs, namely DHAA, which has been identified as a mutagenic agent. Other compounds, including retene (a chemical derived from RAs) and the chlorophenolics formed from the residual lignin in bleaching of chemical pulp using chlorine, are also fairly persistent in receiving waters (Leeuwen *et al.*, 1996).

For the present research work we adopted an *in situ* experimental design, using responses such as cortisol, glucose, and lactate plasma levels in caged *A. anguilla* to pulp mill sediment-contaminated water and offward fishing harbor water.

MATERIAL AND METHODS

Test Animals

European eels (*A. anguilla*) with an average weight of 50 g were captured at the Aveiro Lagoon, Murtosa, Portugal. The eels were acclimatized to laboratory conditions for 1 week prior to the experiment. During the recovery period, eels were kept in 80-L aquaria at 20°C under a natural photoperiod, in recirculating, aerated,

filtered, and dechlorinated tap water or artificial seawater, for experiments 1 and 2, respectively.

Experimental Design

The eels were transported from the laboratory to the exposure sites in recipients without water (30 min), placed into 80-dm^3 net cages, and plunged into the water near to the sediment for 8 and 48 h. The control groups were maintained in the laboratory, at 15±1°C and 7.82±0.6 mg/L dissolved oxygen in experiment 1, and at 20±1°C and 6.15±0.6 mg/L dissolved oxygen in experiment 2, in similar net cages and subjected to the equivalent previous anoxia period (30 min).

At each sampling point the blood was collected from a vein using a heparinized Pasteur pipette. In the laboratory, the blood was centrifuged for plasma isolation using an Eppendorf centrifuge (14,000 rpm).

Experiment 1: BKPME-Contaminated River

The eels were exposed in the Vouga River (northwestern Portugal) at three different downstream sites, located 50 (site 1), 100 (site 2), and 2000 m (site 3) (Fig. 1) from the deactivated BKPME sewage outlet. The experiment was carried out in June (15±1°C water temperature; 8.03±0.6 mg/L dissolved oxygen).

Experiment 2: Experiment: Estuarine Harbor Area

The eels were caged in an offward fishing harbor in the Aveiro Lagoon (northwestern Portugal) (site 1) (Fig. 2) in September (20±1°C water temperature; 6.27±0.6 mg/L dissolved oxygen).

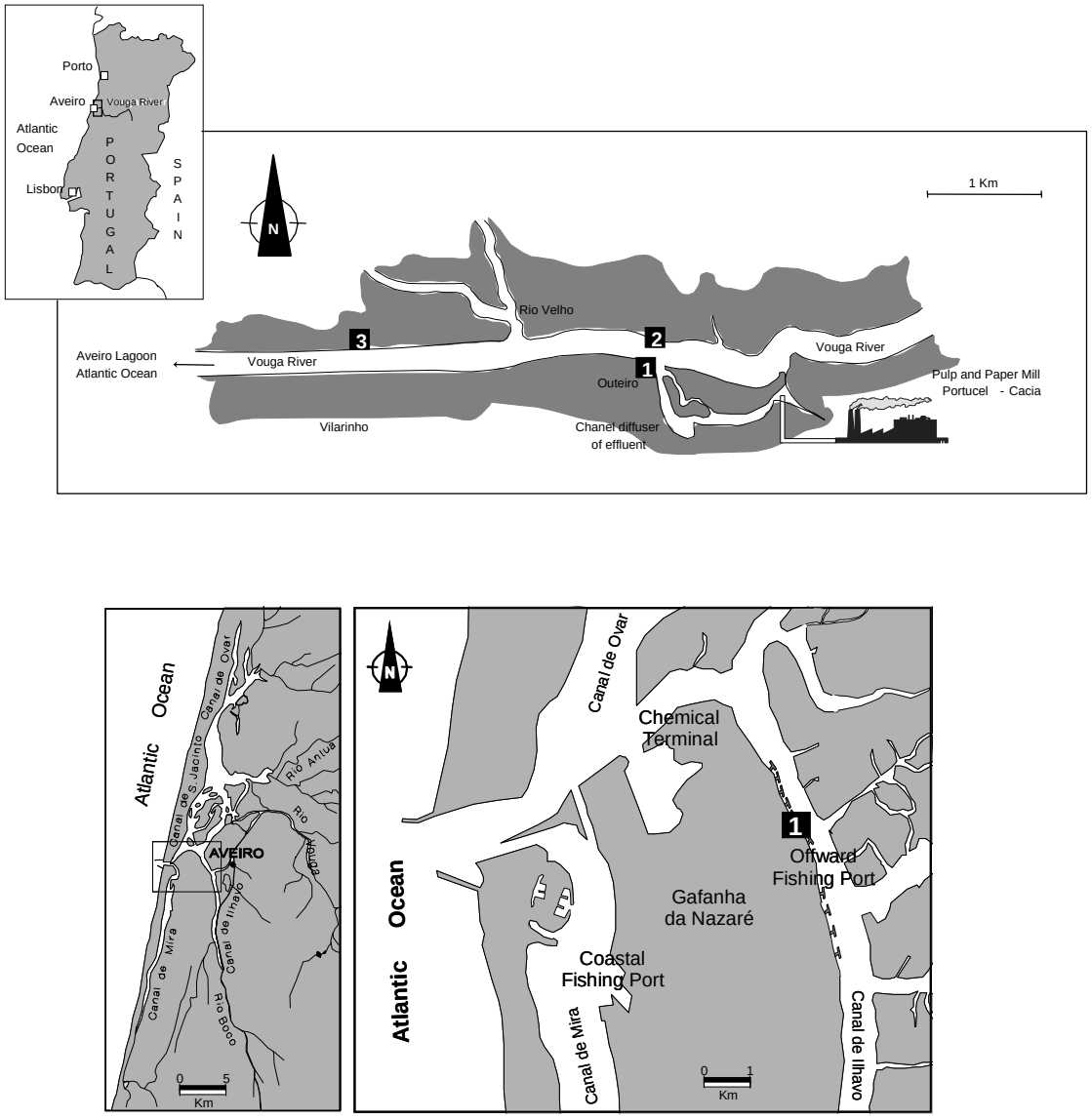

Fig. 1 – Representation of the caging locations on the Vouga River, Cacia, Aveiro, Portugal. Three exposure sites, at the following distances from the effluent source: site 1, 50 m (Outeiro), site 2, 100 m, site 3, 2000 m (Vilarinho).

Fig. 2 – Representation of the caging locations on the Aveiro lagoon, Aveiro, Portugal: site 1, offward fishing harbor.

Biochemical Analysis

Cortisol, Glucose, and Lactate Measurements

Plasma cortisol was determined using a diagnostic enzyme-linked immunosorbent assay (ELISA) direct immunoenzymatic kit (Diametra, Italy, code 10011). Plasma glucose was measured using a diagnostic kit (Granutest, E. Merck-Darmstad No. 1.12194). Plasma lactate was determined with a diagnostic kit (Boehringer Mannheim GmgH, No. 149993) according to the method modified by Noll (1974).

Statistical Analysis

Statistic software (STATISTICA, StatSoft, Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. Variance analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P<0.05$.

RESULTS

Experiment 1: BKPME-Contaminated River

A significant plasma cortisol increase was detected at site 3 after 8 h of exposure (Fig. 3A). The plasma glucose concentration significantly increased at sites 1, 2, and 3 after 8 h of exposure (Fig. 3B). Despite the absence of any statistical significance, a tendency for plasma glucose to increase was observed after 48 h of exposure at site 1. Plasma lactate was elevated after 8 h in site 3 and after 48 h in site 1 (Fig. 3C).

Experiment 2: Estuarine Harbor Area

Plasma cortisol, glucose, and lactate showed significant increases after 48 h exposure in the offward fishing harbor (Fig. 4). Additionally, plasma lactate level also increased after 8 h of exposure.

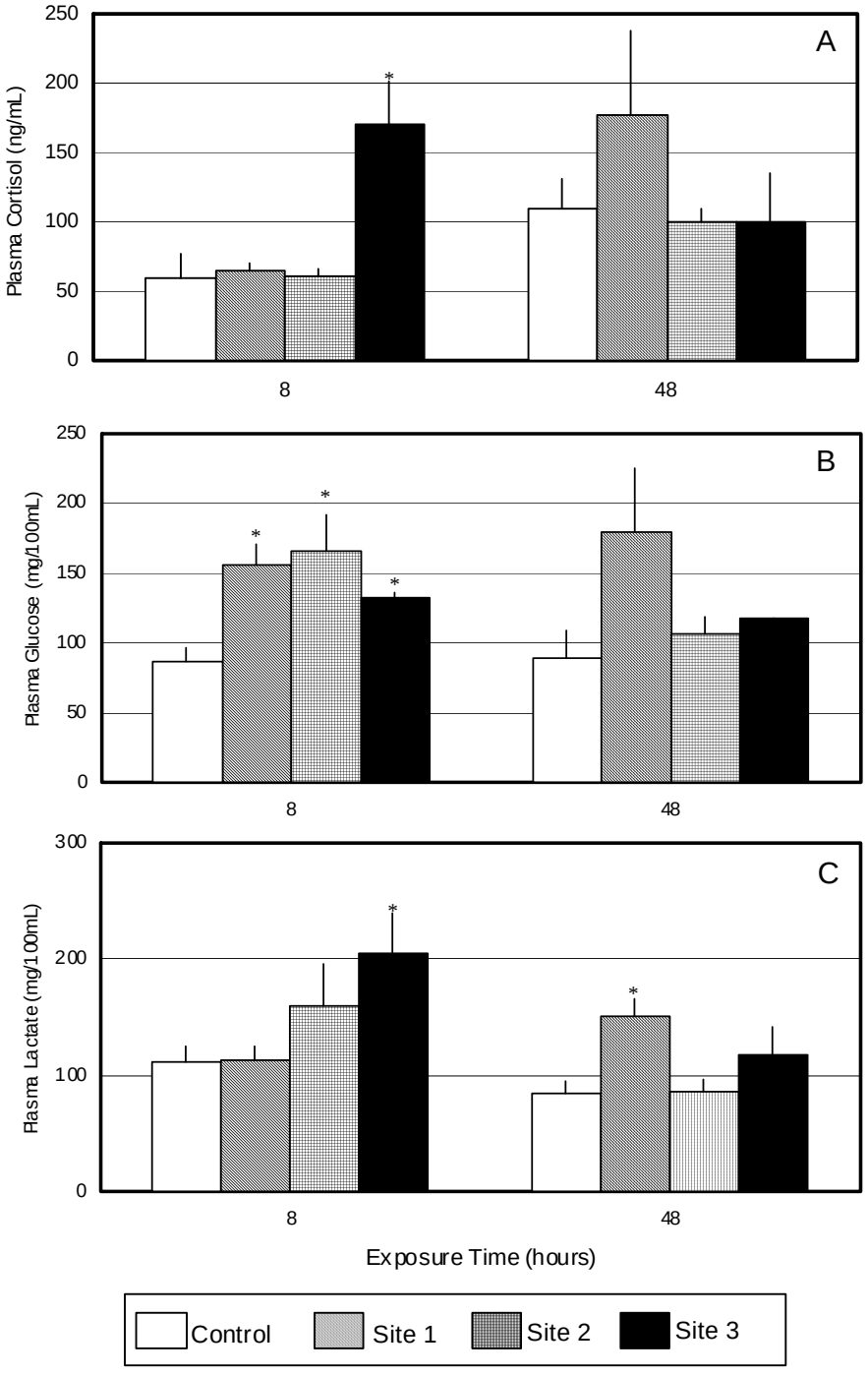

Fig. 3 - Experiment 1. (A) Plasma cortisol levels, (B) plasma glucose levels, and (C) plasma lactate levels in European eels (*A. anguilla*) after 8 and 48 h of exposure to a BKPME-contaminated river. Values represent the means and SD (*n*=5 per treatment). Differences from control: **P*<0.05.

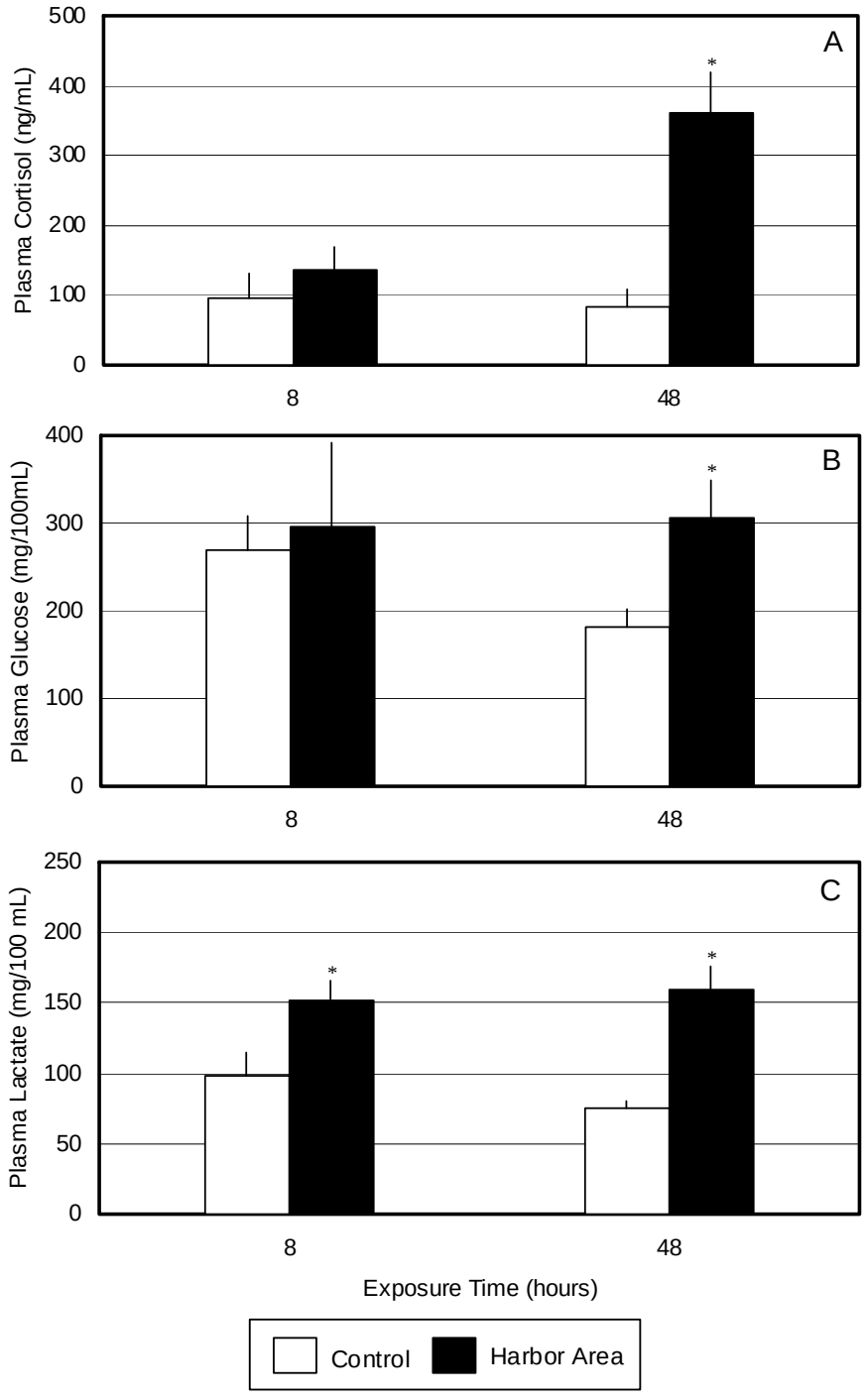

Fig. 4 - Experiment 2. (A) Plasma cortisol levels, (B) plasma glucose levels, and (C) plasma lactate levels in European eels (*A. anguilla*) after 8 and 48 h of exposure to an offward fishing port. Values represent the means and SD (n=5 per treatment). Differences from control: *P<0.05.

DISCUSSION

Experiment 1: BKPME-Contaminated River

The results of experiment 1 support the idea that acute exposures to xenobiotics typically increase the plasma cortisol levels as part of a generalized stress response, which includes effects on the carbohydrate metabolism, namely increases in plasma glucose and lactate. The same responses were obtained in previous fish studies with pulp and paper mill effluent components such as abietic acid (Teles *et al.*, 2003a), chlorinated RAs (Kennedy *et al.*, 1995), and tetrachloroguaicol (Johansen *et al.*, 1994). However, previous studies with *A. anguilla* revealed a divergent response in terms of plasma cortisol. Plasma cortisol was found to decrease after short-term exposures to BKPME (Santos and Pacheco, 1996) or its components such as DHAA and retene (Teles *et al.*, 2003a). Despite the conflicting results in cortisol response, the plasma glucose and lactate alterations observed in the DHAA and retene exposures (Teles *et al.*, 2003a) were similar to those obtained in the present research work.

The results just outlined regarding fish exposures to BKPMEs and their components reinforce the difficulty in establishing a consistent correlation pattern between the stress responses that are primary (i.e., plasma cortisol alterations) and those that are secondary (i.e., changes in plasma glucose and lactate).

The return to the control levels of plasma cortisol, glucose, and lactate observed after 48 h of exposure at site 3 can be interpreted as a fish adaptation to the chemical stressors. Nevertheless, longer exposures should be carried out to confirm this hypothesis.

The occurrence of a significant cortisol increase at site 3 and the concomitant absence of any alteration at the sites closer to the deactivated effluent source may be explained by the types and degree of contamination in each location. Thus, the results at sites 1 and 2 cannot be regarded as a lack of contamination but as a balance between potential inhibitors and inducers of the cortisol release, avoiding any significant response. In the same way, at site 3 this balance was probably disturbed through inducers, thus producing the observed cortisol augmentation.

An analysis of the present results suggests that even 2 years after the official suspension of the pulp mill effluent discharges, the Vouga River maintains a considerable sediment contamination in the vicinity of the Cacia mill, constituting a risk to fish populations.

A considerable proportion of the constituents of BKPME are lipophilic, causing them to adsorb to suspended solids during biological wastewater treatment (Liu *et al.*, 1996) and in receiving ecosystems (Holmbom *et al.*, 1992), and leading to their persistence in the aquatic environment. Therefore, the stress responses observed in the current experiment might be attributable to a wide range of persistent compounds (e.g. chlorophenolics, RAs, and their derivatives) and the eventual interactions between them. Although it is difficult to pinpoint which compound or class of compounds is responsible for the observed effects, the results of a previous study (Teles *et al.*, 2003a) suggest that abietic acid should be considered as one of them.

Experiment 2: Estuarine Harbor Area

In this experiment, the parameters studied responded similarly to those in experiment 1 - that is, increased plasma cortisol, glucose, and lactate levels.

Taking into consideration the type of contaminants expected in the studied harbor area, the present cortisol results are in conformity with prior short-term exposures to sublethal doses of heavy metals (Bleau *et al.*, 1996; Hontela *et al*., 1996), PAHs, and crude oils (Thomas *et al.*, 1987). Furthermore, Pacheco and Santos (2001) observed increased plasma cortisol (exposures from 3 h up to 3 days) and glucose levels (6 days exposure) in eels exposed to diesel water-soluble fraction. Nevertheless, short-term exposure of eels to naphthalene, a PAH previously detected in the study area (Pacheco *et al.*, 2002), revealed a decrease in plasma cortisol levels (Teles *et al.*, 2003b).

The temporal changes in the assayed biomarkers were clearly different in both experiments. Particularly notable in this harbor area experiment was the absence of the previously described fish acclimatization process, suggesting that the recovery mechanisms depend on the type of contaminants rather than on the fish species.

General Statements

The studied parameters, namely plasma cortisol levels, can be affected by a variety of variables, both biotic (sex, predation, confinement, and handling) and abiotic (photoperiod, temperature, water dissolved oxygen) (Hontela, 1997). Accordingly, the conditions of the laboratory controls adopted in the current study were carefully defined to avoid significant differences in the field groups in terms of handling and other stress factors, as well as in terms of abiotic parameters.

Variables such as fish migration, spatial variability of sediment and water contaminant loading, exposure length impreciseness, and lack of knowledge concerning fish history before moving into an area of concern often confound the interpretation of field data. Thus, caging studies offer several advantages in aquatic toxicology, being a promising approach to evaluate environmental contamination (Soimasuo *et al.*, 1995). Additionally, this kind of *in situ* exposure allows the selection of a desired species, its particular developmental stage, and genetic background (Lindström-Seppä and Oikari, 1990).

The observed plasma cortisol elevation in exposed fish (experiments 1 and 2) can be regarded as a characteristic reaction to chemical stressors. Thus, as previously suggested by several authors (Santos and Pacheco, 1996; Hontela, 1997), this primary stress response can be a useful stress biomarker in fish.

Despite the unanimous interpretation of cortisol elevation as a natural response, one cannot disregard the interference with other biological functions causing deleterious effects to fish. Studies of the immunomodulatory potential of cortisol found that it reduces the antibody-producing cells and circulating lymphocytes (Pickering and Stewart, 1984), as well as interacting with the production of lymphokines (Tripp *et al.*, 1987) in teleosts. The incidence of infection by bacteria, fungi, and protozoans has been found to increase in fish with high plasma cortisol levels (Pickering, 1989). Furthermore, the elevation of plasma cortisol can enhance the toxicity of environmental contaminants such as heavy metals and polychlorinated biphenyls (Miller *et al.*, 2002), and decrease the growth hormone levels (Pickering *et al.*, 1991).

Considering the above findings, it can be suggested that fish populations living in the current studied areas can suffer impairment of several biological functions.

Globally, the results presented here reveal the difficulty in interpreting the responses of aquatic organisms to complex mixtures of pollutants, as well as the need to better understand the general fish stress process, namely the interaction between interrenal function and carbohydrate metabolism.

CONCLUSIONS

The current results indicate that both the BKPME-contaminated river (Vouga River) and the offward fishing harbor waters may constitute an ecotoxicological hazard to fish populations, since they induced stress responses, namely increased plasma cortisol, glucose, and lactate levels. Furthermore, it can be suggested that the chemical stressors produced by the former pulp mill activity were not completely removed after a 2-year cleanup process.

Stress responses in *A. anguilla*, such as changes in plasma cortisol, glucose, and lactate, combined with a caging experimental strategy, during specific adequate exposure periods, should be recommended for environmental monitoring.

ACKOWLEDGEMENTS

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CAPÍTULO XI

Respostas de *Anguilla anguilla* L. engaiolada numa lagoa de água doce (Pateira de Fermentelos - Portugal)

Multibiomarker responses of *Anguilla anguilla* L. caged in a freshwater-wetland (Pateira de Fermentelos - Portugal)

M. Teles, M. Pacheco e M.A. Santos (submetido).

ABSTRACT

Pateira de Fermentelos, considered as a lagoon, is a Cértima River enlargement being an important fishing and recreation place. This freshwater ecosystem receives effluents from different origins namely, industrial, in particular electroplating industrial effluents containing heavy metals, domestic wastes, as well as pesticides and fertilizers resulting from agriculture activities. The aim of the present research work was to monitor the effects induced by the contaminants present in Pateira de Fermentelos water, using *Anguilla anguilla* L. (European eel) as a bioindicator. The following biomarkers were measured: liver ethoxyresorufin-*O*-deethylase (EROD) and alanine transaminase (ALT) activities, plasma levels of cortisol, 17β-estradiol (E_2), thyroid-stimulating-hormone (TSH), free thyroxine (T4), free triiodothyronine (T3), as well as glucose and lactate. Moreover, the frequency of erythrocytic nuclear abnormalities (ENA) was scored. The eels were caged for 48 h at a reference site near the Cértima river spring and at 4 sites of Pateira de Fermentelos differing in their distances to the main known pollution source (Cértima River): A (closest to the lagoon entrance), B, C and D (farthest to the Cértima River). The results of this short-term *in situ* experiment revealed plasma E_2 increase at sites B and D when compared to all the other sites, including reference site. Plasma T3 showed a decrease only at site A when compared to reference site. Plasma cortisol and glucose concentrations were increased at all tested sites, revealing a similar pattern of response. The present results revealed indications of Pateira de Fermentelos water contamination, demonstrating the usefulness of this adopted strategy.

Keywords: *Anguilla anguilla*, Pateira de Fermentelos, Biotransformation, Endocrine Function, Genotoxicity.

INTRODUCTION

Lakes, ponds and dams are predisposed to receive and accumulate contaminants discharging from domestic and industrial sewages, as well as agriculture runoff due to their specific water dynamics and configurations differing from other aquatic ecosystems. Pateira de Fermentelos (PF) is a natural freshwater wetland ecosystem located at the central region of Portugal with an area of about 5 Km^2. This water-body is considered an expansion of the Cértima River, its main tributary stream, communicating with Águeda River. Over the last decades, eutrophication produced by domestic sewage and agricultural fields'nutrient runoff, which was aggravated by the incorrect introduction of new organisms, markedly increased the threat to ichthyc populations. Moreover, input of pesticides and industrial effluents, namely from electroplating industries located along Cértima River, contributed to generate a cocktail of contaminants. However, little attention was paid to this ecosystem and a single study is available on its health assessment, which reported the levels of heavy metals, including nickel, zinc, aluminum and manganese (Almeida, 1998). In this context, there is a need for an effective biomonitoring program.

Aquatic toxicology has been using several analytical techniques in order to measure a wide range of chemicals (Rotchell and Ostrander, 2003). However, in the presence of complex environmental mixtures, it becomes impracticable to quantify all the contaminants making difficult a complete scenario characterization. In this context, a strategy involving biomarkers was demonstrated to be a suitable alternative for monitoring and management of aquatic ecosystems (Flammarion *et al.*, 2002).

Biomarkers enable the evaluation of eventual additive, synergistic or antagonistic interactions in the environment, since chemicals may behave differently when acting individually or in mixtures. Furthermore, biomarkers reduce expensive chemical analysis and the information provided may be used as an "early warning system" (Flammarion *et al.*, 2002; Rotchell and Ostrander, 2003; Marin and Mattozzo, 2004).

Biochemical and physiological biomarkers, in particular, were used in order to prevent irreversible damage in whole organisms, communities and ecosystems (López-Barea and Pueyo, 1998). The induction of liver ethoxyresorufin-*O*-deethylase (EROD) activity, a dependent cytochrome P450 1A monooxygenase, is one of the best-studied responses in fish (Bucheli and Fent, 1995). Hypothalamo-pituitary-thyroid (HPT) and hypothalamo-pituitary-interrenal (HPI) axes also play a central role in a wide range of important homeostatic mechanisms in fish. Thyroid hormones regulate growth and hydromineral balance (Van Anholt *et al.*, 2003), while cortisol is involved in the energy metabolism regulation, anti-inflammatory response, as well as immune competence (Hontela, 1997; Wendelaar Bonga, 1997). Thyroid hormones and cortisol both interact and influence carbohydrate metabolism (Hontela *et al.*, 1995). Alterations in plasma concentrations of these hormones, as well as in glucose and lactate levels reflect endocrine alterations, thus affecting fish physiological competence to cope with environmental changes. Hence, the previously mentioned parameters may also be useful tools in monitoring the impact of anthropogenic stressors on fish.

The interaction of genotoxic compounds with DNA induces structural changes in DNA. Unrepaired changes produce cell lesions leading to tumor formation (Malins *et al.*, 1990). Recent studies on genotoxicity of aquatic contaminants demonstrated the suitability of fish erythrocytic nuclear abnormalities (ENA) assay, based on micronuclei and other nuclear anomalies detection in mature erythrocytes. This ENA test was successfully adopted in different fish species (Ayllón and Garcia-Vasquez, 2001; Pacheco and Santos, 2002; Teles *et al.*, 2005a) and is expanding in application due to its simplicity, rapidity, sensitivity and low cost.

Bearing in mind the suitability of *Anguilla anguilla* L. (European eel) as a bioindicator, in the present study, a battery of 10 biomarkers was evaluated on *in situ* exposed eels, caged at increasing distances from the main known PF pollution source (Cértima River). Hepatic responses were evaluated by measuring liver EROD and alanine transaminase (ALT) activities. The endocrine function was assessed by measuring plasma levels of cortisol, thyroid-stimulating hormone (TSH), free

triiodothyronine (T3), free thyroxine (T4) and 17β-estradiol (E_2). The intermediary metabolism was evaluated as plasma glucose and lactate levels. Finally, ENA frequency was determined as a genotoxicity biomarker. It was intended to estimate the relationship between the studied *A. anguilla* biological responses and the different PF exposure sites as an indication of the overall ecosystem condition. Furthermore, the suitability and sensitivity of the adopted biomarkers battery in the early detection of the freshwater contamination was evaluated.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade, obtained from Sigma-Aldrich and E. Merck-Darmstadt.

Test Animals

The experiment was carried out using *A. anguilla* with an average weight of 49.50 ± 0.6 g, collected from a non-polluted site in the Aveiro lagoon – Murtosa, Portugal. The eels were acclimated to laboratory conditions for 7 days prior to experimentation under standard conditions. Briefly, fish were kept at room temperature and natural photoperiod, in aerated (dissolved oxygen: 8.7±0.5 mg/L), filtered, dechlorinated and recirculating tap water, with 7.4±0.2 pH and 0 ‰ salinity. Fish were fed neither under laboratory adaptation nor during the experimental procedure.

In Situ Experimental Design

Eels were transported from the laboratory to the exposure sites in containers without water, placed into 80-L net cages and plunged into the water for 48 h at 4 PF sites (A, B, C and D), differing in their distances to the main known pollution source (Cértima River) (Fig. 1). One more site was chosen as a reference at the Cértima River spring, without having any known industrial and domestic sewage. Fish cages

were kept around 15 cm from the bottom to avoid a direct contact with the sediment. The experiment was carried out in December (10±1°C water temperature).

Each exposure trial was carried out using test groups of 6 eels each. Blood was collected from the posterior cardinal vein with heparin and blood smears were prepared. Following blood sampling, fish were sacrificed by decapitation, liver excised sampled and immediately frozen in liquid nitrogen. In laboratory, blood was centrifuged using an Eppendorf centrifuge (5 min at 14,000 rpm) for plasma isolation and the liver stored at -80 ºC until homogenization.

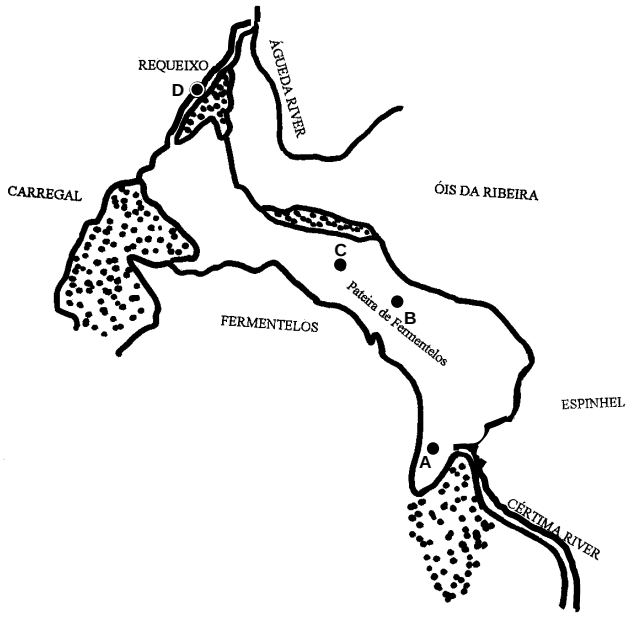

Fig. 1 – Location of exposure sites. Reference (40º20'50'' N; 008º24'08'' W), Site A (40º33'34'' N; 008º30'37'' W), Site B (40º34'24'' N; 008º30'48'' W), Site C (40º34'34'' N; 008º31'06'' W), Site D (40º35'19.55'' N; 008º31'44.19'' W).

Water Physico-Chemical Parameters

Bottom water temperature and dissolved oxygen (DO) were measured in the field using an oxymeter. Water samples were collected and stored in clean, sterile, screw-capped glass containers (1 L) at 4ºC for the following analysis: pH, conductivity, biological oxygen demand (BOD), total solid (TS), total dissolved solid (TDS) and total suspended solids (TSS) using procedures described in APHA (1998).

Ammonium, nitrate, nitrite, phosphate, carbonate hardness (acid binding capacity) and total hardness were performed using Compact laboratory for water testing (Aquamerck, Germany). Depth was also measured at all exposure sites.

Biochemical Analysis

Liver EROD Activity

EROD activity was measured in microsome suspension as described by Burke and Mayer (1974) and adapted by Pacheco and Santos (1998). The reaction was carried out, at 25ºC, in the fluorometer cuvette containing 1 mL 0.5 μM ethoxyresorufin (in 0.1 M Tris–HCl pH 7.4, containing 0.15 M KCl and 20% glycerol) and 25 μL of microsomal suspension. The reaction was initiated by adding 10 μL of NADPH (10 mM) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD-activity was expressed as picomoles per min per mg of microsomal protein.

Liver ALT Activity

ALT activity was measured, in the supernatant resulting from microsomal isolation, according to a colorimetric method based on the measurement of the pyruvate produced by the transamination reaction (Reitman and Frankel, 1957).

Protein Measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornal *et al.*, 1949) using bovine serum albumin as a standard.

Plasma Cortisol, TSH, Free T3, Free T4 and 17β-Estradiol Measurement

The determination of cortisol, TSH, T3 and T4 were performed in plasma, using diagnostic ELISA direct immunoenzymatic kits (Diametra, Italy). The absorbance in each well was measured at 450 nm in a microplate reader (ASYS Hitech).

The cortisol in the sample competes with horseradish peroxidase (HRP)-cortisol for binding onto the limited number of anti-cortisol sites in the microplate wells. The enzyme substrate (H_2O_2) and the TMB-substrate (TMB) are added, and after an appropriate time has elapsed for maximum color development, the enzyme reaction is stopped and the absorbances are determined. Cortisol concentration in the sample is calculated based on a series of standards and the color intensity is inversely proportional to the cortisol concentration in the sample.

The methods for free T3 and free T4 follow the same principles of the cortisol test, requiring immobilized T3 or T4 antibodies, as well as HRP-T3 or HRP-T4 conjugates.

Concerning TSH, an antibody specific to the β-chain of TSH molecule is immobilized on microwell plates and other antibodies to the TSH molecule are conjugated with HRP. TSH from the sample is bound to the plates. The enzymatic reaction is proportional to the amount of TSH in the sample.

The E_2 determination was performed using a diagnostic ELISA direct immunoenzymatic kit (Diametra, Italy). E_2 in the sample competes with horseradish-peroxidase E_2 for binding onto the limited number of anti E_2 sites on the microplates. E_2 concentration in the sample is calculated based on a series of standard; the color intensity is inversely proportional to the E_2 concentration in the sample.

Plasma Glucose and Lactate Measurement

Plasma glucose was measured spectrophotometrically (340 nm) according to the method modified from Banauch *et al.* (1975) based on the quantification of NADH after a glucose oxidation catalyzed by glucose-dehydrogenase. The quantity of NADH formed is proportional to the glucose concentration.

Plasma lactate levels were determined spectrophotometrically (340 nm) according to the method modified from Noll (1974) using lactate-dehydrogenase, ALT and NAD, measuring NADH appearance.

ENA Assay

The blood smears were fixed with methanol for 10 min and stained with Giemsa (5%) for 30 min. In order to evaluate genotoxicity, the erythrocytic nuclear abnormalities were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid (1976), Carrasco *et al.* (1990) and Smith (1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei, lobed nuclei, dumbbell shaped or segmented nuclei and kidney shaped nuclei. The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

Statistical analysis

Statistica software (StatSoft, Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P<0.05$.

RESULTS

Water Physico-Chemical Parameters

Water temperature showed no important variations neither in reference site nor PF sites (Table I). The pH was alkaline at all sites, with an incremental pH trend from site A (minimum) to site C (maximum), followed by a decrease at site D. Conductivity measurement showed no major differences among all the study sites. Concerning DO levels, the observed variation ranged from 6.62 (site D) to 10.34. BOD also showed a variation along all the sites being highest at site D. Among the ammonium, nitrates, nitrites, phosphate, carbonates hardness and total hardness parameters, only nitrites showed marked presence at sites B and D. In terms of TS and TSS, major differences were found since a higher level was detected at reference site. Recorded

depth was minimum at reference site, whereas PF sites showed an increase from site A to C, followed by a decrease at site D.

Table I - Physico-chemical analysis of water at reference site and Pateira de Fermentelos sites (A, B, C, D).

Physico-chemical parameters	Exposure Sites				
	Reference	A	B	C	D
Temperatura (ºC)	10.9	9.3	9.3	9.1	9.1
pH	7.937	7.999	8.175	9.640	8.203
Conductivity (μs/cm)	289	492	435	151	449
DO (mg/l)	10.34	8.86	6.62	8.45	8.84
BOD (mg/l)	2.33	1.38	0.56	1.03	3.73
Ammonium (mg/l)	0.5	0.5	0.8	0.3	0.3
Nitrate (mg/l)	0	0	0	0	0
Nitrite (mg/l)	0.075	<0.025	>0.5	<0.025	0.5
Phosphate (mg/l)	0	0.25	0.125	0.25	0.125
Carbonate hardness (Acid-binding capacity) (mmol/l)	0.9	2.5	2.3	1.0	2.1
Total hardness mmol/l	0.95	2.85	2.4	1.4	2.4
Total Solids (mg/l)	1388.8	294	440	136	188
Total Dissolved Solid (mg/l)	170	256	122	124	14
Total Suspended Solid (mg/l)	1218	38	318	12	174
Depth (m)	1.0	1.5	2.4	2.25	1.50

Biomarkers measurements

Liver EROD and ALT activities (Fig. 2) were not altered. Concerning *A. anguilla* hormonal responses, a significant plasma cortisol increase was observed at all PF sites, when compared to reference site (Fig. 3A). Plasma TSH and T4 (Fig. 3B, C) were also unaltered. On the other hand, plasma T3 was significantly decreased at site A when compared to the reference site, though its general tendency to decrease was observed at all the other PF exposure sites (Fig. 3D). E_2 plasma levels showed significant increases only at sites B and D when compared to the reference site, being

also significantly higher than the other PF sites (A and C) (Fig. 3E). Regarding intermediary metabolism responses, a significant plasma glucose increase was observed at all PF sites, in comparison to reference site, despite the fact that no differences were detected between PF exposed groups (Fig. 4A). However, plasma lactate (Fig. 4B) was unaltered. Finally, ENA frequency (Fig. 5) remained constant.

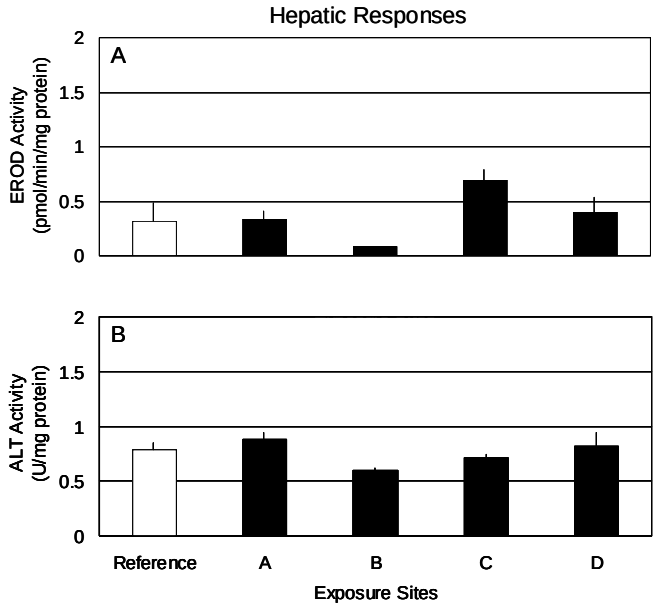

Fig. 2 - *A. anguilla* liver EROD (A) and ALT (B) activities after 48 h exposure at a reference site and contaminated sites (A to D). Values represent the means and SE (*n*=6 per treatment).

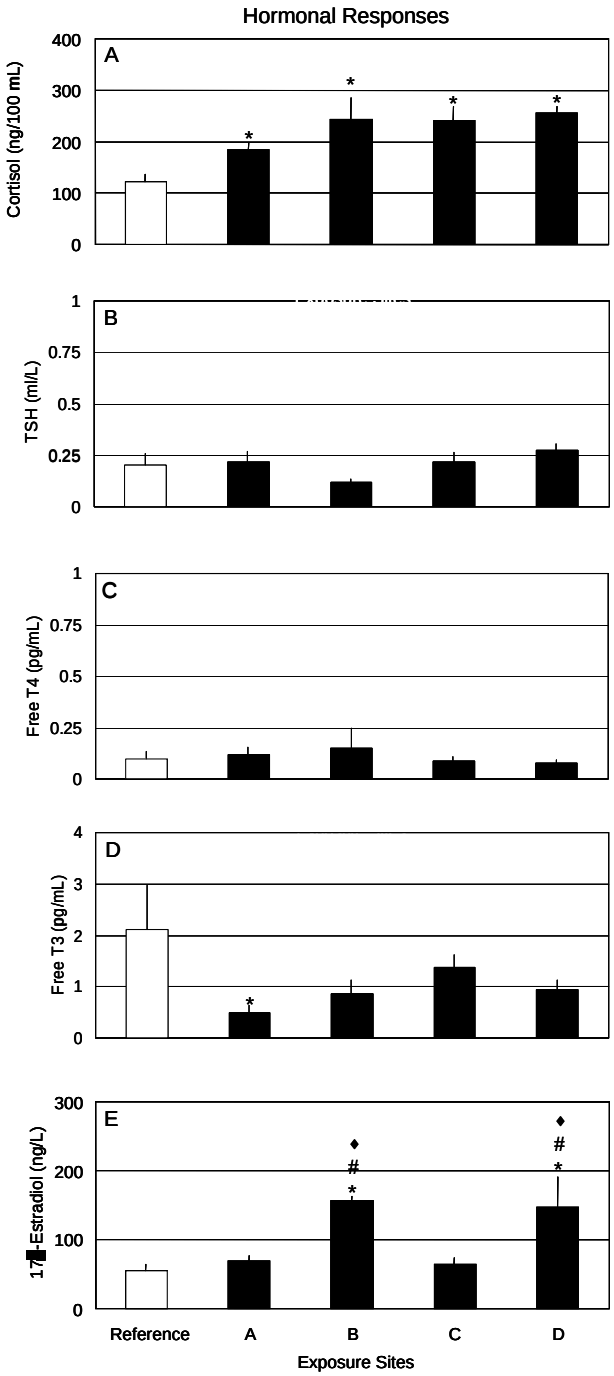

Fig. 3 - *A. anguilla* plasma cortisol (A), TSH (B), free T4 (C), free T3 (D) and 17β-estradiol (E) concentrations after 48 h exposure at a reference site and contaminated sites (A to D). Values represent the means and SE (*n*=6 per treatment). Significant differences are: * *vs.* reference site; # *vs.* site A; ♦ *vs.* site C (*P*<0.05).

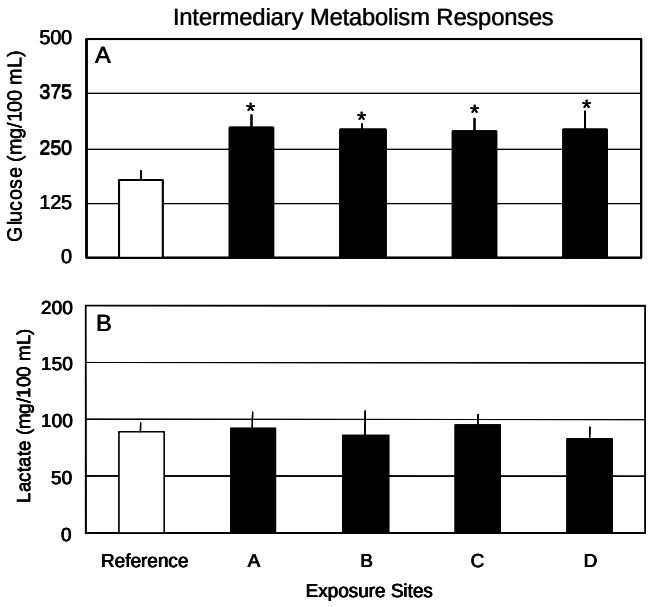

Fig. 4 - *A. anguilla* plasma glucose (A) and lactate (B) concentrations after 48 h exposure at a reference site and contaminated sites (A to D). Values represent the means and SE (*n*=6 per treatment). Differences from reference site: **P*<0.05.

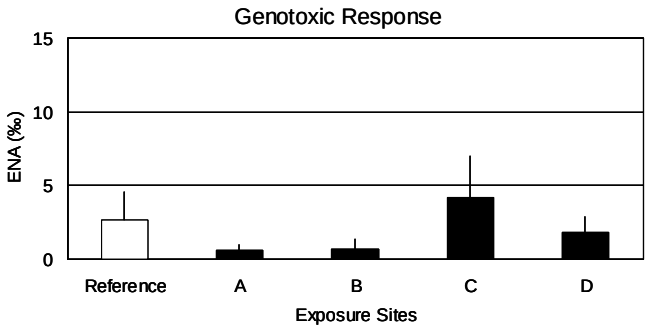

Fig. 5 - *A. anguilla* ENA frequency after 48 h exposure at a reference site and contaminated sites (A to D). Values represent the means and SE (*n*=6 per treatment).

DISCUSSION

Exposure length impreciseness and lack of knowledge concerning fish history before moving into an area of concern often confound the interpretation of field data. Thus, caging studies offer several advantages in aquatic toxicology, being a

promising approach to evaluate environmental contamination (Fenet *et al.*, 1998). Additionally, *in situ* exposures allow the selection of a representative species at a particular developmental stage (Lindström-Seppä and Oikari, 1990).

In the present study, *A. anguilla*, a representative species of PF ichthyofauna, was chosen as a bioindicator due to a considerable knowledge of its physiology and based upon previous research carried out in our laboratory (Santos and Pacheco, 1996; Santos *et al.*, 2004). Moreover, *A. anguilla* resistance and sensitivity to adverse conditions made it particularly applicable to sublethal studies.

The physicochemical analysis has long been employed to assess the water quality. In the current study, water quality parameters are, in general, at acceptable levels considering criteria given in APHA (1998) and in Merck guide lines (Aquamerck, Germany), as well as the *A. anguilla* requirements in particular. However, some exceptions must be considered, as observed for pH, BOD and nitrite levels. Water pH showed a considerably high level at site C (9.64), which may be assumed as an indication of strongly to extremely polluted condition. All the other sites presented pH levels in a range belonging either to unpolluted or slightly polluted state. As a pollution marker, BOD levels revealed site D as the most oxygen demanding, suggesting the existence of a moderate pollution state. Nitrite levels showed a moderately polluted state at sites B and D. In terms of depth, differences were found along PF study sites; however, these differences were inversely related to DO levels, constituting a possible explanation for DO variations. Considering the previous water quality characterization, the studied fish responses are mainly discussed according to the different distances to the main pollution source. Nevertheless, some site-specific responses are also discussed keeping in view the respective water quality variables.

PF exposed eels showed unchanged EROD activity at all exposure sites, suggesting the presence of unsufficient amounts of CYP1A1-modulators in order to alter its biotransformation activity. However, taking into account the contaminant cocktail likely to occur in PF, this result should be carefully interpreted since it cannot be excluded the occurrence of a balance between potential CYP1A1 inhibitors (e.g.

heavy metals and xeno/estrogens) and inducers (e.g. pesticides) preventing any significant response. Accordingly, zinc and nickel, previously determined in PF (Almeida, 1998), demonstrated their liver EROD inhibitory action in fish (Bozcaarmutlu and Arinç, 2004), while pesticides, expected to occur in PF resulting from nearby agriculture runoff, are potential CYP1A inducers (Vindimian *et al.*, 1993).

A. anguilla increased plasma cortisol concentrations at all the PF exposure sites indicating the presence of stressors, though a gradient related to the increased distance from the main pollution source (Cértima River) was not found. Cortisol elevation is known as a natural response to stress, signaling that the animals were physiologically competent and homeostatic regulatory mechanisms are intact. Nevertheless, one cannot disregard the interference with other biological functions that may produce deleterious effects to fish (Brown *et al.*, 1991; Maule *et al.*, 1996). Furthermore, plasma cortisol increase can enhance the toxicity of environmental contaminants such as heavy metals (Miller *et al.*, 2002). A plasma cortisol increase was previously observed in fish exposed short-term to heavy metals (De Boeck *et al.*, 2003) or pesticides (Waring and Moore, 2004), which support current findings due to the type of contaminants expected in the studied area. It can be also suggested that the cortisol hypersecretion measured in the present study may have been preceded by HPI axis stimulation upstream of the interrenal response, as observed by Norris *et al.* (1997) in *Salmo trutta* living in cadmium- and zinc-contaminated waters. Cortisol is also closely related with other metabolic pathways, such as gluconeogenesis resulting in increased plasma glucose release. This correlation is evident in the current research study as after 48 h exposure both plasma cortisol and glucose significantly increased. Accordingly, previous studies revealed an increase in both parameters following exposure of *Oncorhynchus mykiss* to heavy metals (Hontela *et al.*, 1996) or *Cyprinus carpio* to pesticides (Gluth and Hanke, 1985). Furthermore, previous studies focused on intermediary metabolism revealed a plasma glucose increase in *Colisa fasciatus* exposed to heavy metals (Martinez *et al.*, 2004) and *A. anguilla* exposed to pesticides (Sancho *et al.*, 1998).

The elevation of cortisol and glucose levels is frequently accompanied by changes in metabolites such as lactate. However, this pattern of response was not found in the present study since plasma lactate was unaltered. Grutter and Pankhurst (2000) observed similar results in caged *Hemigymnus melapterus*, suggesting that elevated plasma lactate is not a requisite component of the fish stress response. These authors assumed that the stress induced either by chemicals or capture and handling does not always induce anaerobiosis in fish, mainly due to behavior features. Additionally, according to the same authors lactate accumulation may occur but without significant release of lactate ions into the plasma.

Thyroid hormones regulate growth, development and metabolism and are involved in more biological actions than any other hormone (Liu and Chan, 2002). Hence, HPT alterations provide important information about the health status of fish, being reliable candidates as biomarkers of environmental contamination (Hontela, 1997; Teles *et al.*, 2005b). Present data revealed unaltered plasma TSH and T4 levels, concurrently with a general plasma T3 decreasing tendency at all exposure sites, being significant at site A, probably due to its proximity to the main known pollution source. These results agree with previous studies where a plasma T3 decrease was detected after exposure either to heavy metals (Carletta *et al.*, 2002) or pesticides (Thangavel *et al.*, 2005). Since it was previously observed that heavy metals inhibit the conversion of T4 into T3 by 5'monodeiodinase, the T3 plasma decreased level may indicate a lower fish capability of converting T4 into T3. On the other hand, Thangavel *et al.* (2005) justifies the decrease in plasma T3 after pesticide exposure with a reduction on fish metabolic rate, indirectly reducing the toxic impact of the pesticide. Additionally, the indirect action of contaminants through the interference of cortisol may also be considered as an explanation for plasma T3 decrease. It was previously demonstrated that fish treated with cortisol had lower plasma T3 concentrations while T4 was less affected, which was explained by a faster T3 clearance from the blood or a slower conversion of T4 into T3 (Redding *et al.*, 1984). Further research work by the same authors (Redding *et al.*, 1986) and Brown *et al.* (1991) showed that cortisol treatment enhances plasma T3 clearance

without deiodinating activity alteration. Hence, the current results corroborate the impact of the cortisol increase on plasma T3 decrease, probably due to a faster T3 clearance from plasma. Though, it does not allow us to exclude other explanations, namely those related to the contaminants direct action upon the HPT axis. The measurement of TSH in plasma in addition to plasma T4 and T3 provides an overall perspective of the HPT axis status. Despite the decrease in plasma T3 the HPT axis was not significantly affected, since TSH and T4 plasma levels were unaltered.

E_2 measurement in fish plasma can be a useful indicator of estrogens presence in the aquatic environment, considered as endocrine disruptor compounds (EDC) (Imai *et al.*, 2005). Thus, the plasma E_2 increase observed in *A. anguilla* at sites B and D may reflect the presence of E_2 in PF water rather than any EDC capability of increasing endogenous E_2 eel's plasma levels. To our knowledge, there is no evidence that immature eels increase plasma E_2 in a period of 48 h. The alterations on E_2 levels reported for sites B and D are concomitant with the measurement of high levels of nitrites at the same locations. The occurrence of such nitrite levels is indicative of a partial decomposition of organic material resulting from anthropogenic discharges, mainly domestic and livestock wastes corroborating the hypothesis of sewage E_2 input. However, this correlation cannot be completely assumed since it is also known that nitrites occur as a result from an intensive use of nitrogenous fertilizers.

Genotoxicity in the present work, measured as ENA frequency, was unaltered at all exposure sites. However, according to Maria *et al.* (2005) a blood DNA integrity loss, measured as increased DNA strand breaks, was observed in the same species at sites A and C after 48 h exposure, demonstrating the presence of genotoxic chemicals at those particular sites. This apparent disagreement is probably justified by the ability of the DNA strand breaks assay to detect earlier genotoxic events, depending on DNA damage and repair capacity that may precede the ENA appearance. Therefore, in order to evaluate PF genotoxicity using ENA assay, longer exposures are recommended.

The present results revealed indications that PF water was contaminated, as evidenced by an increase in plasma cortisol and glucose levels at all sites. However, study sites differ in terms of contamination, *i.e.* chemical variety and concentrations, since plasma T3 was significantly decreased only at site A and plasma E_2 increased at sites B and D. In conclusion, the previous biomarkers, using *A. anguilla* allied to a caging exposure strategy, are recommended for future environmental monitoring assessments.

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CAPÍTULO XII

Discussão geral

A discussão geral pretende constituir uma análise crítica da globalidade da tese, tendo presentes as orientações definidas inicialmente e dado que cada capítulo da presente tese inclui uma discussão específica do seu conteúdo.

1. EXPOSIÇÕES LABORATORIAIS A CONTAMINANTES ISOLADOS

Os trabalhos em análise neste ponto incluem os capítulos II a VI e correspondem a exposições laboratoriais a compostos individuais. Neste contexto, foram estudados efeitos da exposição a: (i) ácidos resínicos (AR) – ácidos abiético e desidroabiético, e um seu derivado – reteno; (ii) a um hidrocarboneto aromático policíclico (HAP) – naftaleno e a um composto do tipo HAP – β-naftoflavona (BNF); (iii) bem como a uma hormona "natural" – 17β-estradiol (E_2).

A relevância dos AR resulta do facto de serem componentes representativos da contaminação associada a efluentes da produção de pasta de papel (EPP). O naftaleno é o representante mais simples da classe dos HAP, cujos efeitos em peixes estão ainda pouco estudados. A BNF é um composto flavonóide sintético que embora não possuindo uma estrutura química pura de HAP foi utilizado pelo facto de ser um agonista do receptor aril-hidrocarbono (Ah) e apresentar um comportamento indutor de mono-oxigenases P450 tipo HAP (Novi *et al.*, 1998; Gravato e Santos, 2002a). A BNF foi deste modo usada como composto padrão para o estudo da fase I da biotransformação, bem como de outras respostas associadas. A importância do estudo dos efeitos do E_2 em peixes prende-se com o facto de, nos últimos anos, se ter acumulado informação científica sobre a existência de concentrações anormalmente elevadas desta hormona no ambiente aquático (Ying, 2005).

O trabalho relativo à exposição a E_2 contemplou duas formas diferentes de administração – através da diluição na água e por via intraperitoneal (i.p.) – tendo em consideração que os peixes absorvem os xenobióticos, fundamentalmente, a partir da da água (via guelras e pele) e da sua dieta (via intestino).

Os intervalos de concentrações dos contaminantes xenobióticos adoptados nas exposições laboratoriais podem ser considerados, de uma forma geral,

ecotoxicologicamente relevantes, atendendo às concentrações que podem ser encontradas em ecossistemas poluídos.

Nos capítulos II a VI foi avaliada a variação das concentrações plasmáticas de cortisol em *Anguilla anguilla* L. (enguia Europeia) e *Dicentrarchus labrax* L. (robalo) expostos a diferentes contaminantes. A exposição ao ácido abiético foi a única que induziu um aumento de cortisol plasmático (capítulo II), de acordo com a resposta referida como típica, face a exposições de curto prazo a diferentes xenobióticos (Bleau *et al.*, 1996; Pacheco e Santos, 2001a; De Boeck *et al.*, 2003; Waring e Moore, 2004). Contudo, todos os outros compostos testados (ácido desidroabiético, reteno, naftaleno e E_2), excluindo a BNF, induziram um decréscimo dos níveis plasmáticos de cortisol, o que está em aparente desacordo com a resposta frequentemente assumida como típica. Em geral, considerava-se que apenas exposições crónicas a contaminantes podiam causar uma incapacidade do peixe elevar os seus níveis de cortisol plasmático como resposta ao stresse da captura e manuseamento (Hontela *et al.*, 1992, 1995). No entanto, esta ideia foi posteriormente posta em causa pelos trabalhos de Santos e Pacheco (1996) e Pacheco e Santos (2001b) em exposições de curto prazo de *A. anguilla* a EPP ou à fracção solúvel em água do gasóleo. Desta forma, os presentes resultados estão em concordância com os anteriormente referidos, uma vez que, maioritariamente, as exposições mostraram induzir uma desregulação endócrina, expressa através da incapacidade dos peixes elevarem o cortisol no plasma face a situações de stresse. De acordo com os resultados obtidos, deve passar a considerar-se o decréscimo de cortisol plasmático como um padrão alternativo de resposta dos peixes a exposições de curto prazo a contaminantes, o mesmo tendo sido corroborado pelos trabalhos de Benguira *et al.* (2002).

No caso das exposições prolongadas, a incapacidade de elevação do cortisol tem sido explicada pela exaustão do eixo hipotálamo-pituitária-tecido interrenal (HPI) e consequente atrofia da pituitária, possivelmente devido à sua hiperactividade (Hontela *et al.*, 1995). Considerando exposições de curto prazo, Santos e Pacheco (1996) sugerem que determinados contaminantes xenobióticos poderão inibir a libertação de cortisol na corrente sanguínea por parte do tecido interrenal, baseados

na observação de elevados níveis de cortisol nesse tecido concomitantemente com baixos níveis no plasma. Assim, este segundo mecanismo propõe como uma boa hipótese explicativa relativamente aos resultados obtidos no presente trabalho. A globalidade das respostas incluídas nos capítulos II a VI, evidencia um padrão de resposta que depende do composto em causa, não parecendo estar relacionado, de forma tão marcada, com a espécie.

As alterações dos níveis plasmáticos de glucose e lactato foram também determinadas como respostas secundárias de stresse, para todos os compostos anteriormente referidos, excepto para o naftaleno. Os peixes expostos a AR demonstraram um padrão de resposta entendida como típica e que corresponde ao aumento destes dois parâmetros (Santos *et al.*, 1990; Vijayan e Moon, 1992). Contudo, o reteno e a BNF induziram aumentos unicamente na concentração de glucose, enquanto que o E_2 foi o único contaminante que não induziu qualquer alteração nestes parâmetros.

Tendo em consideração o importante papel do cortisol no metabolismo intermediário, torna-se importante o entendimento da sua relação com os níveis plasmáticos de glucose e lactato. Geralmente é referido que a exposição a agentes de stresse induz aumentos destes três parâmetros (Hontela, 1997; Janz, 2000). Este padrão de resposta apenas foi observado após exposição a ácido abiético. Contudo, foram verificadas subidas dos níveis de glucose e de lactato em exposições para as quais não se observou qualquer alteração na concentração plasmática de cortisol, tendo sido por vezes observado descidas dessas concentrações. Desta forma, a análise global da interdependência entre a concentração de cortisol e os parâmetros secundários de stresse revelou ser complexa, não sendo passível de previsão com base num modelo único e pré-estabelecido.

Tendo em consideração que compostos tipicamente indutores das enzimas associadas ao CYP1A mostraram afectar a função da tiróide em mamíferos, decidiu-se estudar o efeito da exposição de *A. anguilla* a BNF sobre o eixo hipotálamo-pituitária-tiróide (HPT) (capítulo IV). Assim, foi observado um decréscimo da concentração de tiroxina (T4) no plasma, mantendo-se contudo inalterados os níveis

da hormona estimuladora da tiróide (TSH) e da triiodotironina (T3). Os resultados parecem dar apoio à hipótese de que o decréscimo de T4 plasmático se deve à sua remoção do plasma, em consequência do aumento da glucoronidação e excreção biliar da T4, devido à indução da UDP-glucoronosil transferase hepática pela BNF (Gravato e Santos, 2002b). A manutenção dos níveis de T3 reflecte uma maior capacidade de estabilização dos seus níveis no plasma comparativamente com a T4. Tendo em consideração o decréscimo de T4, seria de esperar uma resposta compensatória da pituitária no sentido do aumento da TSH. No entanto, essa resposta não se verificou vindo demonstrar a dificuldade ainda existente no entendimento da relação entre estes parâmetros no que respeita às respostas a xenobióticos.

No capítulo VI avaliou-se a capacidade de indução da síntese de vitelogenina (Vtg) em *D. labrax* juvenis após 10 dias de exposição a E_2. Os níveis plasmáticos de E_2 foram também avaliados após exposição, assim como a sua relação com a concentração de Vtg plasmática. A forma como os anteriores parâmetros podem ser afectados pela via de exposição – diluição na água *versus* injecção intraperitoneal (i.p.) foi também avaliada. O aumento das concentrações de E_2 no plasma foi apenas observado na exposição via água, contudo na exposição i.p o seu completo desaparecimento do plasma ocorreu após o período de 10 dias. Esta diferença não pode ser atribuída de forma definitiva à diferença entre as vias de exposição uma vez que na exposição via i.p. foi administrada uma única dose no primeiro dia, enquanto que na exposição via água o E_2 foi adicionado diariamente à água. As duas concentrações mais elevadas de E_2 demonstraram ser capazes de induzir a síntese de Vtg através das duas vias de exposição, indicando que o E_2 foi de facto absorvido pelos peixes tendo atingido o fígado.

A actividade biotransformadora hepática foi também avaliada (capítulos III e V) em exposições laboratoriais a contaminantes isolados. O naftaleno e a BNF foram capazes de induzir um aumento da actividade da 7-etoxiresorufina *O*-desetilase (EROD), comparativamente com a BNF o naftaleno induziu um aumento de EROD mais tardio. Apesar da capacidade indutora da actividade de EROD demonstrada por

estes compostos, eles revelaram também a capacidade de reduzir esta actividade enzimática, especialmente para as concentrações mais elevadas e durante as primeiras horas de exposição. Este efeito está possivelmente relacionado com a ocorrência, numa fase inicial, de elevadas concentrações no tecido hepático, onde essa acção foi detectada. A determinação da alanina transaminase (ALT) no fígado não demonstrou a ocorrência de lesões hepáticas que possam justificar a redução da actividade de EROD pelo que a hipótese de ocorrência de uma acção inibitória sobre a enzima sai reforçada. O desaparecimento da acção inibitória e subsequente expressão de uma acção indutora pode ser explicada por uma adaptação progressiva dos peixes e/ou declínio dos níveis de compostos na água com repercussão nos níveis teciduais. Assim, os resultados apontam para uma dependência da actividade de EROD em função do tempo de exposição e das concentrações de xenobiótico.

A actividade de EROD não foi alterada em *D. labrax* por exposição ao E_2. Contudo, de acordo com estudos anteriores seria de esperar a ocorrência de uma inibição desta actividade, dada a capacidade dos esteróides se ligarem à molécula do CYP1A actuando como inibidores (Navas and Segner, 2000; Elskus, 2004). O naftaleno revelou ser um indutor da actividade da conjugase GST (fase II) no fígado.

No que respeita à genotoxicidade, a resposta da *A. anguilla*, foi claramente diferente em relação aos dois compostos testados no capítulo III. Assim, o naftaleno demonstrou possuir propriedades genotóxicas induzindo um aumento na frequência de anomalias nucleares eritrocíticas (ANE), enquanto que a BNF não produziu qualquer alteração deste parâmetro no mesmo período de tempo (72 horas). Geralmente, considera-se que as características mutagénicas dos HAP estão relacionadas com o número de anéis benzénicos e a sua bioactivação, especialmente por acção da fase I da biotransformação. No entanto, a indução precoce de ANE e o decréscimo simultâneo da actividade de EROD observados, sugerem que o próprio naftaleno poderá ser genotóxico, sem necessitar de bioactivação prévia. Uma vez que o naftaleno é liposolúvel e possui apenas dois anéis aromáticos, a sua entrada na célula é facilitada pelo que um grande número de moléculas poderá rapidamente ser transportada para o núcleo e interagir com o ácido desoxirribonucleico (ADN).

Assim, o declínio da frequência de ANE (16-72 h) acompanhado por um aumento da actividade de EROD reforça a hipótese anterior no que respeita à independência dos acontecimentos, ou seja, a activação da biotransformação parece não ser responsável pela genotoxicidade.

A expressão da resposta genotóxica em termos de frequência de ANE pode ser condicionada pela dinâmica hematológica, ou seja, pelo balanço entre a eritropoiese e o catabolismo dos eritrócitos. Nessa perspectiva, a frequência de eritrócitos imaturos (EI) foi quantificada para as exposições a naftaleno e a BNF com o objectivo de detectar alterações nessa dinâmica. Assim, os resultados de EI demonstraram que o naftaleno não interferiu com a dinâmica hematológica, ao contrário da BNF que causou um decréscimo na frequência de EI. Este último resultado sugere que a ausência de genotoxicidade observada por exposição à BNF deve ser encarada com cepticismo, não se podendo excluir a possibilidade de um resultado falsamente negativo.

A exposição de *D. labrax* a E_2 resultou no aumento da frequência de ANE, demonstrando o seu potencial genotóxico, não tendo este resultado diferido entre as duas vias de exposição testadas. Tal como no caso do naftaleno, não foi encontrada uma correlação entre este efeito e a actividade de EROD.

2. EXPOSIÇÕES LABORATORIAIS A MISTURAS DE CONTAMINANTES

Os trabalhos em análise neste ponto incluem os capítulos VII a IX correspondendo a ensaios laboratoriais quer com misturas simples, quer com exposições sequenciais a diferentes contaminantes, envolvendo três espécies de teleósteos (*D. labrax*, *Sparus aurata* L. - dourada e *A. anguilla*). Neste contexto, foram estudados os efeitos da interação entre: (i) um composto tipo-HAP (BNF) e uma hormona "natural" (E_2) ou entre BNF e um derivado dos etoxilados de alquilfenóis (nonilfenol - NP) em *D. labrax*; (ii) E_2 e NP em *S. aurata*; (iii) BNF e dois metais pesados – crómio (Cr) e cobre (Cu) em *A. anguilla*. A relevância do NP resulta do facto de ser o metabolito mais persistente, resultante da degradação dos alquifenóis etoxilados, enquanto que a importância dos metais pesados resulta essencialmente da sua persistência e resistência à biotransformação e biodegradação. A escolha do E_2 e da BNF foi referida e justificada no ponto anterior.

Em qualquer um destes capítulos (VII a IX) foi determinada a concentração plasmática de cortisol e glucose. Relativamente ao cortisol, observou-se maioritariamente uma ausência de resposta, havendo a registar apenas o seu decréscimo significativo para a exposição de *S. aurata* a E_2+NP (capítulo VII), indicando uma alteração endócrina. Contudo, este efeito não pode ser claramente atribuído à mistura, uma vez que um decréscimo semelhante de cortisol foi também observado após exposição a E_2. A pré-exposição a BNF exerceu uma acção antagonista sobre a capacidade do Cu aumentar a concentração plasmática de cortisol em *A. anguilla* (capítulo IX). Este acontecimento poderá ser devido a uma diminuição da sensibilidade do tecido interrenal à estimulação pela hormona adrenocorticotrofica (ACTH), tal como foi observado anteriormente por Wilson *et al.* (1998) em estudos *in vitro*. Considerando outros estudos com peixes em que se observaram aumentos ou decréscimos de cortisol no plasma após exposição a contaminantes (Santos e Pacheco, 1996; Hontela, 1997), a quase ausência de efeito das misturas testadas neste parâmetro sugere que a susceptibilidade do tecido interrenal dependerá da natureza e concentração dos contaminantes, assim como da espécie usada.

A glucose plasmática aumentou em quase todas as condições de exposição, tendo-se verificado uma acção sinergística para a exposição de *D. labrax* a BNF+NP e de *A. anguilla* a BNF+100 μM Cr. Por outro lado, apesar do aumento significativo de glucose observado para a mistura BNF+E_2, a BNF mostrou limitar a capacidade de elevar a glucose plasmática demonstrada pelo E_2 individualmente. Os aumentos de glucose plasmática, verificados para a mistura BNF+Cu e para BNF+1 mM Cr, não podem contudo ser atribuídos a nenhuma interacção entre os compostos. A exposição de *S. aurata* à mistura de E_2+NP não induziu qualquer alteração neste parâmetro. De um modo geral, os níveis de glucose no plasma confirmaram ser influenciados de forma diferente em resposta a misturas quando comparada com a resposta a compostos individuais. A interacção entre os compostos também parece depender da sua concentração.

Em relação ao outro parâmetro indicador de alterações no metabolismo intermediário (lactato plasmático), determinado nos capítulos VIII e IX, observou-se que, de um modo geral, permaneceu inalterado, tendo-se observado um aumento apenas no caso da mistura de E_2+NP, sem que, contudo, tenha sido perceptível qualquer interacção entre estes compostos. No entanto, observou-se uma acção antagonista da BNF (BNF+Cu), que impediu o aumento de lactato observado em resultado da exposição de *A. anguilla* ao Cu.

A análise global dos três parâmetros indicadores de stresse revelou a glucose plasmática como o parâmetro mais sensível face às situações de contaminação testadas.

A frequência de ANE foi avaliada em todos estes capítulos (VII a IX) como indicador de genotoxicidade. Assim, a exposição de *D. labrax* a BNF+E_2 e a BNF+NP (4 e 8 h) induziram um aumento de ANE. Uma vez que a acção genotóxica da BNF não foi alterada por exposição com o E_2 ou NP, este efeito não poderá ser atribuído a qualquer tipo de interacção, mas sim à BNF. Resultados semelhantes foram anteriormente observados na mesma espécie por Gravato e Santos (2002b). Contudo, a extensão da exposição até 24 h revela também o desaparecimento total das ANE.

A exposição de *A. anguilla* a BNF+Cu (24+24 h) demonstrou o seu potencial genotóxico tendo os compostos interagido positivamente e manifestado uma acção sinergística. Esta acção, mostrou depender especificamente do Cu, uma vez não se observou para o Cr, sendo também dependente da respectiva dose.

A determinação de vários parâmetros hepáticos de biotransformação de fase I (EROD e P450) e fase II (GST), assim como a actividade de ALT e o índice hepato-somático (IHS) foram levadas a cabo nos capítulos VII e VIII. Assim, a interferência de xeno/estrogénios com reconhecido potencial inibidor da actividade de EROD tal como o E_2 ou NP sobre a acção da BNF, um típico indutor desta actividade foi investigada no capítulo VII. Observou-se que ambos os xeno/estrogénios reduziram a acção indutora da actividade de EROD da BNF em *D. labrax*. No capítulo VIII, a mistura E_2+NP teve a capacidade de afectar a expressão constitutiva do CYP1A, medida pela diminuição da actividade de EROD em *S. aurata*. Não obstante, este efeito não pode ser atribuído à mistura, dado que um efeito semelhante foi observado após exposição a E_2. Considerando estes resultados, conclui-se que a capacidade dos peixes metabolizarem HAP ou outros substratos do CYP1A poderá vir a ser alterada pela presença de xeno/estrogénios na água. Esta interferência, para além de constituir um distúrbio na capacidade metabólica dos peixes, poderá também aumentar a possibilidade de interpretações erróneas, acerca da condição dos ecossistemas, com base apenas em dados relativos ao CYP1A.

De uma forma geral, as interferências observadas ao nível da actividade de EROD não se traduziram numa acção semelhante ao nível do conteúdo em citocromo P450, uma vez que este se manteve inalterado, tornando-se assim difícil estabelecer uma relação entre estes dois parâmetros.

Relativamente à actividade hepática da GST em *D. labrax*, a importância da mistura de compostos ficou patente na redução desta actividade observada após exposição a BNF+NP, uma vez que considerados isoladamente não demonstraram essa capacidade (capítulo VII). No capítulo VIII, observou-se em *S. aurata* um aumento da actividade da GST para a mistura de E_2+NP, o que resultou da acção individual do E_2. Este efeito sugere um aumento do catabolismo hepático de

esteróides, dando sequência à acção metabólica de outras famílias do CYP (ex. CYP3A) que não o CYP1A.

Os indicadores da condição hepática (ALT e LSI) foram alterados esporadicamente, não reflectindo interacções entre os compostos (capítulos VII e VIII).

No capítulo IX, investigaram-se as respostas ao nível do eixo HPT perante a exposição sequencial a BNF e a dois metais pesados (Cr e Cu). As alterações registadas dizem respeito a processos ao nível da tiróide, ou a jusante desta, uma vez que, a TSH permaneceu inalterada. A T4 decresceu no plasma de peixes expostos quer a BNF+Cr quer a BNF+Cu, sendo identificável uma acção sinergística entre a BNF e o Cu em que a pré-exposição potenciou o efeito do Cu. Os presentes resultados corroboram dados anteriores em que peixes expostos a um ambiente poluído por HAP e metais pesados apresentavam níveis plasmáticos de T4 significativamente baixos (Hontela *et al.*, 1995).

A importância da pré-exposição a BNF na acção do Cr e do Cu sobre a T3 não foi, de uma forma geral, perceptível; contudo, no caso da exposição a 100 μM Cr a interferência da pré-exposição não pode ser excluída uma vez que os níveis de T3 baixaram relativamente à exposição isolada a 100 μM Cr.

3. EXPOSIÇÕES *IN SITU* A AMBIENTES POLUÍDOS

Este ponto corresponde a exposições *in situ*, englobando os capítulos X e XI, cujas vantagens foram previamente apresentadas no ponto 3 da introdução geral. Foram seleccionados três ecossistemas aquáticos, com natureza e graus de poluição distintos, de forma a permitir o estudo de respostas perante exposição a situações reais de contaminação por misturas de xenobióticos. Assim, escolheu-se uma zona portuária de longo curso, situada na Ria de Aveiro, tipicamente contaminada por HAP, metais pesados e compostos organoestânicos e ainda uma zona do rio Vouga que durante várias décadas foi receptora de efluentes de uma unidade de produção de pasta de papel (capítulo X). No rio Vouga, pretendeu-se avaliar o efeito dos contaminantes remanescentes nos sedimentos sobre *A. anguilla*, após o desvio do efluente para o oceano Atlântico, como uma medida da recuperação do ecossistema. Por fim, escolheu-se um ecossistema de água doce – Pateira de Fermentelos – alvo de efluentes domésticos e industriais, assim como de lixiviados agrícolas, não havendo referência a estudos relativos aos efeitos desta múltipla contaminação sobre a ictiofauna. Assim, o trabalho da Pateira de Fermentelos inseriu-se na primeira campanha de biomonitorização recorrendo a uma espécie representativa da ictiofauna daquele ecossistema.

Em qualquer dos locais de estudo seleccionados foram realizadas exposições de curto prazo, usando *A. anguilla* como organismo indicador, pelas razões apresentadas anteriormente. Foram estudados indicadores de stresse como o cortisol, glucose e lactato plasmáticos em *A. anguilla* exposta nos três ecossistemas referidos (capítulos X e XI). A biotransformação hepática, genotoxicidade, hormonas do eixo HPT e E_2 no plasma foram também avaliados no estudo da Pateira de Fermentelos (capítulo XI). Os indicadores de stresse avaliados mostraram alterações que se enquadram no perfil típico de resposta, ou seja aumento simultâneo de cortisol, glucose e lactato plasmáticos, nos três ecossistemas avaliados, com excepção do lactato que se manteve inalterado no caso da exposição na Pateira de Fermentelos. A simultaneidade no aumento de glucose e cortisol poderá ser indicadora de indução da gluconeogénese, resultante da acção do cortisol. No caso

da exposição a águas contaminadas por EPP observou-se um retorno do cortisol e da glucose plasmáticos para os níveis do controlo das 8 para as 48 h de exposição, o que pode ser interpretado como um processo adaptativo. Contudo, em relação à exposição a águas portuárias não se verificou o processo de adaptação anteriormente descrito. As diferenças observadas sugerem que os mecanismos de recuperação dependem do tipo de contaminantes presentes nos locais de exposição.

Os dados relativos ao rio Vouga revelaram que, mesmo após dois anos do desvio do EPP, persistem no sedimento contaminantes capazes de induzir alterações de stresse nos peixes.

O comportamento destes três parâmetros confirmou a sua capacidade de sinalizar a presença de agentes de stresse de diferentes naturezas, realçando contudo a sua inespecificidade.

Na exposição *in situ* na Pateira de Fermentelos observou-se também uma tendência geral para a diminuição de T3, confirmada com o seu decréscimo significativo no local mais próximo da principal fonte de poluição (Ria Cértima), sem ser contudo acompanhada por alterações nos níveis plasmáticos de TSH e T4. Esta diminuição de T3 no plasma poderá ser devida, directamente, à acção de contaminantes como metais pesados e pesticidas, ou ainda derivar, indirectamente, do aumento do cortisol, tendo sido anteriormente demonstrado que peixes com níveis elevados de cortisol apresentavam decréscimos dos níveis de T3 no plasma (Redding *et al.*, 1986; Brown *et al.*, 1991).

No que diz respeito à presença de E_2 no plasma, observou-se um aumento significativo em dois locais da Pateira de Fermentelos, indicando a presença desta hormona “natural” em níveis elevados na água, sem que seja compreensível a relação com a distância ao principal afluente de contaminação.

Os peixes expostos na Pateira de Fermentelos não revelaram qualquer alteração na actividade de EROD, sugerindo a ausência de moduladores do CYP1A. No entanto, tendo em consideração o “cocktail” de contaminantes que existe na Pateira de Fermentelos, não se pode excluir a possibilidade da ocorrência de um equilíbrio entre potenciais indutores (ex. pesticidas) e inibidores do CYP1A1 (ex.

metais pesados), impedindo uma resposta final detectável. Não foram também detectados danos hepáticos, medidos pela actividade da ALT, nem efeitos genotóxicos, medidos pela frequência de ANE, apesar da diminuição da integridade do ADN sanguíneo ter sido observada (Maria *et al.,* 2006).

Globalmente, os dados obtidos nas exposições *in situ* na Pateira de Fermentelos, forneceram fortes indicações do grau de contaminação deste ecossistema.

4. APRECIAÇÃO GLOBAL E PERSPECTIVAS FUTURAS

Os dados resultantes da presente tese permitiram um melhor conhecimento do potencial tóxico de diferentes contaminantes, de grande relevância ambiental, cuja importância surge acrescida pela escassez de informação disponível no que respeita aos parâmetros estudados. Os presentes dados contribuíram ainda para um melhor entendimento dos processos toxicológicos em peixes, particularmente como resultado da utilização de uma bateria de biomarcadores, distribuídos por diferentes níveis biológicos e pelo facto de se ter explorado a interferência de misturas ou exposições sequenciais a diferentes classes de xenobióticos.

Os resultados comprovaram a existência de interacções de compostos, não sendo estas contudo previsíveis com base apenas nas características das classes dos compostos envolvidos. As interacções mostraram ser também fortemente condicionadas pelas concentrações relativas dos compostos em causa. A diversidade de parâmetros estudados permitiu ainda conhecer melhor a interdependência entre respostas, contribuindo para um conhecimento integrado da resposta dos peixes a contaminantes.

As exposições *in situ* de *A. anguilla* demonstraram a sua aplicabilidade na avaliação do estado de três ecossistemas distintos caracterizados por diferentes tipos de contaminação. Contudo, nem sempre foi perceptível uma relação com a distância à principal fonte de poluição. Assim, este tipo de biomonitorização apesar da sua aplicabilidade poderá suscitar novas questões, sempre que elevadas concentrações de diferentes contaminantes xenobióticos alterarem o sentido das respostas, através da ocorrência de interacções que conduzam a situações de antagonismo ou sinergismo.

Sem pretender diminuir a importância da adopção de biomarcadores, que a presente tese corrobora, os dados obtidos realçam a necessidade da sua complementação com indicadores químicos relativos à quantificação dos contaminantes xenobióticos na água, sedimentos e tecidos dos peixes.

Uma das linhas naturais de evolução do presente trabalho diria respeito à aplicação da bateria de parâmetros utilizada, a diferentes espécies piscícolas

selvagens em estudos de campo. Apesar de todas as limitações que este tipo de abordagem encerra, o completo entendimento das respostas toxicológicas não pode prescindir dessa componente.

As exposições levadas a cabo foram de curto prazo, pelo que para se poder assumir de forma mais definitiva a conclusão acerca dos efeitos dos contaminantes testados, deverá considerar-se dados de exposições mais longas, com amostragens frequentes nesse intervalo de tempo, não incluídas na presente tese e que poderão ser levadas a cabo em trabalhos subsequentes.

As espécies adoptadas como organismos teste demonstraram a sua aplicabilidade no presente contexto, devendo considerar-se para o caso de *A. anguilla* e de *D. labrax* como um facto confirmado. Contudo, a versatilidade fisiológica de *A. anguilla* deverá ser destacada uma vez que tem sido utilizada com sucesso em diversas condições de exposição laboratoriais ou *in situ*, em águas doces, salobras ou salgadas.

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