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Efeitos genotóxicos e bioquímicos de xenobióticos em animais aquáticos

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palavras-chave

Peixes, xenobióticos, contaminação, biomonitorização, Ria de Aveiro, stresse oxidativo, genotoxicidade.

resumo

No presente trabalho, foi estudado um largo espectro de efeitos genotóxicos e bioquímicos na tainha-garrento (*Liza aurata*). Nos Capítulos II e III são descritos os efeitos de exposição de curta duração ao fenantreno, um hidrocarboneto aromático policíclico (HAP). A exposição de curta duração (16 horas) demonstrou a capacidade deste composto induzir a actividade da enzima de fase I da biotransformação, etoxiresorufina O-desetilase (EROD), provocar decréscimos de integridade no ADN hepático e aumento de anomalias nucleares eritrocíticas (ANE). Em termos de respostas de stresse, os níveis plasmáticos de cortisol e glucose aumentaram face à exposição a este HAP. A exposição ao fenantreno induziu o decréscimo da glutationa peroxidase (GPx) nas guelras, enquanto que no fígado a actividade da GPx aumentou. No rim, a actividade da glutationa S-transferase (GST) foi inibida. Nas guelras, verificou-se um aumento da catalase. O fenantreno demonstrou igualmente a capacidade de induzir um aumento dos níveis de glutationa nas guelras e fígado. Estas respostas demonstraram a sensibilidade de *L. aurata*, a este HAP, realçando a especificidade das respostas em termos de órgãos. Apesar dos aumentos das defesas antioxidantes, o potencial tóxico deste composto foi demonstrado pelo aumento da peroxidação lipídica nos três órgãos.

Nos capítulos seguintes, são descritas as respostas de *L. aurata* capturada na Ria de Aveiro, em locais com diferentes perfis de contaminação, inicialmente no Outono de 2005 (Capítulos III a IX) e posteriormente analisando respostas sazonais (Capítulos X e XI).

A análise de respostas de stresse (cortisol, glucose e lactato) revelou que *L. aurata* capturada em Vagos (local contaminado por HAPs) apresentava níveis baixos de cortisol, enquanto que no Laranjo (local contaminado por mercúrio) apresentavam elevados níveis de glucose e lactato. Relativamente às hormonas do eixo hipotálamo – hipófise – tiróide (HHT), foram observados elevados níveis plasmáticos da hormona estimuladora da tiróide (TSH) nos organismos capturados no Laranjo, baixos níveis de tiroxina (T4) nos organismos da Barra (local sujeito a tráfego naval) e baixos níveis de triiodotironina (T3) no Rio Novo do Príncipe (próximo de um antigo efluente de pasta de papel), Laranjo e Vagos. A avaliação das defesas antioxidantes, dano oxidativo e genotóxico nas guelras, rim e fígado revelou diferenças significativas nas respostas dos órgãos. *L. aurata* capturada na Barra apresentou dano oxidativo nas guelras (Capítulo V). No rim foi detectada uma diminuição da integridade do ADN no Rio Novo do Príncipe e Vagos (Capítulo VI), enquanto que no fígado foi observado dano lipídico na Gafanha e Vagos (Capítulo VIII). O dano não esteve sempre associado a um decréscimo das defesas.

As análises da água e do sedimento da Ria de Aveiro (Outono de 2005) revelaram elevadas concentrações de metais (Cd, Hg, Cu e Zn),

principalmente, no Laranjo e Rio Novo do Príncipe. *L. aurata* capturada nestes locais apresentou os níveis mais elevados de metalotioninas hepáticas (Capítulo VII) que parecem responsáveis pela inexistência de danos no fígado (Capítulo VIII).

O dano oxidativo no ADN, avaliado através da quantificação dos níveis plasmáticos de 8-hidroxi-2'-desoxiguanosina (8-OHdG) e o dano clastogénico/aneugénico, avaliado através da quantificação da frequência de ANE, foram estudados, no Outono de 2005, em duas espécies de peixes (*L. aurata* e *Dicentrarchus labrax* - robalo) (Capítulo IX). Os resultados revelaram grande sensibilidade de *D. labrax* em termos de dano oxidativo no ADN na Gafanha, Rio Novo do Príncipe e Vagos, enquanto que *L. aurata* apresentou dano oxidativo apenas no Laranjo. O aumento da frequência de ANE apenas foi detectado em *L. aurata*, em Vagos, não se tendo detectado correlação entre estes dois parâmetros.

O estudo sazonal (Maio de 2006 a Março de 2007) do dano oxidativo no ADN e frequência de ANE em *L. aurata* (Capítulo X) demonstrou a variação destes parâmetros com a estação do ano, apesar de não se ter verificado correlação com os parâmetros hidrológicos determinados. No entanto, no local de referência não se verificaram diferenças sazonais, o que sugere que estes biomarcadores reflectem variações de biodisponibilidade de contaminantes. A análise global dos resultados das diferentes estações do ano revelou que *L. aurata* capturada no Rio Novo do Príncipe e em Vagos apresentou maior susceptibilidade a dano oxidativo no ADN. No entanto, apenas *L. aurata* capturada em Vagos apresentou frequência de ANE superior à do local de referência. Os dados do estudo sazonal revelaram uma correlação entre dano oxidativo e ANE, sugerindo o stresse oxidativo como um possível mecanismo envolvido na formação de anomalias. A integridade do ADN das guelras, rim, fígado e sangue de *L. aurata* foi igualmente estudada ao longo de um ano (Capítulo XI), tendo-se verificado uma grande variabilidade ao longo deste período. Não foi demonstrada sensibilidade a um perfil de contaminação específico, tendo-se verificando variabilidade sazonal no local de referência.

Globalmente, os resultados demonstraram a importância da utilização de uma bateria de biomarcadores na monitorização ambiental e a especificidade da resposta dos diferentes órgãos de *L. aurata*.

keywords

Fish, xenobiotics, contamination, biomonitoring, Ria de Aveiro, oxidative stress, genotoxicity.

abstract

In the present research, a wide range of genotoxic and biochemical effects, were studied in golden grey mullet (*Liza aurata*). In Chapters II and III the effects of short-term exposure to phenanthrene, a polycyclic aromatic hydrocarbon (PAH), are described. The short-term exposure (16 hours) demonstrated the capacity of this compound to induce the activity of the phase I biotransformation enzyme, ethoxyresorufin O-deethylase (EROD), provoke hepatic DNA integrity decrease and increased erythrocytic nuclear abnormalities (ENA). In terms of stress responses, plasma cortisol and glucose levels were increased after exposure to this PAH. Exposure to phenanthrene decreased gill glutathione peroxidase (GPx) activity, whereas in liver GPx activity was increased. In kidney, glutathione S-transferase (GST) activity was inhibited. In gill, an increase in catalase activity was observed. Phenanthrene also demonstrated the capacity to induce increased levels of glutathione in gill and liver. These responses demonstrated *L. aurata* sensitivity to this PAH, highlighting organ-specific responses. Despite the increased antioxidant defences, the toxic potential of this compound was demonstrated by the increase in lipid peroxidation in the three organs.

In the following chapters, the responses of *L. aurata* captured in Ria de Aveiro, at sites with different contamination profiles, are described, initially in autumn 2005 (Chapters III to IX) and afterwards analysing seasonal responses (Chapters X and XI).

The analysis of stress responses (cortisol, glucose and lactate) revealed that *L. aurata* captured at Vagos (site contaminated with PAHs) displayed low cortisol levels whereas at Laranjo (mercury contaminated site) they displayed high levels of glucose and lactate. Concerning hormones from the hypothalamus-pituitary-thyroid (HPT) axis, high levels of plasma thyroid stimulating hormone (TSH) were observed in fish caught at Laranjo, low levels of thyroxine (T4) in fish from Barra (site subject to naval traffic) and low triiodothyronine (T3) levels found at Rio Novo do Príncipe (near former pulp mill effluent), Laranjo and Vagos. The evaluation of antioxidant defences, oxidative and genetic damage in gill, kidney and liver revealed significant differences in organ responses. In gill, oxidative damage was found in Barra (Chapter V); in kidney decreased DNA integrity was found in Rio Novo do Príncipe and Vagos (Chapter VI) whereas in liver lipid peroxidation was found in Gafanha and Vagos (Chapter VIII). Damage was not always associated with decreased defences.

High levels of metals (Cd, Hg, Cu e Zn) were detected in water and sediments of Ria de Aveiro (Autumn 2005), mainly at Laranjo and Rio Novo do Príncipe. *L. aurata* captured at these sites displayed the highest levels of hepatic metallothionein that seem responsible for the lack of damage in liver (Chapter VIII).

The oxidative DNA damage, assessed as plasma 8-hydroxy-2'-

-deoxyguanosine (8-OHdG) and the clastogenic/aneugenic damage, assessed as ENA frequency, were studied in two species (*L. aurata* and *Dicentrarchus labrax* – sea bass) (Chapter IX). Results revealed high sensitivity of *D. labrax* in terms of oxidative DNA damage at Gafanha, Rio Novo do Príncipe and Vagos, whereas the *L. aurata* only displayed oxidative DNA damage in Laranjo. Increased frequency of ENA was only detected in *L. aurata*, at Vagos. No correlation was found between the two parameters.

The seasonal (May 2006 to March 2007) study of *L. aurata* oxidative DNA damage and ENA frequency (Chapter X) revealed variation of these parameters along the different seasons of the year, despite no correlation was found with the assessed hidrological factors. However, at the reference site no seasonal differences were displayed, suggesting that these biomarkers reflected variations in the bioavailability of contaminants. The analysis of data obtained in the different seasons of the year revealed that *L. aurata* captured in Rio Novo do Príncipe and Vagos showed higher sensitivity to oxidative DNA damage. However, only *L. aurata* captured at Vagos displayed higher ENA frequency than the reference site. The data from the seasonal study revealed a correlation between oxidative DNA damage and ENA, suggesting that oxidative stress may be a possible mechanism involved in abnormalities formation. The DNA integrity of *L. aurata* gill, kidney, liver and blood was also assessed over a one year period (Chapter XI), and a high variability was observed. However, DNA integrity did not show sensitivity to a particular contamination profile. Furthermore, seasonal variations were also found in the reference site.

Globally, results demonstrated the importance of selecting a battery of biomarkers in environmental monitoring and the *L. aurata* organ-specific responses.

ÍNDICE

Abreviaturas

Em Português:

$^1O_2$ - Dioxigénio singuleto
8-OHdG - 8-hidroxi-2'desoxiguanosina
A - Adenina
ACTH - Hormona adrenocorticotrófica
ADN - Ácido desoxirribonucleico
ANE - Anomalias nucleares eritrocíticas
C - Citosina
CAT - Catalase
CRH - Hormona libertadora de corticotrofina
DQA - Directiva Quadro da Água
ERN - Espécies reactivas de nitrogénio
ERO - Espécies reactiva de oxigénio
EROD - Etoxiresorufina O-desetilase
G - Guanina
GPx - Glutationa peroxidase
GR - Glutationa redutase
$GS^{\bullet}$ - Glutationilo
GSH - Glutationa reduzida
GSSG - Glutationa disulfeto
GST - Glutationa S-transferase
HAPs - Hidrocarbonetos aromáticos policíclicos
HNE - 4-hidroxinonenal
HHI - Hipotálamo-hipófise-tecido interrenal
HHT - Hipotálamo-hipófise-tiróide
HSPs Proteínas de stresse "heat shock"
$L^{\bullet}$ - Radical lipídico
LH - Ácido gordo insaturado
LO - Aldeído lipídico
$LO^{\bullet}$ - Radical alcoxilo
$LOO^{\bullet}$ - Radical peroxilo
LOOH - Hidroperóxido lipídico
MDA - Malonildialdeído

Em Inglês:

8-OHdG - 8-hydroxy-2'-deoxyguanosine
AAS - Atomic absorption spectrophotometer
ACTH - Adrenocorticotropic hormone
APDC - Ammonium pyrrolidine dithiocarbamate
BaP - Benzo[a]pyrene
BAR - Barra
BHT - Butylated hydroxytoluene
BKPME - Bleached kraft pulp mill effluents
CAT - Catalase
CDNB - 1-chloro-2,4-dinitrobenzene
DMSO - Dimethyl sulfoxide
DNA - Deoxyiribonucleic acid
DPP - Differential pulse polarography
DTNB - 2,5-dithiobis-tetranitrobenzoic acid
DTPA - Diethylenetriaminepentaacetic acid
EDTA - Ethylene diamine tetraacetic acid
ENA - Erythrocytic nuclear abnormalities
EROD - Ethoxyresorufin-O-deethylase
GAF - Gafanha
GGT - γ-glutamyl transpeptidase
GPx - Glutathione peroxidase
GR - Glutathione reductase
GSH - Reduced glutathione
GSHt - Total glutathione
GSSG - Glutathione disulfide
GST - Glutathione S-transferase
$HO^{\bullet}$ - Hydroxyl radical
HPI - Hypothalamo-pituitary-interrenal tissue
HPT - Hypothalamo-pituitary-thyroid
HRP - Horseradish peroxidase
IBR - Integrated biomarker response
LAR - Laranjo

$O_2^{\bullet-}$ Radical superóxido
$OH^{\bullet}$ - Radical hidroxilo
PCBs - Bifenilos policlorados
PL - Peroxidação lipídica
$ROO^{\bullet}$ - Radical peroxilo
Rt3 - Triiodotironina reversa
SOD - Superóxido dismutase
T2 - Diiodotironina
T3 - Triiodotironina
T4 - Tiroxina
T - Timina
TRH - Hormona libertadora da tirotrofina
TSH - Hormona estimuladora da tiróide

LDH - Lactate dehydrogenase
LPO - Lipid peroxidation
m_i - General mean
MIBK - Methyl isobutyl ketone
Min_i - Minimum value
MN - Micronucleus
MT - Metallothionein
NPT - Non protein thiols
$O_2^{\bullet-}$ - Superoxide radical
PAHs - Polycyclic aromatic hydrocarbons
PCA - Principal component analysis
PCs - Principal components
Phe - Phenanthrene
PMS - Post-mitochondrial supernatant
RIO - Rio Novo do Príncipe
ROS - Reactive oxygen species
SE - Standard errors
s_i - Standard deviations
T3 - Triiodothyronine
T4 - Thyroxine
TBA - 2-thiobarbituric acid
TBARS - Thiobarbituric acid reactive substances
TCA - Trichloroacetic acid
TMB - Tetramethylbenzidine
TNB - 5-thio-2-nitrobenzoic acid
TOR - Torreira
TSH - Thyroid stimulating hormone
US EPA - United States Environmental Protection Agency
VAG - Vagos

nota introdutória

A presente tese de Doutoramento está organizada em doze capítulos correspondendo a três partes distintas. Assim, a primeira parte corresponde ao Capítulo I – Introdução geral, no qual se estabelece o contexto do trabalho e se definem os 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. Contaminação do ambiente aquático

O ambiente aquático tem uma enorme importância para o Homem, podendo ser fonte de água (para consumo e actividades antropogénicas), alimento e actividades lúdicas. No entanto, as descargas de resíduos industriais, deposição atmosférica e lixiviação dos solos tornam o ecossistema aquático no principal receptor de contaminantes (orgânicos e inorgânicos). O declínio da qualidade do meio ambiente apresenta-se, assim, como um grave problema para o Homem, constituindo uma séria ameaça para todos os seres vivos. A Directiva Quadro da Água (DQA, 2000/60/CE), adoptada pelo Parlamento Europeu e Conselho Europeu da União Europeia em Outubro de 2000, considera a água não um produto comercial mas "um património que deve ser protegido, defendido e tratado como tal" e estabelece um quadro para a protecção de todos os corpos de água, visando alcançar um bom *status* químico e ecológico para todas as massas de água até 2015.

De entre os poluentes mais frequentes e com maior impacto no meio aquático destacam-se os hidrocarbonetos aromáticos policíclicos (HAPs), hidrocarbonetos clorados como bifenilos policlorados (PCBs) e pesticidas organoclorados, metais e outras classes de compostos resultantes de actividades industriais específicas.

2. Biomarcadores como ferramentas de monitorização ambiental

O objectivo final da monitorização ambiental é a protecção dos sistemas biológicos. Deste modo, é imprescindível estudar os efeitos biológicos da exposição a substâncias presentes no meio ambiente, dado que a mera quantificação destas substâncias não revela a biodisponibilidade das mesmas nem permite estabelecer eventuais efeitos nocivos (Cajaraville et al., 2000; Barsiene et al., 2006). Para além disso, os sistemas biológicos são frequentemente expostos a misturas de contaminantes, os quais podem interagir de diferentes formas (efeitos aditivos, antagonistas ou sinergistas).

Os organismos, quando confrontados com modificações no ambiente (frequentemente hostis aos processos vitais), desencadeiam mecanismos adaptativos que lhes permitem sobreviver, crescer e reproduzir-se, reflectindo as interacções com os contaminantes. Desta forma, os efeitos que estas substâncias estranhas para o organismo (xenobióticos) causam nos organismos destes ecossistemas podem ser utilizados como biomarcadores para avaliar o impacto dos contaminantes na qualidade ambiental (Wells et al., 2001). Um biomarcador pode ser definido como "uma variação bioquímica, celular, fisiológica ou comportamental que pode ser medida no tecido ou amostras de fluidos corporais ou ao nível do organismo e que fornece evidências de exposição a e/ou efeitos de um ou mais poluentes químicos (e/ou radiação)" (Depledge, 1993). Há, no entanto, alguma controvérsia em torno do uso deste termo dado que alguns investigadores consideram biomarcadores apenas respostas ao nível molecular, bioquímico ou fisiológico (Lam, 2009).

De acordo com a Organização Mundial da Saúde (1993), os biomarcadores poderão ser subdivididos em três classes: 1) biomarcadores de exposição, cobrindo a detecção e medida de uma substância exógena, dos seus metabolitos ou do produto de uma interacção entre um agente xenobiótico e algumas moléculas ou células alvo, a qual é medida num compartimento dentro de um organismo; 2) biomarcadores de efeito, que incluem alterações bioquímicas, fisiológicas ou outras mensuráveis nos tecidos ou fluidos corporais que podem ser reconhecidas como associadas com um possível desequilíbrio da saúde; 3) biomarcadores de susceptibilidade, indicando a capacidade inerente ou adquirida de um organismo responder à provocação da exposição a um xenobiótico específico, incluindo factores genéticos e modificações nos receptores que alteram a susceptibilidade de um organismo a essa exposição.

Neste contexto, os biomarcadores podem ser considerados ferramentas integradoras, complementares às análises químicas e ecológicas classicamente usadas na monitorização ambiental (Sanchez et al., 2008). Van der Oost e colaboradores (2003) propuseram vários critérios para avaliar a força e a fraqueza de parâmetros candidatos a biomarcadores:

- Os biomarcadores devem ser fiáveis, relativamente baratos e fáceis de executar. Além disso, devem preferencialmente ser seleccionados métodos não invasivos ou não destrutivos para facilitar a biomonitorização ambiental de espécies protegidas ou ameaçadas de extinção.
- As respostas dos biomarcadores devem ser sensíveis à exposição e/ou efeitos do xenobiótico, de forma a servir como um parâmetro de alerta precoce. Além disso, e para uma melhor compreensão dos resultados dos biomarcadores, devem ser conhecidos os perfis de resposta temporal dos mesmos após a exposição a substâncias químicas.
- O impacto de outros factores nos valores basais e respostas dos biomarcadores deve estar bem estabelecido, a fim de ser possível discernir entre variabilidade natural e induzida por stresse de contaminação. Para esse efeito, a biologia e a fisiologia dos organismos seleccionados deverão ser conhecidas de forma a minimizar as fontes de variação (por exemplo, idade, sexo e estado reprodutivo).
- Os mecanismos que estabelecem a relação entre as respostas biológicas usadas como biomarcadores e a exposição a poluentes devem ser definidos, bem como clarificadas as relações entre as respostas de biomarcadores e os impactos sobre os organismos.

Os biomarcadores podem ser utilizados em três abordagens: 1) biomonitorização passiva, ou seja, comparando a resposta do biomarcador de locais contaminados e referência; 2) biomonitorização activa, com o transplante de espécies sentinelas para o local de estudo num período determinado, a fim de induzir as respostas dos biomarcadores; 3) ensaios de laboratório, onde as espécies são expostas a contaminantes sob condições controladas. As abordagens 1 e 2 são geralmente utilizadas para a avaliação ambiental, enquanto a abordagem 3 é utilizada para investigar relações de causa-efeito (Losso e Ghirardini, 2009).

Alguns autores (Livingstone et al., 2000) consideram crucial a distinção entre biomarcadores gerais – que respondem aos principais tipos de stresse ambiental, fornecendo uma medida quantitativa da *performance* ou condição física do organismo (Widdows e Donkin, 1992) – e específicos – que respondem a

grupos particulares de compostos químicos e, por este motivo, são indicadores do grau de exposição ou dos possíveis efeitos de um composto ou grupo de compostos semelhantes (Livingstone, 1993). No entanto, várias respostas historicamente descritas como altamente específicas (por exemplo, a indução de metalotioninas por metais) podem ser perturbadas por outros compostos químicos (Davies et al., 1994). Além disso, a relação entre a resposta do biomarcador e a exposição a substâncias químicas não é estritamente linear, devido a fenómenos adaptativos ou transitórios, como verificado para os parâmetros antioxidantes (Sanchez et al., 2005). Assim, a aplicação de um conjunto de biomarcadores com base em medições de parâmetros complementares parece ser uma forma valiosa de diferenciar entre os locais limpos e poluídos ou de descrever com precisão os efeitos da contaminação nos peixes (Flammarion et al., 2002; Galloway et al., 2004; Sanchez et al., 2007).

As respostas ao nível bioquímico e molecular tendem a ocorrer primeiro, sendo seguidas por respostas ao nível da célula, tecido, órgão e organismo. Assim, as respostas ao nível bioquímico e molecular podem funcionar como um instrumento de vigilância ambiental (He, 1999; Maroni et al., 2000), ajudando a identificar o início de um possível impacto e permitindo uma acção correctiva antes que os efeitos adversos sejam observados a níveis superiores, ou seja, população, comunidade e ecossistema (Monserrat et al., 2003; Lam, 2009). Como os aspectos comuns entre organismos diferentes se acentuam principalmente ao nível molecular, muitos biomarcadores moleculares possuem a vantagem de poderem ser aplicados a uma ampla variedade de espécies (Lam e Gray, 2003). Por seu lado, os efeitos ao nível do indivíduo são respostas a longo prazo com grande relevância ecológica (Lam, 2009) e, deste modo, mais aplicáveis aos processos de análise de risco ecológico para gestão ambiental.

Nesta perspectiva, os biomarcadores poderão vir a ser usados para responder aos desafios da DQA com vista à melhoria da detecção do impacto de compostos químicos sobre os organismos aquáticos, prevendo-se que, como parte da adaptação da directiva ao progresso científico e técnico e em conformidade com o disposto no artigo 20, possam vir a ser integrados nos programas de monitorização da DQA (Sanchez e Porcher, 2009).

3. Respostas de stresse dos organismos

A resposta ao stresse é considerada um mecanismo adaptativo que permite aos organismos lidarem com stressores, reais ou percebidos, de forma a manterem o seu estado normal ou homeostático (Chrousos, 1998). Os efeitos finais dos stressores na resposta fisiológica de stresse e no ajuste do organismo dependem da duração e severidade dos stressores individuais, da frequência de situações de stresse, bem como do número e duração temporal entre as exposições (Schreck, 2000). Se a intensidade do stressor for demasiado grave ou o stressor duradouro, os mecanismos de resposta fisiológica podem ser comprometidos e o stressor tornar-se prejudicial à saúde e bem-estar dos organismos (Barton e Iwama, 1991).

Nos peixes, as respostas fisiológicas ao stresse ambiental foram categorizadas em respostas primárias, secundárias e terciárias. As respostas primárias, que envolvem as respostas neuroendócrinas iniciais, incluem a libertação de catecolaminas do tecido cromafim (Reid et al., 1998) e a estimulação do eixo hipotálamo – hipófise – tecido interrenal (HHI) que culmina na libertação de hormonas corticosteróides em circulação (Bonga, 1997; Mommsen et al., 1999). As respostas secundárias incluem mudanças dos níveis de iões e metabolitos no plasma e tecidos, de características hematológicas e de proteínas de stresse “heat shock” (HSPs), relacionadas com ajustes fisiológicos, como no metabolismo, respiração, equilíbrio ácido-base, equilíbrio hidromineral, função imunológica e respostas celulares (Iwama et al., 1998; Mommsen et al., 1999). As respostas terciárias englobam aspectos do desempenho animal como um todo, tais como alterações no crescimento, resistência global à doença, comportamento e, finalmente, sobrevivência (Wedemeyer et al., 1990). Este agrupamento é, no entanto, simplista dado que o stresse, dependendo da sua magnitude e duração, pode afectar os peixes em todos os níveis da organização, desde o molecular e bioquímico ao da população e comunidade (Barton, 2002).

4. Sistema endócrino nos peixes teleósteos

O sistema endócrino regula funções fisiológicas, dependentes de hormonas, necessárias para a sobrevivência do organismo e da espécie. Uma mudança brusca nos níveis de hormonas, nos seus receptores e em vários sinais bioquímicos relacionados provocam mudanças significativas na actividade das células e tecidos alvo. Químicos desreguladores endócrinos podem afectar este sistema de diversas formas: imitando ou inibindo a acção de uma hormona através da interacção com seu receptor e mecanismo de acção, alterando a síntese de uma hormona ou de seu receptor, ou alterando a taxa de metabolismo e/ou excreção da hormona.

4.1. Eixo hipotálamo – hipófise – tecido interrenal (HHI) nos teleósteos

O cortisol, principal corticosteróide nos peixes teleósteos, é sintetizado e secretado pelo tecido interrenal situado na porção anterior do rim, o “rim cefálico“. A sua síntese e libertação na circulação sanguínea envolvem um sistema de retrocontrolo (Balm et al., 1994) associado ao eixo HHI. Quando o peixe 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 hipófise anterior que, por sua vez, estimula a síntese e libertação de cortisol pelo tecido interrenal. Uma parte do cortisol libertado circula no plasma sanguíneo, ligado de modo reversível a proteínas, numa forma biologicamente inerte, apenas apresentando actividade fisiológica a sua forma livre. Os receptores para cortisol foram identificados nas brânquias, fígado, cérebro e intestino de peixes teleósteos (Maule e Schreck, 1991; Lee et al., 1992; Pottinger et al., 1994). Os corticosteróides têm efeitos metabólicos que permitem ao animal aumentar a glucose plasmática, fonte energética para mecanismos homeostáticos, activados durante a exposição a stressores. Níveis elevados de cortisol têm efeitos imunosupressivos nos peixes e outros vertebrados (Pruett et al., 1993). O cortisol apresenta igualmente acções permissivas com hormonas

metabólicas, como glucagon, hormonas de crescimento e tiroxina (T4) (De Jesus et al., 1990; Hontela, 1997).

Exposições agudas a diversos contaminantes como metais (Bleau et al., 1996; Hontela et al., 1996; Teles et al., 2005a), pesticidas (Waring e Moore, 2004), constituintes de efluentes de indústria de pasta papel (Teles et al., 2003), fracções solúveis de combustível (Thomas e Rice, 1987) podem aumentar os níveis plasmáticos de cortisol. No entanto, foram igualmente detectados baixos níveis de cortisol plasmático em exposições de curto prazo a ácidos resínicos (Kennedy et al., 1995; Teles et al., 2003), efluentes da pasta de papel (Santos e Pacheco, 1996), assim como à fracção solúvel de combustíveis fósseis (Pacheco e Santos, 2001). A exposição *in situ*, de curta duração, de peixes em zonas portuárias e zonas próximas de efluentes de pasta de papel desactivados resultaram no aumento dos níveis plasmáticos de cortisol (Teles et al., 2004). Peixes capturados em locais contaminados por misturas de HAPs, PCBs e metais (Brodeur et al., 1997; Girard et al., 1998) ou por efluentes de pasta de papel (McMaster et al., 1994; Hontela et al., 1997) demonstraram, em ensaios utilizando a captura como estímulo do eixo HHI, níveis inferiores de cortisol, quando comparados com organismos de locais de referência. Assim, a contínua actividade interrenal pode provocar a atenuação da resposta a stressores adicionais (Hontela et al., 1992; Hontela, 1997; Wilson et al., 1998; Norris et al., 1999; Laflamme et al., 2000) e, eventualmente, levar a sobrevivência reduzida (Hontela, 1997).

A exposição a stressores pode igualmente afectar os níveis de outras hormonas, incluindo a T4 (Brown et al., 1978; Bandeen e Leatherland, 1997), prolactina (Avella et al., 1991; Pottinger et al., 1992) e somatolactina (Randweaver et al., 1993; Kakizawa et al., 1995).

4.2. Eixo Hipotálamo – hipófise – tiróide (HHT) nos teleósteos

Nos teleósteos, as hormonas da tiróide exercem muitas acções fisiológicas, especialmente na regulação metabólica e hidromineral durante o desenvolvimento e crescimento (Peter et al., 2000; Power et al., 2001; Gavlik et al., 2002). Além

disso, foram relatados efeitos estimuladores das hormonas da tiróide no metabolismo oxidativo mitocondrial (Peter e Oommen, 1989; Peter e Oommen, 1993) e lipídico (Sheridan, 1986; Varghese e Oommen, 1999). As hormonas da tiróide e o cortisol podem interagir e influenciar conjuntamente o metabolismo dos hidratos de carbono (Hontela et al., 1995).

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 T4 e triiodotironina (T3). Os folículos da tiróide podem também desenvolver-se em locais secundários como ovários, rim e pericárdio (Janz, 2000). A síntese e libertação destas hormonas no sangue são controladas por um sistema de retrocontrolo associado ao eixo hipotálamo – hipófise – tiróide (HHT). O hipotálamo produz a hormona libertadora da tirotrofina (TRH), a qual controla a síntese da hormona estimuladora da tiróide (TSH) pela hipófise que, por sua vez, estimula a síntese e libertação de T4 e T3 pelos folículos da tiróide (Eales e Brown, 1993; Blanton e Specker, 2007). Nos peixes existe uma síntese muito maior de T4 do que de T3. Estas hormonas circulam no sangue associadas a proteínas transportadoras (Janz, 2000; Power et al., 2001) e apenas a fracção livre se pode difundir para os tecidos periféricos (Eales e Brown, 1993). A T4 é metabolizada em T3, hormona biologicamente mais potente, principalmente em tecidos periféricos (fígado, cérebro, rim e brânquias), por desiodação do anel externo ou, alternativamente, num metabolito inactivo da triiodotironina (rT3), triiodotironina reversa, por desiodação do anel interno. A T3 e rT3 podem ser posteriormente metabolizadas por desiodação do anel interno e do anel externo, respectivamente, originando o metabolito inactivo diiodotironina (T2).

As hormonas do eixo HHT são alvos importantes de desreguladores endócrinos (Schnitzler et al., 2008). A tiróide dos teleósteos apresenta as mesmas propriedades básicas de outros vertebrados. Assim, as respostas associadas à tiróide dos peixes podem ser uma ferramenta valiosa para prever acções tóxicas de contaminantes em outros vertebrados, incluindo seres humanos (Brown et al., 2004). Actualmente, cerca de 116 compostos ambientais são suspeitos de interromper a função da tiróide (Howdeshell, 2002). Estes compostos incluem

desde HAPs, PCBs, metais, pesticidas a simples catiões ou aniões (Waring e Brown, 1997; Zhou et al., 2000; Carletta et al., 2002; Brown et al., 2004; Teles et al., 2005a,b, 2007; Oliveira et al., 2008).

5. Radicais livres e os sistemas biológicos

Os radicais livres podem ser definidos como moléculas ou fragmentos moleculares contendo, em orbitais atómicas ou moleculares, um ou mais electrões desemparelhados (Halliwell e Gutteridge, 1999), os quais geralmente lhes conferem uma considerável reactividade. Os radicais livres de oxigénio ou, de uma forma mais geral, as espécies reactivas de oxigénio (ERO) – como o ião superóxido ($O_2^{\bullet -}$), peróxido de hidrogénio (H_2O_2) e radicais peroxilo ($ROO^{\bullet}$) – bem como as espécies reactivas de nitrogénio (ERN) – como o óxido nítrico ($NO^{\bullet}$) – são produtos do metabolismo celular normal, reconhecidos por desempenharem um duplo papel, nefasto e benéfico (Valko et al., 2006). Os efeitos nefastos dos radicais livres são denominados de stresse oxidativo e nitrosativo e ocorrem nos sistemas biológicos quando existe a produção excessiva de ERO, a principal classe de espécies de radicais geradas nos sistemas vivos (Miller et al., 1990), e ERN e/ou uma diminuição das defesas antioxidantes.

O stresse oxidativo é cada vez mais frequentemente usado como biomarcador de poluição de ambientes aquáticos (Livingstone, 2001; van der Oost et al., 2003; Valavanidis et al., 2006) para avaliar não só os efeitos da exposição, mas também entender o modo de acção dos contaminantes.

5.1. Espécies reactivas de oxigénio (ERO)

A vida na Terra apareceu num ambiente com muito pouco O_2, sendo os organismos essencialmente anaeróbicos. A abundância de CO_2, água e energia solar permitiram que cianobactérias evoluíssem e utilizassem a fotossíntese, resultando na produção de O_2 (Lesser, 2006). Por sua vez, o aumento dos níveis de O_2 na atmosfera levou a que certos organismos começassem um processo

evolutivo, desenvolvendo sistemas de defesas antioxidantes (para se protegerem da toxicidade do O_2) e uma via metabólica mais eficiente, a respiração aeróbica. Contudo, mesmo os organismos aeróbicos actuais sofrem dano oxidativo se expostos a concentrações de O_2 superiores a 21% (Balentine, 1982). Os danos observados variam consideravelmente com o organismo estudado, a idade, o estado fisiológico e a dieta, sendo os respectivos tecidos afectados de diferente forma.

As causas das propriedades tóxicas do oxigénio eram obscuras até à publicação, em 1954, da teoria da toxicidade do oxigénio por radicais livres (Gerschman et al., 1954). Um segundo marco no conhecimento da importância das ERO nos sistemas biológicos foi a descoberta da enzima superóxido dismutase (SOD), em 1969, por McCord e Fridovich, fornecendo assim provas da importância dos radicais livres nos sistemas biológicos. A investigação do papel dos radicais nos sistemas biológicos atingiu um novo patamar quando Mittal e Murad (1977) forneceram evidências de que o radical hidroxilo ($OH^{\bullet}$) estimula a activação da guanilato ciclase e a formação do segundo mensageiro, a guanosina monofosfato cíclica. Desde então, um grande número de evidências tem sido apresentado de que os sistemas vivos não só se adaptaram a co-existir com radicais livres como desenvolveram variados mecanismos para tirar proveito deles em diversas funções, nomeadamente, a defesa contra agentes infecciosos e funcionamento de inúmeros sistemas de sinalização celular (Valko et al., 2007).

O oxigénio é indispensável para a maior parte dos seres vivos, uma vez que é o aceitador final de electrões no processo de respiração celular onde se gera a energia necessária aos organismos aeróbicos. No entanto, a formação de espécies de oxigénio parcialmente reduzidas, como os radicais superóxido ($O_2^{\bullet-}$) e hidroxilo ($HO^{\bullet}$) bem como espécies não radicalares como o peróxido de hidrogénio (H_2O_2), é inevitável. O termo espécies reactivas de oxigénio (ERO) é uma denominação que inclui não apenas radicais de oxigénio ($O_2^{\bullet-}$ e $OH^{\bullet}$), mas também alguns derivados do O_2 que não apresentam electrões desemparelhados, como o peróxido de hidrogénio (H_2O_2), dioxigénio singuleto ($^1O_2$) e ácido hipocloroso (HOCl). As ERO podem ser geradas em vários locais nas células (nomeadamente nas mitocôndrias, no retículo endoplasmático, nos lisossomas,

nas membranas celulares, nos peroxissomas e no citosol) durante processos metabólicos normais, na sequência de processos de transporte de electrões e da actividade catalítica de alguns enzimas, autoxidação de compostos solúveis no citosol, bem como por exposição a radiações e metabolização de xenobióticos. A produção de ERO pode ocorrer intencionalmente como no caso da produção de $O_2^{\bullet-}$, HOCl e H_2O_2 por fagócitos activados (Babior e Woodman, 1990) ou acidentalmente nos sistemas oxidativos catalisados por metais e xenobióticos (Stadtman e Levine, 2000).

Os radicais $O_2^{\bullet-}$ e $HO^{\bullet}$, conhecidos pela capacidade de induzir dano oxidativo em importantes biomoléculas, são as duas ERO mais estudadas. A produção do radical $O_2^{\bullet-}$, considerado a ERO "primária", ocorre essencialmente dentro da mitocôndria (Cadenas e Sies, 1998), na cadeia respiratória mitocondrial por redução monoelectrónica do dioxigénio pela ubiquinona. O anião $O_2^{\bullet-}$ pode igualmente ser formado no citoplasma por acção das enzimas xantina oxidase e aldeído oxidase, durante a auto-oxidação de compostos (como a hemoglobina, catecolaminas, ascorbato e flavinas reduzidas), por acção do enzima NADPH oxidase na membrana citoplasmática e por radiólise da água em meio oxigenado. Os citocromos P450 e b5 do retículo endoplasmático e da membrana nuclear, ao oxidarem de ácidos gordos insaturados e xenobióticos, podem igualmente formar $O_2^{\bullet-}$. Este pode interagir com outras moléculas, directamente ou através de processos catalisados por enzimas ou metais, de forma a gerar ERO "secundárias" (Valko et al., 2005). Muita da toxicidade do $O_2^{\bullet-}$ pode ser explicada pela capacidade de reagir com outras ERO, como H_2O_2, para formar ERO mais reactivas. O radical $O_2^{\bullet-}$ oxida vitaminas antioxidantes (vitamina E e C), catecolaminas e tiois (Fridovich, 1985) e inactiva várias enzimas (catalase, peroxidases, aconitase, fumarase, entre outras)(Kono e Fridovich, 1982; Fridovich, 1995). O $O_2^{\bullet-}$ não reage directamente com polipéptidos, açucares nem com ácidos nucleicos e a sua capacidade de peroxidar lípidos é controversa (Valko et al., 2007).

O radical $HO^{\bullet}$, a forma neutra do ião hidróxido, apresenta um tempo de meia-vida próximo de nano segundos e uma elevada reactividade, que o torna um radical muito perigoso (Pastor et al., 2000). A regulação celular do ferro é

responsável por não haver ferro livre. No entanto, sob condições de stresse, um excesso de $O_2^{\bullet-}$ liberta ferro de moléculas contendo este metal e facilita a produção de $HO^{\bullet}$ a partir de H_2O_2, ao tornar o Fe^{2+} disponível para a reacção de Fenton (Leonard et al., 2004; Valko et al., 2005). O radical $O_2^{\bullet-}$ participa na reacção de Haber-Weiss que combina a reacção de tipo Fenton e a redução de Fe^{3+} pelo $O_2^{\bullet-}$, originando Fe^{2+} e O_2. Deste modo, quando produzido *in vivo*, o radical $HO^{\bullet}$ reage indiscriminadamente, próximo do seu lugar de formação, com componentes celulares como lípidos das membranas biológicas, proteínas e ADN (Richter, 1987; Stadtman e Levine, 2000; Jackson e Loeb, 2001).

6. Biotransformação de xenobióticos

Nos peixes, após a entrada dos xenobióticos no organismo (que pode ocorrer através dos sistemas respiratório e gastrointestinal, bem como pela pele) o sistema circulatório é capaz de os transportar para um local onde exercerão a sua acção tóxica ou para locais de biotransformação (fígado, guelras, mucosa intestinal e rim) onde poderão ser submetidos a uma série de reacções sequenciais conducentes à sua activação, desintoxicação, acumulação (fígado, rim, bílis, tecido adiposo) e excreção (através das superfícies respiratórias e pele bem como pela urina, bílis e fezes). O processo de biotransformação, de um ponto de vista fisiológico/toxicológico, tem como principal função a conversão de xenobióticos lipofílicos em produtos mais hidrossolúveis, facilitando a sua conjugação e respectiva excreção, reduzindo, deste modo, a sua actividade biológica. Este processo pode ser estruturado em reacções de fase I e reacções de fase II (Williams, 1959), 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 numa metabolização adicional dos produtos resultantes das reacções de conjugação da fase II catalisadas por enzimas também envolvidas nas reacções de fase I e/ou fase II (Vermeulen, 1996). Apesar da maioria dos xenobióticos serem submetidos

consecutivamente às reacções de fase I e de fase II, alguns podem ser sujeitos apenas a uma dessas fases.

Os mecanismos de fase I de biotransformação incluem reacções de oxidação, redução ou hidrólise que podem activar, inactivar ou deixar a actividade tóxica de substratos xenobióticos inalterada. As oxidações, por monooxigenases associadas ao citocromo P450, ocorrem no retículo endoplasmático liso das células, através da inserção de um átomo de oxigénio no substrato (Nelson et al., 1996), encontrando-se especialmente concentradas no fígado (Stegeman e Hahn, 1994), embora também detectadas no rim, tracto intestinal e tecido branquial (Varanasi, 1989). Na fase II da biotransformação, os produtos de fase I e outros xenobióticos contendo grupos funcionais (como hidroxilo, amino, epóxido, ou halogéneo) sofrem reacções de conjugação com compostos polares endógenos, tais como o ácido glucurónico, o sulfato, a glutationa ou aminoácidos (glicina, glutamina) (Timbrell, 1991). Os produtos de conjugação, com raras excepções, são menos tóxicos, mais polares e mais facilmente excretáveis que os compostos químicos parentais.

É importante realçar que, apesar da maior parte dos compostos serem desintoxicados e inactivados por estas reacções, alguns, que *per se* não são tóxicos, podem ser bioactivados originando intermediários reactivos com potencial toxicidade. Os xenobióticos podem igualmente causar o aumento da produção de ERO nas células por processos como a inibição do transporte de electrões nas mitocôndrias e consequente acumulação de intermediários reduzidos (Nohl et al., 1981), inactivação de enzimas antioxidantes (Odajima e Yamazaki, 1972; Kono e Fridovich, 1983) e depleção de sequestradores de radicais (Winston e Di Giulio, 1991; Hasspieler et al., 1994). Compostos como metais de transição, quinonas, bipiridilos, herbicidas e compostos nitroaromáticos são capazes de entrar em ciclo redox (que envolve a redução univalente do xenobiótico a um intermediário reactivo, por enzimas como a xantina oxidase e NADPH citocromo P450 redutase, o qual transfere um electrão para o O_2, produzindo $O_2^{\bullet -}$ e regenerando-se o composto parental) e causar stresse oxidativo (Kappus e Sies, 1981). O ciclo redox consome equivalentes celulares redutores, como o NADPH, o que poderá ter consequências ao nível de outros processos metabólicos (Kappus e Sies,

1981). O ciclo pode ser repetido até que o metabolito radical forme um aducto com uma macromolécula endógena (por exemplo, ADN ou enzima) ou seja conjugado e eliminado.

7. Antioxidantes celulares e seus mecanismos de acção

De acordo com Halliwell e Gutteridge (2007), um antioxidante é "qualquer substância que atrase, previna ou remova dano oxidativo de uma molécula alvo". Em organismos aeróbicos saudáveis, a produção de espécies reactivas é contrabalançada pelos sistemas de defesa antioxidante. No entanto, a manutenção de níveis elevados de defesas antioxidantes tem custos energéticos, podendo ser mais rentável a reparação ou substituição de biomoléculas danificadas (Halliwell e Gutteridge, 2007). Para além disso, as defesas antioxidantes podem ser incapazes de interceptar certas espécies reactivas como o radical $OH^{\bullet}$.

Nos sistemas biológicos, a protecção antioxidante pode exercer-se a diversos níveis, ao longo duma sequência oxidativa. Assim, antioxidantes preventivos como a SOD, catalase (CAT) e glutationa peroxidase (GPx) podem prevenir a iniciação de reacções em cadeia, captando radicais iniciadores ou evitando a sua formação, através da quelatação de metais e remoção dos compostos precursores. Enzimas como glutationa redutase (GR) e dihidroascorbato redutase, envolvidos na redução de formas oxidadas de pequenos antioxidantes moleculares ou enzimas responsáveis pela manutenção dos grupos tiólicos das proteínas como tioredoxina redutase, são igualmente considerados antioxidantes de prevenção. O processo oxidativo pode ser interrompido por antioxidantes que reajam com radicais propagadores das reacções em cadeia no meio aquoso (ascorbato, urato e glutationa reduzida - GSH) ou no interior das membranas (tocoferois, flavonoides, carotenoides e ubiquinol) formando um produto estável, designando-se por antioxidantes de quebra de cadeia. Para além da prevenção e da intercepção, a protecção também se pode fazer a nível da reparação. Neste último mecanismo, incluem-se enzimas

reparadoras que têm como finalidade reparar lesões oxidativas nos lípidos, proteínas e ADN, através da remoção e substituição de moléculas lesadas por novas moléculas. Muitos antioxidantes têm mais de um mecanismo de acção e a sua importância relativa depende da natureza do oxidante e do tipo de molécula alvo a ser protegida.

Num organismo, a capacidade antioxidante depende não só do papel específico de cada mecanismo antioxidante, como também da cooperação entre os mesmos. Para além da taxa de produção de ERO, o nível de lesão celular oxidativa motivada pelas ERO está também intimamente dependente da capacidade de defesa orgânica dos diferentes agentes antioxidantes de prevenção ou de intercepção, assim como da capacidade celular de reparação do hipotético dano sofrido. A forma como as defesas se complementam difere não só entre os organismos ou tecidos, mas também entre os compartimentos celulares (Sen, 2001).

7.1. Antioxidantes não enzimáticos

Os sistemas antioxidantes não enzimáticos são essencialmente substâncias de baixo peso molecular tal como as vitamina E e C, β-caroteno e glutationa (Kohen e Nyska, 2002). A vitamina E é uma vitamina lipossolúvel que, por se encontrar nas membranas, previne mais facilmente a peroxidação lipídica (PL). Esta vitamina pode ser encontrada em 8 formas diferentes, das quais o α-tocoferol é a forma mais activa em humanos. As ERO reagem com a vitamina E, formando um radical fenólico pouco reactivo, o qual vai reagir com a vitamina C sendo regenerado à sua forma inicial. Tal como o radical da vitamina E, o radical da vitamina C não é uma espécie reactiva porque o seu electrão desemparelhado é energeticamente estável (Fang et al., 2002; Hensley et al., 2004). Estes radicais são reduzidos a vitamina C e E pela glutationa. No caso do radical vitamina E, a glutationa pode reduzir directamente o radical tocoferol ou indirectamente via redução de semi-desidroascorbato para ascorbato.

A glutationa, o tiol não-proteico mais abundante em quase todas as espécies aeróbicas, ocorre em concentrações intracelulares de 0,5 a 10 mM

(Meister e Anderson, 1983; Wang e Ballatori, 1998). A glutationa é um tripeptídeo (L-γ-glutamil-L-cisteinil-glicina) que intervém directa ou indirectamente em muitos processos fisiológicos importantes. Este composto é sintetizada no citosol a partir dos seus três aminoácidos precursores (L-glutamato, L-cisteína e glicina) em todas as células (Meister e Tate, 1976), sendo o fígado o local de biossíntese de maior importância (DeLeve e Kaplowitz, 1991). Entre as funções conhecidas deste composto tiólico destacam-se a desintoxicação de radicais livres, metais e outros compostos electrofílicos, síntese de proteínas e ADN, manutenção da função imune, transporte de aminoácidos, co-factor essencial de diversas enzimas e forma de armazenamento de cisteína não tóxica (Meister e Anderson, 1983; Ballatori, 1994; Sen e Packer, 1996; Wang e Ballatori, 1998).

O papel importante da glutationa na defesa contra lesões induzidas por radicais tem por base o fornecimento de equivalentes redutores para várias enzimas fundamentais, bem como a capacidade de reagir directamente com radicais como $O_2^{\bullet-}$ e $HO^{\bullet}$ por um processo de transferência de radicais, originando o radical glutationilo, $GS^{\bullet}$ e eventualmente glutationa disulfeto (GSSG). O $GS^{\bullet}$ é um oxidante forte que pode sofrer várias reacções biologicamente relevantes como a dimerização (para formar GSSG); abstracção de átomos de H de ácidos gordos poli-insaturados formando $ROO^{\bullet}$; reacção de conjugação com o dioxigénio para formar radical peroxiglutationilo (o qual pode reagir com a GSH ou então sofrer uma reacção de terminação com outros radicais presentes); conjugação com a própria GSH (originando o radical $GSSG^{\bullet}$, que pode reagir rapidamente com o dioxigénio, formando GSSG e $O_2^{\bullet-}$ pelo que, neste caso, para haver um efeito protector da GSH, é igualmente necessária a presença de SOD para reduzir o $O_2^{\bullet-}$). A exposição de teleósteos a contaminantes ambientais com potencial oxidativo demonstrou a capacidade destes compostos influenciarem os níveis de glutationa (Ahmad et al., 2005; Gravato et al., 2006). Actualmente, os níveis de antioxidantes não enzimáticos (tióis não proteicos e glutationa) têm sido utilizados como biomarcadores de contaminação ambiental no meio aquático (Sayeed et al., 2003; Parvez e Raisuddin, 2006; Ahmad et al., 2008; Maria et al., 2009).

As metalotioninas (MTs) são proteínas citosólicas de baixo peso molecular, ricas em cisteína e desprovidas de aminoácidos aromáticos e histidina, capazes

de se ligarem a metais. O comportamento das MTs é dominado pela química do grupo sulfidrilo (-SH), que lhes confere capacidade antioxidante e de sequestro de radicais livres (Atif et al., 2006). As funções biológicas das MTs incluem a homeostasia de metais fisiologicamente importantes (Cu, Zn), desintoxicação de metais essenciais e não essenciais (como o Cd e Hg) e defesa antioxidante (Roesijadi, 1996; Viarengo et al., 2000; Amiard et al., 2006). O papel das MTs na defesa antioxidante deve-se não só à capacidade de sequestrador de radicais de oxigénio, mas também às dinâmicas de ligação/libertação de metais (Viarengo et al., 2000), limitando a disponibilidade dos catiões em locais indesejados (Langston et al., 1998). Apesar de vários estudos terem evidenciado que as MTs são um bom biomarcador de exposição a metais (Viarengo et al., 1999; Cajaraville et al., 2000; Oliveira et al., 2008), a sua síntese pode ser induzida por stresse oxidativo, assim como por algumas hormonas e tratamentos físicos e químicos (Kagi e Schaffer, 1988).

7.2. Antioxidantes enzimáticos

Entre as defesas antioxidantes enzimáticas, as enzimas com um papel mais relevante são a SOD, GPx e CAT. A SOD é uma metaloenzima que catalisa a dismutação do $O_2^{\bullet -}$. Existem três tipos de SOD consoante o grupo prostético que possuem: a SOD-CuZn, que é a enzima característica do citosol dos eucariontes; o SOD-Mn que existe nos procariontes, eucariontes inferiores e matriz mitocondrial dos eucariontes superiores; e o SOD-Fe característico dos procariontes. Existe ainda uma quarta enzima (enzima de Marklund), em concentração baixa no plasma, ligado ao endotélio vascular, denominada SOD extracelular, que é uma glicoproteína com Cu e Zn, mas distinto da enzima intracelular. Esta enzima também está distribuída heterogeneamente nos tecidos animais em baixas concentrações. O mecanismo provável de catálise na SOD-CuZn envolve os iões cobre que participam na reacção, sofrendo alternadamente reduções e oxidações. O Zn não funciona directamente no processo catalítico, servindo apenas para estabilizar a enzima.

A catalase (CAT) é uma proteína hémica que catalisa a redução do H_2O_2 a H_2O com libertação de O_2. Esta enzima contém 4 subunidades, cada uma com um grupo hémico (Fe(III)-protoporfirina) no centro activo. O centro activo de cada subunidade está estabilizado por uma molécula de NADPH fortemente ligada. A actividade da CAT das células eucariontes está localizada nos peroxissomas, organitos que contêm muitos das enzimas que geram H_2O_2. O mecanismo da reacção envolve a formação dum intermediário com o ferro hémico sob a forma dum complexo oxoferrilo e com um radical catião na proteína, o chamado composto I (Catalase-$[Fe(IV)=O]^{\bullet+}$). No mecanismo de catálise, o ferro hémico cede 2 electrões ao H_2O_2, formando uma espécie intermediária que formalmente é Fe(V)=O. Esta espécie, contudo, está sempre presente como Fe(IV)=O sendo uma carga negativa removida da proteína. Em alternativa, pode formar-se um peróxido férrico (Fe(III)-HOOH), que simultaneamente origina Fe(IV)=O e um radical catião proteico. A partir do composto I, o ciclo reaccional é completado (regresso do Fe ao estado Fe(III)) através da transferência de 2 electrões num único passo provenientes da oxidação de uma molécula de H_2O_2. Em alternativa, o radical catião no composto I pode ser reduzido através da transferência de 1 electrão produzindo Fe(IV)=O, o qual é então reduzido a Fe(III).

A glutationa peroxidase (GPx), fundamentalmente localizada no citosol das células eucariontes mas podendo igualmente ocorrer nas mitocôndrias, é uma enzima que contém selénio no centro activo, sob a forma duma selenocisteína. Esta enzima tem como co-substrato a glutationa (GSH) e catalisa a redução do H_2O_2 a H_2O e a redução peróxidos orgânicos aos respectivos álcoois. O seu mecanismo é muito complexo e só está parcialmente compreendido. A enzima funciona num ciclo com a glutationa redutase que usa equivalentes redutores sob a forma de NADPH para reduzir GSSG. Os sítios ligantes para GSSG da redutase estão situados na interface das duas subunidades que constituem a estrutura quaternária, sendo que cada uma tem um domínio distinto com um sítio ligante para o NADPH. O grupo sulfidrilo de um dos resíduos de cisteína, presentes no local activo da GR, ataca a ligação S-S, libertando uma molécula de GSH e um dissulfeto misto, GSSGR. Um ataque intramolecular do grupo sulfidrilo do segundo resíduo de cisteína liberta a segunda molécula de GSH, formando o

dissulfeto cíclico. A conversão do dissulfeto cíclico em glutationa redutase é realizada à custa da conversão de NADPH para $NADP^+$.

Existem igualmente outras enzimas com actividade de glutationa peroxidase que não são selenoproteínas, sendo designados de glutationa peroxidases não dependentes de selénio. Estas enzimas, GST de classe α (Wang e Ballatori, 1998), também têm actividade de peroxidase – ao nível do citosol e das membranas (microssomal, mitocondrial e citoplasmática) – parecendo ser extremamente importantes em situações em que há deficiência em selénio. Deste modo, as GSTs podem actuar quer como enzimas de fase II (transferases), quer como peroxidases (Shan et al., 1990; Radi, 1993; Wang e Ballatori, 1998). A função transferase da GST está envolvida na desintoxicação de uma grande variedade de compostos, catalisando o ataque nucleofílico da glutationa a compostos apolares que contêm átomos electrofílicos de carbono, azoto ou enxofre (Hayes et al., 2005). Os substratos (electrofílicos) mais comuns das GSTs incluem haletos de alquilo, epóxidos, compostos α,β-insaturados (como quinonas, aldeídos, cetonas e ésteres), haletos de arilo e nitro aromáticos (Armstrong, 1997a; Sheehan et al., 2001). As enzimas citosólicas estão classificadas de acordo com os seus pontos isoeléctricos, especificidade de substrato, sensibilidade a inibidores, reactividade imunológica e composição e sequência de aminoácidos em α, μ, π, θ ou ζ (Meyer et al., 1991; Armstrong, 1997b; Board et al., 1997). Os membros de cada classe apresentam tamanhos de monómeros semelhantes (cerca de 24–28 kDa), partilham uma elevada identidade de sequência de aminoácidos (tipicamente 60–80%) e apresentam especificidades características e sobrepostas de substratos. Por exemplo, a classe α das GSTs é altamente reactiva para hidroperóxido de cumeno (Mannervik et al., 1985; Meyer et al., 1991), a classe μ têm elevada reactividade para epóxidos (Gadagbui e James, 2000), a classe π para o ácido etacrínico e (+)anti -7ß,8a-dihidroxi-9a,10a-epoxi-7,8,9,10-tetrahidrobenzo[a]pireno (Gadagbui e James, 2000), a classe θ para o brometo 1-(2-bromoetil)-4-nitrobenzeno (Meyer et al., 1991). A GST apresenta dois locais activos por dímero, que se comportam independentemente. Cada local activo consiste em pelo menos duas regiões de ligação de ligandos. O local de ligação da GSH é muito específico para este tripéptido, enquanto que o

local de ligação de substratos electrofílicos é menos específico (Danielson e Mannervik, 1985).

Algumas enzimas antioxidantes como a CAT, GR, GPx têm sido propostas como biomarcadores de exposição a poluição em organismos aquáticos (Winston e Di Giulio, 1991; van der Oost et al., 2003; Santos et al., 2004; Valavanidis et al., 2006; Ahmad et al., 2008; Maria et al., 2009).

8. Peroxidação lipídica (PL)

A membrana plasmática constitui um local crítico de reacções radicalares. Os radicais gerados extracelularmente têm de atravessar a membrana, podendo aí desencadear reacções radicalares, quer com os ácidos gordos insaturados quer com aminoácidos oxidáveis de proteínas transmembranares, antes de reagirem com outros componentes celulares. O H_2O_2 atravessa a membrana tão livremente como a H_2O enquanto que o radical $O_2^{\bullet -}$ só a atravessa através de canais aniónicos. Porém, a forma protonada do $O_2^{\bullet -}$, o $HO_2^{\bullet}$, cuja formação é favorecida devido ao pH ácido junto à membrana, tem uma melhor partição na dupla camada lipídica e é mais oxidante.

Os alvos da peroxidação lipídica (PL) são os lípidos das membranas que envolvem as células e os organelos celulares, sendo as membranas das células animais mais susceptíveis a PL por terem, entre outros constituintes, ácidos gordos insaturados como o ácido linoleico, linolénico, araquidónico e docosahexaenoico. Quando os lípidos da bicamada fosfolipídica são alterados oxidativamente, para além da formação de espécies moleculares potencialmente nocivas, é alterada a fluidez da membrana, o que pode levar a alterações nas funções celulares e mesmo a morte celular (Gutteridge, 1995).

A oxidação dos lípidos ocorre através dum conjunto de reacções em cadeia que incluem várias fases: iniciação, propagação e terminação. Este processo é iniciado por radicais que abstraem um átomo de hidrogénio alílico ou bisalílico (átomos de hidrogénio ligados a átomos de carbono em posição adjacente a uma ou duas duplas ligações) dum ácido gordo insaturado (LH). O radical lipídico ($L^{\bullet}$)

resultante sofre um rearranjo das duplas ligações que leva à formação de dienos conjugados. Em seguida, o radical $L^{\bullet}$ reage rapidamente com o O_2 formando o radical peroxilo ($LOO^{\bullet}$) que, se não for neutralizado pelas defesas antioxidantes, faz a abstracção de outro átomo de hidrogénio dum outro ácido gordo, levando à formação de hidroperóxidos lipídicos (LOOH), os quais podem facilmente ser decompostos para formar novamente radicais $L^{\bullet}$. Estes radicais reagem com o dioxigénio para formar mais $LOO^{\bullet}$ continuando, assim, a reacção em cadeia da PL (fase de propagação). Os $LOO^{\bullet}$, derivados de ácidos gordos com três ou mais ligações insaturadas, podem sofrer reacções de ciclização originando endoperóxidos cíclicos, os isoprostanos, que, por decomposição termolítica ou hidrolítica, formam malonildialdeído (MDA) (Fink et al., 1997; Marnett, 1999).

Os hidroperóxidos, resultantes da PL, são decompostos por reacções de oxidação ou redução com iões de metais de transição (Fe^{3+} ou Cu^{2+}) em radicais alcoxilo ($LO^{\bullet}$) e peroxilo ($LOO^{\bullet}$). A velocidade da reacção que origina $LO^{\bullet}$ é, porém, mais elevada podendo iniciar novas reacções em cadeia.

Os radicais alcoxilo também podem sofrer reacções (cisão β), originando alcanos e aldeídos como, por exemplo, o 4-hidroxinonenal (HNE) e o MDA.

Na fase de terminação, inclui-se o conjunto de reacções que levam à eliminação dos radicais lipídicos. Para além das reacções entre dois radicais, é particularmente importante a reacção entre um radical e um antioxidante.

A detecção da PL faz-se medindo os produtos formados como, por exemplo, dienos conjugados, hidroperóxidos, alcanos (etano e pentano), aldeídos (MDA e HNE) e isoprostanos. Normalmente, quantificam-se os níveis de produtos secundários da oxidação, dado que os produtos primários são instáveis. Um dos métodos mais frequentemente usados para a determinação da PL baseia-se na reacção do MDA com duas moléculas ácido tiobarbitúrico (sob aquecimento em condições ácidas).

A maioria dos compostos resultantes da PL são tóxicos e mutagénicos activos (Esterbauer et al., 1990; Porter et al., 1995; d'Ischia et al., 1996), podendo formar aductos de ADN e originar mutações e padrões de expressão génica alterados (Marnett, 1999). O MDA pode provocar o encruzamento e a polimerização de componentes da membrana, alterando as suas propriedades

intrínsecas tais como a plasticidade, o transporte iónico e a actividade de enzimas. Os aldeídos podem, igualmente, difundir-se da membrana e atingir outros alvos na célula exercendo efeitos citopatológicos.

9. Danos no ADN

O ADN nos organismos aeróbicos está sempre a ser danificado e reparado, mesmo na ausência de exposição a substâncias genotóxicas. Os processos causadores de dano incluem fontes endógenas – como instabilidade do ADN (exemplo, depurinação), erros espontâneos durante a replicação e reparação do ADN, numerosas ERO fisiológicas resultantes do metabolismo do oxigénio e produtos da PL – e fontes exógenas, como radiação e xenobióticos. Apesar dos sistemas biológicos estarem protegidos por antioxidantes e por mecanismos de reparação do ADN, que reduzem substancialmente os níveis de dano, existe sempre um nível basal (Gupta e Lutz, 1999).

Muitos compostos xenobióticos ou os seus metabolitos apresentam propriedades genotóxicas, que lhes permitem induzir alterações no material genético. Alguns compostos podem formar uma ligação covalente ao ADN produzindo aductos (Dolcetti et al., 2002; Hellou et al., 2006), os quais podem ser responsáveis por mutações impedindo a replicação fidedigna do ADN ou desencadeando processos de reparação do mesmo, nem sempre efectivos (Maccubbin, 1994). Existem igualmente outras alterações estruturais como quebras de cadeia de ADN, perdas de bases, hipometilação e mutações que sugerem a exposição a compostos genotóxicos.

De entre as diferentes metodologias disponíveis para a avaliação de dano genético, a avaliação das quebras de cadeia de ADN conjuntamente com as anomalias nucleares eritrocíticas (ANE) demonstraram elevada sensibilidade à exposição a compostos xenobióticos, fornecendo informações relativamente a danos moleculares (facilmente reparáveis – quebras de cadeia) e danos sub-celulares (irreversíveis – anomalias) (Oliveira et al., 2008). A integridade do ADN, avaliada pelo método de desenrolamento alcalino, demonstrou sensibilidade dos

peixes a danos genotóxicos induzidos por compostos como HAPs (Maria et al., 2002), constituintes de efluentes da pasta de papel como ácidos resínicos e reteno (Maria et al., 2004a,b; 2005) e metais (Ahmad et al., 2006; Oliveira et al., 2008). Os efeitos genotóxicos dos compostos presentes em zonas portuárias (Maria et al., 2004c), locais contaminados por metais e pesticidas (Maria et al., 2006) e por efluentes de pasta de pasta de papel (Maria et al., 2004d; Maria et al., 2009) foram igualmente demonstrados através desta técnica. As ANE, por seu lado, têm assumido uma grande relevância devido à facilidade e rapidez de execução, sensibilidade, baixo custo e fiabilidade demonstrada em diferentes espécies de peixes expostos a metais (Ayllon e Garcia-Vazquez, 2000; Oliveira et al., 2008), HAPs (Gravato e Santos, 2002; Maria et al., 2002; Pacheco e Santos, 2002), efluentes de pasta de papel (Maria et al., 2004d), efluentes de uma refinaria de petróleo (Çavas e Ergene-Gözükara, 2005) e locais contaminados por metais (Guilherme et al., 2008a).

O ADN é um componente celular chave particularmente susceptível a lesões por ERO (Cerutti, 1985), as quais, na sua maioria, parecem ser mediadas pelo $HO^{\bullet}$. A heterogeneidade das moléculas de ADN permite o ataque de $HO^{\bullet}$ a bases nucleicas e ao esqueleto de desoxirribose (Buxton et al., 1988). O radical $HO^{\bullet}$ pode abstrair átomos de H da desoxirribose, podendo levar à libertação de bases purinas e pirimidinas e à formação de clivagens numa das cadeias do ADN (Halliwell e Gutteridge, 1999). As cisões nas cadeias simples podem ser reparadas através duma série de mecanismos. Pelo contrário, quando as duas cadeias são quebradas no mesmo local, forma-se uma cisão dupla que não pode ser adequadamente reparada. A mutação no ADN é um passo crucial na carcinogénese e elevados níveis de dano oxidativo no ADN foram detectados em vários tumores, implicando este tipo de dano na etiologia do cancro.

A interacção de $OH^{\bullet}$ com nucleobases da cadeia de ADN, como a guanina, leva à formação de C8-hidroxiguanina ou à sua forma nucleósido (8-hidroxi-2'desoxiguanosina - OHdG). Inicialmente ocorre a formação de aductos, radicais que, por eliminação de um electrão, originam a 8-hidroxi-2'desoxiguanosina (8-OHdG). A 8-OHdG sofre tautomerismo ceto-enólico que favorece o produto oxidado 8-oxo-7,8-dihidro-2'-desoxiguanosina. Experiências demonstraram que o

potencial mutagénico da 8-OHdG tem por base a perda da especificidade do emparelhamento de bases e leitura errada de pirimidinas adjacentes (Kasai, 2002). As mutações que podem ocorrer devido à formação da 8-OHdG envolvem transversões GC-TA (Cheng et al., 1992; Lunec et al., 2002), após dois ciclos de replicação, dado que este tipo de dano permite que a guanina emparelhe com a adenina (Ohno et al., 2006). Esta lesão pode ser hidrolisada por uma glicosilase específica e, uma vez que não é posteriormente metabolizada, vai aparecer no plasma e urina, constituindo um bom marcador de stress oxidativo.

Apesar do grande número de estudos sobre danos no ADN induzidos por compostos que geram stresse oxidativo em mamíferos, poucos estudos abordaram o dano oxidativo do ADN em peixes. A literatura disponível sugere que a ocorrência de dano oxidativo pode ser dependente da espécie (Charissou et al., 2004), enfatizando a necessidade de avaliar a sua aplicabilidade como biomarcador em peixes.

10. Ria de Aveiro

A Ria de Aveiro é uma laguna costeira localizada na costa portuguesa (40°38'N, 8"44'W), sendo formada por um complexo de canais caracterizados pela existência de zonas intertidais consideráveis. A Ria apresenta condições naturais adequadas para actividades portuárias, tráfego naval e recreação. Este sistema comunica com o Oceano Atlântico através de um canal artificial (1.3 km de comprimento, 350 m de largura e 20 m de profundidade) apresentando uma largura máxima de 10 Km e comprimento de 45 km. Na Primavera, a área é de 83 km^2 na maré-alta, sendo reduzida para 66 km^2 na maré-baixa. A profundidade média da lagoa é de aproximadamente 1 m, mas no canal localizado perto da entrada da Ria é de cerca de 20 m. Nos outros canais de navegação, onde são frequentemente executadas operações de dragagem, a profundidade é de cerca de 7 m (Dias et al., 2000). As principais fontes de água doce são os rios Antuã (5 m^3/s) e Vouga (50 m^3/s) (Dias et al., 1999). Dois outros rios pequenos, Boco e Caster, contribuem com um fluxo médio inferior a 1 m^3/s (Lopes et al., 2005).

A Ria de Aveiro apresenta uma elevada produtividade, com abundantes populações de invertebrados e peixes, muitos dos quais utilizados como alimento humano. Algumas áreas específicas da Ria têm sido alvo de descargas domésticas e industriais e de actividade agrícola. No entanto, a pesca profissional é uma actividade tradicional, tendo igualmente estado a ser implementada a aquacultura.

A contaminação da Ria de Aveiro resulta de uma enorme variedade de fontes antropogénicas que inclui 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, dada a ocorrência de descargas ocasionais, assim como a persistência de alguns dos contaminantes, a biomonitorização regular dos principais pontos críticos da Ria de Aveiro é de grande relevância.

11. A utilização de peixes em estudos ecotoxicológicos

Os peixes apresentam um elevado grau de heterogeneidade em termos anatómicos, fisiológicos, comportamentais e ecológicos (Lagler et al., 1977; Janz, 2000). Tendo em conta todas as espécies de vertebrados, os peixes representam aproximadamente metade (48%) da diversidade de espécies, sendo 95 % dessa diversidade devida aos teleósteos (Lagler et al., 1977; Janz, 2000). A utilização dos peixes como modelos biológicos de vida aquática tem sido importante para a compreensão dos fundamentos de áreas tão diversas como neurobiologia, endocrinologia, biologia do desenvolvimento e adaptação bioquímica (Powers, 1989; Eisen, 1996).

A variedade de espécies de peixes, assim como de habitats, são vantagens deste modelo biológico, úteis na investigação científica. Em estudos de monitorização ambiental, para ser considerada um bom bioindicador, uma espécie deve (Zhou et al., 2008):

- Poder acumular grande quantidade de poluentes sem morrer;
- Ser representativo de um determinado local;
- Apresentar uma elevada abundância e distribuição, permitindo repetição de amostragens;
- Apresentar uma longevidade que permita comparação entre diferentes idades;
- Apresentar abundância em termos de tecido/células que permitam uma análise a níveis microcósmicos;
- Ser de fácil amostragem e manutenção laboratorial;
- Apresentar posição importante na cadeia alimentar;
- Apresentar uma boa relação dose-efeito.

Dado ser extremamente difícil encontrar um bioindicador que apresente todas estas características, a selecção do bioindicador deve ter em conta os objectivos específicos da biomonitorização (Zhou et al., 2008). Nesta perspectiva, tendo em conta os critérios acima mencionados e os objectivos do presente trabalho, seleccionou-se a tainha-garrento (*Liza aurata*) como organismo principal do presente estudo.

As tainhas pertencem à família Mugilidae, ordem Perciformes, sendo peixes cosmopolitas que se podem encontrar em águas tropicais e temperadas, (Pombo et al., 2005) onde desempenham um importante papel ecológico (Cardona, 2001; Almeida, 2003). Estes organismos são eurialinos, podendo ser encontrados ao longo da costa, em estuários e lagunas, em locais com diferentes salinidades (Cardona, 2006). *L. aurata* tem grande importância económica em algumas zonas tropicais e subtropicais (Hotos et al., 2000; Boglione et al., 2006; Masmoudi et al., 2007), não o tendo, no entanto, em Portugal. As tainhas são caracterizadas por uma grande capacidade de adaptação do regime alimentar de acordo com a disponibilidade trófica (Boglione et al., 2006). Esta espécie alimenta-se essencialmente de organismos bentónicos e detrito/lama. Estudos prévios avaliaram a sensibilidade de *L. aurata* em termos de actividade de EROD, ANE e stresse oxidativo (Pacheco et al., 2005; Guilherme et al., 2008a,b) sugerindo a sua utilização em estudos de biomonitorização.

12. Orientações e objectivos da tese

Neste trabalho, avaliaram-se as respostas da tainha-garrento (*Liza aurata*) à exposição a xenobióticos, utilizando duas metodologias: ensaios laboratoriais e colheitas de organismos residentes na Ria de Aveiro.

Numa primeira abordagem, foi avaliada a resposta da *L. aurata*, em termos de capacidade de biotransformação, respostas de stresse, defesas antioxidantes e danos genéticos, face a uma exposição laboratorial de curta duração a um composto orgânico muito abundante nos sistemas aquáticos contaminados, o fenantreno (HAP de três anéis, incluído na lista dos 16 HAPs prioritários da Environmental Protection Agency – Estados Unidos). Esta avaliação foi efectuada mediante exposição laboratorial, dado que permite um controlo de variáveis relativas à qualidade da água (tais como a temperatura, salinidade, oxigénio dissolvido), assim como outros factores abióticos. Face à sensibilidade demonstrada por esta espécie em termos dos parâmetros determinados, procedeu-se à monitorização de um sistema lagunar costeiro, a Ria de Aveiro, recorrendo a indivíduos residentes. Deste modo, pretendeu-se avaliar a adequabilidade da *L. aurata* como bioindicador em sistemas aquáticos com misturas de contaminantes, tentando, com recursos a biomarcadores de diferentes níveis de organização biológica, identificar relações causa/efeito num ambiente natural. O presente trabalho visou igualmente caracterizar o estado de contaminação deste sistema aquático.

Os organismos utilizados no presente trabalho 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.

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CAPÍTULO II

Citocromo P4501A, respostas genotóxicas e de stresse na tainha-garrento (*Liza aurata*) após exposição de curta duração a fenantreno

Cytochrome P4501A, genotoxic and stress responses in golden grey mullet (*Liza aurata*) following short-term exposure to phenanthrene

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Abstract

This study represents a first approach to short-term effects of phenanthrene (Phe) in fish. The teleost *Liza aurata* was exposed to 0.1 - 2.7 µM Phe during 16 hours. CYP1A induction was assessed as liver ethoxyresorufin-O-deethylase (EROD) activity. Genotoxicity was evaluated in gills and liver as DNA integrity (by alkaline unwinding), whereas in blood the erythrocytic nuclear abnormalities (ENA) frequency was determined. Stress responses were determined as cortisol, glucose and lactate plasma levels. Liver EROD activity was significantly increased by Phe 0.3 - 2.7 µM. Phe genotoxicity in gills was not found, whereas liver DNA integrity significantly decreased after exposure to Phe 0.1 and 0.9 µM demonstrating its genotoxicity which did not correlate with liver CYP1A induction. Phe genotoxicity in blood was demonstrated by a significant ENA increase from 0.1 up to 2.7 µM. In terms of stress responses, plasma cortisol was significantly increased by Phe 0.3 - 2.7 µM, though plasma glucose was only significantly increased by Phe 0.9 and 2.7 µM. The Phe observed effects on *L. aurata* detected at different levels demonstrate a physiological unbalance and a probable ecological risk to ichthyofauna.

Keywords: Phenanthrene; Fish; EROD; Genotoxicity; Endocrine disruption

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment both as natural products and as environmental contaminants. According to the United States Environmental Protection Agency (US EPA) Toxic Release Inventory an estimate of more than 5 million pounds of PAHs were released in the year 2000 (US EPA, 2000). Aquatic organisms, namely fish, living in PAH-contaminated environments may absorb these compounds mainly from the water through gills and by the ingestion of contaminated sediments or food (Varanasi et al., 1989). PAHs easily cross lipid membranes, due to their lipophilicity, and

bioaccumulate in different tissues. However, PAHs concentrations in fish tissues can be significantly reduced by metabolism and excretion (Billiard *et al*., 2002).

Phenanthrene (Phe), a three-ring PAH included in the 16 US EPA priority PAHs (Smith et al., 1989), is present in the aquatic environment as a result of both petrogenic and pyrogenic sources. Water runoff, atmospheric deposition and oil spills are the largest sources of aquatic Phe contamination (Lima et al., 2003; Incardona et al., 2005). Recent studies have shown that Phe inputs in the environment remained constant from 1980s until 1990s when abrupt and substantial increase in concentrations were observed (Lima et al., 2003). Phe water levels in polluted areas ranged from 14.6 µg/L (Vrana et al., 2001) to 1460 µg/L (Anyakora et al., 2005) in a fishing settlement near a crude oil exploration area and considerable levels were detected in aquatic organisms, namely fish (Hellou and Warren, 1997).

Despite the Phe abundance and ubiquity in the aquatic environment, only limited information is available concerning its toxicity in fish. Jee et al. (2004) and Jee and Kang (2003) reported toxic effects of waterborne Phe measured as growth impairment, haematological and acetylcholinesterase activity alterations in olive flounder. Moreover, dose dependent cardiac rhythm changes were observed in fish embryos (Incardona et al., 2004). Several antioxidant parameters were also affected in olive flounder and goldfish following Phe exposure (Jee and Kang 2005; Sun et al., 2006).

PAHs are typical inducers of cytochrome P450 related mixed function oxidases, namely CYP1A. However, a limited number of studies are available on Phe CYP1A modulation showing divergent results. Thus, Bols et al. (1999) observed no induction of CYP1A activity in a rainbow trout liver cell line. Moreover, *in vitro* and *in vivo* EROD (ethoxyresorufin *O*-deethylase) induction by other PAHs was inhibited by Phe (Hawkins et al., 2002). On the other hand, a significant CYP1A induction was observed in cod, flounder (Goksøyr et al., 1986), scup (Stegeman et al., 1998) and tilapia (Shailaja and D'Silva, 2003). Furthermore, Pangrekar et al. (1995; 2003) reported that brown bullhead liver microsomes metabolize Phe.

Fish CYP1A induction associated with DNA damage has been considered as sensitive and suitable approach for pollutant genotoxicity assessment (Goksøyr and Förlin, 1992; Maria et al., 2002). PAH reactive intermediates have the potential to interact with any nucleophilic sites within the cell, including those on proteins and DNA, inducing genetic damage (Stegeman and Lech, 1991).

Aquatic contaminants adversely affect fish endocrine system. Thus, alterations on specific hormonal functions may constitute important stress indicators (Hontela, 1997). Cortisol, a corticoid synthesised by the interrenal cells of the teleost kidney, plays a major role in the physiological response to stress (Waring et al., 1992). Monteiro et al. (2000) found a plasma cortisol increase in flounder after 12 weeks Phe exposure through feeding, suggesting a hypothalamo-pituitary-interrenal HPI axis disruption for the highest dose.

Previous studies (Maria et al., 2002; 2005; Teles et al., 2003) have demonstrated that European eel 16 hours exposure to PAHs such as benzo[a]pyrene, naphthalene and retene significantly increased liver EROD activity, genotoxicity in liver and blood as well as stress responses, namely at the intermediary metabolism level.

Liza aurata has been presented as a good bio-indicator species for monitoring estuarine water contamination by organic xenobiotics within a large lipophilicity range (Pacheco et al., 2005). *L. aurata* is a common European mugilide, widely distributed in both Atlantic and Mediterranean coastal waters, whose feeding behaviour is characterized by extensive foraging in the whole water column with regular contact with the sediment. Hence, this species was adopted in the current study on Phe effects. The aim of the current research work is to study *L. aurata* responses after 16 hours exposure to water born Phe (0.1 - 2.7 µM), in order to assess its impact on CYP1A measured as liver EROD activity, genotoxicity in different tissues evaluated as DNA strand breaks and erythrocytic nuclear abnormalities (ENA). General stress responses were also studied as plasma cortisol, glucose and lactate levels.

2. Material and Methods

2.1. Chemicals: β-nicotinamide adenine dinucleotide (β-NAD), glutamic pyruvic transaminase, L-lactic dehydrogenase and phenanthrene were purchased from Sigma (USA). All the other chemicals were of analytical grade. Marine salt was purchased from Sera Premium (France).

2.2. Experimental Animals: The experiment was carried out using juvenile *L. aurata* collected from the Aveiro lagoon — São Jacinto, Portugal. The sampling site has no known pollution sources and only trace levels of PAHs were detected (Pacheco et al., 2005). *L. aurata* caught in this area did not show any indication of exposure to pollutants (Pacheco et al., 2005). Fish with an average length of 15±5 cm and weighing 25±5 g were transported to the laboratory in oxygenated water and allowed to recover for 7 days in 80 L aquaria (six animals per aquarium – 1.9 g of fish weight per L), at 20 ºC, in aerated and filtered artificial saltwater (34 g/L) being fed with polichaet worms (Nereis sp.) collected at an Aveiro lagoon clean area, each two days. *L. aurata* were not fed during the exposure period to Phe.

2.3. Experimental Design: The experiment was carried out according to the water conditions and fish density adopted during the recovery period. *L. aurata* was exposed to clean water (control group) and 0.1, 0.3, 0.9 and 2.7 µM Phe during 16 hours. Blood was collected from the posterior cardinal vein using a heparinised Pasteur pipette and fish were killed by decapitation. Gills and liver were immediately sampled, frozen in liquid nitrogen and stored at -80 ºC until further treatments. 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 minutes and stained with Giemsa (5%) during 30 minutes.

2.4. Biochemical analysis

2.4.1. CYP1A response: Liver microsomes were obtained according to the methods of Lange et al. (1993) and Monod and Vindimian (1991) as adapted by Pacheco and Santos (1998). Liver EROD activity was measured as described by

Burke and Mayer (1974). Results were expressed as picomoles per minute per milligram of microsomal protein.

2.5. Protein measurement: Liver microsomal protein concentrations were determined according to the Biuret method (Gornal et al., 1949) using bovine serum albumin as a standard.

2.6. Genotoxicity tests: Genotoxicity was tested using the DNA alkaline unwinding assay (gill and liver) and the erythrocytic nuclear abnormalities (ENAs) frequency (blood).

The deep frozen portions of gill and liver were submitted to DNA isolation procedure (Genomic DNA Purification Kit, Fermentas). DNA integrity measurements were performed according to Rao et al. (1996) as previously adapted by Maria et al. (2002). DNA single and double strands were separated by hydroxyapatite (20%) 'batch'' elution and quantified with a DNA-specific fluorescent dye (2.5 nM bisbenzimide), after alkaline unwinding. Data from gill and liver DNA unwinding technique were expressed as DNA integrity (%), determined by applying the following equation:

[(Double stranded DNA)/(Double stranded DNA + Single stranded DNA)]×100.

For the ENA assay, blood smears were fixed with methanol during 10 minutes and stained with Giemsa (5%) during 30 minutes. In order to evaluate genotoxicity, 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.

2.7. Plasma cortisol measurement: The determination of plasma cortisol was performed using a diagnostic ELISA direct immunoenzymatic kit (Diametra,

Italy). The absorbance was measured at 450 nm in a microplate reader (ASYS Hitech). Cortisol plasma concentration is expressed as ng/mL.

2.8. 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 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 formation.

2.9. Statistical analysis: Results are expressed as means ± SE (standard error) corresponding to experimental groups of six fish (n=6). SIGMASTAT 2.03 software was used for statistical analysis. The experimental data were tested first for normality and homogeneity of variance to meet statistical demands and then with the Tukey test (Zar, 1999). Differences between means were considered significant at $P<0.05$.

3. Results

3.1. CYP1A Response

A general EROD activity increase trend was observed after Phe exposure (Figure 1). Thus, EROD activity significantly increased 130, 120 and 233% respectively after 0.3, 0.9 and 2.7 µM Phe exposure. Moreover, 0.3 and 2.7 µM Phe exposed groups revealed EROD levels significantly higher than 0.1 µM.

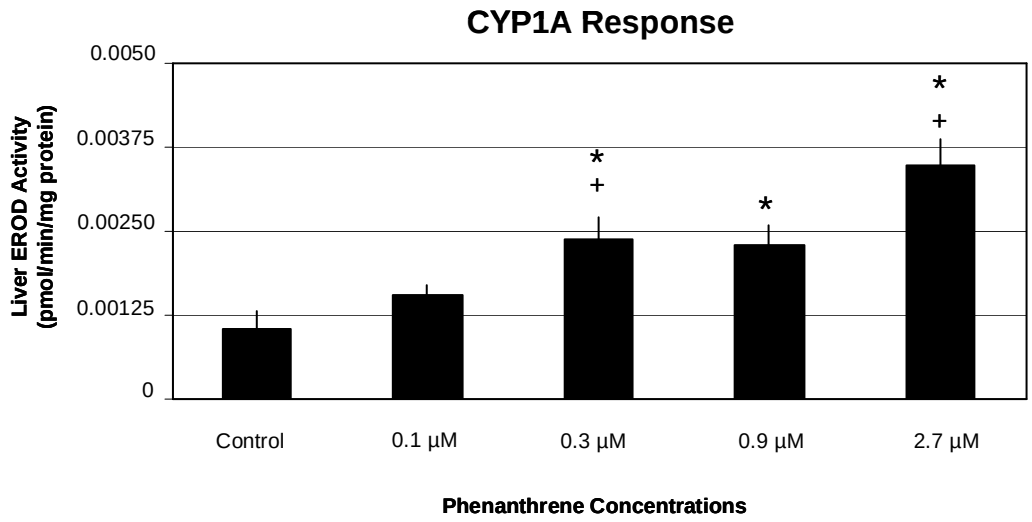

Figure 1 – CYP1A response (liver EROD activity) in *L. aurata* following 16 hours exposure to phenanthrene (Phe). Values represent mean ± standard error. Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe.

3.2. Genotoxic Responses

Gill DNA integrity was not significant altered by any Phe exposure concentration (Figure 2A). Though not significant in comparison with the control, the most pronounced decrease in gill DNA integrity was observed at 0.9 µM Phe exposure, representing a 12% reduction. However, liver displayed a significant DNA integrity loss in 0.1 and 0.9 µM Phe exposed groups when compared with control which represents a 19% and 30% respective decrease (Figure 2B). Though non-significant, a 25% liver DNA integrity decrease was also observed after 2.7 µM Phe exposure.

ENA frequency significantly increased in all Phe exposed groups when compared to control group, despite the absence of a clear Phe dose-response relation (Figure 2C). Thus, ENA frequency increased 220, 233, 133, 167% after 0.1, 0.3, 0.9 and 2.7 µM Phe exposures, respectively. The highest ENA levels were observed for the lowest concentrations (0.1 and 0.3 µM) being 0.3 µM significantly higher than 0.9 µM Phe exposed group.

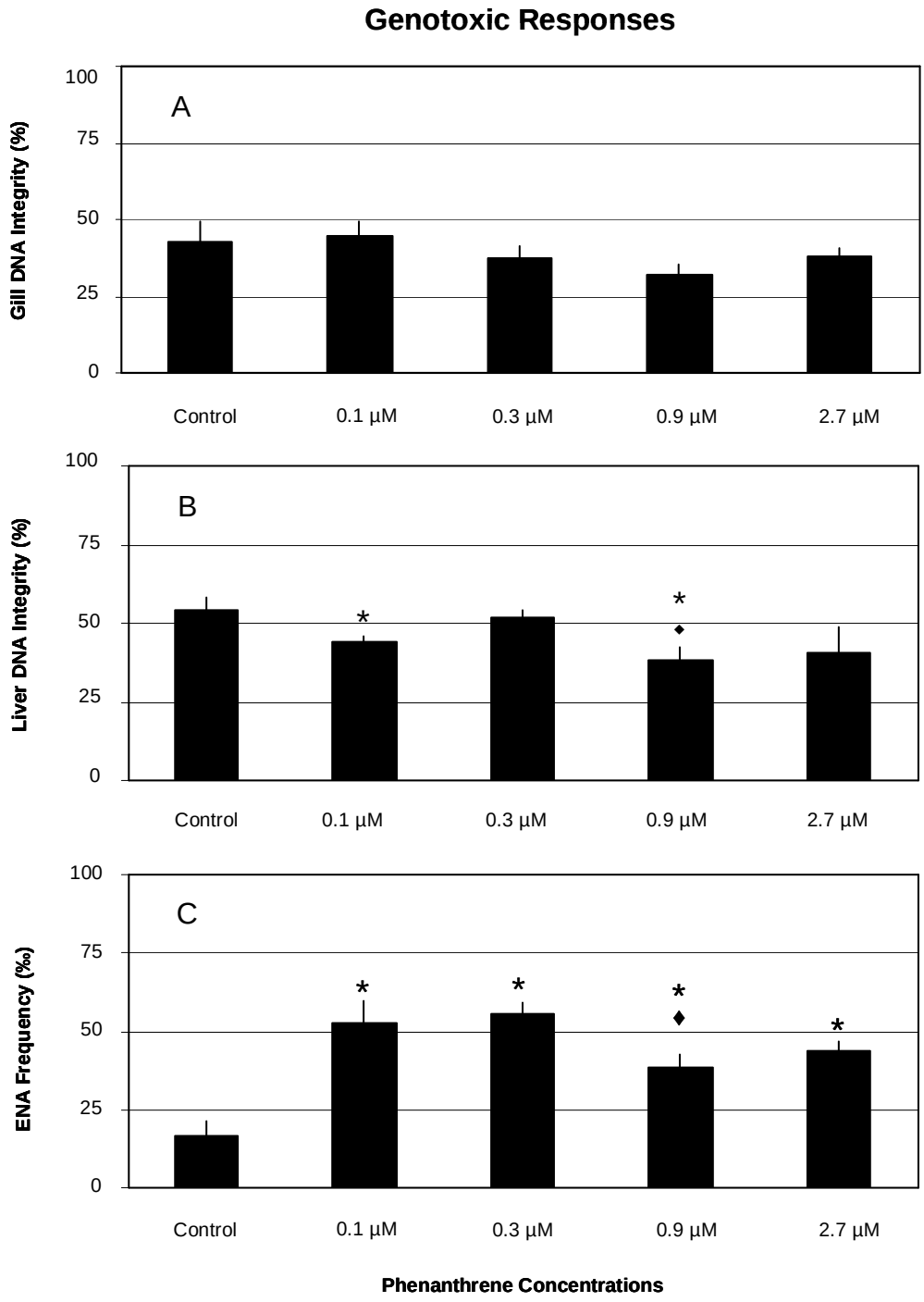

Figure 2 – Genotoxic responses in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Gill DNA integrity; B) Liver DNA integrity; C) Erythrocytic nuclear abnormalities (ENA) frequency. Values represent mean ± standard error (n=6 per treatment). Significant differences ($P<0.05$) are: * *versus* control; ♦ *versus* 0.3 µM Phe.

3.3. Stress Responses

Plasma cortisol was significantly increased by 0.3, 0.9 and 2.7 µM Phe exposure, and a dose-response tendency, i. e. a 43, 60 and 73% increase, was also observed despite its none-significance (Figure 3A).

A significant plasma glucose increase was observed after fish exposure to 0.9 and 2.7 µM Phe when compared to control, corresponding to a 124 and 160% increment, respectively (Figure 3B). Moreover, glucose levels in those groups were also significantly higher than in 0.1 and 0.3 µM exposed groups, being 2.7 µM group also significantly higher than 0.9 µM.

Phe exposure did not significantly alter plasma lactate levels in comparison to control group (Figure 3C). Thus, lactate increased 44%, though not significantly after 0.3 µM exposure, representing its highest level. On the other hand, a significant decrease in plasma lactate levels was observed after exposure to 0.9 µM Phe when compared to 0.1 and 0.3 µM.

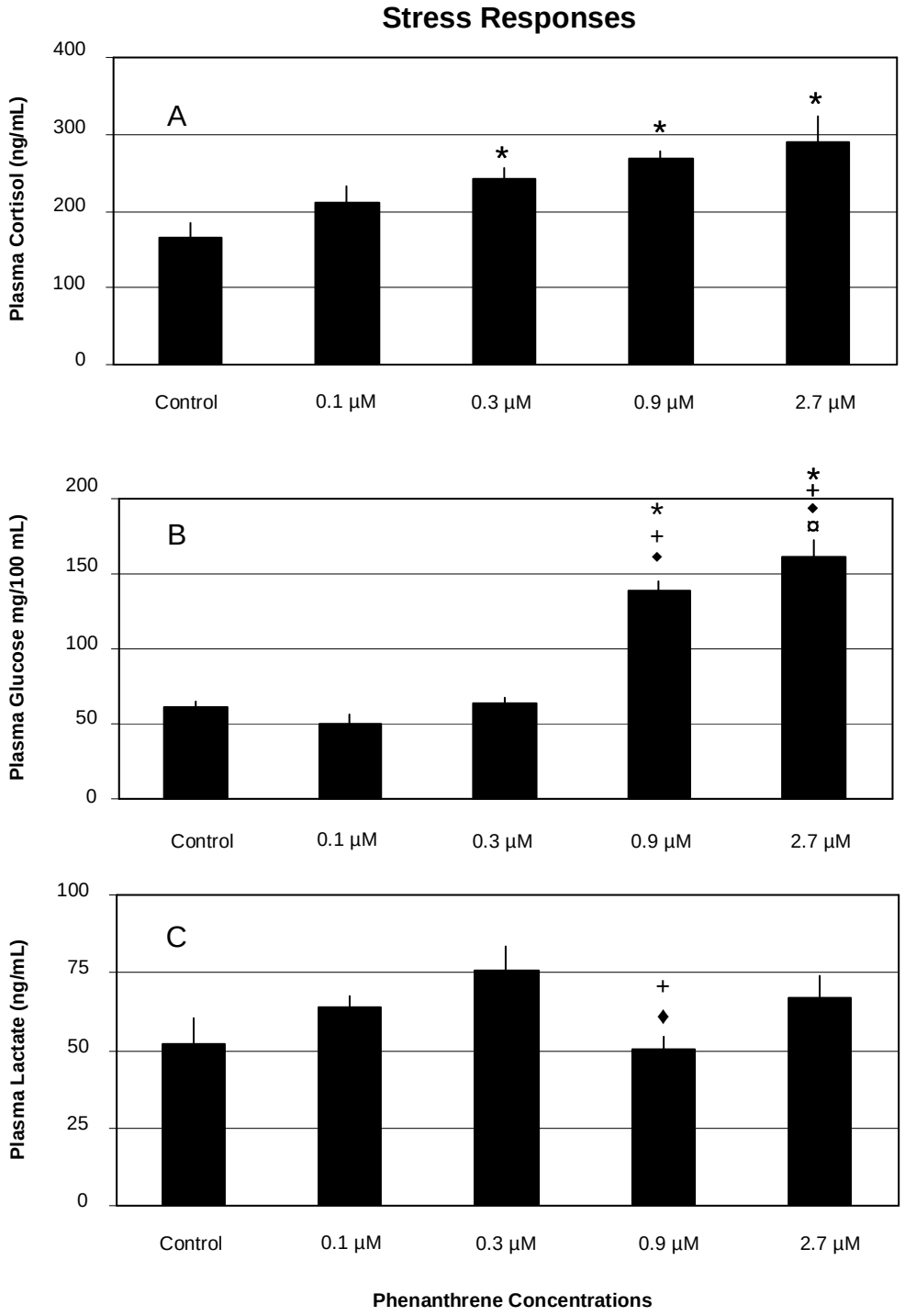

Figure 3 – Stress responses in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Plasma cortisol levels; B) Plasma glucose levels; C) Plasma lactate levels. Values represent mean ± standard error. Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe; ♦ *versus* 0.3 µM Phe: ¤ *versus* 0.9 µM Phe.

4. Discussion

Limited information is available concerning Phe toxicity on fish. Short-term waterborne Phe exposure effect on fish CYP1A activity, genotoxicity and stress responses, so far have not been studied. Sun et al. (2006) showed in goldfish that Phe was rapidly taken up from water and accumulated in the tissues reaching its maximum level after 2 days exposure, having a fast metabolic clearance. The previously reported Phe toxicokinetic studies, together with our previous knowledge concerning other fish species responses to several PAHs supports the adopted 16 hours exposure length in the current study. The concentration range from 0.1 up to 2.7 µM used in the current study can be considered environmentally realistic since it falls within the levels (up to 8.2 µM) detected in natural waters (Anyakora *et al*., 2005).

The available data on fish CYP1A induction by small size PAHs, having four or less fused aromatic rings, such as Phe, pyrene and naphthalene, provided non consensual results being those responses species dependant (Shailaja and D'Silva, 2003). However, the current results showed that liver EROD activity was induced in *L. aurata* by 0.3-2.7 µM Phe exposure. The Phe no observed effect concentration (NOEC) for *L. aurata* at 16 hours exposure, was 0.1 µM. Thus, the previous results confirm Phe CYP1A inducing potential as previously demonstrated in teleost fish by Goksøyr et al. (1986), Stegeman et al. (1998) and Shailaja and D'Silva (2003).

DNA damage is constantly induced and repaired, leading to a steady-state process. Thus, DNA damage observation indicates that the equilibrium has been shifted towards DNA damage accumulation, either through an increase in the number of DNA-damaging events or a decrease in DNA repair (Vock et al., 1998). Living organisms exposure to certain xenobiotics may generate reactive metabolites (phase I biotransformation), that can interact with DNA forming adducts, and/or reactive oxygen species (ROS) (redox cycling routes) capable of inducing single or double DNA strand breaks. DNA single strand breaks are expected to be formed transiently in the course of the DNA–DNA intrastrand crosslinks repair, DNA–protein crosslinks, and due to base modifications (Vock et

al., 1998). DNA single strand breaks have been evaluated by different methodologies, namely by the DNA alkaline unwinding assay, that demonstrated its suitability in the fish genotoxicity assessment (Rao et al., 1996; Maria et al., 2002), although it fails to account for DNA excision processes. In this perspective, micronucleus (MN) assay can constitute a suitable complementary indicator. Micronucleus, caused either by a clastogenic action or by abnormal spindle formation, is only expressed in dividing cells, providing a convenient and reliable index of both chromosome breakage (for which double-strand breaks is a prerequisite) and chromosome loss (Fenech, 2000). Additionally, single-strand breaks can be converted to double-strand breaks if the replication machinery leaves an interrupted daughter strand. It is assumed that other nuclear abnormalities can provide an additional and complementary measure of chromosome damage and rearrangement (Fenech, 2000), which can be scored together with the micronucleus and expressed as total nuclear abnormalities frequency being the basis for the ENA assay (Pacheco and Santos, 1996). In the current study, the Phe genotoxic potential was assessed at a molecular level through the DNA alkaline unwinding assay (gill, liver) and at a cytogenetic level as ENA (blood), considering the DNA damaging events previously described. Fish tissues were selected on the basis of functional criteria: gills as the primary uptake organ exposed to water diluted chemicals, liver which is the main xenobiotic-metabolizing organ and blood due to its function as a vehicle between gill and other target organs.

The DNA strand break data demonstrated clear differences between gills and liver, since no DNA integrity decrease was observed in gills for any exposure concentration, contrasting with liver where DNA integrity decrease was found after 0.1 and 0.9 µM Phe exposure. Thus, liver seems to be more susceptible and vulnerable to Phe genotoxicity. Previous studies that compared these two organs vulnerability to genotoxins revealed divergent and non conclusive results. Hence, Belpaeme et al. (1998) found that liver appeared to be less sensitive than gills, whereas Kilemade et al. (2004) observed no differences between these two organs. Organ specific genotoxic responses are related to their anatomic location, determining its exposure route and distribution of pollutants, as well as their tissue

regeneration rate and defensive capacity either in terms of DNA repair or protection against DNA damaging agents (ex. ROS and reactive metabolites). Gill epithelia have demonstrated high rates of mitosis (Pacheco et al., 1993) providing a rapid regeneration (Zenker et al. 1987) which may be an explanation for the current absence of effects upon gills DNA integrity. Hence, gill tissue regeneration could have been accelerated by the Phe direct cytotoxic action as previously demonstrated by Schirmer et al. (1998) in a rainbow trout gill cell line, after 2 h exposure.

The PAHs genotoxic properties are commonly associated with their biotransformation, especially the initial oxidative metabolism carried out by cytochrome P450 monooxygenases, rather than the parent compound levels in cells. Those reactions have the ability to generate reactive metabolites that can be more toxic than the parent compound, interacting with DNA and causing adducts. Previous studies concerning Phe showed a metabolite profile where benzo-ring dihydrodiols and phenols are the major compounds formed by liver microsomal metabolism in brown bullhead (Pangrekar et al., 2003). The proportion of the putatively most mutagenic/carcinogenic dihydrodiols (with a bay-region double bond) can vary with fish species and previous microsomal inducing background (Pangrekar et al., 2003).

Though Phe metabolism by liver microsomes was not directly assessed in this study, an attempt to correlate DNA damage to CYP1A induction was made. Liver EROD activity and the genotoxicity responses (with the exception of 0.9 µM) do not seem related, suggesting an absence of direct involvement of liver phase I induction upon DNA damage. Sun et al. (2006) suggested that oxidative-reductive reaction can occur and similar redox-active metabolites are produced resulting in the changes in the activities of the antioxidant enzymes as well as the production of ROS, in fish exposed to Phe. Thus, Phe metabolites and ROS production as a probable cause of the observed DNA damage should therefore be considered and measured in order to clarify the observed genotoxic effects.

The ENA frequency was significantly increased for all Phe exposure concentrations (0.1, 0.3, 0.9 and 2.7 µM) demonstrating a strong Phe citogenetic toxicity potential, despite an unclear relation with the concentration. The ENA

assay showed to be more responsive than gill and liver DNA strand breaks assay, probably due to the nature of the DNA damaging events involved. The recovery from genotoxic effects observed as less pronounced ENA increase for the highest Phe concentrations may be related with a variety of factors concerning erythropoiesis, cell division and maturation, as well as cell life span. The induction of nuclear lesions can be masked through a cytotoxic action, which killed the erythrocytes instead of forming non-lethal nuclear lesions (Das and Nanda, 1986; Brunetti et al., 1988). A gradual decrease in frequency of MN with a xenobiotic concentration increase was found by Das and Nanda (1986), being explained by an inhibitory effect on cell division and subsequent hindrance in the passage of the affected cells into peripheral circulation. This effect may result either from inhibition of DNA synthesis (Williams and Metcalfe, 1992) or from direct inhibition of erythropoiesis (Dinnen et al., 1988). Moreover, circulating abnormal cells tend to be removed from the organism faster than undamaged ones (Das and Nanda, 1986) which was corroborated by the occurrence of splenic hemosiderosis concomitantly with ENA expression impairment (Pacheco and Santos, 2002). The responsiveness of the ENA assay in the current study seems to be favoured by the long erythrocyte half-life which was evaluated in approximately 51 days fish (Fischer et al., 1998). Kilemade et al. (2004) study with comet assay, demonstrated that turbot blood is a suitable predictor of DNA damage in the whole organism. *L. aurata* DNA damage detection evaluated as ENA frequency increase, when compared to gill and liver genotoxic responses, is also a good predictor. The blood presents an additional advantage related to its non-destructive sampling procedure and the possibility of repeated sampling. To the best of our knowledge, this is the first study on Phe genotoxicity in fish stressing the risk to ichthyofauna associated to water contamination by this PAH.

Cortisol is the major glucocorticosteroid secreted by teleost 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, including PAHs (Hontela et al. 1992; Santos and Pacheco, 1996; Pacheco and Santos, 2001; Teles et al., 2003). Under acute stress situations, plasmatic cortisol levels tend to increase within a minute to

hour time frame, being followed by a gradual decrease to pre-treatment levels within a day or so, depending upon subsequent maintenance conditions (Mommsen et al., 1999). Under continued stress conditions, fish increase their energy demand and that results in a long fuel substrate mobilisation induced by cortisol (Mommsen et al., 1999). In addition, carbohydrate metabolism alterations have also been observed as a stress response to chemicals, corresponding typically to a plasma glucose and lactate increase (Santos and Pacheco, 1996; Vijayan et al., 1997; Gagnon and Holdway, 1999). However, studies on cortisol and intermediary metabolism responses provided divergent results, indicating the complexity of establishing a single model response (Santos and Pacheco, 1996; Pacheco and Santos, 2001; Teles et al., 2003; 2004).

In the present study Phe induced an increase in both cortisol and glucose plasmatic levels, allowing the NOEC definition respectively as 0.1 and 0.3 µM. The cortisol and glucose increase after 0.9 and 2.7 µM Phe exposure is considered as a part of the typical general stress response. However, plasma lactate did not show any significant alterations proving the difficulty to predict the whole fish stress response based on inflexible standard mechanisms. Current cortisol responses are similar to those found by Monteiro et al. (2000) for moderate Phe doses, despite the important differences in the experimental design, namely in terms of exposure route and duration. Studies concerning alterations on intermediary metabolism in fish exposed to Phe are not available, thus enhancing the relevance of the present study as the first work on the integrated stress response to this ubiquitous contaminant.

L aurata proved to be a sensitive organism for Phe exposure when the assessment of CYP1A induction, stress and genotoxic responses is intended, thus making it a potential bio-indicator species for monitoring the short-term effects of PAH-exposure.

5. Concluding remarks

Short-term exposure of *L. aurata* to Phe demonstrated liver EROD activity induction as well as liver and blood genotoxicity measured as increased DNA strand breaks and ENA frequency, respectively. Genotoxic responses did not show a clear correlation with liver CYP1A induction. Blood genotoxicity assessment by the ENA assay showed to be the most responsive approach. A generalized stress response was also observed, as elevated plasma cortisol and glucose levels. Thus, the observed Phe effects on *L. aurata*, detected at different levels, demonstrated a physiological unbalance and a probable ecological risk to ichthyofauna.

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CAPÍTULO III

Respostas antioxidantes específicas de órgãos da tainha-garrento (*Liza aurata*) após exposição de curta duração a fenantreno

Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene

Oliveira, M., Pacheco, M. Santos, M. A. (2008)
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Abstract

Phenanthrene (Phe) is among the most abundant and ubiquitous polycyclic aromatic hydrocarbons (PAHs) in the aquatic environment as a result of human activities. Even so, the knowledge about its impact on fish health is still limited. In this study, the teleost *Liza aurata* was exposed to 0.1, 0.3, 0.9 and 2.7 µM Phe concentrations during 16 hours. Enzymatic antioxidants such as selenium dependent glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and catalase (CAT), as well as a non enzymatic antioxidant (glutathione - GSH) were quantified in three target organs - gill, kidney and liver. The lipid peroxidation (LPO) was also assessed as a marker of oxidative damage. GPx activity was decreased in gill (0.1 and 0.9 µM), whereas in the liver it was increased (0.3 - 2.7 µM). GST activity was decreased in kidney (0.3 - 2.7 µM) and CAT activity was increased in gill after 0.9 µM exposure. GSH content was significantly increased in gill by the lowest concentration and in liver by all Phe concentrations. Despite the antioxidant defense responses, LPO increased in gill (0.3 - 2.7 µM), kidney (0.1 µM) and liver (0.1 and 2.7 µM). These results revealed organ specific antioxidant defenses depending on the Phe concentration. Liver demonstrated a higher adaptive competence expressed as antioxidant defenses activation, namely GSH and GPx. The lower vulnerability of the kidney to oxidative damage (compared to gill and liver) seems to be related to its higher antioxidant basal levels. Globally, current data highlight the Phe potential to induce oxidative stress and, consequently, to affect the well-being of fish.

Keywords: Phenanthrene; Fish; Antioxidant defenses; Lipid peroxidation

1. Introduction

All aerobic organisms are continually subjected to oxidant challenges from endogenous and exogenous sources, however they evolved antioxidant defenses for their protection (Winston and Di Giulio, 1991), which allow the cells to maintain

a low steady-state of reactive oxygen species (ROS) like superoxide ($O_2^{\bullet -}$) and hydroxyl ($HO^{\bullet}$) radicals as well as hydrogen peroxide (H_2O_2). In this context, thiols have an important role against the pernicious effects of pro-oxidant challenges and glutathione in particular provides a first line of defense against ROS (Ahmad et al., 2000; 2005; Pastore et al., 2003; Li et al., 2007). This non-enzymatic antioxidant participates in many cellular reactions, scavenging ROS directly and indirectly through enzymatic reactions (Fang et al., 2002). As a consequence, reduced glutathione (GSH) is oxidized to GSSG, which is rapidly reduced back to GSH by glutathione reductase (GR) at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) forming a closed system (redox cycle). The reduction of organic hydroperoxides by GSH may be catalyzed by either selenium-dependent glutathione peroxidase (GPx) or by selenium-independent peroxidase activity, which is provided by glutathione S-transferases (GST) of α class (Wang and Ballatori, 1998). Catalase (CAT), though not using GSH has also an important role in cellular protection reducing H_2O_2 to water and oxygen. These systems may prevent oxyradicals formation and/or intercept oxidative propagation reactions promoted by formed oxyradicals (Bainy et al., 1996). However, fish exposure to toxic chemical pollutants may lead to an overwhelmed antioxidant defense that may result in oxidative stress which can be responsible for enzyme inactivation, lipid peroxidation (LPO) and DNA damage (Varanka et al., 2001; Pena-Llopis et al., 2003). Variations in the antioxidant defenses (CAT, GST, GR, glutathione peroxidases Se-dependent and Se-independent and the levels of GSH) can be very sensitive in revealing a pro-oxidant condition and have been used as oxidative stress markers in fish (Ahmad et al., 2000; 2005; Gravato et al., 2006).

The aquatic environment receives large amounts of environmental pollutants that have the potential to cause oxidative stress in fish. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous water contaminants, considered as highly hazardous by the USA and EU (Vives et al., 2004) and have been extensively studied due to the carcinogenic and mutagenic properties of some of them (Aas et al., 2001; Vives et al., 2004). PAHs are taken up by fish due to their lipophilic characteristics (McKeown and March, 1978). Previous studies focusing on PAHs effects in fish found that antioxidant enzyme activities may differ

considerably between organs (Ahmad et al., 2003; Jee and Kang, 2005), highlighting the need to assess antioxidant responses in the main target organs when a comprehensive evaluation of the oxidative stress risk at individual level is intended.

Phenanthrene (Phe), one of the most abundant PAHs in the aquatic environment, is a three-ring compound included in the 16 USA EPA priority PAHs (Smith et al., 1989) that had its environment input levels increased since the 1990´s (Lima et al., 2003). Water runoff, atmospheric deposition and oil spills are the largest sources of aquatic Phe contamination (Lima et al., 2003; Incardona et al., 2005). Studies have shown that in polluted areas high levels of Phe can be found, reaching water concentrations as high as 1460 µg/L (8.2 µM) in marine samples near a crude oil exploration area (Anyakora et al., 2005). Very few studies have focused on the effects of Phe on the antioxidant defences of fish. The available studies demonstrated a potential to generate ROS affecting antioxidant enzymes in *Paralichthys olivaceus* (Jee and Kang, 2005), *Carassius auratus* (Sun et al., 2006; Yin et al., 2007) and *Sparus aurata* (Correia et al., 2007).

The present study focused on the effects of a short-term exposure to Phe environmentally realistic concentrations on enzymatic (GPx, GST, GR and CAT) and non-enzymatic (GSH) antioxidant defenses, using *Liza aurata* as a model species. In addition, LPO was also measured as an indication of ROS induced peroxidative damage. In order to achieve a better understanding on the organ specific responses, the previous parameters were assessed in gill, kidney and liver of fish. These organs were selected on the basis of functional criteria which made them preferential targets, i.e., xenobiotic uptake (gill), haemopoiesis (kidney) and xenobiotic metabolism (liver).

2. Material and Methods

2.1. Chemicals: Phenanthrene was purchased from Sigma-Aldrich (Spain). All the other chemicals were of analytical grade. Marine salt was purchased from Sera Premium (France).

2.2. Experimental Animals: The experiment was carried out using juvenile golden grey mullet (*Liza aurata*) collected from the Aveiro lagoon – S. Jacinto, Portugal. The fishing site has no known pollution sources and only trace levels of PAHs were detected. *L. aurata* previously caught in this area did not show any indication of exposure to pollutants assessed through the analyses of CYP1A activity and genotoxic effects (Pacheco et al., 2005). Fish with an average length of 15±5 cm and weighing 25±5 g were transported to the laboratory in oxygenated water from the fishing site and allowed to recover for 7 days in 80 L aquaria (six animals per aquarium – 1.9 g of fish weight per L), at 20 ºC, in aerated and filtered artificial saltwater (34 g/L) being fed with polichaet worms (Nereis sp.) collected at an Aveiro lagoon clean area, every two days. *L. aurata* were not fed during the exposure period.

2.3. Experimental design: The experiment was carried out according to the water conditions and fish density adopted during the recovery period. *L. aurata* was exposed to 0.1, 0.3, 0.9 and 2.7 µM Phe that had been previously dissolved in 1 mL dimethyl sulfoxide (DMSO), for a period of 16 hours. The same amount of DMSO was added to the control aquarium. Fish were killed by decapitation and gill, kidney and liver were sampled, immediately frozen in liquid nitrogen and stored at -80 ºC until homogenization.

2.4. Biochemical analyses: Gill, kidney and liver were homogenized (1:15) in 0.1 M phosphate buffer (pH 7.4). Part of the liver homogenate was used to determine the extent of LPO and the remaining homogenate was centrifuged for 20 minutes at 13 400 g (4 ºC) to obtain the post-mitochondrial supernatant (PMS).

2.4.1. Glutathione measurement: Glutathione was determined in PMS by the method of Jollow et al. (1974). Briefly, 1 ml PMS was precipitated with 1 ml of sulfosalicylic acid (5.0%). The samples were subjected to centrifugation at 13400 g for 3 min at 4ºC. The assay mixture contained 0.05 ml of the supernatant, 2.35 ml of sodium phosphate buffer (0.1 M, pH 8.0) and 0.1 ml 2,5-dithiobis-

tetranitrobenzoic acid (DTNB, stock = 100 mM in 0.1 M sodium phosphate buffer, pH 8.0) in a total volume of 2.5 ml. After mixture, the yellow colour developed was immediately read at 412 nm on a spectrophotometer (Jasco UV/VIS, V-530). GSH content was calculated as mmol TNB/mg protein using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

2.4.2. Selenium dependent glutathione peroxidase (GPx) activity measurement: The assay was carried out in PMS according to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal (1998). The assay mixture consisted of 1.44 ml phosphate buffer (0.05 M, pH 7.0), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml GR (1 U/ml), 0.1 ml GSH (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml H_2O_2 (0.025 mM) and 0.1 ml of PMS in a total volume of 2 ml. Oxidation of NADPH was recorded spectrophotometrically at 340 nm during 3 minutes. The enzyme activity was calculated as nmol NADPH oxidized/min/mg of protein, using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

2.4.3. GST activity measurement: This activity was determined in PMS by following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974). Briefly, the reaction mixture consisted of 1.89 ml sodium phosphate buffer (0.2 M, pH 7.9), 0.05 ml GSH (8.2 mM), 0.05 ml CDNB (8.2 mM) and 0.01 ml PMS in a total volume of 2 ml. The change in absorbance was recorded at 340 nm during 3 minutes and enzyme activity calculated as mmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

2.4.4. GR activity measurement: The determination was carried out spectrophotometrically following the decrease in absorbance at 340 nm during 3 minutes according to Cribb et al. (1989). Briefly, the assay mixture contained 0.025 ml of PMS fraction and 0.925 ml of NADPH (0.2 mM), GSSG (1 mM) and DTPA (0.5 mM). The enzyme activity was calculated as nmol NADPH oxidized/min/mg of protein using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

2.4.5. CAT activity measurement: This activity was determined in PMS by the method of Claiborne (1985) as described by Giri et al. (1996). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1 ml H_2O_2 (0.019 M) and 0.05 ml PMS in a final volume of 3 ml. Change in absorbance was recorded at 240 nm during 3 minutes. CAT activity was calculated in terms of µmol H_2O_2 consumed/min/mg protein using a molar extinction coefficient of 43.5 M^{-1} cm^{-1}.

2.4.6. Lipid peroxidation measurement: LPO was determined in the previously prepared tissue homogenate by the procedure of Ohkawa et al. (1979) and Bird and Draper (1984), as adapted by Wilhelm et al. (2001) with slight modifications. Briefly, to a 50 µl of homogenate, 3 µl of 4% butylated hydroxytoluene (BHT) in methanol was added and mixed well. After mixing, 0.5 ml of 12% trichloroacetic acid (TCA) in aqueous solution, 0.45 ml Tris-HCl (60 mM, pH 7.4; and 0.1 mM diethylenetriaminepentaacetic acid - DTPA) and 0.5 ml 0.73% 2-thiobarbituric acid (TBA) were added and mixed well. The mixture was heated for 1 hour in a water bath set at boiling temperature. After 1 hour of boiling, the test tubes were removed and cooled to room temperature. The contents from each tube were decanted into 2 ml microtubes and centrifuged at 13 400 g for 3 minutes. The absorbance of each aliquot was measured at 535 nm. The rate of LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed/g tissue using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

2.4.7. Protein measurement: Protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

2.5. Statistical analysis: Results are expressed as means ± SE (standard error) corresponding to experimental groups of six fish (n=6). A statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK). The assumptions of normality and homogeneity of data were verified. A factorial

analysis of variance (Two-Way ANOVA) was performed in order to assess significant effects of Phe in each organ and the differences between organs; this analysis was followed by post-hoc Tukey test to signal significant differences between groups (Zar, 1999). The significance of the results was ascertained at $\alpha = 0.05$.

3. Results

3.1. Glutathione content

The analysis of each organ *per se* demonstrated that gill GSH content was significantly increased by 0.1 µM Phe (81%) when compared to control (Figure 1A), whereas no significant alterations were observed following exposure to higher Phe concentrations. In kidney, no significant changes were observed in GSH levels after Phe exposure (Figure 1B) when compared to the control group despite the slight (non-significant) increase observed after 0.1 and 2.7 µM Phe exposures. Liver GSH content was significantly increased by all Phe concentrations in comparison to control (Figure 1C), displaying an increase trend from 0.1 up to 0.9 µM concentrations. A less marked increase was observed for 2.7 µM Phe also showing a significant difference from 0.9 µM.

Comparing the data for the three organs, liver GSH content was significantly higher than gill in all Phe exposure concentrations excluding the lowest (0.1 µM). Moreover, kidney GSH content was also significantly higher than gill in the 2.7 µM Phe exposed group, as well as in the control group. Comparing liver and kidney the only detected difference was observed at 0.9 µM where liver was significantly higher.

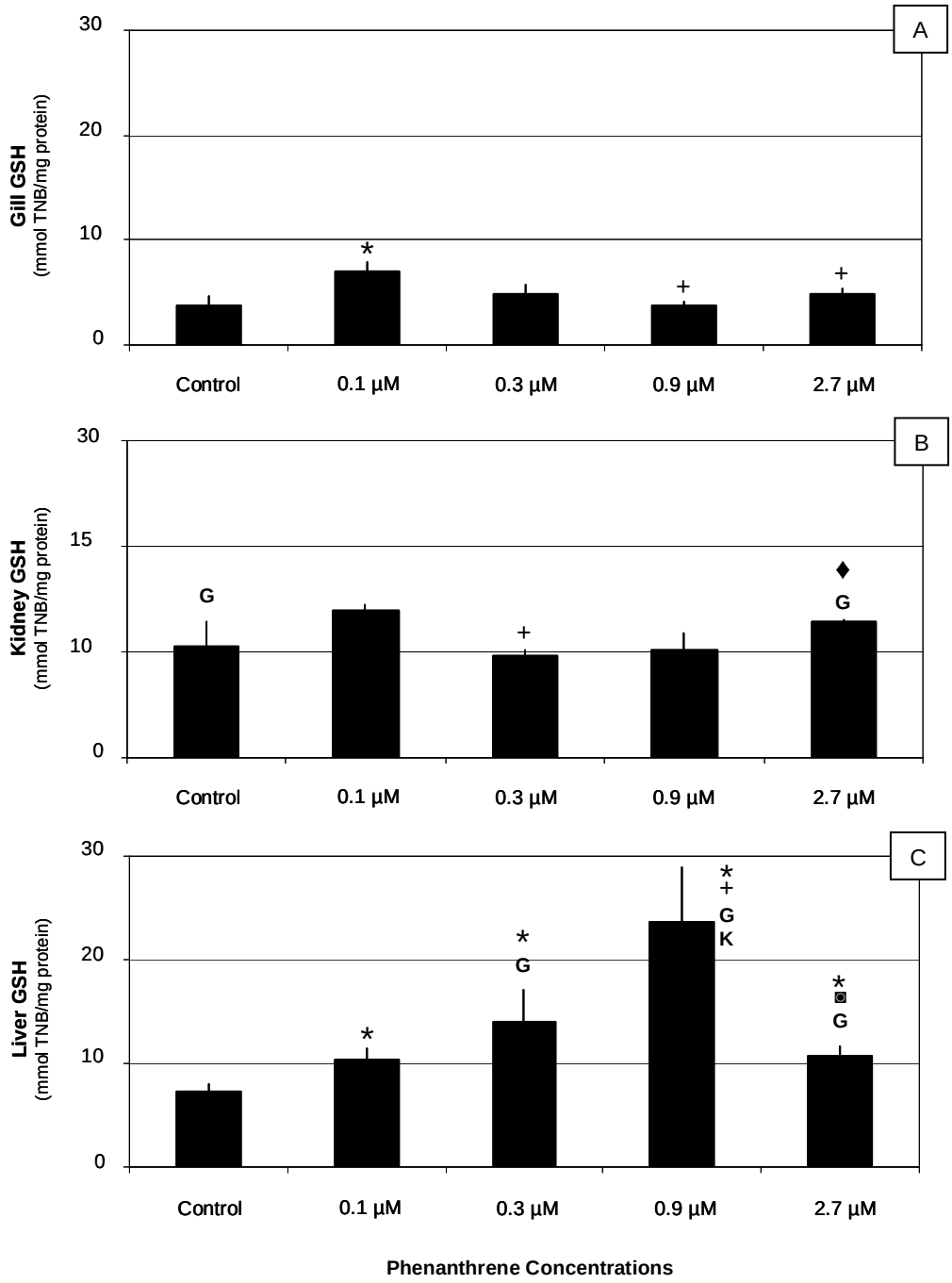

Figure 1 – Glutathione (GSH) content in *L. aurata* following 16 hours exposure to phenanthrene (Phe). A) Gill; B) Kidney; C) Liver. Values represent mean ± standard error (n=6 per treatment). Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe; ♦ *versus* 0.3 µM Phe; ◙ *versus* 0.9 µM Phe; G *versus* gill; K *versus* kidney.

3.2. GPx activity

Considering the response of each organ individually, it was observed that gill GPx activity of fish exposed to 0.1 and 0.9 µM Phe was significantly decreased when compared to the control levels (Figure 2A), whereas kidney showed no significant changes (Figure 2B). On the other hand, liver GPx activity was significantly increased by 0.3, 0.9 and 2.7 µM Phe exposures when compared to control (Figure 2C).

A comparative analysis of GPx activity in the three organs revealed that kidney has significantly higher levels comparing to gill and liver in all Phe exposures (excluding liver at 0.9 µM) as well as in control condition.

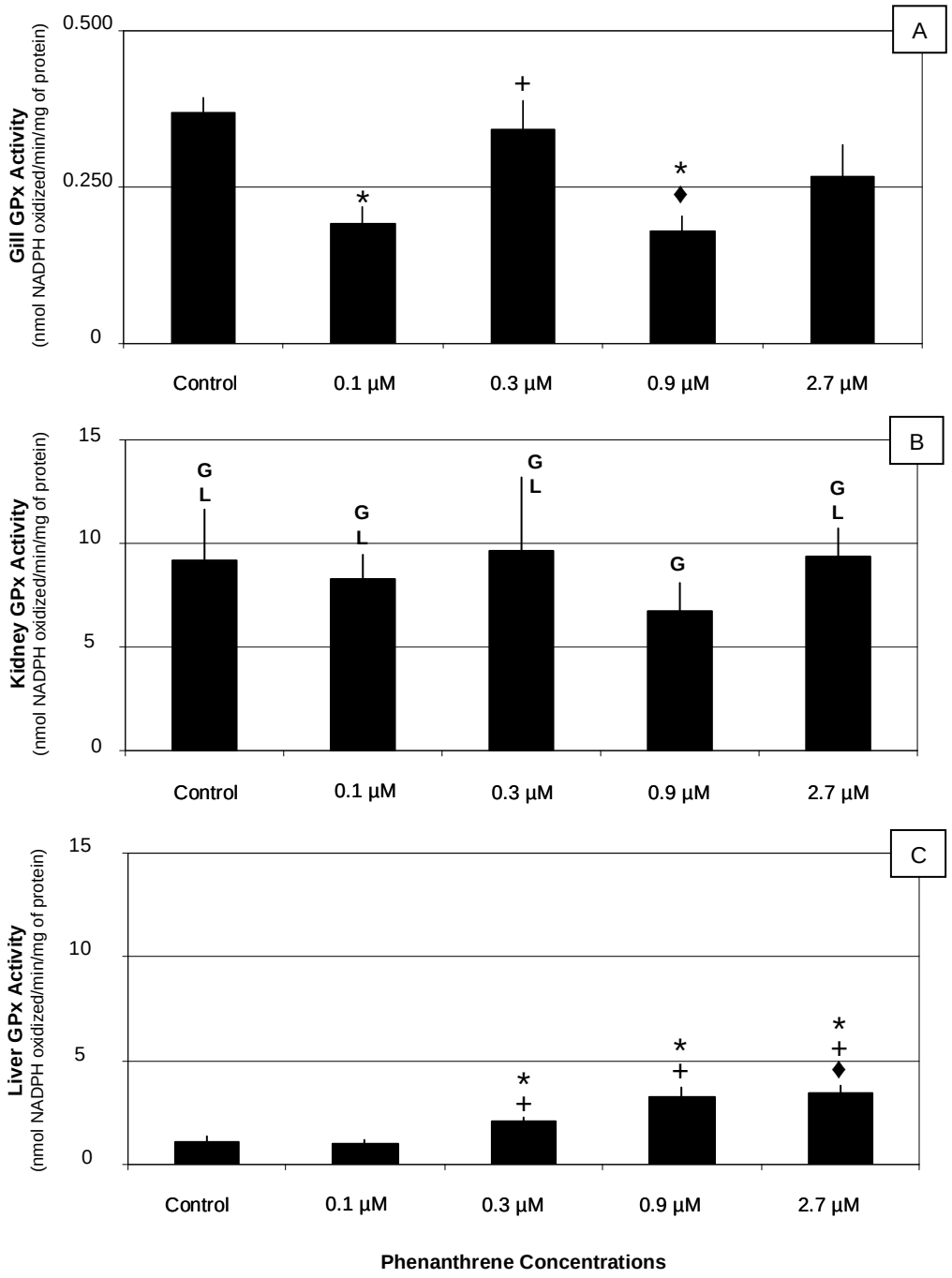

Figure 2 – Glutathione Peroxidase (GPx) activity in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Gill; B) Kidney; C) Liver. Values represent mean ± standard error (n=6 per treatment). Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe; ♦ *versus* 0.3 µM Phe; G *versus* gill; L *versus* liver.

3.3. GST activity

Gill GST activity was not significantly affected by Phe exposure (Figure 3A). Kidney GST activity was significantly decreased after 0.3 (60%), 0.9 (65%) and 2.7 µM (43%) Phe exposures when compared to the control value (Figure 3B). In the liver, Phe exposure induced a slight (non significant) GST activity increase at 0.1 and 0.3 µM concentrations (Figure 3C).

The inter-organ comparison revealed a clearly higher GST activity in liver in comparison to gill for all Phe concentrations though at 0.9 µM it was not statistically significant. Furthermore, liver GST activity was also significantly higher than kidney at 0.3 µM Phe. Comparing the baseline levels (control groups) it is noticeable that gill showed lower GST activity than kidney and liver though this difference was only significant for kidney.

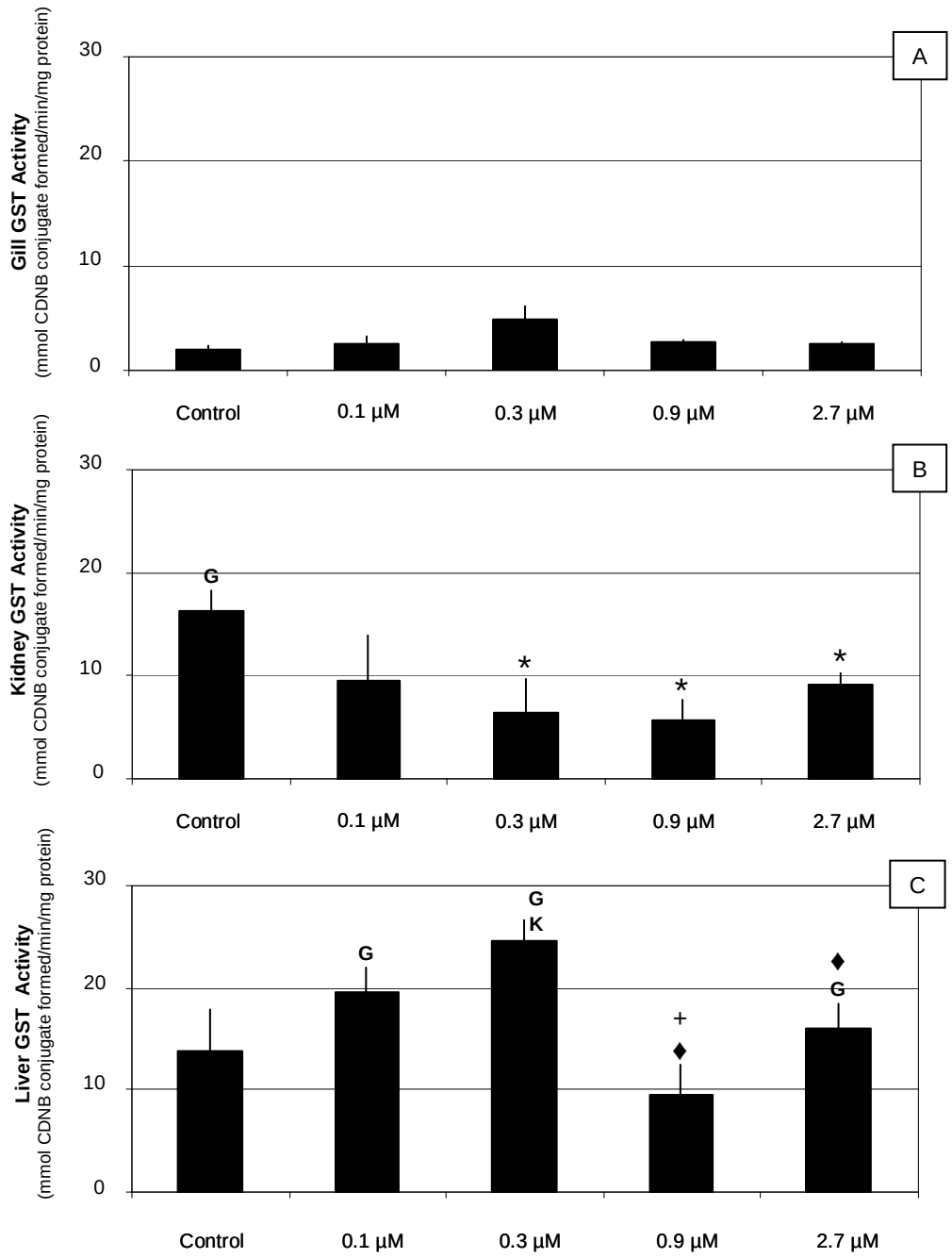

Figure 3 – Glutathione S –transferase (GST) activity in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Gill; B) Kidney; C) Liver. Values represent mean ± standard error (n=6 per treatment). Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe; ♦ *versus* 0.3 µM Phe; G *versus* gill; K *versus* kidney.

3.4. GR activity

Gill, liver and kidney GR activities were not significantly altered by Phe exposure, when compared to the respective control values (Figure 4A, B and C). The inter-organ comparison did not reveal in general differences neither in baseline levels nor in the responses to the different Phe concentrations with the exception of 2.7 µM where liver displayed a significantly higher GR activity than gill.

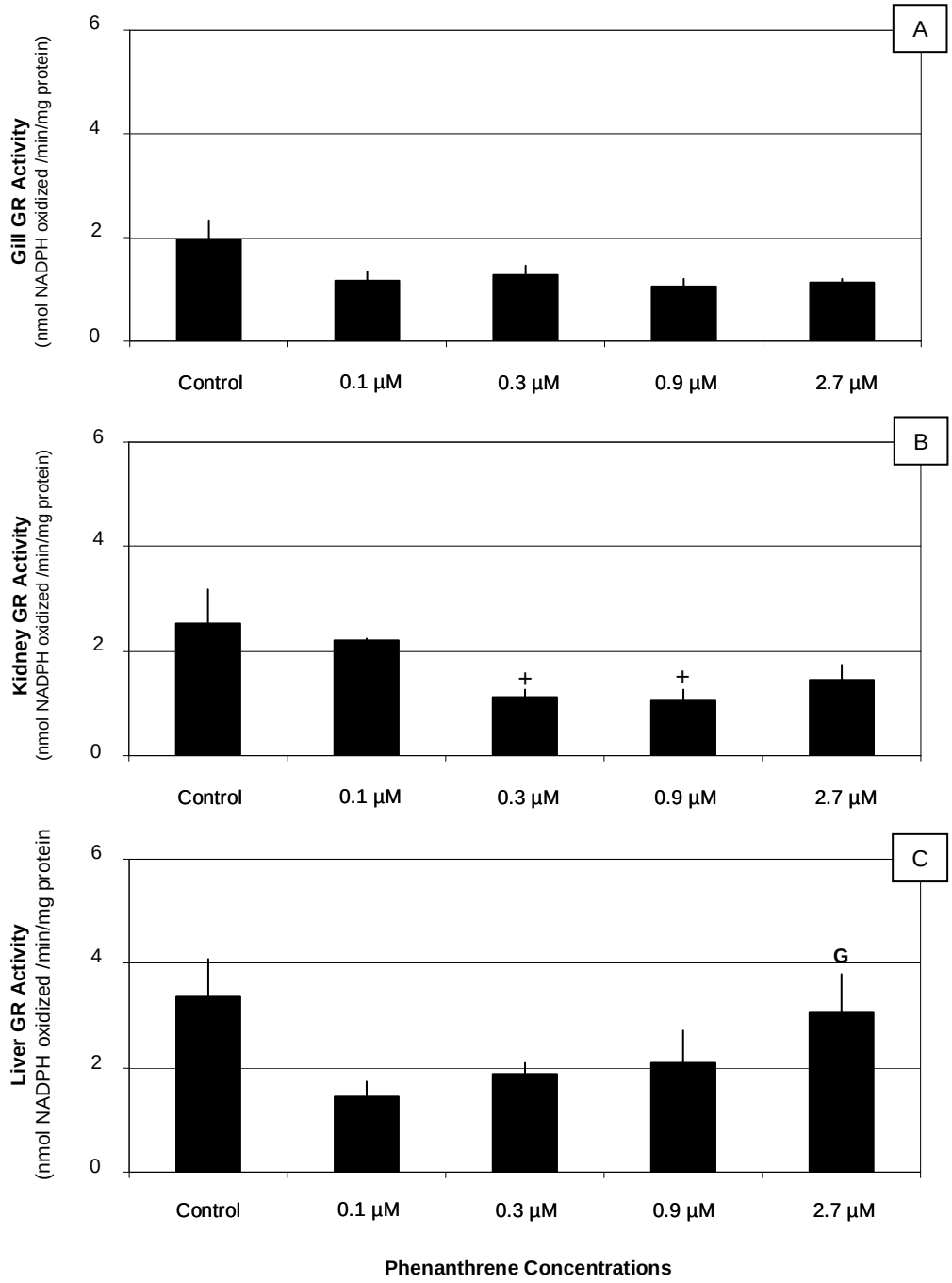

Figure 4 – Glutathione reductase (GR) activity in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Gill; B) Kidney; C) Liver. Values represent mean ± standard error (n=6 per treatment). Significant differences (P<0.05) are: + *versus* 0.1 µM Phe; G *versus* gill.

3.5. CAT activity

Gill CAT activity was significantly increased after fish exposure to 0.9 µM Phe (Figure 5A). Kidney and liver CAT activities showed no significant changes after exposure to any Phe concentration (Figure 5B). Nevertheless, a slight non-significant increase was observed in liver CAT activity after 0.3 µM Phe exposure (Figure 5C).

A comparative analysis of CAT activity in the three organs revealed that liver has significantly higher levels comparing to gill in all Phe exposures as well as in control condition. Similarly, with the exception of 0.1 and 0.3 µM, liver displayed higher CAT activity than kidney.

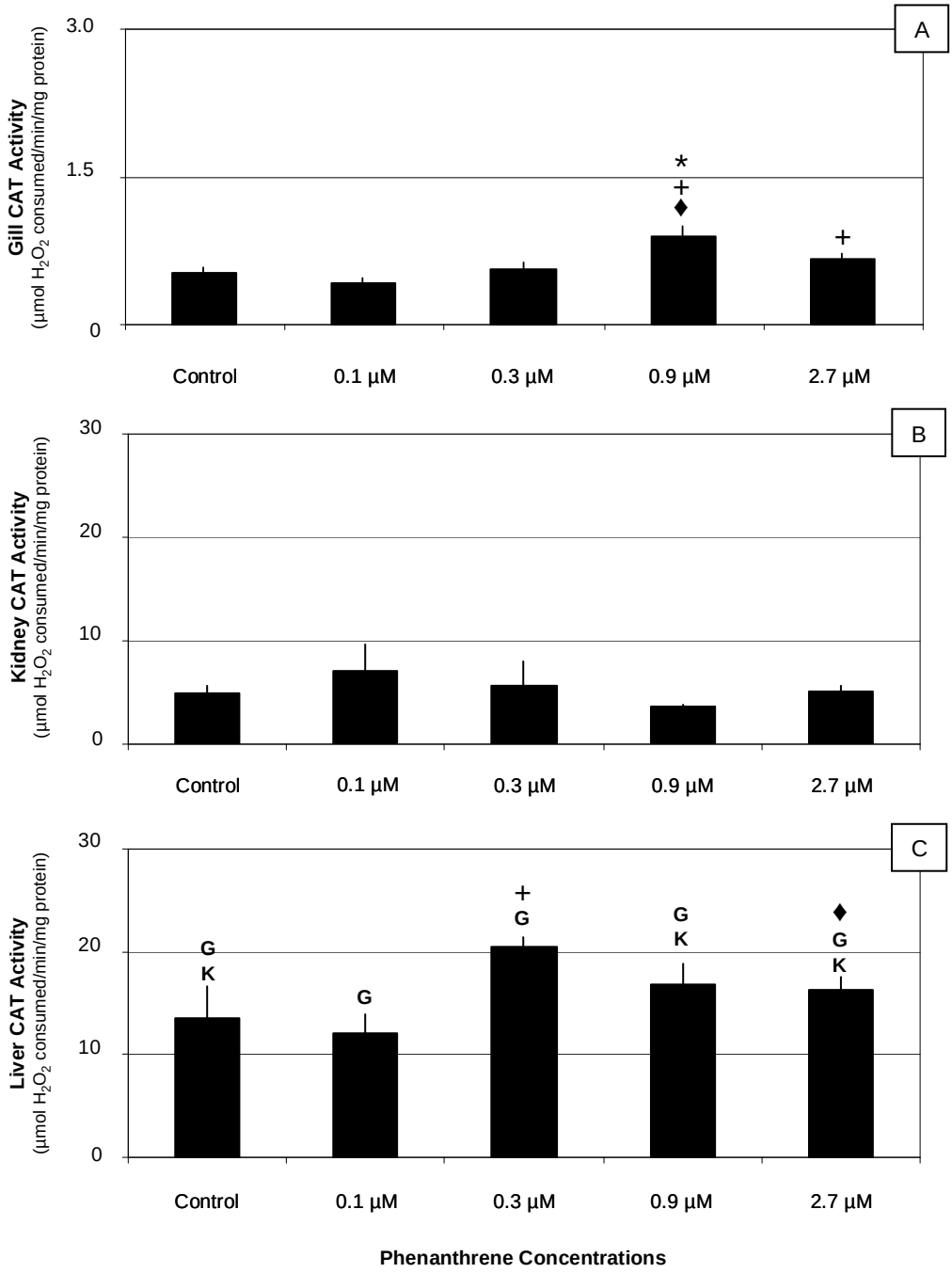

Figure 5 – Catalase (CAT) activity in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Gill; B) Kidney; C) Liver. Values represent mean ± standard error (n=6 per treatment). Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe; ♦ *versus* 0.3 µM Phe; G *versus* gill; K *versus* kidney.

3.6. LPO

Gill LPO increased after exposure to all Phe concentrations with the exception of 0.1 µM (Figure 6A). Kidney LPO was significantly increased only after 0.1 µM Phe exposure (Figure 6B). Fish exposure to 0.1 and 2.7 µM Phe significantly increased liver LPO levels (Figure 6C).

The inter-organ comparison did not reveal differences neither in baseline levels nor in the responses to the different Phe concentrations.

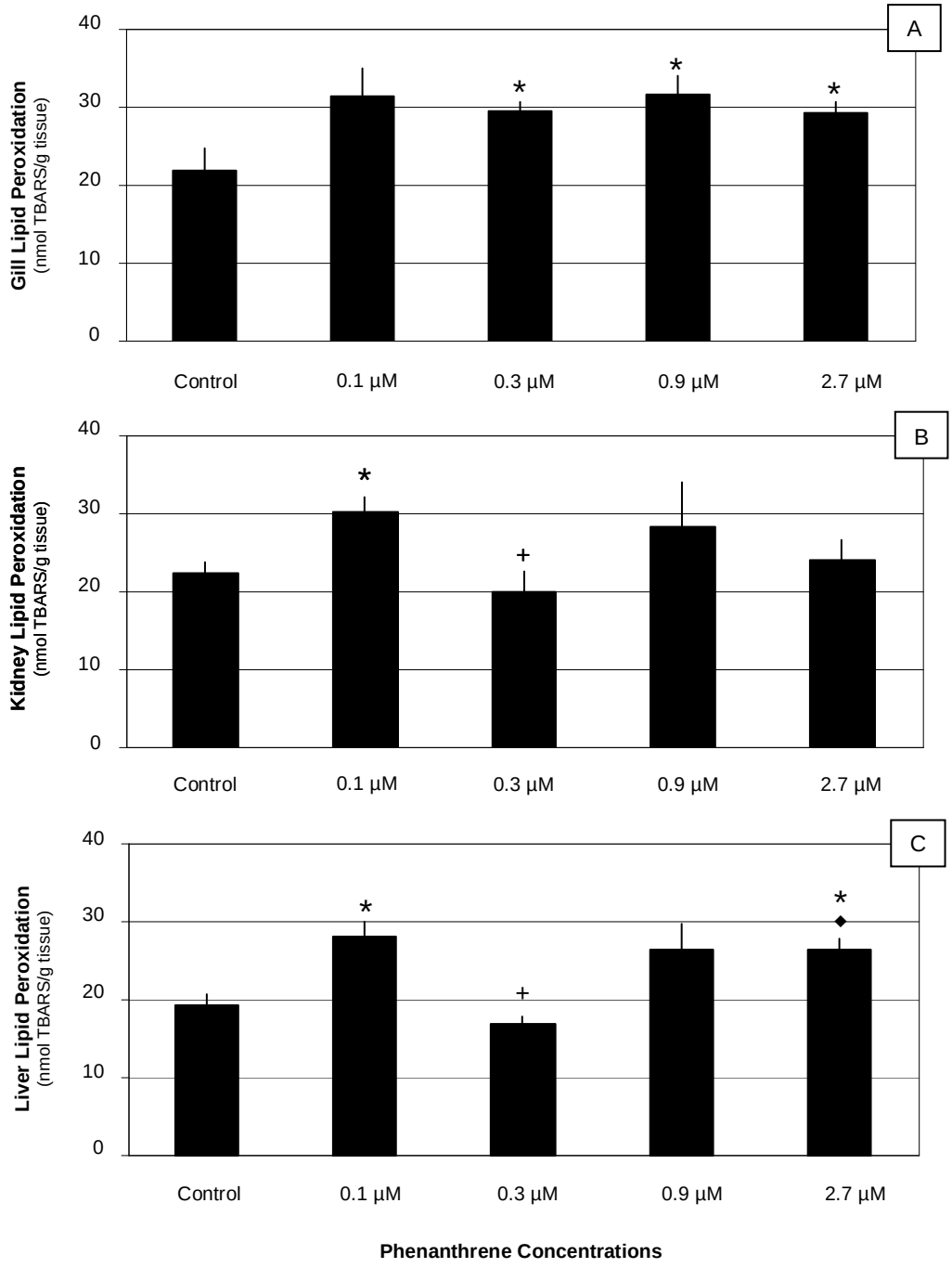

Figure 6 – Lipid peroxidation (LPO) activity in *L. aurata* after 16 hours exposure to phenanthrene (Phe). A) Gill; B) Kidney; C) Liver. Values represent mean ± standard error (n=6 per treatment). Significant differences (P<0.05) are: * *versus* control; + *versus* 0.1 µM Phe; ♦ *versus* 0.3 µM Phe.

4. Discussion

The present study is the first assessment of *L. aurata* antioxidant responses after Phe exposure. *L. aurata* is a common European mugilide, widely distributed in both Atlantic and Mediterranean coastal waters (Oliveira et al., 2007) which demonstrated to be a good bioindicator for monitoring water contamination by PAHs (Pacheco et al., 2005; Oliveira et al., 2007). The currently adopted exposure period (16 hours) demonstrated previously the Phe genotoxic potential in *L. aurata* (liver and blood), as well as the ability to induce stress responses (cortisol and glucose alterations) and cytochrome P450 dependent activity (Oliveira et al., 2007).

Analysis of antioxidant status and other stress responses in tissues of organisms exposed to xenobiotics can help to understand the associated toxic mechanisms and predict the degree of biological damage at higher levels of biological organization (Franco et al., 2006). Though not extensively studied in fish, antioxidant capacities have shown to be organ specific, depending on its anatomic location, determining the exposure route and distribution of xenobiotics (Ahmad et al., 2000). Thus, the current discussion will focus first on each organ individually and then, an attempt to compare those responses will be made in order to better characterize the overall response mechanisms.

4.1. Organ specific responses

4.1.1. Gill responses

Gill is the first organ to be exposed to waterborne contaminants and the primary site for xenobiotics absorption due to its large surface area and permeability (Sancho et al., 1997). Therefore, it can be considered a good candidate to an early assessment of the effects of water diluted chemicals. The current data revealed a GSH content increase after exposure to the lowest Phe concentration which represents an adaptation following Phe exposure, suggesting either an increased synthesis *de novo* as previously demonstrated in fish (Thomas and Wofford, 1984; Gallagher et al., 1992; Thomas and Juedes, 1992) or a

transfer from other organs since it has been demonstrated that most glutathione is synthesized in the liver and then transported to other tissues (Deneke and Fanburg, 1989). However, the absence of this adaptation process in the higher Phe concentrations may be related to the failure of the previous mechanisms due to an inhibition on GSH synthesis pathway in gill or to a GSH availability decrease on the supplier organs where its utilization may have been incremented. In relation to GSH dependent enzymes, it was observed an inhibition for GPx (0.1 and 0.9 µM) whereas no significant alterations were observed for GST. Taking into consideration the GSH levels, the GPx inhibition does not seem explainable by its scarcity. Hence, despite the unclear relation with Phe concentration, GPx activity decrease may be a signal of toxicity probably associated to ROS production induced by Phe or its metabolites. This explanation is supported by the previously demonstrated Phe potential to induce ROS generation (Sun et al., 2006; Ying et al 2007) and the ROS ability to inhibit gill GPx (Monteiro et al., 2006), namely $O_2^{\bullet -}$ (Bagnasco et al., 1991).

Gill GR activity was not significantly altered by any Phe concentration which is not supported by the only available study on fish GR responses to Phe (Jee and Kang, 2005) that reported an enzyme activity increase. However, differences on exposure length (16 hours *versus* 2 and 4 weeks) may justify the observed discrepancy.

CAT activity was increased by 0.9 µM Phe, representing a clear indication of H_2O_2 generation. The only CAT increase occurring concomitantly with a GPx decrease (for 0.9 µM Phe) may suggest a CAT lower susceptibility to ROS and thus, the possibility to act as an alternative to GPx and as a second line of enzymatic defense against H_2O_2. For 0.1 µM Phe, a GPx inhibition was also observed but CAT defense was not mobilized, which can be explained by the additional protection provided by the increased GSH level.

All Phe concentrations, excluding the lowest, displayed peroxidative damage demonstrating that antioxidant defenses were overwhelmed by Phe induced ROS. Moreover, the crucial role of GSH was demonstrated as the only concentration where LPO was not observed coincides with the only GSH content increase.

4.1.2. Kidney responses

Fish kidney plays a vital role in the maintenance of organisms' internal environment, being the key to the extracellular fluid volume, composition and acid–base balance regulation, presenting an additional function related to haematopoiesis. Thus, the assessment of its antioxidant responses is highly relevant since its functions can be disrupted by toxic chemicals causing temporary or permanent derangement of homeostasis (Miller, 2002).

The current kidney GSH levels, as well as GPx, GR and CAT activities were not significantly altered by any Phe concentration. However, GST activity was significantly inhibited after exposure to concentrations higher than 0.3 µM, indicating a toxic effect of Phe, resulting in a decreased ability of *L. aurata* to perform defense reactions associated to GSTs such as the conjugation of Phe reactive metabolites (Jee and Kang, 2005) and reduction of organic hydroperoxides (Cnubben et al., 2001). In the kidney no evidence of antioxidant defenses activation was observed, suggesting that no ROS were produced in this organ as a result of Phe exposure. However, the lowest Phe concentration (0.1 µM) increased LPO, invalidating the previous statement for this particular concentration.

4.1.3. Liver responses

Fish liver is generally the organ where many absorbed PAHs go through a critical metabolic process of activation (oxidation), mediated by cytochrome P450-associated monooxygenases, or inactivation mediated by conjugases, allowing their excretion and elimination. The liver has also been described as the most important organ involved in the redox metabolism (Prieto et al., 2006). In this study, the liver GSH levels (from 0.1 up to 0.9 µM) and GPx activity (whole concentration range) were increased in a dose dependent manner. The observed liver GSH increase is probably a result of an increased synthesis as the GSSG/GSH cycle activation was not evident and even if activated could not explain such increase. The GPx increased activity seems to support the idea that, under these exposure conditions, GPx is the first line of enzymatic defense against H_2O_2. Moreover, a clear relation between GPx increased activity and GSH

increased levels is obvious, demonstrating the well known association between these two parameters. Considering the probable ROS generation by Phe exposure, the simultaneous analysis of current GSH, GPx and LPO data, indicates that the non enzymatic defense by itself is not sufficient to prevent peroxidative damage, as it can be seen for 0.1 µM. The simultaneous increase of GSH and GPx (0.3 and 0.9 µM) yielded a stronger antioxidant defense with no LPO production. Despite the simultaneous increase of GSH and GPx, after 2.7 µM Phe exposure, the less pronounced GSH increment seems to enhance the liver vulnerability towards LPO.

Previous studies demonstrated that Phe effects on liver GST activity depend on the concentration and exposure duration. Hence, for a short term *C. auratus* exposure (0.28 µM) it was observed an inhibition from 24 to 48 hours disappearing after 4 days exposure (Sun et al., 2006). Ying et al. (2007) did not find changes after 4 days exposure to concentrations lower than 0.28 µM whereas in higher concentrations an increase was found. For longer exposures with *P. olivaceus* (2 and 4 weeks), 1 and 2 µM Phe displayed an ability to increase this enzymatic activity (Jee and Kang, 2005).

4.1.4 General statements

The *L. aurata* vulnerability towards Phe induced peroxidative damage, as clearly demonstrated by the present results, agrees with a previous study concerning *P. olivaceus* where Phe toxicity measured as increased LPO was also demonstrated (Jee and Kang, 2005). Considering susceptibility to LPO, the assessed organs can be ordered as follows: gill > liver > kidney. In general, the susceptibility of a given organ is determined by different (i) predisposition to accumulate xenobiotic, (ii) characteristic antioxidants basal levels, (iii) adaptation capacity and consequent antioxidant activation, and (iv) metabolic rates, increasing the potential to produce ROS and challenging the respective defenses. The current results, allow us to understand the organ specific responses mainly on the basis of (ii) and (iii) items.

In terms of GSH, the statistical analysis showed significantly higher levels in liver when compared to gill which is related with a superior adaptation capacity of

the former though its higher basal levels can not be neglected in this comparison. In general, the GSH levels following Phe exposure are not significantly different between kidney and liver. Taking into account that no significant increases were detected in kidney (when compared to the respective control), it can be suggested that liver responded by increasing its GSH level up to an order of magnitude corresponding to the kidney basal levels. Comparing GPx responses in the three organs, clear different patterns are perceptible i.e. a susceptibility to inhibition in gill; no alterations in kidney and increasing tendency in liver. The inhibition observed in gill is probably related to its lower GPx basal levels, increasing the vulnerability in comparison to the other organs. Kidney displayed the higher GPx levels which make its increase less necessary to cope with Phe challenge in opposition to liver where this enzymatic activity was necessary and promoted. Accordingly, the results suggest higher gill susceptibility to Phe toxicity due to a weaker antioxidant defense associated with the observed lower GSH basal levels and lower response capacity. This is confirmed by the current LPO results being also in agreement with previous findings demonstrating a weaker antioxidant potential in gill, compared to other organs after exposure to PAHs and other classes of contaminants such as pesticides (Sayeed et al., 2003; Ahmad et al., 2004). In addition, it should also be considered that gill is the first tissue in contact with waterborne xenobiotics which is particularly relevant in short-term exposures. Liver showed higher response capacity to Phe exposure though insufficient to prevent LPO. Under these circumstances, liver damage may be due to higher metabolic rates rather than to differences in Phe distribution as it is known that chemicals absorbed via gill reach the kidney at least in the same extent than liver (Ahmad et al., 2003), highlighting the role of the previously mentioned item (iv). Moreover, the apparent lower vulnerability of kidney should be associated with the higher basal levels of GSH content and GPx activity.

The present results agree with previous findings that suggest GSH as a first line of defense (Ahmad et al., 2000; 2005; Pastore et al., 2003; Li et al., 2007), as it can scavenge free radicals and reduce H_2O_2. Previous findings concerning GSH level increase in fish exposed to PAHs support this idea (Di Giulio et al., 1993; Ahmad et al., 2003; Pandey et al., 2003). GSH-dependent enzymes are

considered a second line of defense (Pastore et al., 2003). Thus, the conjugation of these two lines of defense (GSH and GPx activity increase) seems to be able to prevent LPO increase in the liver of Phe exposed fish (0.3 and 0.9 µM Phe). The involvement of GPx and CAT in the defense against peroxides is still not fully understood since some studies suggested that CAT was the main H_2O_2-detoxifying enzyme (Lopez-Torres et al., 1990; Bouzyk et al., 1996), whereas other investigations found that GPx was more efficient at H_2O_2 detoxification compared to CAT (Remacle et al., 1992; Dorval, 2003). Jifa et al. (2006) observed nearly no changes in *Lateolabrax japonicus* CAT activity after benzo[a]pyrene (BaP) exposure and found that H_2O_2 produced in BaP metabolism is mainly catabolized by GPx but barely by CAT. The present Phe study revealed a similar pattern of response since CAT (a non GSH dependent enzyme) was less responsive than GPx, i.e. CAT was only increased in gill by 0.9 µM Phe. In this perspective, the higher CAT levels generally found in liver comparing with gill and kidney should be related to the higher hepatic metabolism instead of a Phe induced effect.

The performance of the assayed antioxidants revealed, after 16-hour Phe exposure, that in all the studied organs GSH levels and GPx activity were more responsive compared to GR, GST and CAT.

The response of kidney and liver in terms of LPO increase did not display a clear dose-response relation. However, for liver it can be hypothesized that the lowest Phe concentration represents a condition insufficient to stimulate the defenses but pernicious enough to cause peroxidative damage. On the other hand, increasing the dose the threshold needed for defenses (namely GPx activity and GSH content) activation was achieved enabling the cells to avoid LPO up to a certain extent where damage was again measurable (highest Phe concentration). Concerning kidney, this pattern of responses can not be ignored but the results do not confirm it. In general, the results reinforce the difficulty to predict LPO on the basis of antioxidants depletion as previously stated by Cossu et al. (2000).

The findings of Sun et al. (2006) and Ying et al 2007 that measured increased production of hydroxyl radical in the liver of *C. auratus* after Phe

exposure corroborate the strong indications of a ROS augmented generation provided by the LPO results in the current study.

5. Conclusions

L. aurata short-term (16 hours) exposure to Phe resulted in LPO increase in gill, kidney and liver, revealing overwhelmed antioxidant defenses. Hence, LPO was found higher in gill (followed by liver and kidney) in parallel with its lower antioxidant defense capacity. However, liver demonstrated a higher adaptive competence expressed as antioxidant defenses activation. The kidney lower vulnerability compared to gill and liver seems to be related to its higher antioxidant basal levels.

The *L. aurata* exposure to Phe revealed that oxidative stress is involved in the toxicity of this PAH and GSH content and GPx activity play the main role in fish adaptation under these circumstances.

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CAPÍTULO IV

Respostas de stresse e da tiróide em monitorização aquática - Abordagem integrada de biomarcadores

Fish thyroidal and stress responses in contamination monitoring - An integrated biomarker approach

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Submitted

Abstract

Xenobiotics may affect fish endocrine system, making endocrine responses relevant in pollution monitoring. Five critical sites in Ria de Aveiro (Portugal), having different contamination profiles, were assessed in comparison to a reference site (Torreira), focusing on *Liza aurata* stress (plasma cortisol, glucose and lactate) and thyroidal (plasma thyroid stimulating hormone -TSH, thyroxine - T4 and triiodothyronine -T3) responses. *L. aurata* at Vagos (PAHs contaminated) and Laranjo (mercury contaminated) respectively displayed, low cortisol and high glucose as well as lactate levels. Considering thyroidal responses, high TSH levels were found at Laranjo; low T4 levels were found at Barra (subject to naval traffic) and low T3 levels were found at Rio Novo do Principe (near a former bleached kraft pulp mill effluent), Laranjo and Vagos. The integrated biomarker index ranked impacted sites as: Laranjo > Vagos > Gafanha > Rio Novo do Príncipe > Barra > Torreira. Endocrine responses reflected xenobiotics exposure suggesting environmental risk to fish at Ria de Aveiro. However no clear relation was found between stress and thyroidal responses.

Keywords: *Liza aurata*; thyroid hormones; cortisol; glucose; lactate

1. Introduction

Fish, upon a threatening situation or perceived as so (e.g. presence of predator, toxicant exposure, environmental changes), display a stress response, readjusting their biological activities in an integrated stress response (Barton, 2002). Primary response, is neuroendocrine, involving activation of brain centres, that lead to a rapid release of catecholamines (such as adrenaline) by the interrenal tissue, followed within minutes by pituitary adrenocorticotropic hormone secretion (ACTH), in order to stimulate the secretion of corticosteroids (mainly cortisol) (Wendelaar Bonga, 1997). Secondary responses involve metabolic

alterations such as changes in plasma glucose, lactate, ionic levels, hepatic glycogen consumption and increased metabolic rates (Hori et al., 2008). Tertiary responses include inhibition of growth, reproduction, and immune response and reduced capacity to tolerate additional stress (Wendelaar Bonga, 1997). If fish is repeatedly or chronically stressed, this response becomes attenuated and may result in exhaustion or death (Web et al., 2007).

Cortisol is considered as the primary corticotropic hormone secreted in response to stressors (Schreck et al., 2001; Yada et al., 2002). Thus, plasma cortisol levels are generally accepted as a good indicator of stress in fish (Wendelaar Bonga, 1997; Barton, 2002). Overall, the physiological role of cortisol during stress is to mobilize energy to meet the demand of increased metabolic rates (Mommsen et al., 1999). Thus, cortisol has an important role in the regulation of intermediary metabolism and acclimation to different osmotic conditions (McCormick, 1995; 2001). In this perspective, glucose is an important energy substrate for several tissues and its production is thought to metabolically assist the animal to cope with an increased energy demand caused by stress (Barton and Schreck, 1987; Vijayan and Moon, 1994). Though the immediate glucose production after stress may be due to glycogenolysis, the maintenance of glucose over longer periods of time is due to gluconeogenesis from substrates, including lactate and amino acids (Vijayan et al., 1997a). Studies have shown that cortisol increases hepatic gluconeogenic enzyme activities in fish (Vijayan et al., 1991; 1994a). Vijayan et al. (1997a) suggested that cortisol either directly and/or indirectly contributes to the long-term regulation of glucose to cope with the increased energy demand associated with stress. Plasma lactate increases immediately after stress, primarily due to muscle glycolysis (Milligan and Girard, 1993). This substrate may be used for glucose production and/or glycogen repletion in the liver of stressed fish (Vijayan and Moon, 1992; Vijayan et al., 1994b). The mechanisms by which many xenobiotics alter metabolic substrate availability are unclear, but may be a result of their effects on metabolic enzymes since carbohydrate metabolism pathways are frequently disrupted by pollutants exposure (Scott and Sloman, 2004).

Another important endocrine reaction to xenobiotics exposure involves thyroidal responses. These responses are slower than the previously mentioned stress responses, though their effects are more prolonged. Thyroid hormones play an important role in the maintenance of a normal physiological state in vertebrates. They are known to regulate gene expression, metabolic activity, growth, development and homeostasis of cells and tissues (Janz, 2000). In teleosts, the central control of thyroid hormone is almost limited to production and secretion of thyroxine (T4) (Blanton and Specker, 2007) since the biologically active thyroid hormone, T3 (triiodothyronine), is mainly derived from T4 extrathyroidal deiodination (Eales et al., 1999). The functional activity of thyroid-stimulating hormone (TSH) is limited to regulating T4 release and iodide uptake by the thyroid follicles (Eales et al., 1999). In fish, both T4 and T3 have a negative feedback effect on TSH secretion (Yoshiura et al., 1999). Many toxic chemicals such as polychlorinated biphenyls, planar halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), metals and steroids may alter the circulating levels of thyroid hormones (Rolland, 2000; Brown et al., 2004), affecting several steps, namely synthesis, regulation, metabolism and action of thyroid hormones (Leatherland, 2000).

Ria de Aveiro is a coastal lagoon that, over decades, received considerable influx of anthropogenic pollutants from unidentified and well-known sources, including industrial discharges from chlor-alkali and paper mill plants, harbour activities and domestic effluents. Taking into consideration the vast range of xenobiotics likely to be found in this aquatic system and the limited information that chemical analyses may provide in terms their bioavailability, interactions and toxicity, the biological responses of fish can represent a more powerful tool, detecting possible future chronic pathologies. A previous study, monitoring stress responses in European eel (*Anguilla anguilla*) caged in two sites of Ria de Aveiro (Teles et al., 2004a) detected the presence of xenobiotics able to modulate cortisol and intermediary metabolism responses. Other studies, concerning a wide area of this lagoon, demonstrated the presence of xenobiotics able to cause genetic and oxidative damage in fish (Ahmad et al., 2008; Oliveira et al., 2009). In this

perspective, it is highly relevant to know how the endocrine system of fish in Ria de Aveiro is affected by the xenobiotics present in this lagoon.

Golden grey mullet (*Liza aurata*) has a widespread distribution in Ria de Aveiro and is considered as a sensitive bio-indicator species in terms of stress responses, biotransformation, genotoxicity and oxidative stress (Oliveira et al., 2007, 2008a; Pacheco et al., 2005). Thus, the present study assessed Ria de Aveiro, focusing on *L. aurata* stress responses (plasma cortisol, glucose and lactate) as well as thyroidal responses (plasma TSH, T4 and T3). The integrated biomarker response (IBR) index was used to improve the discriminatory power of the adopted multi-biomarker strategy.

2. Material and Methods

2.1 - Study Area: Ria de Aveiro is a lagoon permanently connected to the Atlantic ocean (Figure 1), about 45 km long and 8.5 km wide. Sampling sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of an unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of PAHs.

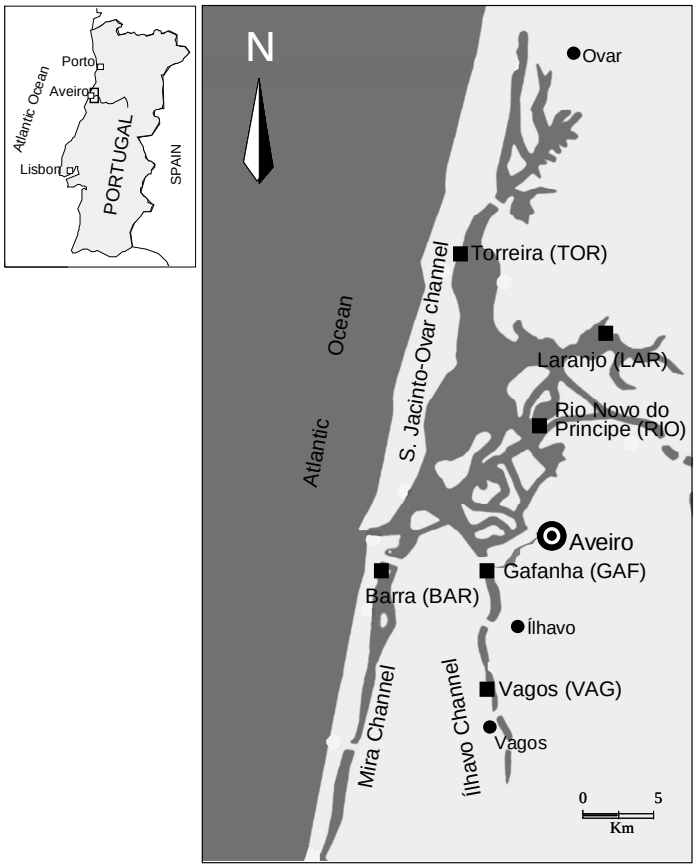

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture sites (■). The respective coordinates are: reference site (TOR)—40º44'02 N, 008º41'44 W; BAR—40º37'42.00"N, 8º44'35.00"W GAF—40º38'38 N, 008º41'42 W; RIO—40º41 '08 N, 008º39'41 W; LAR—40º43'30 N, 008º37'43 W; and VAG—40º33'59 N, 008º40'55 W.

2.2 - Sampling: *Liza aurata* was caught in October 2005, during low tide using a traditional beach-seine net named “chincha”. Juvenile specimens were selected on the basis of their size, having an average length of 14.5±2.5 cm and weighing 21.6±3.7 g. In the laboratory, plasma separation was performed using an Eppendorf centrifuge (13400 g). Samples were stored at –80ºC until analyses.

At each sampling site abiotic parameters were assessed (Table I) according to the APHA (1998) guidelines.

Table I - Physical-chemical parameters of the water from the Ria de Aveiro sampling sites.

	Turbidity (m)	*Dissolved Oxygen (mg/L)*	*Temperature (ºC)*	*pH*	*Salinity*
TOR	0.80	6.88	18.9	8.17	32.5
BAR	1.20	6.16	17.9	8.32	34.0
GAF	0.70	5.78	17.9	8.28	34.0
RIO	1.00	6.37	19.0	8.20	21.0
LAR	0.30	4.96	18.6	7.74	29.0
VAG	0.30	5.18	18.5	7.88	33.0

2.3. Hormonal responses measurement: The determinations of cortisol, TSH, T3 and T4 were performed in plasma, using diagnostic ELISA direct immunoenzymatic kits (Diametra, Italy). Absorbance was measured at 450 nm in a microplate reader (SpectraMax 190). 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 (3,3', 5,5' tetramethylbenzidine)-substrate are added, and after an appropriate time has elapsed for maximum colour development, the enzyme reaction is stopped and the absorbance determined. Cortisol concentration in the sample is calculated based on a series of standards and the colour 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 h-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.

2.4. Intermediary metabolism responses measurement: Plasma glucose was measured spectrophotometrically (Jasco UV/VIS, V-530, 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, alanine transaminase and NAD, measuring the NADH appearance.

2.5. Integrated biomarker response (IBR): A method for combining all the measured biomarkers (with the exception of T4, since no sample from LAR was available to perform this parameter) into one general ‘‘stress index’’ termed ‘‘Integrated Biomarker Response’’ (IBR) (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006).

2.6. Statistical analysis: Results are expressed as means ± SE (standard errors) corresponding to experimental groups of six fish (n=6). A statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK). The assumptions of normality and homogeneity of data were verified. One way ANOVA was performed in order to assess significant effects, followed by Fisher LSD test to signal significant differences between groups (Zar, 1999). The significance of the results was ascertained at $\alpha = 0.05$.

3. Results

3.1. Stress Responses

Plasma cortisol levels were significantly lower at VAG, when compared to the reference site (TOR), displaying 44% lower levels (Figure 2A). Moreover, fish from VAG also displayed significantly lower plasma cortisol levels than BAR, GAF and LAR. In terms of intermediary metabolism, measured as plasma glucose and lactate levels, fish from LAR displayed significant differences to TOR and all the other studied sites (Figure 2B,C). Thus, at LAR plasma glucose and lactate levels were respectively 71 and 179 % higher than TOR.

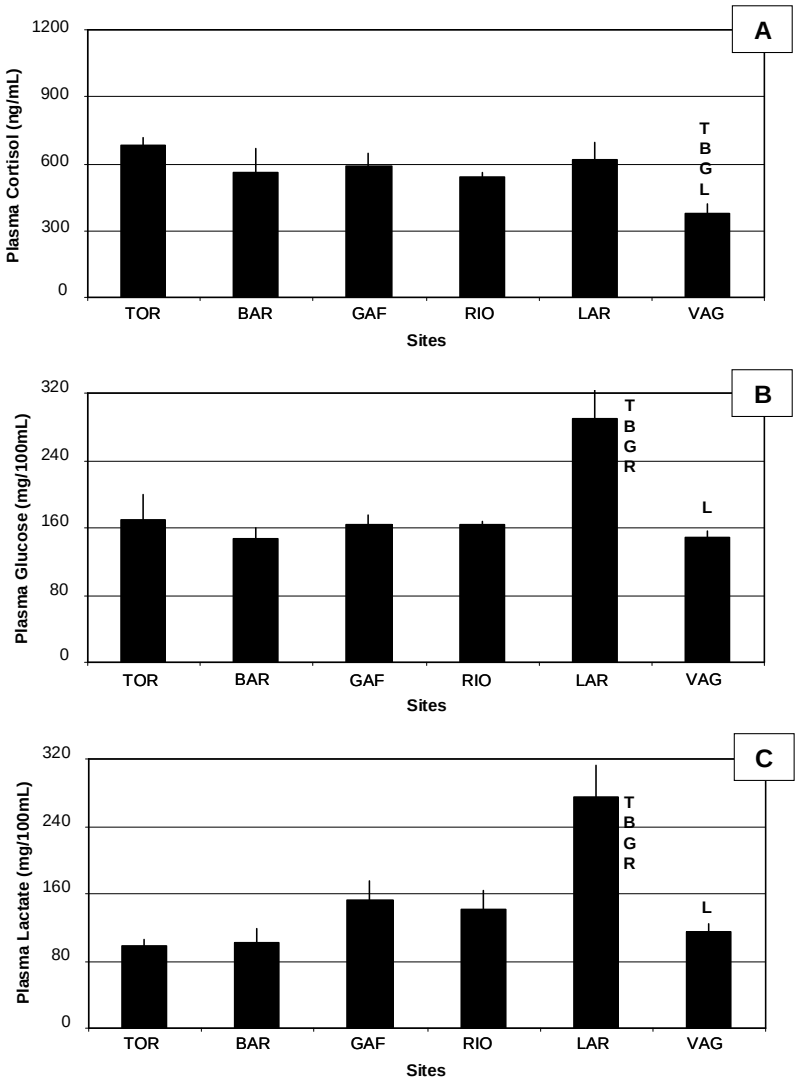

Figure 2 – Stress responses of *L. aurata* collected at different sites in the Aveiro lagoon. A – Plasma cortisol; B – Plasma glucose; C – Plasma latacte. Values represent means ± standard errors (n=6). Significant differences (P<0.05) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR.

3.2. Thyroidal Responses

TSH levels were significantly higher in fish from LAR when compared to TOR as well as to BAR and RIO (Figure 3A). Thus, fish captured at LAR displayed 10% higher TSH levels than fish at TOR.

T4 levels were significantly lower in fish caught at BAR compared to TOR (60%), GAF and VAG (Figure 3B). Though non-significantly, T4 levels in fish from RIO were 33% lower than at TOR whereas at VAG, levels were 31% higher.

T3 levels in fish captured in RIO, LAR and VAG were significantly lower than in TOR, BAR and GAF (Figure 3C). Thus, compared to TOR, T3 levels were 74, 87 and 55% lower at respectively RIO, LAR and VAG. At LAR T3 levels were also significantly lower than VAG.

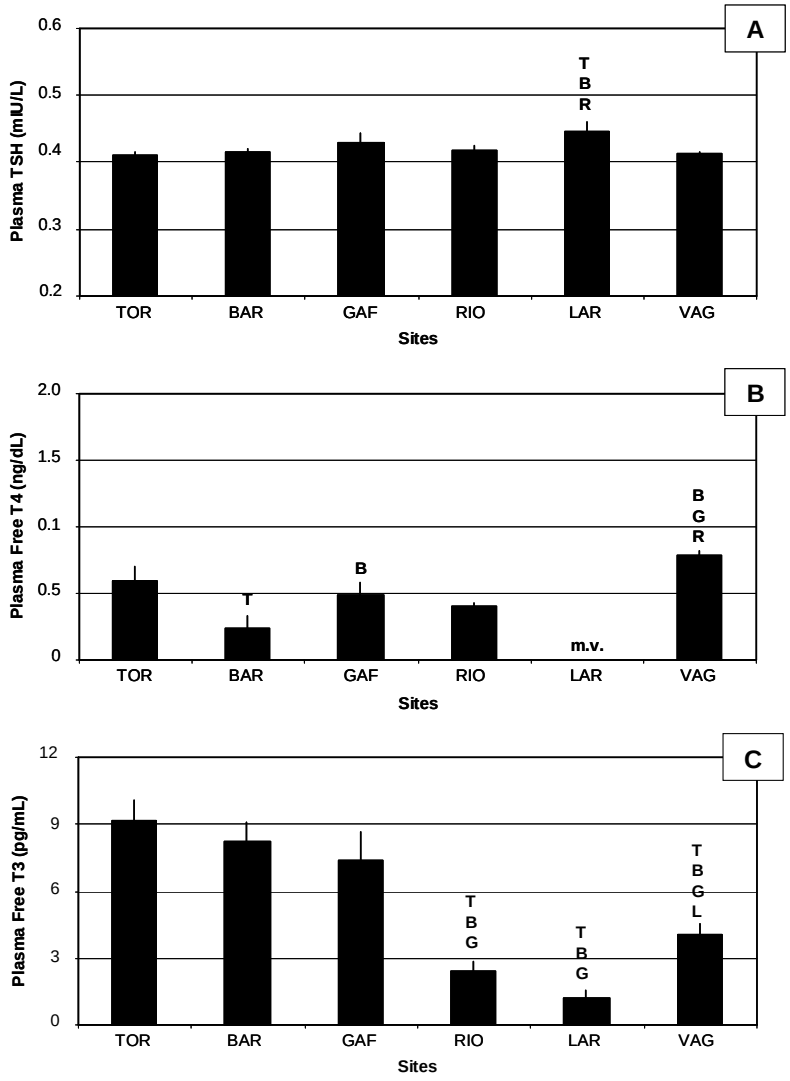

Figure 3 – Thyroidal responses of *L. aurata* collected at different sites in the Aveiro lagoon. A – Plasma TSH; B – Plasma T4; C- Plasma T3 Values represent means ± standard errors (n=6). Significant differences (P<0.05) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR.

3.3. Integrated Biomarker Response

Taking into account that for LAR fish it was not possible to perform T4 quantification, the IBR index was calculated without considering this parameter in order to allow comparison between all sites. Accordingly, the rank of the most affected sites was established as: LAR>VAG>GAF>RIO>BAR>TOR (Figure 4A).

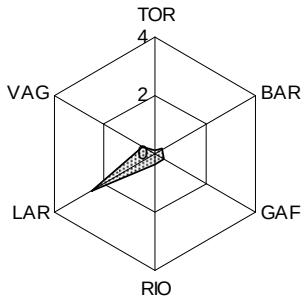

Figure 4 – Integrated biomarker response (IBR).

4. Discussion

According to Pottinger et al. (1996), circulating gonadal steroids may affect hormonal responses namely cortisol. In this perspective, *L. aurata* specimens captured for this study were juvenile and reproductively quiescent.

Fish ability to increase cortisol levels in response to acute stress of capture/handling in a field situation is a potential indicator of physiological competence (Hontela et al., 1997). However, continued exposure to xenobiotics (organic or metals), may lead to low plasma cortisol levels and/or significantly reduced capacity of interrenal tissue to respond to ACTH (Brodeur et al., 1997a,b; Girard et al., 1998; Levesque et al., 2002; Norris et al., 1999). In the current study, *L. aurata* cortisol levels were only different from the reference site at VAG, displaying lower plasma cortisol. Thus, taking into consideration that successful cortisol release relies on a functionally intact hypothalamo-pituitary-interrenal (HPI) axis, the present data may indicate an HPI axis impairment in VAG fish, a site with known high waterborne levels of PAHs (Pacheco et al., 2005). However, it is not

known whether the lower cortisol levels detected at VAG reflect HPI axis exhaustion caused by a chronic stimulation or a cytotoxic effect occurring either during its synthesis or release at the interrenal steroidogenic cells, upstream at pituitary ACTH or hypothalamic cells corticotrophin-releasing hormone synthesis. Nevertheless, this low cortisol at VAG may reduce the capacity of fish to cope with biotic and abiotic stressors normally encountered in their environments (Hontela et al., 1997). The lack of differences in terms of *L. aurata* plasma cortisol levels between the other critical sites (BAR, GAF, RIO and LAR) and TOR, suggest an *L. aurata* adaptation to the different contamination profiles, though other studies (laboratory/field) using different fish species have demonstrated the capacity of xenobiotics to impair the HPI axis. Thus, GAF site located in the vicinity of an harbour water area, has high levels of PAHs, metals and organometallic compounds (Barroso et al., 2000; Pacheco et al., 2005) and has previously demonstrated its ability to increase caged *A. anguilla* cortisol levels after 48 hours exposure (Teles et al. 2004a). Yellow perch (*Perca flavescens*) and pike (*Exos lucius*), caught at sites with contamination profile similar to GAF (i.e. mixtures of contaminants organics and metals) exhibited impairment of the HPI axis, characterized by a reduced ability to elevate blood cortisol after an acute standardized capture and handling stress (Hontela et al., 1992, 1995). RIO area received over several years bleached kraft pulp mill effluents (BKPME) containing complex mixtures of environmentally active compounds. *A. anguilla* laboratory (short-term) exposed to BKPME (Santos and Pacheco, 1996) and *P. flavescens* and *E. lucius* collected from a BKPME area (Hontela et al., 1997) displayed cortisol secretion impairment. LAR, is a site with known high levels of mercury (Abreu et al., 2000), which has been shown to interfere with HPI axis. Juvenile rainbow trout (*Oncorhynchus mykiss*) short-term exposure to mercury stimulated the HPI axis (Bleau et al., 1996). However, decreased plasma cortisol has been found in catfish (*Clarias batrachus*) chronically exposed to mercury (Kirubagaran and Joy, 1991). Moreover, at mercury contaminated sites the capacity to secret cortisol has also been decreased in *E. lucius* (Lockhart et al., 1972) and *P. flavescens* (Brodeur et al., 1997b; Laflamme et al., 2000). Furthermore, *in vitro*

studies demonstrated that mercury may impair cortisol secretion by adrenocortical cells in *O. mykiss* and *P. flavescens* (Leblond and Hontela, 1999).

Changes in carbohydrate metabolism, measured as plasma glucose and lactate, can also be used as general stress indicators in fish. A typical stress response includes plasma glucose and lactate increase (Hontela et al., 1996; Santos and Pacheco, 1996). The plasma glucose concentration in circulation is a function of its production *versus* absorption by tissues. In a stress situation, glucose production provides energy substrates to tissues, in order to cope with the increased energy demand. Lactate has been widely used as a measure of anaerobic metabolism and rapid response to depletion of tissue energy stores. However, the storage or mobilization of metabolic substrates in fish may be disrupted by xenobiotics such as metals (Lockhart et al., 1972; Sjobeck et al., 1984; Bleau et al., 1996; Levesque et al., 2002; Oliveira et al., 2008b), herbicides (Oruc and Uner, 1999), BKPME (Santos and Pacheco, 1996; Teles et al., 2004a), BKPME components - resin acids and retene (Teles et al., 2003), harbour waters (Teles et al., 2004a), organic compounds (Teles et al., 2004b) and mixtures of organic compounds and metals (Teles et al., 2005). Fish chronically affected by water pollution contamination are likely to have higher glucose requirements for adaptation and repair damage (Palermo et al., 2008). However, in the current study, only fish caught at LAR showed effects on intermediary metabolism, such as increased plasma glucose and lactate levels, a typical stress response, suggesting that *L. aurata* exposed to mercury contaminated environments required extra energy to carry out adjustment processes. Mercury induced hyperglycemia was observed in barb (*Barbus conchonius*) laboratory exposed to mercury up to 8 weeks (Gill and Pant, 1981). Hyperglycemic responses have also been found in laboratory short-term exposures to water-soluble fraction of diesel oil in streaked prochilod (*Prochilodus lineatus*) (Simonato et al., 2008), flounder (*Pleuronectes flesus*) (Alkindi et al., 1996) and *A. anguilla* (Pacheco and Santos, 2001). However, in the current study, no significant changes in glucose and lactate were observed at BAR and GAF. The short-term study performed by Teles et al. (2004a) in BKPME outlet area and in harbour waters using caged *A. anguilla* found increased glucose and lactate. Though stress responses of wild and caged

animals as well as different species are not easily comparable, the caged experiment demonstrated the presence of xenobiotics able to modulate stress responses. The lack of differences to the reference site, found in the present study at contaminated sites (BAR, GAF, RIO and VAG) suggests an adaptation by *L. aurata* to those conditions.

Cortisol effect includes gluconeogenic activation, mobilizing substrates such as amino acids, lactate and lipids, as fuel energy. However, in the current study a direct relation between these two levels of stress responses is not clear. 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. According to Vijayan et al. (1997b) besides interrenal cortisol release, other mechanisms might be controlling glucose availability in fish. Our data seem to support this theory.

Thyroid hormones influence the activity of a wider variety of tissues and biological functions when compared to any other hormones (Janz, 2000). Their reduction or absence of production is known to decrease metabolic rate and cause the muscle to become sluggish (Thangavel et al., 2004). Moreover, they have profound effects on lipid, carbohydrate and protein metabolism (Plisetskaya et al., 1983; Varghese and Oommen, 1999; Shameena et al., 2000). Environmental contaminants may interfere with hypothalamo-pituitary-thyroid (HPT) axis through many mechanisms of action, i.e. at the receptor level, in binding to transport proteins, in cellular uptake mechanisms or in modifying the metabolism of thyroid hormones (Boas et al., 2006). In the current study, *L. aurata* plasma TSH levels were only different (higher) from the reference site at LAR. This alteration may have been a response to the observed decreased T3 levels, a physiological unbalance, which may have pernicious effects in fish development if T3 levels are not returned to values similar to TOR. Metal exposure has been shown to affect plasma thyroid hormone levels. Thus, Bleau et al. (1996) observed that *O. mykiss* 4 hours exposure to mercury lead to increased T4 levels despite their return to levels similar to control after 72 and 168 hours. Kirubagaran and Joy, (1994) reported a gradual decrease of plasma T4 as well as T3 after 1 week exposure to methylmercury in *C. batrachus*. Moreover, chronic metal exposure in a

contaminated lake resulted in *P. flavescens* T3 and T4 decreases (Levesque et al., 2003). In fish caught at BAR, despite the significantly low plasma T4 levels, plasma TSH was not different from TOR. These results suggest a hypothalamo-pituitary-thyroid (HPT) axis impairment since a TSH increase would be expected upon a plasma T4 decrease. Plasma T4 levels may be considered a more reliable reflection of thyroid function than T3, since most of circulating T3 (around 80%) is produced by extra-thyroidal T4 deiodination (Sapin and Schlienger, 2003).

Taking into account that *L. aurata* caught at RIO and VAG did not show differences in terms of T4 compared to fish caught at TOR, the observed plasma T3 decrease seems to be related to disruption downstream from thyroid. Hence, plasma T3 decrease may be either due to its increased uptake by peripheral tissues, decreased extrathyroidal tissues 5' -monodeiodinase activity or increased T3 catabolism and plasma clearance rates. Considering that T3 is the biologically active thyroid hormone (Brown et al., 1998), the fish caught at these sites (RIO, LAR and VAG) may have their survival skills seriously impaired. According to the current data, impaired cortisol stress response and impaired thyroid function do not necessarily occur together, which agrees with Gravel et al. (2005) previous findings.

The different patterns of response, observed in the studied sites, suggest different mechanisms of toxic action and confirm different contamination profiles. The IBR ranked the surveyed sites, according to the hormonal and metabolic changes (TSH, T3, cortisol, glucose and lactate) as: LAR>VAG>GAF>RIO>BAR>TOR.

5. Conclusions

The presence of contaminants in Ria de Aveiro, able to interfere with *L. aurata* endocrine system, was clearly demonstrated. Thus, according to the current *L. aurata* responses at LAR, VAG and RIO may have serious effects upon population levels due to diminished survival skills. IBR index points out to a greater impact at LAR.

Despite no clear relation between stress responses and thyroidal hormones was observed in the current research, the importance of assessing both responses in toxicity studies should be emphasized.

L. aurata endocrine responses assessment proved valuable in monitoring sites contaminated by complex mixtures.

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CAPÍTULO V

Avaliação da contaminação de um sistema lagunar costeiro (Ria de Aveiro, Portugal) usando indicadores bioquímicos de defesa e dano nas guelras de *Liza aurata* - Abordagem integrada de biomarcadores

Contamination assessment of a costal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* – An integrated biomarker approach

Oliveira, M., Maria, V.L., Ahmad, I., Serafim, A., Bebianno, M.J., Pacheco, M. Santos, M. A. (2008)
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Abstract

Fish gill importance in toxicants uptake, bioconcentration and excretion allied to meagre knowledge on branchial damage/protection responses substantiate this study. Five critical sites in Ria de Aveiro (Portugal) were assessed in comparison with a reference site (Torreira), focusing on *Liza aurata* gill antioxidant defences *versus* damage (oxidative and genetic). Only in Barra fish displayed damage (lipid peroxidation) though no differences were found in antioxidants. In all other sites, except Rio, antioxidant alterations were found. Thus, fish from Gafanha, Laranjo and Vagos showed higher total glutathione, glutathione peroxidase and catalase. Higher glutathione reductase and glutathione S-transferase activity was also found in the former and last sites, respectively. In Laranjo, metallothionein levels were higher though lower in Gafanha and Vagos. In general, damage was not accompanied by defences weakening confirming that predicting damage based on antioxidants depletion is not straightforward. The integrated biomarker response index ranked sites as: Gafanha>Barra>Laranjo>Vagos>Rio>Torreira.

The integration of antioxidant defence and damage responses in fish, using gill as a key organ, can improve the aquatic contamination assessment.

Keywords: *Liza aurata*; Antioxidant defences; Metallothioneins; Oxidative stress; Genotoxicity

1. Introduction

Aquatic systems are the main recipients of almost all anthropogenic discharges. Given the complex mixture of contaminants found in the aquatic environment, mainly in lagoon systems, their quantification in the water/sediment compartments *per se* gives limited information on the threat to aquatic organisms namely in terms of chemical bioavailability and interactions. Moreover, chemical

analyses are expensive and it is not feasible to measure all classes of chemicals likely to be found in a multi-contamination context. On the other hand, the analysis of early biochemical effects (biomarkers) provides relevant data about possible future chronic pathologies, allowing the prediction of ecological changes. However, a biomarker battery selection is essential, as environmental contamination complexity may induce a variety of responses not necessarily correlated (Viarengo et al., 2000).

Xenobiotics toxicity often depends on their capacity to increase cellular levels of reactive oxygen species (ROS), which can happen either by activation of their synthesis or by cell antioxidant defences imbalance. These responses (namely glutathione content, catalase–CAT, glutathione peroxidase-GPx, glutathione S-transferase–GST and glutathione reductase-GR) can be measured as biomarkers of xenobiotic-mediated oxidative stress (Regoli et al., 2002; Ahmad et al., 2004; Gravato et al., 2006), providing valuable information on environmental status. Metallothioneins (MT) are known to be over-expressed in organisms from environments with high metal concentrations (Viarengo et al., 1999). Nonetheless, studies have shown that MT are also induced by non metal compounds able to cause oxidative stress in fish cells (Pedrajas et al., 1995; Kling et al., 1996). Despite its upregulation by metals, MT may also be used as biomarker of a general stress response to environmental contaminants (Chesman et al., 2007). Lipid peroxidation (LPO) is an oxidative stress sign, indicating that ROS production exceeded antioxidant defences (Ahmad, et al., 2004). This imbalance may also lead to DNA damage which has been proposed as a useful biomarker to assess the presence of genotoxicants. Several *in situ* studies found higher DNA damage in organisms collected from polluted areas, pointing to their utility in biomonitoring programs (Maria et al., 2004a; Maria et al., 2004b; Jha, 2008).

Most environmental monitoring studies using fish focused their attention on liver due to its importance on the metabolism and storage of xenobiotics. However, it is important to know the responses of other tissues. Gill are considered an important route for uptake, bioconcentration and excretion of toxicants and a prime target to contaminants, due to the wide surface area in contact with the external medium and reduced distance between internal and external medium (Sayeed et

al., 2000). Furthermore, gill being between the venous and arterial circulation and receiving nearly all of the cardiac output can also influence the metabolic fate of chemicals taken up by other exposure routes (Levine and Oris, 1999).

Ria de Aveiro, a coastal lagoon in the northwest of Portugal, is a biologically productive system with a significant role in the life cycle of several organisms being used as nursery for many species, namely fish. Over decades, this system received discharges of complex effluents as a result of industrial and harbour activities, as well as urban and agricultural wastes. Preliminary fish biomonitoring studies carried out on Ria de Aveiro (Abreu et al., 2000; Perez Cid et al., 2001; Pacheco et al., 2005; Monteiro et al., 2007) provided limited information due either to few parameters assessed and/or narrow sites selection. Thus, a more comprehensive monitoring of this estuarine system needs to be carried out.

The goal of the present study was the assessment of Ria de Aveiro, as coastal lagoon prototype, focusing simultaneously on antioxidant defences (total glutathione - GSHt, MT, CAT, GPx, GST, GR) and damage (lipids and DNA) responses. The integrated biomarker response (IBR) index was used to improve the discriminatory power of the adopted multi-biomarker strategy.

2. Material and Methods

2.2. Study Area: Ria de Aveiro is a lagoon permanently connected to the ocean (Figure 1), about 45 km long and 8.5 km wide (Dias et al., 2001). Sampling sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of a (theoretically) unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of

the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of PAHs.

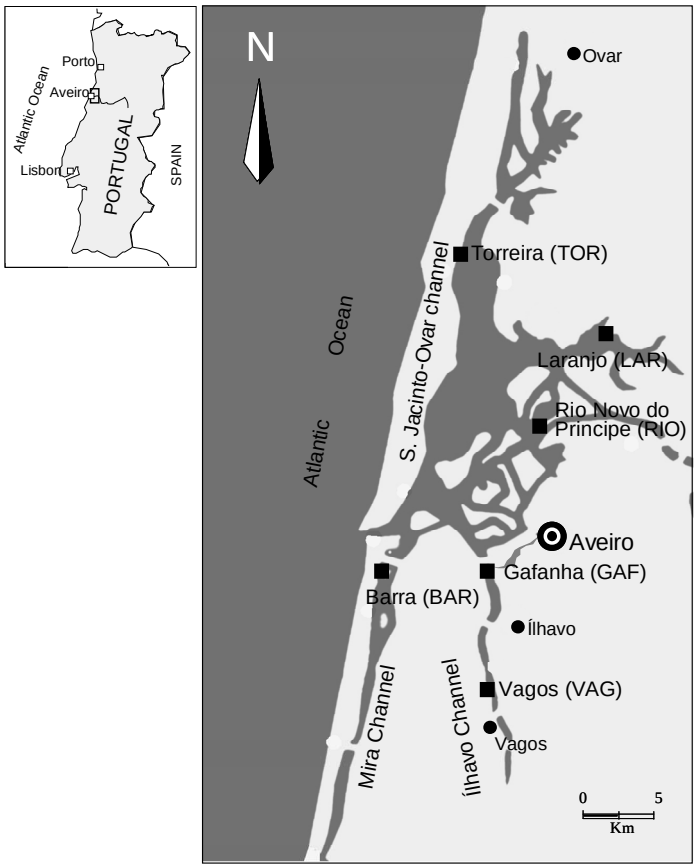

Figure 1 - Map of the sampling sites (■) in the Ria de Aveiro.

2.1.1. Sampling: *Liza aurata* was caught in October 2005, during low tide using a traditional beach-seine net named “chincha”. Juvenile specimens were selected on the basis of their size, having an average length of 14.5±2.5 cm and weighing 21.6±3.7 g. Immediately after catching, fish was sacrificed, gill removed and frozen in liquid nitrogen. In the laboratory, three sets of tissue were separated

for DNA strand breaks, MT and oxidative stress measurements and stored at −80º C until further treatments.

At each sampling site abiotic parameters were assessed (Table I) according APHA (1998) guidelines.

Table I - Physical-chemical parameters of the water from the Ria de Aveiro sampling sites.

	Turbidity (m)	*Dissolved Oxygen (mg/L)*	*Temperature (ºC)*	*pH*	*Salinity*
TOR	0.80	6.88	18.9	8.17	32.5
BAR	1.20	6.16	17.9	8.32	34.0
GAF	0.70	5.78	17.9	8.28	34.0
RIO	1.00	6.37	19.0	8.20	21.0
LAR	0.30	4.96	18.6	7.74	29.0
VAG	0.30	5.18	18.5	7.88	33.0

2.2. Biochemical analyses

One set of tissue was homogenized in phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/15 ml buffer). This homogenate was divided in aliquots for LPO and GSHt measurements, as well as post-mitochondrial supernatant (PMS) preparation. PMS was accomplished by centrifugation in a refrigerated centrifuge at 13400 g for 20 minutes (4ºC). PMS aliquots were stored at –80ºC until analyses.

2.2.1. Non Enzymatic Defences

2.2.1.1. GSHt: Protein content in the homogenate was precipitated with sulfosalicylic acid (5%) for one hour and then centrifuged at 13400 g for 20 minutes (4ºC). GSHt content was determined in the resulting supernatant adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5, dithiobis-tetranitrobenzoic acid producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) (Tietze, 1969; Baker et al., 1990). TNB formation

was measured spectrophotometrically (Jasco UV/VIS, V-530) at 412 nm. Results were expressed as µmol TNB formed/min/mg protein (ε=14.1×10^3 M^{-1} cm^{-1}).

2.2.1.2. MT content: After homogenization in 20 mM Tris buffer (pH 8.6) containing 150 mM of NaCl, homogenates were centrifuged 45 minutes at 30000 g (4ºC). Supernatant aliquots were heat-treated at 80 ºC for 10 minutes and re-centrifuged at 30000 g for 45 minutes (4ºC). MT quantification was made by differential pulse polarography (DPP) according to Bebianno and Langston (1989) using a 646VA Processor autolab type II and an ECO Chemie IME663 mercury drop electrode. Comparisons of peak heights with those of standard additions of purified rabbit MT enabled MT quantification. Accuracy of the DPP analytical method was obtained by internal calibration using commercial rabbit liver MT-I. Recovery studies of our cytosols spiked with the MT-I rabbit liver standards revealed recovery rate from 97 to 103%. Results were expressed as µg MT/g wet weight/mg protein.

2.2.2. Enzymatic Defences

2.2.2.1. CAT activity: Assayed in PMS by the method of Claiborne (1985) with some modifications. Briefly, assay mixture contained 1.99 ml phosphate buffer (0.05 M, pH 7.0), 1 ml H_2O_2 (30 mM) and 0.010 ml PMS. Absorbance was recorded spectrophotometrically at 240 nm (25 ºC). CAT activity was expressed as µmol H_2O_2 consumed/min/mg protein (ε=43.5 M^{-1} cm^{-1}).

2.2.2.2. GPx activity: Assayed in PMS according to the method described by Mohandas et al. (1984) with some modifications. Assay mixture contained 0.720 ml phosphate buffer (0.05M, pH 7.0), 0.050 ml ethylene diamine tetraacetic acid tetrasodium salt hydrate (EDTA) (1 mM), 0.050 ml sodium azide (1 mM), 0.025 ml GR (1 U/ml), 0.050 ml reduced glutathione (4 mM), 0.050 ml β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) (0.8 mM), 0.005 ml H_2O_2 (1.0 mM) and 0.050 ml of PMS. Enzyme activity was quantified by measuring NADPH oxidation at 340 nm (25ºC) and expressed as nmol NADPH oxidized/min/mg protein (ε=6.22×10^3 M^{-1} cm^{-1}).

2.2.2.3. GST activity: Determined in PMS, following the conjugation of GSH with 1-chloro-2,4- dinitrobenzene (CDNB) by the method of Habig et al. (1974) with some modifications. Reaction mixture contained 1.85 ml sodium phosphate buffer (0.1 M, pH 7.4), 0.050 ml reduced glutathione (1 mM), 0.050 ml CDNB (1 mM) and 0.050 ml PMS. Absorbance was recorded at 340 nm (25ºC) and expressed as mmol CDNB conjugate formed/min/mg protein (ε=9.6 mM^{-1} cm^{-1}).

2.2.2.4. GR activity: Assayed by the method of Cribb et al. (1989) with some modifications. Briefly, assay mixture contained 0.025 ml of PMS and 0.925 ml of NADPH (0.2 mM), GSSG (1 mM) and diethylenetriaminepenta-acetic acid (DTPA) (0.5 mM). Enzyme activity was quantified by measuring NADPH disappearance at 340 nm (25ºC) and expressed as nmol NADPH oxidized/min/mg protein (ε=6.22×10^{3} M^{-1} cm^{-1}).

2.3. Peroxidative and genetic damage evaluation

2.3.1. LPO: Determined in the tissue homogenate by the procedure of Ohkawa et al. (1979) and Bird and Draper (1984) with some modifications. Briefly, to 50 µl homogenate, 3 µl of 4% butylated hydroxytoluene in methanol, was added and mixed well. To this aliquot, 0.5 ml of 12% trichloroacetic acid in aqueous solution, 0.45 ml Tris–HCl (60 mM, pH 7.4; and 0.1 mM DTPA) and 0.5 ml 0.73% 2-thiobarbituric acid were added and mixed well. The mixture was heated for 1 hour in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2 ml microtubes and centrifuged at 13400 g for 3 minutes. Absorbance was measured at 535 nm and LPO expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed/mg protein (ε= 1.56×10^{5} $M^{-1}cm^{-1}$).

2.3.2. DNA integrity: Genotoxicity was tested using DNA alkaline unwinding assay. Gill portions were placed in TNES (Tris-HCl 10 mM, NaCl 125 mM, EDTA 10 mM, SDS 1%, pH 7.5) – urea (5 M) buffer with proteinase K

solution (final concentration 0.8 mg/ml). DNA isolation was performed using a genomic DNA purification kit (Fermentas). DNA integrity measurements were performed according to Rao et al. (1996) as adopted by Maria et al. (2002). Data from DNA unwinding technique were expressed as F-value [DNA integrity (%)], determined by applying the following equation:

$$F=\frac{ds}{ds+ss}\times 100$$

where ss is the relative fluorescence (measured with a Jasco FP 750 spectrofluorometer) of the single-stranded eluent of a sample minus the single-stranded control blank fluorescence value, and ds is the relative fluorescence of the corrected double stranded eluent of the same sample.

2.4. Protein content: Determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

2.5. Integrated biomarker response (IBR): Biomarkers were combined into one general ‘‘stress index’’ termed IBR (Beliaeff and Burgeot, 2002). The result is directly dependent on the number of biomarkers (n) in the set and thus, IBR values were presented divided by n as suggested by Broeg and Lehtonen (2006). Results of data standardization procedure needed for IBR calculation were presented in site star plots.

2.6. Statistical analysis: Results were expressed as means ± SE (standard error) corresponding to experimental groups of six fish (n=6). Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). Assumptions of normality and homogeneity of data were verified. One way ANOVA was performed in order to assess significant effects, followed by post-hoc Tukey test to signal significant differences between groups (Zar, 1999). The significance of results was ascertained at $\alpha = 0.05$.

3. Results

3.1. Non Enzymatic Defences

GSHt was significantly higher in GAF, LAR and VAG (Figure 2A) compared to the reference site (TOR). Thus, GAF, LAR and VAG GSHt levels were respectively 3.3, 2.4 and 3.1 fold higher than TOR. Moreover, GSHt content in fish captured in GAF, RIO, LAR and VAG was significantly higher than BAR despite GAF and VAG higher levels than RIO.

MT levels were significantly higher in fish from LAR when compared to TOR and all the other sites (Figure 2B). LAR MT levels were found 1.8 times higher than TOR. On the other hand, fish captured at GAF and VAG displayed MT levels significantly lower than fish from TOR as well as BAR and RIO. The lowest MT levels were found in fish caught at GAF representing a 57% decrease when compared to TOR.

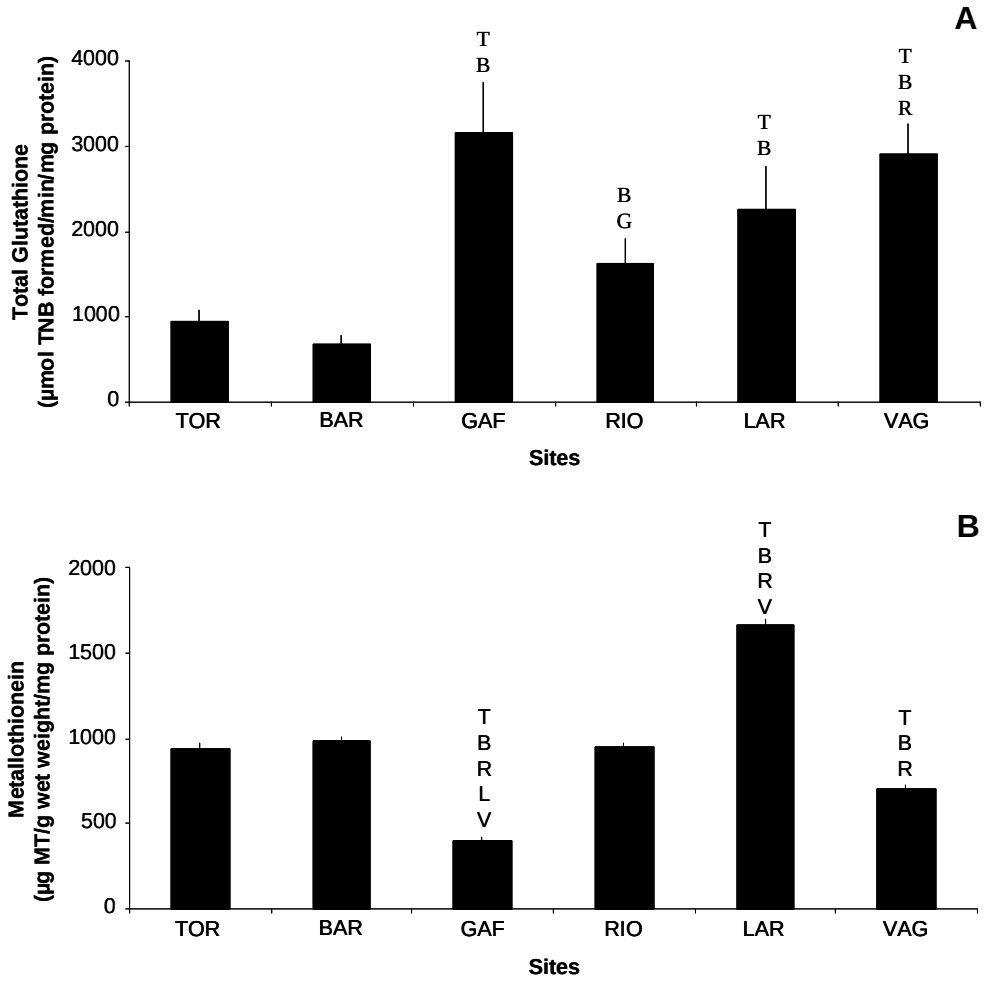

Figure 2 – Non enzymatic defences in the gill of *L. aurata* collected at different sites in the Aveiro lagoon. A – Total glutathione (GSHt); B – Metallothionein (MT). Values represent mean ± standard error (n=6). Significant differences (P<0.05) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR; V *versus* VAG.

3.2. Enzymatic Defences

CAT and GPx showed a similar pattern of response along the different sampling sites as both enzymes revealed higher activities in fish from GAF, LAR and VAG, when compared to TOR as well as to BAR (Figure 3A and 3B). Thus, CAT activity levels were 4.7, 3.5 and 3.9 fold higher in fish from GAF, LAR and VAG, respectively, when compared to TOR. Moreover, CAT activity in LAR and VAG fish was significantly higher than in RIO. Concerning GPx, and comparing to

TOR, its activity was found 3.3, 4 and 2.4 fold higher in fish from GAF, LAR and VAG respectively.

LAR and VAG fish had significantly higher GST activities than TOR and BAR (Figure 3C). Though non significant, GST activity was 2.8 fold higher in GAF fish than in TOR whereas LAR and VAG were respectively, 3.2 and 2.3 higher.

GR activity was significantly higher in fish caught at GAF when compared to TOR (2.2 fold) as well as to all the other sites (Figure 3D).

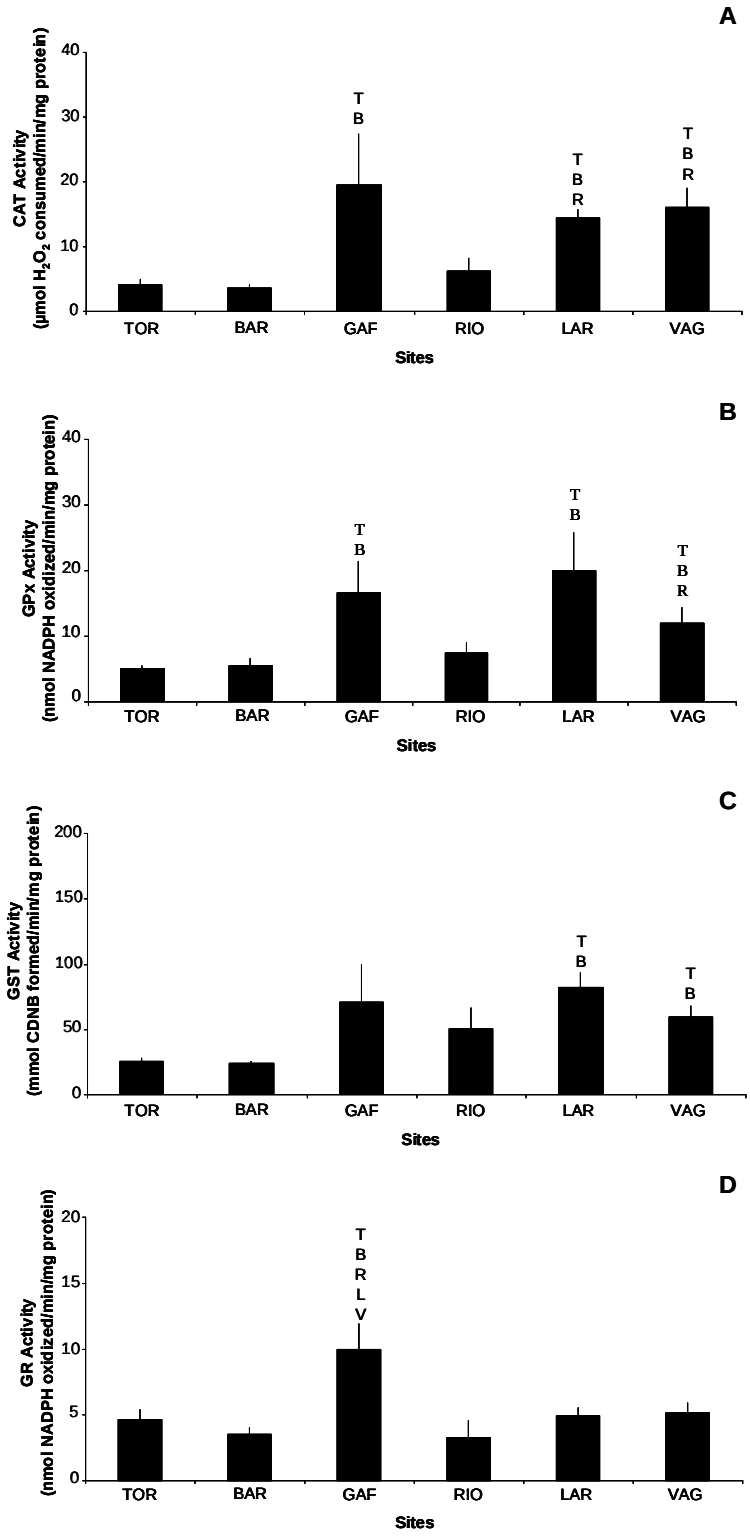

Figure 3 – Enzymatic defences in the gill of *L. aurata* collected at different sites in the Aveiro lagoon. A – Catalase (CAT); B – Glutathione Peroxidase (GPx); C – Glutathione S – transferase (GST); D – Glutathione Reductase (GR). Values represent mean ± standard error (n=6). Significant differences (P<0.05) are: T *versus* TOR; B *versus* BAR; R *versus* RIO; L *versus* LAR.

3.3. Peroxidative and Genetic Damage

TBARS levels were significantly higher in fish from BAR when compared to TOR (2.6 fold) as well as GAF, RIO and LAR (Figure 4A). Furthermore, at GAF and VAG fish had significantly higher TBARS levels than in RIO.

DNA integrity data show that, compared to TOR, no significant genotoxicity was found (Figure 4B). Though non significant, a slightly lower DNA integrity was observed in GAF. Moreover, DNA integrity was found significantly higher in fish from BAR when compared to GAF, RIO and LAR.

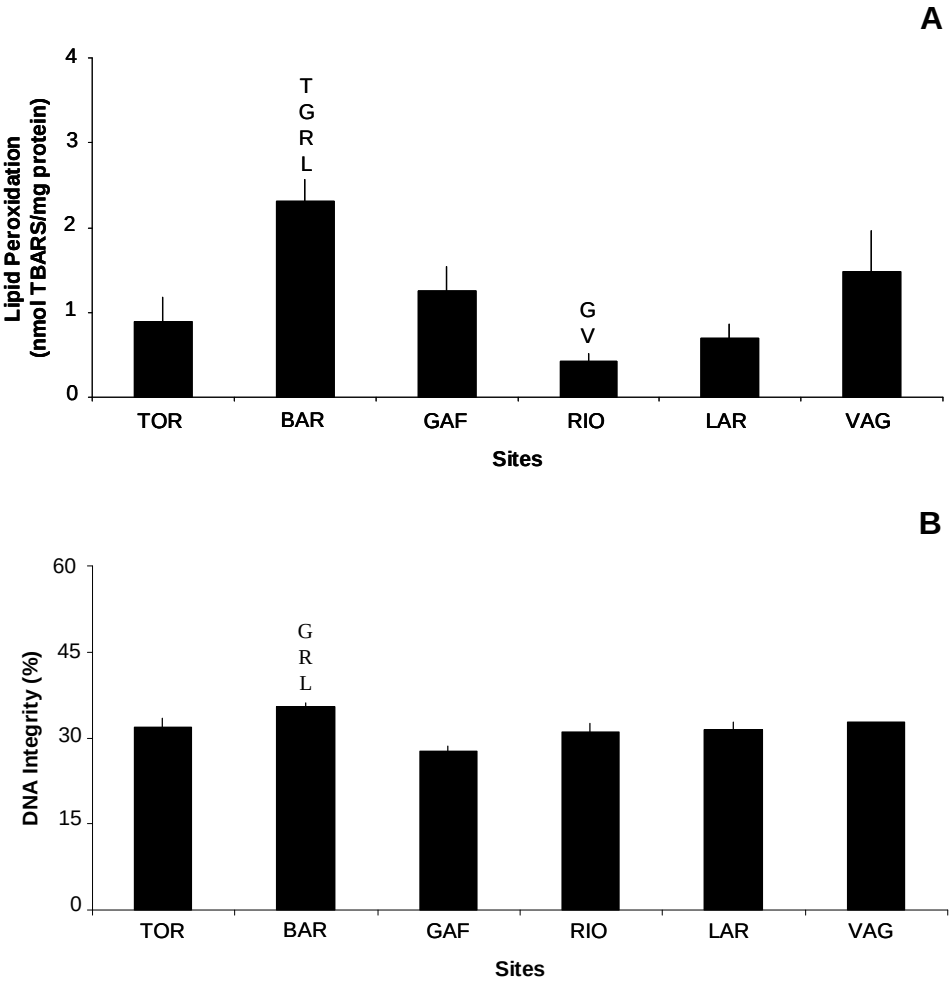

Figure 4 – Peroxidative and genetic damage in the gill of *L. aurata* collected at different sites in the Aveiro lagoon. A – Lipid peroxidation (LPO); B – DNA integrity. Values represent mean ± standard error (n=6). Significant differences (P<0.05) are: T *versus* TOR; G *versus* GAF; R *versus* RIO; L *versus* LAR; V *versus* VAG.

3.4. Integrated Biomarker Response

IBR values ranged from 0.13 in TOR up to 2.19 in GAF (Figure 5A). According to this index, the rank of the most affected sites can be ordered as: GAF>BAR>LAR>VAG>RIO>TOR. The transformed data of all the studied biomarkers are presented as star plot for each site in Figure 5B. Considering the extent and shape of the grey area, it is clear that TOR is the less impacted site, whereas the other sites showed larger areas and site-specific shapes.

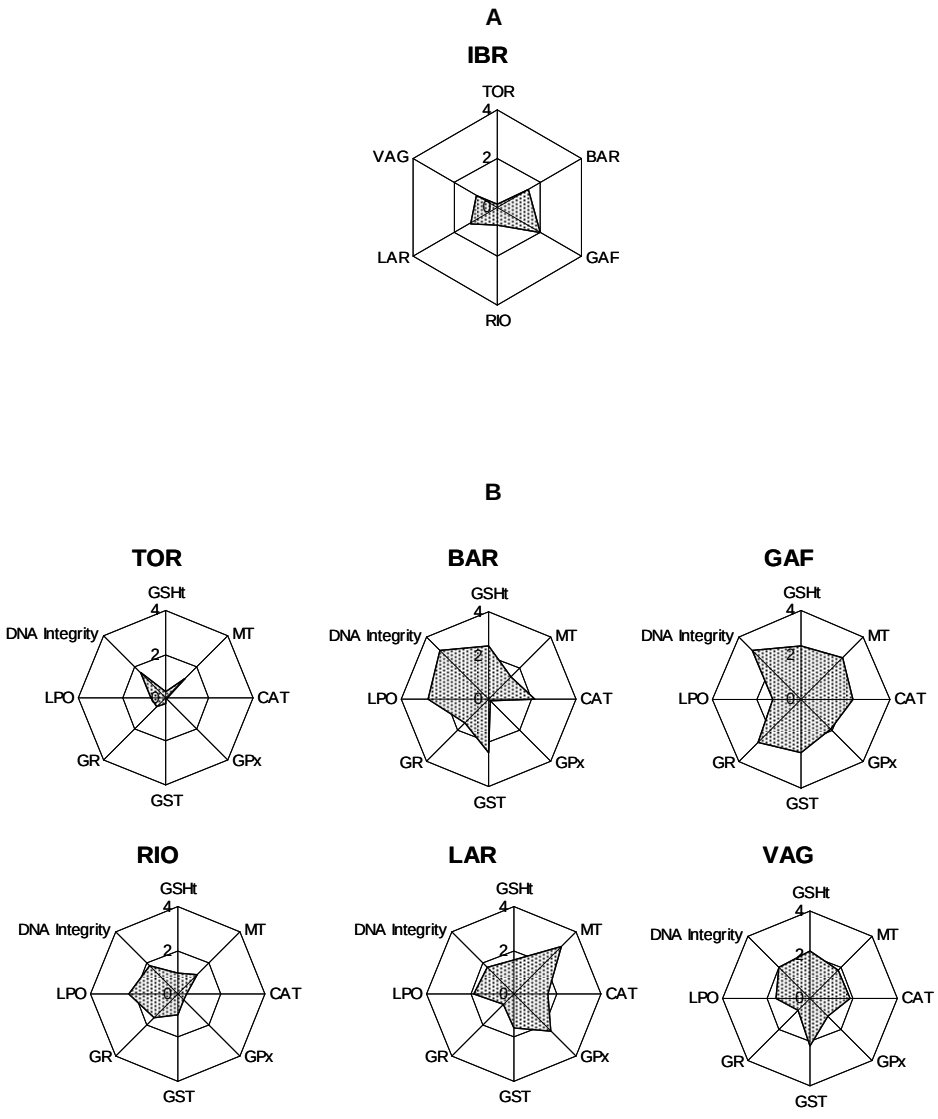

Figure 5 – Integrated biomarker response (IBR) and biomarker star plots A – IBR; B – Biomarker star plots for each site. GSHt – Total glutathione; MT – Metallothionein; CAT – Catalase; GPx – Glutathione Peroxidase; GST – Glutathione S-transferase; GR – Glutathione Reductase; LPO – Lipid Peroxidation.

4. Discussion

In this study, the effects of the environmental pollutants were assessed using gill *L. aurata* responses. The widespread distribution and resistance to different environmental conditions make this species a good bioindicator (Pacheco, et al., 2005; Ferrando et al., 2006; Oliveira et al., 2007).

Abiotic factors may influence biomarker responses to contaminants. Temperature may affect enzymes' catalytic efficiency and binding capacity (Hochachka and Somero, 1984) and temperature variations may disturb the balance oxygen supply/demand and decrease oxygen levels in tissues. The levels of dissolved oxygen can also have an effect on antioxidant defences (Martínez-Álvarez et al., 2005; Lushchak and Bagnyukova, 2006). In our study, hydrological abiotic conditions did not substantially differ between the surveyed sites with the exception of salinity that in RIO was substantially lower than BAR and GAF (21 *versus* 34). Decreases in salinity are usually associated with increased bioavailability and uptake rates of contaminants (Ramachandran et al., 2006; Monserrat et al., 2007). Cailleaud et al. (2007) suggested the ability of salinity stress to interfere with oxidative stress responses in *Eurytemora affinis*. Usually, low salinities originate increased gill oxygen consumption in euryhaline crabs, consequence of energy expenditure related to ionic and osmotic regulation being closely related to ROS generation (Monserrat, et al., 2007). However, in our study salinity in RIO was within *L. aurata* tolerable range and did not seem to influence responses as no activated/inhibited biomarker was found in this site.

4.1. Cell defence mechanisms as early signals of contaminants exposure

GSH and MT are the two major thiol containing molecules in the cell. GSH is the predominant defence against ROS (DeLeve and Kaplowitz, 1991) and protects cells from the pernicious effects of metals and other electrophilic compounds (Wang and Ballatori, 1998). Metal metabolism involves formation of GSH–metal complexes, from which the metal is further transferred to MT apoproteins (Atli and Canli, 2008). In the present study, GSHt was higher in GAF,

LAR and VAG, a clear indication of the presence of contaminants challenging cell defences depending on this thiol. Thus, the protective and adaptive role of GSH, well established in liver of aquatic animals (Otto and Moon, 1995; Pena-Llopis et al., 2001), was also demonstrated in gill. High GSH levels represent a greater cellular ability to destroy free radicals and ROS and may be either a result of de novo synthesis as previously demonstrated in fish (Gallagher et al., 1992; Thomas and Juedes, 1992) or a transfer from other organs. It has been demonstrated that most GSH is synthesized in liver and then exported to other tissues (Deneke and Fanburg, 1989).

MT expression has generally been associated with intracellular metals storage and detoxification. It has been demonstrated that the MT gene promoter region contains not only genetic elements responsive to metals, but also sequences thought to be involved in oxidative stress response (Olsson et al., 1995; Haq et al., 2003). In our study, only LAR, that was subject to intense mercury discharges, showed significantly higher MT levels. MT responsiveness in gill is controversial and some authors stated that gill do not constitute a good organ for MT quantification in fish (Hamza-Chaffai et al., 1997; Olsvik et al., 2001). However, the current *L. aurata* responses demonstrated gill capacity to increase MT as a response to metal exposure. Considering the predictable contamination sources affecting the other sites, results show no MT induction by non metal compounds. Fish from GAF and VAG showed significantly lower gill MT levels suggesting a MT synthesis reduction that may be associated with increased demand of cysteine residues for GSH synthesis during detoxification of organic contaminants (Roméo et al., 1997), which are supposed to be present in high levels in these sites.

CAT and GPx are known to protect the cell by reducing H_2O_2 to H_2O. Furthermore, GSH conjugation with lipid hydroperoxides, catalyzed by GPx and GST, decreases their reactivity. In our study, the general enzymatic antioxidant responses showed that fish are suffering pro-oxidant challenges in GAF, LAR and VAG. Hence, these sites contain compounds that stimulate the production of lipid hydroperoxides, as demonstrated by GPx and GST higher activities (though not significant in GAF), and H_2O_2 depicted by CAT and GPx activities. H_2O_2 is the

main cellular precursor of the hydroxyl radical, the most toxic ROS (Halliwell and Aruoma, 1991). Due to relative poor efficiency of antioxidants towards this ROS, H_2O_2 removal has been indicated as an important strategy for counteracting the toxicity of hydroxyl radicals (Regoli et al., 2000), emphasizing the relevance of the elevated CAT and GPx levels found in the current study. Moreover, the concomitance of CAT and GPx responses may be a further indication of extreme H_2O_2 levels and/or lipid hydroperoxides since, in some cases, only one of these enzymes is activated as observed by Oliveira et al. (2008) in *L. aurata* exposed to phenanthrene. The higher GST activity may also be understood as an increased phase II biotransformation, a sign of conjugation reactions of active electrophilic metabolites or their parental compounds with GSH.

GR plays a major role in GPx and GST reactions as an adjunct in the control of peroxides and free radicals (Bompart et al., 1990), maintaining the proper GSH redox status. Elevated GR activity was observed in organisms exposed to pro-oxidant stressors (Regoli, et al., 2000; Regoli, et al., 2002). The high GR activity in GAF reflects increased glutathione recycling (replenishing GSH in order to avoid its depletion), suggesting that the ratio GSSG/GSH has been increased as consequence of pro-oxidants. This may be associated to the contamination complexity which is probably more obvious in GAF among all the studied sites.

4.2. Contaminant induced damage

Environmental contaminants may inflict damage on membrane lipids, DNA, proteins and carbohydrates directly by the action of parental compounds or their metabolites or indirectly by the generated ROS. LPO, a complex process resulting from free radical reactions in biological membranes forms lipid hydroperoxides which decompose double bonds of unsaturated fatty acids and destructs membrane lipids. LPO intensity is assessed as levels of primary products, conjugated dienes and lipid peroxides, and/or end products of LPO such as malondialdehyde and other aldehydes, which are assayed with thiobarbituric acid and expressed as TBARS (Rice-Evans et al., 1991). Our study revealed higher TBARS levels in BAR, a clear oxidative stress indication.

DNA alterations induced by chemical and physical agents include single and double strand breaks, modified bases, DNA–DNA crosslinks and DNA–protein crosslinks. Strand breaks may also be induced indirectly by an interaction with oxygen radicals, or by the action of excision repair enzymes, and finally as a consequence of apoptosis or necrosis processes (Viarengo et al., 2007). In this study, no significant differences were observed in DNA integrity. However, these results should be carefully interpreted since a previous study with *L. aurata* detected at VAG higher genotoxicity, measured as erythrocytic nuclear abnormalities, in the presence of high waterborne BaP concentrations (Pacheco, et al., 2005). Gill DNA damage expression may be also affected by essential factors such as DNA repair mechanisms and tissue regeneration. Oliveira et al. (2007) suggested that the absence of phenanthrene genotoxicity in *L. aurata* gill could be due to tissue regeneration, accelerated by direct cytotoxic action.

4.3. Protection *versus* damage and uncertainties of its isolated assessment

The lack of measurable defences (non enzymatic and enzymatic) stimulation at BAR in concomitance with higher levels of TBARS may be a result of an environmental condition insufficient to stimulate protection though pernicious enough to cause peroxidative damage.

GAF seems to be one of the most threatened sites taking into account the overall antioxidants activation and decreased MT levels. This interpretation is supported by previous studies that detected high levels of PAHs, metals and organometallic compounds (Barroso et al., 2000; Pacheco et al., 2005). Ahmad et al. (2004), in a previous short term study performed in 2001 using *Anguilla anguilla* caged at GAF, found that gill responses also signalled the presence of pro-oxidants expressed by increased gill phagocytic burst activity, antioxidants depression (GSH, GPx, GST and CAT) and LPO increase. Despite the absence of these toxicity expressions, our data indicate the remaining of risk to fish populations living in GAF area. Hence, the observed gill MT decreased levels at GAF can be regarded as a toxicity indication, increasing fish susceptibility to environmental stressors.

Pulp mill industry was responsible during decades for continuous hazardous discharges affecting the RIO surveyed area. In 2002, 25 months after the end of effluent discharges, Santos et al. (2004, 2006), using caged *A. anguilla*, found high ROS production in gill, concomitantly with decreased GPx, GST, CAT, and GSH as well as increased LPO. Thus, despite the differences between studies (species, distance to source and exposure duration), the present results suggest that RIO area is becoming cleaner since neither defences modulation nor damage was observed.

In LAR, all the protection mechanisms (excluding GR) were activated denoting a physiological adaptation to environmental contamination. GSH and MT have been suggested to play cooperative protective roles against metal toxicity, the former as an initial defence and the last acting at a second stage (Ochi et al., 1988). One of the most important mechanisms for mercury induced oxidative damage is its known sulfhydryl reactivity (Ercal et al., 2001), forming covalent bonds with GSH and cysteine residues of proteins. *In vitro* tests corroborate this statement, highlighting the GSH protective role (Viarengo et al., 1997; Oliveira et al., 2004). Elia et al. (2003) found that moderate doses of mercury were able to increase GST and GPx activities in *Ictalurus melas*, whereas high concentrations inhibited those enzymes. In our study, no enzyme inhibition was observed in LAR suggesting gill defences efficiency on preventing enzyme inhibition and damage (peroxidative and genetic). The absence of enzyme inhibition and damage may also be a result of reduced metal uptake through gill (Olsvik, et al., 2001; Gale et al., 2003) and redistribution of metals to other tissues as soon as they enter branchial cells (De Boeck et al., 2003; Atli and Canli, 2008), in conjugation with efficient scavenging action by GSH and MT (Atif et al., 2005).

VAG also demonstrated the presence of pro-oxidant chemicals. In 2000, VAG had very high levels of PAHs (Pacheco, et al., 2005). Our study confirms that fish from this site are under stress and probably domestic effluents from non point sources are still being released. MT inhibition observed in this site did not have a significant impact in fish since no damage was observed.

In order to compare the overall stress on *L. aurata*, the IBR index was applied. This approach provides a simple tool for a general description of the

‘‘health status’’ of populations, combining the different biomarker signals (Leinio and Lehtonen, 2005). The majority of the available studies using IBR index (Leinio and Lehtonen, 2005; Broeg and Lehtonen, 2006; Damiens et al., 2007) provided consistent indications, reflecting the pollution levels measured at different locations regardless of the considerable variability in the biomarker sets used for the index calculations. IBR results suggest that GAF was the most affected site. The star plots diversity shapes obtained in each site, reflected a dissimilar contamination pattern confirming the available information in the chemical characterization of those sites.

According to the present data, the aquatic system contamination assessment should not focus only in damage parameters but in their conjugation with defence responses since, as observed by Cossu et al. (2000), one may not be predictable on the basis of the other. Different seasons should be considered in future studies as toxic effects can change by the natural characteristic of the water/sediments such as oxygenation, pH and temperature.

5. Conclusions

L. aurata gill biochemical responses were capable of detecting differences between sites and signal critical areas in Ria de Aveiro. Three sites can be labelled as having pro-oxidant contaminants (GAF, LAR and VAG) on the basis of adaptation capacities (defence responses). However, on the basis of peroxidative damage (lipid peroxidation) only BAR is contaminated. The integration of the assessed biomarkers through the IBR index, ranked environmentally impacted sites as follows GAF>BAR>LAR>VAG>RIO>TOR and may be considered a useful complementary tool.

Gill demonstrated its usefulness in biomonitoring studies if this battery of biomarkers is to be considered. Moreover, the conjugation antioxidant defences/damage responses should be assessed in monitoring coastal lagoons.

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CAPÍTULO VI

Monitorização de um sistema lagunar costeiro utilizando respostas de stresse oxidativo e genéticas do rim de *Liza aurata*: Abordagem integrada de biomarcadores

Monitoring pollution of coastal lagoon using *Liza aurata* kidney oxidative stress and genetic endpoints: An integrated biomarker approach

M. Oliveira, I. Ahmad, V.L. Maria, M. Pacheco, M.A. Santos

Ecotoxicology *In press*

Abstract

Despite the importance of fish kidney in several functions (immune, metabolism and excretion of xenobiotics) its use in coastal water biomonitoring focusing on protection and damage is scarce. Five critical sites in Ria de Aveiro (Portugal) (Barra-BAR, Gafanha-GAF, Rio Novo do Principe-RIO; Laranjo-LAR and Vagos-VAG) were assessed in comparison to a reference site (Torreira-TOR), focusing on *Liza aurata* kidney antioxidant defences *versus* damage responses. Non protein thiols were higher at RIO and total glutathione at RIO (near a former bleached kraft pulp mill effluent), LAR (mercury contaminated) and VAG (polycyclic aromatic hydrocarbons contaminated). Catalase and glutathione S-transferase activities were higher at RIO and LAR whereas no differences were found in glutathione peroxidase activity. However, glutathione reductase was higher at BAR (subject to naval traffic), GAF (harbour water area), RIO and LAR. No peroxidative damage was observed despite the decreased DNA integrity at RIO and VAG. The integrated biomarker response index ranked impacted sites as: LAR>RIO>BAR>GAF>VAG>TOR.

Keywords: *Liza aurata*; Antioxidant defences; Oxidative stress; Genotoxicity; Aveiro Lagoon

1. Introduction

The aquatic environment is continuously being subject to toxic contaminants resultant from industrial, agricultural and domestic activities. The monitoring of those contaminants in water, sediment and biota by itself may lead to unreliable assessments of environmental impacts due to the great variety of contaminants, their bioavailability, pharmacodynamic as well as potential exposure routes. However, considering that contaminants uptake may induce a number of subtle biological changes in different tissues, particularly gill, kidney and liver

(Giari et al. 2007), its use may provide more reliable information concerning adverse effects, reflecting the integrated effects of all contaminants to biota at levels below chemical detection limits (Zhou et al. 2008). Nevertheless, tissue-specific responses should be taken into account (Geist et al. 2007). In this perspective, fish kidney responses may provide valuable information concerning environmental pollution (Ortiz et al. 2003) considering its importance in the maintenance of a stable internal environment (Bernet et al. 1999), xenobiotics metabolism (Pesonen et al. 1987; Üner et al. 2001; Ortiz-Delgado et al. 2008), metals storage (Skak and Baatrup 1993; Berntssen et al. 2003) and excretion of xenobiotics metabolites (Hinton et al. 1992). Fish kidney consists of two portions, anterior kidney (head kidney), functionally and anatomically complex, containing hematopoietic, lymphoid, and endocrine tissue and posterior kidney (body kidney) where nephrons are surrounded by hematopoietic and lymphoid tissue dispersed throughout the organ. Compared with other tissues, kidney is more prone to injury since it receives the largest portion of post-branchial blood (becoming a target for gill absorbed xenobiotics) and a high blood flow for elimination of toxic metabolites formed all over the body (Singh et al. 2001).

The toxic effects of xenobiotics or their metabolites on fish often depend on their capacity to increase cellular levels of reactive oxygen species (ROS) (Viarengo et al. 2007) which may damage crucial cellular components, such as lipids, carbohydrates, proteins and DNA if ROS production exceeds antioxidant capability. Endocrine-disrupting effects may also be associated with oxidative stress, in interrenal cells (Dorval et al. 2005), implying that if oxidative species are not rapidly eliminated cell physiological integrity loss may impair its functional capacity and reduce survival. In this perspective, the analysis of antioxidant defences as well as damage responses such as lipid peroxidation (LPO) and genetic damage becomes highly relevant in biomonitoring studies.

Ria de Aveiro, a coastal lagoon located in the northwest of Portugal, has a significant role in the life cycle of several organisms. Over decades, this lagoon has been the main receptor of anthropogenic discharges resulting mainly from chlor-alkali (Pereira et al. 1997) and pulp/paper plants (Pacheco et al. 2005), harbour and dry-dock activities (Barroso et al. 2000; Pacheco et al. 2005),

municipal and domestic effluents (Pacheco et al. 2005) and chemicals used in agriculture (Monteiro et al. 2007). Though in the year 2000 point source discharges were diverted through a submarine outlet 2.5 km far from the sea coast, recent studies have demonstrated the continuation of critical areas (Santos et al. 2004, 2006; Ahmad et al. 2008; Oliveira et al. 2009), revealing the imperative need of an effective biomonitoring programme. Thus, the goal of the present study was the assessment of Ria de Aveiro environmental status, as a coastal lagoon prototype, focusing simultaneously on kidney antioxidant defences (non-protein thiols - NPT, total glutathione - GSHt, catalase - CAT, glutathione peroxidase - GPx, glutathione S-transferase - GST, glutathione reductase - GR) and damage (LPO and DNA integrity loss) responses. The integrated biomarker response (IBR) index was used to improve the discriminatory power of the adopted multi-biomarker strategy.

2. Material and Methods

2.1 - Study Area: Ria de Aveiro (Figure 1) is a coastal lagoon 45 km long (NNE-SSW) and 8.5 km wide, covering a wetland area of approximately 66 (low tide) to 83 km^2 (high tide) which is permanently connected to the ocean through a narrow channel. Sampling sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of an unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Príncipe (RIO), located at the terminal area of the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal

contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of polycyclic aromatic hydrocarbons (PAHs).

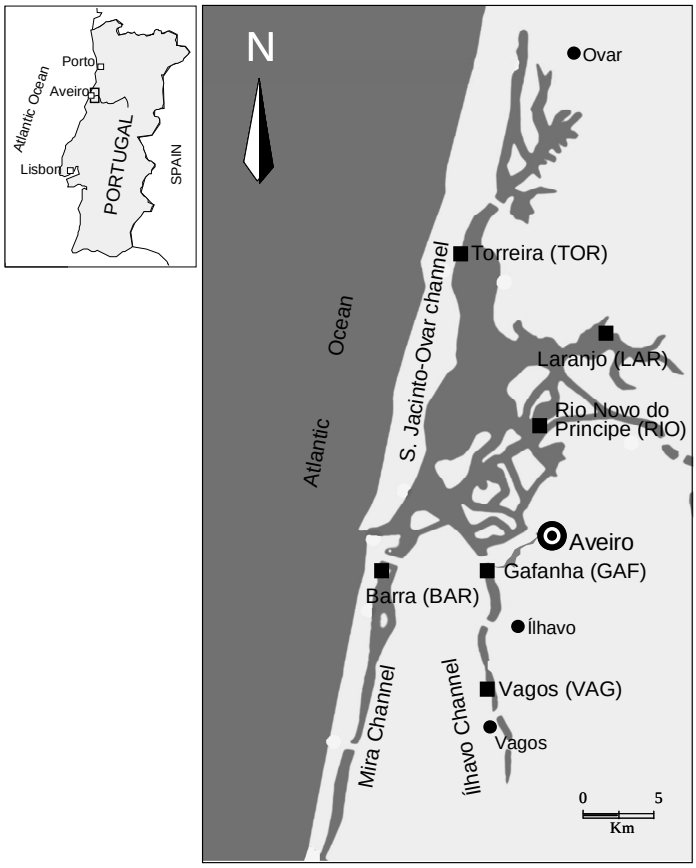

Figure 1 - Map of the sampling sites (■) in the Ria de Aveiro.

2.2 - Sampling: Golden grey mullet (*Liza aurata*) was caught in October 2005, during low tide using a traditional beach-seine net named “chincha”. Six juvenile specimens were selected on the basis of their size. *L. aurata* specimens had an average weight and length of 21.6±3.7 g and 14.5±2.5 cm, respectively. Immediately after catching, fish were sacrificed, kidney removed and frozen in liquid nitrogen. In the laboratory, two sets of tissue were separated for DNA strand breaks and oxidative stress measurements and both stored at −80º C until further treatments.

At each sampling site abiotic parameters were assessed (Table I) as per the guidelines of APHA (1998).

Table I - Physical-chemical parameters of the water from the Ria de Aveiro sampling sites.

	Turbidity (m)	*Dissolved Oxygen (mg/L)*	*Temperature (ºC)*	*pH*	*Salinity*
TOR	0.80	6.88	18.9	8.17	32.5
BAR	1.20	6.16	17.9	8.32	34.0
GAF	0.70	5.78	17.9	8.28	34.0
RIO	1.00	6.37	19.0	8.20	21.0
LAR	0.30	4.96	18.6	7.74	29.0
VAG	0.30	5.18	18.5	7.88	33.0

2.3 – Biochemical Analyses

One set of tissues was homogenized, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/15 ml buffer = 15%). This homogenate was then divided in three aliquots for LPO, non protein thiols (NPT) and glutathione measurements as well as for post-mitochondrial supernatant (PMS) preparation. The samples for NPT and glutathione measurements (0.3 ml) were precipitated with 0.3 ml of sulfosalicylic acid (5.0%) at 4ºC for 1 hour and then subjected to centrifugation at 13400 g (Eppendorf 5415A) for 20 minutes (4 ºC). PMS preparation was accomplished by centrifugation in a refrigerated centrifuge at 13400 g for 20 min at 4 ºC. Samples were divided in microtubes and stored at – 80ºC until analyses.

The other set of tissues, used for DNA integrity assessment, were placed in TNES (Tris-HCl 10 mM, NaCl 125 mM, EDTA 10 mM, SDS 1%, pH 7.5) – urea (5 M) buffer with proteinase K solution (final concentration 0.8 mg/ml). DNA isolation was performed using a genomic DNA purification kit (Fermentas).

2.3.1 - Non Enzymatic Defences

Protein content in the homogenate was precipitated with sulfosalicylic acid (5%) for one hour and then centrifuged at 13400 g for 20 minutes (4ºC). Non-

protein thiols (NPT) were spectrophotometrically (Jasco UV/VIS, V-530) determined in the resulting supernatant at 412 nm by the method of Sedlak and Lindsay (1968) as adopted by Parvez et al. (2003). Total glutathione (GSHt) content was determined in supernatant at 412 nm (25ºC) adopting the enzymatic recycling method (Tietze 1969; Baker et al. 1990).

2.3.2 – Enzymatic Defences

Catalase (CAT) activity was determined in PMS by measuring the consumption of the substrate H_2O_2 at 240 nm (Claiborne, 1985). Glutathione peroxidase (GPx) activity was determined in PMS by measuring NADPH oxidation at 340 nm (25ºC) using H_2O_2 as substrate (Mohandas et al., 1984). Glutathione S-transferase (GST) activity was determined in PMS by following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene at 340 nm (25ºC) (Habig et al., 1974).

Glutathione reductase (GR) activity was quantified in PMS by measuring NADPH disappearance at 340 nm (25ºC) (Cribb et al. 1989). Slight modifications to the described methods were performed as described by Oliveira et al. (2009).

2.3.3 – Peroxidative and genetic damage evaluation

Lipid peroxidation (LPO) was determined in the tissue homogenate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Ohkawa 1979; Bird and Draper 1984; Oliveira et al. 2009). Kidney DNA integrity was assessed using DNA alkaline unwinding assay according to Rao et al. (1996) as adopted by Maria et al. (2002).

2.3.4 - Protein measurement: Protein concentrations were determined according to the Biuret method (Gornall et al. 1949) using bovine serum albumin as a standard.

2.4 - Integrated biomarker response (IBR): All the biomarkers data were standardized according to Beliaeff and Burgeot (2002). Briefly, the mean value (X_i) was calculated for each biomarker at each sampling site. In addition, the general mean (m_i) and standard deviations (s_i) of each biomarker were estimated for all

sampling sites. The value of X_i was then standardized to Y_i, where $Y_i = (X_i - m_i)/s_i$. Then Z_i was computed as $-Y_i$ or Y_i in the case of a biological effect corresponding, respectively, to inhibition or activation. The minimum value (Min_i) of Z_i for each biomarker was calculated for all sampling sites and the score S_i was computed as $S_i = Z_i + |Min_i|$, where $|Min_i|$ is the absolute value. IBR for each sampling site was calculated via de following formula: $IBR = (S_1 \times S_2)/2 + (S_2 \times S_3)/2 + \ldots + (S_{n-1} \times S_n)/2 + (S_n \times S_1)/2$. IBR results are presented divided by the number of biomarkers as suggested by Broeg and Lehtonen (2006).

2.5 - Statistical analysis: Results were expressed as means±SE (standard errors). Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). The DNA integrity data was transformed prior to statistic analyses according to the formula arcsen $\sqrt{p}$. The experimental data were tested first for normality and homogeneity of variance to meet statistical demands and then with the Tukey test (Zar 1999). Differences between means were considered significant at $p<0.05$.

3. Results

3.1 - Non Enzymatic Defences

NPT levels in fish caught at RIO were significantly higher (61%) than in the fish caught at the reference site (TOR) (Figure 2A). Fish caught at BAR, GAF and LAR also displayed higher NPT levels than at TOR (21, 42 and 69%, respectively) though differences were not statistically significant. Moreover, at GAF, RIO and LAR, NPT levels were significantly higher than VAG.

GSHt levels were significantly higher than TOR in fish caught at RIO, LAR and VAG (Figure 2B). Thus, compared to TOR, GSHt levels were respectively 91, 75 and 71% higher at RIO, LAR and VAG. Moreover, *L. aurata* GSHt levels at BAR were significantly lower than RIO and VAG.

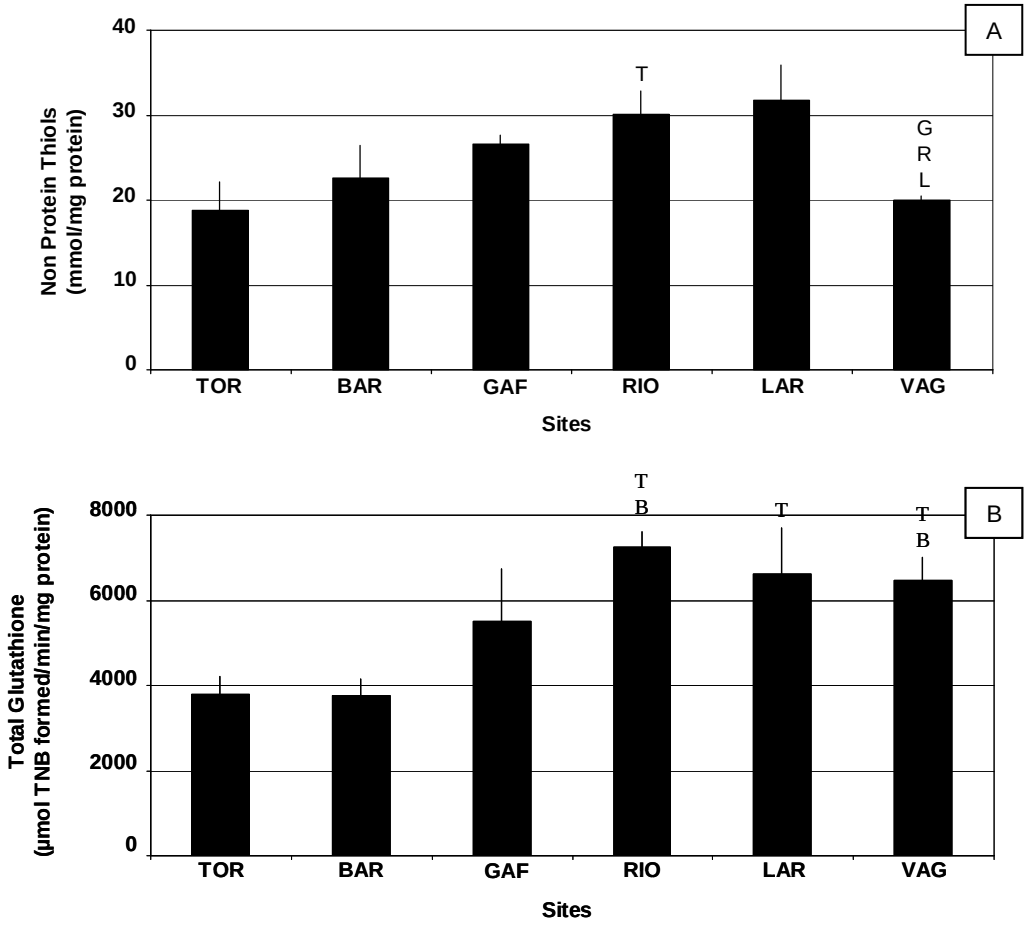

Figure 2 – Non enzymatic defences in the kidney of *L. aurata* collected at different sites in the Aveiro lagoon. A – Non protein thiols (NPT); B – Total glutathione (GSHt). Values represent mean ± standard error. Significant differences ($P<0.05$) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR.

3.2. Enzymatic Defences

CAT levels in fish captured at RIO and LAR were significantly higher than at TOR, 69 and 52%, respectively (Figure 3A). Though not significantly, CAT activity was also higher at BAR (31%) and VAG (38%).

Despite the fact that fish caught at BAR and LAR displayed GPx activities 78% and 93% higher than at TOR, no significant differences were found (Figure 3B).

Fish captured at RIO and LAR displayed significantly higher GST activity than fish from TOR, 159 and 212% respectively (Figure 3C).

The lowest *L. aurata* GR activity was found in fish caught at TOR. Thus, compared to TOR, GR activities were 173, 108, 201, 449, 76% higher at respectively, BAR, GAF, RIO, LAR and VAG, though not significantly different in fish captured at VAG (Figure 3D).

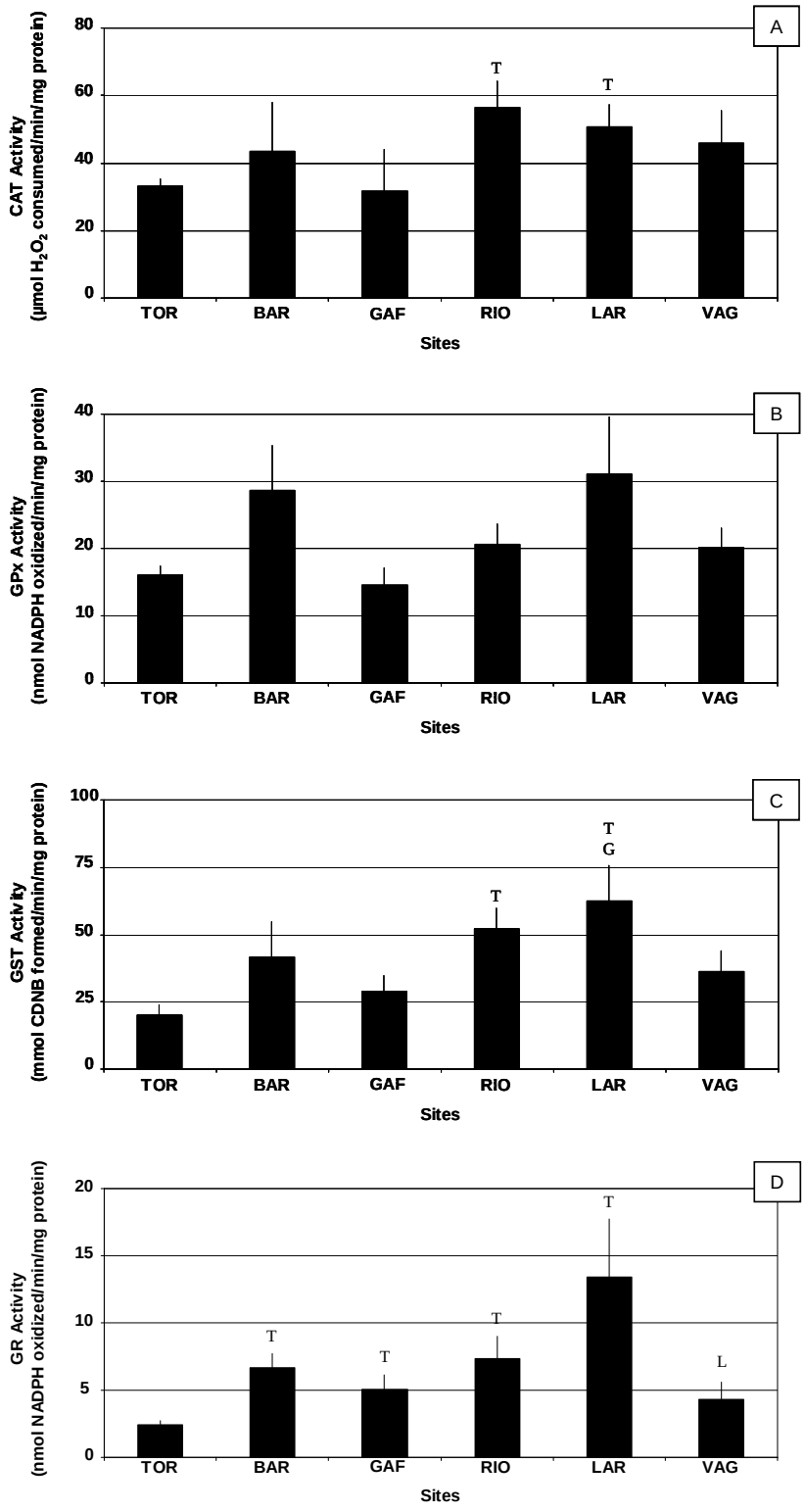

Figure 3 – Enzymatic defences in the kidney of *L. aurata* collected at different sites in the Aveiro lagoon. A – Catalase (CAT); B – Glutathione peroxidase (GPx); C – Glutathione S – transferase (GST); D – Glutathione reductase (GR). Values represent mean ± standard error (n=6). Significant differences ($P<0.05$) are: T *versus* TOR; G *versus* GAF; L *versus* LAR.

3.3. Peroxidative and Genetic Damage

Despite the 73% LPO increase found in fish captured at RIO (when compared to TOR), no significant differences were observed between fish captured at those sites (Figure 4A). At LAR and VAG, though not significantly, fish TBARS levels were respectively 22 and 32% lower than TOR. Kidney genetic damage was manifested through significantly lower DNA integrity levels, found in RIO (13%) and VAG (15%) when compared to TOR (Figure 4B).

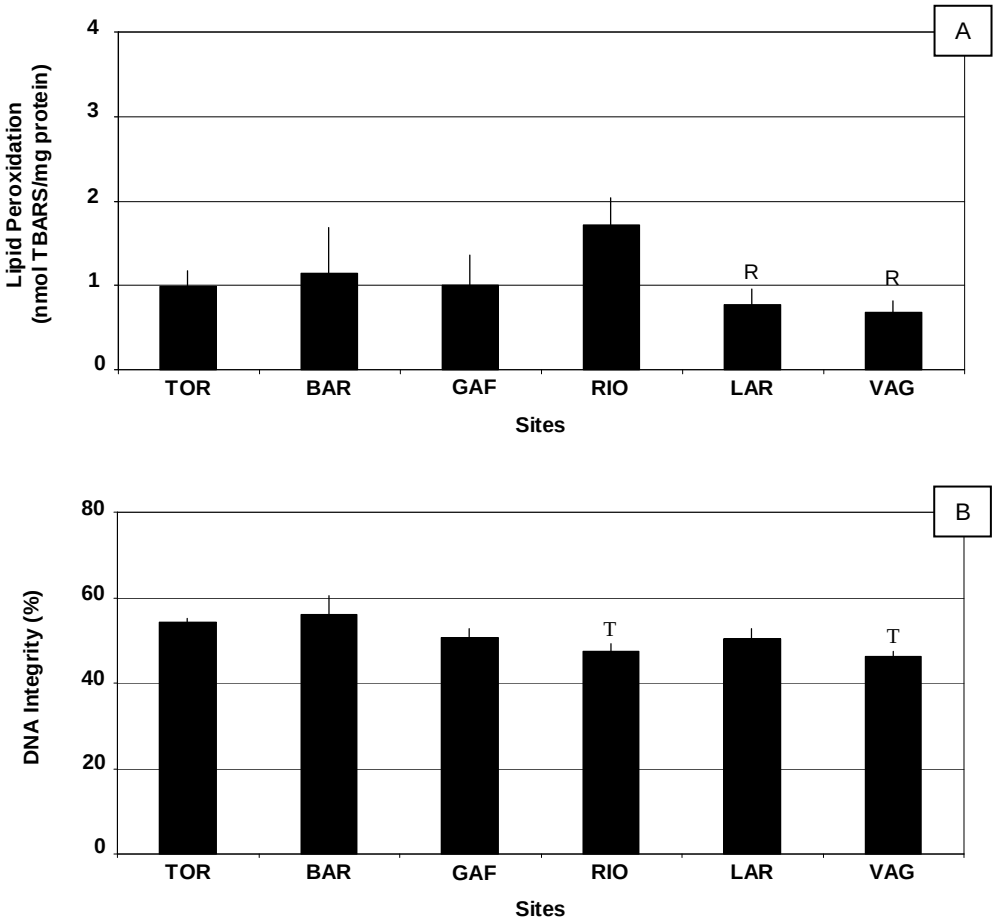

Figure 4 – Peroxidative and genetic damage in the kidney of *L. aurata* collected at different sites in the Aveiro lagoon. A – Lipid peroxidation (LPO); B – DNA integrity. Values represent mean ± standard error (n=6). Significant differences ($P<0.05$) are: T *versus* TOR; R *versus* RIO.

3.4. Integrated Biomarker Response

IBR index signalled LAR as the most affected site. IBR values ranged from 0.05 in TOR up to 1.11 in LAR (Figure 5A). According to this index, the rank of the most affected sites could be ordered as: LAR>RIO>BAR>GAF>VAG>TOR. The transformed data of all the studied biomarkers are presented as star plot for each site in Figure 5B. Considering the extent and shape of the grey area, it is clear that TOR was the less impacted site, whereas the other sites showed larger and site-specific areas.

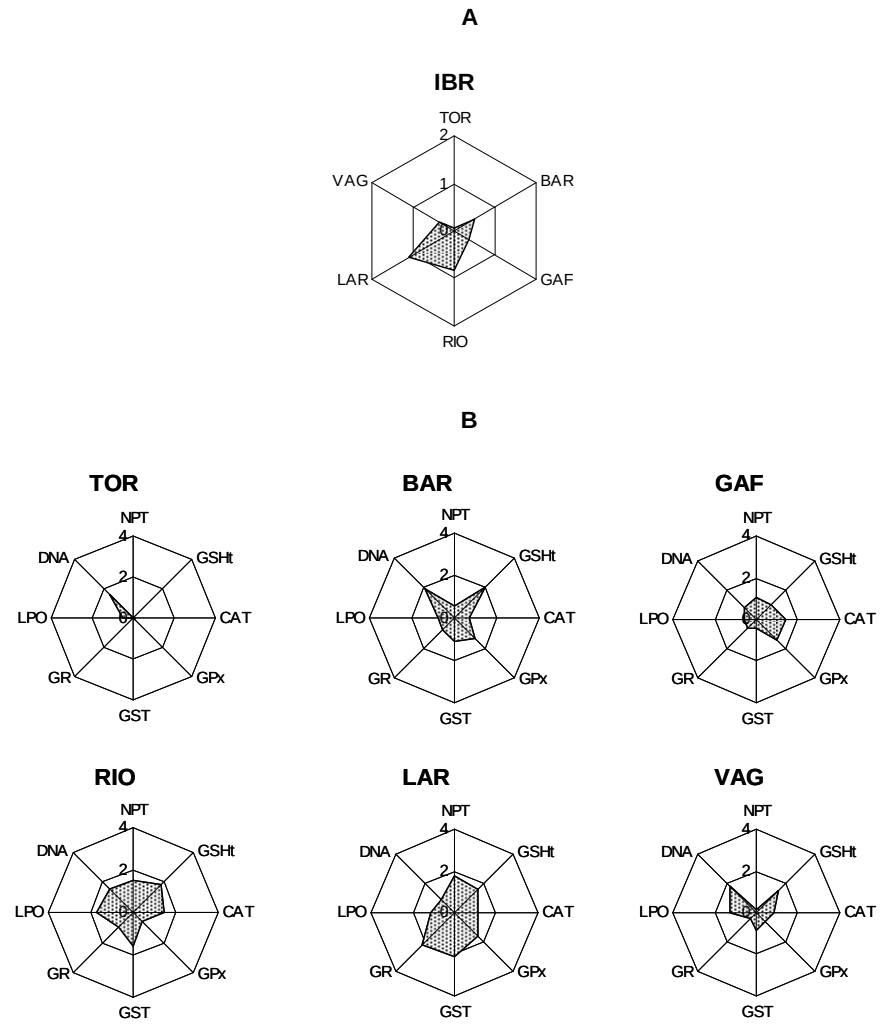

Figure 5 – Integrated biomarker response (IBR) and biomarker star plots: A – IBR; B – Biomarker star plots for each site displaying the general patterns of data variability. NPT – Non protein thiols; GSHt – Total glutathione; GPx – Glutathione peroxidase; CAT – Catalase; GST –

Glutathione S-transferase; GR – Glutathione reductase; LPO – Lipid peroxidation; DNA – DNA integrity.

4. Discussion

Environmental factors may affect several physiological processes involved in the detoxification of hazardous substances in fish and thus the ability to cope with xenobiotics (Heugens et al. 2001). In the present study, hydrological abiotic levels did not substantially differ between sites with the exception of salinity which was substantially lower in RIO when compared to BAR and GAF. The possibility that salinity differences might have influenced fish responses cannot be completely discarded though salinity levels were within the tolerable range for this euryhaline species. In this perspective, discussion will be made based on the predictable contamination scenario at each particular site.

Low molecular weight thiol-containing compounds are ubiquitously distributed in aerobic life forms, coordinating the antioxidant defence network (Dafre et al. 2004). Accordingly, its levels have been used as biomarkers of environmental contamination in the aquatic environment (Sayeed et al. 2003; Parvez and Raisuddin 2006; Ahmad et al. 2008). Fish adapted to waterborne contaminants have shown high levels of non enzymatic antioxidants (Pena-Llopis et al. 2001; Parvez et al. 2006) such as GSH which represents the bulk of non protein thiols, having an important role in detoxification and excretion of xenobiotics (Wang and Ballatori, 1998). Its depletion leads to an imbalance in redox status decreasing cells ability to deal with xenobiotics. Other thiols, such as N-acetyl-L-cysteine, may also protect cells against xenobiotic induced damage (Oliveira et al. 2005). In the current study, GSHt quantification provided more information concerning oxidative stress than the NPT levels which were only elevated at RIO whereas GSHt increased at RIO, LAR and VAG.

CAT, GPx, GST and GR are enzymes important in cell defence against oxidizing environments, helping organisms to adapt to new conditions. The high antioxidant enzyme activities observed at the critical sites suggest the adaptation of *L. aurata* to the pro-oxidant challenges provoked by environmental contaminants signalling increased H_2O_2 and lipid hydroperoxides and/or other

organic xenobiotics levels. The high CAT activity in *L. aurata* caught at RIO and LAR was not associated with high GPx activity which may be an indication that, in *L. aurata* kidney, CAT is the first enzymatic defence to control H_2O_2 although other studies have shown that GPx can be easily increased in kidney in response to oxidative stress (Basha and Rani 2003). The lack of association between these two enzymes in the kidney does not agree with Oliveira et al. (2009) findings for *L. aurata* gill, where both CAT and GPx activities were induced in polluted sites, suggesting a tissue specific response.

GR activity was significantly higher than TOR at all studied sites, except VAG, giving an indication of activated machinery to reduce GSSG into GSH which is energetically less costly than synthesizing *de novo* GSH (Pena-Llopis et al. 2003) and highly relevant considering that most GSH is synthesized in liver. Higher GR activity has been previously observed in the kidney of fish from highly polluted environments (Dautremepuits et al. 2009).

The important role of kidney in the excretion of toxic substances makes it prone to damage. Unlike other tissues, high activity of γ-glutamyl transpeptidase (GGT) (which breaks down glutathione-conjugates to mercapturic acids), can be found in kidney (Dickinson and Forman 2002). GSH catabolism may lead to production of pro-oxidant species (O_2^-, H_2O_2, thiyl radicals), as a result of the interaction of GSH metabolites with trace levels of iron ions present in the cell. In the current study, the lack of kidney LPO suggests efficient antioxidant defences and repair mechanisms in *L. aurata* though peroxidative damage to fish kidney, caused by xenobiotics, both inorganic and organic, has been found in other studies (Ahmad et al. 2003, 2006; Berntssen et al. 2003; Oliveira et al. 2008). Despite the antioxidant defences capability to prevent LPO, DNA integrity loss was found in two sites, RIO and VAG. Nevertheless, the possibility that oxidative stress may have also been involved in the DNA integrity loss can not be excluded since different xenobiotics may display distinct genotoxic mechanisms involving both direct and indirect oxidative DNA damage and impairment of DNA repair capacities (Jha 2008).

According to kidney responses, BAR seems one of the less impacted sites, not displaying damage and only having increased GR activity. Oliveira et al. (2009)

study found no differences to TOR, in terms of gill defences, at BAR, in concomitance with higher TBARS levels and suggested an environmental condition insufficient to stimulate protection though pernicious enough to cause peroxidative damage in gill. High basal antioxidant levels of kidney, compared to gill, associated with increased antioxidant response, may explain the lack of LPO in this study.

Previous studies labelled GAF as one of the most threatened sites in Ria de Aveiro. Thus, high levels of PAHs, metals and organometallic compounds (Barroso et al. 2000; Pacheco et al. 2005) have been found in that area. The only available study using *L. aurata* caught at GAF focusing on antioxidant responses, found a significant induction of *L. aurata* gill CAT, GPx, GR and GSHt and decreased metallothionein (Oliveira et al. 2009) confirming the presence of pro-oxidants in that site. However, in the current study, among the assessed kidney responses, only GR activity was different from TOR. In general, the susceptibility of a given organ is determined by different predisposition to accumulate xenobiotics, characteristic defences basal levels, adaptation capacity and consequent antioxidant activation, metabolic rates, increasing the potential to produce ROS and challenging the respective defences and tissue regeneration ability. Moreover, the routes of xenobiotics exposure determine the most targeted organs. These factors may explain the differences observed in these two studies.

L. aurata caught at RIO displayed increased kidney CAT, GST, GR activities and GSH levels, and decreased DNA integrity, indicating that kidney defences were not effective in genotoxicity prevention. Thus, the present study data suggests the persistence of contaminants, probably associated with the bleached kraft pulp mill effluents, despite its deviation in the year 2000. However, Oliveira et al. (2009) study concerning *L. aurata* gill responses did not find any difference between RIO and TOR. In this perspective, the current kidney data may suggest absorption of contaminants not only waterborne but also more associated with sediments which may enter the organism through ingestion, targeting kidney more than gill. Moreover, kidney responses may also be linked with active metabolites of xenobiotics biotransformed by the kidney and other tissues.

Nevertheless, the current kidney responses demonstrate the persistence of pro-oxidants at RIO.

CAT, GST and GR activities as well as GSHt levels were stimulated in fish captured at LAR, demonstrating an adaptation to mercury pollution which is known to exert oxidative stress via H_2O_2 production (Stohs and Bagchi, 1995). The activated defences provided an efficient protection to *L. aurata* kidney which is more relevant considering that kidney is one of the main accumulating organs for mercury. The kidney antioxidant responses observed in this study at LAR were similar to those observed in a previous study in gill (Oliveira et al. 2009), though gill GPx activity was also increased.

At VAG, *L. aurata* kidney DNA integrity loss was not associated with any defence inhibition and the only parameter significantly different from TOR was GSHt which was higher. Thus, the lack of GPx and GST activation can not be explained by insufficient GSH. It is likely that DNA integrity loss was linked either with xenobiotics direct effect or its metabolites on DNA which in VAG may be attributed to PAHs (Pacheco et al. 2005), domestic effluents and possibly pesticides. Previous studies had also demonstrated genotoxicity to *L. aurata* at VAG, manifested as erythrocytic nuclear abnormalities (Pacheco et al. 2005). Considering the known high levels of PAHs, increased GST activity, a phase II of biotransformation enzyme, would be expected. Thus, an insufficient phase II conjugation may also be linked with the DNA integrity loss. Oliveira et al. (2009) study did not find any *L. aurata* gill peroxidative nor genetic damage at VAG despite the induction of gill antioxidant defences (CAT, GPx, GST and GSHt).

The IBR index approach provides a simple tool for a general description of the ‘‘health status’’ of populations, combining the different biomarker signals (Leinio and Lehtonen, 2005). Its usefulness was previously demonstrated in field studies, regardless of the considerable variability in the biomarker sets used, contamination profiles and species (Broeg and Lehtonen, 2006; Damiens et al. 2007, Oliveira et al. 2009), allowing the study of inter- and intra-regional as well as seasonal differences of responses. The selection of biomarkers used in this index is highly relevant since the use of specific biomarkers, which respond to the same type of pollution, will overemphasise the importance of the presence/absence of a

certain group of compounds. The IBR index has been previously used to integrate oxidative stress responses (Oliveira et al. 2009, Pereira et al. 2009), diminishing the degree of uncertainty on the interpretation of results. However, the information provided by this index should be carefully interpreted since damage biomarkers assume the same importance as defence ones, though their nature represents a more hazardous condition. Furthermore, IBR mathematical expression may lead to a zero or a low value even if some biomarkers are elevated which could lead to false interpretations and oversimplifications (Broeg and Lehtonen, 2006; Baussant et al. 2009). In the present study, IBR index signalled LAR and RIO as the most impacted sites, which seems to agree with the analysis of each biomarker individually. However, VAG that displayed DNA integrity loss is not ranked as highly impacted which suggests the need for an adjustment on this index.

In agreement with our assumptions, environmental monitoring under such complex conditions should not only focus on damage but in its relation with defence responses since cell damage is not always in line with the contamination degree, depending also on the concomitant modulation of defences. Thus, this study demonstrates that Ria de Aveiro still has a high degree of contamination which is able to affect kidney.

5. Conclusions

L. aurata kidney antioxidant responses suggest that this species is able to adapt to environments with different patterns and degrees of contamination. The antioxidant defences demonstrated the existence of strong pro-oxidant conditions, especially at RIO and LAR. In addition, evidences of decreased DNA integrity at RIO and VAG were observed.

L. aurata kidney demonstrated to be well “equipped” to minimize the negative effects of toxic chemicals. Nevertheless, its usefulness in biomonitoring studies was reinforced through the conjugation of antioxidant defences and damage responses, thus being a good alternative to the liver, the organ traditionally used with this purpose.

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CAPÍTULO VII

Níveis de metalotioninas hepáticas na tainha-garrento (*Liza aurata*) – relação com as concentrações ambientais de metais num sistema costeiro em Portugal

Hepatic metallothionein concentrations in the golden grey mullet (*Liza aurata*) - relationship with environmental metal concentrations in a metal-contaminated coastal system in Portugal

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Abstract

This field survey was designed to assess the environmental metal contamination status of Ria de Aveiro (Portugal). To achieve that goal, the levels of Cd, Hg, Cu and Zn in the sediments and water were assessed and Liza aurata hepatic metallothionein (MT) determined. The MT relation with environmental metal levels and hydrological factors was studied. Results revealed a wide distribution of metals both in water and sediments throughout the lagoon, mainly at Rio Novo do Principe (RIO) and Laranjo (LAR), at levels that may affect biota. MT levels were higher at the sites with high metal content (RIO and LAR). A significant positive correlation was found between MT and Cd in the sediments as well as with MT and Hg and Cu in the water. Moreover, a negative correlation between MT and salinity was found. Thus, the current data support MT use as a biomarker of metal exposure emphasizing the importance of hydrological parameters in its levels. Data recommend the continued monitoring of this lagoon system.

Keywords: *Liza aurata*; Metallothioneins; Cadmium; Copper; Zinc; Mercury; Aquatic pollution

1. Introduction

Metal contamination in aquatic systems is a critical environmental issue due to metals toxicity, persistence and accumulation in many aquatic organisms as well as the possibility of their transfer and magnification along the trophic chain, eventually reaching humans. Most of these compounds have high affinity for particulate matter and are readily adsorbed onto suspended particles eventually becoming part of sediments where they will remain even after input cessation. Nevertheless, metals may return to the water column or be transferred to biota through physical, chemical and/or biological processes (Schneider and Davey, 1995; Bervoets et al., 2005).

Normal fish cellular metabolism requires essential metals (such as Cu and Zn) which may be taken up from water, food or sediment. However, non-essential ones (such as Cd and Hg) may also be absorbed and accumulated in tissues (Canli and Atli, 2003). Thus, environmental pollution monitoring frequently includes assessment of metals in sediment, suspended particulate matter or biota. However, the quantification of environmental or tissue concentrations of a variety of metals does not take into account the biological response or significance of the accumulated metals (Chan, 1995) since metals can accumulate to high levels in organisms and yet be metabolically unavailable, thus unable to induce toxicological effects (Vijver et al., 2004; Banni et al., 2007). Metal exposure generally increases total tissue concentrations of metals. However, the increase of essential metals in some tissues may not be significant due to their regulated uptake and distribution (Atli and Canli, 2008).

Metallothioneins (MT) are low molecular weight cytosolic proteins rich in SH groups, known to be over-expressed in organisms from environments with high metal concentrations (Sarkar et al., 2006; Viarengo et al., 2007). In fish, the large amount of MT induction occurs in liver (Canli and Atli, 2003), an organ playing a key role in the storage and inactivation of metals. Considering that MT induction is one of the first detectable signs of the presence of metals (Filipovic and Raspor, 2003; Chesman et al., 2007; Van Campenhout et al., 2008) and the relationship found between metal levels in the environment and MT concentrations in fish tissues (Hylland et al., 1992), they have been used to characterize metal contamination in the environment (Chan, 1995; Linde et al., 2001).

Ria de Aveiro (Portugal), a coastal lagoon, provides an important habitat for a number of fish species, including the golden grey mullet (*Liza aurata*). The feeding habits of this species include benthic organisms and detritus, making it particularly adapted to accumulate sediment-associated contaminants and thus an important bio-indicator species. Previous studies concerning this species, in this complex aquatic system, revealed the existence of critical sites within Ria de Aveiro, capable of inducing oxidative and genetic damage (Oliveira et al., 2009a,b,c) as well as endocrine disruption (Oliveira et al., 2009d). High levels of metals have been detected in this aquatic system (Coelho et al., 2005; Monterroso

et al., 2007) and MT inductions as well as inhibitions were observed in *L. aurata* gill, at some of those critical sites (Oliveira et al., 2009a). Thus, the hepatic MT assessment is highly relevant due to liver central role in metal storage and inactivation. Thus, this study assessed the relation between metal levels found in water and sediment compartments of Ria de Aveiro and *L. aurata* hepatic MT. The influence of hydrological factors on MT levels was also studied.

2. Materials and methods

2.1 - Study area: Ria de Aveiro is a lagoon permanently connected to the ocean (Figure 1), about 45 km long and 8.5 km wide. Sampling sites were selected on a geographic distribution basis taking into account the known and supposed sources of metal contamination as well as the selection of a (theoretically) unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of polycyclic aromatic hydrocarbons (PAHs).

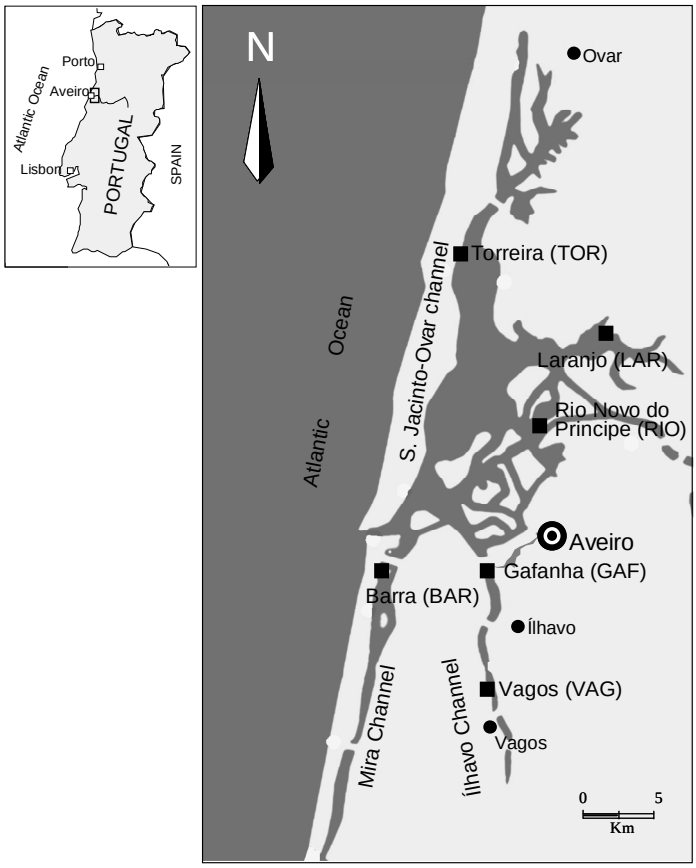

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture sites (■). The respective coordinates are: reference site (TOR)—40º44'02 N, 008º41'44 W; BAR—40º37'42.00"N, 8º44'35.00"W GAF—40º38'38 N, 008º41'42 W; RIO—40º41 '08 N, 008º39'41 W; LAR—40º43'30 N, 008º37'43 W; and VAG—40º33'59 N, 008º40'55 W.

2.2 - Sampling: Liza aurata was caught in October 2005, during low tide using a traditional beach-seine net named “chincha”. Juvenile specimens were selected on the basis of their size, having an average length of 14.5±2.5 cm and weighing 21.6±3.7 g. Immediately after catching, fish were sacrificed, liver removed and frozen in liquid nitrogen. In the laboratory the samples were stored at −80º C until further treatments.

At each sampling site hydrological parameters were assessed (Table I) according to APHA (1998) guidelines. Sediments were collected from the surface (15 cm deep) with a grab and stored at –20ºC until analysis. Sub-surface water samples were collected by hand, using polyethylene gloves and glass bottles of 1 L.

2.3. Metal determination in the water and sediments: Total metal concentrations in the water samples were determined after liquid-liquid extraction with ammonium pyrrolidine dithiocarbamate (APDC) and methyl isobutyl ketone (MIBK) followed by graphite furnace atomic absorption spectrophotometry (PerkinElmer). This procedure was adopted to pre-concentrate the metals present in the water. Certified standard freshwater reference material (SRM 1643d from NIST) was analysed to check the accuracy of metal determinations in the water samples.

Total metal concentrations in the sediments were determined in the < 63 µm fraction, to minimize the effect of grain size on metal distribution. Samples were manually wet sieving using 63 µm nylon sieves and Milli-Q water. After that, this sediment fraction was dried at 40 ºC. For analysis, approximately 2 g of sediments fraction were accurately weighed and placed into 50 ml digestion vessels. After the addition of 5 ml 69% nitric acid (HNO_3), the samples were digested for 2 hours at 80ºC. The digestion mixture was cooled to room temperature and 2 ml 30% hydrogen peroxide (H_2O_2) and 3 ml Milli-Q water carefully added. The digestion vessels were heated for another hour. The residue was allowed to cool and diluted with Milli-Q water to a final volume of 50 ml. Then samples were allowed to settle down for 24 hours and quantitatively transferred to 50 ml vessels for analysis.

The concentration of Cd, Cu, Hg and Zn were determined in the supernatant using a Perkin-Elmer atomic absorption spectrophotometer (AAS) model A Analyst 800. All metals except Cd and Hg were analysed by flame atomic absorption spectrophotometry. Cadmium was analysed by an electrothermal atomic absorption spectrometry and mercury by cold vapour atomic spectrophotometry with an automatic hydride generator system.

2.4. Metallothionein (MT) content: After liver homogenization in 20 mM Tris buffer (pH 8.6) containing 150 mM of NaCl, the homogenates were centrifuged 45 minutes at 30000 g (4ºC). Supernatant aliquots were heat-treated at 80ºC for 10 minutes and re-centrifuged at 30000 g for 45 minutes (4ºC). MT quantification was made by differential pulse polarography (DPP) according to

Bebianno and Langston (1989) using a 646VA Processor autolab type II and an ECO Chemie IME663 mercury drop electrode. Comparisons of peak heights with those of standard additions of purified rabbit MT enabled MT quantification. Accuracy of the DPP analytical method was obtained by internal calibration using commercial rabbit liver MT-I. Recovery studies of our cytosols spiked with the MT-I rabbit liver standards revealed recovery rate from 97 to 103%. Results were expressed as µg MT/g wet weight.

2.5. Statistical analysis: Results were expressed as means ± SE (standard error) corresponding to experimental groups of six fish (n=6). Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). Assumptions of normality and homogeneity of data were verified. One way ANOVA was performed in order to assess significant effects, followed by post-hoc Tukey test to signal significant differences between groups (Zar, 1999). The significance of results was ascertained at $\alpha = 0.05$. Describing factors were grouped using principal component analysis (PCA), reducing the number of axis without loosing relevant information. Principal components (PCs) having eigenvalues of less than one were discarded, and varimax rotation maximising the loading of a variable on one component was then applied on the retained PCs. The resulting components were then used as independent factors in multiple regression analyses taking MT as the dependent variable.

3. Results

3.1. Hydrological parameters

The hydrological parameters, expressed in Table I, show differences in terms of turbidity and salinity. Thus, waters were more turbid at LAR and VAG (0.3 m) and less turbid at BAR (1.2 m). Salinity at RIO was 21 whereas the other sites displayed values between 29 (LAR) and 34 (GAF and BAR).

Table I - Environmental characterization: hydrological parameters and metals concentrations in water and sediments. Distinct letters indicate significant differences between sites ($p<0.05$).

			Sites					
			TOR	**BAR**	**GAF**	**RIO**	**LAR**	**VAG**
Water Physico-Chemical Parameters		Turbidity (m)	0.8	1.2	0.7	1	0.3	0.3
		Dissolved Oxygen (mg/L)	6.88	6.16	5.78	6.37	4.96	5.18
		Temperature (ºC)	18.9	17.9	17.9	19	18.6	18.5
		pH	8.17	8.32	8.28	8.2	7.74	7.88
		Salinity	32.5	34	34	21	29	33
Metal Concentrations	Water ($\mu g.L^{-1}$)	Cd	1.02 ± 0.09^{bc}	0.39 ± 0.12^{c}	1.52 ± 0.10^{b}	0.92 ± 0.13^{bc}	2.91 ± 0.48^{a}	0.85 ± 0.20^{bc}
		Hg	0.46 ± 0.01^{d}	BDL	0.56 ± 0.01^{c}	1.02 ± 0.02^{a}	0.78 ± 0.02^{b}	0.23 ± 0.01^{e}
		Cu	2.80 ± 0.36^{c}	1.25 ± 0.11^{d}	3.82 ± 0.24^{bc}	6.21 ± 0.36^{a}	4.32 ± 0.47^{b}	1.02 ± 0.07^{d}
		Zn	12.05 ± 0.33^{c}	4.07 ± 0.47^{d}	10.10 ± 0.12^{c}	18.72 ± 0.42^{a}	14.52 ± 0.39^{b}	18.05 ± 0.71^{a}
	Sediments ($\mu g.g^{-1}$ d.w.)	Cd	0.16 ± 0.01^{c}	0.14 ± 0.02^{c}	0.15 ± 0.02^{c}	0.91 ± 0.03^{a}	0.31 ± 0.00^{b}	0.12 ± 0.03^{c}
		Hg	0.17 ± 0.08^{c}	0.88 ± 0.25^{b}	0.11 ± 0.02^{c}	0.86 ± 0.08^{b}	2.33 ± 0.20^{a}	0.04 ± 0.02^{c}
		Cu	14.28 ± 0.34^{c}	14.96 ± 0.62^{c}	15.15 ± 0.36^{c}	23.48 ± 0.53^{b}	30.11 ± 0.37^{a}	13.71 ± 0.34^{c}
		Zn	114.63 ± 3.44^{b}	78.44 ± 3.12^{c}	67.75 ± 3.05^{c}	112.85 ± 5.20^{b}	235.77 ± 9.49^{a}	68.08 ± 4.52^{c}

3.2. Metals in water and sediment

The water analyses revealed that RIO displayed significantly higher levels of Hg, Cu and Zn than TOR and the other studied sites (with the exception of Zn levels which were not different from VAG) (Table I). Concerning Cd, the highest levels in water were found at LAR (significantly higher than in all other sites). BAR displayed lower levels of Cd, Hg, Cu and Zn when compared to TOR (though not significantly in the case of Cd). The metal levels in water at GAF were only significantly different (higher) from TOR in the case of Hg. At LAR, the levels of all

the analysed metals were significantly higher than TOR whereas VAG displayed lower Hg and Cu and higher Zn levels in water.

Concerning the levels of metals in the sediments (Table I), the highest levels of Hg, Cu and Zn were found at LAR (significantly higher than all other studied sites). Concerning Cd, the highest levels in the sediments were found at RIO (significantly higher than all other studied sites) and LAR (significantly higher than TOR, BAR, GAF and VAG). Hg levels in sediments at BAR and RIO were also higher than in TOR, GAF and VAG. The levels of Cu in sediments at RIO were also significantly higher than TOR whereas Zn levels at BAR, GAF and VAG were significantly lower than TOR.

3.3. Metallothionein

RIO displayed significantly higher MT levels when compared to all studied sites (Figure 2). Thus, compared to TOR, MT levels were 256% higher at RIO. LAR also displayed significantly higher MT levels than TOR (107%), BAR, GAF, and VAG. At VAG, MT levels were significantly lower than TOR (74%).

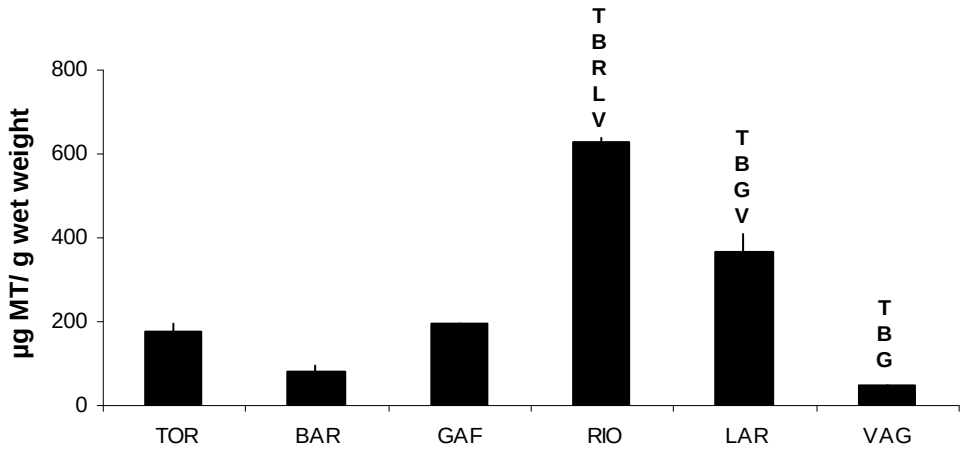

Figure 2 – Hepatic metallothionein (MT) of *L. aurata* collected at different sites in Ria de Aveiro. Values represent mean ± standard error (n=6). Significant differences (P<0.05) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR; V *versus* VAG.

3.4. Principal component analysis and linear multiple regression

Three principal components were defined as explaining the major amount of total variance (89.97%) of the assessed parameters (except MT) (Table II).

Table II – Results of the principal component analysis (PCA) on the dataset: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the PCs are computed (loadings <0.25 were omitted)

	Component		
	1	**2**	**3**
Salinity	***-0,932***	-0,240	-0,118
Cd in Sediments	***0,909***	0,172	0,258
Hg in Water	***0,850***	0,399	-0,060
Temperature	***0,827***	-0,048	-0,243
Cu in Water	***0,804***	0,432	0,193
Zn in Water	***0,770***	-0,139	-0,614
Hg in Sediments	0,114	***0,945***	-0,110
Zn in Sediments	0,238	***0,855***	-0,318
Cu in Sediments	0,469	***0,855***	-0,191
Cd in Water	0,083	***0,789***	-0,491
Turbidity	0,032	-0,150	***0,971***
pH	-0,096	-0,372	***0,897***
Dissolved Oxygen	0,242	-0,397	***0,739***

Thus, the first component which includes salinity, temperature, Cd in the sediments, Hg, Cu and Zn in the water explains 36.19% of total variance. The second component including Hg, Zn and Cu in the sediments and Cd in the water, explains 28.84%. The third components including turbidity, pH and dissolved oxygen explain 24.93% of variance. According to the multiple regression analyses the first two factors account for 98.2 % of MT variability and the first factor alone is responsible for 77%. Cd in the sediments, Hg and Cu in the water and salinity displayed the strongest (significant) correlations with MT (Figure 3).

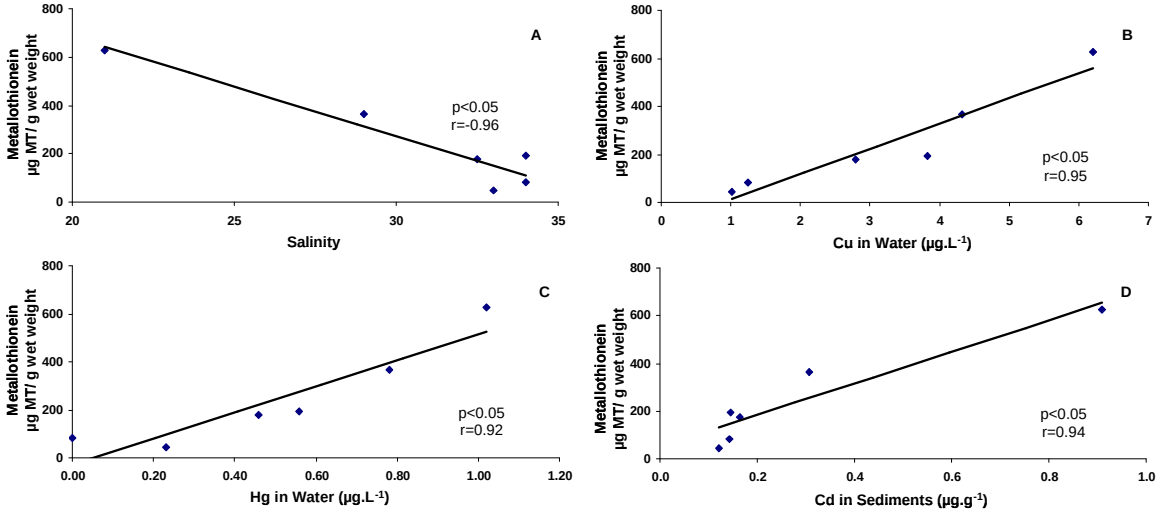

Figure 3 - Correlation between *L. aurata* hepatic MT and A) Salinity; B) Cu in water; C) Hg in water; D) Cd in the sediments. Statistical significance and correlation coefficient are represented by p and r, respectively.

4. Discussion

Biological factors such as age, sex, reproductive status as well as hormone levels may influence MT induction, increasing response variability (Hamza-Chaffai et al., 1995). Moreover, it was demonstrated in *L. aurata* that total length, body weight and age are biometric parameters correlated with MT (Filipovic and Raspor, 2003). In this perspective, *L. aurata* specimens selected for the current study were juvenile with the same age and reproductively quiescent and the stress of capture and handling was reduced as much as possible.

4.1. Metal levels

Metals mobility and reactivity in estuaries are highly influenced by the organic matter content, salinity, redox conditions and pH (Sauve et al., 2000; Liang and Wong, 2003). Sediment metal levels usually exceed those in the water column. However, metals bound to the sediment may enrich interstitial and overlying waters with metals (Bryan and Langston, 1992) upon changes in the environment. In the current study, the levels of two of the most dangerous metals to aquatic organisms, Cd and Hg, listed in the priority substances of EU Directive

2008/105/EC, as well as two essential metals, Cu and Zn, that in high concentrations may also induce toxic effects in aquatic organisms (Oliveira et al., 2004) were quantified in the water and sediments of Ria de Aveiro. The studied metals displayed a distribution throughout the lagoon. Zn displayed the highest levels in sediments, followed by Cu, Cd and Hg. The highest levels of metals in the sediments were found in LAR (for Zn, Cu and Hg) and RIO (for Cd). At RIO, LAR and BAR Hg was higher than the defined Water Framework Directive environmental quality standard for sediments (0.36 µg.g-1). Most of the available studies on metals levels in Ria de Aveiro focused on Hg contamination in LAR, due to the emission history of a chlor-alkali plant (Ramalhosa et al., 2005). Thus, at LAR area, levels of Hg detected in the in sediments reached values as high as 51.7 µg.g-1 (Coelho et al., 2005). In the current study, the highest levels of Hg in the sediments were found in LAR (2.33 µg.g-1) and were within the same order of magnitude of other studies (Pereira et al., 2006; Ramalhosa et al., 2006). The present data also revealed considerable levels of Hg in the sediments of BAR and RIO (0.88 and 0.86 µg.g-1 respectively), probably due to tidal export from Laranjo area (Pereira et al., 1998) and/or from nonpoint sources. Despite most studies on metal levels at LAR focused on Hg, elevated levels of other metals were also found there by Monterroso et al. (2007). The levels of Cd, Cu and Zn found in the current study at LAR sediments were similar to those found by Monterroso et al. (2007).

The metal levels in water reported in the current study can only be compared with other studies in Ria de Aveiro in terms of Hg in LAR, being in the same order of magnitude of previously reported levels (Ramalhosa et al., 2006; Guilherme et al., 2008). Unlike sediments which displayed higher Hg, Cu and Zn levels at LAR, in water, RIO displayed the highest levels of Hg, Cu and Zn. Another clear difference to sediments was found in BAR that displayed very low Hg levels in water (bellow detection limit). Moreover, Cd levels at all studied sites were above the defined environmental quality standard for water of Water Framework Directive (0.21 µg.g-1).

One of the outstanding difficulties encountered in environmental impact assessments is to determine the biological significance of contaminants (Langston

et al., 2002). In this perspective numerical quality guidelines have been developed, identifying concentrations of chemicals of potential concern that may cause or contribute to adverse effects (MacDonald et al., 2000). However, its use should be carefully interpreted since factors like organic matter, grain size and percentage of clay may be confounding elements for direct comparison. Nevertheless, the comparison of the present metal data with some quality guidelines (Long et al., 1995; MacDonald et al., 1996) reveals that the levels detected in the sediments mainly at LAR and RIO and BAR may lead to frequent adverse effects in aquatic organisms. The levels of metals in the water are highly dynamic, being influenced by abiotic factors among which the water flow. Nevertheless, the water levels of Hg at RIO as well as Cu at RIO, LAR and GAF, can lead to damage in aquatic species considering they were higher than the US EPA (2006) estimated highest concentration to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect.

The bioavailability of a metal does not necessarily lead to harmful effects in organisms since they evolved defence mechanisms which allow them to decrease metal reactivity in cells. Among those defences, MT are considered as the most suitable biomarker for Cd, Cu, Zn and Hg exposure (Hylland et al., 1998), reflecting bioavailability and tissue pharmacokinetics of metal uptake (Handy et al., 2003). In this perspective, the study of *L. aurata* liver MT becomes highly relevant, taking into account that liver is the main metal accumulating organ in this species (Usero et al., 2003). However, despite the known presence of metals in Ria de Aveiro and the suitability of fish MT as biomarkers of exposure to metals, the studies focusing on environmental metal levels and fish MT levels are very scarce.

Measurable (constitutive) levels of MT are always expected even in uncontaminated sites, due to MT homeostatic role in transferring essential elements to and from metalloproteins. The observed MT levels ranged from 45.6 to 627.7 (µg.g-1), being in the same order of magnitude of the MT levels found in other species. For example, European flounders (*Pleuronectes flesus*) from the Severn Estuary and Bristol Channel MT levels ranged between 164 and 536 (µg.g-1) (Rotchell et al., 2001) whereas Zorita et al. (2008) found MT levels in red mullets (*Mullus barbatus*) between 270 and 710 (µg.g-1). However, the current

hepatic MT levels were much lower than in *L. aurata* from Eastern Adriatic coastal zone (2320 µg.g-1) (Filipovic and Raspor, 2003). Differences may be linked with different age, weight and size of the surveyed specimens. Supporting this hypothesis, a positive and statistically significant correlation between *L. aurata* total length and body weight with MT and metals in liver cytosol was found by the authors (Filipovic and Raspor, 2003).

In the current study, MT levels were higher at the sites displaying the highest metal levels, supporting its usefulness in environmental monitoring even in complex environments where interference of other xenobiotics can be found. MT use as biomarker of response to metals exposure is more sensitive to metals such Cd and Hg and to a much lower extent to essential metals (Olsvik et al., 2001; Banni et al., 2007; Atli and Canli, 2008;) which have their cytosolic levels closely regulated through homeostatic mechanisms often binding to structures other than MT (Laflamme et al., 2000; Langston et al., 2002; Linde et al., 2005). In this perspective, other sulfhydryl group containing substances such as GSH may also play an important role in cellular protection against metal cytotoxicity (Viarengo and Nott, 1993; Oliveira et al., 2004). Nevertheless, a high positive correlation of MT and Cu cytosolic levels was found in liver of *L. aurata* (Filipovic and Raspor, 2003). Moreover, considering the complex environment that is an estuary, the combined effects of other xenobiotics should also be considered as demonstrated by Romeo et al. (1997) and van den Hurk et al. (2000). Despite the demonstration that simultaneous exposure to metals such as Cd and PAHs may yield higher MT levels than metal exposure alone, our study clearly shows MT induction in environments with high metal levels (RIO and LAR) and low levels in sites with low metal levels (TOR, BAR and VAG). The previous (and only available study) on MT levels of *L. aurata* caught at Ria de Aveiro (Oliveira et al., 2009a), showed higher gill MT levels at LAR and suggested that this site was the most contaminated with metals. Moreover, no significant differences to TOR were found in that study in MT levels from RIO. These results seem to confirm that the hepatic MT levels are more reliable to the environmental metal contamination assessment than gill MT.

4.2. Relation between MTs and abiotic factors

The multiple regression analyses showed that the assessed metals and hydrological parameters grouped in components 1 and 2 could explain 98.2 % of the variability of MTs. Thus, salinity, temperature, Cd in the sediments Hg, Cu and Zn in water were responsible for 77% of MT variability. Of these parameters, linear regression analyses signalled as more correlated Cd in the sediments, Cu and Hg in water and salinity (inversely). It has been generally accepted that the metals toxicity of metals to aquatic animals is influenced by ambient salinity. Thus, metals are usually more toxic at low salinities probably associated with increased uptake rates, as a result of an increase in free metal ion concentrations. In this perspective, a highly significant negative correlation between MT and salinity was also found in the Brazilian flounder (*Paralichthys orbignyanus*) collected from an estuary (Amado et al., 2006). Thus, a determinant role of salinity in the metal bioavailability can be on the basis of the negative correlation currently found between that abiotic parameter and hepatic MT. However, a definitive linkage between these variables is complicated by the occurrence of different patterns of contamination and salinity levels in the sites under comparison.

Thus, the usefulness of *L. aurata* hepatic MT as biomarkers of metal exposure was demonstrated in fish caught at Ria de Aveiro despite the known ability of other xenobiotics to interfere in MT levels and the known existence of different type of xenobiotics in Ria de Aveiro.

Hepatic MT levels were correlated with the levels of metals in both water and sediments. However, the determinant role of salinity in the metal bioavailability was also manifested through its negative correlation with MT.

5. Conclusions

High levels of metals can be found in the sediments and water of Ria de Aveiro which may result in pernicious effects to aquatic organisms. The highest levels of metals in the sediments and water were found in LAR and RIO. Thus, in sediments, LAR for Zn, Cu and Hg and RIO for Cd displayed the highest levels. In

water, RIO displayed the highest levels of Hg, Cu and Zn and LAR the highest levels of Cd.

L. aurata hepatic MT levels can be ranked as: RIO>LAR>GAF>TOR>BAR>VAG. The highest hepatic MT levels displayed by *L. aurata* at the most contaminated sites (RIO and LAR) reveal a strong *L. aurata* defence capacity against metal toxicity.

The high correlation, between environmental levels of metals and *L. aurata* hepatic MT, supports MT applicability as a biomarker of metal pollution in contaminated aquatic systems.

The current study data recommend continued monitoring of Ria de Aveiro.

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CAPÍTULO VIII

Respostas antioxidantes vs. dano no ADN e peroxidação lipídica no fígado da tainha-garrento – Um estudo de campo na Ria de Aveiro (Portugal)

Antioxidant responses vs. DNA damage and lipid peroxidation in golden grey mullet liver – A field study at Ria de Aveiro (Portugal)

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Submitted

Abstract

The present work aimed to investigate golden grey mullet (*Liza aurata*) liver protection vs. damage responses at a polluted coastal lagoon, Ria de Aveiro (Portugal), as a tool to evaluate the human impacts on environmental health at five critical sites in Ria de Aveiro (Portugal) in comparison to a reference site (Torreira). Protection was evaluated by measuring non enzymatic (total glutathione – GSHt and non protein thiols - NPT) and enzymatic (catalase – CAT, glutathione peroxidase – GPx, glutathione S-transferase - GST, and glutathione reductase - GR) antioxidant defenses. Damage was assessed as DNA integrity loss and lipid peroxidation (LPO). No significant differences were found between sites in terms of non enzymatic defences (GSHt and NPT). Enzymatic defences, with the exception of CAT, demonstrated a high susceptibility to inhibition. CAT did not display significant differences between sites. However, GPx at Barra (associated with naval traffic), Gafanha (harbour and dry-dock activities area), Laranjo (metal contaminated associated with chlor–alkali plant) and Vagos (PAH contaminated) was significantly lower than the reference site. GST was lower at Gafanha, Rio Novo do Príncipe (pulp mill effluent area), Laranjo and Vagos whereas GR was lower at Rio Novo do Principe. These low defences were translated into oxidative damage, LPO, only at GAF and VAG. However, no DNA integrity loss was found. Results highlight the importance of the adopted multi-biomarkers as applied in the liver of *L. aurata* in coastal water pollution monitoring. The integration of liver antioxidant defence and damage responses can improve the aquatic contamination assessment.

Keywords: *Liza aurata*; Antioxidant defences; Oxidative stress; Genotoxicity

1. Introduction

A large number of toxic contaminants resultant from increased industrialization and urban development are released into the environment, ultimately reaching estuarine and marine systems. Once absorbed, contaminants may interact with endogenous substances, causing biological effects which may impair the life quality not only of the exposed organisms, but of the whole ecosystem (Ferrando et al., 2006). Considering the complex mixtures of contaminants present in the aquatic systems, whose synergistic/antagonistic effects are hardly interpreted and predicted based on chemical analyses, fish biochemical responses have been increasingly used in monitoring pollution, since they reflect the integrated effects of exposure to all contaminants, even those present at levels below chemical detection limits (Zhou et al., 2008). However, the use of a battery of different biomarkers is essential, as the complexity of environmental contaminants can induce a variety of responses in organisms that are not necessarily correlated (Viarengo et al., 2000).

Normal cellular metabolism produces reactive oxygen species (ROS) that, though crucial for many physiological processes (Droge, 2002), may pose a serious threat at high concentrations. In order to prevent the pernicious effects of ROS and other free radicals, aerobic organisms developed antioxidant defences for their protection (Winston and Di Giulio, 1991). However, exposure to xenobiotics may increase ROS production and overcome antioxidant defences. The biological consequences can vary from a moderate alteration of redox status, to the occurrence of lipid peroxidation (LPO) and genetic damage, destabilization of the main cellular functions with appearance of several pathologies, and eventually death (Gorbi et al., 2005). In this perspective, the analysis of antioxidant status (i.e. antioxidants defences as well as damage responses such as LPO and genetic damage) in biomonitoring studies becomes highly relevant. Oxidative stress biomarkers (Ahmad et al., 2008; Fernandes et al., 2008; Oliveira et al., 2009a; Pandey et al., 2003; Santos et al., 2004) and DNA damage responses (Frenzilli et al., 2009; Siu et al., 2003) have been widely used in environmental

contamination monitoring allowing the detection of exposure to low concentrations of contaminants in a wide range of species.

Ria de Aveiro (Portugal) is a biologically productive coastal ecosystem permanently connected to the sea, with a significant role in the life cycle of several aquatic organisms. Over several decades, this aquatic system received indiscriminate discharges of complex effluents that included, among other pollutants, metals (Abreu et al., 2000), organometallics such as tributyltin (Barroso et al., 2000), polycyclic aromatic hydrocarbons (PAHs) (Pacheco et al., 2005) and other xenobiotics associated with the pulp mill industry. Despite in May 2000 a great portion of the effluents have been diverted to the Atlantic Ocean (2.5 km far from the sea coast), recent studies demonstrated that the risk for aquatic organisms still persists (Ahmad et al., 2008; Oliveira et al., 2009a). In this perspective, the assessment of hepatic defence and damage responses in a resident fish species becomes highly relevant in biomonitoring studies, considering the important role of liver on numerous vital functions such as accumulation, biotransformation and excretion of contaminants, and subsequently the proneness to face high levels of contaminants/metabolites and ROS.

Despite the remarkable increment of scientific studies dealing with pollutant-induced antioxidants modulation, genotoxicity and peroxidative damage in fish, few field studies provided evidence linking all the previous events under realistic field conditions in fish (Ahmad et al., 2008). Thus, the main goal of this study was the assessment of Ria de Aveiro status, as coastal lagoon prototype, focusing simultaneously on antioxidant defences (non protein thiols – NPT, total glutathione - GSHt, catalase - CAT, glutathione peroxidase - GPx, glutathione S-transferase - GST, glutathione reductase - GR) and damage (peroxidative and genetic) responses measured in fish (*Liza aurata*).

2. Material and Methods

2.1 - Study Area: Ria de Aveiro is a lagoon permanently connected to the ocean (Figure 1), about 45 km long and 8.5 km wide (Dias et al., 2001). Sampling

sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of a (theoretically) unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of PAHs.

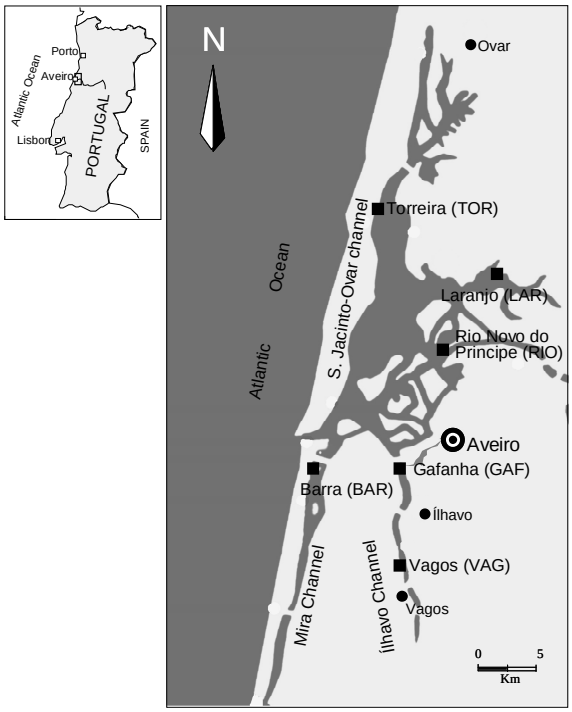

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture sites (■). The respective coordinates are: reference site (TOR)—40º44'02 N, 008º41'44 W; BAR—40º37'42"N, 008º44'35"W; GAF—40º38'38 N, 008º41'42 W; RIO—40º41 '08 N, 008º39'41 W; LAR—40º43'30 N, 008º37'43 W; VAG—40º33'59 N, 008º40'55 W.

2.2 - Sampling: *Liza aurata* was caught in October 2005, during low tide using a traditional beach-seine net named “chincha”. Juvenile specimens were selected on the basis of their size, having an average length of 14.5±2.5 cm and weighing 21.6±3.7 g. Immediately after catching, fish was sacrificed, liver removed and frozen in liquid nitrogen. In the laboratory, three sets of tissue were separated for DNA strand breaks, MT and oxidative stress measurements and stored at −80º C until further treatments.

At each sampling site abiotic parameters were assessed (Table I) according APHA (1998) guidelines.

2.3. Biochemical Analyses

One set of tissue was homogenized in phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/15 ml buffer). This homogenate was divided in aliquots for LPO, GSHt and NPT measurements, as well as post-mitochondrial supernatant (PMS) preparation. The protein content in the homogenate aliquots for GSHt and NPT measurements was precipitated with sulfosalicylic acid (5%) for one hour and then centrifuged at 13400 g for 20 minutes (4 ºC). PMS isolation was accomplished by centrifugation at 13400 g for 20 minutes (4 ºC). Aliquots were stored at –80ºC until analyses.

The second set of liver, used for DNA integrity assessment was placed in TNES (Tris-HCl 10 mM, NaCl 125 mM, EDTA 10 mM, SDS 1%, pH 7.5) – urea (5 M) buffer with proteinase K solution (final concentration 0.8 mg/ml). DNA isolation was performed using a genomic DNA purification kit (Fermentas).

2.3.1. Non Enzymatic Defences

2.3.1.1. Non-protein thiols (NPT): NPT were determined by the method of Sedlak and Lindsay (1968) as adopted by Parvez et al. (2003). The assay mixture contained 0.5 ml of filtered aliquot, 2.3 ml of sodium phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5,5, dithiobis-tetranitrobenzoic acid (DTNB) (stock=100 mM in 0.1 M sodium phosphate buffer, pH 7.4) in a total volume of 3 ml. The optical density

of reaction product was read immediately at 412 nm on a spectrophotometer (Jasco UV/VIS, V-530). Results were expressed as mmol/mg protein.

2.3.1.2. Total glutathione (GSHt): GSHt content was determined adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5, dithiobis-tetranitrobenzoic acid producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) (Baker et al., 1990; Tietze, 1969). TNB formation was measured spectrophotometrically (Jasco UV/VIS, V-530) at 412 nm. Results were expressed as µmol TNB formed/min/mg protein ($\varepsilon=14.1\times10^3\ M^{-1}\ cm^{-1}$).

2.3.2. Enzymatic Defences

2.3.2.1. Catalase (CAT) activity: Assayed in PMS by the method of Claiborne (1985) with some modifications. Briefly, assay mixture contained 1.99 ml phosphate buffer (0.05 M, pH 7.0), 1 ml H_2O_2 (30 mM) and 0.010 ml sample. Absorbance was recorded spectrophotometrically at 240 nm (25 ºC). CAT activity was expressed as µmol H_2O_2 consumed/min/mg protein ($\varepsilon=43.5\ M^{-1}cm^{-1}$).

2.3.2.2. Glutathione peroxidase (GPx) activity: GPx was assayed according to the method described by Mohandas et al. (1984) with some modifications. Assay mixture contained 0.720 ml phosphate buffer (0.05M, pH 7.0), 0.050 ml ethylene diamine tetraacetic acid tetrasodium salt hydrate (EDTA) (1 mM), 0.050 ml sodium azide (1 mM), 0.025 ml GR (1 U/ml), 0.050 ml reduced glutathione (4 mM), 0.050 ml β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) (0.8 mM), 0.005 ml H_2O_2 (1.0 mM) and 0.050 ml sample. Enzyme activity was quantified by measuring NADPH oxidation at 340 nm (25 ºC) and expressed as nmol NADPH oxidized/min/mg protein ($\varepsilon=6.22\times10^3\ M^{-1}\ cm^{-1}$).

2.3.2.3. Glutathione S-transferase (GST) activity: Determined in PMS, following the conjugation of GSH with 1-chloro-2,4- dinitrobenzene (CDNB) by the method of Habig et al. (1974) with some modifications. Reaction mixture contained 1.85 ml sodium phosphate buffer (0.1 M, pH 7.4), 0.050 ml reduced glutathione (1

mM), 0.050 ml CDNB (1 mM) and 0.050 ml PMS. Absorbance was recorded at 340 nm (25 ºC) and expressed as mmol CDNB conjugate formed/min/mg protein (ε=9.6 mM^{-1} cm^{-1}).

2.3.2.4. Glutathione reductase (GR) activity: Assayed by the method of Cribb et al. (1989) with some modifications. Briefly, assay mixture contained 0.025 ml of PMS and 0.925 ml of NADPH (0.2 mM), GSSG (1 mM) and diethylenetriaminepenta-acetic acid (DTPA) (0.5 mM). Enzyme activity was quantified by measuring NADPH oxidation at 340 nm (25ºC) and expressed as nmol NADPH oxidized/min/mg protein (ε=6.22×10^3 M^{-1} cm^{-1}).

2.3.3. Peroxidative and genetic damage evaluation

2.3.3.1. Lipid peroxidation (LPO): Determined in the tissue homogenate by the procedure of Ohkawa et al. (1979) and Bird and Draper (1984) with some modifications. Briefly, to 50 µl homogenate, 3 µl of 4% butylated hydroxytoluene in methanol, was added and mixed well. To this aliquot, 0.5 ml of 12% trichloroacetic acid in aqueous solution, 0.45 ml Tris–HCl (60 mM, pH 7.4; and 0.1 mM DTPA) and 0.5 ml 0.73% 2-thiobarbituric acid were added and mixed well. The mixture was heated for 1 hour in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2 ml microtubes and centrifuged at 13400 g for 3 minutes. Absorbance was measured in the supernatant at 535 nm and LPO expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed/mg protein (ε= 1.56×10^5 $M^{-1}cm^{-1}$).

2.3.3.2. DNA integrity: Genotoxicity was tested using DNA alkaline unwinding assay. DNA integrity measurements were performed according to Rao et al. (1996) as adopted by Maria et al. (2002). Data from DNA unwinding technique were expressed as F-value [DNA integrity (%)], determined by applying the following equation:

$$F = \frac{ds}{ds+ss} \times 100$$

where ss is the relative fluorescence (measured with a Jasco FP 750 spectrofluorometer) of the single-stranded eluent of a sample minus the single-stranded control blank fluorescence value, and ds is the relative fluorescence of the corrected double stranded eluent of the same sample.

2.3.4. Protein content: Determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin as a standard.

2.4. Statistical analysis: Results were expressed as means ± SE (standard error). The DNA integrity data was transformed prior to statistic analyses according to the formula arcsen $\sqrt{p}$. Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). Assumptions of normality and homogeneity of data were verified. One way ANOVA was performed in order to assess significant effects, followed by post-hoc Tukey test to signal significant differences between groups (Zar, 1999). The significance of results was ascertained at $\alpha = 0.05$.

3. Results

3.1. Hydrological parameters

The physico-chemical parameters of the water did not show relevant variations between studied sites (Table I). The highest turbidity levels were found at LAR and VAG (0.3 m) and the lowest turbidity (1.2 m) was found at BAR. Dissolved oxygen ranged from 4.96 at LAR to 6.88 mg/L at TOR. Temperature ranged from 17.9 (at BAR and GAF) to 19.0 ºC (at RIO). pH ranged from 7.74 (at LAR) to 8.32 (at BAR). The lowest salinity (21) was found at RIO whereas the highest was found at BAR and GAF (34). Depth ranged from 1.2 (TOR and LAR) to 6.9 m (GAF).

Table I - Physical-chemical parameters of the water from the Ria de Aveiro sampling sites.

	Turbidity (m)	*Dissolved Oxygen (mg/L)*	*Temperature (ºC)*	*pH*	*Salinity*
TOR	0.80	6.88	18.9	8.17	32.5
BAR	1.20	6.16	17.9	8.32	34.0
GAF	0.70	5.78	17.9	8.28	34.0
RIO	1.00	6.37	19.0	8.20	21.0
LAR	0.30	4.96	18.6	7.74	29.0
VAG	0.30	5.18	18.5	7.88	33.0

3.2. Non-enzymatic responses

L. aurata NPT and GSHt levels did not show significant differences between sites (Figure 2A). Concerning NPT, though not significantly, levels in fish caught at BAR and LAR were respectively 39.8 and 19.6% lower than TOR.

The highest levels of GSHt were found in fish caught at TOR, though not significantly different from the fish caught at the other sites (Figure 2B).

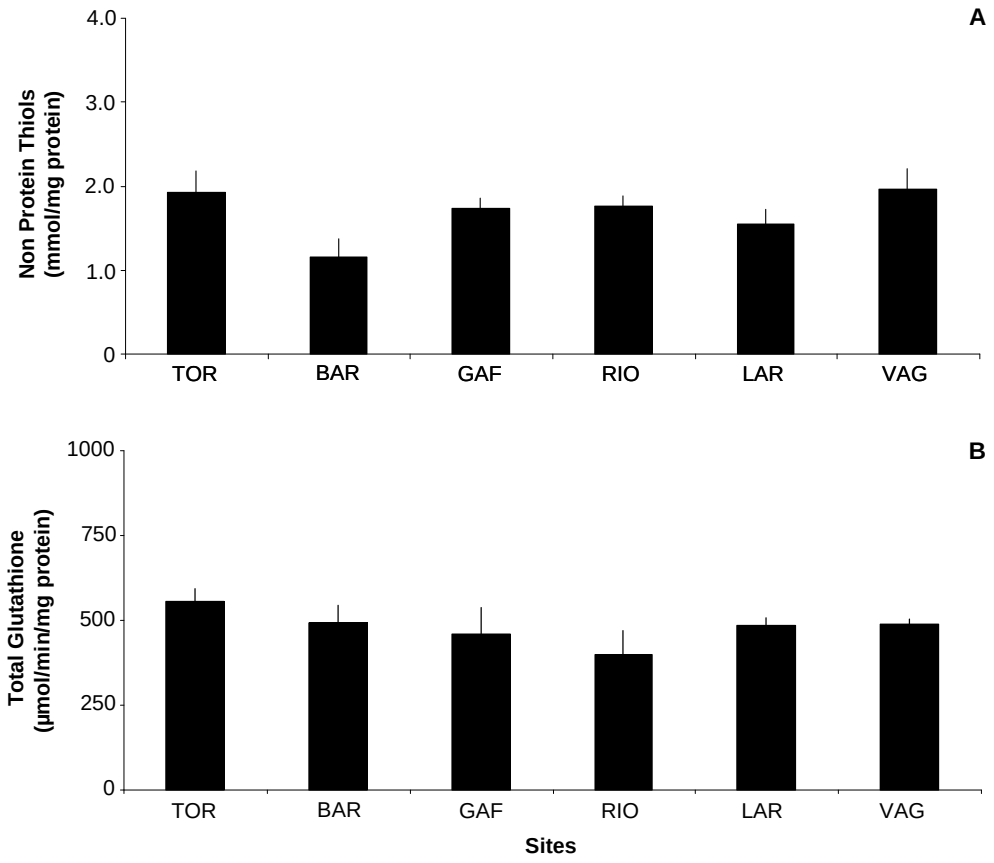

Figure 2 - Non enzymatic defences in the liver of *L. aurata* collected at different sites in the Aveiro lagoon. A – Non protein thiols (NPT); B – Total glutathione (GSHt). Values represent mean ± standard error.

3.3 – Enzymatic responses

Fish captured in this study did not display significant differences in terms of CAT activity (Figure 3A). Overall, the inter-site variability compared to TOR was less than 11%.

In terms of GPx, *L. aurata* captured at BAR, GAF, LAR and VAG displayed significantly lower activities than TOR, corresponding to 61.3, 51.7, 69.3 and 49.9% reduction, respectively (Figure 3B).

L. aurata GST activity was lower than TOR at all studied sites though only significantly at GAF, RIO, LAR and VAG (Figure 3C). Thus, compared to TOR, GST activity was 27.1, 56.5, 58.6, 73.2 and 40.1% lower at BAR, GAF, RIO, LAR

and VAG, respectively. Moreover, at LAR, GST activity was also significantly lower than BAR.

GR activity in *L. aurata* caught at RIO was significantly lower than in fish caught at TOR and LAR (Figure 3D). Though only significant when compared to RIO, GR at BAR, GAF and VAG, was also lower than at TOR, 45.9, 58.0 and 47.0% respectively.

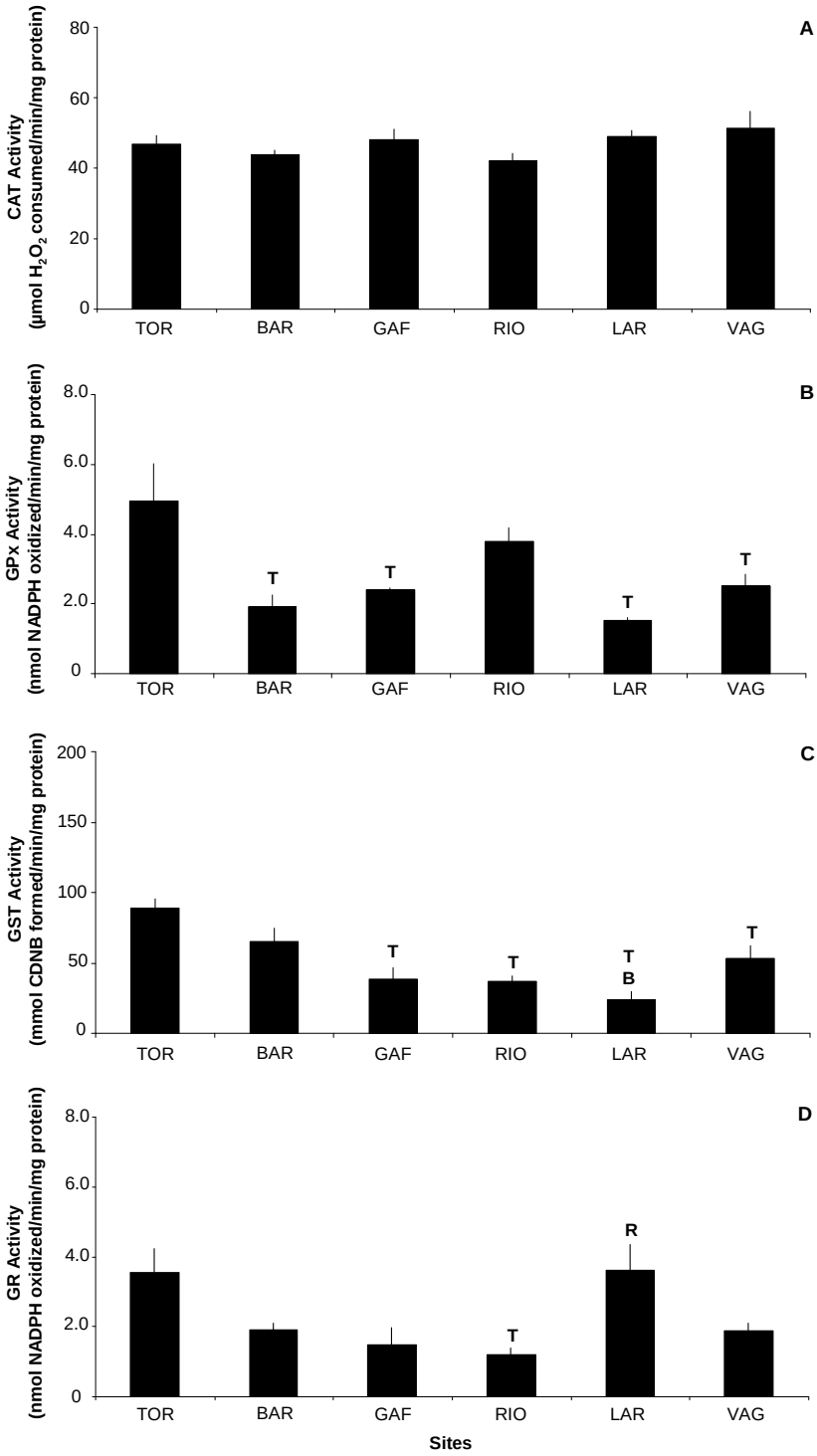

Figure 3 - Enzymatic defences in the liver of *L. aurata* collected at different sites in the Aveiro lagoon. A – Catalase (CAT); B – Glutathione Peroxidase (GPx); C – Glutathione S – transferase (GST); D – Glutathione Reductase (GR). Values represent mean ± standard error. Significant differences (P<0.05) are: T *versus* TOR; B *versus* BAR; R *versus* RIO.

3.4 Damage

TBARS levels in *L. aurata* caught at the five critical sites were higher than in fish caught at TOR though only significantly at GAF and VAG (Figure 4A). BAR, GAF, RIO, LAR and VAG displayed 129, 271, 36, 158 and 367% higher TBARS levels compared to TOR. TBARS levels at VAG were also significantly higher than BAR and RIO.

No significant DNA integrity loss was found at the critical studied sites when compared to TOR (Figure 4B). GAF displayed higher DNA integrity than RIO.

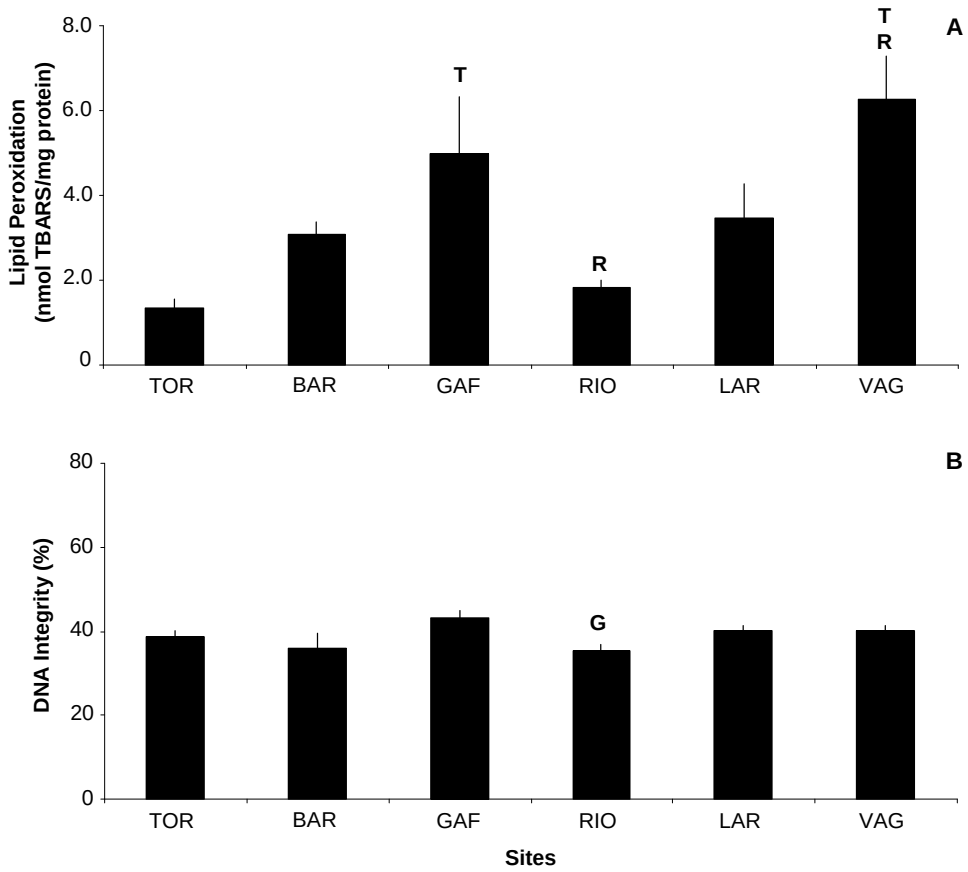

Figure 4 - Peroxidative and genetic damage in the liver of *L. aurata* collected at different sites in the Aveiro lagoon. A – Lipid peroxidation (LPO); B – DNA integrity. Values represent mean ± standard error. Significant differences ($P<0.05$) are: T *versus* TOR; G *versus* GAF; R *versus* RIO.

4. Discussion

Abiotic factors may influence biomarker responses to contaminants. Temperature may affect the catalytic efficiency and binding capacity of enzymes (Hochachka and Somero, 1984) as well as disturb the balance oxygen supply/demand and influencing oxygen levels in tissues which may interfere with antioxidant defences (Lushchak and Bagnyukova, 2006; Martínez-Álvarez et al., 2005). Salinity may influence the bioavailability of contaminants. A decrease in salinity is usually associated with increased bioavailability and uptake rates of contaminants (Monserrat et al., 2007; Ramachandran et al., 2006; Tsuda et al., 1990). However, no correlation between assessed abiotic parameters and the biological responses were observed in the current study. Thus, this discussion will be made on the basis of the predictable contamination scenario at each particular site.

Antioxidant systems are efficient protective mechanisms against ROS. An induction suggests an adaptation to stress resulting from exposure to an environment with pro-oxidants. In contrast, an antioxidant system deficiency will induce a precarious state, making biological species more susceptible to toxic agents (Cossu et al., 1997). In the current study, the non enzymatic defences did not show differences to TOR. Reduced glutathione, which represents the bulk of non-protein thiols, is synthesized in the liver and exported to other tissues (Deneke and Fanburg, 1989) functioning in the detoxification of H_2O_2, other peroxides and free radicals being considered the predominant defence against ROS (DeLeve and Kaplowitz, 1991). It is also involved in the detoxification of a variety of xenobiotics, which are ultimately excreted in the form of mercapturic acids (Pastore et al., 2003). Considering that cells tend to maintain a constant level of reduced glutathione, some authors consider that this biomarker may not be sufficiently sensitive in biomonitoring programs (Viarengo et al., 2007). However, previous biomonitoring studies using *L. aurata* gill and kidney responses found significantly higher GSHt levels at GAF (PAHs and organometallic contaminated), LAR (metal contaminated) and VAG (PAHs contaminated) in gill (Oliveira et al., 2009a) as well as RIO (pulp mill effluent contamination area), LAR and VAG, in

kidney (Oliveira et al. 2009b) when compared to the reference site, which suggests that this biomarker in *L. aurata* can be sensitive to contamination.

CAT and GPx are known to protect cells by reducing H_2O_2 to H_2O. Furthermore, GSH conjugation with lipid hydroperoxides, catalyzed by GPx and GST, decreases their reactivity. In the current study, *L. aurata* liver antioxidant enzymes demonstrated a high susceptibility to contamination since differences to TOR were always reflected in terms of lower enzyme activity. CAT was the only enzyme that did not demonstrate sensitivity to contamination neither in terms of higher activity nor in terms of inhibition. This can be considered unexpected since *L. aurata* gill and kidney demonstrated responsiveness (higher activity) to environmental contamination at GAF, LAR and VAG (gill) (Oliveira et al., 2009a) and RIO and LAR (kidney) (Oliveira et al. 2009b), emphasizing the importance of the tissue selection in biomonitoring, due to tissue specific responses, related with metabolic functions and defences. Moreover, CAT is often one of the earliest antioxidant enzymes to be induced (Cossu et al., 1997), being considered as one of the most conspicuous and responsive enzymes to ROS in vertebrate and invertebrate species (Halliwell and Gutteridge, 1999). CAT activity, observed in the current study, suggests that liver H_2O_2 levels are still within the CAT elimination capacity. Elevated CAT enzyme activities have been reported in several fish species in various laboratory and field studies (Amado et al., 2006; Ferreira et al., 2005; Otto and Moon, 1995; Wilhelm et al., 2001). However decreased CAT activity was also found in field studies (Damasio et al., 2007).

GPx displayed lower activity in the fish caught at all critical sites except RIO. This lower activity may be interpreted as an indication that antioxidant capacity was surpassed by the amount of hydroperoxide products of lipid peroxidation (Remacle et al., 1992). Enzyme activity can be decreased by negative feedback from excess of substrate or damage by oxidative modification (Tabatabaie and Floyd, 1994). Decreased hepatic GPx was also found in seabream (*Diplodus annularis*) caught from a polluted harbour area (Bagnasco et al., 1991). Liver GPx activity also displayed tissue specific responses when compared to results obtained by Oliveira et al. (2009a,b) in the same sites. Those

studies found higher GPx activities at GAF, LAR and VAG in gill and no differences to the reference site in kidney.

GST is a biotransformation enzyme that conjugates GSH with electrophilic organic compounds usually metabolized by cytocrome P-450, like epoxides derived from PAHs. It also reduces of organic hydroperoxides (Cnubben et al., 2001). Fish GST induction has been reported in field studies (Martinez-Gomez et al., 2006; Pandey et al., 2003). However, in the current study, this enzyme was vulnerable to contamination displaying low levels at GAF, RIO, LAR and VAG, sites with different contamination profiles. The lower activity of GPx and GST activities observed in the current study were not associated with lower GSHt levels, which confirms the idea of an impaired activity due to high ROS levels. Low hepatic GST activity has also been found in other studies at contaminated sites (Bagnasco et al., 1991; Wilhelm et al., 2001). *L. aurata* gill and kidney GST activities found by Oliveira et al. (2009a) displayed different response profiles, with no inhibition at any site/tissue and higher activity at LAR and VAG in gill and RIO and LAR in kidney.

GR plays a major role in GPx and GST reactions as an adjunct in the control of peroxides and free radicals (Bompart et al., 1990), maintaining the proper GSH redox status. Elevated GR activity was observed in organisms exposed to pro-oxidant stressors (Regoli, 2000; Regoli et al., 2002). However, in the current study, GR activity was lower in the critical sites (though only different from TOR at RIO), which is contrast with the findings of Oliveira et al. (2009b) for kidney where GR activity was higher at BAR, GAF, RIO and LAR. Oliveira et al. (2009a) also found higher gill GR activity at GAF. The lower GR activities found in the current study suggests lower glutathione recycling (replenishing GSH in order to avoid its depletion). Decreased GR activity might be attributed to the oxidation of cellular NADPH, the major source of reducing equivalent for GR during environmental pollutant-induced formation of toxic ROS (Padmini and Rani, 2009) and is a sign of lower response capacity of liver to pro-oxidants. In this perspective, the ratio GSH/GSSG may be decreased and if new GSH is not synthesized, cells are more vulnerable.

Environmental contaminants may inflict damage on membrane lipids, DNA, proteins and carbohydrates, directly by the action of parental compounds or their metabolites or indirectly by the generated ROS. Despite the low antioxidant enzymes activity displayed by *L. aurata* in the present study, peroxidative damage was only found at GAF and VAG. However, this peroxidative damage was not associated with DNA damage, assessed as DNA integrity loss, suggesting efficient DNA repair mechanism in liver. Unlike proteins and lipids that may be removed via normal turnover of molecules, damage to DNA is required to be repaired once modified since it cannot be replaced. Thus, the absence of DNA integrity loss is an indication of effective repair mechanisms not overwhelmed by contamination exposure. The fact that LAR and RIO did not display peroxidative damage in lipids, despite the observed low enzymatic defence activities, may be linked the protective role of other molecules such as metallothionein. Oliveira et al. (2009c) study found high liver metallothionein levels at RIO and LAR which have been proposed as an oxyradical scavenger (Atif et al., 2006). Moreover, at VAG metallothionein levels were lower than TOR, suggesting a higher susceptibility to oxidative stress.

According the present liver responses, BAR area, though subjected to naval traffic associated contamination, is one of the least polluted sites of Ria de Aveiro, only displaying GPx activity depletion.

GAF, a site with high levels of PAHs (Pacheco et al., 2005) and organometallic compounds (Barroso et al., 2000), seems seriously contaminated as demonstrated by GPx and GST low activities, probably due to inhibition by highly reactive lipid hydroperoxides, induced by xenobiotics exposure. The oxidative pressure was demonstrated by the high levels of TBARS. Nevertheless, the deficient phase II conjugation was not reflected in terms of DNA damage, suggesting efficient DNA repair mechanisms.

RIO, subjected during decades to continuous hazardous discharges of pulp mill industry, displayed low GST and GR activities suggesting a high oxidative pressure to *L. aurata* liver, though insufficient to induce damage (peroxidative and genetic).

LAR, a mercury contaminated site (Oliveira et al. 2009c; Pereira et al., 1998), also demonstrated the capacity to decrease antioxidant enzymes, namely GPx and GST. Despite the high sulfhydryl reactivity of mercury which allows it to bind with GSH (Oliveira et al., 2004; Viarengo et al., 1997), no differences were observed in terms of non enzymatic defences. However, high liver metallothionein levels were detected in *L. aurata* captured at this site (Oliveira et al. 2009c), suggesting activated defence systems. Nevertheless, GPx and GST activities were decreased. Elia et al. (2003) found that moderate doses of mercury were able to increase GST and GPx activities in catfish (*Ictalurus melas*), whereas high concentrations inhibited those enzymes.

L. aurata liver responses signalled VAG as one of the most polluted sites, since GPx and GST inhibitions were associated with peroxidative damage. These responses are attributable to high PAHs levels as demonstrated by Pacheco et al. (2005). The potentially oxidative nature of VAG conditions was emphasized by the observed *L. aurata* liver metallothionein decreased levels (Oliveira et al. 2009c), which may be associated with increased demand of cysteine residues for GSH synthesis during detoxification of organic contaminants (Roméo et al., 1997).

The current data confirms that the antioxidant enzymes, usually responsive to pro-oxidant environments may also display suppressed activities due to oxidative damage and a loss in compensatory mechanisms (Sun et al., 2007; Zhang et al., 2004). *L. aurata* chronically exposed to contaminated sites was unable to set up adequate antioxidant defences, probably due to severe injury to their hepatocytes (Wilhelm et al., 2001), especially at GAF and VAG.

5. Conclusions

L. aurata liver biochemical responses signalled critical areas in Ria de Aveiro. Two sites can be labelled as having pro-oxidant contamination (GAF and VAG) on the basis of peroxidative damage. However, all other sites demonstrated the capacity to impair *L. aurata* enzymatic antioxidants. BAR seemed the less pernicious site since only GPx activity was decreased.

Though, antioxidant enzyme inhibition was not always associated with damage, as observed in LAR, lipid peroxidation, found at GAF and VAG, was associated with decreased activity of GPx and GST. In terms of susceptibility to damage, hepatic lipids were more sensitive than DNA integrity.

Overall, this study confirms that the conjugation antioxidant defences/damage responses should be assessed in coastal lagoons monitoring.

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CAPÍTULO IX

Dano oxidativo no ADN e respostas clastogénicas/aneugénicas na tainha-garrento e robalo de uma laguna costeira contaminada

Golden grey mullet and sea bass oxidative DNA damage and clastogenic/aneugenic responses in a contaminated coastal lagoon

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Submitted

Abstract

Several xenobiotics or their metabolites have redox-cycling properties and potential to induce oxidative stress and DNA damage. The current work aimed to study, under environmental conditions, oxidative DNA damage (8-hydroxy-2'-deoxyguanosine (8-OHdG)) and its association with chromosomal damage measured as erythrocytic nuclear abnormalities (ENAs), in *Liza aurata* and *Dicentrarchus labrax*, caught at a costal lagoon (Ria de Aveiro, Portugal) having sites with different contamination profiles. The quantified parameters were also used to assess the lagoon's environmental status. Five critical sites were assessed comparing to a reference site. *L. aurata* displayed higher 8-OHdG levels and ENAs frequency respectively at Laranjo and Vagos. *D. labrax* 8-OHdG levels were higher at the sites where quantified whereas no differences were found in terms of ENAs. No correlation was found in both species between 8-OHdG and ENAs. Despite no direct linkage between the two biomarkers was found, this study demonstrates species and site dependent genotoxic responses.

Keywords: *Liza aurata*; *Dicentrarchus labrax*; Genotoxicity; 8-hydroxy-2'-deoxyguanosine; Erythrocytic nuclear abnormalities

1. Introduction

Normal aerobic metabolism, generates reactive oxygen species (ROS) that, though crucial for many physiological processes (Droge, 2002), may pose serious threat to aerobic organisms at high concentrations. There are many sources of endogenous ROS which include the mitochondrial electron transport chain, plasma membrane-associated oxidases such as NADPH oxidases, peroxisomes, as well as liver microsomes. Some soluble oxidases, dehydrogenases, and dioxygenases in the cytoplasm can also generate ROS during catalytic cycling (Aniagu et al., 2006). In order to prevent the pernicious effects of ROS and other

free radicals, aerobic organisms developed antioxidant defences for their protection (Winston and Di Giulio, 1991). However, exposure to xenobiotics may increase ROS production and overcome antioxidant defences resulting in oxidative stress and among other effects, DNA damage. Most environmental pollutants exert their effects via both genotoxic and metabolically toxic mechanisms, operating simultaneously (Depledge, 1998; Rodríguez-Ariza et al., 1999). Nucleotides exposure to ROS can originate a variety of altered bases (Cadet et al., 2003; Neeley and Essigmann, 2006) of which guanine is the most susceptible to oxidation (Steenken and Jovanovic, 1997). The oxidation of guanine in the C8 position results in the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), one of the predominant and most studied oxidative DNA lesions. Although the consequences of 8-OHdG base modification for cell integrity are not completely known, it was demonstrated that the absence of base repair, may lead to strand misreading, mutations, altered gene expression, strand breaks, microsatellite instability and loss of heterozygoty, chromosomal aberrations, cytostasis, cytotoxicity or neoplastic growth (Croteau and Bohr, 1997; Evans et al., 2004).

Detection of micronucleus together with other nuclear abnormalities in fish erythrocytes (ENA assay) is an established cytogenetic assay providing an index of accumulated genetic damage during cells life span, being successfully applied in different species exposed to various classes of environmental genotoxins (Pacheco and Santos, 1996, 2001; Ayllón and Garcia-Vazquez, 2001; Pacheco et al., 2005; Gravato and Santos, 2002a; Bolognesi et al., 2006). Micronuclei are small cellular chromatin bodies that appear when a whole chromosome or a fragment fails to migrate with one of the two daughter nuclei formed during mitosis. Micronuclei may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell-cycle checkpoint and/or DNA repair genes (Bonassi et al., 2007). The sensitivity of the micronucleus assay in fish erythrocytes has been always debatable due to its low prevalence in several species (Cavalcante et al., 2008) and lack of significant induction found after fish exposure to known genotoxicants (Carrasco et al., 1990; Ayllón and Garcia-Vazquez, 2000). The different types of nuclear anomalies are related to specific genotoxic events associated to the different mechanisms of action of the

mutagenic agents (Bolognesi et al., 2006). Despite the mechanisms responsible for nuclear abnormalities are not completely understood some nuclear abnormalities are interpreted as nuclear lesions analogous to micronuclei that can be induced by genotoxic compounds even if micronuclei are not induced (Ayllón and Garcia-Vazquez, 2000). It was observed that in European icthyofauna species such as *Liza aurata* micronucleus are not easily found despite a significant ENAs expression (Guilherme et al., 2008). Several studies have demonstrated a direct relationship between genomic instability and some of these abnormalities, such as nuclear buds and micronuclei (Serrano-Garcia and Montero-Montoya, 2001; Fenech and Crott, 2002). Thus, the formation of nuclear abnormalities may represent a way to eliminate any amplified genetic material from the cell nucleus (Shimizu et al., 1998, 2000). It has been suggested that problems in segregating tangled and attached chromosomes or gene amplification via the breakage–fusion–bridge cycle could cause lobed nuclei attachments during the elimination of amplified DNA from the nucleus (Çavas and Ergene-Gözükara, 2005; Çavas, 2008).

Ria de Aveiro is a coastal lagoon located in the northern region of Portugal that has a significant role in the life cycle of several species. However, over decades this aquatic system received considerable influx of anthropogenic pollutants from unidentified and well-known sources, including industrial discharges from chlor-alkali and paper mill plants, harbour activities and domestic effluents. The studies performed in Ria de Aveiro using fish pointed out the existence of critical areas based on fish physiological and genetic responses, detecting significant inter-site differences (Pacheco et al., 2004, 2005; Ahmad et al., 2004, 2008; Maria et al., 2004a, b; Santos et al., 2004, 2006; Teles et al., 2004; Monteiro et al., 2007; Guilherme et al., 2008; Oliveira et al. 2009).

Though several studies have shown that unrepaired 8-OHdG may have pernicious consequences, to the author's knowledge no studies have focused on the relation between oxidative DNA damage (an easily repairable lesion) and nuclear abnormalities (an irreparable DNA lesion) in fish. The DNA damage capacity of oxidative stress associated with poor understanding of the mechanisms responsible for nuclear abnormalities formation ranks as highly

relevant the study of possible linkage between oxidative DNA damage and nuclear abnormalities in environmental conditions. Moreover, the utility of oxidative DNA damage in the monitoring of environmental pollution using fish though potentially useful biomarker (Rodríguez-Ariza, et al., 1999) requires further validation in the monitoring of complex environments. Thus, the current research work aimed to study, under environmental conditions, the involvement of oxidative stress on DNA damage and its eventual association with damage at a higher level such as chromosomal damage measured as ENA formation in two species with different feeding and swimming behaviours (*Liza aurata* and *Dicentrarchus labrax*). In addition, it was intended to apply the quantification of these two levels of genetic damage on environmental biomonitoring.

2. Material and Methods

2.1 - Study Area: Ria de Aveiro (Figure 1) is a coastal lagoon 45 km long (NNE-SSW) and 8.5 km wide, covering a wetland area of approximately 66 (low tide) to 83 km2 (high tide) which is permanently connected to the ocean through a narrow channel (Dias et al., 2001). Sampling sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of a (theoretically) unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving

municipal and domestic effluents with high levels of polycyclic aromatic hydrocarbons (PAHs).

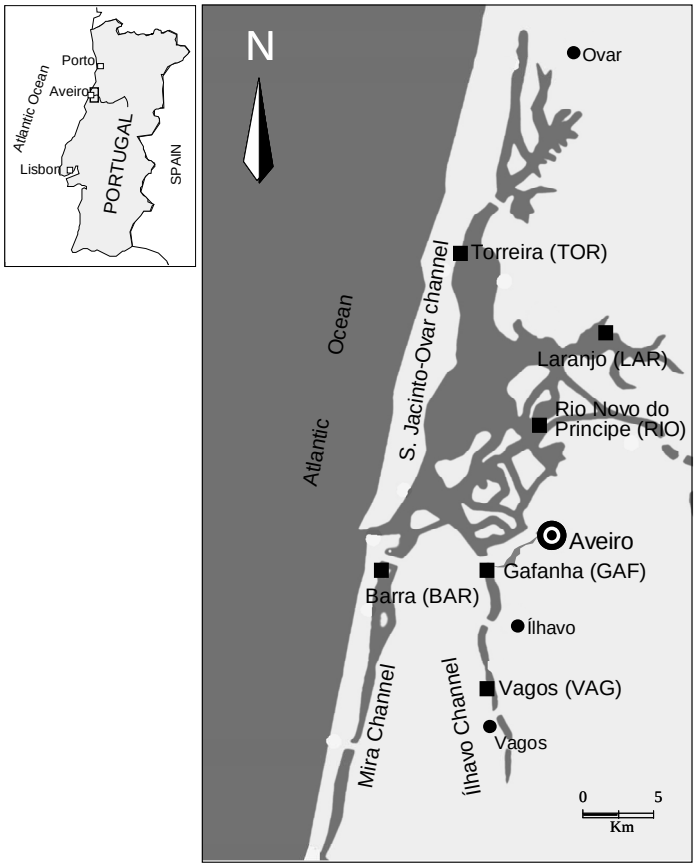

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture sites (■). The respective coordinates are: reference site (TOR)—40º44'02 N, 008º41'44 W; BAR—40º37'42.00"N, 8º44'35.00"W GAF—40º38'38 N, 008º41'42 W; RIO—40º41 '08 N, 008º39'41 W; LAR—40º43'30 N, 008º37'43 W; and VAG—40º33'59 N, 008º40'55 W.

2.2 - Sampling: In October 2005, six juvenile sea bass (*D. labrax*) were caught using a fishing rod and six juvenile golden grey mullet (*L. aurata*) captured using a traditional beach-seine net named “chincha”, at each selected sampling. *D. labrax* and *L. aurata* specimens, selected on the basis of their size, had respectively, an average length of 13.5±2.7 and 14.5±2.5 cm and weighed 24.6±4.9 and 21.6±3.7 g. At BAR, it was not possible to catch sufficient number of *D. labrax* to perform analyses.

Blood was collected from the posterior cardinal vein using a heparinised Pasteur pipette and used for smear preparation to perform cytogenetic assessment (ENA assay) and the remaining volume was kept cool for oxidative DNA damage determination (8-OHdG). The blood volume of *D. labrax* collected at LAR did not allow 8-OHdG quantification at this site.

At each sampling site, abiotic parameters were assessed (Table I) as per the guidelines of APHA (1998).

Table I - Physical-chemical parameters of the water from the Ria de Aveiro sampling sites.

	Turbidity (m)	*Dissolved Oxygen (mg/L)*	*Temperature (ºC)*	*pH*	*Salinity*
TOR	0.80	6.88	18.9	8.17	32.5
BAR	1.20	6.16	17.9	8.32	34.0
GAF	0.70	5.78	17.9	8.28	34.0
RIO	1.00	6.37	19.0	8.20	21.0
LAR	0.30	4.96	18.6	7.74	29.0
VAG	0.30	5.18	18.5	7.88	33.0

2.3 – Genotoxic Responses

2.3.1 - 8-hydroxy-2'-deoxyguanosine (8-OHdG) assessment: Upon arrival in laboratory, blood samples were centrifuged (13400 g) to isolate plasma. Interfering substances were excluded by plasma filtering (cut off molecular weight 10 000 dalton) and 7 times dilution (to reduce deviation). The 8-OHdG was assessed through a competitive *in vitro* enzyme linked immunosorbent assay (ELISA) using an IBL International GmbH (Germany) kit. Results were expressed as ng/ml.

2.3.2 - Erythrocytic nuclear abnormalities (ENA) frequency: Blood smears were fixed with methanol during 10 minutes and stained with Giemsa (5%) during 30 minutes. 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). Nuclear lesions were scored as: MN, lobed nuclei (L), binucleates or segmented nuclei (S) and kidney shaped nuclei (K). Blebbed and lobed nuclei were considered in a single category — lobed nuclei — and not differentially scored as suggested by other authors due to some ambiguity in their distinction. The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

2.4 - Statistical analysis: Results are expressed as means ± SE (standard error) corresponding to experimental groups of six fish (n=6). Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). The assumptions of normality and homogeneity of data were verified. Factorial ANOVA was performed in order to assess significant effects at each site and differences between species; this analysis was followed by post-hoc Fisher LSD test to signal significant differences between groups (Zar, 1999). Significance of results was ascertained at $\alpha = 0.05$. The relationship between 8-OHdG and ENAs was studied using linear regression analyses. The correlation coefficient (r) was calculated and its statistical significance (p) determined from the table of critical values for correlation coefficient, r.

3. Results

L. aurata 8-OHdG plasma levels, compared to the reference site (TOR), were 2.6, 2.9, 1.7, 5.9 and 3.6 times higher at respectively BAR, GAF, RIO, LAR and VAG though only significant at LAR (Figure 2A). Moreover, at LAR *L. aurata* displayed significantly higher 8-OHdG plasma levels than in RIO. The total ENAs score (Figure 2B) revealed that *L. aurata* caught at VAG had significantly higher ENAs frequencies compared with all the studied sites. Compared to TOR, ENAs frequency was 28.5 times higher at VAG. No micronuclei were detected in *L. aurata* at any of the studied sites.

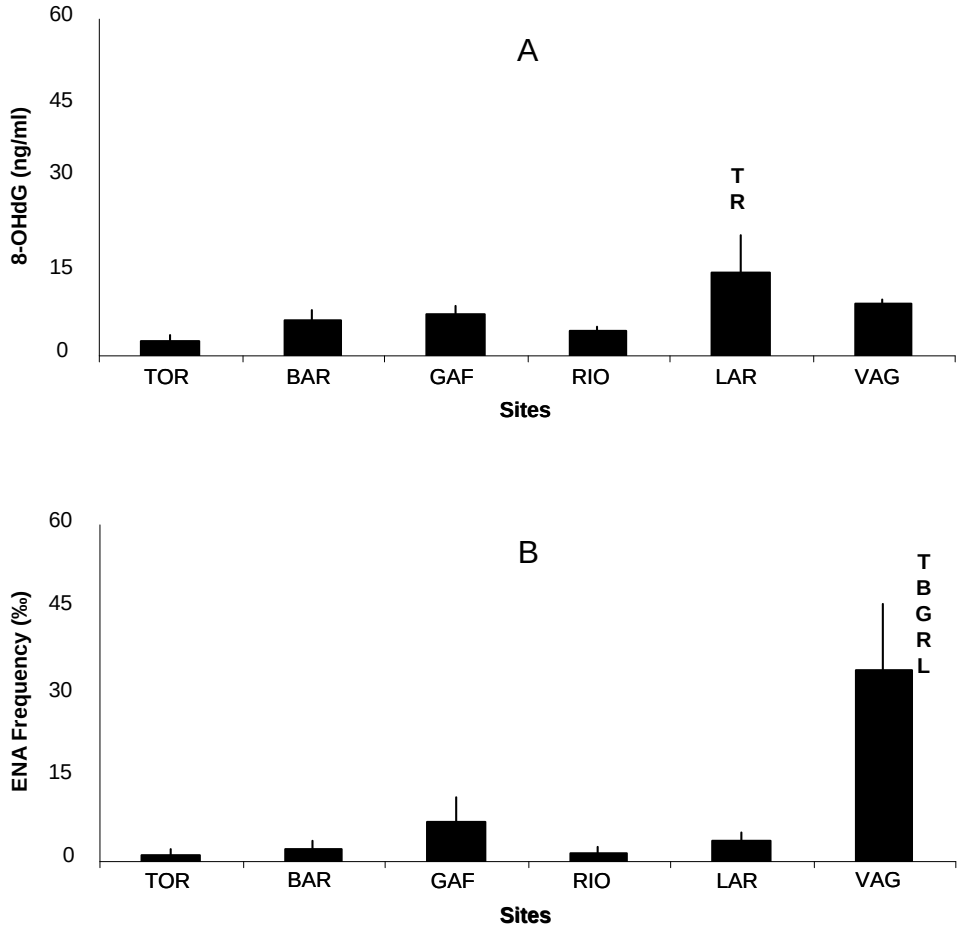

Figure 2 – Genotoxic responses in *L. aurata* caught at different sites in the Ria de Aveiro. A) Levels of 8-hydroxy-2'deoxyguanosine (8-OHdG); B) Erythrocytic nuclear abnormalities (ENAs) frequency. Values represent mean ± standard error. Significant differences ($P<0.05$) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR.

D. labrax 8-OHdG plasma levels were significantly higher at GAF, RIO and VAG when compared to TOR (Figure 3A). Thus, 8-OHdG levels at GAF, RIO and VAG were respectively 6.8, 6.1 and 4.5 times higher than at TOR. At LAR, due to the insufficient volume of blood collected, 8-OHdG could not be quantified in *D. labrax*. No significant differences to TOR were found in terms of total *D. labrax* ENAs frequency (Figure 3B). At RIO, *D. labrax* ENAs frequency was significantly lower than at GAF and VAG. As in *L. aurata*, *D. labrax* displayed a negligible frequency of micronuclei, being detected among all studied sites, 1‰ micronucleus at GAF.

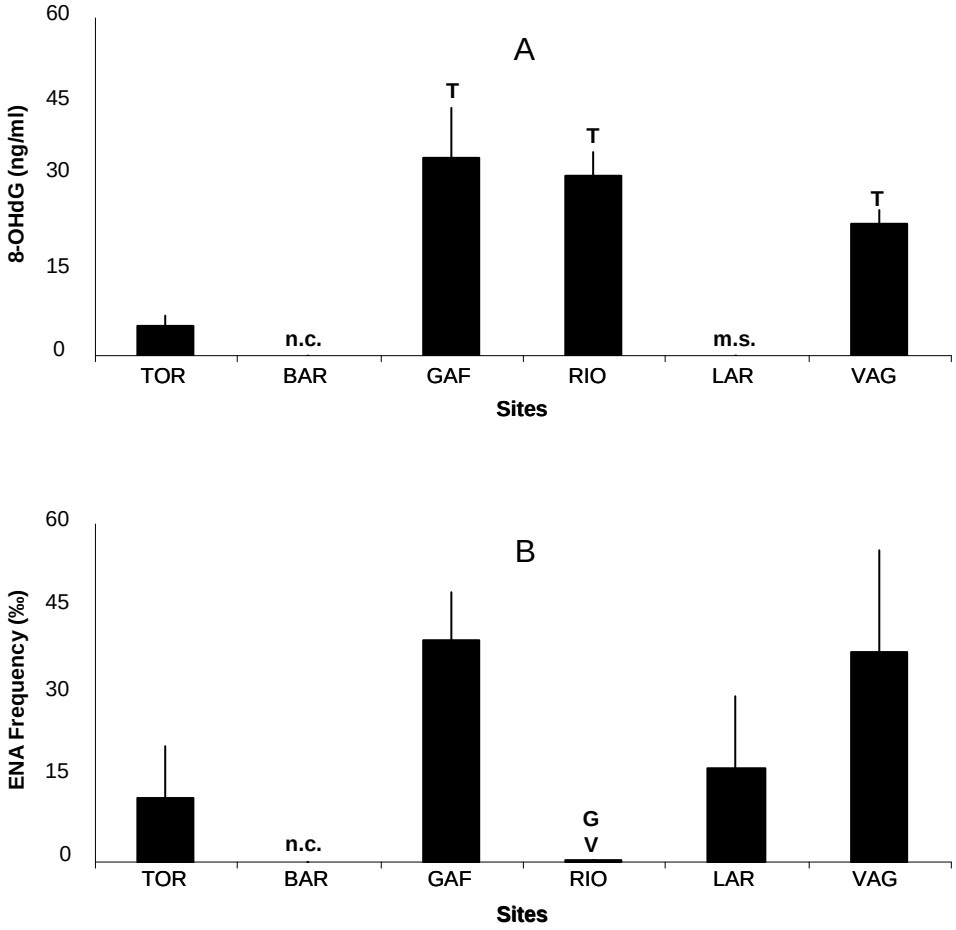

Figure 3. Genotoxic responses in *D. labrax* caught at different sites in the Ria de Aveiro. A) Levels of 8-hydroxy-2'deoxyguanosine (8-OHdG); B) Erythrocytic nuclear abnormalities (ENAs) frequency. Values represent mean ± standard error. Significant differences ($P<0.05$) are: T *versus* TOR; G *versus* GAF; V *versus* VAG; n.c. – not caught; m.s. – missing sample.

The analyses of the possible correlation between 8-OHdG and the total and individual nuclear abnormalities revealed no correlation for both species.

Comparing the two species, 8-OHdG levels in RIO and GAF were significantly higher in *D. labrax*. Moreover, at GAF, ENAs levels were also significantly higher in *D. labrax*.

4. Discussion

In recent years there has been increasing apprehension regarding genotoxic/carcinogenic hazards and numerous studies reported the occurrence of malignancies in aquatic organisms (Jha, 2004). The conservation of most mechanisms of intracellular xenobiotics concentration, regulation, metabolic transformation and detoxification found in mammals, as well as generation of DNA lesions (Wirgin and Waldman, 1998) make fish attractive study models. Moreover, they are widely spread in the aquatic environment, and top consumers. Thus, in order to evaluate the presence and impact of genotoxicants in a multi-polluted aquatic environment, fish have been widely used.

L. aurata is a common European mugilide that feeds mainly on small benthic organisms and detritus/mud, being particularly adapted to accumulate sediment-associated contaminants whereas *D. labrax* is a highly adaptable and opportunistic feeder with less frequent contact with sediments than *L. aurata*. The widespread distribution and resistance to different environmental conditions make these species good candidates for the role of bioindicators. Previous studies have demonstrated the usefulness of ENAs assay to detect genotoxic xenobiotics using *L. aurata* (Oliveira et al., 2007, 2008; Guilherme, et al., 2008) and *D. labrax* (Gravato and Santos, 2003a, b). Despite damage to DNA by compounds that generate oxidative stress is well-established in higher vertebrates, fewer studies addressed this topic in fish (Rodríguez-Ariza, et al., 1999) and, to our knowledge, no studies have focused on oxidative DNA damage in *D. labrax* and *L. aurata*.

In the current study, the hydrological abiotic parameters showed, in general, no important differences between sites with the exception of salinity that in RIO was substantially lower than in BAR and GAF (21 *versus* 34). Decreases in salinity are usually associated with increased bioavailability and uptake rates of contaminants (Monserrat et al., 2007). Thus, the possibility that the salinity differences may have affected responses can not be excluded, though salinity was within these species tolerable range as both species are euryhaline (Nebel et al., 2005; Khodabandeh et al., 2008). Hence, the current discussion will be made on

the basis of the predictable contamination scenario at each particular site, analyzing both species.

Oxidative DNA Response

It is important, for genotoxicity environmental assessment, to understand the influence of several xenobiotics interactions on oxidative DNA stress. Moriwaki et al. (2008) found that interactions between metal ions had various influences (suppressive, additive and synergistic effects) on formation of 8-OHdG and single and double-strand breaks. The available literature, suggests that the occurrence of DNA oxidative lesions can be species-dependent (Charissou et al., 2004) and compared to laboratory studies, the situation in the field becomes more complex due to several confounding factors (Jha, 2004). *L. aurata*, 8-OHdG levels were only significantly higher at LAR (a mercury contaminate site) supporting the idea that mercury is able to cause oxidative DNA damage. Chen et al. (2005) observed significant increase in urinary 8-OHdG concentrations of mercury-exposed humans. The mechanism of Hg-induced DNA damage and cytotoxicity may involve an Hg-induced elevation of ROS, although other mechanisms such as enzymes inhibition (DNA repair, glutathione metabolism and antioxidant enzymes) may also contribute (Schurz et al., 2000; Tran et al., 2007). Oliveira et al. (2009) study found that *L. aurata* at LAR was under oxidative pressure demonstrated by higher *L. aurata* gill antioxidant defenses (glutathione peroxidase (GPx), glutathione reductase (GR) and catalase as well as non protein thiols and metallothioneins).

D. labrax 8-OHdG at LAR, were not determined in this study due to insufficient sample to perform analysis not allowing comparison with *L. aurata*. However, at GAF, RIO and VAG, *D. labrax* 8-OHdG levels were significantly higher than reference site. The higher *D. labrax* 8-OHdG levels found in GAF can be considered as expectable since previous studies have demonstrated the presence of high levels of PAHs and organometallic compounds (Barroso et al., 2000; Pacheco, et al., 2005). Exposure to PAHs *in vitro* and *in vivo* has been found to be accompanied by an increase in DNA damage such as 8-OHdG or thymine glycol (Luch, 2005). The pro-oxidant nature of GAF contaminants was

recently demonstrated in *D. labrax* captured at GAF showing lower gill DNA integrity and higher LPO (lipid peroxidation) than reference site (Ahmad et al., 2008). Moreover, GPx, GR and glutathione S-transferase activity, as well as non-protein thiols levels were increased. The absence of significantly higher 8-OHdG levels in *L. aurata*, can be considered unexpected considering that in Oliveira et al. (2009) study, *L. aurata* gill responses signalled this site as one of the most polluted. The 8-OHdG levels of *D. labrax* caught at RIO, a site near a pulp/paper mill effluent outlet that discharged to this water course during nearly five decades, suggest the continuation of pro-oxidant substances though Ahmad et al. (2008) found no evidence of pro-oxidants in *D. labrax* gill. Thus, the oxidative DNA damage observed in the current study suggests a high sensitivity of 8-OHdG in *D. labrax*. However, *L. aurata* from RIO did not show any oxidative DNA damage in agreement with Oliveira et al. (2009) study where no differences to TOR were found in terms of *L. aurata* gill DNA damage, LPO and antioxidant enzymes. VAG is a site with known high levels of organic xenobiotics, namely BaP (Pacheco et al., 2005). In agreement with this, 8-OHdG levels found in *D. labrax* caught at VAG were higher than at TOR, signalling oxidative pressure probably associated with the PAHs levels. *D. labrax* gill responses in Ahmad et al. (2008) study, signalled VAG as the second most polluted site in the Ria de Aveiro with a slightly (non significant) decreased DNA integrity, increased LPO and antioxidant defences. Considering *L. aurata* gill antioxidant responses, Oliveira et al. (2009) also ranked VAG as a polluted site. However, this was not confirmed in the present study by the oxidative DNA damage levels.

The non-consensual results obtained for these two species was also found among other species, in laboratory or field studies, suggesting species dependent responses and the complexity in the mechanisms that cause 8-OHdG as well as the difficulty in establishing association with other biomarkers of oxidative stress. Thus, Nishimoto et al. (1991) found increased 8-OHdG hepatic levels in English sole (*Parophrys vetulus*) after nitrofurantoin treatment. Despite its known potential to promote ROS generation in flounder (*Platichthys flesus*) (Lemaire et al., 1994), no increase in 8-OHdG was found in turbot (*Scophthalmus maximus*), dab (*Limanda limanda*) and sole (*Solea solea*) exposed to the same agent

(Mitchelmore et al., 1996). No increase in 8-OHdG levels was found in the liver of channel catfish (*Ictalurus punctatus*) and brown bullhead (*Ameriurus nebulosus*) after 72 hours exposure to the pro-oxidant menadione (Di Giulio et al., 1995) and 48 hours to tert-butyl hydroperoxide (Ploch, et al., 1999). Rodríguez-Ariza (1999) found that 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) content did not always respond in gilthead seabream (*Sparus aurata*) tissues after fish exposure to model pollutants (paraquat, dieldrin and copper chloride) known to induce oxidative damages to other fish biomolecules, though other biomarkers signalled oxidative stress. However, in the same study, moderate levels of urban and industrial pollution caused significantly higher 8-oxodG content in hepatic DNA than those from a close pristine area. Higher contents of oxidized purine bases and ring-opened by-products were found in English sole (*Parophrys vetulus*) from areas containing PAHs and PCBs (Malins and Haimanot, 1990; 1991; Malins et al., 1996). Machella et al. (2004) observed that BaP induced liver DNA damage in *A. anguilla*, generating a significant increase of 8-oxo-dG despite the absence of a clear dose-dependent relationship. Even though many previous laboratory studies have shown significant dose–response relationship and some authors defend that 8-OHdG is a reliable marker for oxidative DNA damage (Kasai, 1997), others still defend that 8-OHdG is unlikely to be a good genotoxic biomarker in field studies (Siu et al., 2008). According to the current study data, 8-OHdG levels may be a valuable biomarker, though species specific responses should be carefully considered. In this perspective, *D. labrax* seem more responsive than *L. aurata*.

Clastogenic/Aneugenic Response

L. aurata ENAs frequency was higher than the reference site in VAG in agreement with Pacheco et al. (2005) study, where high ENAs were observed in *L. aurata* caught at the same season. However, in *D. labrax*, no differences to TOR were found in terms of ENAs suggesting that the absence of abnormalities, as observed in the current study at all sites for *D. labrax*, does not necessarily indicate the absence of genotoxicants. Despite previous studies have demonstrated in the laboratory the potential of PAHs (Pacheco et al., 1997; Gravato and Santos 2002b; Maria et al., 2002a,b), resin acids (Pacheco et al.,

1997; Gravato and Santos 2002a; Maria et al., 2004c,d) and metals (Oliveira et al., 2008) to induce ENAs, the complex mixture of xenobiotics in environmental conditions may result in the absence of ENAs since induction of nuclear lesions by genotoxic agents may be masked through a cytotoxic action that kills erythrocytes instead of forming non-lethal lesions (Das and Nanda, 1986; Brunetti et al., 1988), inhibits DNA synthesis (Williams and Metcalfe, 1992) or from direct inhibition of erythropoiesis (Dinnen et al., 1988). In Guilherme et al. (2008) study, *L. aurata* caught in September 2005 at LAR, revealed significantly higher ENA frequency than the reference site which was not observed in the current study. Despite the clear differences between studies (the current study was 1.3 km farthest to the mercury source and the site considered as references site was not the same), the different ENA response profile may also be explained by changes in abiotic conditions namely total precipitation (20.1-30.0 vs 140.1 – 180.0 mm in September and October respectively) which may have changed drastically the mercury bioavailability.

Several studies suggest the use of micronuclei as a biomarker in polluted sites (Bolognesi et al., 2004, 2006; Barsiene et al., 2006; Matsumoto et al., 2006; Jiraungkoorskul et al., 2008). However, in the current study, wild *D. labrax* and *L. aurata* micronuclei did not respond to pollution unlike other nuclear abnormalities in agreement with previous studies where nuclear abnormalities were increased after xenobiotics exposure, though micronuclei were not (Ayllón and Garcia-Vazquez, 2000, Guilherme et al., 2008). Carrasco et al. (1990) also did not find a significant association between micronuclei in wild white croaker (Genyonemus lineatus) and chemical sediments pollution.

General Discussion

The different responses of these two biomarkers may be related with the nature of these two assays. Whereas 8-OHdG detects primary repairable and thus reversible DNA lesions, ENAs frequency detects more persistent lesions that cannot be repaired (Hartmann et al., 2001). The two studied parameters revealed no significant correlation between any of the nuclear abnormalities studied and the 8-OHdG levels in plasma. To our knowledge, no studies have demonstrated in fish

a correlation between 8-OHdG and ENAs. Fenech et al. (1999) observed that 8-OHdG was not induced in human lymphocytes at cytotoxic doses of H_2O_2 in culture medium despite the significant correlations between H_2O_2 concentration and micronucleated cells frequency. Bolognesi et al. (1997) found significantly higher 8-OHdG levels in Swiss CD1 mice kidney simultaneous with a significant increase in micronucleus frequency in bone marrow after exposure to the herbicide Roundup.

This study reveals clear differences between the selected species which may be explained by species-specific biochemical and physiological profiles. The differences found in species responses may also be explained by different feeding behaviour. Though both species can feed all over the water column, *L. aurata* also ingests mud, being more exposed to the contaminants present in the sediments. However, considering the observed lower susceptibility to oxidative DNA damage, this closer contact to the sediments may have lead to adaptation mechanisms which provide a higher protection against oxidative DNA damage. In this perspective, basal antioxidant defences in association with the responsiveness towards oxidative challenge and efficient DNA repair enzymes are crucial for DNA protection. Given that oxidative DNA damage may be easily repairable, other types of DNA damage should also be assessed. In this perspective, ENAs is a good choice as a biomarker in fish providing a fast response at low costs. The absence of correlation observed in the present study does not necessarily prove that these genotoxicity expressions are not linked as they are separated by a sequence of events (e.g. repair mechanisms) that may mask the evidences of the association. In future studies, the assessment of DNA repair enzymes activity upon exposure to xenobiotics, may provide valuable information concerning the degree of contamination since the detected DNA damage results from a balance between its damage and repair.

5. Conclusions

D. labrax and *L. aurata* demonstrated different susceptibility to genetic damage. In terms of 8-OHdG, *D. labrax* was more sensitive signalling the sites where it was collected - GAF, RIO and VAG, as having oxidative DNA damaging chemicals. On the other hand, *L. aurata* only signalled LAR.

On a cytogenetic level, assessed through ENA frequencies, *L. aurata* was more sensitive, showing cytogenetic damage at VAG whereas *D. labrax* showed no differences with the fish caught at the reference site.

Thus, 8-OHdG levels and ENAs frequency were species dependent and the correlation between the two parameters needs further validation.

The employment of 8-OHdG and ENA assays for genotoxicity assessment may provide valuable complementary information, since they are related to genotoxic chemicals at different levels: the 8-OHdG reflecting DNA damage at the molecular level whereas ENAs reflect chromosome-level damage.

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CAPÍTULO X

Avaliação de lesões oxidativas no ADN e anomalias nucleares eritrocíticas em peixes selvagens (*Liza aurata*) como abordagem integrada para avaliação da genotoxicidade

Evaluation of oxidative DNA lesions and erythrocytic nuclear abnormalities in wild fish (*Liza aurata*) as an integrated approach for genotoxicity assessment

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Submitted

Abstract

Genetic lesions (8-hydroxy-2'-deoxyguanosine - 8-OHdG and erythrocytic nuclear abnormalities - ENAs) were seasonally quantified in the blood of *Liza aurata* caught at Ria de Aveiro (Portugal), a multi-contaminated aquatic system. Thus, five critical sites were assessed and compared to a reference site (Torreira). Oxidative DNA damage was found in Gafanha (harbour water area), Laranjo (metal contaminated) and Vagos (PAHs contaminated) in spring; Rio Novo do Principe (near former paper mill effluent) in autumn; Rio Novo do Principe and Vagos in winter. ENAs were higher than Torreira at VAG (spring and winter). Torreira did not display seasonal variation neither in terms of 8-OHdG nor total ENAs. A positive correlation between 8-OHdG and ENAs was found, suggesting oxidative stress as a possible mechanism involved in ENAs formation. This study clearly demonstrates the presence of DNA damaging substances in Ria de Aveiro and recommend the use of 8-OHdG and ENAs as biomarkers of environmental contamination.

Keywords: *Liza aurata*; Genotoxicity; 8-hydroxy-2'-deoxyguanosine; Erythrocytic nuclear abnormalities

1. Introduction

Genetic lesions in aquatic organisms are valuable tools to assess acute and chronic exposure to genotoxic substances, being able to detect exposure to low concentrations of contaminants in a wide range of species [1]. Moreover, as genotoxicants may induce changes in DNA that are passed on to future generations, this kind of biomarker may be used in a predictive way, avoiding irreversible ecological consequences [2].

DNA in aerobic organisms is constantly damaged, even in the absence of genotoxic carcinogens [3], due to its susceptibility to reactive oxygen species

(ROS) [4] that may be endogenously formed as part of physiological processes. However, xenobiotics exposure may increase ROS production leading to overwhelmed antioxidant defences and, among other pernicious effects, DNA damage. ROS are able to attack not only DNA bases but also deoxyribosyl backbone of DNA, reacting five times faster with nucleobases [5]. The oxidation of guanine in the C8 position results in the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a predominant and one of the most studied oxidative DNA lesions due to its easy formation and mutagenicity [6]. The absence of base repair may lead to strand misreading, mutations, altered gene expression, strand breaks, microsatellite instability and loss of heterozygoty, chromosomal aberrations, cytostasis, cytotoxicity or neoplastic growth [7,8]. Interestingly, the study of 8-OHdG levels in fish exposed to xenobiotics has not been extensively explored.

8-OHdG is an easily repairable genetic alteration unlike chromosomal damages which represent a more serious and irreversible lesion. The detection of micronucleus together with other nuclear abnormalities in fish erythrocytes (erythrocytic nuclear abnormalities - ENAs assay) provides an index of accumulated genetic damage during cells life span. Micronuclei, small cellular chromatin bodies that appear when a whole chromosome or a fragment fails to migrate with one of the two daughter nuclei formed, during mitosis, may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell-cycle checkpoint and/or DNA repair genes [9]. Despite the mechanisms responsible for other nuclear abnormalities are not completely understood, some nuclear abnormalities are interpreted as nuclear lesions analogous to micronuclei that can be induced by genotoxic compounds even if micronuclei are not induced [10]. ENAs assay has been successfully applied to detect exposure to genotoxic xenobiotics in a large number of species [11-14].

Ria de Aveiro, a coastal lagoon located in the northwest of Portugal, received during 5 decades discharges of complex effluents of industrial and harbour activities, as well as urban and agricultural wastes. In spite of the fact that after May 2000 most of the contamination has been diverted to 2.5 km far from the sea coast through a submarine outlet, the persistence of critical sites in this lagoon

and the presence of oxidative stress/genotoxicity inducers has been reported, affecting one of the most representative fish species - *Liza aurata* [14-17].

Thus, the objective of this study was the assessment of the genetic responses, 8-OHdG and ENAs, of a native species, *L. aurata*, seasonally caught at different sites of a multi-contaminated coastal lagoon (Ria de Aveiro, Portugal). The use of the selected genetic endpoints, assessed in blood, aimed to study the association between easily induced and repairable lesions such as 8-OHdG, observed at a molecular level, with an irreparable lesion at a sub-cellular level (ENAs). Ultimately, their usefulness in biomonitoring of a multi-contaminated environment and their relation with hydrological parameters were studied.

2. Material and Methods

2.1 - Study Area: Ria de Aveiro (Figure 1) is a coastal lagoon 45 km long (NNE-SSW) and 8.5 km wide, covering a wetland area of approximately 66 (low tide) to 83 km^2 (high tide) which is permanently connected to the ocean through a narrow channel [18]. Sampling sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of a (theoretically) unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of the Vouga River, 6.5 km distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of PAHs.

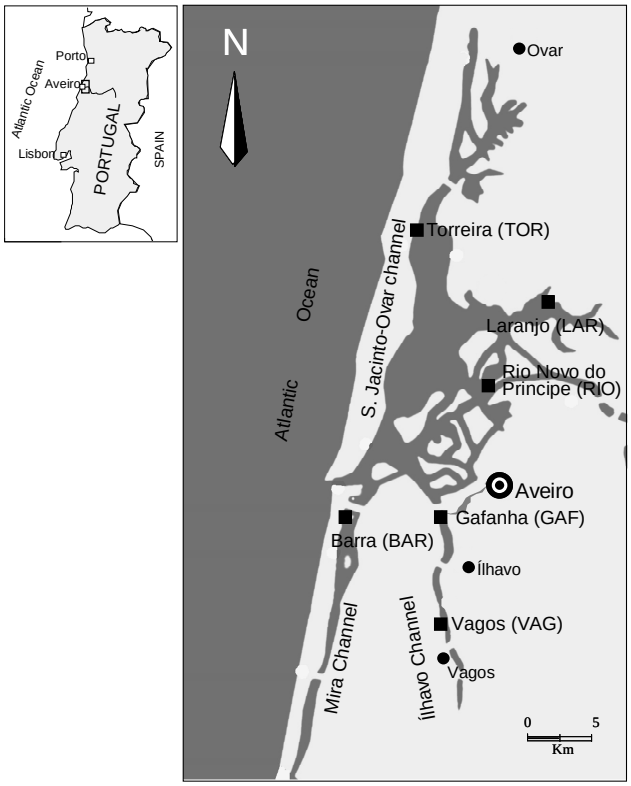

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture sites (■). The respective coordinates are: reference site (TOR)—40º44'02 N, 008º41'44 W; BAR—40º37'42.00"N,8º44'35.00"W GAF—40º38'38 N, 008º41'4 2 W; RIO—40º41'08 N, 008º39'41 W; LAR—40º43'30 N, 008º37'43 W; and VAG—40º33'59 N, 008º40'55 W.

2.2. - Sampling: Golden grey mullet (*L. aurata*) seasonal samplings were carried out between May 2006 and March 2007 in spring, summer, autumn and winter, using a traditional beach-seine net named “chincha”. *L. aurata* specimens, selected on the basis of their size, had an average length of 14.0±3.0 cm and weighed 21.4±3.6 g. Blood was collected from the posterior cardinal vein using a heparinised Pasteur pipette and used for smear preparation to perform cytogenetic assessment (ENAs assay) and the remaining volume was kept cool for oxidative DNA damage determination (8-OHdG).

At each sampling site, abiotic parameters were assessed (Table I) as per the guidelines of APHA [19].

Table I - Hydrological parameters in the studied sites of Ria de Aveiro. n.d. – not determined

Season	Site	Depth (m)	Turbidity (m)	Dissolved O_2 ($mg.L^{-1}$)	Temperature (ºC)	pH	Salinity
Spring	TOR	1.10	0.3	3.51	23.3	8.351	n.d.
	BAR	0.80	0.4	3.52	22.3	8.351	30
	GAF	3.00	1.0	4.10	21.4	8.207	28
	RIO	4.20	0.8	3.10	22.1	7.765	0
	LAR	1.10	0.6	3.28	23.4	7.742	17
	VAG	2.80	0.7	n.d.	24.6	7.655	11
Summer	TOR	1.20	0.5	5.03	25.1	8.396	26
	BAR	2.70	2.5	1.80	22.4	8.205	24
	GAF	5.60	1.0	3.64	20.1	8.249	30
	RIO	0.90	0.6	2.27	24.1	8.056	25
	LAR	1.20	0.3	1.07	22.0	7.446	25
	VAG	3.60	0.5	2.21	24.0	7.842	26
Autumn	TOR	1.00	0.5	17.70	17.7	8.122	23
	BAR	2.35	0.3	15.75	15.6	8.022	5
	GAF	1.50	n.d.	12.12	14.5	7.865	8
	RIO	4.75	0.2	4.26	16.3	7.947	0
	LAR	1.30	0.7	3.52	16.6	7.511	6
	VAG	n.d.	0.3	6.19	16.3	7.441	0
Winter	TOR	n.d.	n.d.	9.40	14.5	8.132	15
	BAR	2.55	0.5	9.20	15.9	7.748	n.d.
	GAF	4.10	0.4	9.44	14.9	8.195	21
	RIO	1.50	0.5	8.31	14.0	8.322	0
	LAR	1.00	0.2	8.58	12.0	7.184	22
	VAG	2.00	0.2	7.50	14.0	7.253	0

2.3. Genotoxic Responses

2.3.1. 8-hydroxy-2'-deoxyguanosine (8-OHdG) assessment: Upon arrival in laboratory, blood samples were centrifuged (13400 g) to isolate plasma. Interfering substances were excluded by plasma filtering (cut off molecular weight 10 000 dalton) and 7 times dilution (to reduce deviation). The 8-OHdG was assessed through a competitive *in vitro* enzyme linked immunosorbent assay (ELISA) using an IBL International GmbH (Germany) kit. Results were expressed as ng/mL.

2.3.2. Erythrocytic nuclear abnormalities (ENAs) assay: Blood smears were fixed with methanol during 10 minutes and stained with Giemsa (5%) during 30 minutes. The nuclear abnormalities were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid [20], Carrasco et al. [21] and Smith [22] adapted by Pacheco and Santos [23]. Nuclear lesions were scored as: micronuclei, lobed nuclei, binucleates or segmented nuclei and kidney shaped nuclei. Blebbed and lobed nuclei were considered in a single category — lobed nuclei — and not differentially scored as suggested by other authors due to some ambiguity in their distinction. The final result was expressed as the mean value (‰) of the sum for all the individual lesions observed.

2.4. Statistical analysis: Results are expressed as means ± SE (standard error) corresponding to experimental groups of six fish (n=6). Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). The assumptions of normality and homogeneity of data were verified. Factorial ANOVA was performed in order to assess significant effects at each site and seasonal differences. This analysis was followed by post-hoc Tukey test to signal significant differences between groups [24]. Significance of results was ascertained at $\alpha = 0.05$. The relation between 8-OHdG and ENAs, as well as the relations between biological responses and hydrological parameters, was assessed using multiple regression analysis.

3. Results

3.1. 8-OHdG assessment

In spring, *L. aurata* 8-OHdG levels were significantly higher than the reference site (TOR) at GAF, LAR and VAG (Figure 2A). However, in summer, no significant differences to TOR were observed. In autumn, at RIO, *L. aurata* displayed significantly higher 8-OHdG levels when compared to TOR, whereas in winter oxidative DNA damage was significantly higher at RIO and VAG.

The study of seasonal variations within sites revealed no significant differences at TOR, whereas the other sites revealed clear variations. Thus, at BAR, 8-OHdG levels were significantly higher in winter than in summer and autumn. At GAF, *L. aurata* displayed significantly lower oxidative DNA damage at spring, when compared to the other studied seasons. At RIO, in autumn and winter, 8-OHdG levels were significantly higher than spring and summer. At LAR and VAG, fish caught in spring displayed lower oxidative DNA damage (compared with the other seasons). Moreover, at LAR, in summer, 8-OHdG levels were lower than in autumn and winter. At VAG, in winter *L. aurata* displayed significantly higher levels than summer and autumn.

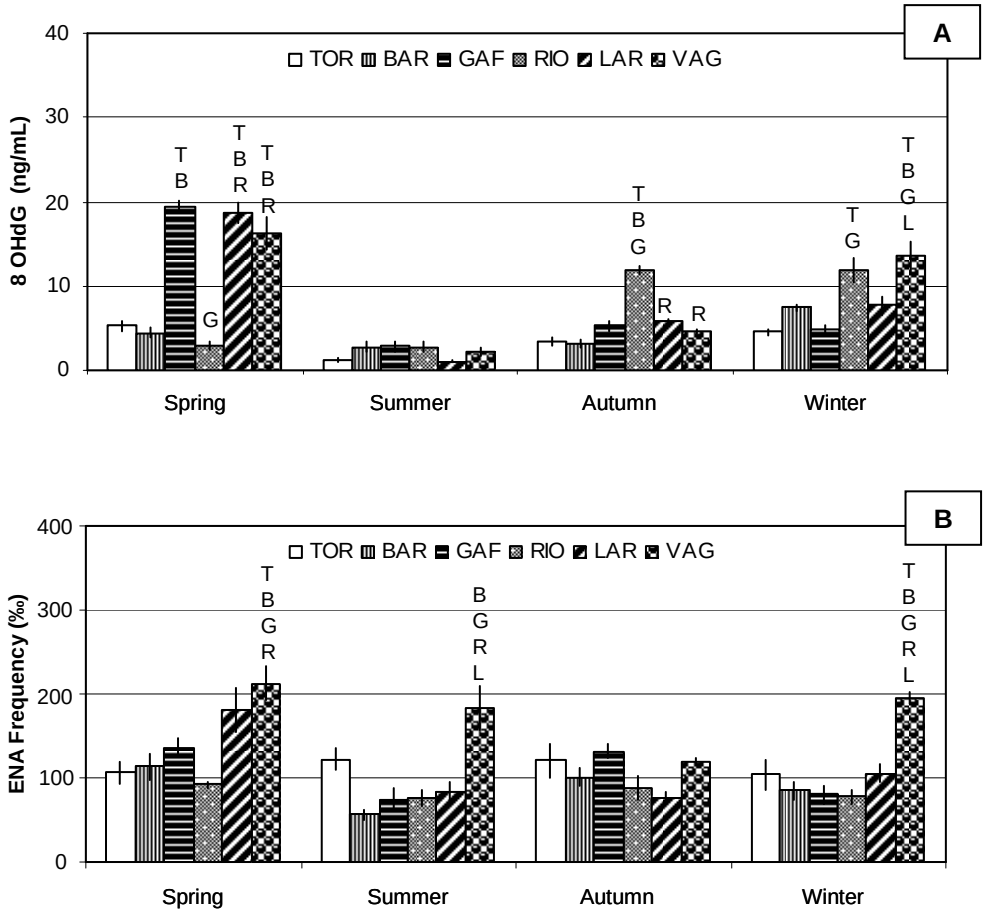

Figure 2 - A) Levels of plasma 8-hydroxy-2'deoxyguanosine (8-OHdG) in *L. aurata* seasonally caught at different sites in the Ria de Aveiro. B) Erythrocytic nuclear abnormalities in *L. aurata* seasonally captured at different sites in the Ria de Aveiro. Significant differences ($P<0.05$) are: T *versus* TOR; B *versus* BAR; G *versus* GAF; R *versus* RIO; L *versus* LAR.

3.2. ENAs frequency

In terms of ENAs frequency, *L. aurata* caught at VAG in spring and winter displayed higher ENAs frequency than at TOR (Figure 2B). Moreover, *L. aurata* caught at VAG also displayed significantly higher ENAs frequency than BAR, GAF and RIO in spring and than BAR, GAF, RIO and LAR in winter. In summer, though not significant when compared to TOR, VAG also displayed higher ENAs frequency than BAR, GAF, RIO and LAR.

In terms of seasonal variations, no significant changes were found at TOR, BAR, GAF and RIO. At LAR, *L. aurata* displayed significantly higher ENAs frequency in spring when compared to the other seasons. At VAG, in autumn, *L. aurata* displayed significantly lower ENAs frequency than in spring and winter.

In terms of individual nuclear abnormalities, no significant differences to TOR were found in terms of kidney shaped abnormalities in any studied season (Table II). The only significant differences between sites were found in spring when *L. aurata* kidney shaped nuclei were significantly higher at BAR than at GAF and VAG. Seasonal differences were found at TOR and BAR. Thus, at TOR spring levels were significantly higher than in autumn and winter whereas at BAR spring levels were significantly higher than in summer, autumn and winter. Lobed nuclei were significantly higher than TOR in spring at LAR and VAG and winter at VAG. Furthermore, at VAG, lobed nuclei were significantly higher than BAR, GAF and RIO in spring and BAR, GAF, RIO and LAR in summer and winter. Seasonal differences were found at LAR and VAG. Thus, at LAR lobbed nuclei frequency was significantly higher in spring when compared to summer and winter. At VAG and LAR, lobed nuclei in spring were also significantly higher than in autumn. No significant differences were found in any season in terms of segmented and micronucleated nuclei.

Table II - Mean frequency (‰) of each nuclear abnormality category (±SE) in peripheral blood erythrocytes of *L. aurata* seasonally captured at different sites in the Ria de Aveiro

	Site	Kidney shaped	Lobed	Segmented	Micronuclei
Spring	TOR	10.50±3.59	96.00±12.02	96.00±0.25	0.25±0.25
	BAR	14.00±3.03	99.50±17.42	99.50±0.00	0.25±0.25
	GAF	4.75±1.89^{B}	130.00±9.27	130.00±0.50	0.00±0.00
	RIO	6.25±0.85	85.25±3.75	85.25±0.25	0.00±0.00
	LAR	6.00±2.68	175.50±25.06^{TR}	175.50±0.50	0.00±0.00
	VAG	2.80±0.58^{B}	207.80±19.53^{TBGR}	207.80±0.93	0.60±0.24
Summer	TOR	4.50±1.44	117.50±14.03	117.50±0.25	0.25±0.25
	BAR	2.00±1.08^{Sp}	53.50±5.01	53.50±0.00	0.50±0.29
	GAF	3.50±1.66	70.75±12.8	70.75±0.00	0.00±0.00
	RIO	3.20±1.02	72.20±9.52	72.20±0.58	0.20±0.20
	LAR	1.50±1.19	82.00±10.46^{Sp}	82.00±0.00	0.00±0.00
	VAG	4.60±3.16	178.40±26.68^{BGRL}	178.40±0.00	0.00±0.00
Autumn	TOR	1.60±0.81^{Sp}	118.80±19.55	118.80±0.00	0.00±0.00
	BAR	3.00±0.84^{Sp}	97.40±10.35	97.40±0.20	0.40±0.24
	GAF	2.60±0.60	129.00±7.48	129.00±0.40	0.00±0.00
	RIO	3.20±1.02	84.20±13.56	84.20±0.00	0.00±0.00
	LAR	3.00±0.77	72.60±11.94^{Sp}	72.60±0.37	0.00±0.00
	VAG	2.00±0.71	117.00±3.05^{Sp}	117.00±0.24	0.00±0.00
Winter	TOR	2.00±1.14^{Sp}	101.80±17.13	101.80±0.00	0.00±0.00
	BAR	2.00±1.52^{Sp}	82.60±9.86	82.60±0.20	0.20±0.20
	GAF	1.00±0.63	79.00±10.91	79.00±0.20	0.00±0.00
	RIO	1.00±0.00	77.00±7.91	77.00±0.20	0.00±0.00
	LAR	0.00±0.00	105.80±9.88	105.80±0.00	0.00±0.00
	VAG	4.00±1.38	190.80±6.87$^{TBGRLV;Au}$	190.80±0.00	0.00±0.00

3.3. Responses Correlation

To study of the relation between 8-OHdG and ENAs was performed through linear regression analysis which revealed that 8-OHdG could explain 29.5% of the variance of total ENAs ($P<0.05$). The relation between 8-OHdG and ENAs was positive and significant (Figure 3A). Linear regression revealed significant

coefficients between 8-OHdG (Figure 3B) and lobed nuclei. No significant correlations were found between hydrological and biological parameters.

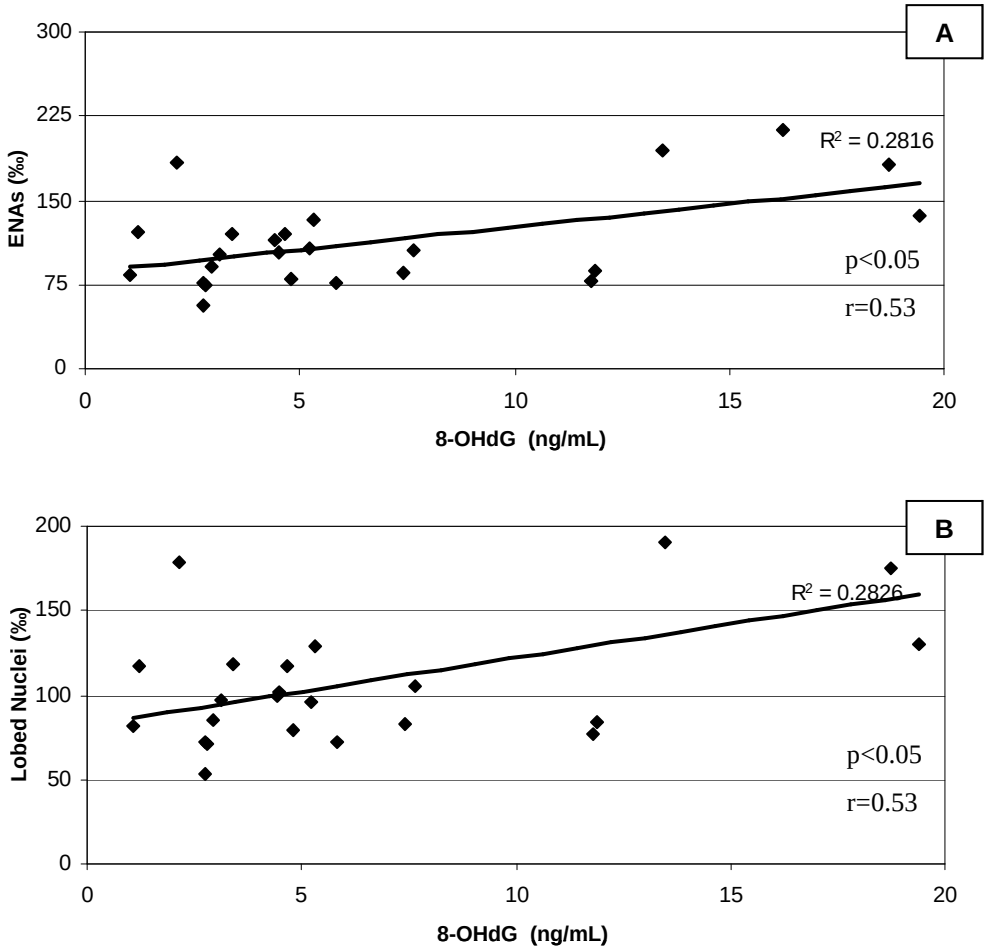

Figure 3 - Correlations between 8-hydroxy-2'-deoxyguanosine (8-OHdG) and erythrocytic nuclear abnormalities (ENAs) frequency in *Liza aurata*. A) 8-OHdG and total ENAs; B) 8-OHdG and lobed nuclei. Statistical significance and correlation coefficient are represented by p and r, respectively.

4. Discussion

The maintenance of DNA integrity is of supreme importance to all organisms. For this reason, living organisms possess mechanisms to protect their genetic material. Nevertheless, exposure to genotoxicants may overcome

defences and lead to genetic damage. On the other hand, the susceptibility to DNA damage may be influenced by physiological aspects of the tested organisms. Studies have found correlation between DNA damage and fish length and weight [25], sex and age [26]. Thus, *L. aurata* specimens selected for this study were reproductively quiescent juveniles with approximately the same age and size, in order to minimize possible influence of age and reproductive status in the selected biomarkers. The selection of blood to perform this study was based on its important role in the transference of the xenobiotics absorbed through gill, skin and gut to other tissues which makes blood cells a target to their toxic effects. Moreover, fish erythrocytes being nucleated and having a mitotic index higher than other cells such as hepatocytes, allow the study of genetic endpoints, such as ENAs, that require cell division.

The *L. aurata* plasma 8-OHdG levels suggest high levels of ROS that seem to have overwhelmed cells antioxidants defences in fish caught at GAF, LAR and VAG in spring, at RIO in autumn and RIO and VAG in winter. Thus, with the exception of BAR, all critical sites displayed, at least in one season, higher oxidative DNA damage than TOR. However, no discernible seasonal pattern to explain the oxidative DNA damage can be found in agreement with the lack of significant correlations between abiotic factors and 8-OHdG.

The lack of 8-OHdG and ENAs increase at BAR, a site subject to naval traffic and the associated contamination (i.e. PAHs and organometallic compounds), may be related with efficient defences. Nevertheless, peroxidative damage has been previously detected in the gill of *L. aurata* caught at BAR [15].

GAF is a site with high levels of PAHs, metals and organometallic compounds [12,27]. The high pro-oxidant potential of GAF contaminants was demonstrated in spring though without a concomitant increase of nuclear abnormalities, suggesting an efficient oxidative DNA damage repair and efficient defences against clastogenic/aneugenic events. In the following seasons (summer, autumn and winter) no DNA damage was found. Oxidative DNA damage had been previously detected in *Dicentrachus labrax* caught in autumn 2005 at GAF though not detected in *L. aurata* [15].

RIO, an area subjected to pulp/paper mill effluents during five decades and that has Hg and Cd [28], demonstrated the capacity to cause oxidative DNA damage in autumn and winter, seasons with a high water flow from River Vouga, that may be mobilizing and rendering more bioavailable the sediment associated contaminants resultant from pulp mill effluents. However, 8-OHdG higher levels were not associated with higher nuclear abnormalities. The capability of RIO contaminants to induce high 8-OHdG levels in autumn had previously been demonstrated in wild *D. labrax* though not demonstrated for *L. aurata* [15]. Moreover, the capacity of ENAs induction near pulp mill effluent area (RIO) was demonstrated in short-term experiments using caged *Anguilla anguilla* [29].

LAR is a site with high levels metal contamination, mainly mercury, associated with an historical effluent discharge from a chlor-alkali plant [30,31]. High levels of 8-OHdG were only found in spring, though *L. aurata* oxidative DNA damage had been found in autumn [17]. The oxidative DNA damage found in spring was not associated with ENAs frequency increase. However, Guilherme et al. [14] study with *L. aurata* caught in 2005 at LAR area (1.3 km closer to the Hg source than in the current study) revealed significantly higher ENAs frequency in summer and autumn when compared to that study reference site. Thus, the current study suggests that at this site, despite the high levels of metals such as Hg and Cd [28], *L. aurata* defences such as gill and liver metallothionein were effective in the prevention of metal induced clastogenic/aneugenic effects. Supporting this hypothesis, high levels of the liver and gill metallothionein were found in *L. aurata* caught in LAR [15,28].

VAG, a PAHs contaminated site [12], displayed high 8-OHdG and ENAs levels in spring and winter and no differences to TOR in summer and autumn. Though in the current study, no significant differences to the reference site in terms of ENAs frequency were found in autumn, the previous Ria de Aveiro biomonitoring studies using *L. aurata*, performed in autumn 2000 [12] and 2005 [17], found significantly higher ENAs frequency in VAG. Thus, the lack of differences to TOR may be related with altered bioavailability of xenobiotics linked with environmental conditions. Nevertheless, the current results agree with the

previous mentioned studies since VAG was, among the studied sites, the only displaying significantly higher ENAs frequency than the reference site.

The susceptibility to oxidative DNA damage varies with species and periods of the year, probably associated with seasonal effects in fish metabolism and antioxidants baseline levels, bioavailability of xenobiotics as well as oxidative DNA repair mechanisms. Studies have demonstrated that the repair of 8-OHdG is not stimulated by a preceding exposure to a “priming” dose of either an oxidant or an alkylating agent though oxidant and alkylating agents caused an adaptive response of the cellular glutathione levels under the same conditions [32]. Moreover, the repair of oxidative base modifications may be impaired under conditions of high oxidative stress [32]. This factors make the assessment of 8-OHdG an important early damage biomarker, providing information on a recent exposure to a DNA damaging agent, which represents an advantage over ENAs that require cell division to be expressed. However, these molecular responses may be easily repaired unlike ENAs, which represent a permanent damage that can only be fixed through cell removal. The lack of ENAs expression in the studied sites (with the exception of VAG) suggests a effective protection mechanisms in local specimens and/or increased splenic erythrocytic catabolism and reduced erythropoiesis rate [12].

Taking into account the known sources of contamination it may be inferred the sensitivity of *L. aurata* DNA to oxidative damage, assessed as 8-OHdG, upon exposure to environments with PAHs (VAG), PAHs and organometalic compounds (GAF), pulp mill effluent associated compounds as well as metals (RIO), Hg and Cd (LAR). However, seasonal variations were found, which may be related with both biotic and abiotic factors. Concerning ENAs frequency, this biomarker revealed a high sensitivity only to PAHs exposure (VAG).

Although the expression of the toxic potential of contaminants using different biomarkers may not always be linked up or correlated, due to possible attenuation or threshold values at different levels of biological organization [33], the current study results suggest a significant and positive linkage between 8-OHdG and ENAs frequency.

5. Conclusions

Ria de Aveiro has been subject to genotoxic pressure of xenobiotics. The levels of 8-OHdG and total ENAs, at the reference site, did not show seasonal variability, suggesting that 8-OHdG and total ENAs may be seen as reliable reflection of xenobiotic exposure, with 8-OHdG displaying sensitivity to a wider range of contaminants. The genetic damage, observed in the critical sites, though not correlated the physical-chemical parameters of the water, demonstrated seasonal variation. High levels of 8-OHdG was found in spring, at GAF, LAR and VAG, in autumn at RIO and in winter at RIO and VAG. However, no oxidative DNA damage was found in summer. VAG was the only site displaying ENAs (in spring and winter).

The significant correlation between 8-OHdG and total ENAs suggests a genotoxic mechanism in ENAs formation linked with oxidative stress.

The presence of xenobiotics in Ria de Aveiro demonstrated by oxidative DNA damage and erythrocytes nuclear abnormalities, recommend the continued monitoring of this lagoon system.

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CAPÍTULO XI

Variação sazonal da integridade do ADN nos tecidos de *Liza aurata* numa laguna costeira multi-contaminada (Ria de Aveiro, Portugal)

Seasonal *Liza aurata* tissue-specific DNA integrity in a multi-contaminated coastal lagoon (Ria de Aveiro, Portugal)

M. Oliveira, V.L. Maria, I. Ahmad, M. Pacheco, M.A. Santos

Submitted

Abstract

In this study, the DNA integrity of golden grey mullet (*Liza aurata*) collected in differently contaminated sites of a coastal lagoon, Ria de Aveiro (Portugal), was assessed, over the period of one year, using the DNA alkaline unwinding assay, in four different tissues (gill, kidney, liver and blood) and compared to a reference site. The four tissues displayed different DNA integrity basal levels, clearly affected by seasonal factors. Gill and kidney were respectively, the most and least sensitive tissues. All sites demonstrated the capacity to interfere with DNA integrity. The sites displaying the highest and lowest DNA damage capability were respectively Barra (subject to naval traffic) and Vagos (contaminated with polycyclic aromatic hydrocarbons), respectively. In terms of seasonal variability, autumn seems to be the more critical season (more DNA damage) unlike summer when no DNA damage was found in any tissue. Data recommend the continued monitoring of this aquatic system.

Keywords: *Liza aurata*; DNA strand breaks; Gill; Kidney, Liver, Blood

1. Introduction

The aquatic environment is often the ultimate recipient of large a large number of contaminants, mostly resultant from human activities, a large proportion of which potentially genotoxic and carcinogenic. The quantification of contaminants in the environment though frequently used in environmental studies may not be provide reliable information on the risk to biota, considering the possible presence of unknown substances, the presence of substances in concentrations beyond detection limits, the potential interactions between contaminants as well as differences in bioavailability that may lead to discrepancies between the predictable risk of environmental contaminants, based on chemical analyses, and the actual effects. In this perspective, biological

systems, that are the target of toxicant action, may provide important information not readily available from chemical analyses (Jha et al., 2000). Biota exposure to contaminants may disrupt normal cellular processes and lead to structural modifications to DNA which may cause subsequent problems for the cell. A high prevalence of unrepaired DNA lesions may lead to incomplete transcription, cellular dysfunction, growth inhibition, aging, weakened immunity and diseases in the organism itself (Woo et al., 2006). The analysis of DNA alterations in aquatic organisms has been shown a suitable method for evaluating the presence of genotoxic contamination, allowing the detection of exposure to low concentrations of contaminants in a wide range of species (Siu et al., 2003; Frenzilli et al., 2009). Thus, its use in monitoring multi-contaminated environments is recommended.

Some of the most common DNA lesions are single strand breaks which have been classified as potentially pre-mutagenic lesions (Emmanouil et al., 2007). DNA strand breaks may occur due to direct DNA damage caused by exogenous agents, the indirect action of pro-genotoxic agents following biotransformation, oxidative stress and inhibition of DNA synthesis and repair (Eastman and Barry, 1992; Speit and Hartmann, 1995; Lee and Steinert, 2003). Many contaminants may cause damage to DNA by more than one mechanism. Thus, DNA strand breaks may provide information on the presence of a wide range of genotoxic chemicals such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and metals (Everaarts, 1995; Mitchelmore and Chipman, 1998) individually or in mixtures (Bihari and Fafandel, 2004).

The genotoxic potential of chemicals depends on the exposure route as well as the properties of the target tissue, namely in terms of accumulation capacity, metabolic activity and basal antioxidant defense levels, cell type heterogeneity, cell cycle, turnover frequency and DNA repair efficiency (Lee and Steinert, 2003; Raisuddin and Jha, 2004; Jha et al., 2005). In this perspective, fish tissues such as gill, kidney, liver and blood may respond differently to environmental contaminants, displaying different susceptibility.

A previous biomonitoring study focusing on the responses of a native fish species, golden grey mullet (*Liza aurata*) caught at differently contaminated sites of a coastal lagoon Ria de Aveiro (Portugal) revealed no significant differences

between the critical sites and reference site in gill DNA integrity (Oliveira et al., 2009a), despite the significant DNA integrity loss found in kidney at two sites (Oliveira et al.2009b). Despite the known presence of genotoxicants in Ria de Aveiro, Oliveira et al. (2009c) found seasonal variations of oxidative DNA damage and erythrocytes nuclear abnormalities. With the exception of the previously mentioned studies, the DNA damage dynamics in *L. aurata* has not been studied in complex environments with different contamination profiles despite the knowledge that environmental conditions may affect xenobiotics bioavailability and input as well as food availability to fish (Noyes et al., 2009) which may have a dramatic influence on enzyme systems that activate or detoxify genotoxicants. Thus, the objective of this study was to assess the tissue specific (gill, kidney, liver and blood) DNA integrity variations of *L. aurata* seasonally caught at differently contaminated sites of Ria de Aveiro (Portugal) and select the most reliable tissue for genotoxicity assessment in a multi-contaminated environment. Additionally we aimed to rank the assessed sites in terms of the presence of genotoxicants.

2. Material and Methods

2.1 - Study Area: Ria de Aveiro (Figure 1) is a coastal lagoon 45 km long (NNE-SSW) and 8.5 km wide, covering a wetland area of approximately 66 (low tide) to 83 km2 (high tide) which is permanently connected to the ocean through a narrow channel (Dias et al., 2001). Sampling sites were selected on a geographic distribution basis taking into account the various types and sources of contamination as well as the selection of a (theoretically) unpolluted reference point. Sampling sites were: Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel), far from the main polluting sources and thus assumed as reference site; Barra (BAR), the initial part of the Mira channel close to the lagoon entrance and subject to considerable naval traffic; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Rio Novo do Principe (RIO), located at the terminal area of the Vouga River, 6.5 km

distant from a pulp/paper mill effluent outlet, that discharged to this water course during nearly five decades (until the year 2000); Laranjo (LAR), close to a chlor-alkali plant (6 km), an important source of metal contamination (mainly mercury); Vagos (VAG), located at the terminal part of the Ílhavo channel, receiving municipal and domestic effluents with high levels of PAHs.

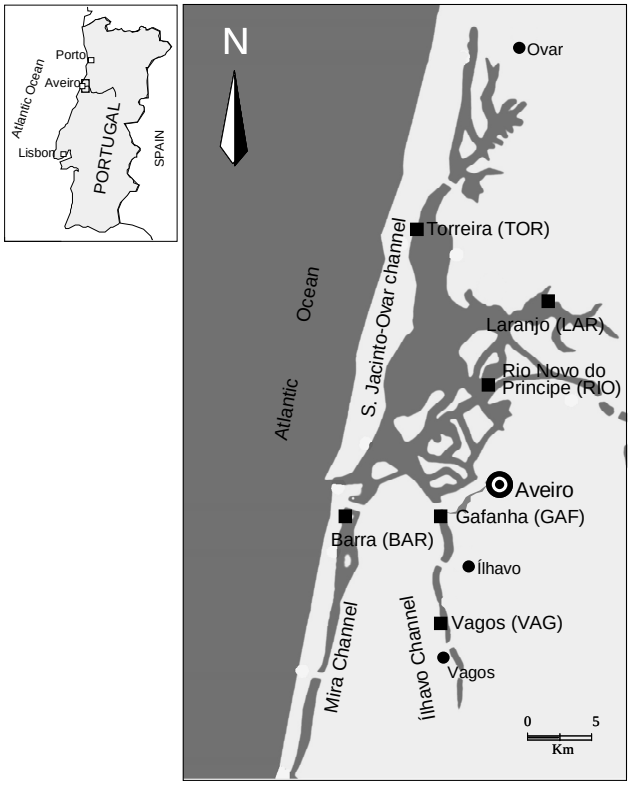

Figure 1 - Map of Ria de Aveiro (Portugal) with locations of fish-capture sites (■). The respective coordinates are: reference site (TOR)—40º44'02 N, 008º41'44 W; BAR—40º37'42.00"N, 8º44'35.00"W GAF—40º38'38 N, 008º41'42 W; RIO—40º41 '08 N, 008º39'41 W; LAR—40º43'30 N, 008º37'43 W; and VAG—40º33'59 N, 008º40'55 W.

2.2 - Sampling: *L. aurata* seasonal samplings were carried out between May 2006 and March 2007 in spring, summer, autumn and winter, using a traditional beach-seine net named “chincha”, at each selected sampling site. *L. aurata* specimens (n=6), selected on the basis of their size, had an average length of 14.0±3.0 cm and weighed 21.4±3.6 g. Immediately after catching blood was collected from the posterior cardinal vein using a heparinised Pasteur pipette, fish

was sacrificed and gill, kidney, liver were removed. All tissues were immediately frozen in liquid nitrogen.

At each sampling site, abiotic parameters were assessed (Table I) as per the guidelines of APHA (1998).

Table I - Hydrological parameters in the studied sites of Ria de Aveiro. BDL – Bellow detection limits. n.d. – not determined

Season	Site	Depth (m)	Turbidity (m)	Dissolved O_2 ($mg.L^{-1}$)	Temperature (ºC)	pH	Salinity
Spring	TOR	1.10	0.3	3.51	23.3	8.351	n.d.
	BAR	0.80	0.4	3.52	22.3	8.351	30
	GAF	3.00	1.0	4.10	21.4	8.207	28
	RIO	4.20	0.8	3.10	22.1	7.765	0
	LAR	1.10	0.6	3.28	23.4	7.742	17
	VAG	2.80	0.7	n.d.	24.6	7.655	11
Summer	TOR	1.20	0.5	5.03	25.1	8.396	26
	BAR	2.70	2.5	1.80	22.4	8.205	24
	GAF	5.60	1.0	3.64	20.1	8.249	30
	RIO	0.90	0.6	2.27	24.1	8.056	25
	LAR	1.20	0.3	1.07	22.0	7.446	25
	VAG	3.60	0.5	2.21	24.0	7.842	26
Autumn	TOR	1.00	0.5	17.70	17.7	8.122	23
	BAR	2.35	0.3	15.75	15.6	8.022	5
	GAF	1.50	n.d.	12.12	14.5	7.865	8
	RIO	4.75	0.2	4.26	16.3	7.947	0
	LAR	1.30	0.7	3.52	16.6	7.511	6
	VAG	n.d.	0.3	6.19	16.3	7.441	0
Winter	TOR	n.d.	n.d.	9.40	14.5	8.132	15
	BAR	2.55	0.5	9.20	15.9	7.748	n.d.
	GAF	4.10	0.4	9.44	14.9	8.195	21
	RIO	1.50	0.5	8.31	14.0	8.322	0
	LAR	1.00	0.2	8.58	12.0	7.184	22
	VAG	2.00	0.2	7.50	14.0	7.253	0

2.3. DNA integrity assessment: DNA integrity was tested using DNA alkaline unwinding assay. Tissues were placed in TNES (Tris-HCl 10 mM, NaCl 125 mM, EDTA 10 mM, SDS 1%, pH 7.5) – urea (5 M) buffer with proteinase K solution (final concentration 0.8 mg/ml). DNA isolation was performed using a genomic DNA purification kit (Fermentas). DNA integrity measurements were performed according to Rao et al. (1996) as adopted by Maria et al. (2002). Data from DNA unwinding technique were expressed as F-value [DNA integrity (%)], determined by applying the following equation:

$$F = \frac{ds}{ds+ss} \times 100$$

where ss is the relative fluorescence (measured with a Jasco FP 750 spectrofluorometer) of the single-stranded eluent of a sample minus the single-stranded control blank fluorescence value, and ds is the relative fluorescence of the corrected double stranded eluent of the same sample.

2.4 - Statistical analysis: Results are expressed as means ± SE (standard error). The DNA integrity data was transformed prior to statistic analyses according to the formula arcsen $\sqrt{p}$. Statistical data analysis was done using Statistica software (StatSoft, Inc., Tulsa, OK, USA). The assumptions of normality and homogeneity of data were verified. Factorial ANOVA was performed in order to assess significant effects at each site and seasonal differences. This analysis was followed by post-hoc Tukey test to signal significant differences between groups (Zar, 1999). Significance of results was ascertained at $\alpha = 0.05$. The relationship between DNA integrity loss and abiotic factors was studied using multiple regression analyses.

3. Results

3.1. Hydrological parameters

The hydrological parameters for each sampling season including temperature, dissolved oxygen, salinity, pH, turbidity and depth are depicted in Table I.

3.2. Gill DNA integrity

In gill, *L. aurata* DNA integrity was only different from the reference site (TOR) in spring and autumn. Thus, in spring, at BAR and VAG, *L. aurata* gill DNA integrity was significantly higher than in fish from reference site (TOR), RIO and LAR (Figure 2). Fish from GAF also displayed significantly higher DNA integrity than RIO and LAR. In autumn, gill DNA integrity was significantly lower than TOR at BAR, GAF, RIO and LAR. At BAR, GAF and LAR *L. aurata* gill DNA integrity levels were also lower than VAG. In summer and winter, no significant differences were found between sites.

The seasonal variations at each site were also studied. In general, the highest gill DNA integrities (though not always significantly) were found in winter. Thus, at TOR, fish gill DNA integrities in summer and winter were significantly the lowest and highest, respectively. At BAR, LAR and VAG, gill DNA integrity displayed similar seasonal patterns, with significantly higher integrities at spring and winter when compared to summer and autumn. No significant differences were found between neither spring and winter nor summer and autumn. At GAF, in spring and winter gill DNA integrity was significantly higher than summer and autumn (that displayed the significantly lowest DNA integrity). Concerning RIO, gill DNA integrity was significantly higher in winter (when compared with the other studied seasons) and spring (compared to summer and autumn).

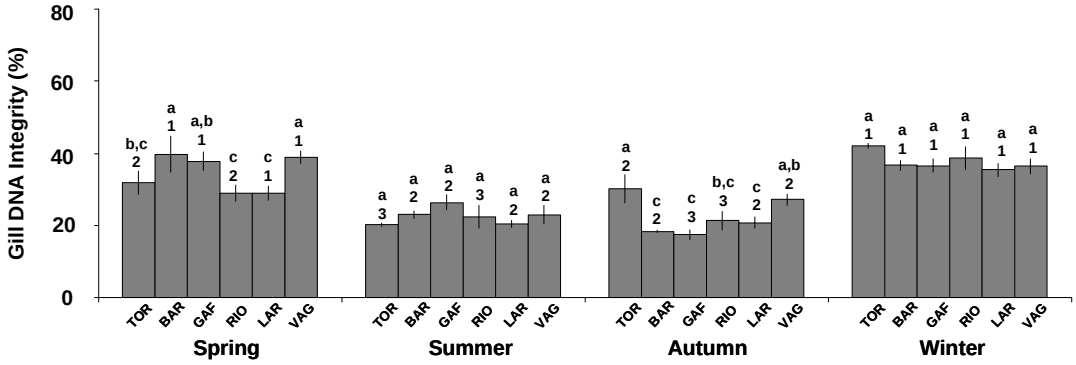

Figure 2. Gill DNA integrity in *L. aurata* seasonally captured at different sites in the Ria de Aveiro. Values represent mean ± standard error. Distinct letters and numbers indicate, respectively, significant ($p<0.05$) differences between sites and seasons.

3.2. Kidney DNA integrity

In spring, at GAF and VAG, kidney DNA integrity was higher than TOR as well as BAR, RIO and LAR (Figure 3). However, comparing to TOR, as well GAF, RIO, LAR and VAG, significant kidney DNA integrity loss was found at BAR. In summer, no kidney DNA integrity loss was found. In fact, kidney DNA integrity was significantly higher than TOR at all studied sites except BAR.

In autumn, significant differences to TOR were only found at BAR which displayed higher kidney DNA integrity than TOR, RIO and VAG. Kidney DNA integrity at GAF was also higher than at VAG.

In winter, kidney DNA integrity was significantly lower than TOR at GAF.

Concerning seasonal differences, TOR displayed the highest DNA integrities in autumn and winter which were significantly higher than spring and summer. At BAR, kidney DNA integrity was, in spring and autumn, respectively, significantly lower and higher than in the other seasons. At GAF, in autumn, kidney DNA integrity was significantly higher than in the other studied seasons. At RIO and LAR, the lowest kidney DNA integrities were found in spring. At RIO, kidney DNA integrity in autumn and winter was higher than in summer whereas at LAR, kidney DNA in autumn was significantly higher than all studied seasons. At VAG,

kidney DNA integrity was significantly lower in summer when compared to the other studied seasons.

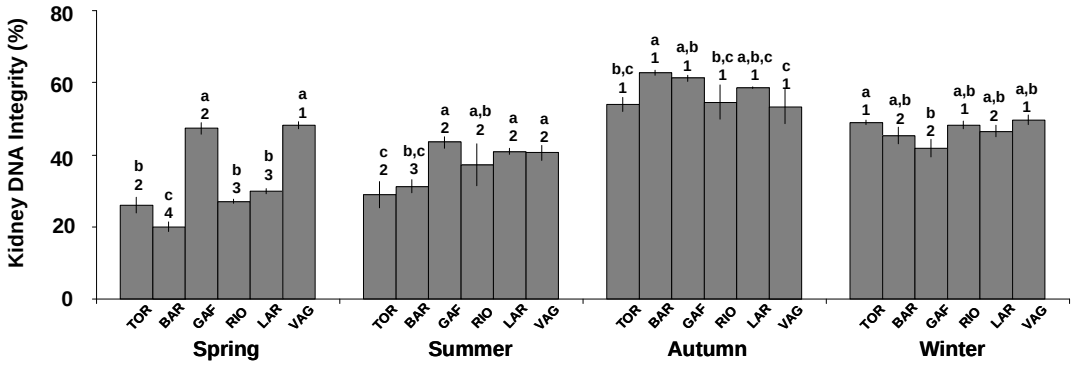

Figure 3. Kidney DNA integrity in *L. aurata* seasonally captured at different sites in the Ria de Aveiro. Values represent mean ± standard error. Distinct letters and numbers indicate, respectively, significant ($p<0.05$) differences between sites and seasons.

3.3. Liver DNA integrity

In spring, *L. aurata* caught at VAG displayed higher DNA integrity than the fish caught at TOR (Figure 4). No significant differences between sites were found in summer and winter. In autumn, DNA integrity in the fish caught at GAF, LAR and VAG was significantly lower than at TOR. Moreover, at GAF, LAR and VAG liver DNA integrity was also lower than at BAR.

A general seasonal pattern was found in liver DNA integrity at all sites. DNA integrity in spring was significantly lower than in the other seasons. At TOR, BAR, RIO and VAG, the liver DNA integrity of *L. aurata* caught in autumn and winter was significantly higher than in summer. At GAF and LAR, the liver DNA integrity of the fish caught in winter was significantly higher than in summer.

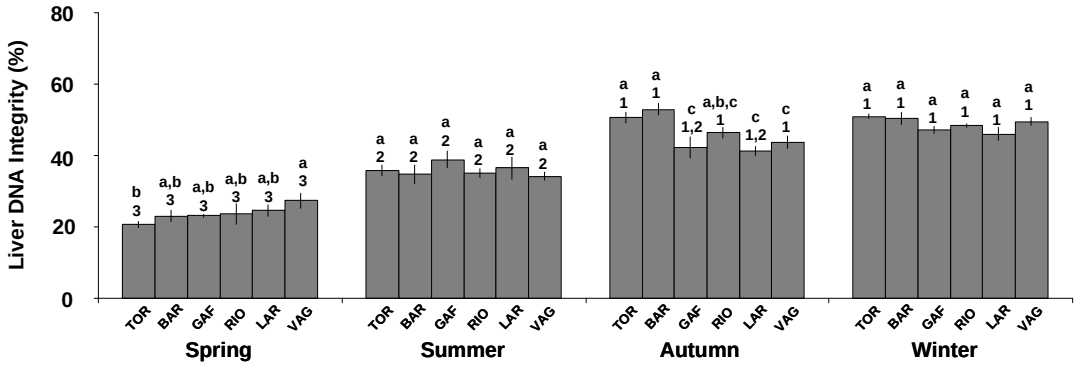

Figure 4. Liver DNA integrity in *L. aurata* seasonally captured at different sites in the Ria de Aveiro. Values represent mean ± standard error. Distinct letters and numbers indicate, respectively, significant ($p<0.05$) differences between sites and seasons.

3.4. Blood DNA integrity

In spring, *L. aurata* blood DNA integrity at BAR, RIO and LAR was significantly lower than at TOR as well as BAR and VAG (Figure 5). However, in summer, blood DNA integrity at BAR and VAG was significantly higher than TOR, GAF, RIO and LAR. In autumn, DNA integrity at BAR, GAF, LAR and VAG were significantly higher than TOR. At BAR, GAF and LAR, blood cells DNA integrity was significantly higher than RIO. Fish at BAR also displayed significantly higher DNA integrity than GAF and LAR. In winter, no significant differences between sites were found.

Seasonal variations were also found in blood cells DNA integrity. Thus, at TOR, *L. aurata* displayed significantly higher DNA integrity in spring, when compared to the other seasons. In summer and autumn, DNA integrity was also significantly lower than in winter. At BAR, DNA integrity was significantly higher in autumn than in the other studied seasons. At GAF, in spring DNA integrity was significantly higher than in summer and winter and in autumn DNA integrity was higher than in summer. No seasonal differences were found at RIO and VAG. At LAR, blood cells DNA integrity in winter was significantly higher than in summer.

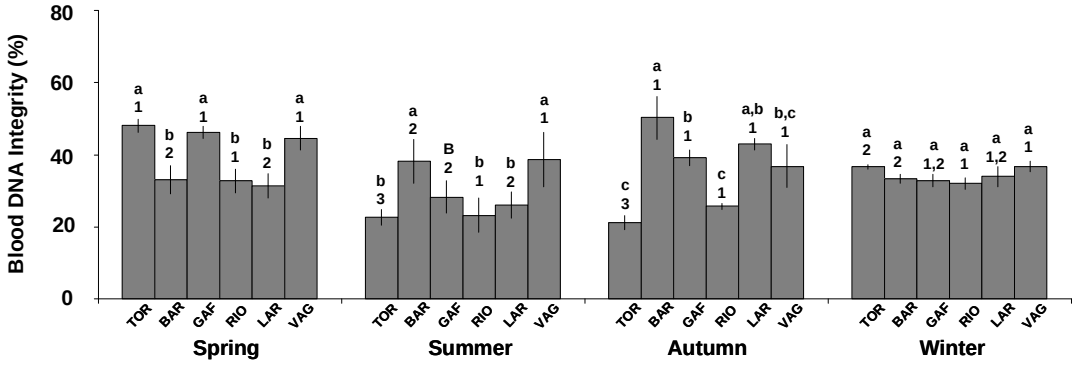

Figure 5. Blood DNA integrity in *L. aurata* seasonally captured at different sites in the Ria de Aveiro. Values represent mean ± standard error. Distinct letters and numbers indicate, respectively, significant ($p<0.05$) differences between sites and seasons.

3.5. Linear Multiple Regression Analysis

Multiple regression analysis revealed that temperature and salinity could explain 65.8 and 47.8% of the variance of respectively, liver and kidney DNA integrity ($p<0.05$). The relation between temperature and liver DNA integrity was negative and significant. However, salinity revealed no significant impact on liver DNA integrity.

4. Discussion

Structural alternations in the DNA of organisms exposed to environmental contaminated sites provide an indication of occurrence and bioavailability of genotoxicants able to pass toxicokinetic barriers (Shugart, 2000). Thus, DNA damage responses may be very useful in biomonitoring aquatic systems with complex mixtures of contaminants though physiological aspects of the tested organisms may influence responses. In this perspective, the *L. aurata* specimens selected for this study were reproductively quiescent juveniles with approximately the same age and size in order to decrease possible variability on DNA integrity

due to length and weight (Wirzinger et al., 2007), as well as sex and age (Akcha et al., 2004).

Overall, all sites demonstrated the capability to induce DNA integrity alterations. BAR, GAF and LAR were able to decrease DNA integrity in three tissues, RIO in two tissues and VAG in one tissue. However, higher DNA integrities, compared to TOR, were found in four tissues at VAG, three tissues at BAR, two tissues at GAF and LAR, one tissue at RIO. Accordingly, the sites with genotoxicants more able to overwhelm *L. aurata* defenses and DNA repair mechanisms seem to be BAR (that is a site subject organic contamination associated with naval traffic), GAF (harbor water area), LAR (metal contaminated site) followed by RIO (subject during decades to pulp mill effluent) and VAG (PAHs contaminated).

All studied sites, including the reference site (TOR), displayed seasonal differences emphasizing the importance of assessing fish responses under different environmental conditions. Overall, when compared to the reference site, all sites demonstrated the capacity to induce DNA integrity loss and increase, showing a capacity not specific for a particular contamination profile. The variation of DNA integrity found in the reference site did not seem explainable by the presence of contamination since its value as a reference site has been demonstrated in several studies, using different species and biomarkers (Oliveira et al.; Ahmad et al., 2008; Mohmood et al., 2008; Maria et al., 2009). In the present study, with the exception of blood, the highest DNA integrities at TOR were found in the colder seasons (autumn and winter for liver and kidney and winter for gill) which seems to support the idea that DNA damage baseline may be increased by high temperatures (Andrade et al., 2004). Regression analysis, confirmed the important role of temperature in kidney and liver DNA integrity though that was not confirmed for gill. The temperature effects on fish metabolism, cell replication rates and DNA repair may be possible explanations for the observed variations (Venier et al., 1997). Blood displayed a completely different pattern of response neither correlated with temperature nor any hydrological parameter. Other studies have also found a positive correlation between water temperature and DNA integrity in other aquatic species such as zebra mussels

(Buschini et al., 2003). Autumn seems to be the more critical season, considering that it was the season in which *L. aurata* displayed damage at more sites, i.e. gill at 4 sites and liver at 3 sites, probably due to a conjugation of factors such as increased bioavailability of sediment associated contaminants linked with increased turbidity. In spring, damage was only found in kidney (at 1 site – GAF) and in blood, that only displayed damage in this season (at BAR, RIO and LAR). However, none of the studied tissues displayed significant DNA integrity decrease in summer. The lower re-suspension of sediment associated contaminants may result in lower bioavailability of contaminants. Wirzinger et al. (2007) also found the lowest DNA damage in summer in three-spined sticklebacks (*Gasterosteus aculeatus*). Winter was the season when DNA integrity was less responsive (both in terms of increased and decreased integrity) only displaying a DNA integrity decrease at 1 site - GAF. The lower variability in terms of DNA integrity in winter may be related with a decreased activity of fish with low water filtration through gill presenting a lower metabolism and consequent decreased uptake of contaminants. In the four studied tissues DNA damage was never found in two consecutive seasons at the same site. Seasons associated with more extreme temperatures (summer and winter) displayed less damage.

Higher susceptibility to short-term exposure to a PAH (phenanthrene) in terms of oxidative stress was found in *L. aurata* gill followed by liver and kidney (Oliveira et al., 2008). However, DNA integrity loss was found in liver and not in gill (Oliveira et al., 2007) probably related with higher cell regeneration rate of gill. Kilemade et al. (2004) found that juvenile turbot (*Scophthalmus maximus*) gill DNA was the more sensitive to damage caused by inter-tidal contaminated sediments exposure, followed by liver and blood. On the other hand, Belpaeme et al. (1998) found in *S. maximus* exposed to ethyl methanesulphonate that gill cells seemed more sensitive than blood and the responsiveness of liver cells was even lower. Overall, the present data suggests that *L. aurata* kidney is the tissue least vulnerable to DNA damage since DNA integrity loss, though displayed in two seasons (spring and winter), was only found at two sites (BAR in spring and GAF in winter) with similar contamination profile (associated with naval traffic and harbour activities, respectively). This supports the hypothesis of Kilemade et al.

(2004) that haematopoietic tissues could be the least sensitive or responsive cell type. Gill was the most sensitive tissue towards DNA damage, displaying damage at more sites, which may be considered expected since they are the first organ to be exposed to waterborne contaminants displaying low levels of basal defenses when compared to kidney (Oliveira et al., 2008). The DNA integrity loss found in blood cells at RIO and LAR without concomitant damage in other tissues does not support the idea that damage in blood may be reflected in other tissues or vice-versa. Kilemade et al. (2004) found correlations between blood and liver DNA damages as well as between blood and gill and suggested that blood could act as a suitable predictor of DNA damage in the whole organism.

The data from this study confirms the tissue specific sensitivity to contaminants that was highly variable with seasons. The use of genotoxic responses of resident species in biomonitoring studies, though logistically easy may be also influenced by physiological adaptations which potentially render chronically exposed specimens less responsive to genotoxic impacts (Frenzilli et al., 2009; Katsumiti et al., 2009) as observed for example in VAG site, that is contaminated with genotoxicants (Pacheco et al., 2005). The higher DNA integrity found in critical sites compared to the reference site may be the result of a more efficient DNA repair system, stimulated by the chronic exposure.

Thus, the genotoxic effects measurements utilizing DNA strand breaks in resident species may provide misleading results as observed for example in summer, where no DNA integrity loss was found despite the known presence of contaminants. The lack of differences between clean and polluted sites was also found in other studies. Akcha et al. (2004) and Large et al. (2002) found no differences in mussels (Mytilus sp.) DNA strand breaks despite the different extents in contamination between sites. Everaarts et al. (1993) found a high incidence in strand breaks in an uncontaminated area in hardhead catfish (*Arius felis*) and no differences in DNA integrity in relation to contaminated sites. Bombail et al. (2001) also did not find differences between sites in butterfish (*Pholis gunnellus*) caught along a pollution gradient. This may be due to higher defenses basal levels and activation of cellular defenses, which are thought to prevent accumulation of electrophilic metabolites and free radicals and hence partially

protect DNA and other cellular macromolecules against oxidation and adduct formation (Rocher et al., 2006). Furthermore, repair enzymes, cell turnover, regulated absorption, detoxification, excretion and storage may also play a determinant in the different responses displayed by *L. aurata* tissues.

5. Conclusions

All sites demonstrated the capacity to induce DNA integrity loss and increase when compared to the reference site. *L. aurata* DNA integrity was most affected at BAR whereas VAG displayed the least DNA damage.

As expected, gill, kidney, liver and blood displayed different basal levels, which were clearly affected by season. Gill and kidney seem to be, respectively, the most and least sensitive tissues.

The use of resident *L. aurata* DNA integrity assessment in biomonitoring studies may provide misleading results since physiological adaptations (more efficient defenses and repair mechanisms) may lead to DNA integrities even higher than in the reference site. The highly dynamic nature of DNA integrity, dependent on an array of processes that may lead to its damage or repair, may limit its usefulness in pollution monitoring if not included in a set of biomarkers namely including more permanent indications of damage.

6. Acknowledgements

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CAPÍTULO XII

Discussão geral

Tendo em conta que cada capítulo inclui a discussão específica do seu conteúdo, esta discussão geral pretende constituir uma análise crítica da globalidade da tese, tendo presentes as orientações definidas inicialmente.

1. Exposições laboratoriais

Os trabalhos em análise neste ponto incluem os Capítulos II e III, que correspondem à exposição, de curta duração (16 horas), de *L. aurata* a uma gama de concentrações de fenantreno (0.1, 0.3, 0.9 e 2.7 µM). Nestes capítulos foram descritas respostas de stresse (níveis plasmáticos de cortisol, glucose e lactato), capacidade de indução de uma enzima de fase I da biotransformação (etoxiresorufina O-desetilase - EROD), efeitos genotóxicos (quebras de cadeia de ADN nas guelras e fígado e anomalias nucleares eritrocíticas – ANE), defesas antioxidantes (níveis de glutationa e actividades de glutationa S-transferase - GST, glutationa redutase - GR e catalase - CAT) bem como peroxidação lipídica (PL) nas guelras, rim e fígado.

Os resultados constantes nestes capítulos revelaram efeitos potencialmente nefastos do fenantreno para *L. aurata*. O aumento dos níveis plasmáticos de cortisol e glucose, considerado resposta típica de stresse, indicou que para concentrações de fenantreno iguais ou superiores a 0.3 µM, foram desencadeados mecanismos adaptativos. O aumento dos níveis plasmáticos de glucose pode ser compreendido como uma resposta ao aumento das necessidades energéticas resultantes dessa adaptação. O aumento dos níveis de cortisol e glucose não foi, no entanto, acompanhado por um aumento dos níveis plasmáticos de lactato, evidenciando a complexidade dos modelos de respostas a xenobióticos. Estes resultados tornam-se mais relevantes tendo em conta a inexistência de estudos sobre os efeitos do fenantreno ao nível do metabolismo secundário em peixes, nomeadamente nos níveis de glucose e lactato plasmáticos.

O aumento da actividade da EROD hepática de *L. aurata* após 16 horas de exposição ao fenantreno, para concentrações iguais ou superiores a 0.3 µM, constitui igualmente um resultado relevante em termos do conhecimento de

possíveis mecanismos de toxicidade deste hidrocarboneto aromático policíclico (HAP) para esta espécie. Em termos de quebras de cadeia do ADN, a exposição ao fenantreno revelou resultados diferentes para guelras e fígado. Nas guelras não foram detectadas quebras de cadeia (possivelmente devido a um aumento da taxa de regeneração celular causada pelos efeitos citotóxicos do fenantreno) ao contrário do fígado, onde a integridade do ADN diminuiu para as concentrações 0.1 e 0.9 µM. Apesar do metabolismo do fenantreno não ter sido directamente estudado, a genotoxicidade deste HAP não pareceu estar apenas relacionada com a activação da fase I da biotransformação, dado que foi detectado decréscimo da integridade do ADN hepático e aumento de ANE após exposição a 0.1 µM de fenantreno, concentração que não induziu a actividade da EROD. Assim, a toxicidade de fenantreno poderá estar relacionada não só com efeitos tóxicos directos do fenantreno, mas também com a capacidade deste composto induzir metabolitos reactivos e radicais. Este facto parece ser apoiado pelos resultados da PL no fígado, que foi observada após exposição a fenantreno 0.1 (concentração de fenantreno não indutora da EROD) e 2.7 µM (concentração indutora de EROD).

A susceptibilidade das guelras, rim e fígado de *L. aurata* a PL, induzida por exposição ao fenantreno, pôde ser ordenada da seguinte forma: guelras>fígado>rim. As guelras apresentaram os níveis basais de defesas antioxidantes mais baixos e uma capacidade de os aumentar inferior à do fígado. A GSH demonstrou uma grande importância nas guelras, evidenciada pelo facto de não se ter verificado dano apenas para a concentração de fenantreno indutora do aumento deste tiol (0.1 µM). No fígado, a exposição ao fenantreno resultou num aumento de GSH para todas a concentrações de xenobiótico. Por outro lado, o rim apresentou os níveis de defesas basais mais elevados entre os órgãos estudados, não se tendo verificado activação de qualquer defesa. A CAT demonstrou uma menor sensibilidade a inibição por exposição ao fenantreno que a GPx, tendo a sua actividade sido induzida apenas nas guelras e para uma das concentrações capazes de inibir a GPx (0.9 µM). Estes resultados sugerem uma primeira linha de defesa nas guelras constituída pela GSH e por GPx.

A sensibilidade das guelras, fígado e rim de *L. aurata* à exposição de curta duração ao fenantreno sugere a utilização destes órgãos em biomonitorização ambiental, facto que poderá ajudar a uma melhor compreensão dos mecanismos de acção tóxica dos xenobióticos.

2. Biomonitorização da Ria de Aveiro

Face aos resultados laboratoriais obtidos nos Capítulos II e III, à complexidade da contaminação na Ria de Aveiro e às possíveis vias de exposição dos peixes aos contaminantes (água, sedimento e alimento), foi analisada uma bateria de biomarcadores em espécimes de *L. aurata* colhidos em locais daquele sistema estuarino com diferentes perfis de contaminação. Os locais seleccionados foram: Barra, local próximo da entrada da Ria, sujeito a tráfego naval; Gafanha, zona de actividade portuária em contacto com o canal principal da cidade de Aveiro; Rio Novo do Príncipe, localizado na área terminal do Rio Vouga, a 6.5 Km de um efluente desactivado de uma fábrica de pasta de papel que descarregou para este local durante mais de cinco décadas; Laranjo, local próximo do complexo químico de Estarreja e sujeito durante décadas a descargas contínuas de uma indústria de soda cáustica, que originou níveis elevados de metais, essencialmente mercúrio; Vagos, local sujeito a descargas de efluentes municipais e domésticos, apresentando elevados níveis de HAPs. Estes locais "críticos" foram comparados com a Torreira, um local longe de fontes localizadas de poluição.

Os parâmetros avaliados incluíram respostas hormonais (níveis plasmáticos de cortisol, tirotrofina - TSH, tiroxina - T4 e triiodotironina - T3), parâmetros do metabolismo secundário (níveis plasmáticos de glucose e lactato), defesas antioxidantes não enzimáticas (tióis não proteicos, glutationa total e metalotioninas) e enzimáticas (CAT, GPx, GST, GR), dano oxidativo no ADN (níveis plasmáticos de 8-hidroxi-2'desoxiguanosina - 8-OHdG), integridade do ADN (quebras de cadeia) e lesões citogenéticas (ANE).

Os níveis plasmáticos de cortisol, glucose, lactato, TSH, T4 e T3 foram avaliados no Outono de 2005 (Capítulo IV). Em Vagos, *L. aurata* apresentou

níveis de cortisol inferiores aos dos animais capturados na Torreira (local de referência), o que sugeriu uma possível menor capacidade de resposta a estímulos. *L. aurata* colhida nos outros locais estudados não apresentou alterações significativas nos níveis deste corticosteróide (comparativamente aos organismos do local de referência), sugerindo uma capacidade de adaptação às condições nesses locais. No entanto, os níveis plasmáticos de glucose e lactato no Laranjo, comparativamente ao local de referência, assinalaram uma maior mobilização de substratos energéticos, muito provavelmente associada às adaptações e reparação de danos induzidos por xenobióticos presentes neste local. Tal como no Capítulo II, estes resultados confirmaram a complexidade da relação entre os níveis de cortisol, glucose e lactato.

As respostas das hormonas associadas ao eixo hipotálamo – hipófise – tiróide (HHT) sugeriram igualmente a presença de contaminantes. No Laranjo, os níveis plasmáticos de TSH de *L. aurata* apresentaram-se mais elevados que no local de referência, provavelmente como consequência dos baixos níveis de T3 verificados neste local. No entanto, no Rio Novo do Príncipe e em Vagos, os baixos níveis de T3 observados não se reflectiram em alterações nos níveis plasmáticos de TSH, o que sugeriu efeitos de xenobióticos sobre outros órgãos que não a tiróide, como aumento de absorção por tecidos periféricos, decréscimo da desiodação hepática e/ou aumento do catabolismo de T3 plasmático. Os níveis de T4, em *L. aurata*, apresentaram-se inferiores na Barra em relação aos observados no local de referência, sem que tivesse ocorrido um aumento dos níveis de TSH. Em virtude da relevância biológica da T3, os dados sugerem uma menor capacidade de sobrevivência dos peixes no Rio Novo do Príncipe, Laranjo e Vagos.

As respostas antioxidantes, PL e integridade do ADN de *L. aurata* foram igualmente avaliadas no Outono de 2005. Os resultados confirmaram a diferente susceptibilidade dos órgãos estudados às condições dos diferentes locais. As respostas antioxidantes nas guelras (Capítulo V) sinalizaram um maior potencial pró-oxidante, manifestado pela presença de níveis superiores de defesas (glutationa total, CAT e GPx) neste órgão em determinados locais, tal como Gafanha, Laranjo e Vagos. Esta activação das defesas antioxidantes demonstrou

ser eficiente dado, que nestes locais, as guelras não apresentaram aumento de PL nem diminuição de integridade do ADN. No entanto, foram observados na Barra, níveis superiores de substâncias reactivas ao ácido tiobarbitúrico, apesar de não se terem verificado diferenças nas defesas antioxidantes relativamente ao local de referência. A inibição dos níveis de metalotioninas em locais onde ocorreu aumento de outras defesas, Gafanha e Vagos, foi um aspecto particularmente importante observado nas guelras. Este facto poderá ser atribuído à mobilização de um maior número de grupos sulfidrilo destinados às defesas antioxidantes e à consequente indisponibilidade para a síntese de metalotioninas.

As respostas antioxidantes do rim (Capítulo VI), ao contrário das guelras, sugeriram um maior potencial pró-oxidante no Rio Novo do Príncipe, traduzido por níveis mais elevados de tióis não proteicos e glutationa total bem como actividades mais elevadas de CAT, GST e GR. O rim de *L. aurata* capturada no Laranjo apresentou, tal como nas guelras, níveis elevados de defesas antioxidantes. No entanto, não se verificou o aumento de PL no rim em qualquer dos locais estudados, apesar de ter sido observado o decréscimo de integridade, do ADN no Rio Novo do Príncipe e Vagos.

A quantificação de metais na água e sedimento dos locais estudados no Outono de 2005 (Capítulo VII) confirmou a existência de elevados níveis de metais na Ria de Aveiro, essencialmente no sedimento, que poderão ter consequências graves para os peixes expostos, principalmente se ocorrer a mobilização dos contaminantes no sedimento, aumentando a biodisponibilidade. Assim, os dados revelaram uma maior contaminação metálica do sedimento no Laranjo e Rio Novo de Príncipe, locais onde a tainha apresentou níveis mais elevados de metalotioninas hepáticas. No entanto, os níveis de metalotioninas determinadas nas guelras (Capítulo V) apenas se apresentaram elevados no Laranjo. Assim, a determinação destas proteínas citosólicas no fígado reflectiu melhor os níveis de metais no ambiente. Contrariamente às guelras e ao rim, no fígado não se verificou a elevação dos níveis de defesas antioxidantes comparativamente ao local de referência (Capítulo VIII). Assim, verificou-se a inibição de GPx (Barra, Gafanha, Laranjo, Vagos), GST (Gafanha, Rio Novo de Príncipe, Laranjo e Vagos) e GR (Rio Novo do Príncipe), facto que poderá estar

relacionado com a função deste órgão na biotransformação de xenobióticos e consequente formação de metabolitos, alguns potencialmente reactivos. As actividades inferiores da GPx e GST não estiveram associadas a decréscimos de glutationa total, o que sugere uma inactivação enzimática provavelmente associada a níveis elevados de ERO. No entanto, apenas foi detectada PL em peixes capturados na Gafanha e em Vagos, possivelmente devido ao efeito protector das metalotioninas hepáticas no Rio Novo do Príncipe e Laranjo.

Os níveis de dano peroxidativo no ADN (avaliado pela quantificação dos níveis plasmáticos de 8-OHdG) e efeitos citogenéticos (ANE) foram igualmente quantificados no Outono de 2005 (Capítulo IX) em *L. aurata* e *D. labrax* e comparada a susceptibilidade das duas espécies. *L. aurata* demonstrou uma menor sensibilidade a dano peroxidativo no ADN que *D. labrax*, dado que para *L. aurata* apenas foram detectados níveis plasmáticos de 8-OHdG superiores aos do local de referência no Laranjo, enquanto que para *D. labrax* foram detectados níveis plasmáticos superiores de 8-OHdG nos locais onde foi possível fazer a colheita (Gafanha, Rio Novo do Príncipe e Vagos). Contudo, as ANE detectadas em *L. aurata* sinalizaram Vagos como um local com propriedades genotóxicas, ao contrário de *D. labrax* cujos valores de ANE não diferiram entre os locais estudados.

Em suma, as respostas biológicas de *L. aurata* capturada no Outono de 2005 indicaram a presença de substâncias com a capacidade de induzir decréscimo nos níveis plasmáticos de cortisol (Vagos), aumento dos níveis plasmáticos de glucose e lactato (Laranjo), diminuição dos níveis de T4 (Barra) e T3 (Rio Novo do Príncipe, Laranjo e Vagos), aumento de PL nas guelras (Barra) e fígado (Gafanha e Vagos), aumento dos níveis plasmáticos de 8-OHdG (Laranjo), diminuição da integridade do ADN no rim (Rio Novo do Príncipe e Vagos) e ANE (Vagos). No entanto, o dano não esteve sempre associado a decréscimo das defesas antioxidantes, enfatizando a importância da conjugação da avaliação de respostas de defesa e dano. Os dados dos diferentes biomarcadores recomendam, assim, a contínua monitorização dos locais estudados.

Face aos dados obtidos no Outono de 2005, procedeu-se ao estudo da variação sazonal, entre Maio de 2006 e Março de 2007, dos níveis plasmáticos de

8-OHdG e frequência de ANE (Capítulo X) e integridade do ADN nas guelras, rim, fígado e sangue (Capítulo XI). Os níveis plasmáticos de 8-OHdG em *L. aurata* apresentaram variações sazonais, não se tendo observado um padrão sazonal evidente. A Primavera foi a estação do ano em que *L. aurata* apresentou níveis elevados de 8-OHdG em mais locais (Gafanha, Laranjo e Vagos), comparativamente ao local de referência. No Inverno, foram detectados níveis elevados de 8-OHdG no Rio Novo do Príncipe e Vagos e no Outono apenas se detectou dano oxidativo no ADN no Rio Novo do Príncipe. Assim, a susceptibilidade a dano oxidativo no ADN pareceu mais elevada nos peixes capturados no Rio Novo do Príncipe e em Vagos. No entanto, a frequência de ANE apenas se apresentou superior à do local de referência em Vagos, na Primavera, Verão e Inverno. Os dois parâmetros apresentaram uma correlação positiva que pareceu indicar o stresse oxidativo como uma das causas envolvidas na formação de ANE. A comparação entre os dados de 8-OHdG e ANE obtidos no Outono de 2005 e 2006 sugere que ocorreram alterações nas condições dos locais estudados que afectaram a biodisponibilidade dos contaminantes para *L. aurata*. Esta hipótese parece suportada pelos parâmetros hidrológicos que sugerem uma maior ressuspensão dos sedimentos no Rio Novo do Príncipe no Outono de 2006 ao contrário do Laranjo que apresentou uma menor turbidez.

A avaliação sazonal da integridade do ADN de *L. aurata* revelou variações significativas entre estações do ano, em todos os locais estudados, incluindo no local de referência. A Barra demonstrou a capacidade de induzir decréscimos de integridade nas guelras, rim e sangue; a Gafanha nas guelras, rim e fígado e o Laranjo nas guelras, fígado e sangue. No Rio Novo do Príncipe, foram observados decréscimos de integridade de ADN nas guelras e sangue e em Vagos apenas se detectou menor integridade de ADN no fígado. Todos os locais demonstraram a capacidade de induzir aumentos e decréscimos de integridade do ADN. No local de referência, com excepção do sangue, as integridades do ADN mais elevadas foram observadas nas estações mais frias (Outono e Inverno para o fígado e rim e Inverno para as guelras), sugerindo que a integridade basal do ADN pode ser diminuída com o aumento da temperatura. O sangue apresentou um perfil de resposta que não se relacionou com a temperatura nem

com nenhum dos outros parâmetros hidrológicos estudados. Em termos de estações do ano, os dados sugeriram que o Outono parece ser a estação mais crítica, na qual *L. aurata* apresentou dano em mais locais (4 locais para as guelras e 3 para o fígado). Pelo contrário, no Verão não foram observados decréscimos de integridade em nenhum dos locais críticos estudados. De entre guelras, rim, fígado e sangue, o rim pareceu ser menos susceptível a quebras de cadeia do ADN e as guelras revelaram maior vulnerabilidade. Ao contrário do observado para a 8-OHdG e ANE, a integridade do ADN variou no local de referência, facto que se apresenta como uma importante limitação na utilização deste parâmetro em estudos de biomonitorização com recurso a organismos residentes, indiciando a susceptibilidade a outros factores que não a presença de contaminantes.

Deste modo, verifica-se que continuam a existir locais críticos na Ria de Aveiro, apesar do desvio das principais fontes de poluição (industriais e domésticas) no ano de 2000. Apesar da possibilidade de continuarem a ser libertados contaminantes para a Ria, muitos destes efeitos serão consequência da acumulação de contaminantes persistentes nos sedimentos. Este estudo revelou a importância de serem avaliadas as respostas bioquímicas e fisiológicas em diferentes órgãos, que podem reflectir a susceptibilidade dos peixes a uma grande gama de compostos e fontes de exposição. *L. aurata* demonstrou ser um bom organismo bioindicador, abundante em todos os locais estudados e sensível aos diferentes perfis de contaminação. A sua limitada mobilidade no contexto dos peixes confere um elevado grau de confiança de que os efeitos avaliados dizem respeito à exposição nos locais estudados.

3. Considerações finais e perspectivas futuras

De uma forma geral, os resultados do presente trabalho sugerem a necessidade de futuros estudos, quer em termos de monitorização de locais contaminados na Ria de Aveiro, quer no laboratório para estudar, mediante condições controladas, mecanismos de acção tóxica de contaminantes.

Os efeitos da exposição de curta duração de *L. aurata* ao fenantreno sugerem a necessidade de um estudo de maior duração com quantificação de

metabolitos, de forma a permitir compreender os mecanismos de acção tóxica. De igual modo, seria relevante tentar estabelecer a ponte entre as respostas precoces e sensíveis ao stresse, a nível sub-individual, e as respostas a longo prazo a níveis supra-individuais. Nesta perspectiva, a avaliação de alguns parâmetros comportamentais, ecologicamente mais relevantes (como por exemplo a velocidade natatória), poderá ser uma ferramenta útil para avaliar o reflexos de, por exemplo, decréscimos dos níveis de T3 e cortisol nos peixes. Dado que os organismos no meio ambiente estão expostos a misturas de contaminantes, seria igualmente relevante avaliar o efeito do fenantreno em misturas com outros xenobióticos (orgânicos e inorgânicos), estudando eventuais sinergismos e antagonismos.

Relativamente à Ria de Aveiro, os resultados aconselham a continuação da monitorização das respostas dos organismos residentes. A exposição *in situ* nos locais críticos da Ria de Aveiro de organismos colhidos em locais de referência poderá ajudar a compreender os mecanismos de adaptação das espécies residentes. A alternativa seria a colheita de amostras de água e/ou sedimento dos locais contaminados e a exposição laboratorial de organismos do local de referência, mediante condições devidamente controladas. Futuros estudos de biomonitorização deverão proceder à quantificação dos contaminantes nos tecidos, de forma a ser possível estabelecer uma relação causa-efeito.

Os resultados revelam que seria extremamente relevante determinar a actividade de enzimas de reparação do ADN nos organismos residentes, as quais poderão estar a impossibilitar a detecção de danos genéticos.