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Abstract

Estuaries are highly productive systems with high natural variability and affected by various anthropogenic pressures. Considering this complexity, several physiological and biochemical responses of fish were evaluated as indicators of habitat quality in estuaries. Condition indices, growth estimates and biomarkers of exposure and of effects to contaminants in fish were used due to their ability to integrate habitat quality, life-history, inter-specific, temporal and spatial patterns. Global growth and condition patterns of several fish species (larvae and juveniles) were related with latitudinal distribution, habitat and life-history strategies. Morphometric indices (K and HSI) and lipid content were good indicators of fish nutritional condition. Juveniles growth rates in different species were used to assess habitat quality, using an RNA:DNA based model and an ecophysiological framework that considered the interactions of metabolism, bioenergetics and environmental variability. The relation between environmental variability and variation patterns of condition indices and biomarkers of exposure to contaminants was assessed on a temporal scale of days to weeks. Despite the relationship observed between temperature and biochemical condition indices, natural variability had no effects on biomarkers at this scale. On an experimental approach, the effects of copper exposure induced responses of biomarker of exposure and of effects, including condition indices. A multi-specific and multi-metric approach revealed spatial differences related with strong species patterns and degree of environmental contamination. Despite the sensitivity of the biomarkers of exposure to and of effects of contaminants, habitat quality assessment should take natural variability into consideration due to its possible relation with some markers at different scales, and to its strong influence on growth and condition patterns of fish in estuarine environments.

Key-words: estuaries; fish; growth; condition indices; biomarkers; contamination.

Resumo

Os estuários são sistemas altamente produtivos, com elevada variabilidade natural, afectados por pressões antropogénicas. Perante esta complexidade, várias respostas fisiológicas e bioquímicas de peixes foram avaliadas como indicadores da qualidade do habitat estuarino. Índices de condição, medidas de crescimento e biomarcadores de exposição e de efeitos de contaminantes foram utilizadas como integradores da qualidade ambiental, tendo sido avaliados padrões de história de vida, inter-específicos, temporais e espaciais. Padrões globais de crescimento e condição de várias espécies de peixes (larvas e juvenis) foram relacionados com a distribuição latitudinal, habitat e estratégias de vida. Índices morfométricos (K e HSI) e conteúdo lipídico foram bons indicadores da condição nutricional de peixes. Taxas de crescimento de juvenis de diferentes espécies foram utilizadas na caracterização da qualidade do habitat, modeladas com recurso à razão ARN:ADN, e ainda com base num modelo ecofisiológico que relaciona o metabolismo, bioenergética e variabilidade ambiental. A relação da variabilidade ambiental com os padrões de variação de índices de condição e biomarcadores de exposição a contaminantes foi avaliada numa escala temporal de dias e semanas. Apesar da relação observada entre temperatura e índices bioquímicos de condição, não foram observados efeitos da variabilidade natural nos biomarcadores a esta escala. Numa abordagem experimental, os efeitos da exposição ao cobre induziram respostas ao nível de biomarcadores de exposição e de efeitos, nos quais se incluiram índices de condição. Uma abordagem multi-específica e multi-métrica revelou diferenças espaciais relacionadas com fortes padrões específicos e com os níveis de contaminação ambiental. Apesar da sensiblidade revelada pelos biomarcadores de exposição e de efeitos da contaminação, a variabilidade natural deve ser necessariamente considerada na avaliação da qualidade ambiental pela sua possível relação com alguns marcadores a diferentes escalas e pela sua marcada influência nos padrões de crescimento e condição de peixes em meio estuarino.

Palavras-chave: estuários; peixes; crescimento; índices de condição; biomarcadores; contaminação.

Resumo alargado

Os estuários são ecossistemas caracterizados por uma elevada produtividade ecológica, marcados por extensos padrões de variabilidade natural a várias escalas temporais e sujeitos a inúmeras pressões antropogénicas.

O presente estudo tem como objectivo principal avaliar o potencial de respostas fisiológicas e bioquímicas em peixes como indicadores da qualidade do habitat em ambiente estuarino, integrando simultaneamente os stresses naturais e antropogénicos. Face à complexidade de processos e interacções abióticas e bióticas que caracterizam os estuários, a resposta a esta questão exige uma abordagem integrativa das suas várias dimensões, com recurso a diferentes ferramentas e metodologias que actuam a diferentes níveis de organização biológica.

A presente tese é composta por dez capítulos, dos quais oito referem-se a artigos científicos, publicados ou submetidos e em revisão em revistas internacionais com arbitragem científica indexadas no Science Citation Index. Os restantes capítulos referem-se ao capítulo inicial de introdução geral e ao capítulo final, onde são apresentadas a discussão geral e as considerações finais.

Na introdução geral, capítulo 1, foi realizado um enquadramento do tema da presente tese, no que concerne: a dicotomia variabilidade natural vs stress antropogénico presente nos estuários; a legislação actual que prevê o desenvolvimento de metodologias para avaliar a qualidade ambiental nestes sistemas; e a utilidade dos peixes e das suas respostas a nível sub-individual como indicadores da qualidade ambiental.

Uma revisão global explorando a forma como os padrões de crescimento e condição na fase larvar e juvenil estão relacionados com as estratégias de vida (segundo o modelo desenvolvido por Winemiller e Rose 1992), a utilização do habitat e a distribuição latitudinal das espécies de peixes é apresentada no capítulo 2. Na base desta comparação está uma ampla compilação de dados ecológicos relativos às fases larvar e juvenil de 46 espécies de peixes, nomeadamente percentagem do comprimento máximo alcançado no primeiro ano de vida, idade de maturação, factor de condição K (ou Fulton), razão ARN:ADN, conteúdo lipídico e conteúdo proteico. A análise multivariada permitiu uma segregação coerente das espécies de acordo com a distribuição geográfica e a utilização do habitat das mesmas, assim como a separação dos três tipos de estratégias de vida definidos pela metodologia utilizada. Assim, espécies de peixes que ocorrem em ambientes mais complexos e variáveis, como recifes de coral e estuários, apresentaram taxas de crescimento e índices de condição superiores comparativamente a espécies que ocorrem em ambientes mais estáveis ou previsíveis, como os ambientes de água-doce ou regiões polares, que por sua vez apresentaram um desenvolvimento mais tardio e maiores reservas energéticas.

O valor de vários indicadores lipídicos de condição nutricional em peixes foi testado numa experiência laboratorial, discutida no capítulo 3. De acordo com o período experimental, os indicadores que mais fielmente retrataram os diferentes níveis de alimentação foram os morfométricos (factor de condição K e índice hepatosomático HSI) e o conteúdo em lípidos totais no fígado de juvenis de linguados (*Solea solea*). A análise de classes de ácidos gordos também evidenciou padrões interessantes, nomeadamente a preservação de ácidos gordos polinsaturados, muitos dos quais são essenciais para peixes. Quanto à análise de ácidos gordos individuais, não foi possível detectar padrões de variação consistentes com o stress nutricional, o que se atribui à influência do perfil em ácidos gordos da dieta e à complexidade de processos de incorporação, oxidação e síntese destas moléculas.

No capítulo 4 foi desenvolvido um modelo de estimação da taxa de crescimento em juvenis de robalo (*Dicentrarchus labrax*) e de linguado do Senegal (*Solea senegalensis*), por meio de uma calibração experimental da razão ARN:ADN face a diferentes temperaturas e salinidades. Os modelos desenvolvidos foram aplicados numa avaliação da qualidade do habitat para juvenis de ambas as espécies recolhidos em cinco estuários da costa Portuguesa, nos anos de 2005 e 2006. A estimativa da percentagem da taxa de crescimento individual (realizada por comparação com a taxa máxima ou de referência observada) permitiu avaliar as diferenças inter-estuarinas e inter-anuais para ambas as espécies relacionadas com a variabilidade ambiental.

Na sequência da estimativa de taxas de crescimento de peixes em ambiente estuarino, foi parametrizado e aplicado um modelo ecofisiológico, Ecophys.Fish (Neill et al. 2004), que integra os efeitos e interacções da variação abiótica no metabolismo e processos bioenergéticos em peixes (Capítulo 5). O crescimento é determinado com base na energia metabólica disponível depois de mobilizada a energia para actividades de rotina (processamento dos alimentos, excreção). As estimativas de crescimento obtidas reflectiram com um elevado grau de fidelidade os valores de estudos prévios utilizados para a parametrização inicial do modelo. A aplicação do modelo a uma série de dados de quatro anos para o estuário do Tejo revelou padrões de variação no crescimento de juvenis de linguados (*S. solea* e *S. senegalensis*) associados à variabilidade ambiental inter-anual.

No capítulo 6 foi explorada a relação entre variáveis abióticas (temperatura e salinidade) e a variação de índices de condição numa escala temporal de dias e semanas. Foram amostradas três espécies de peixes no estuário do Tejo (*D. labrax, S. senegalensis* e o caboz, *Pomatoschistus microps*) e uma na zona costeira adjacente (o carapau, *Trachurus trachurus*), com o objectivo de comparar os padrões de condição de espécies em ambientes com níveis contrastantes de variabilidade natural. Foram descritas correlações positivas entre a temperatura e os índices de condição bioquímicos (razão ARN:ADN e conteúdo proteico). No entanto, nenhuma relação foi estabelecida com a salinidade. Também não foram observadas diferenças nos padrões de condição entre as espécies de habitats contrastantes, o que certamente se deve à capacidade de resiliência das espécies estuarinas face à elevada variabilidade ambiental previamente descrita.

Na abordagem experimental descrita no capítulo 7 comparam-se várias respostas de exposição e de efeitos do cobre em juvenis de *S. senegalensis.* Neste caso, um conjunto de índices de condição foram considerados também como respostas de efeitos. Vários biomarcadores assinalaram a exposição ao cobre, nomeadamente a indução significativa de metalotioninas (MT), o aumento da peroxidação lipídica e a redução significativa da razão ARN:ADN. A taxa de crescimento de peixes sujeitos a contaminação manteve-se positiva, apesar de inferior ao grupo de controle, o que foi associado à mobilização das reservas lipídicas.

Os efeitos da variabilidade ambiental foram novamente investigados no capítulo 8. Foi realizada uma avaliação da variação diária e semanal de um conjunto de biomarcadores de exposição a contaminantes em três espécies de peixes no estuário do Tejo (*D. labrax, S. senegalensis* e *P. microps*) considerando a variação concomitante da temperatura e salinidade. A actividade das enzimas antioxidantes (catalase, CAT; e superoxide dismutase, SOD) apresentou menor variabilidade temporal que a actividade das enzimas de biotransformação (glutationa S-transferase, GST; e 7-Etoxiresorufina O-desetilase, EROD). Contudo, à excepção da correlação positiva entre a temperatura e a actividade da CAT em juvenis de *S. senegalensis*, não foram estabelecidas outras relações entre as variáveis ambientais e os biomarcadores. A maior especificidade das enzimas de biotransformação face à exposição a contaminantes foi corroborada pelas respostas concordantes de EROD e GST.

No capítulo 9 foi avaliada a qualidade ambiental de habitats estuarinos com uma abordagem integradora dos capítulos anteriores: multi-específica (*D. labrax, S. senegalensis* e *P.microps*), multi-métrica [enzimas antioxidantes (SOD, CAT, glutationa peroxidase total (TGPx) e selénio-dependente (SeGPx); enzimas de biotransformação (GST, EROD); metalotioninas (MT); nível de peroxidação lipídica (LPO); razão ARN:ADN; conteúdo proteico e lipídico] e com medidas da contaminação ambiental [concentração de metais e hidrocarbonetos aromáticos policíclicos (PAH) no sedimento]. Várias fontes de pressão antropogénica foram identificadas com base nos níveis de contaminação do sedimento em três locais do estuário do Tejo e da Ria de Aveiro. As diferenças inter-específicas nas respostas biológicas sobrevieram aos padrões de variação espacial, sublinhando a importância das diferenças ecológicas entre espécies e da qualidade de habitat relativa para cada espécie. Não obstante, locais com maior nível de contaminação e toxicidade foram associados a uma indução significativa de respostas de biomarcadores antioxidantes e de biotransformação, bem como a uma redução da condição dos peixes amostrados nestas áreas.

Por último, no capítulo 10 é realizada uma discussão geral, onde são integrados os principais resultados e conclusões dos capítulos anteriores e delineadas perspectivas futuras de investigação, no contexto do objectivo geral do estudo de associar as respostas fisiológicas e bioquímicas de peixes à qualidade ambiental, integrando a variabilidade natural e as pressões antropogénicas.

LIST OF PAPERS

This thesis is comprised by the papers listed below, each corresponding to a Chapter, from 2 to 9. The author of the thesis was responsible for conception and design of the work, field surveys, sample collection and processing, laboratory analytical procedures, data analysis and manuscript writing of all the papers. Remaining authors collaborated in some or several of these procedures. All papers published or in press were included with the publishers' agreement.

CHAPTER 2: Are fish early growth and condition patterns related to life-history strategies?
Vanessa Fonseca, Henrique N Cabral
Published in Reviews in Fish Biology and Fisheries (2007) 374(2-3):199-215
DOI 10.1007/s11160-007-9054-x

CHAPTER 3: Lipid-based indicators of nutritional condition in juvenile sole *Solea solea*
Vanessa Fonseca, Luís Narciso, Henrique N Cabral
In review in Journal of Applied Icthyology

CHAPTER 4: Growth performance of juvenile *Dicentrarchus labrax* and *Solea senegalensis* in estuaries along the Portuguese coast assessed via experimental calibration
Vanessa Fonseca, Rita P Vasconcelos, Patrick Reis-Santos, Susanne Tanner, Henrique Cabral
In review in Journal of Fish Biology

CHAPTER 5: Ecophys.Fish perspectives on growth of juvenile soles, *Solea solea* and *Solea senegalensis*, in the Tagus estuary, Portugal
Vanessa Fonseca, William H Neill, John M Miller, Henrique N Cabral
Published in Journal of Sea Research (2010) 64(1-2):118-124
DOI: 10.1016/j.seares.2009.10.007

CHAPTER 6: Short-term variability of condition indices in fish from estuarine and shallow coastal areas of the Portuguese coast: response to environmental variability
Vanessa Fonseca, Rita P Vasconcelos, Susana França, Marisa Batista, Susanne Tanner, Henrique Cabral
In review in Estuarine, Coastal and Shelf Science

CHAPTER 7: Effect of copper exposure on growth, condition indices and biomarker response in

juvenile sole *Solea senegalensis*
Vanessa Fonseca, Ângela Serafim, Rui Company, Maria João Bebianno, Henrique Cabral
Published in Scientia Marina (2009) 73(1): 51-58
DOI: 10.3989/scimar.2009.73n1051

CHAPTER 8: Short-term variability of multiple biomarker response in fish from estuaries: influence of environmental dynamics
Vanessa Fonseca, Susana França, Rita P Vasconcelos, Ângela Serafim, Ruy Company, Belisandra Lopes, Maria João Bebianno, Henrique N Cabral
In review in Aquatic Toxicology

CHAPTER 9: Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*
Vanessa Fonseca, Susana França, Ângela Serafim, Rui Company, Belisandra Lopes, Maria João Bebianno, Henrique N Cabral
In review in Chemosphere

CHAPTER 1

General introduction
Aims and importance
Thesis outline

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General introduction

Estuaries are highly productive ecosystems that sustain valuable biological and economic resources (Costanza et al. 1997; McLusky and Elliott 2004). These transitional environments support fundamental ecological links between freshwater and marine environments, with a diverse fish assemblage of resident, occasional, early-life stage and migratory organisms (Beck et al. 2001; Able 2005; Franco et al. 2008). From a functional perspective, estuaries are particularly important for fish as they provide various habitats for feeding, refuge from predators, nursery areas, reproduction and migration routes for species that are resilient to the highly dynamic environment (Haedrich 1983; McLusky and Elliott 2004; Elliott et al. 2007). Estuarine systems are characterized by highly dynamic environmental conditions, such as varying water temperature, salinity and dissolved oxygen concentration, chiefly due to the influence of tidal regimes, freshwater input and seawater intrusion. Hence, estuaries are widely regarded as naturally stressful environments, where only species that can accommodate or adapt to the inherently dynamic conditions can thrive (McLusky and Elliott 2004, 2007).

In addition to natural variability, estuaries are also impacted by multiple anthropogenic stressors, derived from the intense human activities and interests typically established in these areas, including domestic, agricultural and industrial effluent discharges, port activities, bank reclamation, dams and fishing (Kennish 2002; Vasconcelos et al. 2007; Dauvin 2008). Accordingly, increased awareness of the potential risks posed by the permanent pressures and overexploitation of these ecosystems resources led to integrated legislative protective action worldwide. These include the European Union (EU) Water Framework Directive (WFD, 2000/60/EC) and the EU Marine Strategy Framework Directive (MSFD, 2008/56/EC), the National Land and Water Resources Audit in Australia (Heap et al. 2001), the Clean Water Act in the United States of America (USEPA 2002) and the Water Act in South Africa (Adams et al. 2002), all aiming at the sustainable and integrated management of water resources.

In general terms, the EU Water Framework Directive aims at: preventing further deterioration of water resources; enhancing protection and improvement of the aquatic environment (e.g. through the reduction of discharges and pollution levels); and mitigating the effects of floods and droughts. According to the WFD all EU member states are required to assess the ecological quality status of their rivers, lakes, groundwater, transitional and coastal waters, and achieve a good ecological status in all water bodies by 2015 (WFD, 2000/60/EC). In this context the definition of good ecological status refers to low levels of distortion on the biological quality elements resulting from anthropogenic activity, when compared to undisturbed reference conditions. Applying this definition to fish, renders that a habitat of good quality for fish is associated with an assemblage with high densities, high species richness, presence of species sensitive to disturbance and suitable conditions for growth, survival and reproduction, taking into account the habitat reference conditions.

Fish, and their responses, have been extensively used as indicators of habitat quality in assessment tools developed for scientific and management purposes (e.g. Deegan et al. 1997; Delpech et al. 2010). The value of fish as biological quality elements relies on some key features: i) their wide dispersion in the aquatic environment and diversity of functional guilds, which covers the spectrum of anthropogenic stressors; ii) their major ecological role in food-webs, carrying energy from lower to higher trophic levels; iii) their relatively long life-span, which accounts for long-term effects of stressors on a biological endpoint; iv) they are a direct resource for men, which heightens the level of public conscious relative to conservation needs and cost-benefit analysis (Whitfield and Elliott 2002, van der Oost et al. 2003).

Earlier approaches to habitat quality assessment for fish looked into species presence or absence data, abundance, production, growth and survival, whenever data was available (e.g. Karr 1981; Rogers 1992; Able et al. 1999). All these descriptors have a significant ecological meaning, ranging from the individual to the community level. However, increasing levels of biological organization are more difficult to relate to specific anthropogenic pressures (Adams 2002). As a consequence, biological responses at the sub-organism level that exhibit faster response time and higher specificity or known mechanistic responses to particular stressors have become a rapid emerging topic, with new techniques and biochemical processes being applied to assess environmental disturbance (Adams 2002, van der Oost et al. 2003).

Biomarkers are generally defined as measures of change in biological responses, at the sub-individual level (e.g. molecular, physiological), linked to a potential anthropogenic hazard, which may be physical, chemical or biological (see review in van der Oost et al. 2003). These can be classified into biomarkers of exposure and biomarkers of effects (some authors also consider biomarkers of susceptibility, which are beyond the scope of this thesis). Biomarkers of exposure refer to biological responses that enable the detection of xenobiotics; while biomarkers of effects include biochemical, physiological or other sub-organism biological changes that can be linked to a stress or health impairment, and thus can also serve as biomarkers of exposure (van der Oost et al., 2003). A wide range of biochemical and physiological measures have been extensively used has biomarkers, such as the activity level of antioxidant enzymes, biotransformation enzymes, metallothioneins which are usually considered biomarkers of exposure; whilst the level of molecular oxidative damage (e.g. lipid peroxidation) and even measures of condition and general health can be used as biomarkers of effects.

Establishing direct relationships between biomarkers of exposure to pollutants and additional ecological consequences at the individual or higher biological level represents a difficult challenge, one which still demands for further research into individual adaptability and the influence of natural environmental conditions (Adams 2002; van der Oost et al. 2003). Hence, it is important to understand how exposure biomarker responses to contamination interact with fish growth and condition, and to assess potential deleterious effects at such relevant endpoints. In addition, fish growth and condition indices have been extensively used as measures of habitat quality, since they are integrative measures of the whole environment (Phelan et al. 2000; Lloret and Planes 2003; Fonseca et al. 2006; Amara et al. 2007; Vasconcelos et al. 2009). Growth and condition indices integrate environmental variability and allow for an assessment of fish health in a given habitat, by considering how fish respond to abiotic and biotic variables, food quality and availability and also pollution. The utility of assessing fish health and condition in a habitat quality assessment context is accentuated by the difficulty to discriminate between the effects of natural variability and anthropogenic induced stress, which has been recently described as the Estuarine Quality Paradox (Dauvin 2007; Elliott and Quintino 2007). Accordingly, Elliott and Quintino (2007) pointed out that to overcome this issue, it would be necessary to quantify the effects of natural stress and subtract this from the anthropogenic stress, or to use alternative methods to detect anthropogenic stress against a background of natural stress.

Aims and importance

The aim of the present thesis was to investigate the use of fish physiological and biochemical responses as indicators of habitat quality in the estuarine environment.

Highly dynamic environmental settings allied with significant anthropogenic stressors present in estuaries provide a complex context for the assessment of estuarine habitat quality for fish. Therefore, different yet complementary approaches were used to resolve various aspects of this issue, i.e. effects of natural variability, effects of contaminants, realistic temporal scales, selection of adequate biomarkers, confounding factors in biomarker analysis, multi-species differences in biochemical and physiological responses. These several dimensions were addressed with laboratory calibration experiments, models application, field assessments and multi-species and multi-biomarkers approaches.

In view of the present thesis context, good habitat quality is associated with favorable conditions for fish growth, low energy expenditure and high metabolic scope for fish activities (e.g. growth, reproduction) as well as low pollution levels that do not elevate biochemical defense and detoxification mechanisms above baseline values.

The value of this study relies on its multiple approaches and methodologies, which allow for a real and ecologically meaningful framework of habitat quality assessment for fish. The expected results include the ability to assess growth and fish condition in time-varying scenarios, the selection of adequate biomarkers and species to evaluate habitat quality in different contexts, and the identification of major confounding factors in biomarker analysis considering these factors variation in a significant time scale.

Growth estimations and condition indices were first used as measures of fish nutritional condition, growth and overall fish health, and hence of habitat quality for fish. These indices can be viewed as integrative of the whole environment, including chemical exposure. Since exposure to contaminants can have deleterious effects on fish health, condition indices were also tested in this study as biomarkers of effects in both experimental and field assessments. This represents an innovative perspective, seldom explored in current research studies.

Overall, the core idea was to go beyond physical-chemical characterization and species numerical assessment of habitat quality, by exploring physiological and biochemical endpoints. These biological endpoints, with higher pressure specificity and early response time, can signal stressors and account for early and reversible biological effects taking into account the natural variability in estuarine conditions.

Ultimately, understanding how fish first respond and integrate environmental quality in an estuarine environment is of the utmost importance for the evaluation of habitat quality and the protection of this economical and ecologically important resource, in view of present and future legislative and management perspectives.

Thesis outline

The present thesis comprises eight scientific papers published or in review in peer reviewed international journals, each corresponding to a chapter.

A review of how fish early growth and condition patterns are related to life-history strategies is discussed in Chapter 2. A broad comparison of early-life features in forty-six fish species, specifically first year length percentage, age at maturation, condition factor K (Fulton), RNA:DNA ratio, and lipid and protein content, was used to evaluate degree of species segregation related with latitudinal distribution, habitat use patterns and life-history strategies [based on the framework developed by Winemiller and Rose (1992)].

In Chapter 3 the dynamics of fish lipid content, including measures at the individual and molecular level, is evaluated through a laboratory experiment. A set of lipid-based indices of condition, from morphometric indices (condition factor K and hepatosomatic index) to individual fatty acid percentages, are evaluated as potential indicators of feeding level.

Further experimental calibration is described in Chapter 4, as a means to estimate juvenile fish growth in estuaries. Growth rate estimates and growth performance based on a nucleic acid model were determined for two marine migrant fish species in five systems along the Portuguese coast during two consecutive years. Overall, habitat quality for both species was analysed based on juveniles' growth performance.

In Chapter 5, estuarine environmental dynamics, fish metabolism and bioenergetics are integrated into a mechanistic model developed to simulate fish growth in time-varying environments, the Ecophys.Fish (Neill et al. 2004). This ecophysiological framework was parameterized for two sole species, and applied to a four year data series for the Tejo estuary, in order to characterize habitat quality based on juveniles metabolic scope for growth and estimated growth rates.

The influence of short-term abiotic environmental variability on several condition indices, in four fish species inhabiting estuarine and shallow coastal areas, was explored in Chapter 6. Variation of condition indices was measured on a scale of consecutive days to weeks, and related to the variation of water temperature and salinity on a concordant time scale. The potential differences in fish condition indices due to the contrasting environments were also discussed.

In Chapter 7 a wide array of biomarkers of exposure to and effects of contaminants were tested in an experimental setting, designed to observe the combined responses of these biomarkers to chemical stress (copper contamination). Fish biomarker responses included lipid peroxidation, morphometric and biochemical condition indices as measures of biological effects, whilst metallothioneins were used as specific biomarkers of exposure to metal toxicity.

The effects of short-term temporal variability of environmental conditions on biomarkers of exposure were determined on Chapter 8. A set of adaptation responses, with different degrees of specificity to contaminants, were determined in three fish species, sampled on estuarine sites on a daily and weekly scale, and the relation with abiotic variables (temperature and salinity) assessed.

In Chapter 9 an integrated multi-biomarker and multi-specific approach was applied to assess habitat quality in two estuarine systems, integrating previously applied metrics and results. Habitat chemical characterization, measured in the form of metals and polycyclic aromatic hydrocarbons concentration, was used as covariate in the analysis on the spatial variability patterns in fish biomarker responses. Species specific differences in biomarker responses were also discussed in terms of life-history traits and habitat use.

A general discussion of the major results from these studies is presented in the final Chapter 10. In this chapter, an integrative view of the main conclusions is presented within the context of the use of fish as indicators of habitat quality, particularly in estuarine systems, as well as future research perspectives.

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CHAPTER 2

Are fish early growth and condition patterns related to life-history strategies?

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Are fish early growth and condition patterns related to life-history strategies?

Abstract: Life-history studies provide a global framework for comparison of fish species responses and trade-offs facing ecological and environmental constrains. A broad comparison among fishes' early growth and condition traits is performed in order to determine ecological patterns of early development regarding latitudinal distribution, habitat use and life-history strategies. Based on Winemiller and Rose (1992) classification of life-history strategies, data on early growth and condition indices of 46 fish species worldwide was analysed. Available information on fishes' early features, namely first year length percentage (relative to species maximum theoretical length), age at maturation and Fulton's condition index (K), provided a good segregation of species by latitudinal distribution and habitat use, and evidenced the categories of the three-endpoint model. Higher larvae and juvenile growth rates and condition indices (K, mean RNA–DNA ratios and protein contents) were associated with tropical and temperate fish species that occur in complex or variable habitats (respectively coral reefs and estuaries). These species selected for the opportunistic and periodic strategies, investing highly in rapid growth in order to increase survival probability to counter high mortality rates during early stages or unstable habitat conditions. Later age at maturation, slower larvae and juvenile growth as well as lower mean condition indices were consistent with fish species from more stable or predictable environments, as polar regions and freshwater habitats, which selected for the equilibrium strategy. Nonetheless, differences in energy allocation strategies during early stages were not observed, evidencing the scarcity of available data regarding condition indices and/or the importance of integrating life-history intermediate strategies. Future research into condition indices and other physiological processes, for a broader set of species and for a wider latitudinal and habitat range including seasonal variability (particularly for species from tropical and polar regions), is essential to better understand or test current theories of species ecological patterns. The use of direct quantitative measures of young fishes' metabolic investment and fitness constitutes a new approach for life history studies, and should be fundamental for predicting species' responses to acute environmental or human constrains, especially in a global climate change scenario that is expected to affect distribution and abundance of fish species worldwide.

Key-words: fish; early growth; condition; life history strategy

1. Introduction

Life-history traits and strategies of organisms have long faced natural selection, and the comparative study of patterns emerging from their evolution is the goal of life-history theory (e.g. Stearns 1976, 1992; Roff 1992). Life-history studies also examine how traits are intercorrelated and constrained by ecological factors and constitute an important tool to predict demographic responses to disturbances at variable spatial and temporal scales (Winemiller 2005). The different environmental conditions and habitats colonized by fish species sustain a certain degree of phenotypic plasticity within life-history patterns (Stearns 1989). However, not all strategies are expected to be maintained throughout consecutive generations, and an evolutionary convergence is believed to occur among phylogenetically close-related species and even between distributional areas (Vila-Gispert et al. 2002).

Fishes' response to environmental and ecological constrains is complex, depending on the intricate relations between environmental conditions and the individual ability to adapt. Fish exhibit various adaptations or strategies that result from trade-offs among growth, reproduction and survival (Arendt 1997). For example delaying the age at maturation allocates more energy for juvenile growth (Rochet 2000); and assuming higher predation risks under suitable growth conditions, can also maximize growth rates with the payoff of better overcoming size dependent overwinter mortality (Schindler 1999).

Following Winemiller and Rose classification (1992) three life-history strategies of fish species can be identified as the endpoints of a trilateral continuum based on trade-offs among fish survival, fecundity and age at maturation. The opportunistic strategy maximizes the intrinsic rate of population growth through a reduction in mean generation time. This strategy is characterized by small-sized individuals with early maturation, that continuously release small eggs in order to colonize highly disturbed or constantly changing habitats. The periodic strategy concerns highly fecund fishes with some degree of delayed maturation that can exploit predictable spatial and temporal environmental patterns, in which habitat conditions are more suitable for growth and survival of the massive number of offspring produced. The equilibrium strategy comprises small to medium sized fish with delayed maturation that produce small clutches of large eggs and that present well developed parental care (Winemiller and Rose 1992). This model's assumptions regarding fish early-life stages are that equilibrium strategists have higher juvenile survival, while periodic and opportunistic strategists have higher larval and juvenile growth rates.

Given the importance of early-life stages in population dynamics (May 1974), such that even small differences in early growth and survival rates can affect the number of recruits entering the adult stock (e.g. Houde 1987, 1989), and the high energetic costs associated with high development or growth rates (Kamler 1992), fishes' strategies during early development are crucial for species success. Mortality rates at early stages are generally high and related to size-dependent factors, namely starvation and predation pressures (Miller et al. 1988; Kamler 1992; Houde 1994). Thus survival probability in early-life stages should increase with rapid growth, since larger fish have higher food consumption rates and lower vulnerability to predators when compared to smaller individuals (e.g. Peterson and Wroblewski 1984; Miller et al. 1988; Hoey and McCormick 2004). Larvae and juvenile fish in better condition are also presumably less likely to die of starvation or predation, since nutritional standing and energy storage act as buffers against environmental variability (Kerrigam 1996; van Dijk et al. 2005). Several measures of growth and condition of larvae and juvenile fish have been used to assess individual and population status as well as habitat quality (e.g. Buckley et al. 1999; Yamashita et al. 2003; Gilliers et al. 2004; Fonseca et al. 2006). These measures comprise growth indices (RNA:DNA ratios, protein specific growth rate, otolith increments), morphometric indices (Fulton's K) and storage indices (lipid content) that relate to the individual ability to respond and interact with the environment at different time scales (Suthers 1998).

Because life-history studies provide a basis for comparison and grouping of fish species considering their responses to environmental variation, our goal is to determine ecological patterns of fish species during their early development regarding latitudinal distribution, habitat use and life-history strategies using Winemiller and Rose (1992) classification. Even though a latitudinal gradient in species metabolic pace is expected due to the regulatory effect of temperature, phylogenetic, ecological and habitat constraints should also affect species responses.

Therefore, the Winemiller and Rose model (W&R model) is analysed, particularly the early development strategies, in a new perspective for life-history studies, based on early-life growth and condition data by considering direct quantitative measures of young fishes' metabolic investment and fitness. It is also hypothesised, based on the W&R model, that fish inhabiting more stable environments and that favour young fish survival can potentially allocate more energy for storage than fishes facing more variable environments and that invest on rapid growth. Thus there should be a difference between growth related indices and energy storage indices when considering different life-history strategies.

2. Materials and methods

2.1 Ecological and life-history dataset

Data on ecological and life-history traits were collected from literature sources for 46 fish species or genus worldwide (see Appendix). Fish early-life stages were characterized by larvae and juvenile growth (mm day^{-1}); length percentage at the end of the first year relative to the species maximum theoretical length (MTL); age at maturation (years); Fulton's condition index (K); RNA:DNA ratio for larvae and juvenile fish; and lipid and protein body content (% dry-weight).

Three major categorical descriptions were considered for species ordination purposes: (i) latitudinal distribution defined by climatic regions (polar, temperate, subtropical, tropical, and deepwater species); (ii) juvenile fish habitat (estuarine and shallow coastal waters, freshwater, marine, and coral-reef species); (iii) and life-history strategies based on the three-endpoint model by Winemiller and Rose (1992) (opportunistic, periodic and equilibrium strategists). Briefly, for the latter classification: equilibrium strategists present higher values of juvenile survival and delayed maturation, periodic strategists relate both to high fecundity and age at maturation, while the opportunistic strategists are characterized by low fecundity, low juvenile survival and early maturation. Each species strategy was considered as a weighed collection of five life-history traits that accounted for a good distinction of species strategies in previous work: parental care; egg size; number or spawning bouts per year; clutch size and length at maturation (Winemiller and Rose 1992). These five variables were considered of equal importance, as well as their degrees of variation concerning information on species life-history traits (e.g. degree of parental care as offspring placement or nest protection and care by parents; for further detail see Winemiller and Rose 1992). Larvae and juvenile growth as well as age at maturation were excluded from this classification in order to avoid redundant information in the analysis that evaluates the relationship between species' strategies and significant early life-history traits. Thus, our assumption was that if the model provides a good prediction, then the species ordination should evidence the different life-history strategies, habitat distribution and habitat use, associated with early-life features. The choice of species was limited by data availability and to coherent determinations of early growth and condition variables among species and studies. Despite variability in protocols mainly for condition measures, only similar and thus comparable data was used, taking into account several important factors: fish development stage, environmental or experimental conditions characteristic of each species natural environment, sample type and size and equal measurement units or ratios. In the present work, measures of condition are not used for assessment of species in better condition, since no direct comparisons of indices' values are intended or interpreted as so. Instead, they are viewed as measures of metabolic activity and energy storage that result from fishes' tactics facing specific environmental and ecological constraints. Values selected for condition indices were those characterized as indicators of good health or condition for each species by the respective authors, so that all species were equitably compared and appropriate for the study's goal.

2.2 Statistical analysis

A principal components analysis (PCA) was performed on ln-transformed fish data in order to identify patterns of association among species and early-life traits according to habitat type, latitudinal distribution and life-history strategies. For each category (e.g. marine habitat, temperate region, equilibrium strategy) of fish guilds the mean value of the nine early-life variables was determined. These categories were then compared using non-parametric Kruskal–Wallis tests followed by *a posteriori* multiple comparisons (Zar 1996), since the data set failed to meet the assumptions of parametric tests, even after transformation. The differences between categories would not account for the total variability of the data set; nonetheless given the limitations of the available data these differences might explain a great part of the species aggregation patterns. The significant variables per fish guild (i.e. variables which best describe the variability of the data from the latitudinal distribution, habitat use and life history strategies) were then analysed separately in three new ordination analyses (PCA). These different PCA will also determine which guild or categories type by latitude, habitat and life-history strategy will allow a clearer species segregation.

In order to test differences between growth investment and energy storage of fish species with different life-history strategies, Spearman correlation coefficients were determined among all variables. The PCAs were produced using the CANOCO 4.5 software, while the remaining analyses were performed using the STATISTICA 6.0 software.

3. Results

Some patterns of association among species and life-history traits were observed from PCA analysis (Fig. 1). The PCA analysis accounted for 45% of the total variation when considering the first two principal components. The diagram put in evidence some relations among

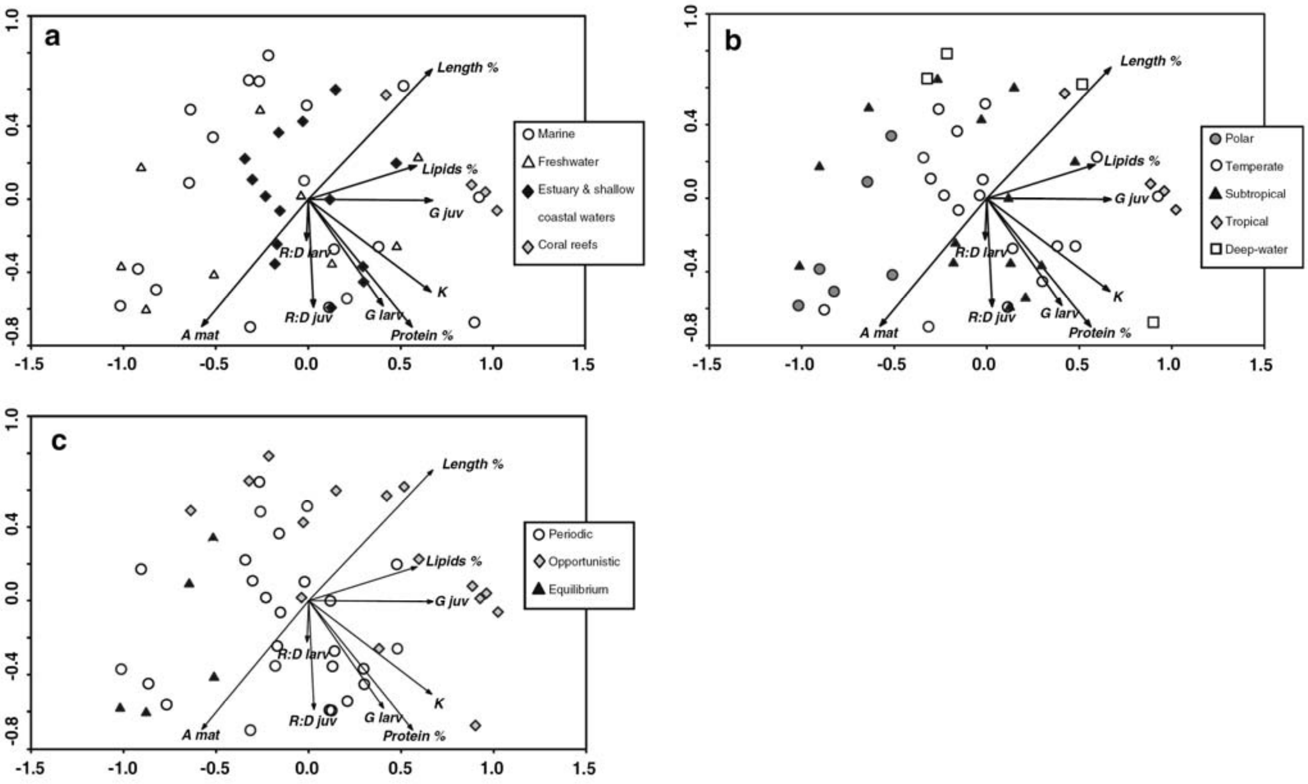

Figure 1. Fish species ordination (PCA) based on their ln transformed data of early growth and condition. Species characterization by **(a)** habitat type (○marine; △freshwater; ◆ estuary and shallow coastal waters; ◇ coral reefs), **(b)** latitudinal distribution (● polar; .○ temperate; .▲ subtropical; ◇ tropical; □ deepwater), and **(c)** life-history strategy (○ periodic; ◇ opportunistic; ▲ equilibrium).

variables, namely the opposite linear setting of age at maturation and of the first year length percentage of the species MTL. Larvae growth rates and bulk protein percentage, as well as, the RNA:DNA ratios of larvae and juvenile fish appear to be close related. The PCA evidenced general trends on the data set based on the categorical classification of species ecology. Figure 1a illustrates juvenile fish habitats differentiation based on early life-history characteristics, and distinguishes between reef-associated species on one side, estuarine and shallow coastal waters species in a central ordination, and on the other end marine and freshwater fish species. Considering the species latitudinal distribution (Fig. 1b), species from the tropical region are set together on one end of the diagram whereas polar species were positioned on the other end. In between are mainly species from temperate regions along with most subtropical fish species. Species that inhabit deep pelagic environments present a more scattered pattern in the diagram. The life-history classification of fish species in the ordination plot (Fig. 1c) also highlights a gradient of life-history strategies, with equilibrium, periodic and opportunistic strategists comparatively spaced out from each other.

The mean values of early-life traits examined for each categorical variable emphasize the different strategies or tactics adopted by fish in response to environmental and other ecological constraints (Table 1). In what concerns habitat differences some trends are observed. Higher values of first year growth and Fulton's K are found for young fishes from coral reef areas and from estuarine and shallow coastal areas, which contrasts with their earlier mean age at

Table 1. Mean values (and standard errors) of early-life traits relative to each categorical variable: habitat, latitudinal distribution and life-history strategy.

	Larvae growth	Juvenile growth	Fulton's K	RNA:DNA Larvae	RNA:DNA Juvenile	Total protein %	Total lipids %	Length % MTL	Age at maturation
Habitat									
Estuary and shallow coastal waters	0.35 (0.04)	0.60 (1.40)	1.40 (0.13)	4.49 (0.61)	4.38 (0.77)	14.90 (5.16)	10.65 (0.59)	31.42 (4.12)	2.64 (0.23)
Freshwater	0.34 (0.08)	0.39 (0.10)	0.91 (0.17)^{a}	2.85 (0.63)	3.42 (0.65)	11.97 (2.46)	10.24 (4.96)	28.25 (7.40)	5.36 (1.92)^{a}
Marine	0.60 (0.27)	0.60 (0.09)	1.35 (0.30)^{a}	3.75 (0.48)	4.88 (1.35)	27.93 (9.50)	19.02 (4.55)	27.64 (3.43)	3.38 (0.55)^{a}
Coral reef	0.60 (0.09)	0.58 (0.09)	2.78 (0.55)	–	–	29.51 (3.82)	18.72 (2.80)	49.19 (7.16)	1.16 (0.16)
Latitudinal distribution									
Deep-water	–	0.70 (0.28)	0.96 (0.23)	–	–	10.18 (4.12)	25.85 (9.88)	29.51 (4.50)	2.23 (0.67)
Polar	0.37 (0.08)	0.26 (0.07)	0.58 (0.27)^{bc}	2.43 (0.71)	3.90	9.90	20.83 (6.09)	13.53 (3.98)^{b}	5.43 (1.64)^{bc}
Subtropical	0.37 (0.05)	0.55 (0.07)	1.19 (0.19)	4.55 (0.87)	3.19 (0.59)	20.98 (6.46)	9.79 (5.85)	29.85 (4.96)	2.89 (0.37)
Tropical	0.60 (0.09)	0.58 (0.09)	2.78 (0.55)^{b}	–	–	29.51 (3.82)	18.72 (2.80)	49.19 (7.16)^{a}	1.16 (0.16)^{b}
Temperate	0.32 (0.05)	0.58 (0.07)	1.51 (0.19)^{c}	3.86 (0.25)	5.69 (0.85)	24.45 (7.71)	9.75 (2.37)	32.09 (3.80)^{b}	3.93 (0.99)^{c}
Life-history strategy									
Equilibrium	0.29 (0.10)	0.23(0.06)^{ab}	0.61 (0.19)	2.43 (0.71)	3.90	12.50 (2.60)	16.93 (5.92)	12.82(3.16)^{cd}	8.34 (3.18)^{de}
Opportunistic	0.68 (0.27)	0.60 (0.09)^{a}	1.78 (0.33)	4.31 (0.32)	2.85 (2.25)	28.57 (7.60)	15.01 (2.05)	43.17 (4.02)^{c}	2.14 (0.39)^{d}
Periodic	0.37 (0.04)	0.59 (0.05)^{b}	1.41 (0.14)	4.10 (0.46)	4.48 (0.59)	17.95 (3.74)	9.95 (3.13)	27.20(2.93)^{d}	4.03 (0.23)^{e}

Values sharing like characters are significantly different, within each column (a posteriori tests; *P* < 0.05) maturation. It was also noticed that freshwater species showed the lowest condition indices. According to the Kruskal–Wallis tests performed, only freshwater and marine species were significantly different for Fulton's K and age at maturation ($H = 9.23$, $P < 0.05$).

Categorization of species based on their latitudinal distribution underlined the previous pattern of fast growth, high condition indices (K and bulk protein content) and early maturation for tropical fishes. Subtropical and temperate fishes also had similar trends, although the mean values observed were less diverse (Table 1). Fish species from deep marine water also had fairly high growth indices and particularly high body lipid percentage (ca. 26%). On the other hand, polar fishes evidenced low growth rates, with one-year individuals reaching only around 14% of their MTL, high lipid reserves, and delayed age at maturation (Table 1). In fact, the Kruskal–Wallis tests for Fulton's K, age at maturation and first year length percentage evidenced significant differences for fishes from polar regions relative to fishes from tropical and temperate regions ($H > 9.8$, $P < 0.05$, in all tests).

The categorization of species life-history strategy emphasized some distinctive traits such that equilibrium species presented lower larvae and juvenile growth rates along with late maturation. Equilibrium strategists from periodic and opportunistic strategists were significantly different for juveniles' growth rates, first year length percentage of the MTL and age at maturation ($H > 6.8$, $P < 0.05$, in all tests). The opposite set of traits was described for both opportunistic and periodic species, i.e. higher young growth rates and early maturation. The significant difference between the two strategies in the length percentage attained by one year-old individuals relative to species MTL: opportunistic fish species grew up to around 43% of their MTL and periodic fishes only grew, on average, about 27% ($H = 15.3$, $P < 0.05$).

The PCA analyses performed using only the significant traits for each category type previously identified improved significantly species segregation, and explained between 75% and 87% of the data variability in the first two axis. The species habitat plot clearly shows positive correlations of fishes from coral reefs and fishes from estuarine and shallow coastal waters with Fulton's condition index. Freshwater species evidenced a negative correlation with K, but were positively correlated with age at maturation (Fig. 2).

Young fishes latitudinal distribution by climatic regions outlines the positive relationship of tropical fishes with first year length percentage and Fulton's K. It is also noticeable that polar species are positively correlated to age at first maturation and negatively correlated with both K and first year length percentage. Fishes from intermediate latitudes show a more scattered pattern (Fig. 3).

The analysis that considered life-history strategies (Fig. 4) also evidenced gradients: opportunistic strategists were associated with high juvenile growth rates and first year length percentage of the MTL, and were negatively correlated with age at maturation. Equilibrium strategists showed the exact opposite associations with these early-life traits. The periodic strategy was set between the other two strategies in the PCA ordination diagram (Fig. 4).

The Spearman correlation analysis only evidenced a significant correlation between juvenile growth rates and the first year length percentage of the species MTL ($r = 0.382$; $P < 0.01$), thus

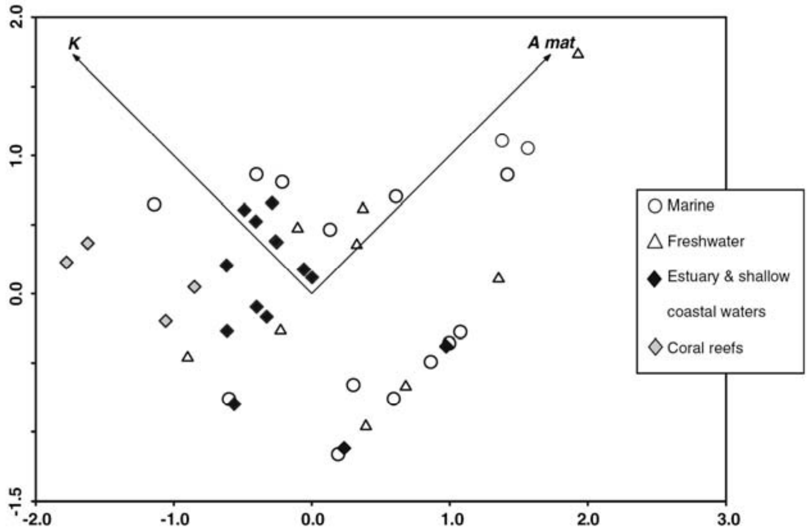

Figure 2. Fish species ordination (PCA) according to the early-life traits that described significant differences between species habitat use (○marine; △ freshwater; ◆ estuary and shallow coastal waters; ◇ coral reefs): Fulton's condition index (K) and age at maturation (A mat).

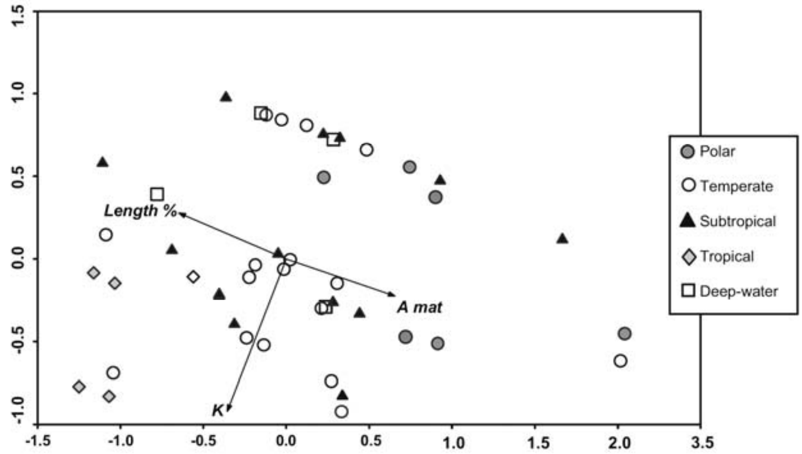

Figure 3. Fish species ordination (PCA) based on first year length percentage (relative to species maximum theoretical length- Length %), Fulton's condition index (K) and age at maturation (A mat), according to significant differences evidenced in species classification by latitudinal distribution (●polar; ○ temperate; ▲ subtropical; ◇ tropical; □ deep-water).

no negative correlation was determined for growth related indices and energy storage indices as hypothesized.

4. Discussion

Trade-offs between growth and other life-history traits have been described for several fish species as the result of phylogenetic and environmental constraints (e.g. Rochet 2000; Mangel and Stamps 2001; Vila-Gispert et al. 2002; Kamler 2005). In the present work some patterns of fish early growth and condition strategies have emerged from species latitudinal distribution range, habitat use and life-history strategy. Age at maturation, first year length percentage of the species MTL and Fulton's K allowed a clear distinction between fish species from different climatic regions, habitats and life strategies.

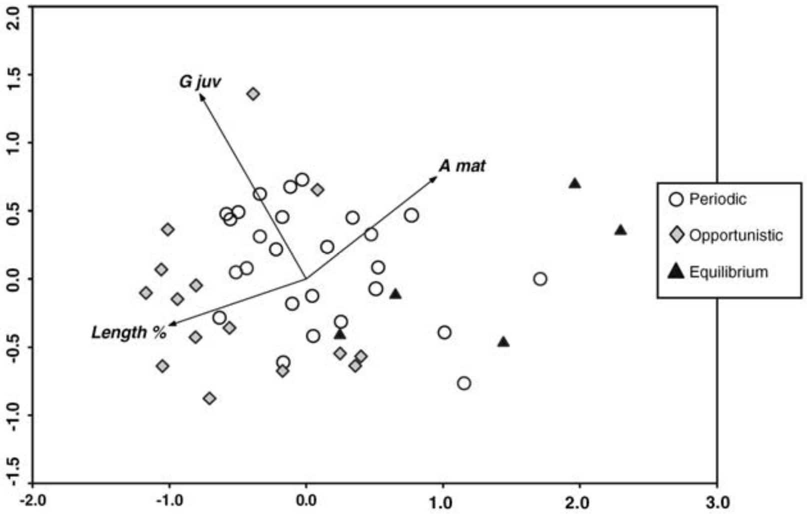

Figure 4. Fish species ordination (PCA) based on the first year length percentage (relative to species maximum theoretical length - Length %), juvenile growth rate (G juv) and age at maturation (A mat), according to significant differences described in the species categorization by life-history strategies (○ periodic; ◇ opportunistic; ▲ equilibrium).

Higher growth rates of larvae and juvenile fish were associated with temperate and tropical regions, along with complex (reefs) or fairly unstable habitats (estuaries). Several reasons might account for such pattern. At temperate latitudes there is a marked seasonal variation, somewhat predictable at a large temporal scale that could be explored by fish species in order to synchronize reproduction with most favourable conditions for larval growth and survival (Boyce 1979). Moreover, high variability at settlement is a strong factor in population regulation. Thus rapid growth should improve survival probability of young temperate fishes, which is selected as an effective strategy. In fact, for some temperate fish, slow growth has been related to lower survival probability, as mortality rates declined with fish size (Peterson and Wroblewski 1984; Houde 1987; Fortier and Quinonez-Velasquez 1998). Estuaries are highly productive and dynamic environments influenced by frequent changes in tide regime, seasonal variation in climate and river discharges (McLusky and Elliot 2004). Not many fishes can tolerate these natural stress conditions, but those who do benefit from the high food availability and refuge from predators (Miller et al. 1985). Estuaries are used as nursery areas by juveniles of many marine fish species and the favourable conditions for growth result in high growth rates of young fishes described by several authors (e.g. Cabral 2003; Yamashita et al. 2003; Islam and Tanaka 2005).

Tropical reefs comprise some of the densest and species diverse habitats in the world (Blaber 2000). Although reefs are fairly stable habitats that only occur within certain physical and environmental limits (Blaber 2000), they are frequently subjected to natural disturbances (Sims and Jones 2000) and coral reef fishes experience severe mortality during early-life stages (e.g. Jones 1990; Carr and Hixon 1995; Caley 1998; Webster 2002). Many tropical reef associated species, as those studied in the present work, possess complex social schemes (e.g. courtship behaviour, nest guarding, sex reversal), which strongly influence the reproductive probability of each individual, generally favouring larger sized fishes (Jones 1987).

In these conditions faster growth seems to confer selective advantages for young fishes. Wilson and Meekan (2002) found that fast growing cohorts of *Stegastes partitus* larvae (a coral reef species) had higher survival than slow growing cohorts, and thus contributed more to the replenishment of benthic populations than slow growing cohorts of this species.

Fishes from higher latitudes presented slower growth during early stages. Polar marine regions are characterized by well-oxygenated waters with narrow ranges of cold temperatures (Rose et al. 2000), and therefore may be considered a stable habitat as suggested by the reduction of erythrocyte and haemoglobin types in icefishes' blood from these latitudes (di Prisco et al. 1990; Kunzmann 1991). Many fish species from these areas also present antifreeze physiological mechanisms (De Vries 1988; Kock 1992), whose production and regulation should leave less energy available for growth and reproduction (Rose et al. 2000).

The condition indices determination as proxy measures for individual growth and fitness is a recent subject area that relates fish's responses with environmental variability. However, it still evidences the need for species and laboratory's intercalibration in order to permit more objective species and areas' comparisons (Buckley et al. 1999; Suthers 1998). In this first approach species condition was compared as differences in fishes' metabolic investment and energy storage strategies during early-life. Our results revealed important differences in young fishes Fulton's K when distinguishing between species from different habitats and climatic regions. As observed for growth, K was positively associated with fishes inhabiting estuarine and coral reef habitats, from tropical and temperate regions that ultimately selected for periodic and opportunistic strategies. For example, higher Fulton's K and total lipid content was related with increased survival of a Caribbean damselfish (*Stegastes partitus*) shortly after settlement (Booth and Hixon 1999). Similarly, Hoey and McCormick (2004) found that predation at reef settlement was selective for a tropical reef fish (*Pomacentrus amboinensis*) that grew slowly during late larvae phase, were low in total lipids, but had a high Fulton's K (thus the importance of determining several condition measures). Mean RNA:DNA values also revealed some differences (although not statistically significant), mainly for fish larvae. Higher RNA:DNA ratios were observed for species that presented faster growth, thus supporting the idea of higher metabolic investment for protein synthesis, which is also deeply related to the effect of water temperature on the RNA activity. The establishment of more RNA:DNA-temperature growth models (e.g. Buckley 1984; Peck et al. 2003; Caldarone 2005; Mercaldo-Allen et al. 2006) or regression models for RNA variation with fish size considering species from different latitudes and habitats should give further insight into this relationship.

Protein and lipids mean percentages did not differ between categories except for deep-water species, for which a high lipid content is associated with high wax esters percentage for buoyancy requirements during vertical migrations (e.g. Sabatés et al. 2003). In this case, the discrimination of lipid classes is essential to identify the biological roles of lipids associated with energy demands. However, differences in lipid composition related to different life strategies have been observed for five notothenioid fish species from high-Antarctic seas (Hagen et al. 2000).

Fishes from higher latitudes are known to have slower metabolic processes, which have implications on energy budgets and life-history strategies (Johnston 1993). The marked seasonal patterns in climate that strongly determine growth and metabolism offset in these species could also explain the negative relationship between most condition indices and polar fishes in the present study.

The lack of data for most condition indices and especially for species from tropical and polar latitudes does not allow any further conclusions regarding species life-patterns. This fact could explain why only Fulton's K, that presented the more complete dataset, evidenced significant differential patterns of species distribution and life strategy. Nonetheless, the inclusion of the condition of young fishes in any analysis of lifehistory patterns or population dynamics is justified by the importance of including the quality of early life stages, since recruitment variability and year class strength have been related to recruits' growth and condition (Bailey et al. 1995; Meekan and Fortier 1996). Size related success or 'bigger is better' has been readdressed to comprehend faster growth in increased survival probability (McCormick and Molony 1993; Suthers 1998). However, the ecological importance of the several condition indices still needs further investigation as suggested by Suthers (2000) and by our results, and should also consider a broader set of species.

The species life-history strategies analysis emphasized distinctive traits described in the three-endpoint model by Winemiller and Rose (1992). Opportunistic strategists evidenced faster early growth and were negatively correlated with age at maturation. These species also presented higher protein and lipids content, possibly due to higher energy requirements to face high mortality rates or to support drastic changes in environmental conditions. Tropical reef species and some estuarine fishes are examples of species with this strategy. Most marine fish species were considered periodic strategists based on high growth indices, although lower than those observed for the opportunistic end-point, and on the relatively delayed age at maturation when considering the opportunistic strategy. Equilibrium species presented lower larvae and juvenile growth rates as well as later age at maturation. Fishes from freshwater habitats and polar regions were mainly associated with the equilibrium strategy. These associations are in accordance with previous analysis of life-history variation among fish species from Europe and America (Vila-Gispert et al. 2002).

The early growth assumptions considered in the W&R model are supported by the current results; nonetheless differences in species energy allocation during early stages, based on growth expenditure versus energy storage, were not observed. The concept that young fish don't have differentiated energy allocation strategies regardless of their metabolic pace is not consistent with previous findings for different fish species. For example, Morgan et al. (2002) found differences in fatty acids accumulation in juvenile Atlantic salmon *Salmo salar*, related to individuals' size and migration timing. Sogard and Spencer (2004) described trade-offs between somatic growth and lipid storage for juvenile sablefish *Anoplopoma fimbria* under unfavourable conditions.

Two reasons can account for the lack of correlation between growth and storage indices in the present work: insufficient data available on young fish condition covering a more diversified set of species, and/or the inadequate classification of species life-history strategy, since the W&R model is a continuum that acknowledges the existence of intermediate strategies. Intermediate strategies consist of traits and tactics usually associated with two different strategies, which evidences the flexibility of this classification and the need to consider each species individually. As an example, the coral reef species analysed in the present work evidenced not only fast early-growth indices and early maturation associated with the opportunistic strategy, but also some degree of parental care (e.g. nest guarding, eggs deposition on specific substrate) usually associated with the equilibrium strategy.

The early-life ecological patterns described by our results sustain W&R model predictions, as well as many other studies on ecophysiology, growth, condition, ecological limits and trade-offs of various fish species worldwide. The high degree of species segregation obtained for the fish guilds of latitudinal distribution, habitat use and life-history strategy evidence the importance of early-life stages for individual success and species perseverance. The results also highlight the importance of considering lower levels of biological organization, such as physiological measures of young fishes' energetic investment and trade-offs, in future studies regarding ecological processes or establishing management priorities. What was also evident was the need to further investigate the condition and other physiological processes for a broader set of species and a wider range of habitats, in order to better understand and support the current concepts on patterns of species responses to ecological and environmental constrains. Seasonal variation in higher latitudes is another important factor not usually considered, but that is known to introduce variability in fishes' physiological patterns of energy storage and expenditure (e.g. Hernaman and Munday 2005; van Dijk et al. 2005), therefore requiring further studies.

Life-history strategy identification can also help predict populations' response to acute environmental or human constrains, which allows for a better management of fish stocks and habitat quality (Winemiller 2005). In the present global climate change scenario, the expected impacts of increasing water temperature and sea level rise on fish and fisheries will be reflected in the distribution and abundance of fish species, affecting migratory patterns and fish communities worldwide (Roessig et al. 2004). These effects should be particularly strong on species associated with vulnerable habitats (tropical reefs, estuaries and coastal lagoons) and with relatively small temperature ranges (e.g. polar seas) (Kock 2005; Roessig et al. 2004). Predicting species responses to these environmental changes will depend on our knowledge of their ability to adapt, i.e. of their life-history strategies and tactics, and especially on the integration of this information in future conservation and management plans.

Appendix

List of the fish species analysed, categories assignment and respective sources of the collected data. Fish species were classified based on juvenile habitat (F - freshwater; ES - estuarine and shallow coastal waters; M - marine; C - coral reefs), climatic region (DW - deep water; P - polar ST - subtropical; T - temperate; TP - tropical) and life-history strategies (Eq - Equilibrium; Op - opportunistic; Pe - periodic).

Species	Categories	Main sources
Acipenser transmontanus Richardson, 1836	F, T, Eq	Miller and Beckman (1992), Hung et al. (1997), Froese and Pauly (2006)
Ammodytes spp.	ES, T, Op	Buckley (1984), Froese and Pauly (2006)
Anguilla japonica Temminck and Schlegel, 1847	ES, ST, Pe	Kawakami et al. (1999), Dou et al. (2003), Froese and Pauly (2006)
Anoplopoma fimbria (Pallas, 1814)	M, DW, Op	Clarke et al. (1999), Sogard and Spencer (2004), Froese and Pauly (2006)
Benthosema glaciale (Reinhardt, 1837)	M, DW, Op	Sabatés et al. (2003), Froese and Pauly (2006)
Clupea harengus Linnaeus, 1758	M, T, Op	Buckley (1984), Clemmesen (1993), Clemmesen (1994), Mathers et al. (1994), Suneetha et al. (1999), Froese and Pauly (2006)
Coregonus spp.	F, T, Pe	Steinhart and Eckmann (1992), Malzahn et al. (2003), Froese and Pauly (2006)
Diaphus kapalae Nafpaktitis Robertson and Paxton, 1995	M, ST, Op	Suthers (1996), Rissik and Suthers (2000), Froese and Pauly (2006)
Dicentrarchus labrax (Linnaeus, 1758)	ES, ST, Pe	Mustafa et al. (1991), Folkyord et al. (1996), Froese and Pauly (2006)
Diplodus sargus (Linnaeus, 1758)	ES, ST, Pe	Lloret and Planes (2003), Froese and Pauly (2006)
Gadus morhua Linnaeus, 1758	ES, T, Pe	Buckley (1979, 1984), Suthers et al. (1992), Clemmesen and Doan (1993), Lochmann et al. (1997) Couture et al. (1998), Morais et al. (2001), Caldarone et al. (2003), Peck et al. (2003), Froese and Pauly (2006)
Gobionotothen gibberifrons (Lönnberg, 1905)	M, P, Eq	North (1998), Morales-Nin et al. (2002), Froese and Pauly (2006)
Hygophum benoiti (Cocco, 1838)	M, DW, Op	Olivar and Palomera (1994), Sabatés et al. (2003), Froese and Pauly (2006)
Kareius bicoloratus (Basilewsky, 1855)	ES, T, Pe	Malloy et al. (1996), Yamashita et al. (2003) Froese and Pauly (2006)
Lateolabrax japonicas (Cuvier, 1828)	ES, ST, Pe	Ai et al. (2004), Islam and Tanaka (2005), Froese and Pauly (2006), Islam et al. (2006)
Limanda limanda (Linnaeus, 1758)	ES, T, Pe	Gilliers et al. (2004), Froese and Pauly (2006)
Macquaria novemaculeata (Steindachner, 1866)	F, ST, Pe	Suthers et al. (1996), Froese and Pauly (2006)
Mallotus villosus (Müller, 1776)	M, P, Pe	Ferron and Leggett (1994), Elliot and Legget (1998), Froese and Pauly (2006)
Maurolicus muelleri (Gmelin, 1789)	M, DW, Op	Goodson et al. (1995), Ikeda (1996), Sabatés et al. (2003), Froese and Pauly (2006)

Appendix (cont.)

Species	Categories	Main sources
Melanogrammus aeglefinus (Linnaeus, 1758)	M, T, Pe	Buckley (1984), Peck et al. (2003), Caldarone (2005), Froese and Pauly (2006)
Morone saxatilis (Walbaum, 1792)	ES, T, Pe	Buckley (1984), Martin and Wright (1987), Secor and Dean (1989), Cooper (1998), Froese and Pauly (2006)
Notolepis coatsi Dollo, 1908	M, P, Eq	Morales-Nin et al. (2002), Froese and Pauly (2006)
Oncorhynchus mykiss (Walbaum, 1792)	F, T, Pe	Ferguson and Danzmann (1990), Mathers et al. (1993), Weber et al. (2003), Froese and Pauly (2006)
Paralichthys dentatus (Linnaeus, 1766)	ES, T, Pe	Buckley (1984), Malloy and Targett (1994), Froese and Pauly (2006)
Paralichthys olivaceus (Temminck and Schlegel, 1846)	ES, ST, Pe	Fukuda et al. (2001), Gwak and Tanaka (2001), Gwak et al. (2003), Cho (2005), Froese and Pauly (2006)
Paramonacanthus otisensis Whitley, 1931	M, ST, Pe	Suthers et al. (1996), Froese and Pauly (2006)
Pimephales promelas Rafinesque, 1820	F, T, Op	Jennings (1996), Weber et al. (2003), Froese and Pauly (2006)
Pleuronectes platessa Linnaeus, 1758	ES, T, Pe	Chritensen and Korsgaard (1999), Gilliers et al. (2004), Hovenkamp (1990), Hovenkamp and Witte (1991), Paulsen and Støttrup (2004), Froese and Pauly (2006)
Pomacentrus amboinensis Bleeker, 1868	C, TP, Op	Kerrigan (1994), Kerrigan (1996), Lewis (1997), Hoey and McCormick (2004), Froese and Pauly (2006)
Pomacentrus nagasakiensis Tanaka, 1917	C, TP, Op	Kerrigan (1996), Froese and Pauly (2006)
Pomatoschistus spp. and *Gobius* spp.	ES, ST, Op	Esteves et al. (2000), Froese and Pauly (2006)
Psetta maxima (Linnaeus, 1758)	M, T, Pe	Cunha et al. (2003), Cho et al. (2005), Imsland et al. (2001, 2002), Peron-Le Ruyet et al. (2002), Froese and Pauly (2006)
Pseudopleuronectes americanus (Walbaum, 1792)	ES, T, Pe	Buckley (1982, 1984), Mercier et al. (2004), Montgolfier et al. (2005), Froese and Pauly (2006)
Rutilus rutilus (Linnaeus, 1758)	F, T, Op	van Dijk et al. (2005), Froese and Pauly (2006)
Salvelinus alpines (Linnaeus, 1758)	F, P, Eq	Blanar et al. (2005a, b), Miglavs and Jobling (1989a, b), Froese and Pauly (2006)
Sardina pilchardus (Walbaum, 1792)	ES, ST, Op	Chícharo (1998), Chícharo et al. (1998), Chícharo et al. (2003), Ramirez et al. (2004), Froese and Pauly (2006)
Sciaenops ocellatus (Linnaeus, 1766)	ES, ST, Pe	Westerman and Holt (1994), Rooker and Holt (1996), Rooker et al. (1997), Johnson et al. (2002), Froese and Pauly (2006)
Solea senegalensis Kaup, 1858	ES, ST, Pe	Cabral (2003), Parra and Yúfera (2001), Rueda-Jasso et al. (2004), Fonseca et al. (2006), Froese and Pauly (2006)

Appendix (cont.)

Species	Categories	Main sources
Solea solea (Linnaeus, 1758)	ES, ST, Pe	Richard et al. (1991), Cabral (2003), Amara and Galois (2004), Gilliers et al. (2004), Fonseca et al. (2006), Froese and Pauly (2006)
Stegastes partitus (Poey, 1868)	C, TP, Op	Booth and Hixon (1999) Hoey and McCormick (2004), Froese and Pauly (2006)
Tautoga onitis (Linnaeus, 1758)	ES, ST, Pe	Sogard et al. (1992), Steimle and Shaheen (1999), Phelan et al. (2000), Kuropat et al. (2002), Mercaldo-Allen et al. (2006), Froese and Pauly (2006)
Theragra Chalcogramma (Pallas, 1814)	M, P, Pe	Canino et al. (1991), Brodeur et al. (2000), Froese and Pauly (2006)
Thunnus maccoyii (Castelnau, 1872)	M, T, Pe	Carter et al. (1998), Froese and Pauly (2006)
Trematomus scotti (Boulenger, 1907)	M, P, Eq	Morales-Nin et al. (2002), Froese and Pauly (2006)
Upeneus tragula Richardson, 1846	C, TP, Op	McCormick and Molony (1993), McCormick and Kerrigan (1996), Froese and Pauly (2006)

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CHAPTER 3

Lipid-based indicators of nutritional condition in juvenile sole *Solea solea*

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Lipid-based indicators of nutritional condition in juvenile sole *Solea solea*

Abstract: The effects of feeding level on juvenile *Solea solea* (Linnaeus, 1758) lipid-related indicators of nutritional condition were assessed via experimental calibration over a 25 days trial. Morphometric indices (K and HSI), liver lipid content and fatty acid (FA) clusters were good indicators of feeding level, while individual FA showed highly dynamic variation patterns. Additionally, liver indices were more sensitive to feeding stress than muscle measurements. HSI determination (and K) is fairly straightforward, with lower costs and simple procedures. Nevertheless, liver lipid content was also an important indicator of feeding level and constitutes a direct measure of food utilization and energy storage capacity. The individual variability and the complex dynamics of FA with feeding level are disadvantages for the use of FA analysis even for early-warning signalling of feed limitation. Overall, *S. solea* nutritional condition can be suitably assessed by morphometric indices and lipid content, especially considering liver measurements (HSI and liver lipid content), provided that fish development stage is accounted for (i.e. gonad development and reproductive cycle).

Key-words: *Solea solea*; lipid content; fatty acids; feeding level.

1. Introduction

Lipid reserves are essential energy sources for fish, influencing their growth (Santos et al. 1993), reproductive success (Silverstein et al. 1998), stress resistance capability (Adams 1999) and ultimately survival probability (Sogard and Olla 2000). Larvae and juvenile fish are particularly dependent on energy allocation processes while undergoing severe development changes (Post and Parkinson 2001). Total lipid content as an indicator of fish nutritional status and long-term growth potential has been broadly used for assessment of habitat quality, since lipid reserves can be directly related to food availability and quality (e.g. Lloret and Planes 2003; Shulman et al. 2005; Amara et al. 2007). Other biochemical indicators of condition, such as protein content and RNA to DNA ratio, encompass shorter response time to nutritional stress compared to lipid content (e.g Weber et al. 2003). Nevertheless, lipids as the major form of energy storage in fish can represent added ecological and physiological significance in terms of assessing long-term growth potential and survival probability, especially during early-life stages (e.g. Lochmann et al. 1995).

Fish totally or partially deprived of food generally evidence a decrease in lipid reserves, mainly composed of triacylglycerols, specifically with the prioritized mobilization of monounsaturated fatty acids (MUFA) and some saturated fatty acids (SFA), countered with the preservation of essential fatty acids (FA) (Jezierska et al. 1982; Zamal and Ollevier 1995). At this finer scale, specific deficiencies in terms of lipid classes or even individual (FA) have been found to result in reduced growth, diminished stress resistance and behavioural changes in different fish species (Navarro and Sargent 1992; Logue et al. 2000). However, the majority of these studies focus on improving aquaculture conditions and fish commercial feeds, not providing insight into the effects of natural food availability and quality on the dynamics of young fish lipid reserves. Nevertheless, there is some evidence of the influence of habitat trophic structure on lipid dynamics, even at the FA level. Fish tissues' FA composition may reflect dietary input (e.g. Santos et al. 1993) and polyunsaturated FA (PUFA) analysis has been suggested as a promising tool to examine trophic relationships of estuarine and marine fishes (Turner and Rooker 2005). Therefore, it is important to consider a comprehensive approach into fish lipid reserves' dynamics with food availability, ranging from lipid body content to individual FA variation patterns, in order to assess the potential value of lipid indicators of wild fish nutritional condition. Lipid content has been considered a more sensitive measure of feeding conditions when compared with weight indices (Norton et al. 2001). Hence it is hypothesized that lipid content and morphometric indices decrease with increasing food deprivation, and that major FA classes can also reflect nutritional status based on prioritized maintenance of essential FA, given that food sources remain the same.

Common sole, *Solea solea* (Linnaeus, 1758), is a marine flatfish species with high commercial value, widely distributed along the Northeastern Atlantic (Quéro et al. 1986), that uses estuarine and shallow coastal nursery areas during its juvenile life stage (e.g. Cabral and Costa 1999; Le Pape et al. 2003). Common sole feeds mainly on benthic invertebrate prey (Cabral 2000), and presents high vacuity indices (around 70%) and low daily food consumption rates for Portuguese estuaries (Cabral 2000; Vinagre and Cabral 2008), which suggest the occurrence of some periods of food deprivation. The present study aims to determine the effects of feeding level on lipid reserves content and FA composition in juvenile *Solea solea*, through a laboratory calibration experiment and comparison with field collected soles. In the process, several lipid-related indices will be compared and evaluated in terms of suitable description of fish nutritional status.

2. Materials and methods

2.1 Fish collection and experimental procedures

Juvenile common sole *S. solea* (size range 68 – 87 mm), were sampled at a nursery area in the Sado estuary in July 2007, using a beam trawl. Upon collection, juvenile soles were transported alive to the laboratory where they were kept in 50 L tanks connected in parallel to a recirculation system composed of a strongly aerated 200 L sump, filled with biological media, a 40 W ultra-violet filter and a protein skimmer, until the beginning of the experiment (one week acclimation period). The tanks contained UV-treated and filtered seawater at room temperature 20.0ºC (± 0.9) and were subjected to natural photoperiod.

Fish were divided in three experimental sets to test three feeding levels: satiation (representing ca. 4.0 % of total wet mass of soles in each tank), intermediate (ca. 2.0 % of fish total wet mass) and starvation (food deprivation). There were three tanks per treatment each containing five soles. Fish were fed *ad libitum* with natural prey items caught during the experimental period, namely the polychaete *Hedistes diversicolor* (O.F. Müller, 1776) and siphons of the bivalve *Scrobicularia plana* (da Costa, 1778) (fatty acid profile in Table 1). Soles were sampled on days 5, 10, 15, 20 and 25 (three individuals per treatment per day, one individual from each tank), anaesthetized in 1:3000 solution of tricaine methanesulphonate (MS 222 Sandoz) and sacrificed with a cut on the anterior vertebral spine. For all individuals' total length (to the nearest mm), total weight and liver weight (to the nearest 0.01 g) were determined. Liver and muscle samples (0.2 – 0.8 g) were initially frozen in liquid nitrogen, and latter freeze-dried and stored at – 20ºC until subsequent analyses. Field soles for comparison purposes were collected at the end of the experimental trial. Three individuals were selected, anaesthetized, sacrificed and sampled as described above.

Table 1. Mean percentage (standard deviation between brackets) of most prevalent fatty acids methyl esters (FAME) in experimental prey items, *Hediste diversicolor* and *Scrobicularia plana*.

FAME	Prey items	
	H. diversicolor	*S. plana*
16:0	16.74 (2.22)	13.18 (1.76)
18:0	6.37 (0.61)	5.74 (0.31)
16:1n-7	3.42 (0.56)	0.30 (0.26)
18:1n-7	4.92 (0.87)	1.98 (0.78)
18:1n-9	6.74 (1.51)	7.71 (2.12)
18:2n-6	7.08 (2.51)	2.98 (0.97)
20:4n-3	2.98 (0.66)	4.64 (0.10)
20:5n-3	15.55 (2.68)	17.67 (2.60)
22:5n-3	4.78 (2.64)	3.02 (1.08)
22:6n-3	5.77 (1.21)	9.37 (3.04)
SFA	29.35 (3.25)	25.44 (2.68)
MUFA	26.55 (0.54)	26.5 (1.33)
PUFA	42.56 (3.85)	46.9 (3.20)
Branched	1.54 (0.33)	1.16 (0.17)
n-3:n-6	5.31 (1.38)	4.27 (1.78)

2.2 Morphometric indices and lipid analyses

Two morphometric condition indices were calculated for all juvenile soles: the condition factor, $K = W.L^{-3}$, where W is the wet weight (in grams) and L is the total length (in cm) (Ricker 1975); and the hepatosomatic index, $HSI = W_L.W^{-1}$, where W_L is the liver weight (in g).

Lipid extraction was carried out according to Bligh and Dyer (1959), with tissue homogenization (ca.100 mg dry weight) in a mixture of chloroform–methanol–water (2:2:1.8).

After saponification and esterification of the lipid extracts (Metcalfe and Schmitz 1961), the fatty acid methyl esters (FAME) were analysed in a Varian 3400 gas chromatograph, equipped with an autosampler and fitted with a flame ionization detector. The separation was carried out, with helium as carrier gas, in a fused silica capillary column Chrompack CPSil/88 (50 m x 0.32 mm id), programmed from 180ºC to 200ºC at 4ºC min^{-1}, held for 10 min at 200ºC, and heated to 210ºC for 14.5 min, with a detector at 250ºC. A split injector (100:1) at 250ºC was used. FAME were identified by comparison of their retention time with those of chromatographic Sigma standards. Gas Liquid Cromatography (GLD) data acquisition and handling was done using a

Varian integrator 4290 connected to the GLC. Peak quantification was carried out with a Star Chromatography workstation.

2.3 Statistical analyses

Differences in fish morphometric indices, lipid content and FA composition between feeding levels and experiment days were tested using two-way analysis of variance (ANOVA), followed by *post-hoc* Tukey tests, whenever null hypotheses were rejected. FA composition of fish tissues was analysed both at the individual and group level, where FA groups or clusters were determined according to the degree of saturation and structure, namely saturated FA (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA), and branched FA. Only the most representative individual FA, which accounted for more than 3.0 % of total FAME, were considered in the analyses. Results for field-collected fish were compared with experimental sole data observed at the end of the trial (day 25) by one-way analysis of variance (ANOVA), followed by *post-hoc* Tukey tests.

Fatty acid data were arcsine-transformed prior to the analysis of variance, in order to correct for the FA percentage binomial distribution (Zar 1996).

Principal components analyses (PCA) were then used to explore multivariate association patterns between feeding levels, time, general morphometric and lipid indices and FA variables, which had previously showed significant differences. At one point FA clusters and most prevalent individual FA were analysed together, and despite some redundancy, the PCA performed well, and the added information of including both levels of information was considered an interpretative advantage.

Analyses of variance were performed using Statistica software. PCA analyses were performed with CANOCO software. A significance level of 0.05 was considered in all test procedures.

3. Results

Morphometric condition indices, condition factor K and the hepatosomatic index (HSI), and lipid content of juvenile soles differed significantly with feeding level over the experimental trial (Table 2). Juvenile soles fed to satiation showed higher values of condition factor K and HSI when compared with the starvation and the intermediate feeding levels (Fig. 1a and 1b).

Lipid content, expressed as weight percentage of muscle and liver tissues, showed a similar trend, with significant higher percentages in the satiation group (Fig. 1c and 1d; Table 2). HSI and liver lipid content allowed for the distinction between the three feeding levels (Table 2).

Forty individual fatty acids (FA) were identified during analysis, but only 10 represented individually more than 3.0 % of total FAME. Differences in FA composition were observed in the muscle of young soles, both at the group and individual FA levels (Table 2). Figure 2 showed a slight increase in the relative percentage contribution of starved soles SFA from day 15, complementary to a slight decrease of PUFA, while satiated soles evidenced the opposite

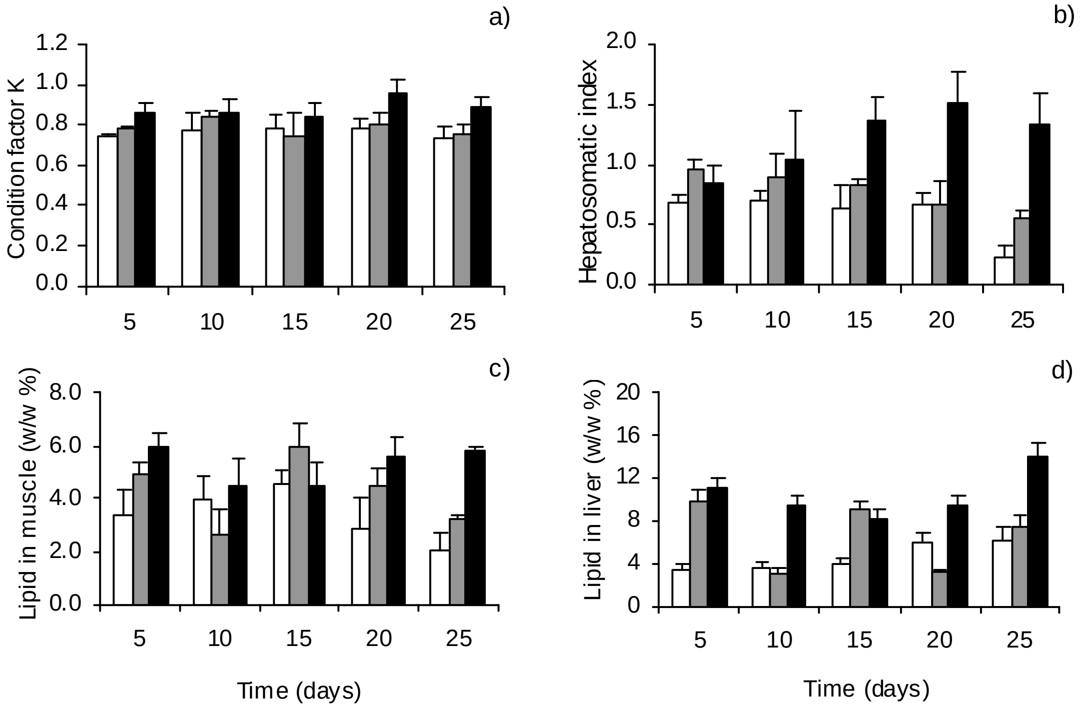

Figure 1. Mean (standard deviation above bars) of: a) condition factor K; b) hepatosomatic index (HSI), c) lipid percentage in the muscle and d) in the liver of juvenile sole with varying feeding levels (starvation □; intermediate ▨ and satiation ■) over a 25 days trial.

pattern. However, PUFA was the only FA group that evidenced significant differences between the intermediate and the satiation feeding levels, which resulted from increased percentage of individual FA of the n-3 series in the intermediate level, namely n-3 arachidonic and docosahexaenoic acids (Table 2; 20:4n-3 and 22:6n-3). The n-3:n-6 ratio increased moderately in the intermediate and starvation levels compared to soles fed to satiation, but without statistical significance (Fig. 2d, Table 2).

Hepatic FA profile varied significantly with time, feeding regime and with the interaction of these two factors, at the individual FA and cluster level (Table 2). All FA clusters evidenced significant differences between feeding levels (Table 2). Liver PUFA in satiated soles was lower in relative percentage compared to other feeding groups, and the inverse situation was observed in SFA and MUFA levels (Fig. 3a, 3b and 3c). Time effects were complex at all FA levels (Table 2), nevertheless after day 15 there was an increase in liver PUFA of the intermediate group, but especially in starved soles (Fig. 3c). The 10 most representative FA belonging to all FA clusters - the SFA palmitic and stearic acids (16:0 and 18:0), the MUFA palmitoleic, octadecenoic and oleic acids (16:1n-7, 18:1n-7 and 18:1n-9), and the PUFA linoleic, n-3 arachidonic, eicosapentanoic, docosapentaenoic and docosahexaenoic acids (18:2n-6; 20:4n-3; 20:5n-3; 22:5n-3 and 22:6n-3) - varied significantly, frequently between the starvation and the satiation feeding levels.

There were no clear patterns of the effects of time in the FA variation at the individual or cluster levels in both tissues (Table 2). Nevertheless, liver FA variation with time was more dynamic than in the muscle, with liver PUFA, 18:1n-9, 18:2n-6 and 22:6n-3 differing between every experimental day, but without a clear trend.

Table 2. Two-way ANOVA results on the effects of time and feeding level (Starvation - Starv.; Intermediate - Inter.; and Satiation - Sat.) on morphometric indices (K and HSI), lipid (LIP in w/w %) and FA (% FAME) content of *S. solea* muscle (*M.*) and liver (*L.*) tissues. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant differences from *post hoc* Tukey tests results ($P < 0.05$) between experimental days and between feeding levels are also represented by different letters.

Index	Time (days)						Feeding level				Feeding x Time
	F (df 4)	5	10	15	20	25	F (df 2)	Starv.	Inter.	Sat.	F (df 8)
K	1.71						15.97 ***	a	a	b	0.90
HSI	2.25						43.65 ***	a	b	c	4.41 **
LIP % *M.*	5.32 **			a		b	4.95 *	a		b	1.67
LIP % *L.*	4.50 **		a			b	34.39 ***	a	b	c	3.45 **
PUFA *M.*	1.11						5.06 *		a	b	1.55
16:1n-7 *M.*	3.32 *	a	a			b	3.57 *		a	b	2.98 *
18:1n-9 *M.*	3.44 *			a		b	5.16 *	a	b		2.79 *
20:4n-3 *M.*	1.21						4.27 *	a		b	0.78
22:6n-3 *M.*	1.60						13.24 ***	a	a	b	1.45
SFA *L.*	11.21 ***	a	a	b	a	b	29.1 ***	a	b	c	6.01 ***
MUFA *L.*	13.65 ***	a	ab	a	b	c	7.26 ***	a	b	c	4.91 ***
PUFA *L.*	10.61 ***	a	b	c	d	e	12.06 ***	a	b	c	7.11 ***
Branched *L.*	9.79 ***	a	ab	b	b	c	3.62 *	a	b		4.81 ***
n-3:n-6 *L.*	5.72 **	a	a	a	b	b	10.47 ***	a	b	c	9.69 ***
16:0 *L.*	4.79 **	a	b	b	b	b	5.39 **	a	a	b	3.34 **
18:0 *L.*	6.43 ***	a	ac	ac	b	c	8.42 ***	a	a	b	5.14 ***
16:1n-7 *L.*	6.98 ***	a	a	b	b	a	8.35 ***	a	a	b	6.34 ***
18:1n-7 *L.*	4.45 **	a	b	a	a	a	6.05 **	a	b	b	3.90 **
18:1n-9 *L.*	10.61 ***	a	b	c	d	e	7.84 ***	a	b	c	6.64 ***
18:2n-6 *L.*	12.31 ***	a	b	c	d	e	9.74 ***	a	a	b	10.33 ***
20:4n-3 *L.*	3.54 *	a	a	a		b	4.43 *	a	a	b	3.72 **
20:5n-3 *L.*	3.76 *	a	b	b	b	b	5.28 **	a	b	c	9.08 ***
22:5n-3 *L.*	3.65 *	a	b	b	b	b	5.77 **	a	b	c	7.09 ***
22:6n-3 *L.*	10.72 ***	a	b	c	d	e	8.76 ***	a	b	c	9.02 ***

The liver n-3:n-6 ratio also varied significantly with time and feeding regimes, with young soles fed to satiation evidencing lower values from day 15 to day 25 (Fig. 3d). The relative content of PUFA of the n-6 series increased in the satiation groups, and in addition to liver PUFA of the n-3 series percentage decrease, resulted in lower ratio levels.

Juvenile sole collected in the Sado estuary presented high mean K and HSI values, comparable to the satiation soles on day 25, but differed significantly from soles subjected to starvation (in both indices) and to the intermediate feeding level (only for HSI) (Table 3). The high percentage of lipid content in the muscle and liver of field-collected soles was also similar to the satiation treatment on day 25, and also differed from the starvation and intermediate groups (Table 3).

The muscle FA profile of field-collected soles (only considering FA clusters) was not different from muscle FA of soles at the end of the experiment, except for the difference between the PUFA percentage of starved and field soles (Table 3). However, the liver FA profile evidenced significant differences in the relative importance of MUFA and PUFA clusters,

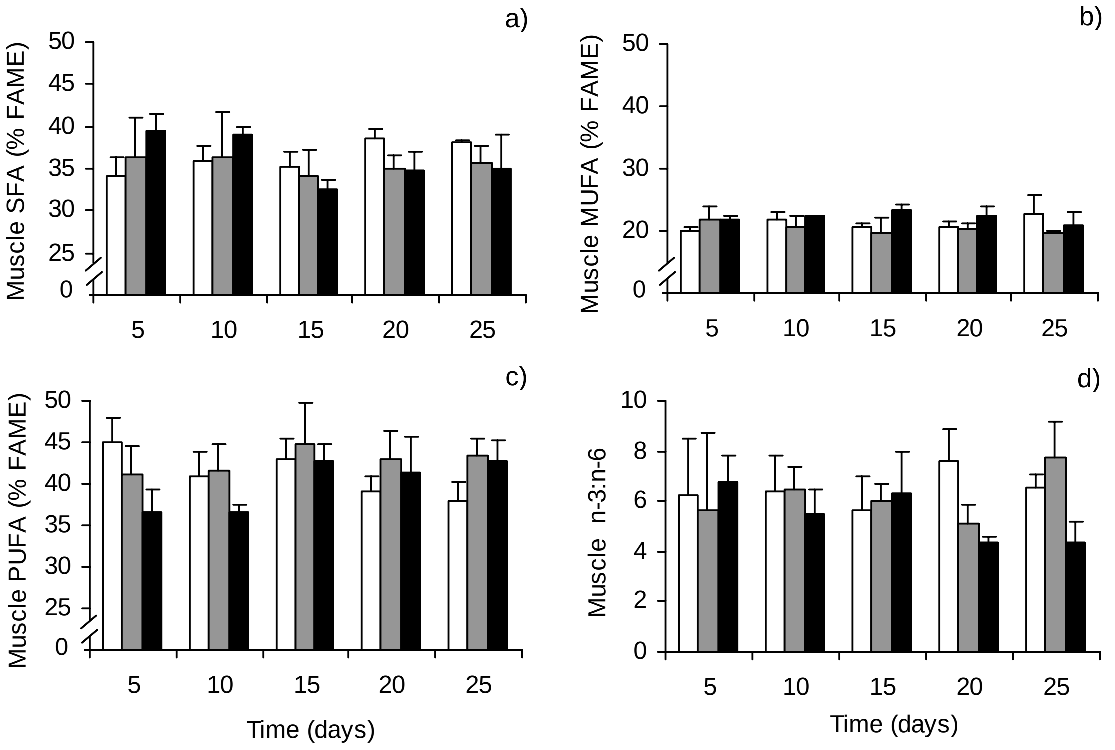

Figure 2. Mean percent composition (standard deviation above bars) fatty acid clusters in juvenile sole muscle: a) SFA, b) MUFA and c) PUFA and d) n-3:n-6 ratio variation with feeding level (starvation □; intermediate ▨ and satiation ■) over a 25 days trial.

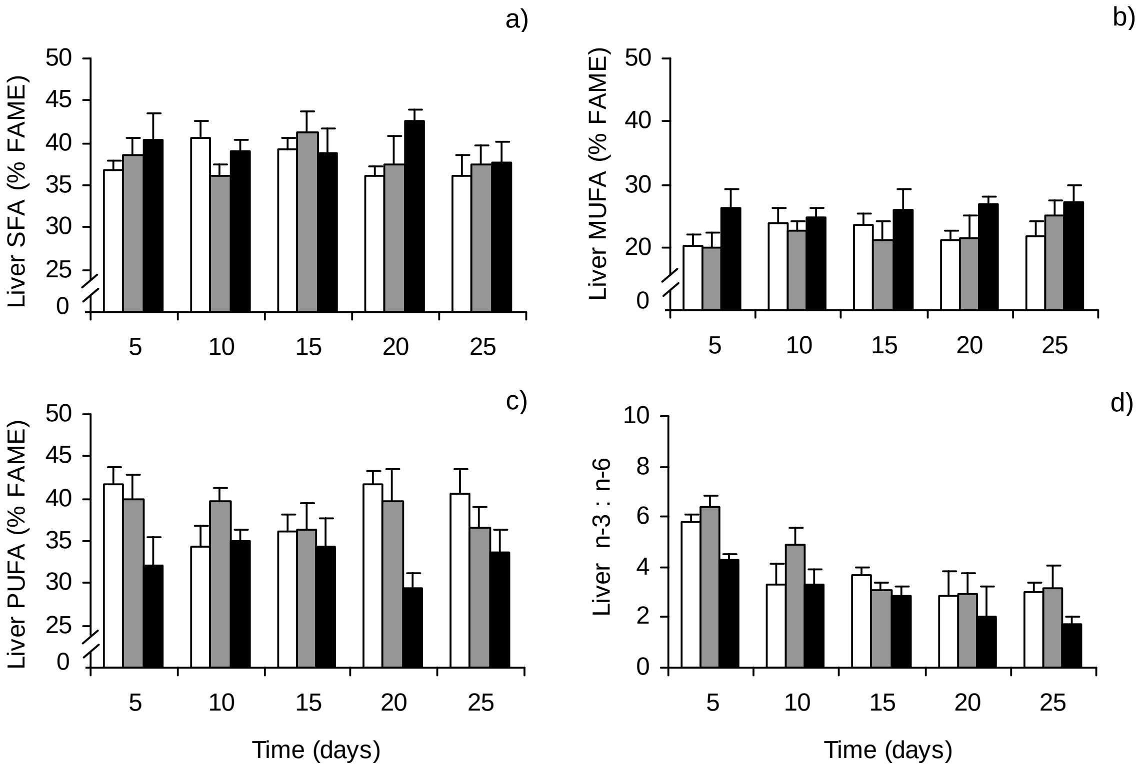

Figure 3. Fatty acid clusters' mean percent composition (standard deviation above bars) in juvenile sole liver: a) SFA, b) MUFA and c) PUFA and d) n-3:n-6 ratio variation with feeding level (starvation □; intermediate ▨ and satiation ■) over a 25 days trial.

Table 3. Mean values (standard deviation between brackets) of morphometric indices (K and HSI), lipid (LIP in w/w %) and FA content (% FAME) in muscle and liver tissues of juvenile *S. solea*. Analyses of variance results (F, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) considering experimental feeding levels (Starvation - Starv.; Intermediate - Inter.; and Satiation - Sat.) and field-collected (Field) soles on day 25. Significant differences from *post hoc* Tukey tests are also represent by different superscript letters ($P < 0.05$).

Index	Muscle					Liver				
	F (df 3)	Field	Starv.	Inter.	Sat.	F (df 3)	Field	Starv.	Inter.	Sat.
K	11.30***	0.89^{a} (0.08)	0.73^{b} (0.06)	0.75 (0.05)	0.89^{a} (0.05)					
HSI						21.43***	1.40^{a} (0.11)	0.22^{b} (0.10)	0.55^{c} (0.06)	1.34^{a} (0.25)
LIP %	3.13*	4.40^{a} (0.82)	2.04^{b} (0.68)	3.26^{a} (0.13)	5.80^{a} (0.81)	15.86***	11.67^{a} (1.64)	6.17^{b} (1.24)	7,49^{b} (1.00)	14.12^{a} (1.25)
SFA	0.50	34.49 (1.66)	38.07 (0.43)	35.66 (2.76)	34.99 (4.09)	1.95	36.96 (4.57)	36.17 (3.55)	37.48 (2.05)	37.75 (4.19)
MUFA	1.59	19.85 (0.66)	22.59 (4.36)	19.66 (0.50)	20.92 (2.14)	7.57***	17.98^{a} (1.30)	21.22^{b} (1.27)	25.04^{c} (3.17)	27.18^{c} (3.22)
PUFA	3.42*	43.71^{a} (0.93)	38.06^{b} (3.04)	43.39 (2.79)	42.64 (2.58)	17.41***	43.24^{a} (3.13)	40.67^{b} (2.52)	36.61 (1.89)	33.83^{b} (3.92)
Branched	2.45	1.95 (0.18)	1.28 (0.89)	1.29 (0.52)	1.45 (0.39)	4.91**	1.82 (0.13)	1.94 (0.57)^{a}	0.87 (0.16)^{b}	1.24 (0.28)
n-3:n-6	3.25*	5.30 (1.55)	6.56^{a} (0.68)	7.77^{a} (1.97)	4.36^{b} (0.84)	10.16***	7.21^{a} (3.53)	3.24^{b} (0.59)	2.98^{b,c} (0.71)	2.01^{c} (0.36)
16:0	1.54	18.78 (1.77)	20.72 (3.70)	21.35 (0.89)	21.11 (2.67)	22.28***	16.90^{a} (1.29)	23.26^{b} (1.28)	23.63 b (0.76)	24.05^{b} (2.34)
18:00	5.94**	9.57^{a} (0.37)	8.70^{b} (0.13)	8.20^{b} (1.37)	8.71^{b} (0.36)	17.95***	11.21^{a} (1.82)	10.20^{a} (1.21)	8.40^{a} (0.94)	6.93^{b} (1.90)
16:1n7	0.76	3.30 (0.11)	3.47 (0.05)	3.67 (0.04)	2.32 (1.05)	5.63**	2.98^{a} (1.22)	4.28 (1.03)	5.62 (1.97)	6.46^{b} (2.57)
18:1n-7	1.65	3.17 (0.23)	3.30 (0.35)	3.16 (0.31)	3.48 (0.07)	4.07**	2.88 (0.21)	3.57 (0.53)	3.63 (0.56)	3.95 (1.19)
18:1n-9	9.92***	7.87^{a} (0.28)	11.73^{b} (1.36)	10.11^{b} (1.32)	9.81^{b} (0.49)	4.33**	6.05^{a} (2.44)	10.71^{b} (2.57)	12.78^{b} (1.04)	13.23^{b} (3.36)
18:2n-6	3.49*	2.96 (1.43)	1.74^{a} (0.46)	1.72^{a} (0.61)	3.56^{b} (0.77)	7.10***	1.61^{a} (0.81)	4.08^{b} (0.89)	4.66^{b} (1.03)	5.87^{b} (1.15)
20:4n-3	4.76**	4.65^{a} (0.11)	5.91^{b} (0.17)	5.88^{b} (0.59)	5.40^{b} (0.09)	1.39	5.50 (1.40)	6.18 (0.54)	6.20 (1.29)	5.28 (2.73)
20:5n-3	2.11	6.50 (0.51)	5.05 (1.41)	6.16 (0.14)	6.57 (0.72)	16.64***	6.65^{a} (1.28)	3.42^{b} (0.42)	3.51^{b} (0.48)	2.67^{b} (0.89)
22:5n-3	3.97*	6.01^{a} (0.11)	4.13^{b} (0.66)	6.10^{a} (0.15)	5.03 (0.86)	8.39***	5.62^{a} (1.41)	3.24 (0.61)	3.56 (0.52)	2.58^{b} (0.82)
22:6n-3	8.47***	18.29^{a} (1.71)	17.62^{a} (0.79)	19.32^{a} (2.84)	16.20^{b} (1.77)	10.94***	19.26^{a} (2.40)	13.66^{b} (2.66)	13.21^{b} (2.34)	10.41^{b} (1.36)

compared to the experimental feeding regimes, which presented higher MUFA and lower PUFA values than field soles.

Muscle n-3:n-6 ratio of field soles was similar to all experimental groups, while liver n-3:n-6 of field soles was significantly higher than all experimental sole's ratios observed at the end of the feeding trial (Table 3). Significant differences were also observed for many of the individual FA of field soles compared to the experimental groups, especially in the liver, indicating that field-fish FA profile contribution was distinctive (Table 3).

Principal components analysis considered significant variables at two distinct levels of biological organization, namely a higher level considering the individual and tissue specific responses with K, HSI and lipid content (Fig. 4), and a molecular level with FA composition (Fig. 5). In figure 4 the satiation and field groups are associated with higher K, HSI and liver lipid content, and clearly to one side from the intermediate and starvation groups. Thus the first two

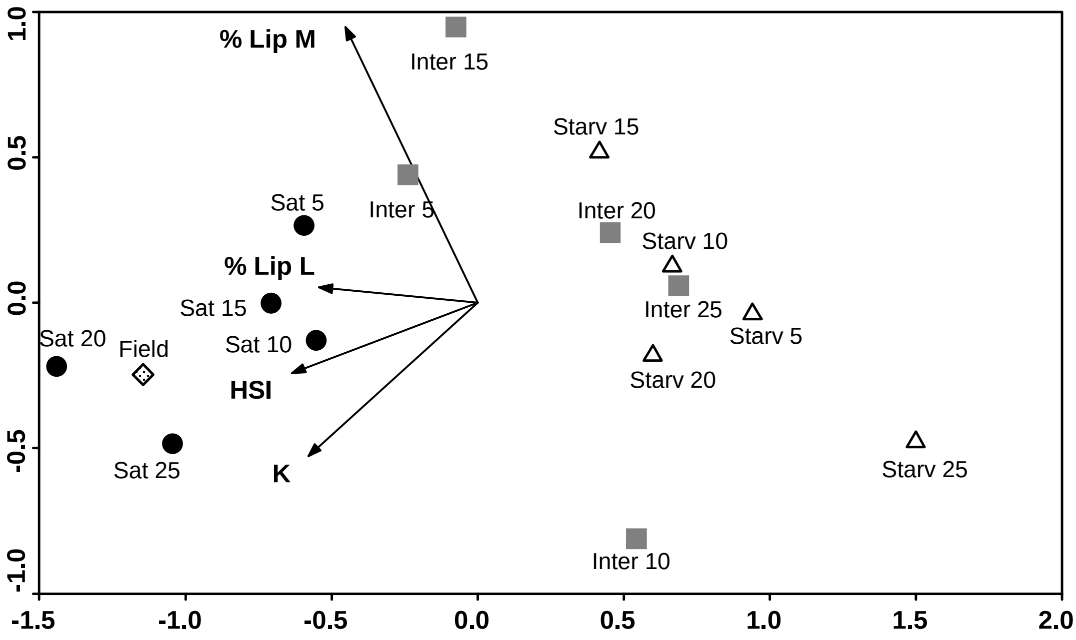

Figure 4. PCA ordination diagram (first two axes) of *S. solea* juveniles based on morphometric indices (K and HSI) and muscle and liver lipid content (% Lip M. and % Lip L.) variation with experimental feeding levels (starvation △; intermediate ■ and satiation ●; 5 to 25 experimental days). Field-collected soles are also represented (◇).

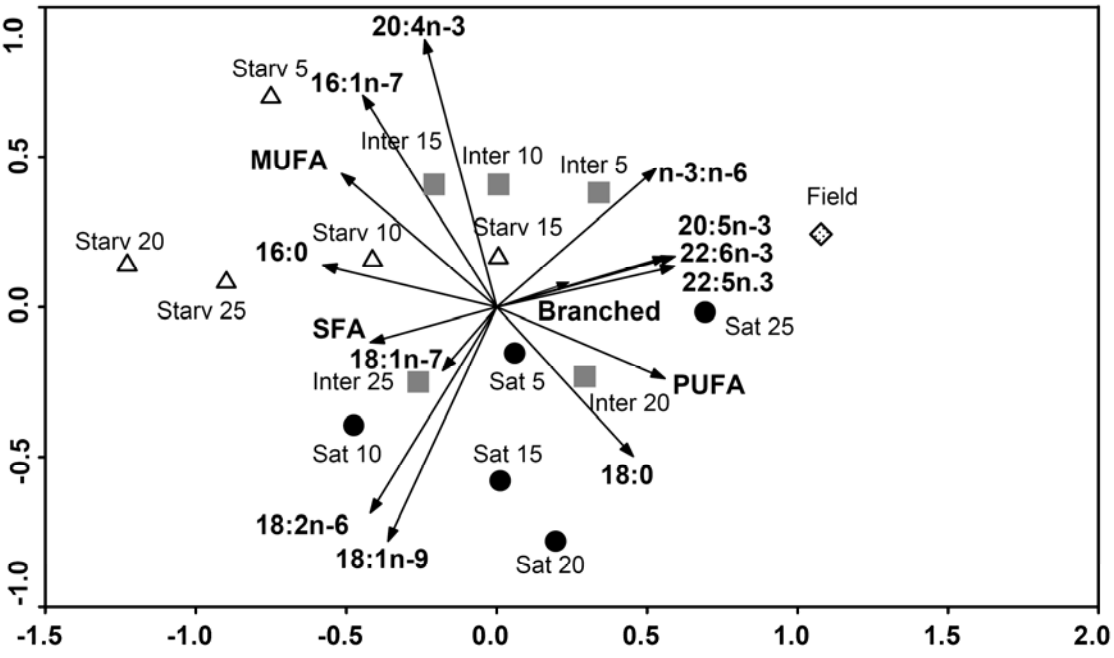

Figure 5. PCA ordination diagram (first two axes) of *S. solea* juveniles based on liver FA clusters and most prevalent individual FA variation with experimental feeding levels (starvation △; intermediate ■ and satiation ●; 5 to 25 experimental days). Field-collected soles are also represented (◇).

axes of the PCA explained 84.9 % of the total variation in morphometric indices and lipid content of soles. The second PCA analysis accounted for 73.1 % of the total variation in FA composition considering the first two axes. The resulting diagram (Fig. 5) suggested a positional sequence from starvation, to intermediate and to satiation feeding levels, associated with the opposite trends in SFA and MUFA relative to PUFA content, although groups' separation level was lower than for the previous diagram. Field-collected soles were set apart from the experimental groups but were associated with high PUFA content, particularly of the n-3 series.

4. Discussion

Morphometric indices, muscle and liver lipid content and FA profile of juvenile soles varied with feeding level over a 25 days experiment. Higher condition factor K and hepatosomatic index (HSI) were observed in the satiation treatment and in field-collected soles compared to individuals from the intermediate and starvation feeding levels. Variations in K and HSI have been reported for fish subjected to food constrains, both under laboratory and field conditions (Blanchard et al. 2005; van Dijk et al. 2005), although field studies usually encompass a broader response period (i.e. months). Morphometric indices are usually less sensitive to nutritional stress than physiological measures, since weight changes require longer to be perceived (Ferron and Leggett 1994). In this case, liver lipid content and HSI values were more responsive to nutritional stress than condition factor K or muscle lipid content, suggesting that liver indices would be better indicators of food availability in soles under field conditions.

Variation in fatty acid (FA) composition with time and feeding regime was less marked in the muscle than in the liver of young soles, both at the individual and group of fatty acids level. Moreover, fatty acid profiles of experimental soles showed different trends in the muscle and liver tissues. Muscle SFA percentages increased slightly during starvation, while PUFA had a parallel decrease, contradicting the commonly accepted idea that essential FA (HUFA or PUFA) are preserved during starvation periods and SFA or MUFA are mobilized for energy requirements (Jezierska et al. 1982; Rondán et al. 2004). Nevertheless, essential FA preservation was observed in the liver, the main organ of lipid storage in lean fish. Most prevalent liver PUFA of the n-3 increased in FA relative content of soles subject to starvation and intermediate feeding levels, namely n-3 arachidonic, eicosapentanoic and docosahexaenoic acids (20:4n-3; 20:5n-3 and 22:6n-3). Logue et al. (2000) described lower abiotic stress resistance of *S. solea* larvae following administration of a diet deficient in n-3 PUFA. Therefore, the preservation of these FA under feeding stress should be important for assuring some degree of stress response capability, even at the juvenile stage.

Fish of the satiation group evidenced more pronounced increases in the SFA and MUFA contents, which should account for the increased tissue lipid storage. Additionally most prevalent PUFA of the n-6 series increased, due to the high levels of linoleic and arachidonic acids (18:2n-6 and 20:4n-6) originating from diet sources, *H. diversicolor* and *S. plana*.

Field-collected soles had, in general, a distinctive individual FA contribution from all experimental groups suggesting that diet effects from other minor prey items were present (i.e.

amphipods, microphytobenthos ingested with sediments), and influenced FA expenditure and conservation dynamics. Nonetheless, the polychaete *H. diversicolor* and the bivalve *S. plana* are the main food items in juvenile *S. solea* diet reported for Portuguese estuaries (Cabral 2000), and despite the individual variability in the FA percentages, they were rich in essential FA of the n-3 and n-6 series. Overall, most prevalent FA were responsible for the differences described in FA clusters, but no individual FA showed an unique pattern with feeding level or time that could be used to flag feeding stress, and thus be used as an indicator of food availability. Intricate relationships have been described in FA requirements for a similar sole species, *Solea senegalensis* Kaup 1858, with Morais et al. (2005) reporting high absorption efficiency and tissue retention rates of DHA (22:6n-3), suggesting selective conservation during larvae development, while other studies reported a lack of correlation between dietary DHA and growth and survival of sole larvae (e.g. Villalta et al. 2005). Other potential sources of variability in present FA results could be related to alternative energy mobilization pathways used by organisms, e.g. other physiological fuels such as protein catabolism and glycogen metabolism, as well as FA dynamics between polar and neutral lipids (McCue 2010).

The n-3:n-6 ratio was used as a tool to understand the PUFA dynamics, but varied only in soles' liver. Higher values were observed for the starvation and intermediate feeding levels mainly due to liver FA preservation of n-3 series PUFA, while in the satiation level lower ratio values were mainly due to a significant accumulation of liver n-6 PUFA originating from the diet. An increase in this ratio has been related to lower feeding rates by other studies (e.g. Rondán et al. 2004). However, field-collected soles presented values near the upper limit of the range of experimental ratios, potentially signalling dietary differences for which major contributors were PUFA of the n-3 series in both quantity and diversity. This again suggests that smaller diet contributors and even indirectly ingested organisms could be an important source of FA for this species.

Overall, individual and tissue specific indices, namely morphometric indices (K and HSI) and tissues lipid content allowed for a consistent separation of all feeding levels and field soles. Soles' satiation and field groups were positively correlated with higher K, HSI and lipid content, while intermediate and starvation groups were negatively correlated with these indices. At a molecular level, liver FA clusters and most prevalent individual FA also evidenced differences between feeding levels, with the starvation and intermediate groups associated with higher SFA and MUFA contents, while the satiation group was mainly associated with higher PUFA content. Field-collected soles were set apart from the experimental groups but were associated with high PUFA content, particularly of the n-3 series. Even so, the groups' separation level was lower compared to the previous analysis, which may result from the fact that molecular responses to stresses are more sensitive and immediate than the responses at higher organizational levels (Adams 2002), and in this case resulted in more complex variation patterns with time.

HSI determination (and K factor) is obviously the most straightforward method, with fewer costs and simpler procedures, compared to biochemical measures, lipid-related or otherwise. In the present work, in addition to HSI determination, lipid content, particularly in the liver, was an important indicator of feeding level, and represents a more direct measure of food utilization related to energy storage capacity. Lipid measurements possibly require more time-consuming and costly procedures compared to other common biochemical indicators of fish condition (RNA:DNA, protein content), however it's relevancy in fish condition assessment when considering energy allocation strategies and growth and survival probability are good arguments for its continued use (Lloret and Planes 2003; Weber et al. 2003). The complex dynamics of FA with feeding level, associated with labor-intensive and costly techniques, constitute apparent disadvantages in the use of FA analysis, even for early-warning signalling of feed limitation.

Characteristic and repeatable patterns of nutritional stress-induced modifications of FA profiles are mainly from aquaculture studies, and it has been suggested that application of these indices to wild animals still needs further research (McCue 2010). In order to improve knowledge on the variability of FA profiles with nutritional stress in field collected fish, FA analysis during periods of food limitation in natural conditions would give valuable information.

In conclusion, *S. solea* nutritional condition and food availability under field conditions can be easily assessed by morphometric indices and lipid content, with particular emphasis on using liver measurements (HSI and liver lipid content), provided that fish development stage is considered (i.e. gonad development and reproductive cycle).

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CHAPTER 4

Growth performance of juvenile *Dicentrarchus labrax* and *Solea senegalensis* in estuaries along the Portuguese coast assessed via experimental calibration

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In review in Journal of Fish Biology

Growth performance of juvenile *Dicentrarchus labrax* and *Solea senegalensis* in estuaries along the Portuguese coast assessed via experimental calibration

Abstract: Juvenile sea bass (*Dicentrarchus labrax*) and Senegalese sole (*Solea senegalensis*) instantaneous growth rates were estimated through experimental calibration of nucleic acid-based indices. 72.0 % of the variability in growth of juvenile *D. labrax* was explained by the simple linear regression G = - 0.0377 + 0.0165(R:D) ($P < 0.001$, n = 54), whereas RNA:DNA ratio (R:D) and temperature (T, in ºC) explained 63.0 % of the variability in growth of juvenile *S. senegalensis* in a multiple linear regression, G = - 0.0319 + 0.0098(R:D) + 0.0021(T) ($P < 0.001$, n = 72). Model-based growth rate estimates and growth performance of juvenile sea bass and Senegalese sole differed amongst estuaries and years considered (2005 and 2006). For both species mean growth performance was usually below 60.0 %, yet *D. labrax* reference or maximum growth rate was derived from field individuals from the Tejo estuary in 2006, while *S. senegalensis* maximum growth rate was attained under laboratory conditions. In 2006 mean growth performances were higher than in 2005, potentially due increased environmental stress from drought conditions. Overall, species specific differences were evident from the differences in model development and in estuarine growth performance, and should be accounted for in habitat quality assessment.

Key-words: model; growth; RNA:DNA ratio; temperature; habitat quality; sea bass; Senegalese sole.

1. Introduction

Growth of juvenile fish is assumed to be a comprehensive indicator of habitat quality and individual health, as it integrates environmental conditions (e.g. food resources, salinity, temperature) and individual performance (e.g. Able et al. 1999; Meng et al. 2000). The importance of habitat quality assessments relies on the influence of habitat quality on juvenile fish individual growth potential and survival probability (Sogard 1997), ultimately with a strong impact on recruitment success to the adult population (Houde 1987; Gibson 1994). Measures of fish growth and condition have thus been frequently used to assess habitat quality (e.g. Phelan et al. 2000; Lloret and Planes 2003; Fonseca et al. 2006; Amara et al. 2007; Vasconcelos et al. 2009). Nevertheless, determining habitat quality of essential areas for fish is a complex process that depends not only on the existing natural and anthropogenic conditions, but also on the individuals' ability to respond to their environment (Gibson 1994; Adams 2002).

Since different species can integrate habitat conditions in a diversified manner depending on their life-history and biological constrains (Fonseca and Cabral 2007), for example through trade-offs among growth, energy reserves and individual behaviour (Arendt 1997; Sogard and Spencer 2004), habitat quality for different species can only be determined on a comparative basis. Moreover, growth-based assessments of habitat quality should identify the underlying factors that determine growth rate differences among habitats (Searcy et al. 2007).

Experimental analysis of fish growth rates under varying conditions (e.g. temperature, food supply) enables growth estimation to be used as a reliable measure of the habitat value for wild fish (e.g. Fukuda et al. 2001; Mercaldo-Allen et al. 2006). Growth calibration experiments can also aid in establishing a reference or maximum growth rate, achieved under optimal feeding and abiotic conditions, for comparison with other growth estimations and thus determine individual and population growth performance or the fraction of realized growth.

Nucleic acid based indices, namely RNA content or the ratio of RNA to DNA content (RNA:DNA), have been used as growth proxies for several fish species in both field and laboratory calibrations, the latter usually aiming at the development of growth models (Rooker and Holt 1996; Kuropat et al. 2002; Yamashita et al. 2003; Gilliers et al. 2006; Mercaldo-Allen et al. 2006, 2008; Vinagre et al. 2008). The underlying premise is the fact that protein synthesis, and thus growth rate, varies in proportion to RNA cell concentration, which is influenced by food availability and protein requirement, while DNA cell content remains relatively constant (Bulow 1970; Buckley and Bulow 1987). Ultimately, nucleic acid based growth models, which usually consider the influence of water temperature, represent a simplified and expedite method for recent growth assessment (Buckley et al. 1999).

Along the Portuguese coast, several economically important marine fish species utilize estuarine nursery areas during their early life. Vasconcelos et al. (2009) described differences in quality of these estuarine nursery areas for five fish species along the Portuguese coast, based on RNA:DNA and the morphometric Fulton's K indices. In the present study, two laboratory rearing experiments were performed with juveniles of two of these commercially important fish species, sea bass *Dicentrarchus labrax* (Linnaeus, 1758) and Senegalese sole *Solea senegalensis* Kaup,1858, in order to define or calibrate the relationship between measured nucleic acid indices and directly determined growth rates under different conditions of salinity, temperature and feeding level. The aim was to develop growth models based on this calibration and test their applicability to growth rate estimation in wild estuarine juveniles. Furthermore, the models' growth rates estimations were used to define a maximum threshold or reference growth rate in order to determine individuals' field growth performance. Overall, habitat quality for sea bass and Senegalese sole of the main Portuguese estuaries was analysed based on juveniles' growth performance.

2. Materials and methods

2.1 Laboratory calibration experiments

Juvenile *S. senegalensis* and *D. labrax* were collected with a beam trawl in the Tejo estuary (Fig. 1) in July 2006. Fish were transported alive to the laboratory and held in two sets of six 120 L aquariums (one set per species) during a two week acclimation period and a nine day trial period. A total of 72 sole and 54 sea bass were evenly distributed among the six aquariums used for each species trial (12 soles and 9 sea bass per aquarium, respectively). Fish were anaesthetized (with MS220), tagged, weighed (initial wet weight W_i, measured to the nearest 0.01 g) and total length measured (initial L_i, measured to the nearest mm). Holding conditions

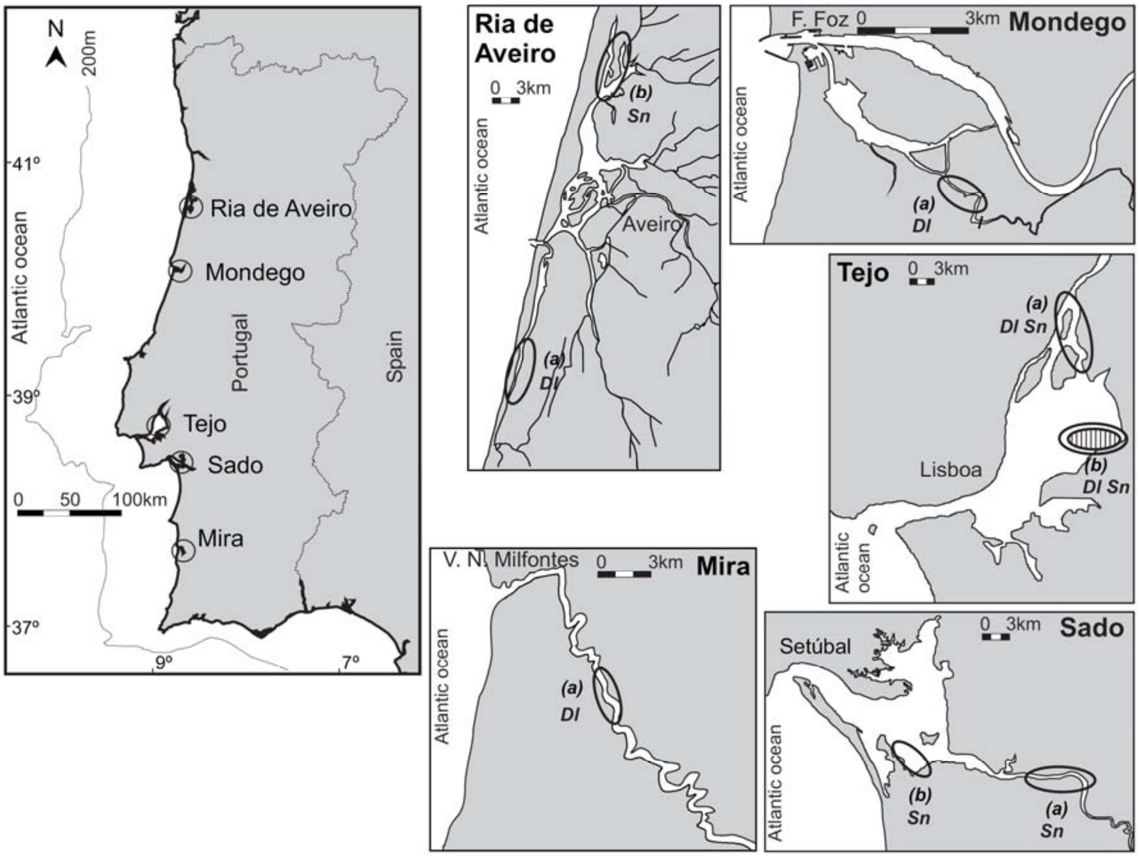


Figure 1. Location of estuarine systems of the Portuguese coast where juvenile *Dicentrarchus labrax* (*Dl*) and *Solea senegalensis* (*Sn*) were sampled during July 2005 and 2006: specimens for laboratory calibration experiments were collected in sites marked with ▥ , and specimens used for field calibration were collected in sites marked with ⬭ .

were adjusted separately during the first week in order to achieve the previously defined experimental values for temperature (15 ºC and 25 ºC) and salinity (15 and 25). During the acclimation period, fish were fed daily with natural prey items, polychaete *Hediste diversicolor* (Müller, 1776) for both species, and brown shrimp *Crangon crangon* (Linnaeus, 1758) for sea bass (Cabral 2000; Cabral and Costa 2001).During the trial two feeding levels were used, *ad libitum* feeding and starvation. The experimental design of the trial consisted of the following conditions combinations for each feeding level: T = 15 ºC and S = 15; T = 25 ºC and S = 15; T = 25 ºC and S = 25 (where T refers to temperature and S to salinity). Three *D. labrax* and four *S. senegalensis* were collected on days 3, 6 and 9 from each aquarium and anaesthetized and then sacrificed. Individuals were weighed, measured and muscle tissue samples (from the dorsal anterior area) were dissected and immediately frozen at - 80 ºC. Muscle samples were then lyophilized and stored at - 20 ºC until nucleic acids quantification.

2.2 Nucleic acids quantification

Nucleic acids quantification was performed on two replicate individual muscle samples, of ca. 0.02 - 0.04 g dry weight. Nucleic acid quantification was carried out by the fluorometric method described in Caldarone et al. (2001) as adapted to a cuvete assay by Fonseca et al.

(2006). Muscle samples were homogenized through short-term ice-sonication in N-lauroylsarcosine Tris-EDTA buffer (0.1%, pH 7.5), centrifuged (at 6000 rpm for 10 min at 0 ºC), and 300 µl aliquots of the supernatant were used for the quantification of RNA and DNA fluorescence with ethidium bromide (spectrofluorophotometer RF-1501 Shimadzu, emission wavelength 590 nm, excitation wavelength 360 nm). RNA fluorescence was determined as the difference between total nucleic acids fluorescence and DNA fluorescence following treatment with RNase A (bovine pancreatic ribonuclease A 0.12 mg ml^{-1}, from bovine pancreas, 20 U ml^{-1}, Sigma). Standard curves were previously determined using pure calf-thymus DNA (Calbiochem) and 18S- and 28S-rRNA (Sigma), and the ratio between the two slopes from each standard curve was 4.04.

2.3 Fish collection in estuaries

Five estuarine systems along the Portuguese coast were sampled (Ria de Aveiro, Mondego, Tejo, Sado and Mira) in July 2005 and 2006. These systems are distributed along a small latitudinal gradient (Fig. 1), and present distinct hydrologic and geomorphologic characteristics (see Vasconcelos et al. 2007). 0-group juveniles *Solea senegalensis* and *Dicentrarchus labrax* were collected using a beam trawl in the selected estuarine systems, in areas with high juvenile densities. To avoid within-month temporal variation, sampling was carried out within the shortest time frame possible (a fortnight). Upon collection, fish were stored and transported on ice to the laboratory and preserved frozen until dissection. Simultaneously with fish sampling surveys water temperature and salinity of sampled estuarine areas were measured.

Individual fish length (total length, measured to the nearest mm) and weight (wet weight, measured to the nearest 0.01g) were determined. For each site and sampling period 15 to 30 individuals of each species were selected. These specimens were representative of the length frequency distributions of 0-group juveniles (60 - 100 mm and 40 - 90 mm for *D. labrax* and *S. senegalensis*, respectively) occurring in the sampling periods, and corresponded to the size range of experimental individuals. For nucleic acid quantification, a sample of muscle from the dorsal anterior area was removed from each individual, and stored at -80 ºC. After lyophilisation, samples were stored at -20 ºC and nucleic acid quantification was determined as mentioned above.

2.3 Data analysis and growth performance estimation

Daily instantaneous growth rates (G d^{-1}), expressed in wet weight change per day, of juvenile *D. labrax* and *S. senegalensis* were calculated with the following equation: $G = (\ln W_f - \ln W_i) / t$, where W_i and W_f are the initial and final wet weight in grams of each fish and t is the time interval in days.

A Pearson product moment correlation was used to test associations between measured variables, namely RNA:DNA ratios, RNA and DNA concentrations, instantaneous daily growth rates (*G*), water temperature and salinity. Stepwise multiple linear regression analyses were used to generate growth models for each species considering as independent variables RNA:DNA ratio, RNA and DNA concentrations, water temperature, salinity and an interaction term between RNA:DNA and temperature as independent variables. Residuals from the best-fit models, with higher explanatory power were analysed against these variables.

Exploratory analyses revealed low, but significant, relationships when tested with linear regression (r^2 = 0.039, n = 263 and r^2 = 0.028, n = 214, P < 0.05) between fish length and RNA:DNA ratios in both *D. labrax* and *S. senegalensis* juveniles. Therefore, length corrected RNA:DNA indices were calculated by subtracting the slope of the linear regression between length and RNA:DNA ratios, multiplied by the fish length from the original index data (Gillanders and Kingsford 2003). All analyses were then performed for both the original data and length-corrected indices, however no improvements or significant differences were observed for the latter when compared to the original data, and given that field collected individuals were within the laboratory fish size range, only original data was presented.

The difference between experimental and field conditions was expressed as the difference (ΔG) between maximum growth rates attained by juvenile fish in the laboratory and individually estimated growth rates under field conditions, i.e. $\Delta G = G_{MAXLAB} - G_{FIELD}$. This difference was used to evaluate the RNA:DNA-growth model predictions and was mathematically translated into: $\Delta G = (\beta0 + \beta1X1_{LAB} + \beta2X2_{LAB} + ... + \beta jXj_{LAB}) - (\beta0 + \beta1X1_{FIELD} + \beta2X2_{FIELD} + ... + \beta jXj_{FIELD})$; where β are the regression model coefficients, and X are the independent variables that best explained growth variability, considering for laboratory conditions the X_{LAB} values of all X_{LAB} that resulted in the highest observed growth rates, while field values were individually tested. If obtained $\Delta G < 0$ then laboratory conditions were limiting fish growth, conversely if $\Delta G > 0$ then wild fish were not growing at their full potential under estuarine conditions when compared to the experimental values observed.

Accordingly, growth performance (G_{PF} %) of field-collected fish in both years was determined as the mean quotient of field growth rates (G_{FIELD}) to reference or maximum growth rates from either laboratory or field estimates (G_{REF}): $G_{PF} = G_{FIELD}/G_{REF} \times 100$.

Spatial and temporal variation in RNA:DNA ratios, instantaneous growth rate estimates and growth performance of juvenile fish from the different estuaries in both years were tested with one–way analysis of variance (ANOVA) followed by *post-hoc* Tukey tests, or with Student's *t*-test, when applicable. Since growth rates were estimated based on the determined RNA:DNA ratios these were highly correlated and results were redundant, therefore only growth rates' significant differences were presented. All analyses were performed using Statistica software and a 0.05 (or lower) significance level was considered in all test procedures.

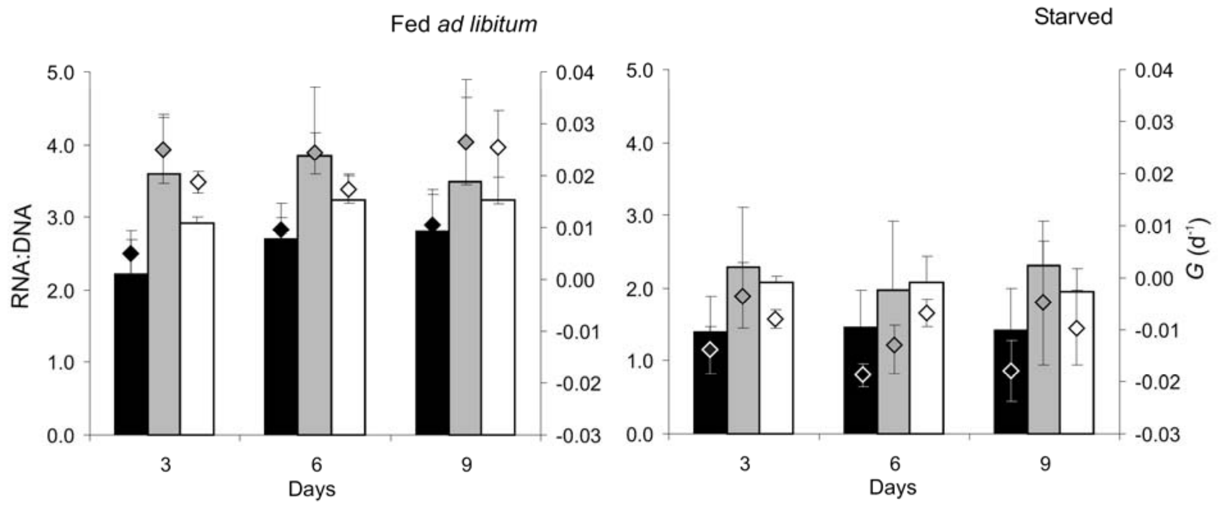
3. Results

3.1 Laboratory calibration experiments and development of growth models

Instantaneous growth rates observed during the calibration experiments ranged from -0.026 d^{-1} to 0.035 d^{-1} in *D. labrax* and from -0.046 d^{-1} to 0.072 d^{-1} in *S. senegalensis* (Fig. 2). Individual RNA:DNA ratios varied between 1.10 and 4.93 in *D. labrax* and between 1.79 and 8.05 in *S.*

senegalensis (Fig. 2). The starvation treatment resulted in lower RNA:DNA ratios and lower growth rates or even mass loss in juveniles from both species (Fig. 2). Pearson product moment correlation results showed a significant positive correlation between RNA:DNA and weight-based instantaneous growth rate (r = 0.83, n = 54 in *D. labrax* and r = 0.54, n = 72 in *S. senegalensis,* P < 0.001) for both species. RNA concentration also had a positive correlation with instantaneous growth rates, although with lower correlation coefficients (r = 0.23, n = 54 in *D. labrax* r = 0.39, n = 72 in *S. senegalensis*, P < 0.01). Temperature was negatively correlated with RNA concentration in *S. senegalensis* (r = -0.08, n = 72, P < 0.05), yet had a positive correlation with RNA:DNA in *D. labrax* (r = 0.28, n = 54, P < 0.05) and with growth rate in juvenile *S. senegalensis* (r = 0.18, n = 72, P < 0.05).

a) *Dicentrarchus labrax*



b) *Solea senegalensis*

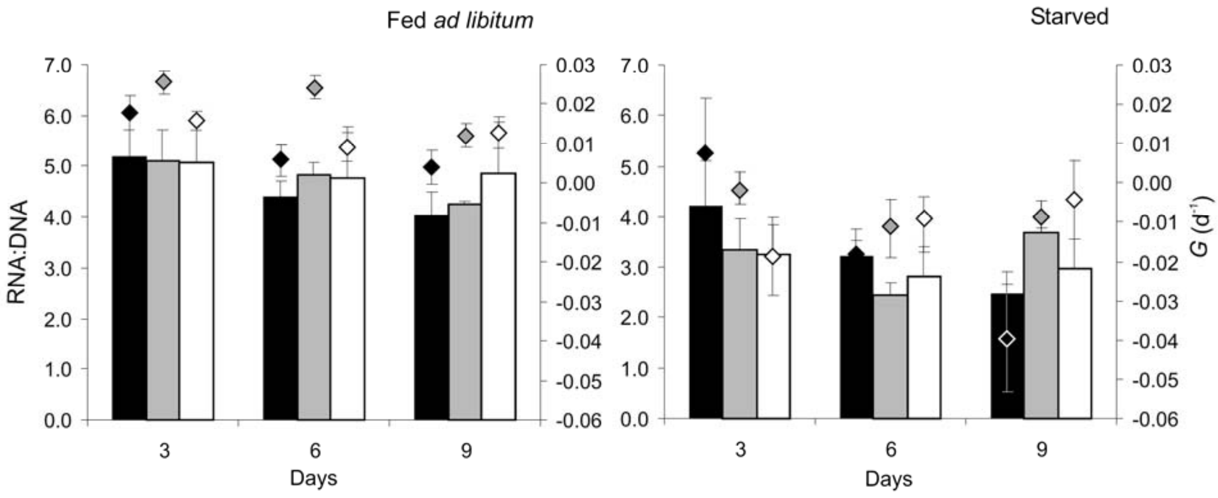


Figure 2. Mean (and standard deviation) RNA:DNA ratio (bars) on the primary axis and instantaneous growth rate ($G\ d^{-1}$) on the secondary axis (symbols ◇) of juvenile **(a)** *Dicentrarchus labrax* and **(b)** *Solea senegalensis* during the experimental calibration. The experimental design included two feeding levels, *ad libitum* and starvation, and tested three different temperatures (T, in ºC) and salinity (S) treatments: T = 15ºC and S = 15 (white bars and symbols ☐); T = 25ºC and S = 15 (grey bars and symbols ▨); T = 25ºC and S = 25 (black bars and symbols ■).

RNA:DNA ratio (R:D) was the first variable included in the stepwise multiple regression analyses for both species, and in the case of *S. senegalensis* water temperature (T, in degrees Celsius) was the next variable selected, while the remaining variables were not significant. The resulting model equation for estimating juvenile *D. labrax* instantaneous growth rate (*G*) was: $G = -0.0377 + 0.0165(R{:}D)$ ($r^2 = 0.72$, n = 54, $P < 0.001$). The model equation for estimating instantaneous growth rate of juvenile *S. senegalensis* was: $G = -0.0319 + 0.0098(R{:}D) + 0.0021(T)$ ($r^2 = 0.63$, n = 72, $P < 0.001$). Models residuals did not reveal any clear pattern when plotted against all independent variables considered in the present study.

3.2 Fish growth performance in estuaries

Mean values of water temperature and salinity in the estuaries sampled during July 2005 and 2006 are presented in Table 1. Mean water temperature and salinity were close to the highest values tested in the laboratory calibration experiment (temperature 25 ºC and salinity *ca*. 25) with the exception of the Sado estuary in 2006, which was near the lower salinity level experimentally tested (ca. 15). Both years were characterized by air temperatures above local average, yet in terms of rainfall these years were very distinct and were considered an extremely dry (2005) and a regular year (2006), respectively (Instituto de Meteorologia 2006, 2007), and higher water temperatures were mostly observed in 2006.

Table 1. Mean (with standard deviation between brackets) water temperature (ºC) and salinity in Portuguese estuaries sampled in July 2005 and 2006.

Estuary	Year	Temperature (ºC)	Salinity
Ria Aveiro	2005	25.13 (3.02)	24.13 (6.49)
	2006	25.21 (2.76)	20.54 (3.32)
Mondego	2005	24.36 (2.61)	21.01 (1.69)
	2006	23.93 (2.92)	19.24 (5.36)
Tejo	2005	24.60 (3.20)	20.38 (5.60)
	2006	27.09 (3.15)	24.07 (4.58)
Sado	2005	23.65 (2.71)	27.73 (2.23)
	2006	27.10 (1.18)	13.06 (5.59)
Mira	2005	23.65 (1.71)	26.72 (1.23)
	2006	26.67 (0.37)	22.96 (4.40)

Spatial and temporal differences were observed in RNA:DNA ratios, and consequently in model estimated instantaneous growth rates of estuarine-collected fish (Table 2). Juvenile *D. labrax* RNA:DNA ratios varied between 0.94 and 5.47 in 2005 and between 0.65 and 6.03 in 2006, with higher mean values in the Mondego and Mira estuaries and lower in Ria de Aveiro. Instantaneous growth rates, ranging from -0.022 d^{-1} to 0.053 d^{-1}, were significantly different amongst estuaries in 2005 (ANOVA, df = 3, $P < 0.001$), with significantly lower growth rates estimated for individuals occurring in the northern estuary (Ria de Aveiro), and higher estimates observed in the Mondego estuary. A similar pattern was depicted in 2006, with *G* varying from -0.033 d^{-1} to 0.062 d^{-1}, although with no significant differences (Table 2). *S. senegalensis*

Table 2. Mean (and standard deviation in brackets) wet weight (g), total length (mm), RNA:DNA ratio and instantaneous growth rate (d^{-1}) of juvenile *Dicentrarchus labrax* and *Solea senegalensis* sampled in each estuary in July 2005 and 2006. Spatial and inter-annual significant differences, per species, in juvenile fishes' instantaneous growth rates are indicated by similar letters (from post-hoc comparisons Tukey tests or Student's *t*-test, whenever applicable).

Species	Estuary	Year	Wet weight (g)	Total length (mm)	RNA:DNA	G (d^{-1})
D. labrax						
	Ria Aveiro	2005	6.12 (2.79)	87.77 (11.44)	2.74 (1.16)	0.007 (0.012) ^{a,b}
		2006	4.11 (2.50)	70.76 (15.29)	3.78 (0.64)	0.025 (0.009) ^{b}
	Mondego	2005	4.34 (1.61)	73.44 (12.40)	3.83 (0.92)	0.028 (0.010) ^{a}
		2006	5.02 (1.86)	70.48 (11.35)	4.03 (0.57)	0.029 (0.005)
	Tejo	2005	5.50 (1.30)	77.07 (12.97)	3.17 (0.61)	0.015 (0.010) ^{c}
		2006	6.09 (2.93)	81.27 (13.46)	3.90 (0.98)	0.027 (0.016) ^{c}
	Mira	2005	4.15 (1.66)	71.29 (6.13)	3.17 (1.64)	0.015 (0.015) ^{d}
		2006	5.43 (1.54)	80.44 (5.94)	4.15 (0.80)	0.031 (0.008) ^{d}
S. senegalensis						
	Ria Aveiro	2006	5.14 (3.21)	70.01 (12.28)	4.63 (0.87)	0.028 (0.007)
	Tejo	2005	4.11 (2.17)	67.71 (14.13)	3.56 (0.77)	0.019 (0.011) ^{a,c}
		2006	4.36 (1.23)	64.51 (6.37)	4.02 (0.99)	0.025 (0.008) ^{b,c}
	Sado	2005	4.12 (1.89)	61.10 (15.21)	4.29 (0.85)	0.024 (0.007) ^{a,d}
		2006	5.38 (2.95)	77.9 (10.84)	4.87 (0.97)	0.033 (0.008) ^{b,d}

RNA:DNA ratios ranged from 2.27 to 6.08 in 2005 and from 2.69 to 6.98 in 2006, with higher mean values in the Sado estuary and lower values in the Tejo estuary. Model application resulted in estimated instantaneous growth rates between 0.007 d^{-1} and 0.040 d^{-1} in 2005 and 0.014 d^{-1} and 0.052 d^{-1} in 2006. Differences in juvenile growth rates estimated in both years occurred between the Tejo and the Sado estuary (Student's *t*-test, df = 58, $P < 0.01$ in 2005; ANOVA, df = 2, $P < 0.05$ in 2006) (Table 2).

In general mean, RNA:DNA ratios and growth rates were higher in 2006 when compared to 2005 for both juvenile sea bass (Student's *t*-test, df = 219, $P < 0.001$) and Senegalese sole (Student's *t*-test, df = 142, $P < 0.001$).

The difference between maximum laboratory observed and field-collected fish growth rates (ΔG) was positive for all *S. senegalensis* juveniles and for the majority of *D. labrax* juveniles (Fig. 3). However, 10 individuals from the Mondego, Tejo and Mira estuaries presented higher estimated growth rates, which accounted for a small percentage of negative ΔG (Fig. 3). Hence, the reference growth rate considered for *D. labrax* juveniles was an average of the maximum field-estimated growth rates, 0.060 d^{-1}. *D. labrax* mean growth performance varied between 16.1 % and 46.5 % in 2005 and between 37.2 % and 54.7 % in 2006. Highest mean growth performance was observed in the Mondego and Mira estuaries (in 2005 and 2006, respectively) and the lowest mean growth performance percentage was observed in Ria de Aveiro in both years (ANOVA, df = 3, $P < 0.05$)(Fig. 4a). Growth performance of *S. senegalensis* juveniles ranged from 35.9 % to 47.0 % and from 48.6 % to 63.1 % in 2005 and 2006, respectively. A similar variation pattern was observed in both years, with higher mean growth performance percentages in the Sado estuary and lower mean percentages observed in the Tejo estuary (Student's *t*-test, df = 58, *P* < 0.01 in 2005; ANOVA, df = 2, *P* <0.01 in 2006) (Fig. 4b).

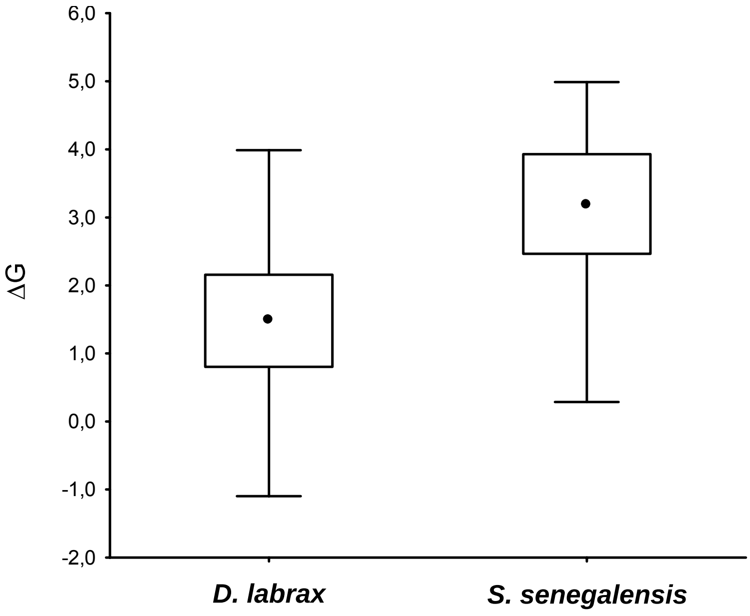


Figure 3. Difference between maximum growth rates of experimental and field-collected *Dicentrarchus labrax* and *Solea senegalensis* juveniles (ΔG). ΔG median (●), 25% and 75% percentiles (☐) and range are represented. Negative ΔG indicates higher growth rates of estuarine individuals when compared to maximum observed rates in the experimental calibration.

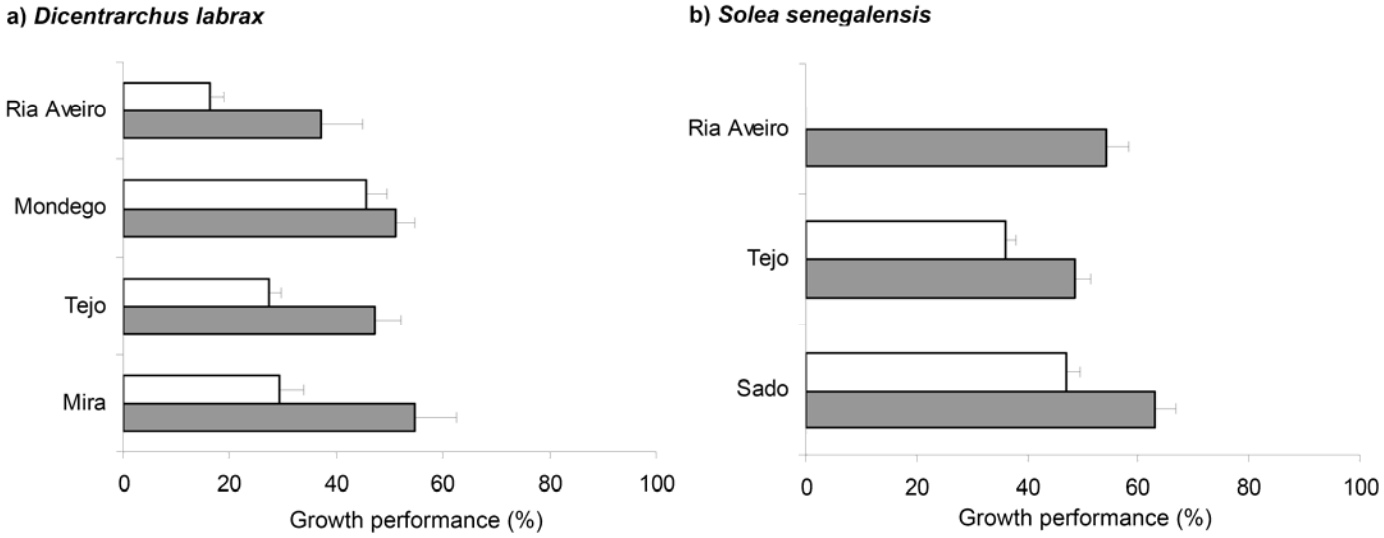


Figure 4. Mean (and standard deviation) growth performance (in %) of juvenile **(a)** *Dicentrarchus labrax* and **(b)** *Solea senegalensis* collected in five Portuguese estuaries in July 2005 (☐) and 2006 (▨).

4. Discussion

4.1 Laboratory calibration experiments and development of growth models

In general, instantaneous growth rates of juvenile *D. labrax and S. senegalensis* were highest in the treatment with water temperature of 25 ºC and salinity 15. Juvenile from both species responded to the different feeding levels, which resulted in a wide range of RNA:DNA values and instantaneous growth rates. Food deprivation led to lower RNA:DNA ratios and to individuals' weight loss, thus accounting for negative instantaneous growth rates observed in both species. Several studies have also described the RNA:DNA index sensitivity to food quantity and quality (e.g. Gwak and Tanaka 2001; Johnson et al. 2002; Islam and Tanaka 2005).

Sudden salinity changes have been found to limit growth and RNA:DNA ratios in fish larvae (Kim et al. 2008). Nonetheless, in the present experiments salinity had no apparent effect on fish growth rate or RNA:DNA ratios in both species. This could be due to the fact that juvenile *D. labrax* and *S. senegalensis* are euryhaline species and well adapted to the salinity range considered (15 to 25), which likely enables them to endure daily tidal variation. Possible effects of varying salinity regimes, such as tidal variations, were not tested and should be considered in future studies.

Fish length had a low but significant correlation with RNA:DNA, however, it did not met the required significance level to be included in the model, nor did a length standardized RNA:DNA ratio result in better model predictions. Fish length may account for some of the models' unexplained growth variability, however this effect can be minimized by considering the current size range in future model applications.

RNA:DNA index was the only biochemical feature highly correlated with instantaneous growth rates, and the main variable explaining juvenile fish growth variability in both species (72 % in *D. labrax* and 63 % in *S. senegalensis*). Juvenile *D. labrax* and *S. senegalensis* RNA concentration was also positively correlated with growth rate but explained less variability of individuals' growth rate.

Temperature had a low negative correlation with RNA concentration in *S. senegalensis*. This negative relationship has been reported previously, suggesting that at lower temperatures an increase in RNA concentration may compensate for lower RNA activity (Goolish et al. 1984), while at higher temperatures growth rate increase could be related to higher RNA activity and not to a significant increase in RNA synthesis (Buckley 1984; Malzahn et al. 2003). In the present work temperature was also positively correlated with fish growth rates in *S. senegalensis* and with RNA:DNA in *D. labrax.* Hence, the best multiple regression growth-models analysed for *S. senegalensis* juveniles included a water temperature term, which was not the case for *D. labrax*.

Since temperature may have an indirect effect on growth rate through an interaction with RNA:DNA, of an interaction term between RNA:DNA and temperature (R:DxT) was tested for both species, following a similar approach by Buckley et al. (2008) with several fish species larvae. Nonetheless, this approach did not improve previous model predictions. Senegalese sole and sea bass growth models' differences may result from differences in species metabolic pace or acclimation rates in relation to temperature. Sea bass is a highly active fish species with significant higher metabolic rates when compared to sole species (Lefrançois and Claireaux 2003). This may result in a more rapid adaptation to different temperatures within the given experimental range. In this case, temperature had indirect effects on *D. labrax* growth through direct effects on RNA:DNA ratios.

Some of the unexplained variability in growth rates may be an artefact of laboratory conditions that do not accurately simulate the variability in environmental conditions. In addition, other influential aspects may be introduced, such as fish confinement and thus possible density-dependent effects or behavioural changes, which may influence individual growth rate. These are well acknowledged limitations to laboratory experiments; however they provide insightful knowledge into fish responses to the environment and represent a fair compromise between the lack of model calibration and large scale mesocosm experiments.

Many other calibration studies have demonstrated a strong relationship between RNA:DNA and juvenile growth rates of various fish species (e.g. Malloy and Targett 1994; Fukuda et al. 2001; Peck et al. 2003; Mercaldo-Allen et al. 2006; Mercaldo-Allen et al. 2008), which resulted in different RNA-DNA based growth models that either included (*e.g.* Peck et al. 2003; Mercaldo-Allen et al. 2006) or excluded an independent temperature term (Mercaldo-Allen et al. 2008). The latter authors suggested that the relationship between water temperature, RNA:DNA and growth rates may be different when comparing juvenile and larvae fish stages. Further investigation is needed to clarify this relationship.

Overall, a collective growth predictive model for juvenile fishes appears less feasible than for larvae as tested by Buckley et al. (2008), probably due to increased biological specialization inherent to species ontogeny and life traits, as well as to higher individual variability.

4.2 Fish growth performance in estuaries

The difference between juvenile growth response to defined laboratory experimental settings and field conditions, expressed as the difference between maximum growth rates attained by juvenile fish under each situation (ΔG), was used to evaluate the R:D-growth model estimates. ΔG was positive in all *S. senegalensis* individuals, suggesting that laboratory conditions were not limiting juvenile growth. On the other hand, a small percentage of estuarine *D. labrax* juveniles from the Tejo estuary presented higher instantaneous growth rates than experimental individuals, which resulted in the establishment of the maximum field estimated growth rates as the reference growth rate for growth performance determination. In this case, laboratory conditions could be limiting maximum growth of juvenile sea bass or lower maximum growth rates were due to individual variability. Nevertheless, growth performance of individuals within trial conditions reached *ca*. 92.0 % when compared to field reference growth rate, and the R:D-growth model explained about 72.0 % of the growth variability, which is considerably high and allows for a good degree of confidence in future model applications.

The two species have a high commercial importance in Portuguese fisheries, and the quality of the nursery areas that sustain the juveniles may influence the viability of the coastal stocks (Beck et al. 2001). Therefore, assessing juveniles' growth and condition for either species is extremely important due to the fact that the reported differences in growth rate estimation and condition could amount to different conclusions regarding habitat quality and importance for each species. Vasconcelos et al. (2009) emphasizes the importance of a multi-species approach using condition indices to globally determine the nursery value in estuarine systems. As in the latter work, spatial and temporal differences were observed in RNA:DNA ratios for both sea bass and Senegalese sole juveniles. Hence estimated growth rates and juvenile growth performance also differed in both species for some of the estuaries and years considered, and were not concurrent between the two species. The mean fraction of maximum growth realized by juvenile *D. labrax* and *S. senegalensis* in estuaries was generally lower than 60.0 % in all systems for both 2005 and 2006. Nevertheless, in 2006 mean growth performances were always higher than in 2005, with greater inter-annual differences being observed for juvenile *D. labrax* when compared to the closer annual percentages in *S. senegalensis* juveniles. The year 2005 was considered a drought year and it appears that both species' juveniles were sensitive to this environmental feature, potentially due to its effects on food availability and quality or to other abiotic interactions that could have limited growth. Although there is no field data to support this conclusion, Dolbeth et al. (2008) suggested that reduced estuarine fish production in the Mondego estuary was related to an extreme drought event in 2005, and Smith et al. (2008) found evidence of decreased estuarine habitat quality for juvenile fishes under a drought scenario.

D. labrax and *S. senegalensis* individuals were not growing at their full potential in estuaries, most likely due to abiotic or biotic limitations from the physiological cost associated with estuarine life (Yamashita et al. 2003). Furthermore, a drought event in 2005 could have also influenced the lower mean growth performance, despite high individual variability (where slower growing individuals may never attain maximum growth rates observed in other faster growing individuals).

High individual variability in growth performance observed in the Senegalese sole and sea bass in estuaries was evident from the wide range of reported percentages. Reference or maximum growth rates (100.0 % growth performance) were described for *D. labrax* juveniles from the Tejo estuary, while simultaneously some individuals were not growing at all in different estuaries (0.0 % growth performance observed in ca. 5.0 % of total individuals analysed in 2005). A considerable sampling effort was important in order to include a significant number of individuals that could be representative of the entire population, and is also required for future work.

Overall, the fraction of maximum growth realized by juvenile *D. labrax* and *S. senegalensis* allowed for species, estuarine and annual differentiation in habitat quality considering conditions for fish growth enhancement. Growth performance in *S. senegalensis* individuals was more homogeneous throughout the period and sites studied than in *D. labrax* juveniles, highlighting the potential problems of defining habitat quality priorities for different species from conclusions drawn from only one or even more very distinct species. Nonetheless, the specific growth models developed in the current work represent a considerable step forward in assuming and resolving these differences.

In the future, it would be also be interesting to test the growth performance method under different pressure gradients, in order to examine the sensitivity of fish response to different stressors and to further test its utility as a monitoring tool.

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CHAPTER 5

Ecophys.Fish perspectives on growth of juvenile soles, *Solea solea* and *Solea senegalensis*, in the Tagus estuary, Portugal

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Ecophys.Fish perspectives on growth of juvenile soles, *Solea solea* and *Solea senegalensis*, in the Tagus estuary, Portugal

Abstract: Ecophys.Fish, an ecophysiological framework to simulate fish growth in time-varying environments, was parameterized for two sole species, *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* Kaup, 1858. The model gave reliable predictions of soles' growth and metabolic rates from published data under controlled environments. Differences in model parameters reflected species different environmental optima and were in accordance with their distributional range - northern range of *S. solea* and a southern range of *S. senegalensis*. Field application of this model to resolve the effects of varying habitat conditions on juvenile soles' growth in the Tagus estuary (Portugal), during the spring–summer period from 2003 to 2006, highlighted spatial and temporal differences in soles' metabolic scope for growth and estimated growth rates. Higher growth estimates were obtained for *S. solea* and *S. senegalensis* in Vila Franca de Xira during 2006, and for *S. senegalensis* in Alcochete during 2003, 2004 and 2006, and were fairly well explained by natural variation in abiotic conditions. Overall, the Ecophys.Fish model gave accurate field predictions of each sole species' growth rate and proved to be a useful tool for monitoring and assessment of habitat quality for juvenile sole.

Key-words: flatfish; juvenile sole; growth; metabolic scope; Ecophys.Fish

1. Introduction

Metabolism can power the activities of organisms and it is only through metabolism that the environment has its effects on those activities - e.g. feeding, digestion, excretion, swimming, and growth (Fry 1947, 1971). According to Fry's “physiological classification of the environment” there are five categories of environmental factors: lethal (e.g. predators); loading or masking factors that increase maintenance costs (e.g. salinity); controlling factors that determine the inherent metabolic pace at both maximum and minimum activity levels (e.g. temperature); limiting factors that restrict maximum metabolism (e.g. oxygen); and directive factors that guide individual regulatory behaviour and physiological acclimatory processes on both maximum and minimum metabolism(e.g. photoperiod). These factors affect the available metabolic power to sustain activities above the obligatory minimum for life (standard metabolic rate, SMR) and below the physiological maximum (active or maximum metabolic rate, MMR). The difference between MMR and SMR is termed metabolic scope (Fry 1947, 1971). In this context, the metabolic scope for growth (MSg) can be considered as the available power for growth after all routine activities have been performed corresponding to the difference between MMR and routine metabolic rate (RMR).

Growth in fish is assumed to be a comprehensive measure of habitat quality and fish health, as it integrates the effects of environmental factors (e.g. food resources, salinity, temperature) and individual fitness (e.g. Able et al. 1999; Meng et al. 2000; Fonseca et al. 2006). Metabolic scope for growth is strongly correlated with growth rates, and can be used to predict fish growth given respirometric determination of the metabolic scope (Neill and Bryan 1991). Building on this concept, Neill et al. (2004) developed an ecophysiological framework for simulating fish growth under varying conditions of multiple environmental variables, the Ecophys.Fish model. The model mechanistically describes the interaction of Fry's classes of environmental factors and their joint effects on growth, coupling metabolism and bioenergetics. It depicts the way the available energy is partitioned within the fish so it can perform all activities (including food processing and excretion) according to abiotic constraints on the metabolic capacity. Model components have been tested in specific scenarios, which measured fish growth and metabolic performance with varying temperature and oxygen (Del Toro-Silva et al. 2008) and with simultaneous variation in temperature and feed energy (Fontaine et al. 2007), and proved effective in both cases.

Yamashita et al. (2001) described the ecophysiology of flatfish in nursery grounds based on Fry's environmental classification and discussed the possibility of an ecophysiological model to predict growth and subpopulation production. Using growth as an evaluative endpoint, the Ecophys.Fish model can also be used for habitat quality assessment under realistic time-varying environments. Del Toro-Silva (2008) adapted the Ecophys.Fish model for southern flounder (*Paralichthys lethostigma* Jordan and Gilbert, 1884) and applied it to evaluate habitat quality of nursery areas in North Carolina estuaries.

In the present study, Ecophys.Fish was parameterized for two sole species, the common sole *Solea solea* (Linnaeus, 1758) and the Senegalese sole *Solea senegalensis* Kaup, 1858. These sole species are very similar in terms of morphology and ecology, but have different distributional ranges: the common sole has a more northern distribution ranging from the North Sea to North Africa, including the Mediterranean, while the Senegalese sole occurs from the Bay of Biscay to Senegal (Quéro et al. 1986). Both species use the Tagus estuary (Portugal) as an important nursery ground (Costa and Bruxelas 1989; Cabral and Costa 1999), occurring in sympatry in this area providing the opportunity to assess how the model performs under field conditions, i.e. to determine the metabolic scope for growth and estimate juvenile sole growth in the Tagus estuary. Although estuarine conditions are highly dynamic, in nursery areas juvenile fishes usually benefit from abundant food resources, favourable temperature ranges and low predatory pressure (Miller et al. 1985). Ecophys.Fish model was thus used as a tool to resolve the effects of varying habitat conditions on juvenile soles' growth in the Tagus, during the spring–summer period from 2003 to 2006.

Information for constructing the models was retrieved from several studies focusing on these species metabolic rates and growth patterns under controlled conditions (e.g. Fonds 1975; Howell and Canário 1987; van den Thillart et al. 1994; Lefrançois and Claireaux 2003; Salas-Leiton et al. 2008).

2. Materials and methods

2.1 Model description and parameterization

The Ecophys.Fish model has two functional modules dealing with fish metabolism and bioenergetics. The metabolism subroutine describes how time-varying environmental factors, namely temperature, salinity, pH, food and dissolved oxygen influence metabolic rates and thus the metabolic scope for growth. Standard metabolic rate (SMR) is a function of the loading effects of salinity, temperature (as a controlling factor) and fish weight, where modelled fish partially compensate for temperature change. SMR is defined as: SMR=S^*exp(q1Taccl)*exp(q2Tstress); where S is the intercept value, which is a function of salinity, fish weight and temperature, elevated as an exponential function both of acclimation temperature (Taccl and q1, the steady state component and rate constant) and of the difference between ambient and acclimation temperatures (Tstress and q2, the transient state component and rate constant) in order to account for both the Ahrennius effect and thermal acclimation. Maximum metabolic rate (MMR) is determined by the limiting effects of dissolved oxygen (DO) with regard to the controlling effects of temperature and pH and the effects of fish weight. Thus, MMR=*MMSO***DO*a**pH*factor**DO*lim**DO*accl**W*effect, where *MMSO* is the intercept of marginal metabolic scope (a determinant of intrinsic metabolic efficiency); *DO*a is ambient dissolved oxygen concentration; *pH*factor is a dimensionless pH transformation to account for a crude Bohr effect; *DO*lim is a power-hyperbolic function of the temperature-dependent DO that limits MMR; *DO*accl is the DO acclimation component that accounts for DO changes at a given rate proportional to SMR; and *W*effect is the effect of fish weight on metabolic rates. In addition, routine metabolic rate (RMR) is defined as 2.0*SMR based on Winberg's empiricism, which results in a metabolic scope for growth (MSg) equal to the difference between MMR and 2.0*SMR.

In the bioenergetics subroutine, the fish partitions the consumed food energy between obligatory expenditures, namely standard and routine activities, feed-processing costs, wastes and the residual energy is then converted to new biomass - fish growth. Under the model a fish eats all appropriate food encountered or until available digestive or metabolic capacity becomes insufficient to support further food processing, thus representing a metabolic limitation of food intake. Neill et al. (2004) describe in full detail the above intertwined relationships between environmental factors and fish physiological processes.

Ecophys.Fish model parameterization for *S. solea* and *S. senegalensis* (with STELLA® software) was based on published data describing the two species' respective environmental preferences and limits (e.g. salinity, temperature, dissolved oxygen), as well as biological constrains (e.g. feeding rate) (Table 1). The model's inputs are time series of temperature, pH, dissolved oxygen, salinity, food availability and energy content, and initial size and energy density of juvenile fish (Neill et al. 2004). Metabolic rate data were also compiled from previous studies on sole species, providing estimates of oxygen uptake rates during standard, routine or maximum activity (Table 1). In the complete absence of requisite data, some model parameters were retained as in the original version of the model, such as the functions describing the variable acclimation rate coefficients for DO and temperature changes. Model parameters were progressively adjusted and tested by simulating growth and respirometry trials documented in these published studies. The models' adequacy or goodness of fit was

Table 1. Main sources of reference data for Ecophys.Fish model parameterization for *Solea solea* and *Solea senegalensis*.

Model parameterization	*S.solea*	*S. senegalensis*	References
Temperature			
Topt (°C)	20	25	Fonds (1975); Fonds et al. (1989); Howell (1997); Cabral and Costa (1999); Amara (2004); Imsland et al. (2003); Lefrançois and Claireaux (2003).
Tinfl (°C)	12	16	
q1 (1/°C)	0.052	0.054	
Dissolved oxygen and metabolic rates			
DOlim, intercept (mg O_2 L^{-1})	0.175	0.35	Howell and Canário (1987); van den Thillart et al. (1994); Aragão et al. (2003); Lefrançois and Claireaux (2003); Davoodi and Claireaux (2007); Salas-Leiton et al. (2008).
DOlim, Max (mg O_2 L^{-1})	1.75	2.00	
Smino (mg O_2 g^{-1} h^{-1})	0.037	0.042	
Hill parameter	1.9	2.25	
Salinity			
SalOpt	10	20	Imsland et al. (2003); Arjona et al. (2005); Vinagre et al. (2006); Vinagre et al. (2007).
Feeding rate			
FeedRateMax (g_{feed}/g_{fish}*day)	0.20	0.25	Cabral (2000); Vinagre et al. (2007); Vinagre and Cabral (2008).
Growth rate			Fonds (1975); Day et al. (1997); Dias et al. (2004); Rodiles et al. (2005); Rodríguez et al. (2005); Palazzi et al. (2006); Pinto et al. (2007); Costas et al. (2008); Salas-Leiton et al. (2008).

For detailed description of model parameters see Neill et al. (2004).

checked by linear regression analysis of observed (references in Table 1) and simulated growth rates (as weight percent change per day relative to fish initial weight).

2.2. Field application

The Tagus estuary (38°40′N 9°15′W) is a large system (325 km^2), partially mixed and with a tidal range of about 4 m. Intertidal and saltmarsh areas account for ca. 40% of the total estuarine area. Mean river flow discharge is 400m^{3}s^{-1} although it varies considerably with season and year (Bettencourt and Ramos 2003). The upper part of the estuary is shallow and fringed by salt marshes. The two main nursery areas for sole (Vila Franca de Xira - VFX and Alcochete - ALC) identified by Costa and Bruxelas (1989) and Cabral and Costa (1999) are located in the upper estuary (Fig. 1). In VFX nursery area both sole species occur, but in ALC nursery only *S. senegalensis* is present (Cabral and Costa 1999). The uppermost area of ca. 46.46 km^2 (VFX) is deeper (mean value 4.4 m) and has a higher proportion of fine sand in the sediment, while in the other area of ca. 24.75 km^2 (ALC) the mean depth is 1.9 m and the sediment is mainly composed of mud. Intertidal mudflats encompass 23% of VFX and 87% of ALC areas (Cabral and Costa, 1999).

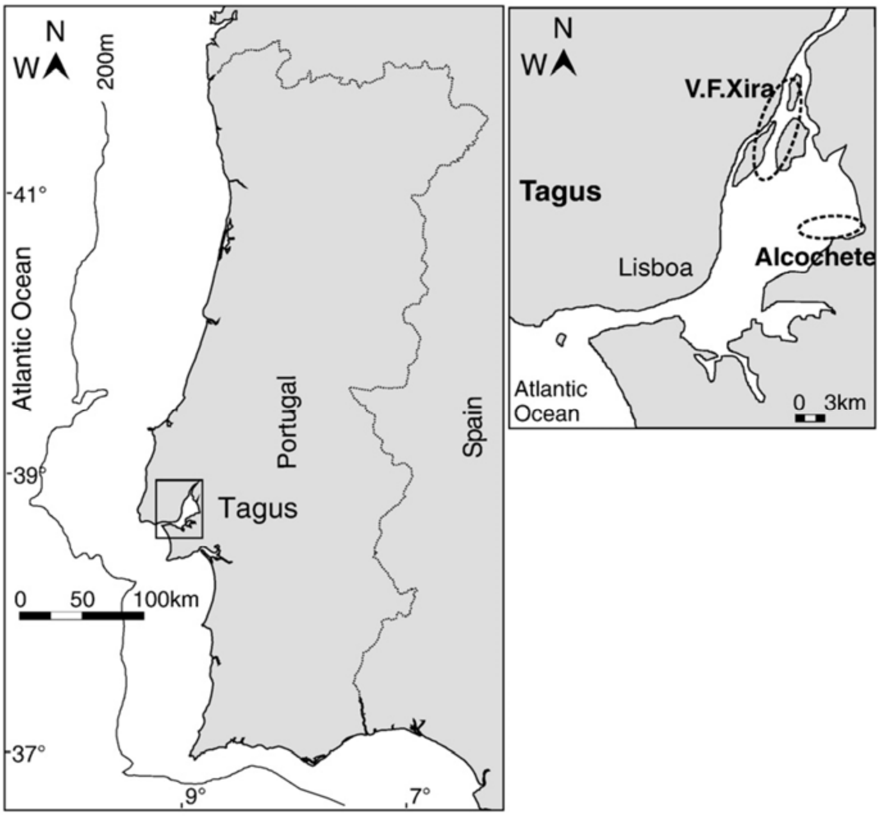

Figure 1. Fig. 1. The Tagus estuary and the two nursery areas for juvenile *S. solea* and *S. senegalensis*, Vila Franca de Xira and Alcochete.

Juvenile sole were collected monthly in the two nursery areas, using a beam trawl, between May and September 2003 to 2006. Fish total length and body weight were determined. Weight-frequency data were analysed by modal progression analysis using the Bhattacharya method (Bhattacharya 1967), with the software FISAT II, version 1.1.2 (FAO 2002). Observed growth rates of young soles (as weight percent change per day relative to fish initial weight) were determined only for the first cohort as follows: obs $Gr=(Wt_f-Wt_i)/(Wt_i*(t_f-t_i))\times100$; where Wt_f and Wt_i correspond to total weight (in grams) at the initial (t_i) and final days (t_f) of the considered period.

For each nursery area, time series of environmental variables (on an hourly basis), namely water temperature, pH, salinity and dissolved oxygen, were obtained from data bases of the Portuguese Water Institute (INAG http://snirh.inag.pt) and the MOHID hydrological operational model (Fernandes et al. 2004; http://www.mohid.com/tejo%2Dop/), and from field measurements with a multi-parameter probe (WTW) and temperature data loggers (HOBO H20-001). Food availability was based on major prey items' density (i.e. polychaetes and bivalves) in the nursery areas, determined from sediment samples collected with a van Veen grab-sampler, and included in the model based on preliminary laboratory data accounting for prey density and juvenile sole feeding rate (Vinagre et al. 2007). The energy content of prey items was based on calorimetric measurements (e.g. Fidalgo e Costa et al. 2000).

Ecophys.Fish was parameterized by running simulations of juvenile sole growth observed under specified environmental conditions. The best simulation was considered for each year, per area and species (for the first cohort), when environmental and species data gave coherent and accurate estimates of observed growth rates obtained from modal progression analysis (linear regression analysis of observed vs simulated rates of weight change).

These simulation models were used to compare juvenile sole performance (growth wise) between nursery areas, years and species (when in sympatry). Individual growth rates (as rate of weight change relative to initial weight) were simulated for equal periods, with collected fish ranging from 1 to 3 g (20 to 30 individuals of each species, per site and year; for a total of 258 individual simulations). The effect of soles' initial weight distribution on growth rate simulations was tested through analyses of variance (ANOVA). The absence of significant differences ($F_{10,258}$=0.86, $P > 0.05$) indicated that the major source of variation in fish growth rates was due to species and environmental conditions. To avoid unduly inflating goodness of fit due to the high correlation of metabolic scope for growth and growth rates, statistical analyses are only presented for growth estimates.

Significant differences in mean growth rate estimates between the two sole species were evaluated by a t-test. Analyses of variance were used to assess growth-rate variation between years for each species and area, followed by *a posteriori* Tukey tests whenever the null hypothesis of equality of variances was rejected. Additionally, differences between the two nursery areas were determined, with a t-test, only for *S. senegalensis* due to its simultaneous use of both areas. All statistical analyses were performed using Statistica software and a significance level of 0.05 was considered in all test procedures.

3. Results

3.1 Model parameterization

Experimental conditions reporting sole oxygen uptake rates were accurately simulated with the Ecophys.Fish model parameterization for both species (Fig. 2a; R^2=0.90). In the process, further adjustments made to the ecophysiological model also gave reliable simulations of observed growth rates of juvenile soles from previous studies (Fig. 2b and c; R^2=0.98 and R^2=0.94 for *S. solea* and *S. senegalensis* respectively).

Model parameter differences between species were consistent with differing environmental optima, namely lower salinity and temperature optimum for *S. solea* as well as higher maximum feeding rate with higher temperature for *S. senegalensis* (Table 1). In the context of metabolism these differences translated into moderately different parameters describing the loading effects of salinity on standard metabolism (e.g. Smin — minimum of the salinity component on SMR at optimum salinity) and the limiting effects of oxygen on active metabolism (e.g. DOlim, the temperature-dependent DO below which MMR becomes DO-dependent), considering the controlling effects of pH and temperature (Table 1). Therefore, the metabolic rate variation with temperature for both sole species was similar at lower temperatures; yet, *S. senegalensis* had higher metabolic rates (both standard and active rates) with increasing temperature (Fig. 3a). The outcome of these metabolic differences was a shift in the metabolic scope for growth towards higher temperatures in *S. senegalensis* (Fig. 3b).

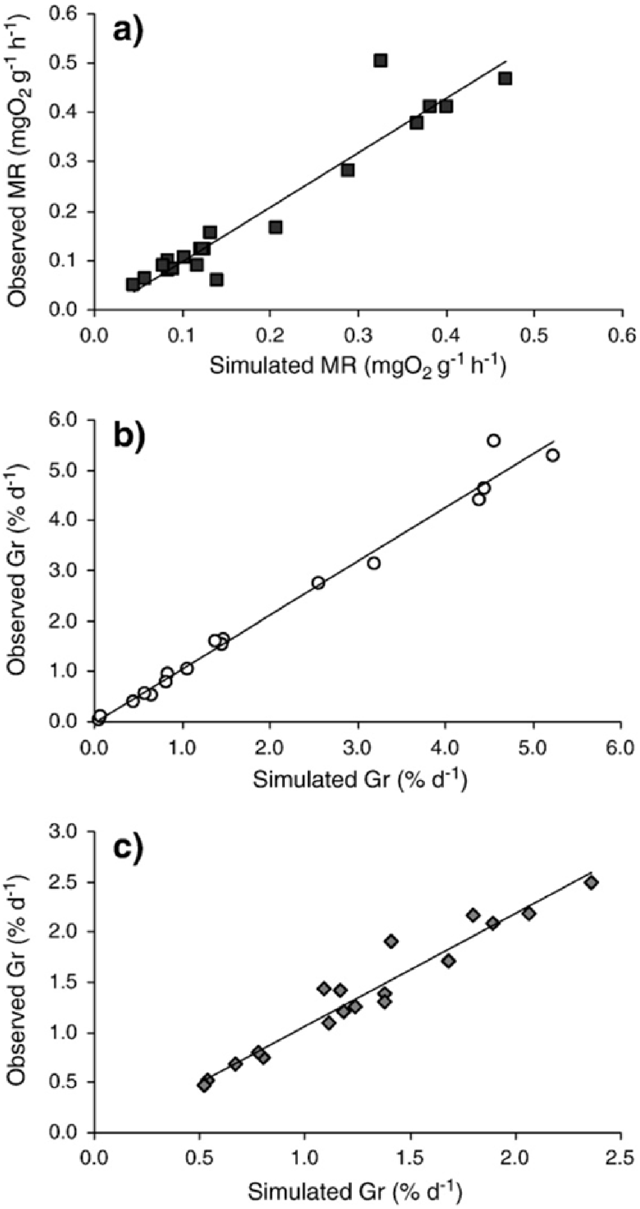

Figure 2. Linear regressions of observed vs simulated metabolic and growth rates: **a)** metabolic rates for a fish, with a weight of 1g, corrected for the weight effect on metabolic rates (Fry 1971; Brett and Groves 1979) and with data for both species combined (slope=1.10, intercept=−0.02, R^2=0.90, n=20; **b)** growth rates for *S. solea* (slope=1.07, intercept=−0.04, R^2=0.98, n=17); and **c)** growth rates for *S. senegalensis* (slope=1.12, intercept=−0.06, R^2=0.94, n=19). For references on observed rates see Table 1.

Thus, maximum metabolic scope for growth occurs at 20 °C for *S. solea* and at 25 °C for *S. senegalensis*.

3.2. Field application

Temperature values observed from 2003 to 2006 are amongst the highest values recorded over the past 50 years in Portugal (source IM - Portuguese Meteorology Institute). Additionally, 2003 and 2005 were considered dry years due to low rainfall values (source INAG).

Comparing the abiotic conditions in the two nursery areas, but excluding salinity that is markedly different between areas, other variables - namely temperature, dissolved oxygen, rainfall and pH – had low site variability (Table 2). Moreover, all environmental variables

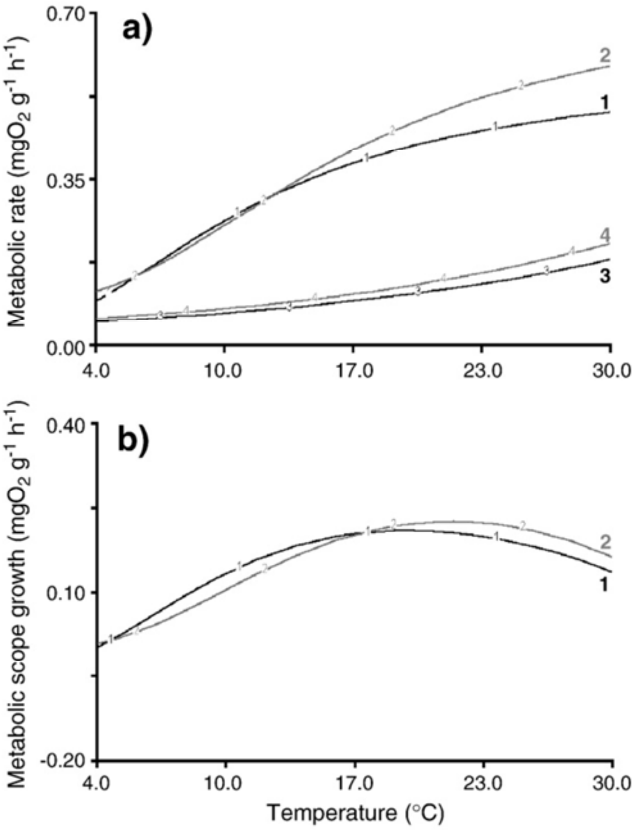

Figure 3. Model parameterized metabolic rates and metabolic scope for growth in soles: **a)** maximum and standard metabolic rates variation with temperature, for *S. solea* (MMR - 1, SMR - 3) and *S. senegalensis* (MMR - 2, SMR - 4); **b)** metabolic scope for growth variation with temperature for *S. solea* (1) and *S. senegalensis* (2).

were within the ranges considered not to have deleterious effects on soles growth (references in Table 1). Food resources were also not limiting; in fact, there was an abundance of important prey items for sole reported for Portuguese estuaries (i.e. polychaete *Hediste diversicolor* (O.F. Müller, 1776) and bivalve *Scrobicularia plana* (da Costa, 1778)) (Cabral 2000).

In order to improve model simulations when applying field conditions and comparing to observed growth rates, further adjustment of parameters was necessary, and resulted in changes in the MMSO parameter, which is the residual intercept of the marginal metabolic scope. As defined by Neill et al. (2004), MMSO describes the inherent metabolic efficiency of the fish-environment system after the presumed effects of temperature, pH, dissolved oxygen, salinity, and fish size have been taken into account. The initial model supposed an MMSO of 0.28 for both species; it was changed to 0.29 for *S. solea* during 2006; and to 0.30 for *S. senegalensis* in VFX during 2006 and for the three years considered in ALC. After these changes in MMSO the regression of model-simulated vs field-observed growth rates were in very good agreement (Fig. 4; R^2=0.91). Based on this good fit, comparative estimations of juvenile soles rates of weight change, for similar periods and weight classes (initial weight from 1 to 3 g), indicated significantly higher growth estimates for *S. senegalensis* juveniles occurring in the same area as *S. solea* (Fig. 5; $t_{190,192}=-3.95$, $P < 0.001$). Year effects on

Table 2. Mean, range and standard deviation (in brackets) of environmental conditions for each site (Vila Franca de Xira - VFX; and Alcochete - ALC) from May to September of 2003 to 2006.

Site	Year	Temperature (°C)	Rainfall (mm)	Salinity	DO (mg l^{-1})	pH
VFX	2003	21.30 (3.02)	5.92 (2.42)	18.20 (2.85)	7.80 (1.40)	7.33 (0.28)
		14.26–30.17	2.00–15.40	3.40–21.50	7.10–8.21	7.10–7.91
	2004	20.27 (2.76)	11.96 (5.37)	14.10 (3.32)	8.78 (2.39)	7.38 (0.47)
		12.39–28.02	0.50–24.60	2.35–19.10	6.25–9.30	6.95–8.00
	2005	20.69 (2.61)	5.62 (2.72)	12.52 (2.13)	8.69 (1.30)	7.48 (0.23)
		15.12–28.15	0.70–15.70	4.71–16.98	6.22–10.05	6.90–7.60
	2006	21.47 (2.92)	19.12 (11.60)	11.20 (3.89)	9.30 (1.02)	7.73 (0.35)
		14.84–28.69	0.20–52.50	2.39–15.76	7.40–9.38	6.99–8.40
ALC	2003	21.61 (3.20)	4.56 (1.36)	23.40 (4.03)	6.01 (1.41)	8.07 (0.07)
		13.48–30.22	2.10–8.80	8.40–25.92	5.53–6.78	8.00–8.20
	2004	20.43 (3.15)	10.73 (7.19)	22.70 (2.79)	6.44 (2.39)	8.07 (0.06)
		12.23–29.94	0.00–34.20	4.62–25.79	5.18 –12.70	7.95–8.20
	2005	21.51 (2.71)	5.92 (4.15)	27.73 (5.32)	5.93 (0.69)	8.09 (0.64)
		14.88–29.97	0.90–22.40	9.37–32.5	4.59–6.49	7.00–8.10
	2006	21.04 (3.10)	14.50 (8.56)	21.66 (2.85)	5.23 (1.81)	8.01 (0.32)
		13.82–29.03	1.60–36.50	7.50–28.35	4.84–9.60	7.58–8.15

juvenile soles' growth rate estimates were observed for both species in the sympatric area (Fig. 5; $F_{3,107}$=53.3 and $F_{3,85}$=25.2 for *S. solea* and *S. senegalensis*, respectively, $P < 0.001$), with significantly lower estimates in 2005 and significantly higher estimates in 2006 for both species (Tukey tests, $P < 0.001$). *S. senegalensis* estimated rates of weight change in the ALC area were not different between years ($F_{2,66}$=0.81, $P > 0.05$), but were significantly higher than estimates from the conspecifics in the VFX area ($t_{149,151}$=−9.16, $P < 0.001$). Simulated metabolic scope for growth evidenced the same pattern as growth rate estimates, with mean values varying from 0.175 mg O_2 g^{-1} h^{-1} for *S. solea* juveniles in 2005 to 0.207 mg O_2 g^{-1} h^{-1} for *S. senegalensis* in ALC 2006 (Fig. 5).

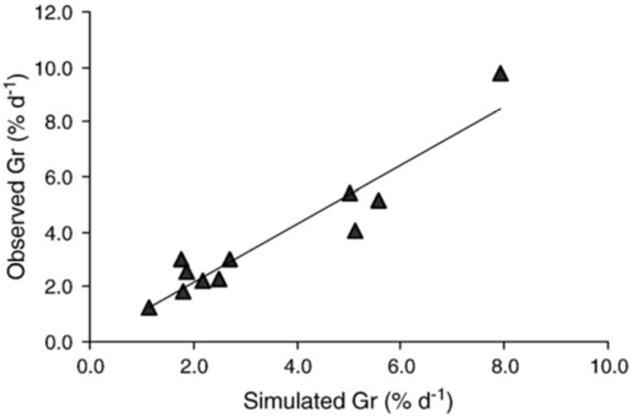

Figure 4. Linear regression of observed vs simulated growth rates from field-collected soles. Observed values are for the two sole species, in both nursery areas from 2003 to 2006; slope=1.08, intercept=−0.03, R^2=0.91, n=11.

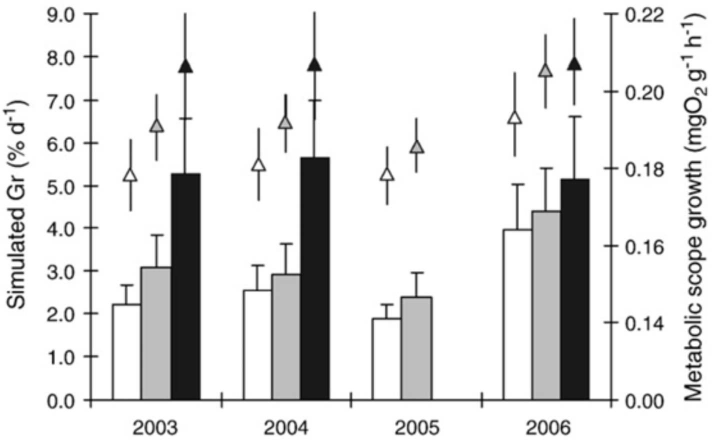

Figure 5. Simulated growth rates (Gr) and metabolic scope for growth (Msg, secondary axis) of soles in the Tagus estuary. Bars represent mean and standard deviation of simulated growth rates for *S. solea* from VFX (□) and *S. senegalensis* from VFX (▨) and ALC (■) during 2003 to 2006. Mean and standard deviation of Msg values for *S. solea* from VFX (△) and *S. senegalensis* from VFX (◭) and ALC (▲) are also showed for the same years.

4. Discussion

Modeling individual or population performance in a dynamically varying environment requires well established mechanisms of individual physiological integration of environmental conditions, which is extremely complex (Yamashita et al. 2001). There have been several approaches to this question that resulted in various models, such as bioenergetic integration of ecosystem-scale models of trophic interactions (Kitchell et al. 1974) or the dynamic energy budget (DEB) theory (Kooijman 2000; van der Veer et al. 2003) which describes energy k-rule partitioning between organisms' maintenance, growth and reproduction. Temperature and diet are considered the most important external regulators; still DEB models add surface area and structural volume influence on metabolic rates to assess individual growth in environmental dynamic conditions. The Ecophys.Fish model shares some common features with the earlier models, namely the bioenergetic processes and the temperature control on metabolic rates through the Arrenhius effect, but includes Fry's “physiological classification of the environment”, and therefore encompasses acclimation processes and interactions of multiple abiotic variables. This model explicitly accommodates temporal changes in environmental temperature and dissolved oxygen, by considering physiological acclimation processes in the form of modified exponential lags in fish metabolic response (Neill et al. 2004). The use of variable acclimation rate coefficients introduces biological realism to the model in the sense that it invokes the process of continuously modified physiological state by the individuals' environmental history. Although physiological acclimation is important for fish performance particularly in highly dynamic environments such as estuaries, it is still a fairly overlooked process with few data on different species conditioned physiological states, particularly their interaction with fluctuating environmental conditions (Fry 1971).

In this context, a frequent difficulty in ecological modeling is the sparsity of data and the lack of directed experimentation to test specific model assumptions, which makes it harder to parameterize models for different species and to independently evaluate the models (Neill et al. 2004). In the present work model parameterization for sole species entailed an extensive bibliographic data collection, which was fairly restricted for *S. senegalensis* ecology and metabolism.

The major differences between model parameters for the two sole species were due to difference in environmental optima, as described in previous studies. *S. solea* prefers lower temperatures and salinity (Cabral and Costa 1999; Imsland et al. 2003) than *S. senegalensis*, which in turn has higher maximum feeding rates with increasing temperature (Vinagre et al. 2007). Description of metabolic relationships for the sole species reflected these differences in model parameters that accounted for the effects of salinity and temperature on standard and active metabolic rates. In fact, at lower temperatures metabolic rates were quite similar for both species, but that of *S. senegalensis* increased with increasing temperatures (at higher salinities), which translated into a shift in the metabolic scope for growth of this species towards higher temperatures. The improved metabolic performance of *S. senegalensis* at higher temperatures, when compared with the common sole is in accordance with the two species' distributional ranges - a more northern distribution for *S. solea* and a more southern range for *S. senegalensis*.

The parameterized model for each sole species accurately simulated growth and metabolism observed under experimental conditions in several earlier studies (e.g. Fonds 1975; van den Thillart et al. 1994; Lefrançois and Claireaux 2003; Rodríguez et al. 2005; Salas-Leiton et al. 2008), which supports the models' utility under controlled environmental conditions. Nevertheless, current data on oxygen uptake rates of sole have not been effectively measured at temperatures above 24°C, which represents a knowledge gap near the upper lethal tolerance limits and needs to be addressed in the future.

Ecophys.Fish model's goodness of fit for juvenile soles' growth simulations was comparable or superior to the model performance predicting red drum and blueguill growth (Neill et al. 2004; Fontaine et al. 2007). Comparing metabolic parameters of the sole species' model and red drum and blueguill, originally used in the Ecophys.Fish parameterization (Neill et al. 2004) is beyond the scope of this work. Yet it is noteworthy that there were similarities in parameters describing acclimation and some physiological processes across species, namely the transient state rate constant (q2), DO and thermal acclimation rate coefficients, the Winberg factor, pH gain and feed processing energy expenditure (sda). Potentially some of the similarity resulted from the lack of current data, which does not allow for improved parameter estimates (e.g. acclimation rates coefficients), yet others like the Winberg factor may represent a common feature across fish species. Nevertheless, metabolism-related parameter values were usually lower for sole species (e.g. DOlim, Smino) and resulted in lower metabolic rates, which is in agreement with the metabolic differences between flatfish and roundfish reported in Duthie (1982).

The Ecophys.Fish model parameterized for each sole species under field conditions also gave consistent estimates of juvenile fish growth rates (as weight percent changes per day) compared to observed values, after the slight adjustment to the MMSO parameter. Potentially small changes in feed quality rather than in the MMSO parameter could have resulted in similar growth patterns; however, field data did not support this idea since there was no evident difference in food quantity or quality observed over the four years considered. MMSO is a measure of the intrinsic metabolic efficiency of the fish-environment system after the model integration of all other variables that affect metabolism (Neill et al. 2004). The increase in MMSO implies a more efficient metabolic performance in a given milieu, which might be the case for *S. solea* and *S. senegalensis* in VFX during 2006, and for *S. senegalensis* in ALC during 2003, 2004 and 2006.

Accordingly, spatial and temporal differences in soles' metabolic scope for growth (MSg) and estimated growth rates were fairly well explained by natural variation in abiotic conditions. Dry years (2003 and 2005) had an adverse effect on young soles' growth for both species occurring in VFX, particularly *S. solea*. During spring and summer 2006 juvenile soles presented the highest MSg and growth rates linked to high temperatures and rainfall simultaneously, which seems to have enhanced soles' metabolic performance. Considering *S. senegalensis*, there was a significant difference in fish performance between the two nursery areas. In agreement with the Ecophys.Fish autoecological approach (Neill et al. 2004), salinity seems to be the main factor explaining MSg and growth rate differences between sites. Another possibility is that species sympatry in VFX would induce inter-specific competition, thus reducing species scope for growth, a biotic interaction which is not represented in the model if food is not limiting. However, in this particular case, previous studies have not found evidence of species competition. On the contrary, Cabral and Costa (1999) and Cabral (2003) described different patterns of habitat use for juveniles of the two sole species, related to differences in spawning behaviour and to oscillations in environmental conditions along the estuary that would minimize inter-specific competition for the same resources.

Another factor not considered in the present study was migratory movements from and to the nursery areas, including individuals' interchange between them, which would render problematic comparisons regarding fish performance between sites. Nonetheless, flatfish are demersal fish with negative buoyancy and are considered rather poor swimmers, unable to sustain high or maximum activity through long periods (Priede and Holliday 1980; Duthie 1982; van den Thillart et al., 1994), which does not support the idea of constant transfers between distant nursery areas. Also, and through the analyses of stable isotopes, Vinagre et al. (2008) described high site fidelity of 0-group *S. solea* and *S. senegalensis* in the Tagus nursery areas.

An admitted weakness in our accurate simulation of sole performance in the Tagus is the lack of independent support for the required adjustments in the model parameter MMSO. Only with respirometry data can such corroborating data be obtained. We have no such data for the years and locations upon which this study focused. Thus, we can only argue that the variation in requisite MMSO values is reasonable, and urge that future studies include respirometry of the type advocated by Neill et al. (2004).

Further developments of the Ecophys.Fish ecophysiological framework would be interesting, especially if including biotic interactions (e.g. predation, competition, and parasitism) and mobility (e.g. responses to stimuli and habitat choice), as well as the effects of diseases or environmental contaminants on fish metabolic performance. Still, for juvenile sole growth in the Tagus nursery areas the model's predictions were remarkably accurate. In this context, it is possible to apply the model as a tool for monitoring and assessment of habitat quality for juvenile sole.

It would also be interesting to assess how the model and the species would perform in other geographic regions, as well as in different scenarios (e.g. pollution, climate change).

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CHAPTER 6

Short-term variability of condition indices in fish from estuarine and shallow coastal areas of the Portuguese coast: response to environmental variability

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In review in Estuarine, Coastal and Shelf Science

Short-term variability of condition indices in fish from estuarine and shallow coastal areas of the Portuguese coast: response to environmental variability

Abstract: Short-term variability in condition indices, condition factor K, RNA:DNA and protein content and instantaneous growth rates were determined in four fish species from contrasting environments. *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps* were collected in two sites of the Tejo estuary, while *Trachurus trachurus* individuals were collected in an adjacent shallow coastal area, all in Spring-Summer 2009. Variation of condition indices at the week scale (sampling periods with fortnight intervals) was more frequent in all species than daily variability (consecutive days in each sampling period). Water temperature was significantly correlated with biochemical indices (RNA:DNA and protein content) in all fish species, while salinity showed no relationship with variation of indices. Despite higher variability of abiotic conditions in estuarine sites, there were no clear differences in condition patterns of fish species from the two environments, emphasizing that estuarine-inhabiting species are resilient to the dynamic estuarine environment. Yet, higher individual variability in fish condition was observed during the first weeks of sampling and tended to decrease towards the final week. This resulted in a more homogeneous condition status of fish populations, particularly for *T. trachurus*, which could be related to more stable environmental conditions in the coastal area. Overall, biochemical indices were sensitive measures of short-term environmental variability, which should be accounted for in monitoring plans.

Key-words: temporal variation; condition factor K; RNA:DNA ratio; protein content; instantaneous growth rate; temperature; salinity.

1. Introduction

Condition indices and growth have been comprehensively used as indicators of fish nutritional status and health, and hence in assessing habitat quality for different species (Fonseca et al. 2006; Amara et al. 2007; Vasconcelos et al. 2009). These indices encompass different biological response levels, from morphometric to biochemical measures, considering weight changes (e.g. condition factor K), energy reserves allocation (e.g. lipids or protein content) or rates of metabolic activity (such as RNA and protein synthesis) (e.g. Weber et al. 2003). Depending on the biological response level, condition indices have different responsiveness to environmental variability, such that biochemical indices respond more rapidly and are thus more sensitive to short-term variations than morphometric measures (Ferron and Legget 1994; Suthers 1998). Furthermore, condition indices are influenced by many factors, namely species-specific constrains, life-stage, abiotic (such as water temperature, dissolved oxygen), biotic (e.g. prey availability) and anthropogenic factors (Buckley et al. 1999; Amara et al. 2007; Fonseca and Cabral 2007).

Overall, assessing how environmental variability influences individual responses is a complex process that requires coherent time scales between environmental changes and indices response periods. This can be particularly important in highly dynamic environments such as estuaries, where significant variation in physical-chemical variables exists, e.g. salinity, temperature and river flow regime. In contrast, adjacent coastal areas are less variable environments, potentially less stressful for organisms (McLusky and Elliott 2007). In this context, short-term condition indicators in natural systems are a promising tool to comprehend mechanisms by which organisms respond to rapid environmental change, linking environmental settings with fish condition at the time of sampling (Buckley et al. 1999; Dahlhoff et al. 2004).

Nucleic acid based indices, such as the ratio of RNA to DNA cellular content (R:D), have been frequently used as indicators of fish nutritional status and growth rate on a scale of days to weeks. This index gives a measure of the synthetic capacity of the cell and usually correlates with nutritional status and protein requirements (Bulow 1987; Buckley et al. 1999). The rationale for R:D use is that it reflects variations in protein synthesis rates, and thus growth, since RNA content varies with both food availability and environmental conditions, while the amount of DNA remains stable (Bulow 1987; Buckley et al. 1999). Total protein content in fish muscle is also related to growth on a short time scale and considered as a potential source of energy under severe nutritional stress (Foster et al. 1993; Weber et al. 2003). In addition to R:D and protein content, an easy-to-use morphometric index, the condition factor K, which assumes that heavier fish for a given length are in better condition (Ricker 1975), has been extensively applied to assessing fish fitness.

Several economically important marine fish species use estuarine and shallow coastal areas along the Portuguese coast as nursery grounds during their early life. In the present study, four widely distributed fish species with different life strategies and habitat use patterns were selected: European sea bass *Dicentrarchus labrax* (Linnaeus 1758) and Senegalese sole *Solea senegalensis* Kaup, 1858, two marine species which utilize estuaries as nursery areas during the juvenile period (Cabral and Costa 1999; Cabral and Costa 2001; Vasconcelos et al. 2010); common goby *Pomatoschistus microps* (Krøyer 1838), a dominant estuarine resident species with a short life span (ca. 2 years) (Arruda et al. 1993; Leitão et al. 2006); and Atlantic horse mackerel *Trachurus trachurus* (Linnaeus, 1758), a marine species which uses shallow coastal areas as nurseries (Cabral et al. 2000, 2003).

The aim of the present study was to determine how short-term natural environmental variability influenced a set of growth and condition indices in populations of different fish species inhabiting contrasting environments. Considering the highly dynamic nature of estuarine environments, where abiotic conditions vary significantly on short time-scales, the temporal frame considered varied on a scale of days to four weeks.

2. Materials and methods

2.1 Fish collection and sample preparation

Fish collection was carried out between May and July 2009, with sampling performed during three consecutive days (referred to hereafter as daily variation) at every fortnight (referred to hereafter as weekly variation) over a total period of four fortnights. The start of sampling period varied with species estuarine habitat use, since for *D. labrax* and *S. senegalensis* it followed colonization of the nursery grounds by juveniles. Sampling of *P. microps* started simultaneous with the first sampling event for the remaining species and extended for the determined period of four fortnights.

Collection of *D. labrax*, *S. senegalensis* and *P. microps* was carried out using a beam trawl in two sites of the Tejo estuary recognized as important nursery grounds - Vila Franca de Xira (VFX) and Alcochete (ALC) (Cabral and Costa 2001; Vasconcelos et al. 2010), while *T. trachurus* were collected with a beach seine at an adjacent coastal area acknowledged as a nursery for this species - Costa de Caparica (CC) (Cabral et al. 2000, 2003) (Fig. 1). Upon collection, fish were transported to the laboratory, where they were immediately sacrificed, total length (Lt, in mm) and weight (Wt, in g) recorded and target tissue (muscle) dissected, frozen in liquid nitrogen and stored at -80 ºC until analyses.

Total length of sampled *D. labrax*, *S. senegalensis* and *T. trachurus* (Table 1) indicated that these individuals were age 0+ juveniles from 2009, with *D. labrax* and *S. senegalensis* from the first cohort that colonized the estuary (Arruda 1984; Cabral and Costa 1999; Cabral and Costa 2001). *P. microps* were age 0+ late juveniles to early age 1 adults (Arruda et al. 1993; Leitão et al. 2006).

Daily water temperature was measured throughout the entire sampling period with temperature data loggers (HOBO H20-001) permanently positioned in both estuarine areas, while data for the adjacent coastal area was obtained with a multi-parameter probe (WTW) and

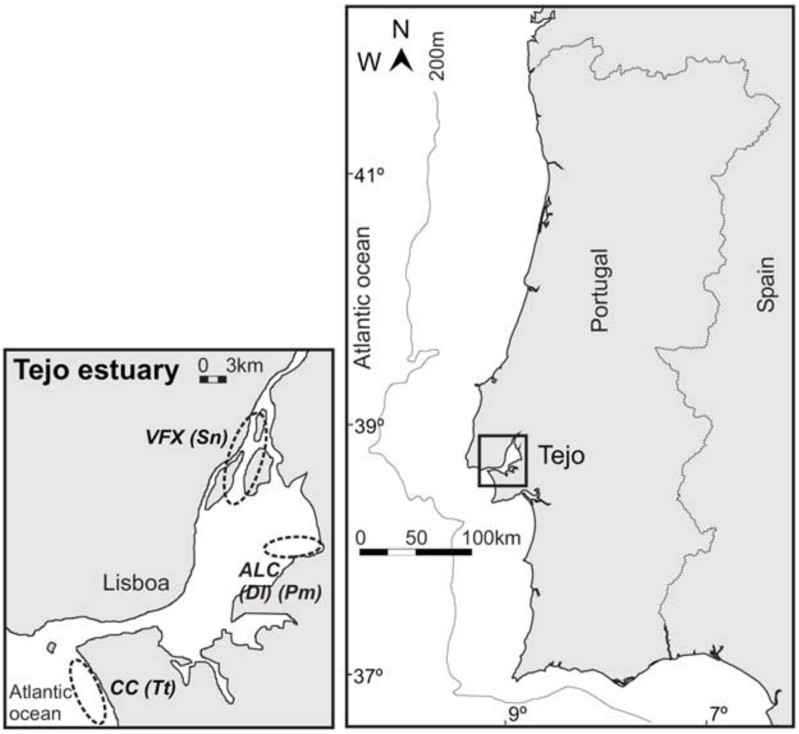


Figure 1. Location of sites sampled within the Tejo estuary (*VFX* - Vila Franca de Xira and *ALC* - Alcochete) and in an adjacent shallow coastal area (*CC* – Costa da Caparica) between May and July 2009. Also shown, fish species collected in each site: *Dicentrarchus labrax* (*Dl*) and *Pomatoschistus microps* (*Pm*) in Alcochete, *Solea senegalensis* (*Sn*) in Vila Franca de Xira and *Trachurus trachurus* (*Tt*) in Costa da Caparica.

Table 1. Mean (and standard deviation between brackets) total length (in mm) and weight (in g) of *Dicentrarchus labrax*, *Solea senegalensis*, *Pomatoschistus microps* and *Trachurus trachurus* sampled between May and July 2009. Total number of individuals (N) sampled is also shown.

Species	N	Total length (mm)	Total weight (g)
D. labrax	176	64.11 (15.84)	3.03 (1.75)
S. senegalensis	173	71.95 (12.39)	3.32 (1.92)
P. microps	178	35.65 (5.51)	0.40 (0.16)
T. trachurus	175	81.58 (16.57)	5.26 (3.37)

from databases of the Portuguese Meteorological Institute. Salinity was measured only on sampling days with a multi-parameter probe (WTW).

2.2 Condition indices and growth

Individual condition factor (K) was determined from morphometric data, according to the formula K = (100 x Wt) / Lt^3, where Wt is total weight (g) and Lt is total length (cm) (Ricker 1975).

Weight-frequency data were analysed by modal progression analysis using the Bhattacharya method (Bhattacharya 1967), with the software FISAT II, version 1.1.2 (FAO 2002). Instantaneous growth rates (G) expressed in wet weight change per day (d^{-1}), were determined for each species: $G = (\ln W_f - \ln W_i) / (t_f - t_i)$, where W_i and W_f correspond to total weight (in g) at the initial (t_i) and final days (t_f) of the considered period.

RNA to DNA ratio (R:D) was determined in two replicate individual muscle samples, of ca. 30 - 80 mg wet weight, according to the fluorometric method described in Caldarone et al. (2001). Muscle samples were homogenized through short-term ice-sonication in N-lauroylsarcosine Tris-EDTA buffer (0.1%, pH 7.5), centrifuged (at 6000 rpm for 10 min at 0 ºC), and 125 µl aliquots of the supernatant were used for the quantification of RNA and DNA fluorescence with ethidium bromide (microplate-reader Biotek Synergy HT, emission wavelength 590 nm, excitation wavelength 360 nm). RNA fluorescence was determined as the difference between total nucleic acids fluorescence and DNA fluorescence following treatment with RNase A (bovine pancreatic ribonuclease A 0.12 mg ml^{-1}, from bovine pancreas, 20 U ml^{-1}, Sigma). Standard curves were previously determined using pure calf-thymus DNA (Calbiochem) and 18S- and 28S-rRNA (Sigma), and the ratio between the two slopes from each standard curve was 7.98.

Protein content (Prot) in individual muscle samples was determined according to Lowry et al. (1951). Aliquots from the R:D prepared samples reacted with Folin's and Copper reagent and the absorbance of the complex was read at 750 nm (microplate-reader Biotek Synergy HT) and compared with a standard curve previously constructed with a dilution series of bovine serum albumin (Sigma).

2.3 Statistical analysis

Significant correlations were observed between fish weight and condition indices for all species. Therefore, weight-adjusted indices (R:D', Prot' and K') were calculated by subtracting the slope of the linear regression between weight and each index for each species multiplied by the fish weight from the original index data (Gillanders and Kingsford 2003).

Short-term variability in fish condition indices, namely R:D', Prot' and K', was tested with nested analysis of variance (ANOVA), followed by *post-hoc* Tukey tests, considering two time scales: effects at the week level and daily effects nested into weeks. Differences between weekly estimations of G' were tested with one-way ANOVA, followed by *post-hoc* Tukey tests whenever significant differences with ANOVA were observed. Prior to these analyses, data was logarithm transformed when necessary in order to meet assumptions of normality and homogeneity of variances.

Correlations between environmental variables (water temperature - mean for the three days prior to fish collection, and salinity - mean for the days of fish collection) and fish condition indices (R:D', Prot' and K') and growth rate (G'), and between all biological variables (total length and weight) were determined for each fish species with Pearson product moment correlation.

All analyses were performed using Statistica software and a 0.05 significance level was considered in all test procedures.

3. Results

Both sites from the Tejo estuary had higher variability in temperature and salinity than the site in the adjacent coastal area (CC) (Table 2).

Biochemical condition indices, RNA to DNA ratio (R:D') and muscle protein content (Prot'), presented low temporal variability at the daily scale (i.e. considering consecutive days) while weekly variation (i.e. referring to once in a fortnight sampling period) was high for all species (Fig. 2). *D. labrax* presented R:D' significantly lower in week two (Fig. 2a; $F = 7.73$, $P < 0.001$) and Prot' significantly lower in the third week (Fig. 2a; $F = 28.31$, $P < 0.001$). Significant daily variation among consecutive days was only observed in weeks one and four for R:D' (Fig. 2a; $F = 4.51$, $P < 0.001$), and in week one for Prot' (Fig. 2a; $F = 6.11$, $P < 0.001$). R:D' and Prot' in *S. senegalensis* juveniles varied significantly only at week level, with lower values in the final week (Fig. 2b; $F > 6.46$, $P < 0.001$). In *P. microps* Prot' differed significantly at the week level, with lower values registered in the last week (Fig. 2c; $F = 26.84$, $P < 0.001$), while R:D' ratios did not differ at either time scales considered (Fig. 2c; $F < 2.51$, $P > 0.05$). Weekly variation in both R:D' and Prot' was observed in *T. trachurus* individuals, with significantly lower R:D' values in week 2 and lower Prot' values in week three (Fig. 2d; $F > 3.45$, $P < 0.05$).

Table 2. Mean (and standard deviation between brackets) depth, water temperature and salinity in sites sampled within the Tejo estuary (ALC – Alcochete; VFX – Vila Franca de Xira) and in an adjacent shallow coastal area (CC – Costa da Caparica) between May and July 2009.

Site	Depth (m)	Temperature (ºC)	Salinity
ALC	2.12 (0.60)	22.38 (2.45)	16.75 (3.56)
VFX	4.61 (1.70)	22.67 (2.56)	6.31 (2.12)
CC	8.50 (3.30)	18.52 (2.12)	35.51 (1.61)

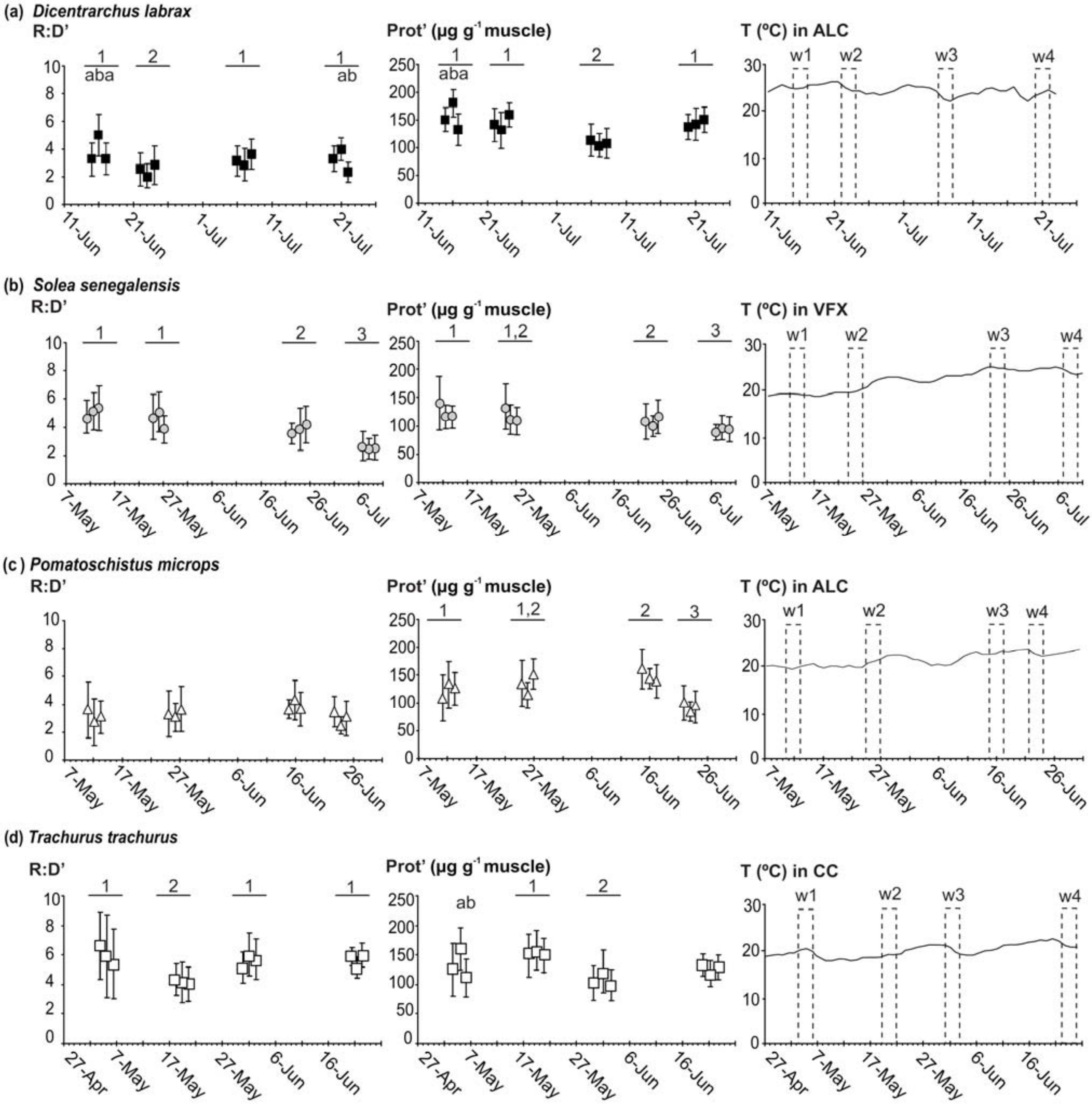
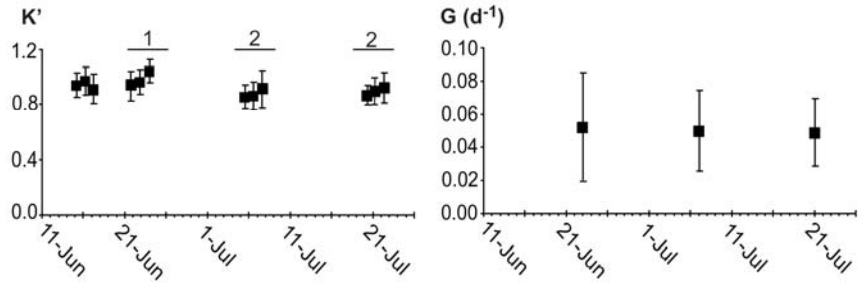


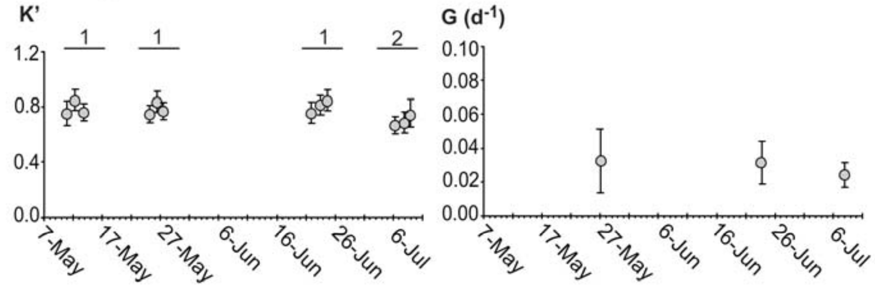
Figure 2. Mean (and standard deviation) RNA to DNA ratio (R:D') and protein content (Prot'; µg mg^{-1} muscle) values in **(a)** *Dicentrarchus labrax*, **(b)** *Solea senegalensis*, **(c)** *Pomatoschistus microps* and **(d)** *Trachurus trachurus*. Results of nested analysis of variance are also represented, considering effects at the week level (different numbers indicate significant differences between weeks) and daily effects nested into weeks (different letters indicate significant differences between consecutive days). Mean daily water temperature is also shown for the collection site and period of each species.

Morphometric condition factor K' only varied significantly at the week scale for *D. labrax* (Fig. 3a; $F = 4.68$, $P < 0.01$) and *S. senegalensis* (Fig. 3b; $F = 9.20$, $P < 0.001$) during the full sampling period. Instantaneous growth rates, G, only varied significantly between the two latter sampling periods for *T. trachurus* (Fig. 3d; $F = 14.73$, $P < 0.05$).

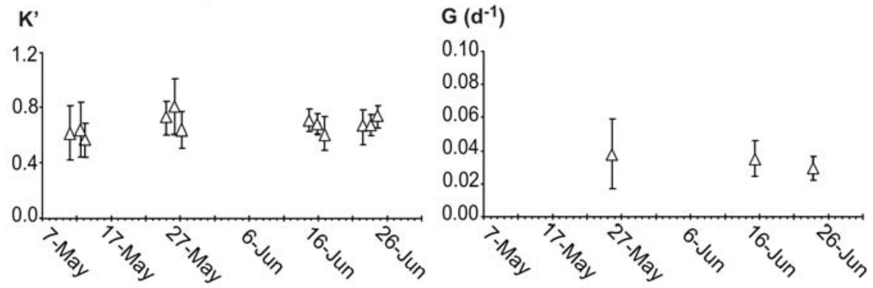
Moreover, standard deviation of R:D', Prot' and to a lesser degree of G', tended to decrease by week four in *D. labrax*, *S. senegalensis* and *T. trachurus* individuals.



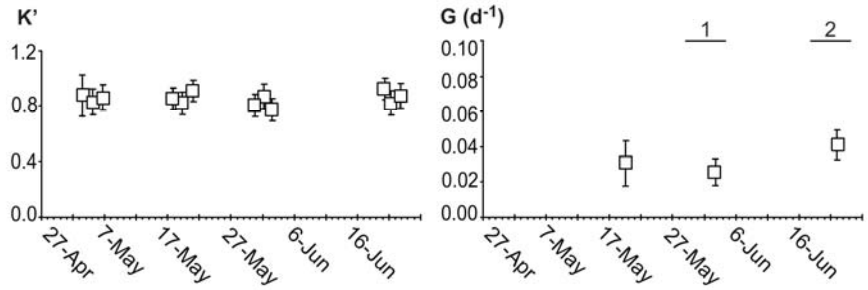
(a) ***Dicentrarchus labrax***



(b) ***Solea senegalensis***



(c) ***Pomatoschistus microps***



(d) ***Trachurus trachurus***

Figure 3. Mean (and standard deviation) condition factor (K') and instantaneous growth rate (G) (wet weight change d^{-1}) in **(a)** *Dicentrarchus labrax*, **(b)** *Solea senegalensis*, **(c)** *Pomatoschistus microps* and **(d)** *Trachurus trachurus*. Results of analysis of variance are also shown: for K' with a nested design considering effects at the week level (different numbers indicate significant differences between weeks) and daily effects nested into weeks (different letters indicate significant differences between consecutive days); and for G with a one way design for weekly effects (different numbers indicate significant differences between weeks).

A positive correlation between R:D' and Prot' was only observed for *S. senegalensis* (r = 0.22, $P < 0.01$) and *P. microps* (r = 0.27, $P < 0.01$). Mean water temperature was positively correlated with mean R:D' values in *D. labrax* (r = 0.56, $P < 0.05$), *P. microps* (r = 0.62, $P < 0.05$) and *T. trachurus* (r = 0.69, $P < 0.05$). Mean temperature and Prot' were also positively correlated in *D. labrax* juveniles (r = 0.70, $P < 0.05$) and inversely, a negative correlation between mean temperature and R:D' values was observed for *S. senegalensis* (r = -0.58, $P < 0.05$). Nonetheless, no significant correlation was observed between salinity and biochemical condition indices in any of the species.

No significant relations were observed between K' and G, or between these weight-based measurements and any of the biochemical indices or abiotic factors (temperature and salinity) for all the species considered.

4. Discussion

Short-term variability in condition indices was observed in sampled fish species occurring in both estuarine and shallow coastal areas. Condition indices observed for juvenile *D. labrax* and *S. senegalensis* in the present study were within the range reported in previous studies in the same sites, which were previously identified as important nursery grounds for these species (Fonseca et al. 2006; Vinagre et al. 2008; Vasconcelos et al. 2009). There are no previous records on condition indices for *P. microps* and juvenile *T. trachurus* available for comparison.

Significant size effects on both biochemical (R:D' and Prot') and morphometric (K') indices were identified, seeing that the time frame considered was long enough to allow for significant size composition changes in all species. Size-corrected indices, in this case weight corrected, were used to explore other sources of variability influencing condition measures, particularly the effects of environmental variability and their temporal patterns, removing the influence of allometric effects on the variability of the indices (Suthers 1998), thus adjusting for differences in individual growth rates (Buckley et al. 1999).

Variation of condition indices at the weekly scale (considering a fortnight sampling period) was more frequent in all four species than daily variability (in consecutive days) in R:D', Prot' and K'. Daily variability of biochemical indices was only observed in few occasions in two fish species (*D. labrax* and *T. trachurus*). Biochemical condition indices have been described as more sensitive measures of overall fish condition when compared to traditional morphometric measures, particularly on a short-time scale of days to weeks (Ferron and Leggett 1994; Chícharo et al. 1998). This is in agreement with the higher variability observed at daily and even at weekly scale in R:D' and Prot' indices when compared to K'. This difference in the temporal response of indices, or latency as termed by Ferron and Leggett (1994), usually accounts for the poor correlation between indices at the individual level, particularly between morphometric and biochemical indices, as observed in the present study and previously reported for different fish species (e.g. Tanner et al. 2009; Vasconcelos et al. 2009). Nonetheless, considering biochemical indices alone, low but significant positive correlations between R:D' and Prot' were observed in *S. senegalensis* and *P. microps* individuals in the time frame considered, while in *D. labrax* and *T. trachurus* a fortnight delay or lag in Prot' response following the R:D' response was observed.

A similar decreasing pattern throughout the study period was observed between fish condition indices and instantaneous growth rates. Nevertheless, instantaneous growth rates were not correlated with any of the condition indices, despite the fact that RNA cell content and protein synthesis can be good proxies for short-term growth determination (e.g. Fukuda et al. 2001; Mercaldo-Allen et al. 2006). The observed lack of correlation can be explained by the fact that growth estimation was based on mean population weight changes, in contrast with condition indices which were determined at the individual level. Alternative individual growth measurements, such as daily otolith increments or RNA:DNA-based growth models for each species could potentially improve this relation (Buckley et al. 1999), although they could not be applied in the present study.

Significant temporal variation patterns in condition have been described in several fish species and life-stages. Seasonal (Haines 1980; Ramírez et al. 2004), monthly (Fonseca et al. 2006; Vasconcelos et al. 2009), and even diel variability (Chícharo et al. 1998; Gwak 2000) has been reported for condition indices in both juvenile and larvae fish. These patterns constitute an additional source of variation in fish condition, which can influence sampling design and interpretation of results. From a practical point of view, characterizing habitat suitability for fish species may be biased through the effect of short term unfavorable environmental conditions, which may hamper fish condition and render a poor habitat classification in the absence of further sampling events.

The short temporal scale approach of the current work evidenced the influence of short-term variations in environmental settings, particularly temperature, on biochemical indices in natural fish populations. Temperature was positively correlated with R:D’ in three fish species, *D. labrax*, *P. microps* and *T. trachurus*. In these species observed periods of temperature decrease and concurrent lower R:D’ values were generally followed by decreased protein content and generally lower growth rates. The positive correlation also observed between Prot’ and temperature for *D. labrax* juveniles, which was characterized by a higher correlation coefficient than the correlation between R:D’ and temperature, is due to a direct effect of temperature on protein content, influencing protein turnover rates, added to the indirect effect of temperature on Prot’ that is determined through regulation of RNA cellular concentration. On the other hand, the negative relationship between R:D’ and temperature observed in *S. senegalensis* juveniles, suggested that higher R:D’ levels at lower temperatures are a compensatory mechanism for lower RNA activity at lower temperatures with increased RNA synthesis (Goolish et al. 1984).

Contrary to temperature, salinity had no visible effect on fish condition. Kim et al. (2008) found that drastic salinity shifts limited growth and R:D ratios in river pufferfish (*Takifugu obscurus*) larvae. However, despite the elevated diel and seasonal variations in salinity in sampled estuarine sites, estuarine resident and marine migrant species are well adapted to salinity variations, whilst salinity in coastal areas is fairly constant.

Other abiotic (e.g. dissolved oxygen, contaminants) and biotic factors (e.g. predation pressure) not measured in the present work could also account for part of the variability in condition indices, and should be considered in future studies aiming at establishing causal-effect relationships with condition indices.

No noteworthy differences were observed in condition variation patterns between fish species from coastal and estuarine areas, i.e. between a more stable and a more dynamic environment. Even though there was a close link between fish condition and water temperature and despite the potential physiological costs for juveniles of marine species associated with estuarine life (Yamashita et al. 2003), species inhabiting estuaries are generally considered to be resilient and well adapted to the dynamic nature of the estuarine environment (Elliot and Quintino 2007).

Higher individual variability in condition (standard deviation of condition indices) was observed over the first weeks of sampling and tended to decrease towards the final week. This suggests the incidence of a selective drive that resulted in more homogeneous populations of older individuals, condition wise. In addition, this decrease was particularly pronounced in *T. trachurus* juveniles which could be related to higher environment stability of this habitat (compared to estuarine sites), where a unidirectional drive would account for a more homogenous population. Inversely, estuarine species would benefit from higher individual variability and theoretically a wider range of available responses to the dynamic environmental conditions. Nonetheless, current data do not allow for a copious assessment of this prospective selection nor of its direction. Still, previous studies with individual variability and fish population models described that successful fish populations, by the end of the first growing season, were predominantly composed of individuals that were larger to begin with or that experienced fast growth rates (see review by Tyler and Rose 1994). Other studies have also linked higher growth and condition with increased survival probability in fish early life stages, since less time is spent in less favorable sizes, hence indicating selective advantages for these individuals (e.g. Meekan and Fortier 1996; Buckley and Durbin 2006). At this point, it is not possible to determine potential effects of such selective processes on the general health and resilience of fish populations if facing a drastic change in environmental conditions, which will ultimately depend on the species plasticity or ability to adapt.

Overall, biochemical indices proved to be sensitive measures of short-term environmental variability, specifically on a week scale, which should be accounted for in monitoring and habitat quality assessment plans. The rapid response of these biochemical condition indices could also be useful in view of future scenarios of increased frequency of drastic climatic events (Easterling et al. 2000), concomitantly with other structural and functional responses at higher biological levels as suggested in the review by Chícharo and Chícharo (2008).

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CHAPTER 7

Effect of copper exposure on growth, condition indices and biomarker response in juvenile sole *Solea senegalensis*

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Effect of copper exposure on growth, condition indices and biomarker response in juvenile sole *Solea senegalensis*

Abstract: Juvenile *Solea senegalensis* were exposed to different concentrations of waterborne copper (Cu) for 15 days in static conditions with continuous aerated artificial salt water at 20ºC (± 0.8ºC) with a normal photoperiod (10 h/14 h light/dark) and daily feeding. Several measures of exposure and effects were determined: 1) biomarkers - metallothioneins and lipid peroxidation level; 2) mass indices - growth rate and morphometric condition indices; and 3) biochemical condition indices - RNA:DNA ratio and lipid and protein content in fish tissues. Copper exposure triggered the response of the biomarkers and resulted in reduced growth and condition (RNA:DNA and lipid content), but the morphometric indices did not vary. The physiological costs of Cu contamination on condition suggested that lipid reserves were allocated as an energy source to enable exposed fish to respond to Cu toxicity as well as to maintain positive growth rates and protein synthesis throughout the experiment, although with lower growth rates than the control fish. This study showed the importance of selecting suitable biomarkers according to contaminant source, fish species and their life-history stage. In addition, the use of several biomarkers of exposure, growth and specific condition indices can improve fish health determination and should be considered in evaluations of the effects of environmental contaminants on fish.

Key-words: fish growth; fish condition; metallothioneins; lipid peroxidation; juvenile sole; biomarkers

1. Introduction

Aquatic ecosystems are increasingly threatened by the presence of organic and inorganic anthropogenic pollutants (Daskalakis and O’Connor 1995). Fish behaviour and physiological responses to specific and multiple stressors have been extensively used to determine individual health and population status, and to assess habitat quality (e.g. Lloret and Planes 2003; Marchand et al. 2003; Fonseca et al. 2006). Few studies have integrated indicators of exposure to contamination and effects on fish’s health and condition, and most have reported unclear or limited responses of individual growth and condition to contaminant exposure (De Boeck et al. 1997; Wu et al. 2002; Humphrey et al. 2007). However, recent work has outlined the importance of applying a multibiomarker approach to assess the causes and effects of stressors on marine systems (e.g. Adams 2005; Broeg and Lehtonen 2006).

Biomarkers are considered, in a broad sense, as measurements of changes in biological responses linked to a potential hazard, which may be chemical, physical or biological (van der Oost et al. 2003). When assessing the adverse effects of environmental contamination, exposure biomarkers are commonly used as early signs or functional measures of toxicity of specific contaminants or suites of contaminants (Shugart et al. 1992; Adams 2002). However, a direct relationship between biomarkers’ response to pollutants and further ecological consequences at the individual or higher level is difficult to establish, and still needs further integration of both individual adaptability and the influence of natural attributes (van der Oost et al. 2003).

Trace metals are widespread pollutants in estuaries and coastal areas. Common biomarkers of exposure induced by trace metal toxicity are metallothioneins (MT), thiol-rich proteins that bind to the toxic metals and enable their excretion (Roesijadi 1996), as well as antioxidant enzymes (e.g. superoxide dismutase [SOD], catalase [CAT]). These defence mechanisms have energetic and physiological costs for the individual and often result in trade-offs with other biological processes, namely reproduction and growth (Eastwood and Couture 2002; Marchand et al. 2004). Fish growth and condition are linked to the ecological status of the environment (Beyers et al. 1999; Buckley et al. 1999), yet they may vary with non-pollutant stresses, masking the effects of pollution on the ecosystem, which emphasises the importance of experimental investigation and background information. It is also imperative to understand how the biochemical mechanisms that induce the biomarkers' responses influence individual growth and condition. A wider ecological scope of the stressors' cost to the individual may be attained by the simultaneous determination of several biological endpoints that correspond to different response levels (Den Besten 1998; Adams 2002), from the molecular to the whole individual level.

In the present study, juvenile Senegalese sole, *Solea senegalensis* Kaup, 1858, were exposed to naturally occurring copper (Cu) concentrations observed in Portuguese estuaries. This essential trace metal (toxic at higher concentrations) is very common in estuaries and coastal waters, and several studies have demonstrated its deleterious effects on growth rates, condition and ion balance of different fish species (Marr et al. 1996; De Boeck et al. 1997; Blanchard and Grosell 2006). Thus, Cu was selected as an example of trace-metal toxicity in fish, and the different biomarkers determined were chosen based on: (1) their sensitivity to Cu exposure (biomarkers of exposure – levels of MT and lipid peroxidation); (2) the information on general condition and growth (morphometric indices and growth rates); (3) the information on physiological condition related to growth and to the energy pathways for juvenile fish. The aims of this study were to determine how sublethal copper exposure simultaneously influences growth, condition indices and biomarker response in juvenile sole, and to determine the usefulness of these indicators for future habitat quality assessment studies.

2. Materials and methods

2.1 Experimental procedures

Cultured juvenile *Solea senegalensis* (2.3-6.7 g ± 0.01 g wet weight, 5.7-8.0 cm ± 0.1 cm total length) from the same spawning batch were exposed to three sublethal copper treatments at the concentrations of 5, 25 and 100 µg Cu L-1, plus a clean water control group, over a 15-day experiment period. The lower copper concentration was chosen based on naturally occurring values determined by water quality control measurements in Portuguese estuaries (source INAG - National Water Institute - 2005) and also reported for other European estuaries (Hall and Anderson 1999). The other two copper concentrations were chosen in order to simulate episodic contamination events. A total of 51 fish were analysed, 3 at the beginning of the experiment and 48 fish divided into four groups of 12 individuals and subjected to the Cu treatments, plus the control trial. Two separate 14 L tanks were used per treatment, each containing 6 fish individually tagged, measured and weighed at the beginning of the experiment, following a two week acclimation period. During the acclimation and the experimental period fish were fed with a very common natural prey, the polychaete *Hediste diversicolor*. The experiment was carried out in static exposure conditions, with continuous aerated artificial salt water at 20ºC (± 0.8ºC), normal photoperiod (10 h/14 h light/dark) and daily feeding. Salt water (18 g L-1) was prepared with tap water (pH 7.3 ± 0.5) and artificial salt (Instant Ocean) throughout the 15- day trial and for the two-week acclimation period, to ensure daily water renewal. Copper exposure was carried out by the dilution in distilled water of copper sulphate hydrate (Sigma) and Cu concentrations were monitored during the experimental period by atomic absorption spectrophotometry. Fish were collected on days 3, 5, 10 and 15 (3 fish per treatment per day) and were immediately sacrificed with a cut on the anterior spine. Fish length and body and liver weight were determined. Dissected liver and muscle samples were initially frozen in liquid nitrogen, and then stored at - 80ºC for subsequent analysis.

2.2 Analytical procedures for determining growth, condition indices and biomarker response

Instantaneous growth rates (G) were determined as $G = (\ln W_2 - \ln W_1)/(t_2 - t_1)$, where W1 and W2 are fish total body weight at times t_1 (beginning of the experiment) and t_2 (time of collection).

The morphometric condition indices determined for juvenile sole were the condition factor, $K = W/L^3$, where W is the wet weight (in grams) and L is the total length (in centimetres) (Ricker 1975), and the hepatosomatic index, $HSI = W_{Liver} / W$, where W_{Liver} is the liver weight.

Biochemical condition indices, namely RNA:DNA ratio and protein and lipid content, were determined in duplicate for individual muscle samples. RNA, DNA and protein measurements were determined from two replicates of ca. 150 mg wet weight each from the anterior-dorsal portion of the muscle, whereas the remainder of the muscle was used to determine lipid content (average 400 mg wet weight). Nucleic acid quantification was carried out by the fluorometric method described in Caldarone et al. (2001), adapted to a cuvette assay (Fonseca et al. 2006). Muscle samples were homogenised in N-lauroylsarcosine Tris-EDTA buffer (0.1%, pH 7.5), centrifuged, and aliquots of the supernatant were used for the quantification of RNA and DNA fluorescence with ethidium bromide (emission wavelength 590 nm, excitation wavelength 360 nm). RNA fluorescence was determined as the difference between total nucleic acid fluorescence and DNA fluorescence following treatment with RNase A (Sigma). Standard curves were previously determined using pure calf-thymus DNA (Calbiochem) and 18S- and 28S-rRNA (Sigma), and the ratio between the two slopes from each standard curve was 4.04.

Protein content determination was based on the Lowry procedure (Lowry et al. 1951). Aliquots of the samples' supernatant used for RNA:DNA quantification reacted with Folin's and

Copper reagent, and the complex's absorbance was read at 750 nm and compared with a standard curve previously constructed with a dilution series of bovine serum albumin (Calbiochem).

Lipid muscle content was determined with an adaptation of the sulphophosphovanilin method described by Knight et al. (1972). Prior to lipid analyses, a gravimetric assay (n = 3) was used to determine the ratio of saturated and unsaturated fatty acids in sole muscle, in order to construct a representative calibration curve. The standards used were oleic and palmitic acid (Sigma) in a 75:25 ratio. Lipid extraction followed the homogenisation of the remaining muscle tissue with methanol:ethanol:water (2:2:1.8 ratio).

Metallothionein concentration (MT) was determined on individual liver samples, after the cytosol had been separated from the residual fraction (30000 g, 45 min, 4ºC), heat-treated at 80ºC for 10 min, and subsequently centrifuged (30000 g, 45 min, 4ºC). Aliquots of the heat-treated cytosol (25 µl) were used to quantify the MT level by differential pulse polarography (DPP) according to the method described by Bebianno and Langston (1989).

Lipid peroxidation was determined in liver samples according to the method described by Erdelmeier et al. (1998) which measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) produced during the decomposition of polyunsaturated fatty acid peroxides of membrane lipids. The concentration of lipid peroxidation was expressed as µmoles of MDA and 4-HNE g^{-1} total protein.

2.3 Statistical analyses

Differences in fish growth rates, condition indices and biomarker response between Cu treatments and periods were tested by two-way analysis of variance (ANOVA), followed by *post-hoc* Tukey tests whenever null hypotheses were refuted. Correlation analyses were used to test associations between all variables: instantaneous growth rates, condition indices and biomarkers. All analyses were performed using Statistica software and a 0.05 (or lower) significance level was considered in all test procedures.

3. Results

Morphometric indices, condition factor K and HIS did not show significant differences between copper treatments over the 15 days of the experiment (Fig. 1a and 1b respectively, Table 1; $P > 0.05$). Mean instantaneous growth rates in mass were affected by Cu exposure with significant differences observed between the control and the 25 µg L^{-1} and 100 µg L^{-1} Cu concentrations (Fig. 1c, Table 1; $F_{3;48} = 5.44$, $P < 0.01$). Lower growth rates were first observed for higher Cu concentrations, but by day 15 all Cu treatments had limited mean instantaneous growth rates.

Biochemical condition indices also reflected Cu contamination, as mean RNA:DNA ratios and lipid content were significantly lower in fish exposed to Cu (Fig. 1d and 1f), although lipid content differed only between the control and the highest Cu concentration, whereas mean

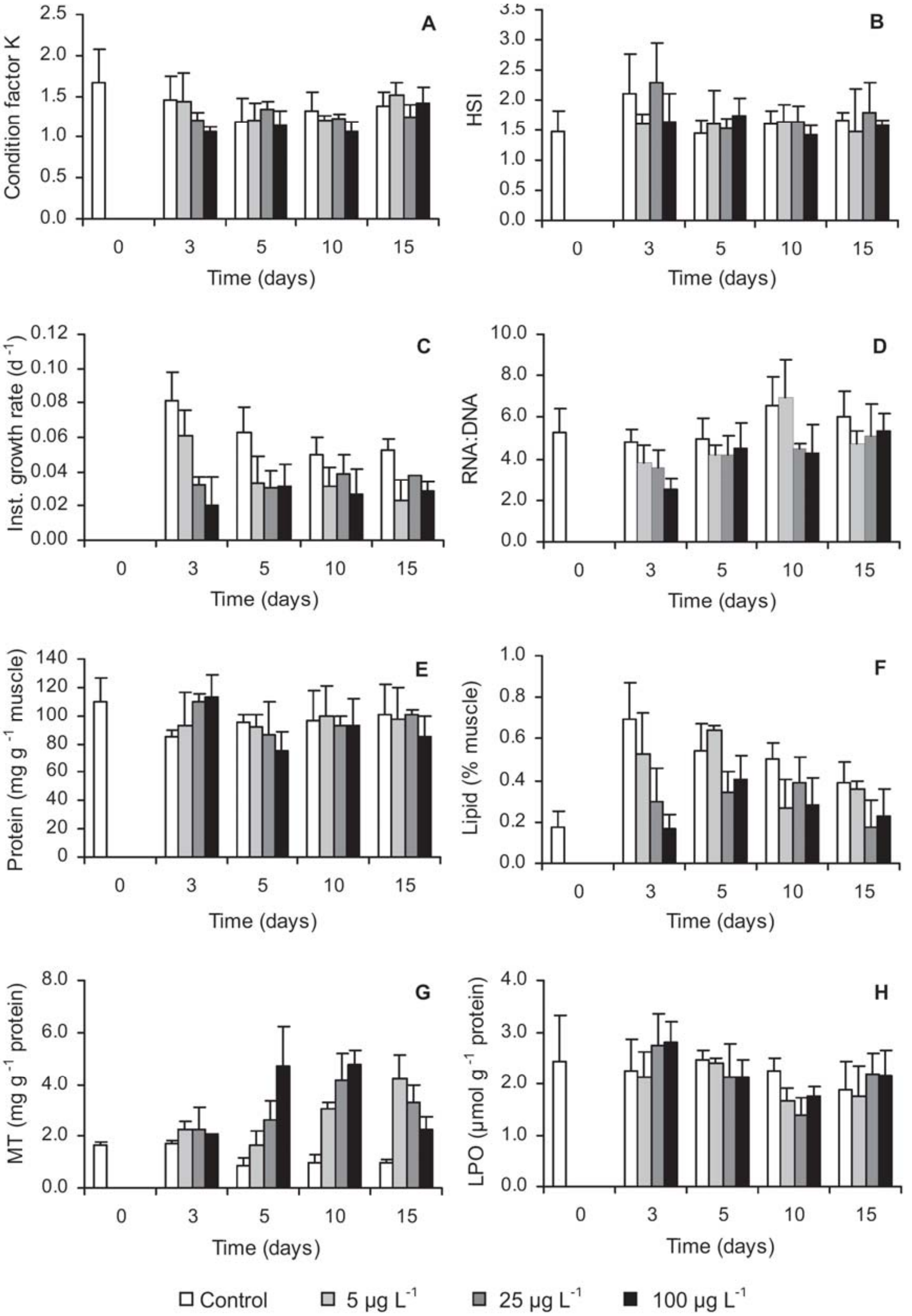

Figure 1. Biomarker variation with Cu treatments (5, 25 and 100 µg L^{-1}) and control group, over the 15-days exposure period (mean values and standard error, n = 51): **(A)** condition factor K; **(B)** hepatosomatic index HSI; **(C)** instantaneous growth rate in mass (d^{-1}); **(D)** RNA:DNA ratio; **(E)** protein content (mg g^{-1} muscle); **(F)** lipid percentage (% muscle); **(G)** metallothionein level (MT, mg g^{-1} protein) and **(H)** lipid peroxidation (LPO, µmol g^{-1} protein).

Table 1. Results of the two-way ANOVA statistical analysis considering the effects of exposure time, Cu concentration and the interaction effects on the biomarkers levels.

	Time (days)				Cu concentration ($\mu g\ L^{-1}$)				Interaction Time x [Cu]
	3	5	10	15	0	5	25	100	
K	-	-	-	-	-	-	-	-	-
HSI	-	-	-	-	-	-	-	-	-
Growth	-	-	-	-	a	-	b	b	-
RNA:DNA	a	-	b	b	a	-	-	b	-
Protein	-	-	-	-	-	-	-	-	-
Lipid	-	-	-	-	a	-	-	b	-
MT	a	a	b	-	a	b	b	b	***
LPO	a	-	b	-	-	-	-	-	-

Different letters indicate significant differences, from *post hoc* comparison Tukey tests (a,b); *** - significant interaction effects (p<0.001); ‘-‘ no significant differences observed.

RNA:DNA was significantly lower than the control for all Cu treatments (Table 1; $F_{3;48}$ = 3.09 and $F_{3;48}$ = 4.46, P < 0.05, respectively). RNA:DNA values also varied significantly with time, since higher values were observed towards the end of the experiment, by days 10 and 15, relatively to day 3 (Table 1; $F_{3;48}$ = 5.92, P < 0.05). Muscle DNA concentration remained relatively constant over the experimental period, with no significant differences between treatments (P > 0.05). Total protein content did not vary significantly with Cu exposure during the 15-day trial (Fig.1e; Table 1, P > 0.05).

Exposure biomarkers, metallothioneins (MT) and lipid peroxidation level (LPO) indicated Cu-mediated damage, although with different response levels. MT response to Cu exposure was more extensive, since MT values varied significantly with both Cu concentration and time, with higher MT levels generally being observed at the highest Cu concentrations and for the longest exposure times, until day 10 (Fig. 1g, Table 1; $F_{3;48}$ = 28.80, $F_{3;48}$ = 6.58, $F_{9;48}$ = 7.65, respectively, P < 0.001 for all tests). Interaction effects of Cu concentration and exposure time on MT level indicated the most significant differences by day 10, when MT levels from all Cu treatments were significantly higher than those from previous days, as well as by day 15, when MT levels from all Cu treatments differed from all controls (Tukey tests, P < 0.05). Lipid peroxidation only showed early time effects, as LPO levels by day 3 were significantly higher than by day 10 (Table 1; $F_{3;48}$ = 6.33, P = 0.01), although no significant differences were observed between treatments. MT and LPO levels also showed inverse response patterns, with an increased MT level up to day 10 followed by a decrease until day 15, whereas LPO decreased from day 3 to day 10 and increased up to day 15 (Fig. 1g and 1h).

Cu concentrations were negatively correlated with RNA:DNA and instantaneous growth (r = -0.30 and r = -0.32; respectively, P < 0.05 for both), and were positively correlated with MT level (r = 0.44, P < 0.05). Other significant correlations were observed between instantaneous growth rates and RNA:DNA ratios (r = 0.51, P < 0.05) and between instantaneous growth and lipid content (r = 0.41, P < 0.05).

4. Discussion

The present multibiomarker approach identified deleterious effects of sublethal copper exposure on growth and physiological condition of juvenile Senegalese sole. The biochemical indices and the biomarkers measured responded to Cu-induced stress during the 15-day trial, but the morphometric indices did not, indicating that a wider time frame would be necessary to account for any variations following trace metal exposure, or even that these indices may not be suitable. However, Levesque et al. (2002) observed differences in seasonal patterns of the condition factor (K) and the hepatosomatic index (HSI) in yellow perch (*Perca flavescens*) chronically exposed to field metals. Eastwood and Couture (2002) also found a seasonal negative correlation between liver Cu content and the scaling coefficient (slope of the linear regression between fish body weight and length) in yellow perch, but observed no variation in the HSI.

On the other hand, changes in mass, measured as instantaneous growth rates, showed the adverse effects of Cu exposure, with growth rates being negatively correlated with Cu concentrations. Control group growth rates were in agreement with values observed by other authors in cultured juvenile Senegalese sole (Rueda-Jasso et al. 2004). The initial decrease in instantaneous growth rates in fish exposed to higher Cu concentrations, compared to the control group growth rates, was maintained towards the end of the experiment, and the control values were never reached. Similar results have been found in other studies (e.g. Marr et al. 1996; Lundebye et al. 1999), whereas yet others have found that an initial reduction in growth of exposed fish was followed by a recovery period (e.g. Seim et al. 1984; De Boeck et al. 1997). Growth is a direct measure of individual fish health, but it is not specific to fish stress responses to pollutants, as several other biotic and abiotic stressors can affect it. Environmental conditions must be considered when one is integrating growth responses and pollution stress. Also, measuring growth in field studies is labour-intensive and time-consuming, so it is necessary to use proxy measures when direct growth measures are difficult to attain.

RNA:DNA ratio showed negative dose and time-response effects due to Cu exposure. It is well known that Cu contamination may lead to DNA oxidative damage through the increase in cellular reactive oxygen species (ROS), which influences the RNA:DNA ratio assumption of DNA concentration constancy (Bullow 1970). Humphrey et al. (2007) described lower RNA:DNA values in barramundi (*Lates calcarifer*) from a highly contaminated estuary in North Queensland, but their study estimated liver nucleic acid, which undermines the use of this biomarker as a proxy for somatic growth. De Boeck et al. (1997) questioned the use of RNA:DNA as a biomarker in toxicological studies since RNA:DNA and growth were poorly correlated in juvenile common carp exposed to copper. However, in both these studies DNA concentration (liver and muscle) also varied significantly with the degree of contamination, which was not the case in the present study. Additionally, our study shows a positive correlation between growth rates and RNA:DNA, suggesting that RNA:DNA ratio indicates growth impairment in juvenile Senegalese sole subjected to sublethal Cu concentrations. Nye et al. (2007) also found that RNA:DNA was a good indicator of growth and condition in 14-day post-hatch larvae of *Fundulus heteroclitus* exposed to polycyclic aromatic hydrocarbons (PAHs), but also described the influence of other factors on RNA:DNA, namely life-stage and maternal influence.

Another assumption regarding the use of the RNA:DNA ratio is that the RNA concentration increases with protein synthesis involved in somatic growth (Bullow 1970). However, in cases of exposure to contaminants, an increase in RNA might be related to the induction of protein detoxification systems but also to an increase in protein turnover rate, which together would lead to high RNA:DNA ratios but decreased growth rates. The question is how significant is the increase in muscle RNA that is directly linked to cell response to contaminants, especially taking into account that the liver is the main organ for the detoxification of xenobiotics, and whether this increase overshadows the potential decrease in RNA production due to lower growth investment in fish facing toxic stressors. One way to look at this problem is to effectively measure growth and protein synthesis. As mentioned above, growth was positively correlated to RNA:DNA, indicating that RNA variability is in any case linked to growth, although it could also be linked to other protein defence mechanisms not measured in this study. However, protein content was not significantly different between copper treatments and control, nor was it associated with growth or RNA:DNA . This result may reflect a possible technical fault, such as inaccurate detection sensitivity in protein determination, or protein synthesis could have been similar for control and exposed fish due to different requirements, namely for somatic growth that was observed in both cases and additionally for toxicity defence mechanisms in Cu exposed fish. Therefore, RNA:DNA ratio may be a valuable condition indicator in toxicology studies, if the initial assumptions are met throughout the study, such as fish in comparable early life-stages, preservation of the DNA concentration throughout the contamination period, and the relation between RNA variability and growth previously established. Ultimately it should be complemented with another growth indicator, as suggested by several authors (e.g. Ferron and Leggett 1994; Suthers 1998).

The variation in muscle lipid content between the highest Cu concentration treatment and control suggests differential energy allocation strategies, since lipid reserves of control fish were generally higher than fish subjected to Cu treatments. A general decrease with time was also observed in lipid content of fish exposed to Cu. Both observations could be linked to oxidation processes derived from Cu exposure, or on the other hand imply that lipid reserves were mobilised to respond to the toxicity stress, but also to maintain positive growth rates and thus protein synthesis even under Cu contamination. Previous studies have reported similar depletion in lipid reserves of fish subjected to metal trace exposure (Rowe 2003). Additionally, higher lipid percentages were also observed for all treatments and the control group when compared to the initial reference percentage at day 0, except for the higher Cu concentration. This may be due to the change in diet from pre-experimental conditions, as trial fish were fed daily with a richer lipid diet, and despite the Cu exposure their lipid storage was higher than at the beginning of the experiment.

The biomarkers of exposure response, metallothioneins (MT) and lipid peroxidation (LPO) indicated fish Cu contamination, although only the MT level showed significant responses to different Cu treatments and periods of exposure. MT levels were within the range of previous studies on metal exposure, including Cu, in *Solea senegalensis* (Riba et al. 2004). LPO only showed differences between days of exposure. Given that lipid peroxidation was quite low for Cu-treated fish and showed a general pattern of decrease, it does not seem to be the main factor responsible for the above-mentioned decrease in lipid content of exposed fish, thus reinforcing the idea of lipid expenditure to sustain the toxicity defence mechanisms and the positive growth rates observed, although at slower rates than those observed for control fish. Moreover, these biomarkers showed inverse response patterns, such that the decrease in MT induction towards the end of the experimental period may be due to several factors: i) exceeded Cu toxicity in the liver resulting in a diminished MT induction; ii) a biphasic pattern of MT induction with continued Cu exposure; iii) precipitation of Cu-MT complex in granules; or iv) an increase in other antioxidant response mechanisms (Sanchez et al. 2005). However, the inverse LPO response suggests an increase in oxidative stress concomitant with MT decrease.

The lowest Cu concentration tested in the current work matches environmental values commonly observed in many European estuarine systems (Hall and Anderson 1999). Although our results indicate that for this level of copper exposure no significant effects on fish condition were observed (only MT level indicated Cu contamination) in a short time frame, the ecological implications due to a permanent exposure were not tested, and future work should explore this issue.

The results emphasised the need for a multi-biomarker approach for habitat quality assessment and toxicology studies, since common indicators of fish health, such as growth and condition, reflected the adverse effects of pollutants (Cu in this case). This interaction may influence conclusions drawn from these fitness indicators in a long-term data analysis, if the alteration of physiological processes due to pollution is not taken into account.

In conclusion, exposure to high concentrations of copper in the water had direct physiological costs for juvenile Senegalese sole, expressed as decreased growth and lower physiological condition (RNA:DNA values), even under optimal feeding conditions. Nonetheless, lack of variation in protein content could suggest that protein requirements were both for somatic growth and protein detoxification systems. Lipid reserve decreases seemed to sustain the toxicity defence mechanisms and the positive growth rates of exposed fish, although at slower rates than those observed for the control fish.

Acknowledgements

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CHAPTER 8

Short-term variability of multiple biomarker response in fish from estuaries: influence of environmental dynamics

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In review in Aquatic Toxicology

Short-term variability of multiple biomarker response in fish from estuaries: influence of environmental dynamics

Abstract: A multi-biomarker and multi-species approach was applied in a dynamic environment with a significant chemical contamination (trace metals and polycyclic aromatic hydrocarbons) to explore short-term variability of biomarkers and effect of environmental variables (temperature and salinity). *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps* were collected in two sites of the Tejo estuary on a short-time scale, of days to weeks. Short-term variability in biomarker responses was observed in the three species although no pattern was discerned over time. Antioxidant enzymes activity [superoxide dismutase (SOD) and catalase (CAT)] showed low variability at both scales (days and weeks) for all species, indicating some constancy or baseline level in antioxidant responses. Moreover only CAT activity in *S. senegalensis* was correlated with temperature, therefore significant exposure to contaminants appears to be triggering antioxidant adaptation responses. Higher short-term variability was observed in biotransformation enzymes activity [phase I ethoxyresorufin O-deethylase (EROD) and phase II glutathione S-transferase (GST)] at both temporal scales and for all fish species. Moreover, significant correlation between them in *D. labrax* and *S. senegalensis* suggests a concomitant response to contaminants exposure. However, this parallel and the lack of correlation between biotransformation enzymes and environmental variables (temperature and salinity) on concordant time scales, suggest a high specificity of the four analysed biomarkers to chemical exposure (such as PAH), rather than high variability due to environmental dynamics. Present results reinforce the value of these markers in environmental monitoring as well as the importance of considering several biochemical responses in order to attain a more complete view of the toxicity risks and their effects.

Key-words: biomarkers; short scale temporal variation; *Dicentrarchus labrax*; *Solea senegalensis*; *Pomatoschistus microps*; estuaries.

1. Introduction

Biomarkers are commonly used measures of biological changes at the sub-individual level linked to environmental stressors of chemical, physical or biological nature (see review in van der Oost et al. 2003). Considering the ubiquity of various chemical contaminants in the aquatic environment, several biomarkers of exposure to and of /effects of contaminants have been extensively used as early warning signs of exposure, with the potential to: flag the presence of specific classes of toxicants (Shugart et al. 1992; Adams 2002), and account for the primary deleterious effects following exposure to pollutants (van der Oost et al. 2003). Nevertheless, a straightforward relationship between biomarker response to contaminants and further biological consequences at the individual or higher biological levels is difficult to establish, chiefly due to the adaptability of individuals and to the influence of natural variability (Adams 2002; van der Oost et al. 2003). Highly variable environments, such as estuaries, add to the difficulty of distinguishing between effects of anthropogenic stressors and variable natural conditions, such as temperature, salinity or dissolved oxygen, despite the resilience of organisms adapted to estuaries (Elliott and Quintino 2007). In order to correctly assess biomarker responses, it is fundamental to account for background environmental noise in a significant time-scale, concordant with natural variability (Wu et al. 2005; Kopecka and Pempkowiak 2008).

In the present work the activity level of several biomarkers was assessed in three fish species on a short-time scale (of days to weeks) under natural environmental conditions. Multiple biomarker responses were considered, namely: (1) antioxidant defence mechanisms in the form of enzymes that mitigate the toxicity of reactive oxygen species (ROS), specifically superoxide dismutase (SOD) and catalase (CAT), and (2) biotransformation enzymes, phase I ethoxyresorufin O-deethylase (EROD) and phase II glutathione S-transferase (GST), which metabolize xenobiotics and facilitate their excretion (Winston and Di Giulio 1991; Stegeman et al. 1992; Whyte et al. 2000). These biochemical responses were analysed in three fish species that inhabit the Tejo estuary, and are also abundant in numerous other estuaries: common goby *Pomatoschistus microps* (Krøyer 1838) - a widespread and dominant estuarine resident species (Arruda et al. 1993; Leitão et al. 2006); European sea bass *Dicentrarchus labrax* (Linnaeus 1758) and Senegalese sole *Solea senegalensis* Kaup, 1858, two marine species that rely on estuarine nursery grounds during their juvenile stage (Costa and Bruxelas 1989; Cabral and Costa 2001; Vasconcelos et al. 2010). Biomarker responses in these species have been previously used in bioassays and contaminant monitoring studies (e.g. Gravato and Santos 2003; Fernandes et al. 2007; Monteiro et al. 2007; Fonseca et al. 2009; Vieira et al. 2009).

The aim of the study was to examine the short-term variability of the selected biomarkers activity, in three different fish species, in a highly dynamic environment. This should allow to explore the relationship between environmental abiotic variables (temperature and salinity) and the measured biochemical responses. The present multi-biomarker and multi-species approach under natural conditions with a significant degree of chemical contamination, constitutes an integrative approach to habitat quality for fish, in a meaningful temporal scale that accommodates abiotic variation patterns in estuaries. The Tejo is one of the largest European estuaries and it is highly explored and affected by intense and diverse human activities (Vasconcelos et al. 2007) and constitutes an adequate setting to explore this issue.

2. Materials and methods

2.1 Study area and fish sampling

Fish collection was carried out between May and July 2009: sampling was performed in three consecutive days (hereafter referred to as daily variation), at every fortnight (hereafter referred to as weekly variation) over a period of four fortnights. The start of the sampling period varied with species, since estuarine use pattern differs: for *D. labrax* and *S. senegalensis* it immediately followed the colonization of the nursery grounds by juveniles; for *P. microps* sampling started simultaneously with the first sampling event for the remaining species and extended for four fortnights.

Fish collection was carried out using a beam trawl in two sites of the Tejo estuary recognized as important nursery grounds for several species (Cabral and Costa 2001; Vasconcelos et al. 2010). Two species were sampled in Alcochete (ALC) - *Dicentrarchus labrax* and *Pomatoschistus microps,* and *Solea senegalensis* was sampled in Vila Franca de Xira (VFX) (Fig. 1). Upon collection, fish were transported to the laboratory, where they were immediately sacrificed, total length (Lt, in mm) and weight (Wt, in g) recorded and livers collected, frozen in liquid nitrogen and stored at -80 ºC.

Length composition of selected *D. labrax* and *S. senegalensis* individuals corresponded to early age-0+ juveniles that colonized the estuary in the previous month(s) (Cabral and Costa 1999; Cabral and Costa 2001), while *P. microps* corresponded to age-1 adults (Arruda et al. 1993; Leitão et al. 2006) (Table 1). An equal sex-ratio was guaranteed in selected *P. microps.*

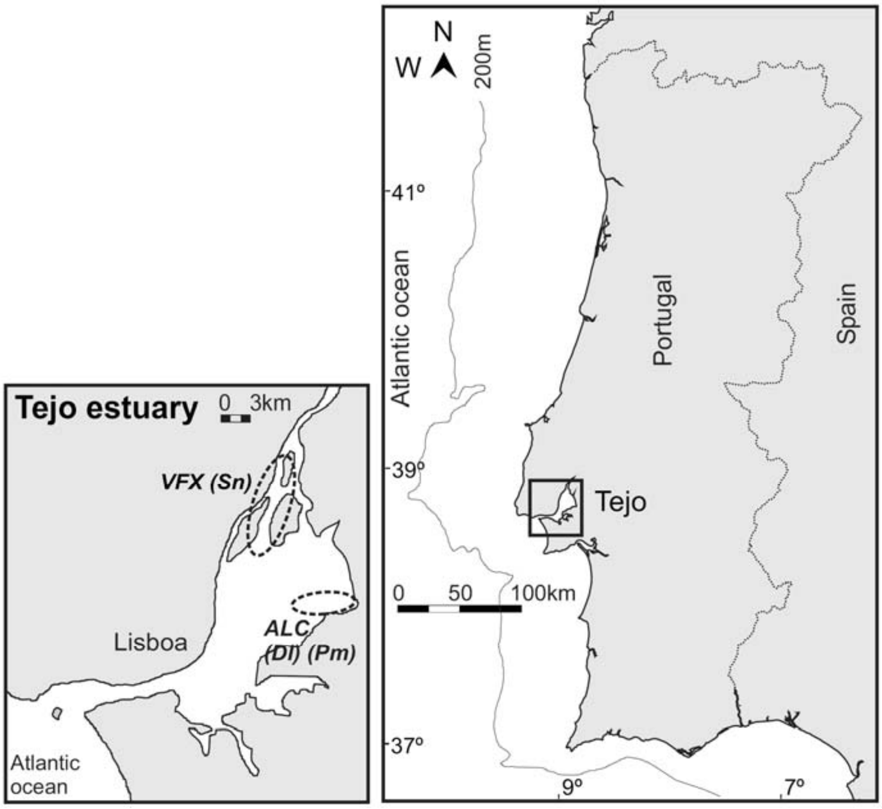


Figure 1. Location of sites sampled within the Tejo estuary (Vila Franca de Xira - VFX, and Alcochete - ALC) between May and July 2009. Also shown, fish species collected in each site: *Dicentrarchus labrax* (*Dl*), and *Pomatoschistus microps* (*Pm*) in Alcochete and *Solea senegalensis* (*Sn*) in Vila Franca de Xira.

Table 1. Mean (and standard deviation between brackets) total length (mm) and weight (g) of *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps* sampled between May and July 2009 in the Tejo estuary and selected for biomarkers analysis.

Species	Total length (mm)	Total weight (g)
D. labrax	63.07 (5.84)	3.01 (2.19)
S. senegalensis	69.20 (2.39)	3.15 (2.12)
P. microps	32.45 (1.50)	0.39 (0.20)

2.2 Habitat environmental characterization

Daily water temperature was measured throughout the entire survey period with temperature data loggers (HOBO H20-001) permanently positioned in both estuarine areas.

Salinity was measured only on fish sampling days with a multi-parameter probe (WTW), as well as depth.

Sediment chemical characterization was based on the concentrations of several trace metals (cadmium - Cd; chromium - Cr; copper - Cu; mercury - Hg; nickel - Ni; lead - Pb and zinc - Zn) and polycyclic aromatic hydrocarbons (PAH). For this purpose, three replicate sediment samples were collected from each site at the beginning of the study period, with an adapted van Veen grab.

For trace metals analysis, sediment samples were wet sieved through a 0.063 mm mesh sieve - in order to minimize the effect of grain size on metal distribution - and the mud fraction (grain size < 0.063 mm) dried in an oven at 40 ºC. The percentage of mud content in the sediment was also determined (dry weight). Approximately 2 g of dried sediment were digested in 5 ml of 69% nitric acid (HNO_3) for 2 h at 80 ºC. The digestion mixture was cooled to room temperature and 2 ml of 30% hydrogen peroxide (H_2O_2) and 3 ml of Milli-Q water were added. Samples were further diluted with Milli-Q water to a final volume of 50 ml. The concentrations of Cd, Cu, Hg, Ni, Pb, Cr 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 analyzed with flame atomic absorption spectrophotometry. Cadmium was analyzed with electrothermal atomic absorption spectrometry and mercury with cold vapour atomic spectrophotometry with an automatic hydride generator system.

PAH concentration was determined in sediment samples with ca. 10 g, which were refluxed in 50 mL of 6% potassium hydroxide (KOH) methanol solution for 4 h. Samples were then centrifuged (2000 *g*, 5 min) and the supernatant decanted into a separating funnel. Extracts were then washed with 50 ml of hexane and further concentrated to 5 ml in a rotary evaporator. Finally, samples were purified by liquid chromatography in a silica-alumina column. Individual quantification of PAH was made using a HPLC-UV with a Spectra System UV3000HR detector. Individual PAH (ng g^{-1} dw) were identified and quantified by comparison of retention times (254 nm) and library spectra of reference compounds (EPA 610 PAH Mix, Sigma). Detection limit ranged from 0.01 to 0.24 ng g^{-1} dw, for the several individual PAH.

2.3 Biomarkers determination

The selected biomarkers were determined in three replicate liver samples pooled from 5-10 individuals (ca. 200 mg), per sampling day and per fish species. Briefly, after weighting, livers were flushed with icecold 1.15% potassium chloride (KCl) and homogenized in 1:5 w/v of cold 100mM KH_2PO_4/K_2HPO_4 (monobasic potassium phosphate/dibasic potassium phosphate) buffer (pH 7.4), containing 150mM KCl and supplemented with 1mM dithiothreitol (DDT), 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 1mM ethylenediaminetetra acetic acid (EDTA). Homogenates were centrifuged at 1500*g* for 15 min and the fatty layer removed. The obtained supernatant was then centrifuged at 12000*g* for 20 min. All assays were carried out with the resulting supernatant (post-mitochondrial supernatant).

Superoxide dismutase (SOD) activity was determined according to MacCord and Fridovich

(1969), by measuring the absorption of the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at a wavelength of 550 nm. One unit of SOD is the amount of the enzyme that inhibits by 50% the reduction of cytochrome c. SOD activity was expressed as U mg^{-1} of total protein concentration.

Determination of catalase (CAT) activity followed the method described by Aebi (1974), by measuring the decrease in absorbance at 240 nm due to H_2O_2 (hydrogen peroxide) consumption. The CAT activity was determined as the difference in the absorbance per unit of time ($\varepsilon = -0.04\ mM^{-1}\ cm^{-1}$), and expressed as mmol $min^{-1}\ mg^{-1}$ of total protein concentration.

EROD activity was determined through a methodology adapted from Fernandes et al. (2002, 2007), with samples incubated at 30 ºC for 10 min in a final volume of 1 ml containing 100mM K_2HPO_4/KH_2PO_4 (pH 7.4), 0.25 mM NADPH and 4.15 mM 7-ethoxyresorufin. The reaction was stopped by adding 2 ml of ice-cold acetone. Samples were centrifuged at 3000 rpm for 10 min and 7-hydroxyresorufin fluorescence was determined at 537 nm/583 nm excitation/emission wavelengths. Activity was calculated as the amount of resorufin (pmol) generated per milligram of protein per minute of reaction time.

Glutathione S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, as described in Fernandes et al. (2002). The final reaction mixture containing 80 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 1 mM CDNB and 1 mM reduced glutathione in a total volume of 1 ml. The change in absorbance, at room temperature, was recorded at 340 nm and the enzyme activity was calculated as mmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 $mM^{-1}\ cm^{-1}$.

Total protein content was measured by the method of Lowry et al. (1951), using Folin's reagent and bovine serum albumin (BSA) as standard.

All determinations were performed in a Biotek Synergy HT microplate reader.

2.4 Statistical analysis

Short-term variability in fish biomarker responses, specifically SOD, CAT, EROD and GST, was tested with nested analysis of variance (ANOVA) followed by Post-hoc multiple comparison Tukey tests, considering two temporal effects: (1) week; and (2) day (nested into weeks). Prior to these analyses, data was log transformed whenever necessary in order to meet assumptions of normality and homogeneity of variance. Significant differences in environmental variables between sites were tested with Student's *t*-test.

Pearson product moment correlation was used to test pairwise associations, separately for each species: between biomarkers and environmental variables (mean daily water temperature and mean daily salinity). A correlation analysis was also applied to compare biomarker responses in *D. labrax* and *P. microps,* when sampling of the two species in site ALC coincided in the same dates.

All analyses were performed using Statistica software and a 0.05 significance level was considered in all test procedures.

3. Results

3.1. Habitat abiotic and chemical characterization

Mean depth and salinity differed between sites, with higher salinity and lower depth in ALC and lower salinity and higher depth in VFX (Table 2). Mean water temperature was similar between sites, and daily temperature increased from early May to late July in both sites, whereas salinity showed little variation throughout the study period (Fig. 2).

Concentration of trace metals in the sediment was also similar between sites, except for Ni and Hg which were higher in VFX and ALC respectively (Table 2; *t* = 2.58, *P* < 0.05). Total PAH concentration was higher in sediments from VFX compared to ALC (Table 2; *t* = 2.91, *P* < 0.05). The majority of PAH in the sediment at both sites were 2 and 3 rings PAH (Table 2; above 80.0%). Lower concentrations of pyrolytic high molecular weight PAH were also present.

Table 2. Mean (and standard variation between brackets) environmental variables in two sites in the Tejo estuary (Vila Franca de Xira - VFX and Alcochete - ALC) sampled between May and July 2009: water temperature (ºC), salinity, depth (m), mud content (grain size <0.063 mm) (% of dry weight of total sediment), trace metals in mud fraction of sediment (Cd, Cu, Hg, Zn, Ni, Pb and Cr) (mg kg^{-1}) and PAH concentration in sediment [total concentration of PAH (in ng g^{-1} dw); and percentage of total PAH represented by different PAH, namely 2 and 3 ring, 4 ring, and 5 and 6 ring). Significant differences between sites (*t*-test) are represented with an asterisk * (*P* < 0.05).

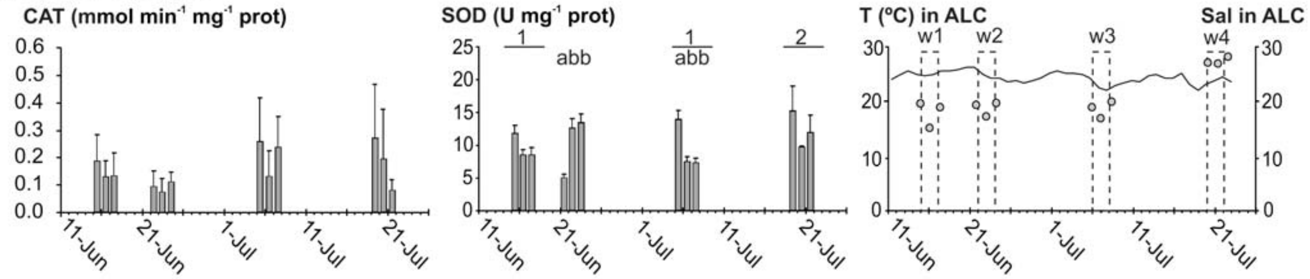
Environmental variable	Vila Franca de Xira (VFX)	Alcochete (ALC)
Temperature (ºC)	22.67 (2.56)	22.38 (2.45)
Salinity *	6.31 (2.12)	16.75 (3.56)
Depth (m) *	4.61 (2.50)	2.12 (0.60)
Mud content in sediment (%)	89.19 (6.91)	98.37 (2.16)
Cd (mg kg^{-1})	3.86 (0.31)	4.02 (0.27)
Cu (mg kg^{-1})	27.54 (0.84)	26.87 (1.23)
Hg (mg kg^{-1}) *	0.27 (0.01)	0.38 (0.02)
Zn (mg kg^{-1})	177.13 (5.81)	184.77 (5.67)
Ni (mg kg^{-1}) *	33.10 (1.24)	28.31 (1.97)
Pb (mg kg^{-1})	60.42 (2.18)	61.11 (2.57)
Cr (mg kg^{-1})	3.79 (0.32)	4.08 (1.18)
Total PAH (ng g^{-1} dw) *	134.31 (13.03)	93.86 (19.65)
2+3 ring (% Total PAH)	98.81 (2.49)	81.17 (2.85)
4 ring (% Total PAH)	1.06 (0.08)	17.71 (0.67)
5+6 ring (% Total PAH)	0.13 (0.01)	1.12 (0.04)

3.2 Biomarker responses

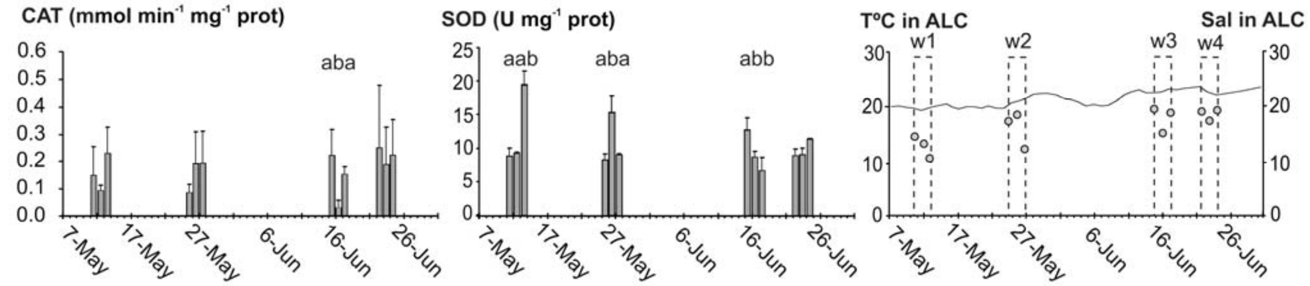
Temporal variability of antioxidant enzymes differed with enzyme and fish species. CAT activity presented low temporal variability, with no differences for *D. labrax* (Fig. 2a; *F* < 1.31, *P* > 0.05), and daily and weekly significant variation was only observed on one occasion for *P. microps* and *S. senegalensis*, respectively (Fig. 2b and c; *F* = 4.24 and *F* = 5.07, *P* < 0.05).

SOD activity varied significantly at daily and weekly time scales for all species. In *D. labrax* SOD activity was significantly higher in week four (Fig. 2a; *F* = 4.11; *P* < 0.05), yet significant daily variation was observed during weeks two and three (Fig. 2a; *F* = 10.10, *P* < 0.01).

(a) ***Dicentrarchus labrax***



(b) ***Pomatoschistus microps***



(c) ***Solea senegalensis***

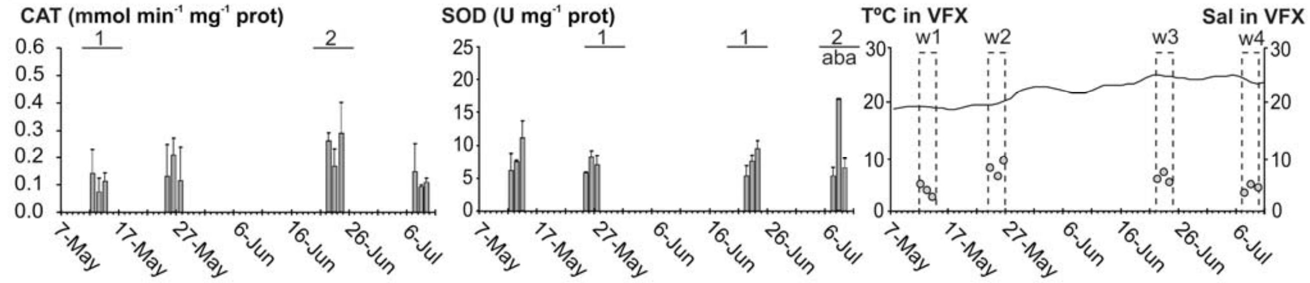


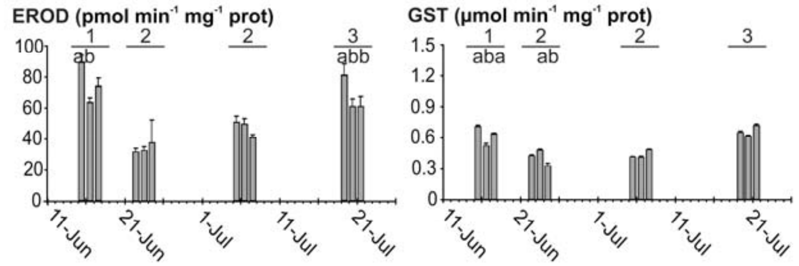
Figure 2. Mean (and standard deviation) antioxidant enzymes activity: SOD (U mg^{-1} prot) and CAT (mmol min^{-1} mg^{-1} prot), in **(a)** *Dicentrarchus labrax*, **(b)** *Pomatoschistus microps* and **(c)** *Solea senegalensis*. Results of nested analysis of variance are also represented: considering effects at the week level (different numbers indicate significant differences between weeks); and daily effects nested into weeks (different letters indicate significant differences between consecutive days). Mean daily water temperature (T in ºC) and salinity (Sal) are also shown for the collection site and period of each species.

P. microps individuals evidenced significant daily variation in SOD activity among consecutive days during the first three weeks (Fig. 2b; F = 23.87, P < 0.001). SOD activity in juvenile *S. senegalensis* liver varied significantly at the daily scale (Fig. 2c; F = 16.68, P < 0.001) and at the week scale, with lower levels in weeks two and three compared to week four (Fig. 2c; F = 4.88, P < 0.01). No significant correlations were observed between antioxidant enzymes activity (CAT and SOD) for all fish species.

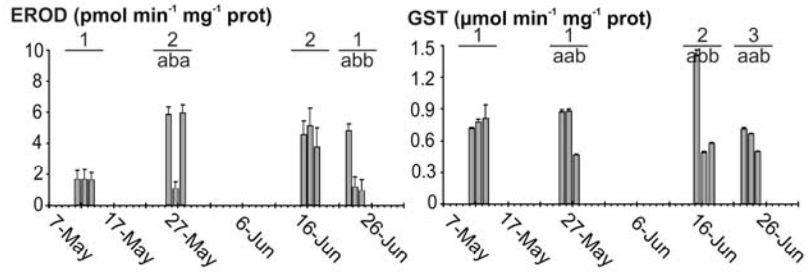
Biotransformation enzymes had higher short-term variability compared to antioxidant responses. Significant differences in EROD activity were observed for all fish species among consecutive days (Fig. 3; F > 10.90, P < 0.001) and among weeks (Fig. 3; F > 16.23, P < 0.001). A similar temporal variability pattern was recorded for GST activity, with significant differences at the daily (Fig. 3; F > 23.83; P < 0.001) and weekly scales (Fig. 3; F > 18.25, P < 0.001). In addition, significant positive correlations between EROD and GST activities were observed in *D. labrax* and *S. senegalensis* individuals (respectively r = 0.72 and r = 0.81, P < 0.05).

Considering associations between biomarker responses and abiotic variables, only CAT

(a) *Dicentrarchus labrax*



(b) *Pomatoschistus microps*



(c) *Solea senegalensis*

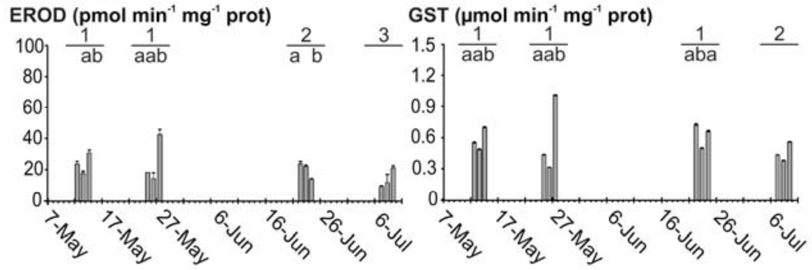


Figure 3. Mean (and standard deviation) biotransformation enzymes activity: EROD (pmol min^{-1} mg^{-1} prot) and GST (µmol min^{-1} mg^{-1} prot), in (a) *Dicentrarchus labrax,* (b) *Pomatoschistus microps* and (c) *Solea senegalensis*. Results of nested analysis of variance are also represented: considering effects at the week level (different numbers indicate significant differences between weeks) and daily effects nested into weeks (different letters indicate significant differences between consecutive days).

activity in *S. senegalensis* livers was positively correlated with water temperature (r = 0.58, P < 0.05).

A positive correlation was also observed between biomarker responses in *D. labrax* and *P. microps* (r = 0.41, P < 0.05) - considering the only coincident sampling occasion for the two species at the same site (weeks one and two for *D. labrax* and weeks three and four for *P. microps* in ALC).

4. Discussion

Short-term variability in multiple biomarker responses was observed in three fish species from the Tejo estuary, although no clear pattern could be discerned in biomarker responses over time.

Significant chemical load namely from trace metals and polycyclic aromatic hydrocarbons (PAH) were measured in sediment samples from both sites of the Tejo estuary. Sediment trace metal concentrations were within the same range reported in previous studies in this estuary (França et al. 2005; Pedro et al. 2008; Vale et al. 2008). PAH composition points to mixed sources of PAH inputs into the system, with higher percentage of low molecular weight PAH (2 to 3 rings) of petrogenic origin (uncombusted petroleum-derived PAH) compared to pyrolytic high molecular weight PAH (4 to 6 rings) resulting from the combustion of petroleum-like products and other organic matter (wood and coal) (Neff et al. 1979; Yunker et al. 2002). Accordingly, earlier work described significant anthropogenic stressors in the Tejo estuary, from industrial, agricultural and urban discharges and emissions, as well as from intense shipping and port activities (França et al. 2005; Vasconcelos et al. 2007; Vale et al. 2008).

The sediment chemical characterization in both estuarine sites served as a reference point to determine potential toxicity stress and to identify sources of anthropogenic contamination present in each site that could induce adaptive biochemical responses in natural fish populations.

In general, the activity levels of biomarker responses were within range or somewhat higher than previously reported values for these species that corresponded to biochemical adaptations to various chemical stresses, in both field and laboratory assays (Fernandes et al. 2007; Monteiro et al. 2007; Solé et al. 2008; Vieira et al. 2008; Fonseca et al. 2009; Maria et al. 2009; Vieira et al. 2009).

The activity of antioxidant enzymes (CAT and SOD) showed low temporal variability at both scales considered (days and weeks) for all species, which is indicative of some constancy or baseline level in antioxidant responses. This constant response could result from a permanent contact with stressful conditions either of chemical or environmental nature, resulting in oxidative damage. Antioxidant defence mechanisms can be triggered by different biotic and abiotic factors, other than contaminants exposure, such as temperature, dissolved oxygen, diet and feeding behaviour, all of which can influence cellular ROS production (Martínez-Álvarez et al. 2005). Nevertheless, in the present work, only CAT activity in *S. senegalensis* was positively correlated with water temperature. Therefore a significant exposure to contaminants appears to be triggering higher values of antioxidant adaptation responses.

Higher short-term variability was observed in the activity of biotransformation enzymes (EROD and GST) when compared to antioxidant enzymes, at both daily and weekly time scales, for all fish species. Furthermore, EROD and GST activities were positively correlated in *D. labrax* and *S. senegalensis* individuals, indicating concordant metabolism of xenobiotic chemicals from both phase I and phase II enzymes. Akin to antioxidant responses, several confounding factors have been described to influence the activity of biotransformation enzymes, including temperature, salinity, diet, reproductive stage (van der Oost et al. 2003; Gourley and Kennedy 2009). However, the lack of correlation between biotransformation enzymes and environmental variables (temperature and salinity) measured on concordant time scales, and the concomitant levels of EROD and GST activity, suggest a high specificity of the four analysed biomarkers to chemical exposure (such as PAH), rather than high variability due to environmental dynamics.

Temperature and salinity have been described as modulating factors of individuals' metabolic and physiological processes, since temperature is a controlling factor of the metabolic pace determining enzymatic reactions and oxygen uptake rates, while salinity influences basic metabolic expenditure through osmoregulation control (Fry 1971; Sampaio and Bianchini 2002; Yamashita et al. 2003; Fonseca et al. 2010). Hence, previous studies have linked these abiotic variables to biomarker responses on different time scales. Sleiderink et al. (1995) found that, in addition to polychlorinated biphenyls (PCB) exposure, temperature and nutritional status influenced EROD activity in dab (*Limanda limanda*, Linnaeus, 1758) from the North Sea. Higher SOD activity in European sea bass has been associated with chemical toxicity and also with higher salinity and water temperature (Roche and Bogé 1996). Kopecka and Pempkowiak (2008) attributed seasonal variation of CAT activity in flounder (*Platichthys flesus*, Linnaeus, 1758) to temperature and possible salinity effects on GST activity. On the other hand, studies have reported little or no effects of these abiotic variables on biomarkers response, as follows. Acute temperature increase has been shown to increase cytochrome P-450 enzyme system mediated responses in fish, yet given a sufficient acclimation period activities return to baseline levels (Andersson and Förlin 1992; Lange et al. 1998). Kopecka and Pempkowiak (2008) have also described that correlations between biomarker activities in flounder (including EROD, GST and CAT) and abiotic factors (temperature, salinity and dissolved oxygen concentration) were less important than the effects of reproduction and pollution on biomarker induced responses, which is in line with present results.

In addition to their influence on physiological processes, temperature and salinity can also affect bioavailability of contaminants through effects on binding, complexation and partioning processes between chemicals and the sediment/water phase (Eggleton and Thomas 2004). Despite the potential link with biomarker responses analysed in the present study, the bioavailability of contaminants is still poorly understood, as it is difficult to integrate the various factors affecting fate of contaminants, particularly at short-time scales since frequent sediment remobilization is induced by several natural events and anthropogenic activities (e.g. daily tidal currents, bioturbation, dredging and fishing activities) (Eggleton and Thomas 2004).

Other potentially confounding factors that can vary at short-time scales, such as dissolved oxygen, were not assessed in this work, Nevertheless, non limiting oxygen conditions and significant prey availability have been described in recent studies in these sites of the Tejo estuary (Fonseca et al. 2010), which leads to the assumption that both variables would not have influenced present biomarker responses.

In view of the short-time scales considered, other acknowledged confounding factors such as fish nutritional status, gender, age and spawning season should not influence present biomarker responses, since fish were in good condition (Fonseca et al. in review a) and equal sex-ratio was considered in mature *P. microps*.

To some extent biomarker responses of the three species were concordant, with similar response patterns in *D. labrax* and *P. microps* occurring at the same time in Alcochete (ALC) and a correlation between EROD and GST activities in both *D. labrax* and *S. senegalensis* juveniles. Yet, the different levels of biomarker responses between species can be attributed to differences in exposure to contaminants from different habitat use, metabolic capacity or even life-stage (Filho et al. 1993; van der Oost et al. 2003; Fonseca et al. in review b).

Present results provide evidence reinforcing the importance of considering several biochemical responses in order to attain a more complete view of the toxicity risks and their effects, in spite of the difficulty to distinguish between adverse effects of highly variable environments and of anthropogenic stressors (Elliott and Quintino 2007).

Overall, considering the determined sediment contamination levels, biomarker responses in local fish populations could be expected to signal biochemical adaptation to pollution. Nonetheless, antioxidant responses appeared to be less specific to toxicity stress since a rather constant response was observed throughout the study period. Biotransformation enzymes evidenced higher short-term variability and significant correlation between them (in *D. labrax* and *S. senegalensis* juveniles), suggesting a concomitant response to contaminants exposure. Nevertheless, habitat quality assessments in terms of chemical load based on biomarker responses should always include measures of abiotic variables, in order to guarantee that any acute variation in these parameters is not wrongly interpreted as pollutants' increase toxicity..

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CHAPTER 9

Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*

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In review in Chemosphere

Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*

Abstract: Several biomarker responses were determined in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps* from two estuaries of the Portuguese coast, Ria de Aveiro and Tejo. Both estuaries have significant anthropogenic influences from multiple sources (industrial, agricultural, shipping activities), which was evident from sediment chemical characterization concerning metals (Cu, Zn, Ni, Pb and Cr) and polycyclic aromatic hydrocarbons (PAH) concentrations. Spatial variability in adaptation responses was observed across species for most biomarkers of exposure (SOD, CAT, TGPx, SeGPx, EROD, GST, MT) and effects biomarkers (LPO, R:D, protein and lipid content). In general, the interspecific differences in biomarker responses surpassed the spatial patterns, due to differences in species' behavior and habitat use. Nevertheless, similarities were also observed considering both chemical load and biomarker responses. In highly polluted sites fish showed in general a significantly antioxidant enzymatic induction, associated with lower R:D values, while fish from the least impacted site had little enzymatic induction and better condition indices (high R:D and low LPO values). EROD activity was also higher for all species in the Tejo estuary, despite generally higher TPAH measured in Ria de Aveiro, most likely due to a higher proportion of 4 and 6-rings PAH in the Tejo, considered more toxic than low molecular weight PAH. In conclusion, this multi-biomarker approach considering multiple species provided a better understanding of the diverse responses and effects of exposure to contaminants and the effective risk it poses for different fish species.

Key-words: biomarkers; *D. labrax*; *S. senegalensis*; *P. microps*; estuaries; anthropogenic pressure

1. Introduction

Coastal transition ecosystems (e.g. estuaries and coastal lagoons) sustain valuable ecologic and economic resources (Costanza et al. 1997), however they are often vulnerable to various stressors derived from human activities (i.e. agriculture, industry, human settlements, fishing, port activities) (Kennish 2002).

The increasing presence of organic and inorganic contaminants in estuaries renders monitoring and risk-assessment procedures essential to ensure the preservation of their biological function (Adams 2002) and has led to the search for improved monitoring methods that can express the biological and ecological implications of pollution beyond environmental chemical characterization (van der Oost et al. 2003).

Biomarkers are considered useful and early measures of exposure to and/or effects of contaminants in aquatic organisms (Shugart et al. 1992; Adams 2002; van der Oost et al. 2003) and have been frequently used to assess habitat quality (e.g. Fernandes et al. 2002; Monteiro et al. 2007; Maria et al. 2009). Nevertheless, the presence of complex mixtures of xenobiotics in the environment and of other potentially confounding factors (i.e. life stage, abiotic natural variability) may result in further difficulties in the interpretation of biomarkers response patterns (Adams 2002; van der Oost et al. 2003).

A multi-biomarker approach, consisting of the combined use of different biomarkers that can both signal exposure to contaminants and quantify their effects on the organisms' health, enables a more comprehensive and integrative assessment of environmental quality (Adams 2005; Broeg and Lehtonen 2006; Humphrey et al. 2007). Moreover, determining biochemical responses in natural populations of different fish species encompasses diverse forms of biological integration of environmental toxicants, which can result from different physiological processes or species ecology, thus adding ecological relevance to a study (Solé et al. 2009).

In the present study, a multi-biomarker approach was considered for three fish species with different life-history traits and strategies: common goby *Pomatoschistus microps* (Krøyer 1838), a dominant estuarine resident species with short life span (ca. 2 years) (Arruda et al. 1993; Leitão et al. 2006); and European sea bass *Dicentrarchus labrax* (Linnaeus 1758) and Senegalese sole *Solea senegalensis* Kaup, 1858, two marine species with high economic value and which utilize estuaries as nursery grounds during the juvenile period (Costa and Bruxelas 1989; Cabral and Costa 2001; Vasconcelos et al. 2010).

The Ria de Aveiro and Tejo, are estuarine systems located along the Portuguese coast, highly explored and impacted by human activities, with significant and diverse sources of aquatic contaminants due to population settlement, industrial, agricultural and shipping activities (Pacheco et al. 2005; Vasconcelos et al. 2007; Vale et al. 2008). Accordingly, several biomarkers of exposure and effect were chosen in order to account for a wide range of pollutants: 1) biotransformation enzymes [phase I ethoxyresorufin O-deethylase (EROD) and phase II glutathione S-transferase (GST) activities], that metabolize xenobiotics; 2) antioxidant enzymes activities (superoxide dismutase - SOD; catalase - CAT; and glutathione peroxidase - GPx), that reduce cellular damage (e.g. lipid peroxidation - LPO) resulting from reactive oxygen species (ROS) which in turn increase with exposure to various contaminants; 3) stress proteins, specifically metallothioneins (MT), inducible by both essential and non-essential metals; and 4) morphological and physiological measures of fish general condition related to growth potential and energy reserves [condition factor K; nucleic acids ratio (R:D); protein and lipid content].

The main objective of the present work was to determine the biochemical responses of different fish species in contaminated estuarine environments, based on the spatial variability patterns (at site and estuary level) described for each species' set of biomarkers response. The multi-biomarker approach intended to explore a wide range of fish responses to pollution in a realistic context, including biomarkers of exposure (e.g. metallothioneins; antioxidant enzymes activity) and general condition indices or biomarkers of effect (e.g. R:D ratio and lipid peroxidation, respectively) in natural populations of different fish species.

2. Materials and methods

2.1 Study area and environmental chemical characterization

Two estuarine systems along the Portuguese coast - Ria de Aveiro and Tejo - and three estuarine sites per estuary (Ovar (Ra1 – 40.72ºN, 8.68ºW), Murtosa (Ra2 – 40.73ºN, 8.66ºW) and Mira channel (Ra3 – 40.53ºN, 8.77ºW) in Ria de Aveiro; and Vila F Xira (Tj1 – 38.88ºN, 9.01ºW), Alcochete (Tj2 – 38.76ºN, 8.93ºW) and Montijo (Tj3 – 38.68ºN, 9.01ºW) in Tejo), were sampled in October 2008. This period was selected since it corresponded to the late estuarine colonization period of *D. labrax* and *S. senegalensis* juveniles, and to the occurrence period of early adults of *P. microps*. The sites were selected per estuary based both on the presence of known anthropogenic pressures and on the composition of the fish community – i.e. in order to ensure that the three fish species were present (França et al. 2009; Vasconcelos et al. 2010).

Chemical characterization of each site was based on the quantification of trace metals (Cadmium, Cd; Copper, Cu; Zinc, Zn; Nickel, Ni; Lead, Pb and Chromium, Cr) and polycyclic aromatic hydrocarbons (PAH) concentrations in the sediment. Three replicate sediment samples were collected in each site with a van Veen grab and used for chemical characterization. Trace metals were determined in the mud fraction of the sediment (grain size < 0.063 mm) to minimize the effect of differences in grain size composition of samples on metal distribution. Briefly, samples were wet sieved in 63 µm nylon sieves using Milli-Q water and the mud fraction was dried at 40 ºC. The percentage of mud content in the sediment was also determined (dry weight). Approximately 2 g of the dried and grinded sediment fraction were digested with 5 ml of 69% nitric acid (HNO_3) for 2 h at 80 ºC. The digestion mixture was cooled to room temperature and 2 ml of 30% hydrogen peroxide (H_2O_2) and 3 ml of Milli-Q water were added. The mixture was heated for another hour, before the residue was allowed to cool and further diluted with Milli-Q water to a final volume of 50 ml. Samples were allowed to settle for 24 h and quantitatively transferred to 50 ml vessels for analysis.

The concentrations of Cd, Cu, Ni, Pb, Cr and Zn were determined in the supernatant using a Perkin-Elmer atomic absorption spectrophotometer (AAS) model A Analyst 800. All metals, except Cd, were analyzed by flame atomic absorption spectrophotometry. Cadmium was analyzed with an electrothermal atomic absorption spectrometry.

PAH concentration was determined in three replicates of 10 g of sediment, which were refluxed in 50 ml of 6% KOH (potassium hydroxide) methanol solution for 4 h. Samples were then centrifuged (2000 g, 5 min) and the supernatant was decanted into a separating funnel. Extracts were then washed with 50 ml of hexane and further concentrated to 5 ml in a rotary evaporator. Finally, samples were purified by liquid chromatography in a silica-alumina column. Aliphatic hydrocarbons were elluted with 50 ml n-hexane. Mono and di-cyclic hydrocarbons were eluted with 25 ml of n-hexane/dichloromethane (9:1) and PAH from three to six rings with 25 ml of the same mixture (4:1). The aromatic fractions were combined and taken to complete dryness by a combination of rotary evaporation and a gentle nitrogen stream. Individual quantification of PAH was made using a HPLC-UV with a Spectra System UV3000HR detector. Individual PAH (ng g-1 dry weight) were identified and quantified by comparison of retention times (254 nm) and library spectra of reference compounds. A standard mixture containing 16 individual PAH (EPA 610 PAH Mix, Sigma) namely naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene (Phen), anthracene (Ant), fluoranthene (Flu), pyrene

(Pyr), benzo(a)anthracene (BaA), chrysene (Chris), benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene (BPer) and indeno-(1,2,3-cd)pyrene (IndP) was used. Detection limit ranged from 0.01 to 0.24 ng g^{-1} d.w., for individual PAH. PAHs were not detected in procedural blanks.

Additionally, PAH diagnostic ratios, namely Phen/Ant, Flu/Pyr, Ant/Ant+Phen, Flu/Flu+Pyr, BaA/BaA+Chris and IndP/IndP+BPer, were used to identify sources of PAH input to the environment, based on Baumard et al. (1998) and on Yunker et al. (2002) (ratios thresholds are shown in Table 1).

Table 1. Origin of polycyclic aromatic hydrocarbons (PAH) based on diagnostic molecular indices of specific PAH, according to Baumard et al. (1998) and Yunker et al. (2002).

PAH molecular ratio	Origin		
	Petrogenic	Pyrogenic	Biomass combustion
Phe/Ant [a]	> 10	< 10	
Flu/Py [a]	< 1.0	> 1.0	
Ant/Ant + Phe [b]	< 0.1	> 0.1	
Flu/Flu + Py [b]	< 0.4	0.4 – 0.5	> 0.5
BaA/BaA + Chr [b]	< 0.2	> 0.35	
IP/IP + Bghi [b]	< 0.2	0.2 – 0.5	> 0.5

[a] Baumard et al. (1998)
[b] Yunker et al. (2002)

2.2. Fish collection and sample preparation

Fish collection was carried out using a beam trawl in the selected estuarine areas. Upon collection, fish were transported to the laboratory, where they were immediately sacrificed, total length (Lt, in mm) and weight (Wt, in g) recorded and target tissues (muscle and liver) dissected, frozen in liquid nitrogen and stored at -80º C until analyses.

Biochemical measurements in the muscle were done in 10 to 30 individuals per species from each study site based on the length frequency distributions of collected fish, in order to account for the effect of fish size. The condition factor (K) was determined from morphometric data, according to the formula $K = (100 \times Wt) / Lt^3$, where Wt is total weight (g) and Lt is total length (cm) (Ricker 1975). Biomarkers in the liver were determined in pooled samples of these individuals, except for *P. microps*, which due to small liver sizes, required more individuals to ensure minimal sample weight (ca. 100 individuals).

2.3. Biomarkers determination

To determine the levels of enzymatic activity, microsomal and cytosolic fractions were prepared as described in Lavado et al. (2004). Briefly, after weighting, livers were flushed with ice-cooled 1.15% KCl (potassium chloride) and homogenized in 1:5 w/v of cold 100 mM KH_2PO_4/K_2HPO_4 (monobasic potassium phosphate/dibasic potassium phosphate) buffer (pH 7.4), containing 150 mM KCl and supplemented with 1mM dithiothreitol (DDT), 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetra acetic acid (EDTA).

Homogenates were centrifuged at 1500 *g* for 15 min and the fatty layer removed. The obtained supernatant was then centrifuged at 12000 *g* for 20 min. The resulting supernatant was further centrifuged at 100000 *g* for 60 min to obtain the cytosolic and microsomal fractions. Microsomal pellets were resuspended in a ratio of 0.5 ml buffer g^{-1} liver tissue in 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4), containing 150 mM KCl, 20% (w/v) glycerol and supplemented with 1 mM DDT, 0.1 mM PMSF and 1 mM EDTA. Microsomal and cytosolic proteins were measured by the method of Lowry et al. (1951), using Folin's reagent and bovine serum albumin (BSA) as standard.

Superoxide dismutase (SOD) activity was determined in liver cytosol, using the method described in MacCord and Fridovich (1969), by measuring the absorption of the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at a wavelength of 550 nm. One unit of SOD is the amount of the enzyme that inhibits by 50% the reduction of cytochrome c. SOD activity was expressed as U mg^{-1} of total protein concentration.

Catalase (CAT) activity was measured in liver cytosol, following the method described in Greenwald (1985) by the decrease in absorbance at 240 nm due to H_2O_2 (hydrogen peroxide) consumption. The CAT activity was determined as the difference in the absorbance per unit of time ($\varepsilon = -0.04$ mM^{-1} cm^{-1}), and expressed as mmol min^{-1} mg^{-1} of total protein concentration.

Glutathione peroxidases (total, TGPx, and Se-dependent, SeGPx) activities were measured in liver cytosol at 340 nm following NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (Lawrence and Burk 1976). Cumene hydroperoxide and H_2O_2 were used as substrates for the determination of total and Se-dependent glutathione peroxidase activity. The difference in the absorbance per unit of time (the rate of blank reaction was subtracted from the total rate) was taken as the measure of GPx activity ($\varepsilon = -6.22$ mM^{-1} cm^{-1}), which were expressed as μmol min^{-1} mg^{-1} of total protein concentration.

EROD activity was determined in the microsomal fraction of the liver as described in Fernandes et al. (2002). Hepatic microsomes (200 mg) were incubated at 30 ºC for 10 min in a final volume of 1 ml containing 100 mM K_2HPO_4/KH_2PO_4 pH 7.4, 0.25 mM NADPH and 4.15 mM 7-ethoxyresorufin. The reaction was stopped by adding 2 ml of ice-cold acetone. Samples were centrifuged at 3000 rpm for 10 min and 7-hydroxyresorufin fluorescence was determined using a Hitachi 650-10 spectrofluorometer at 537/583 excitation/emission wavelengths. Activity was calculated as the amount of resorufin (pmol) generated per milligram of protein per minute of reaction time.

Glutathione S-transferase (GST) activity was measured in the cytosolic fraction of liver, using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, as described in Fernandes et al. (2002). The final reaction mixture containing 80 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 1 mM CDNB and 1 mM reduced glutathione in a total volume of 1 ml. The change in absorbance, at room temperature, was recorded at 340 nm and the enzyme activity was calculated as mmol CDNB conjugate formed per milligram of protein per minute using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Metallothionein (MT) concentration was determined on liver samples, after the cytosol had been separated from the residual fraction (30000 *g*, 45 min, 4 ºC), heat-treated at 80 ºC for 10 min, and subsequently centrifuged (30000 *g*, 45 min, 4 ºC). Aliquots of the heat-treated cytosol (25 µl) were used to quantify the MT levels by differential pulse polarography (DPP) using a 646VA Processor autolab type II and an ECOChemie IME663 mercury drop electrode, according to the method described by Bebianno and Langston (1989).

Lipid peroxidation was determined in liver samples according to the method described by Erdelmeier et al. (1998) which measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) produced during the decomposition of polyunsaturated fatty acid peroxides of membrane lipids. The concentration of lipid peroxidation was expressed as µmoles of MDA and 4-HNE g^{-1} total protein.

RNA to DNA ratio (R:D) was determined on two replicate individual muscle samples, of ca. 30 - 80 mg wet weight, according to the fluorometric method described in Caldarone et al. (2001). Muscle samples were homogenized through short-term ice-sonication in N-lauroylsarcosine Tris-EDTA buffer (0.1%, pH 7.5), centrifuged (at 6000 rpm for 10 min at 0 ºC), and 125 µl aliquots of the supernatant were used for the quantification of RNA and DNA fluorescence with ethidium bromide (microplate-reader Biotek Synergy HT, emission wavelength 590 nm, excitation wavelength 360 nm). RNA fluorescence was determined as the difference between total nucleic acids fluorescence and DNA fluorescence following treatment with RNase A (bovine pancreatic ribonuclease A 0.12 mg ml^{-1}, from bovine pancreas, 20 U ml^{-1}, Sigma). Standard curves were previously determined using pure calf-thymus DNA (Calbiochem) and 18S- and 28S-rRNA (Sigma), and the ratio between the two slopes from each standard curve was 8.21.

Protein content in muscle and liver samples was determined according to Lowry et al. (1951). Aliquots reacted with Folin's and Copper reagent, and the complex's absorbance was read at 750 nm and compared with a standard curve previously constructed with a dilution series of bovine serum albumin (Sigma).

Muscle lipid content was based on total fatty acids methyl esters (FAME) determination on individual samples (ca. 50 - 200 mg dry weight depending on the remaining muscle available), followed by weight normalization according to lipid percent composition in phospholipids and triacylglicerols. FAME were analysed in a CP 3800 Varian gas chromatograph, equipped with an auto-sampler and fitted with a flame ionisation detector (FID), as described in Bandarra et al. (2009). Species' appropriate conversion factors were determined with thin-layer chromatography, on five selected individuals per species. Additionally, lipid content in these selected individuals was also determined with the Bligh and Dyer (1959) method in order to assure consistency of the results. Lipid content was expressed as % of muscle wet weight after adjustment, based on wet and dry weight determinations prior and after sample lyophilisation.

2.4. Statistical analyses

Exploratory data analysis (one-way analysis of variance) was used to search for differences in species' weight and length measurements between estuarine sites. The lack of differences indicated similar weight and length composition of fish populations, and thus no further adjustments were made to account for size effects on biomarkers response.

Nested analyses of variance were used to determine significant differences between sites and between estuaries in mud percentage, trace metal and PAH concentrations in the sediment, and in each species biomarkers' responses. Data were previously log10 transformed, and assumptions of normality and homogeneity of variances were met. Whenever significant differences were observed, Tukey *post-hoc* comparison tests were used to distinguish spatial patterns.

A Pearson product moment correlation was used to test associations between each species biomarkers' responses and sediment contamination, measured by trace metals and total PAH load.

A correspondence analysis, using trace metals and PAH loads as covariates, was also used to outline species spatial response patterns, associated with the measured biomarkers.

A significance level of $P < 0.05$ was considered in all test procedures.

3. Results

3.1. Environmental chemical characterization

The Tejo estuary presented higher concentrations of the analysed metals in the sediment, measured in the finer sediment fraction (grain size < 0.063 mm), when compared to Ria de Aveiro, although high metal concentrations were observed in Ra1 (Table 2). In general Ra3 site had the lowest metal concentrations in the sediment, as well as lower percentage of mud in sediment (Table 2).

In total, 16 individual PAH were identified in sediments from all sites (data not shown). Total PAH concentration was higher in sediments from Ria de Aveiro relatively to Tejo, except at one site of Ria de Aveiro (Ra3), where the lowest concentrations were found (Table 2). Overall, concentrations of 2 and 3 rings PAH were higher than those of 4 to 6 rings PAH, indicative of an uncombusted oil/petroleum contamination (petrogenic) (Table 2). However, pyrolytic high molecular weight PAH with 5 and 6 rings were also present, representing between 25% and 35% of total PAH concentration, mainly in the Tejo estuary. The presence of both petrogenic and pyrogenic PAH (mixed sources) is also evident from the diagnostic ratios Phe/Ant; Flu/Py; Ant/Ant+ Phe; Flu/Flu + Py; BaA/BaA+Chr and IP/IP+Bghi (Table 1). Values of these ratios are indicative of petroleum/crude hydrocarbons inputs and combustion PAH sources, which in turn are mainly from incomplete fossil fuels' combustion and in Tj3 are also from products of incomplete combustion of biomass (Table 2).

3.2. Fish biometrics

Length and weight composition of selected fish samples were homogeneous between estuarine sites (Table 3). Based on length compositions, *D. labrax* and *S. senegalensis* individuals corresponded to age 0+ juveniles that colonized the estuarine habitat in 2008 late

Table 2. Mean (and standard deviation in brackets) mud content in the sediment (percentage of grain size < 0.063 mm), metals and PAH concentrations in sediments from estuarine sites in Ria de Aveiro (Ra1 - Ovar, Ra2 - Murtosa, Ra3 - Mira channel) and Tejo (Tj1 - Vila Franca de Xira, Tj2 - Alcochete, Tj3 - Montijo). Nested analyses of variance were used to determine differences in mud %, metals and PAH loads between estuaries (*), and sites ($P < 0.05$). Significant differences between sites (Tukey *post hoc* comparison tests) are represented by different superscript letters ($P < 0.05$). Origin of PAH is also characterized by several diagnostic molecular indices (see table 1).

	Ria de Aveiro			Tejo		
	Ovar Ra1	Murtosa Ra2	Mira channel Ra3	Vila F Xira Tj1	Alcochete Tj2	Montijo Tj3
% Mud sed. (<0.063 mm)*	80.01 ^{a}	56.99 ^{b}	48.17 ^{b}	88.34 ^{a}	99.26 ^{c}	90.20 ^{a}
	(4.26)	(9.21)	(8.57)	(8.25)	(1.24)	(5.24)
Cd (mg kg^{-1}) *	1.03 ^{a,c}	1.06 ^{a,c}	0.14 ^{b}	1.25 ^{a,d}	0.97 ^{c}	1.37 ^{d}
	(0.09)	(0.01)	(0.04)	(0.01)	(0.11)	(0.21)
Cu (mg kg^{-1}) *	39.18 ^{a,b}	32.90 ^{a,b}	26.51 ^{a}	48.40 ^{b}	48.30 ^{b}	66.35 ^{c}
	(3.08)	(11.47)	(4.19)	(10.86)	(15.63)	(4.69)
Zn (mg kg^{-1}) *	257.84 ^{a}	173.04 ^{b}	96.18 ^{c}	202.17 ^{a,b}	243.11 ^{a,b}	269.26 ^{a}
	(7.63)	(56.89)	(9.99)	(43.62)	(18.21)	(12.36)
Ni (mg kg^{-1}) *	24.42 ^{a}	23.96 ^{a}	16.72 ^{b}	32.42 ^{c}	33.90 ^{c}	33.78 ^{c}
	(3.75)	(3.11)	(2.55)	(2.19)	(1.64)	(0.53)
Pb (mg kg^{-1}) *	28.80 ^{a}	30.10 ^{a}	25.26 ^{a}	56.64 ^{b}	72.90 ^{c}	128.44 ^{d}
	(1.90)	(7.46)	(3.00)	(1.78)	(22.55)	(10.82)
Cr (mg kg^{-1}) *	42.41 ^{a}	43.87 ^{a}	50.39 ^{a}	87.80 ^{b}	75.52 ^{c}	91.96 ^{b}
	(3.15)	(5.30)	(9.53)	(4.32)	(3.09)	(1.65)
Total PAH (ng g^{-1} dw)	269.01 ^{a}	347.13 ^{a}	16.13 ^{b}	248.86 ^{a}	162.40 ^{c}	169.58 ^{c}
	(68.41)	(43.96)	(0.29)	(57.03)	(45.20)	(42.28)
2+3 ring (% TPAH)	94.79	79.12	31.20	83.08	59.94	55.45
4 ring (% TPAH)	4.30	12.84	43.63	6.78	11.21	9.35
5+6 ring (% TPAH)	0.91	8.03	25.17	10.14	28.85	35.21
Phe/Ant	0.57	0.15	0.07	0.08	0.19	0.33
Flu/Py	0.27	0.24	0.76	0.76	0.86	1.06
Ant/Ant+ Phe	0.64	0.87	0.93	0.93	0.84	0.75
Flu/Flu + Py	0.22	0.19	0.43	0.43	0.46	0.51
BaA/BaA+Chr	0.54	0.85	0.50	0.20	0.86	0.61
IP/IP+Bghi	0.00	0.10	0.22	0.00	0.00	0.14

spring-early summer (Cabral and Costa 1999; Cabral and Costa 2001), while *P. microps* were early first year adults (Arruda et al. 1993; Leitão et al. 2006).

Condition factor K did not differ between estuaries and sites for all species (Table 3).

3.3 Biomarkers responses

SOD, SeGPx and TGPx activities varied significantly between estuarine sites in *D. labrax* ($F = 5.83$ and $F = 4.46$, $P < 0.05$; and $F = 20.42$, $P < 0.001$ respectively). Higher SOD activity was observed in Ra2 and lower activity in Ra1, while higher TGPx activity was observed in Ra1 (Fig. 1). SeGPx level was generally higher in Tj1, but no significant differences were observed between estuarine systems (Fig. 1). Only TGPx activity differed between estuaries for *D. labrax* ($F = 9.24$, $P < 0.01$), with higher levels in Ria de Aveiro (Fig. 1).

Significant differences at the estuary and site level were observed in TGPx ($F = 4.70$ and $F = 4.25$, $P < 0.05$) and SeGPx ($F = 27.26$, $P < 0.001$; and $F = 4.43$, $P < 0.05$) activities in *S. senegalensis*' liver; while CAT activity only differed between sites ($F = 4.87$, $P < 0.001$), with a maximum activity observed for distinct sites in every case (Ra2; Tj3 and Tj1 respectively;

Table 3. Fish biometrics: mean (and standard deviation in brackets) length (mm), weight (g) and condition factor K of *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps* in estuarine sites in Ria de Aveiro (Ra1 - Ovar, Ra2 - Murtosa, Ra3 - Mira channel) and Tejo (Tj1 - Vila Franca de Xira, Tj2 - Alcochete, Tj3 - Montijo).

Fish biometrics	Ria de Aveiro			Tejo		
	Ovar Ra1	Murtosa Ra2	Mira channel Ra3	Vila F Xira Tj1	Alcochete Tj2	Montijo Tj3
D. labrax						
n	15	11	13	10	18	10
Length (mm)	142.73	143.33	134.46	115.86	122.50	128.67
	(16.49)	(38.08)	(29.10)	(14.61)	(20.73)	(24.34)
Weight (g)	33.72	70.76	28.22	16.22	11.61	17.35
	(11.30)	(35.69)	(18.67)	(4.75)	(8.06)	(11.77)
K	1.14	1.07	1.08	1.04	0.96	1.22
	(0.19)	(0.17)	(0.18)	(0.18)	(0.10)	(0.03)
S. senegalensis						
n	11	10	16	13	23	11
Length (mm)	96.01	116.83	95.17	115.92	99.69	117.70
	(42.91)	(40.99)	(34.33)	(28.90)	(16.36)	(28.91)
Weight (g)	13.20	19.73	11.77	18.78	13.25	19.24
	(16.88)	(15.71)	(16.87)	(17.92)	(5.58)	(11.01)
K	0.91	0.98	0.89	1.02	1.04	1.05
	(0.08)	(0.09)	(0.09)	(0.10)	(0.08)	(0.05)
P. microps						
n	30	30	30	27	30	29
Length (mm)	34.27	36.33	32.83	36.11	37.30	31.76
	(2.94)	(2.97)	(5.85)	(4.33)	(4.41)	(5.28)
Weight (g)	0.37	0.41	0.36	0.47	0.46	0.37
	(0.11)	(0.10)	(0.17)	(0.17)	(0.16)	(0.16)
K	0.92	0.85	0.76	0.79	0.85	0.92
	(0.10)	(0.13)	(0.11)	(0.19)	(0.12)	(0.11)

Fig.1). No clear estuarine pattern was observed for *S. senegalensis* antioxidant enzymes activity.

In *P. microps* the activity of SOD, SeGPx and CAT differed between sites ($F = 25.68$ and $F = 12.07$, $P < 0.001$; $F = 3.80$, $P < 0.05$), yet only SOD levels varied among estuaries ($F = 29.50$, $P < 0.001$) (Fig. 1). As for the other species, antioxidant enzymes response patterns in *P. microps* were not uniform between sites, since SOD and TGPx activity was significantly lower in Ra3, while CAT activity was significantly lower in Ra1, and SeGPx in Ra2 and Tj1 (Fig. 1).

EROD activity was generally higher in the Tejo estuary for all species, particularly for *S. senegalensis* ($F = 7.01$, $P < 0.05$) and *P. microps* ($F = 13.49$, $P < 0.01$) (Fig. 2). Site variability was also observed, with higher activity observed in Tj1 for *D. labrax* and *S. senegalensis* ($F = 3.24$ and $F = 3.10$, $P < 0.05$).

GST activity was also higher in *D. labrax* and *S. senegalensis* from Tj1 ($F = 4.70$, $P < 0.05$; and $F = 14.74$, $P < 0.001$), along with Ra2 and Ra3 for the latter species. Lower GST levels were observed in Ra1 for *S. senegalensis* and *P. microps* ($F = 10.12$, $P < 0.001$) (Fig. 2).

D. labrax and *S. senegalensis* from Ra1 presented the highest MT levels compared to other sites ($F = 10.85$ and $F = 18.51$, $P < 0.001$), followed by MT concentrations from the Tejo estuary (Tj2 and Tj3) (Fig. 2). MT response did not vary in *P. microps* populations from the two estuarine systems (Fig. 2).

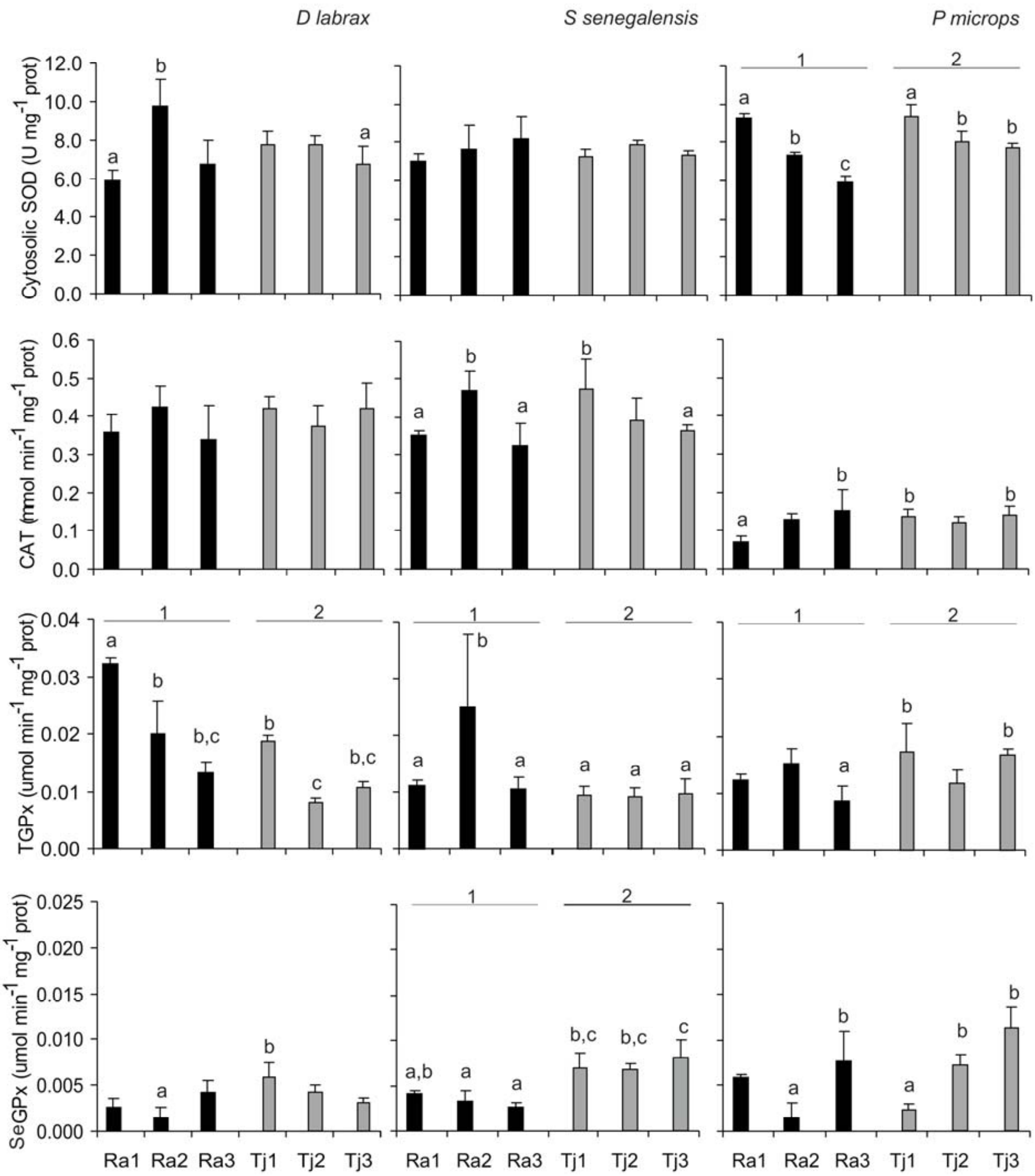

Figure 1. Antioxidant enzymes activity, SOD (U mg^{-1} prot), CAT (mmol min^{-1} mg^{-1} prot), TGPx (µmol min^{-1} mg^{-1} prot) and SeGPx (µmol min^{-1} mg^{-1} prot), in *Dicentrarchus labrax, Solea senegalensis* and *Pomatoschistus microps* from estuarine sites along the Ria de Aveiro (Ra1, Ra2, Ra3; represented by black bars) and Tejo (Tj1, Tj2, Tj3; represented by grey bars). Results of nested analysis of variance are also represented: between estuaries (different numbers indicate significant differences) and between sites (different letters indicate significant differences).

Considering biomarkers of effect and general condition indices, all species presented a significant spatial variability concerning liver lipid peroxidation, with higher LPO in *S. senegalensis* and *P. microps* (F = 21.24 and F = 14.79, P < 0.001) from the Tejo estuary (Fig. 3). However, no consistent spatial pattern was observed across species, with higher LPO values occurring at Ra2 for *D. labrax* (F = 7.38, P < 0.01), at Tj2 for *S. senegalensis* (F = 16.51, P < 0.001) and Tj1 for *P. microps* (F = 14.12, P < 0.001).

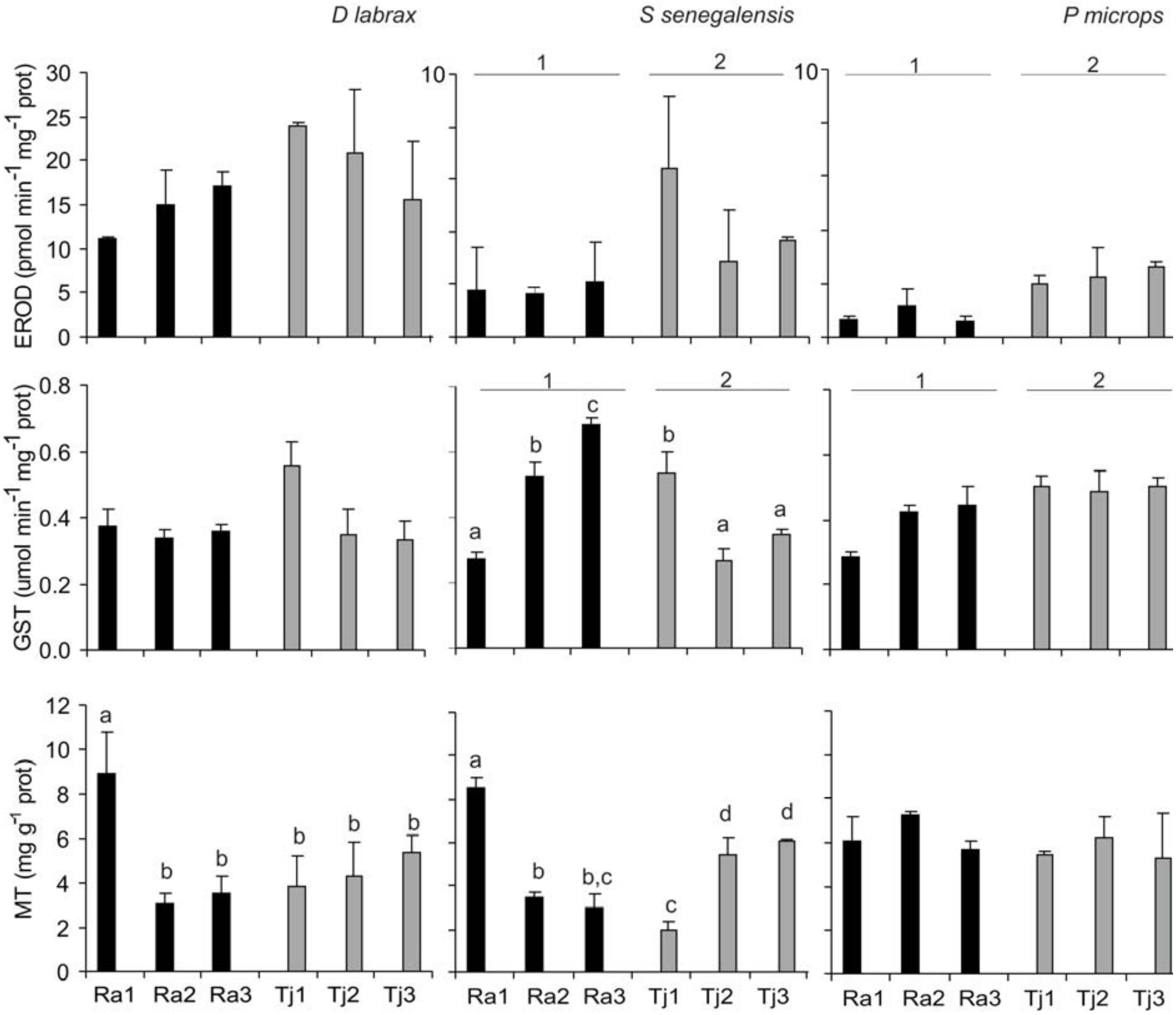

Figure 2. Biotransformation enzymes [EROD (pmol min^{-1} mg^{-1} prot); and GST (µmol min^{-1} mg^{-1} prot), and metallothioneins activity [MT (mg g^{-1} prot)], in *Dicentrarchus labrax, Solea senegalensis* and *Pomatoschistus microps* from estuarine sites along the Ria de Aveiro (Ra1, Ra2, Ra3; represented by black bars) and Tejo (Tj1, Tj2, Tj3; represented by grey bars). Results of nested analysis of variance are also represented: between estuaries (different numbers indicate significant differences) and between sites (different letters indicate significant differences).

R:D, muscle protein and lipid content in *D. labrax* varied between sites (Fig. 3). R:D and protein content were lower in Ra1 (F = 31.13 and F = 6.58, P < 0.001), and R:D and lipid content were higher in Ra3 (F = 4.67, P < 0.01). *D. labrax* from Ria de Aveiro presented higher lipid content compared to the Tejo estuary (F = 5.60, P < 0.01) (Fig. 3).

Similarly, *S. senegalensis* also showed significant differences between sites for R:D, protein and lipid content (F > 5.39, P < 0.01), although higher values of each biomarker were observed for different sites. R:D was higher in Tj2, protein content was higher in Ra2 and lipid content was higher in Ra1 (Fig. 3).

P. microps from Ra2 and Ra3 had significant higher R:D values compared to other sites (F = 12.74, P < 0.001), while protein content was higher for *P. microps* from Ra2 and Tj2 (F = 15.63, P < 0.001). Lipid content in *P. microps* did not differ among the different sites (Fig. 3).

Few significant correlations between biomarkers were observed considering species spatial

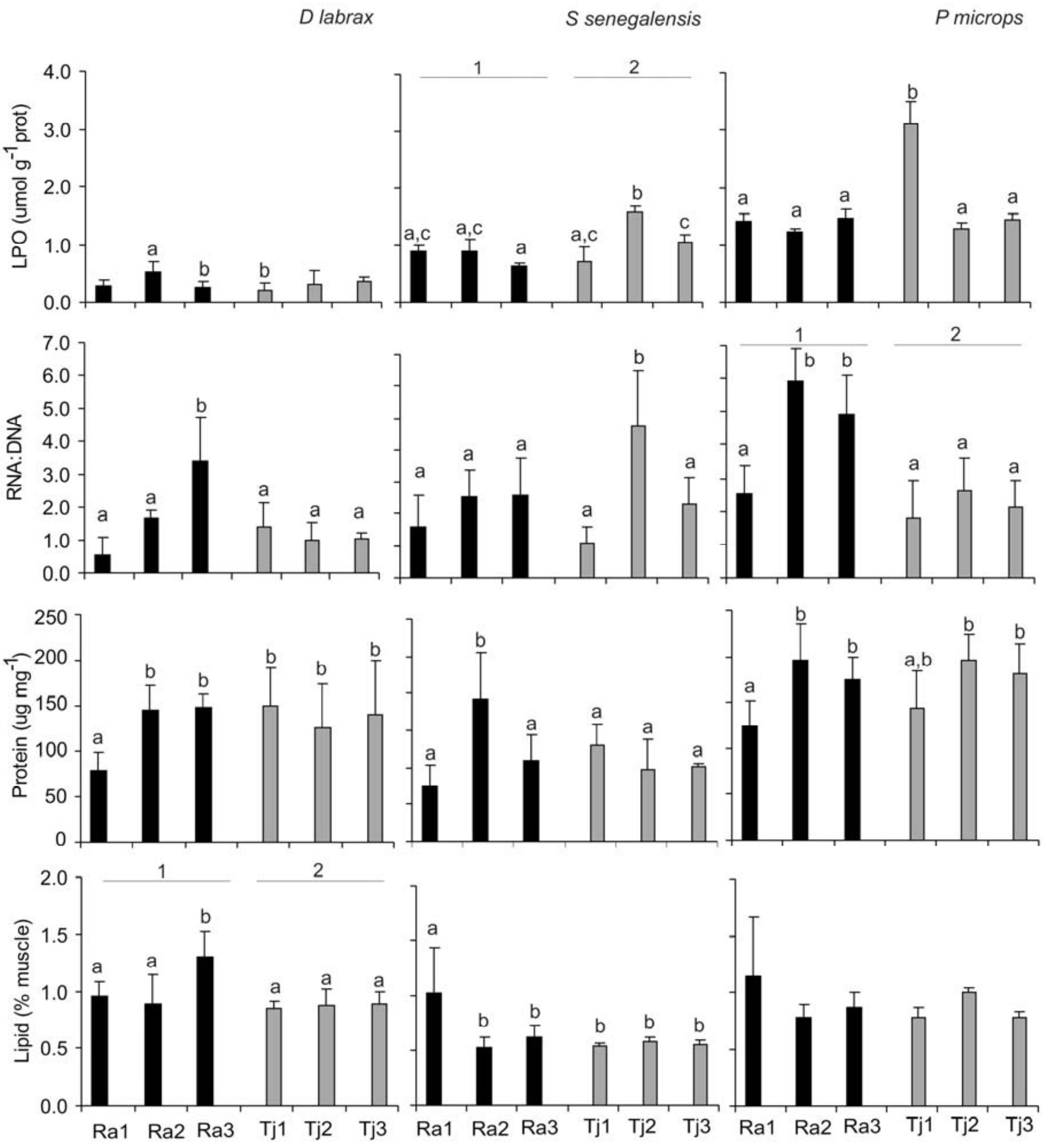

Figure 3. Biomarkers of effect responses, LPO (μmol g^{-1} prot), R:D, protein content (μg mg^{-1} muscle) and lipid content (% muscle), in *Dicentrarchus labrax, Solea senegalensis* and *Pomatoschistus microps* from estuarine sites along the Ria de Aveiro (Ra1, Ra2, Ra3; represented by black bars) and Tejo (Tj1, Tj2, Tj3; represented by grey bars). Results of nested analysis of variance are also represented: between estuaries (different numbers indicate significant differences) and between sites (different letters indicate significant differences).

response patterns. R:D had a positive correlation with protein and lipid content in *D. labrax* (r = 0.89 and r = 0.74, $P < 0.05$). For *P. microps*, R:D also had a positive correlation with protein content (r = 0.74, $P < 0.05$). In *S. senegalensis*, no significant correlations were observed.

Correlations between biomarkers' response and sediment chemical characterization (i.e. metal and PAH concentrations) also varied between species. *D. labrax* SOD and CAT activities were positively correlated with Cd, Cu and Zn sediment load (r = 0.70, r = 0.76 and r = 0.79, $P <$ 0.05). In addition, R:D was negatively correlated with Cd and TPAH concentrations (r = -0.85

and $r = -0.86$, $P < 0.05$).

S. senegalensis SOD was positively correlated with TPAH concentration ($r = 0.81$, $P < 0.05$), while R:D evidenced a negative correlation with TPAH ($r = -0.78$, $P < 0.05$). For *P. microps* SOD was negatively correlated with TPAH ($r = -0.83$, $P < 0.05$).

The CA ordination diagram based on the biomarkers' responses for the three species, with metals and TPAH concentrations as covariables, explained 77.3 % of the variation in the first two axes (Fig. 4). Samples were clustered in three groups according to species, with no clear spatial pattern, except for *P. microps* where estuaries were plotted separately. *S. senegalensis* and *P. microps*, were set closer together while *D. labrax* was set further apart.

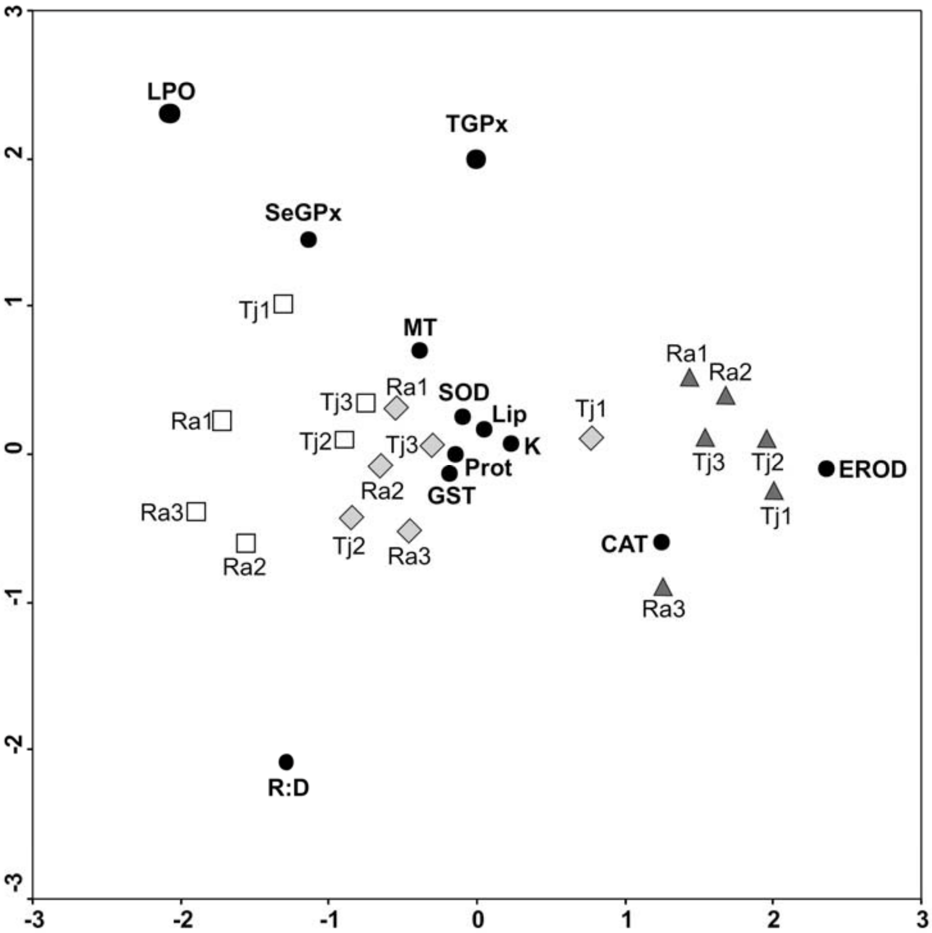

Figure 4. Correspondence analysis diagram based on *Dicentrarchus labrax* (▲), *Solea senegalensis* (◇) and *Pomatoschistus microps* (□) biomarker responses (●; SOD; CAT; TGPx; SeGPx; EROD; GST; MT; LPO; R:D; Prot and Lip). Estuarine sites sampled in Ria de Aveiro (Ra1, Ra2, Ra3) and Tejo (Tj1, Tj2, Tj3) are also represented.

4. Discussion

4.1. Environmental chemical characterization

The chemical characterization of sampled sites in the Ria de Aveiro and Tejo systems showed the presence of substantial and diverse contaminants in the sediments, namely metals and PAH and their origin was traced primarily to human activities. Previous studies in the these areas described the incidence of these and other pollutants (e.g. pesticides, mercury), as well as their main anthropogenic sources, namely industrial and agricultural discharges, domestic/urban effluents from two major cities and their peripheries, shipping and dry-dock activities (Canário et al. 2003; França et al. 2005; Sousa et al. 2007; Vasconcelos et al. 2007; Vale et al. 2008; Oliveira et al. 2009).

The metal concentrations found in this study are within the range of previous values reported for the same estuaries (França et al. 2005; Vale et al. 2008; Oliveira et al. 2009), yet for PAH concentrations there are no prior data available for comparison in these areas. Additionally, PAH composition and diagnostic molecular indices identified mixed sources of PAH input into sediments, of both pyrogenic (combustion derived PAH, such as emissions from motor vehicles or wood and coal combustion) and petrogenic (petroleum-derived PAH, e.g. oil spills, motors' leakage) origins (Baumard et al. 1998; Yunker et al. 2002). It is also noteworthy that despite lower concentrations of PAH in Tejo, higher molecular weight PAH (5 and 6 rings) represented a significant percentage of TPAH (25 % to 35 %), and these PAH have been described as more toxic to fish than 2 to 3 ringed PAH (Gowland et al. 2002; Seruto et al. 2005). Therefore, shipping and port activities, urban emissions and, in the case of Tj3, some products of biomass combustion were the main sources of PAH in both estuaries.

Overall, Ra3 had lower metals and PAH concentration in the sediment and was the least impacted site. Despite known agricultural runoff, this most likely resulted from the absence of nearby industries and smaller population settlements and from the coarser sediment grain size that reduces contaminants retention in the sediment. This is in agreement with previous work that identified the Mira channel (Ra3) as the least contaminated part of the Ria de Aveiro estuary, based on sediment toxicity bioassays with amphipods (Castro et al. 2006). Higher industrial and urban stressors occur near sites Tj1, Tj3, Ra1 and Ra2, while agriculture is the main stressor in Tj2. Both estuaries support significant shipping activity, fishing ports and traditional fishing activity within estuarine areas.

4.2 Biomarkers responses

The complex mixture of xenobiotic in estuaries has resulted in specific adaptation or defence responses from several fish species in recent years, including the ones used in the present study (e.g. Monteiro et al. 2007; Maria et al. 2009). Morphometric condition factor K did not vary between sites or estuaries for all species most likely due to the narrow size range of analysed individuals, intentionally selected in order to avoid size effects on biochemical responses. In addition, morphometric changes may require longer exposure periods than the ones considered in this study, since both sea bass and Senegalese sole colonized the estuaries in the 5 to 6 months prior to sampling. Given the short time frame of most toxicity assays, the absence of responsiveness of K factor to field contamination was somewhat expected (Fonseca et al. 2009), however lower K values have been reported in fish from metal polluted habitats, on a seasonal scale (e.g. Levesque et al. 2002).

The three species evidenced complex biomarkers responses to environmental contamination, exhibiting differences at the activity level and spatial patterns of the adaptation responses. Nevertheless, significant similarities across species were also found. The three species have been individually used in previous studies considering biomarkers' response to contamination, in both laboratory (e.g. Gravato and Santos 2003; Fonseca et al. 2009; Vieira et al. 2009) and field conditions (e.g. Fernandes et al. 2007; Monteiro et al. 2007; Costa et al. 2009) yet not simultaneously in the same study.

Enzymatic antioxidant response in *D. labrax* was characterized by a higher variability in SOD and TGPx among sites, with higher activities in two different Ria de Aveiro sites (Ra2 and Ra1, respectively), by opposition to CAT and SeGPx activities that were more constant across sites. Despite the lower variability, CAT and SOD activity had a positive correlation with Cd, Cu and Zn concentration in the sediment, suggesting that these metals increase the ROS levels in this fish species and the antioxidant system responds to an increase of environmental stress. Maria et al. (2009) described lower antioxidant enzymatic activities in sea bass from Ria de Aveiro in October 2005, nonetheless they also reported a significant inhibition of the antioxidant system associated with complex mixture of environmental contaminants in several sites in the Ria de Aveiro system. Another study considering wild sea bass biomarker responses from the south coast of Portugal described an antioxidant enzymatic activity range comparable to the present study (Fernandes et al. 2007).

S. senegalensis antioxidant enzymes activities reported in the present study were within range or slightly higher than experimental control values from toxicity studies looking into this species' responses to PAH and hypochlorite exposure (Solé et al. 2008; López-Galindo et al. 2010). As for *D. labrax*, the maximum antioxidant enzymes activities were not site concordant, occurring at Tj1, Ra2 and Tj3 for CAT, total and Se-dependent GPx, which suggests an adaptation of this species to local oxidative stress conditions with different xenobiotics and/or different concentrations exposure. Site-specific biomarkers response patterns in the Ria de Aveiro system, resulting from various xenobiotic combinations, has been previously described in the gills of different fish species such as *D. labrax* (Ahmad et al. 2008) and *Liza aurata* (Oliveira et al. 2009), and for *P. microps* (Monteiro et al. 2007).

Similarly to *D. labrax* and *S. senegalensis*, antioxidant enzymes in *P. microps* showed a high spatial variability, with significant SOD, CAT and SeGPx induction in both Tejo and Ria de Aveiro sites. SOD activity was significantly lower in the least impacted site (Ra3), while CAT activity was significantly lower in a highly contaminated site (Ra1), without further site variability, suggesting an effective inhibition of CAT activity in Ra1. SeGPx was significantly higher in less impacted sites, however, TGPx activity compensated with higher values in more polluted sites. Vieira et al. (2008) reported that a petrochemical mixture induced less oxidative stress when compared to two individual PAH exposure in *P. microps*, by not inducing CAT, SOD and glutathione reductase (GR) activities. There is no known field data on *P. microps* antioxidant enzymes activity, however, Vieira et al. (2008, 2009) performed PAH and metal toxicity bioassays with *P. microps* captured in the Minho estuary (a fairly low impacted estuary on the North coast of Portugal). Considering the response in unexposed fish as a rough indicator of the reference background antioxidant defence biomarkers, the present SOD and TGPx activities were within the range of previous works, while CAT activity was higher in this study (4 fold). Furthermore, SOD was negatively correlated with TPAH concentration in the sediment, suggesting some degree of SOD inhibition by organic contaminants in *P. microps*.

Assuming that EROD is a specific biomarker of PAH-like compounds exposure, it would be expected that individuals from Ria de Aveiro had higher EROD activity compared to Tejo, given that Ria de Aveiro sediments contained higher PAH concentration (except for Ra3). However, higher EROD induction was observed for all species in the Tejo estuary, potentially due to different TPAH composition between estuaries. As mentioned above, PAH from Ria de Aveiro were mainly composed of 2 and 3 rings PAH (around 85 %), while TPAH composition of the Tejo sediments had a higher percentage of 4 to 6 rings PAH (25% to 35%), which are generally considered to be more toxic to fish species compared to low molecular weight PAH - usually of petrogenic origin (Gowland et al. 2002; Seruto et al. 2005). Another possibility would be an EROD inhibition in Ria de Aveiro by trace metals, which has been previously described in several fish species (Roméo et al. 1994; Guilherme et al. 2008; Vieira et al. 2009), yet Tejo sediments had higher metal concentration and higher EROD activity was found for all species.

Spatial patterns in GST activity were complex, given that higher enzymatic activity was observed both in highly polluted sites (e.g. Tj1 for sea bass and sole) but also in the least polluted site (Ra3 for sole). Additionally, results suggest a strong GST inhibition by organic compounds in *S. senegalensis* and *P. microps* from Ra1, where the highest PAH concentrations were measured. Therefore, different factors may be determining GST variation pattern, namely species-specific responses, since *S. senegalensis* showed high spatial variability which was not observed in the other species, and variability in local concentration of contaminants, causing a GST inhibition in the more polluted sites, particularly Ra1, and an induction in the least polluted site (Ra3). Previous studies have also described this observed bell-shaped pattern in GST activity for different species, where the enzyme activity increases with increasing contaminant exposure until a certain concentration where enzymatic activity progressively decreases (Elia et al. 2003; Vieira et al. 2009). Moreover, both GST inhibition and induction after exposure to different PAH and metals has been reported (Sanchez et al. 2005; Vieira et al. 2008).

A significant induction of metallothionein in *D. labrax* and *S. senegalensis* was observed compared to prior MT concentrations measured in field and laboratory toxicity assays (Fernandes et al. 2007; Fonseca et al. 2009; Jiménez-Tenório et al. 2009; Maria et al. 2009). The lowest MT levels were observed in Ra3, where lower metal contamination was also measured, but also in more polluted sites (Ra2 and Tj1), where MT response appears to be inhibited. The significant induction of MT in sea bass and sole individuals in Ra1 could be related to an additional oxidative stress response to other contaminants not considered in this study, since metal concentrations were not remarkably high in Ra1 compared to the other sites. *P. microps* MT levels were significantly higher compared to other species and remained constant between sites, and in the absence of previous studies for comparison, an ubiquitous MT response to general stress conditions in *P. microps* is assumed.

Oxidative damage in fish liver, measured as lipid peroxidation (LPO) showed spatial variability in all species, and also between estuaries for *S. senegalensis* and *P. microps*, the latter exhibiting higher LPO values than sea bass, and in the Tejo estuary. Compared to previous studies, LPO levels in *D. labrax* were in the upper range of observed concentrations for this species in Ria de Aveiro (Maria et al. 2009). Comparable LPO values were observed for *S. senegalensis* and *P. microps*, although maximum concentrations, particularly in Tj1, far exceed previous laboratory measurements (Fonseca et al. 2009; Vieira et al. 2009), clearly indicating the existence of oxidative damage to cellular membranes in these species.

R:D values were within the previously reported range for *D. labrax* and *S. senegalensis* in several Portuguese estuaries (Vasconcelos et al. 2009). However, low R:D values and protein content were observed in all species from Ra1, a site which was already identified as significantly polluted. Contrarily, fish from Ra3, the least impacted site, had significantly higher R:D ratios, protein content, and even the highest lipid content in *D. labrax*. Higher site variability in R:D and protein content, with peak values in Tj2 and Ra2, were found for *S. senegalensis* and *P. microps*. R:D ratios were positively correlated with protein content in *D. labrax* and *P. microps*. Muscle lipid content was fairly constant, although higher values were observed for Ra1 in both *S. senegalensis* and *P. microps*, probably due to local environmental conditions. Taking into account that these indices are not specific to environmental contamination as they integrate the whole environment, it cannot be excluded that spatial variability patterns are influenced by local abiotic conditions (e.g. temperature, salinity), food quality and availability and estuarine community composition (i.e. occurrence and abundance of competitor or predator species). Despite that at time of collection, abiotic conditions between sites were not significantly different (data not shown), the possible influence of precedent conditions on spatial patterns must be considered. Nevertheless, R:D ratios were negatively correlated with sediment Cd levels *in D. labrax*, and with TPAH concentrations, in both *D. labrax* and *S. senegalensis*, which is in agreement with Fonseca et al. (2009) that described a negative dose and time response of R:D in *S. senegalensis* exposed to Cu, with similar R:D values observed in Cu exposed fish when compared to Ra1 and Tj1.

Both estuaries had significant chemical contamination, hence estuarine differences in species' biomarker responses were meagre, with most sites signalled as contaminated depending on the species and biomarkers considered.

Overall, interspecies' differences in biomarker responses seem more important than spatial response patterns in the ordination analysis. Nevertheless, *P. microps* and *S. senegalensis* were set closer together, likely due to their predominantly benthic behavior, particularly in comparison to *D. labrax*, that despite feeding on epibenthic fauna, is a much more active species with a largest distribution in the water column. The fact that *D. labrax* has a higher mobility and potentially a wider range of habitat use, may also account for the lower spatial variability in biomarker responses.

A significant effort was done to achieve a holistic view of the adaptative responses of natural fish populations to environmental contamination. However, the complexity of environmental chemical mixtures and their interactive effects on different fish species, including other pollutants not measured in the present work, may be responsible for some of the observed patterns. Furthermore, the chemical burden in fish tissues was not analyzed, thus the effects of environmental pollutants cannot be directly inferred.

In conclusion, a multi-biomarker approach considering several species can provide a better understanding of the effects of contaminants exposure and the real risk it poses for different fish species. A significant variability in spatial adaptation patterns was observed across species for most biomarkers considered. Nevertheless, some responses were consistent among sites, especially in Ra3 that can be globally characterized as the least impacted site based on both chemical (metals and TPAH) load and on species biomarker responses. On the other hand, in highly polluted sites (Tj1 and Ra1), antioxidant enzymes responses can either be induced or inhibited depending on other factors, and lower R:D values were observed. Species feeding behavior, life cycle and habitat use influenced spatial response patterns, with *S. senegalensis* and *P. microps* evidencing greater spatial variability, while *D. labrax* responses were more homogeneous between estuarine sites. Overall, selection of appropriate species for monitoring habitat quality through assessment of contaminant-induced effects is fundamental, and despite the added logistical difficulties, the value of a multi-specific approach is unquestionable for an ecosystem-based approach.

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CHAPTER 10

General discussion

Future perspectives

General discussion
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General discussion

The main objective of the present study was to evaluate the potential of fish biochemical and physiological responses as indicators of habitat quality in estuaries, considering both natural and anthropogenic stressors.

Estuaries are highly productive ecosystems characterized by high natural variability, particularly on a short temporal frame, in terms of water temperature, salinity, dissolved oxygen and even sediment mobility, and are frequently impacted by various pressures of anthropogenic origin (Haedrich 1983; Kennish 2002; McLusky and Elliott 2007; Vasconcelos et al. 2007). Given the complexity of these ecosystems, assessing habitat quality requires a comprehensive effort to integrate the different processes, variables and pressures that influence fish health.

Measures of fish growth and condition provide information on population health and are intrinsically related to environmental settings (Buckley et al. 1999; Fonseca et al. 2006; Amara et al. 2007). The estimation of growth and condition has evolved considerably over the past three decades, from length or size-based estimates of growth rates and morphometric condition indices to biochemical proxies for growth, quantification of lipid reserves or otolith daily-rings analysis (Buckley et al. 1999; Lloret and Planes 2003; Vinagre et al. 2008).

An extensive review of how fish early growth and condition patterns are related to life-history strategies, habitat and geographical distributions was presented in Chapter 2. The outcome supported the initial hypothesis that fish inhabiting more variable environments (e.g. coral reefs, estuaries) would select for higher growth rates and condition in larvae and juveniles in order to counter high mortality rates during early stages (Houde 1989; Wilson and Meekan 2002), while species facing more stable environments (polar waters, freshwater systems) would select for slower development and higher energy reserves. The high degree of segregation of species according to their life-history strategies, habitat and latitudinal distribution based on measures of growth, age at maturation and condition factor K supported the established assumptions, but also emphasized the lack of available data regarding fish condition. This chapter presented an innovative approach to life-history studies, by applying specific quantitative measures of fish metabolic investment and fitness during early-life stages to a simple life-history framework developed by Winemiller and Rose (1992). It is also the first comparison on a global scale of condition and early growth patterns, which are generally assessed on a local scale (Buckley et al. 2008). Further development of the life-history strategy approach can be a useful tool to predict population responses to acute environmental or human constrains, since species strategies give an idea of species ability to adapt.

The first measures of fish condition tested in the present thesis were lipid-based indices in response to nutritional stress (Chapter 3). The experiment results outlined the relationship between morphometric indices (condition factor K and hepatosomatic index HSI) and lipid content, particularly in the fish liver. Fatty acid (FA) clusters also presented some interesting trends related to feeding level (Jezierska et al. 1982; Zamal and Ollevier 1995). In contrast, individual FA dynamics depended significantly on diet, as also shown for instance by Santos et al. (1993), and moreover it was highly complex disabling their use as early-warning signs of food limitation. From a logistical perspective, morphometric indices have the advantage of being simple to determine with low costs, thus they should be applied to assess fish condition whenever possible. Lipid content determined gravimetrically (Bligh and Dyer 1959) requires a few more time and technical resources, yet it has the advantage of integrating food consumption and conversion into energy storage, while FA quantification is by far the most expensive and labor-intensive methodology, which did not offer additional value compared to the other indices for this particular goal. Lipids as the main form of energy storage in fish can affect long-term growth potential and survival probability (Lochmann et al. 1995; Lloret and Planes 2003). Hence, lipid content was used when possible throughout this thesis (Chapters 3, 7 and 9), although with different methodologies due to logistic constrains, respectively: gravimetric determination (Bligh and Dyer 1959); spectrophotometric determination (Knight et al. 1972) and FAME analysis (Bandarra et al. 2009) followed by weight normalization according to lipid percent composition in phospholipids and triacylglicerols. Overall, all methods gave reliable estimates of tissue lipid content. However, the gravimetric and particularly the spectrophotometric methodologies were simpler procedures and had lower technical requirements, which is a significant advantage for its routine use.

Since different species integrate responses to the surrounding environment in a diversified manner, depending on their life-history strategy and biological constrains (Fonseca and Cabral 2007), for example through trade-offs between energy reserves, growth and individual behavior (Arendt 1997; Sogard and Spencer 2004), habitat quality for different fish species can only be determined on a comparative basis (Vasconcelos et al. 2009). Hence, growth performance of two fish species in five estuaries along the Portuguese coast was estimated via experimental calibration of nucleic acid-based indices (Chapter 4). Nucleic acid indices can serve as proxies for growth, based on the assumption that protein synthesis varies in proportion to RNA cellular concentration, which in turn is influenced by food availability and protein requirement (Bulow 1970; Buckley and Bulow 1987). Following recent studies on this topic (Gwak and Tanaka 2001; Mercaldo-Allen et al. 2006, 2008), the laboratorial experiment enabled the development of a growth model under varying temperature, salinity and feeding level, which has the advantage of calibrating field values and allows establishing a maximum growth reference under optimal conditions (Buckley et al. 2008). Analysis of growth performance, measured as the percentage of maximum or reference growth achieved by individual fish, allowed for a more reliable identification of spatial and temporal patterns associated with environmental variability, as evidenced for example with the decrease in growth performance observed during a drought event (Smith et al. 2008).

A novel approach to growth estimation in ecological modeling considering the effects of the environment on fish metabolism and bioenergetics was tested in Chapter 5. This ecophysiological framework (Ecophys.Fish, Neill et al. 2004) accurately estimated juvenile sole growth rates related to habitat variability over a period of four years in the Tejo estuary. The Ecophys.Fish model is extremely relevant in the estuarine context as it integrates the effects of time-varying environment on fish metabolism and bioenergetics, and growth rate is based on metabolic scope for growth, i.e. energy available for mass gain after all routine activities have been performed (e.g. food processing, excretion). Acclimation processes are also an important component of the modeled interactions that add realistic value to growth estimations since it accommodates a continuously modified physiological state by the individuals' environmental history. This aspect lacks in previous models such as the bioenergetic approach by Kitchell et al. (1974) and the dynamic energy budget theory (DEB) developed by Kooijman (2000). In spite of the good estimates of fish growth, indicating that this is a promising tool for monitoring and assessment of habitat quality, the model requires extensive data input on environmental conditions and knowledge on species ecology and metabolic rates. Moreover, integrating biotic interactions (e.g. predation, parasitism) and the effects of contaminants on fish metabolic performance in order to accommodate the whole environment have been suggested as valuable future developments to these models.

As previously addressed, fish inhabiting estuaries face daily variability in environmental conditions, such as water temperature and salinity, due to tidal influence and freshwater input. Whilst the short-term effects of environmental variability on fish metabolism were integrated in chapter 5, as described above, in Chapter 6 a field study was used to investigate the relation between environmental variability and condition indices. At the short-time scale analysed, only temperature was significantly correlated with biochemical indices (RNA:DNA and protein content), while salinity showed no influence on indices variation, potentially due to the absence of any drastic changes in freshwater input. On the other hand, the applied sampling design aimed at evaluating variation in condition indices at a fine temporal scale of consecutive days to weeks, which has not been described earlier. Moreover, this was conducted in different fish species from both estuarine and shallow coastal areas. Other studies have described different temporal patterns in condition indices of larvae and juveniles, namely at seasonal (Haines 1980; Ramírez et al. 2004), monthly (Fonseca et al. 2006, Vasconcelos et al. 2009) and even diel scale (Chícharo et al. 1998, Gwak 2000). In the present study, significant variation of condition indices was more frequent at the week scale than daily variability in all species. Higher abiotic variability in estuarine sites compared to the shallow coastal site sampled was not reflected in fish condition patterns, emphasizing the resilient capacity of estuarine-inhabiting species to the dynamic environment (Elliott and Quintino 2007). Nevertheless, individual condition tended to be more homogeneous towards the end of the study period, particularly for *T. trachurus,* the species collected in the adjacent coastal area, which could be related to more stable environmental conditions, but this requires further research. In conclusion, biochemical indices were sensitive measures of short-term environmental variability, which should be accounted for when assessing habitat quality.

Following the evaluation of fish growth rates and condition indices as measures of habitat quality, biochemical responses to contaminants were considered in the set of fish responses used to assess habitat quality. Several biomarkers of exposure to and of effects of contaminants, the latter including condition indices, were tested in the final three chapters.

In Chapter 7, a laboratory experiment was designed to test the combined responses of a set of measures of exposure and effects of copper exposure: namely metallothioneins and lipid peroxidation level, previously described as biomarkers of exposure and effects of trace metal toxicity (Stegeman et al. 1992; Roméo et al. 1997); and measures of fish growth rate and condition, which were tested as biomarkers of effects. Copper exposure triggered metallothioneins and elevated lipid peroxidation (Riba et al. 2004; Sanchez et al. 2005) in addition to reduced growth (Marr et al. 1996) and condition (RNA:DNA and lipid content). Moreover, results suggested that lipid reserves were mobilized to maintain growth rates and protein synthesis in exposed fish, although with lower growth rates than control fish. Few studies have tested the influence of contaminants exposure on fish condition, with contrasting results (De Boeck et al. 1997; Wu et al. 2003; Humphrey et al. 2007), which in this study proved to be useful in order to integrate the effects of chemical toxicity in fish health.

The variability of several biomarkers over a short-temporal scale was measured in three fish species whilst simultaneously addressing their relation to a dynamic environment (temperature and salinity) with a significant chemical load (trace metals and polycyclic aromatic hydrocarbons) (Chapter 8). The lack of correlation between environmental variables and the activity of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT] and biotransformation enzymes [ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST)] indicated that significant variation in biomarker responses were associated with chemical stress at this temporal scale. Previous studies have suggested the link between biomarker responses and environmental variables (temperature and salinity) considering different time scales (Sleiderink et al. 1995; Roche and Bogé 1996; Kopecka and Pempkowiak 2008). In addition, EROD and GST activities were positively correlated, which suggested a specific response to chemicals exposure that induced biotransformation activity (e.g. PAH). Higher specificity of biotransformation enzymes compared to antioxidant mechanisms has been reported and can be explained by the wider range of factors that may induce oxidative stress compared to those that can induce biotransformation processes (van der Oost et al. 2003). Overall, results emphasized the utility of these adaptation responses in signalling chemical stress in the estuarine environment over a short-time scale. Moreover, the importance of considering a multi-biomarker approach to better portrait existing toxicity risks was also evidenced.

In Chapter 9, an integrative approach of the previously used biomarker responses, including condition indices, was applied to determine spatial differences in estuarine habitat quality for three fish species. Significant chemical load in trace metals and polycyclic aromatic hydrocarbons (PAH) resulting from various anthropogenic activities (e.g. industry, agriculture and shipping activities) had been previously identified for several areas within the Tejo and Ria de Aveiro (Canário et al. 2003; Vasconcelos et al. 2007; Oliveira et al. 2009). Spatial patterns in biomarkers response were surpassed by interspecific differences, due to differences in species behavior and habitat use. Solé et al. (2009) also described autoecological differences in biomarker responses in several marine fish species. Nevertheless, significant antioxidant enzymatic induction, associated with lower R:D values, and higher LPO values were associated with estuarine sites with higher pollution load. Also, EROD activity showed high specificity to PAH exposure, with higher activities in sites containing higher proportion of more toxic PAH (4 and 6-rings PAH), which is in accordance to Gowland et al. (2002) and Seruto et al. (2005). In summary, the multi-biomarker and multi-specific approaches provided a better understanding of the diverse responses and effects of exposure to contaminants in a highly dynamic environment.

Overall, considering the fish condition and growth estimates determined throughout the thesis for different estuarine sites and years, habitat quality status was fairly good, yet adverse effects on fish condition were related to drought events and biochemical responses signaled xenobiotic exposure. Another important aspect of the study was the use of natural fish populations (except for Chapter 7), which considers the different physiological processes from different species biology and ecology, thus adding ecological relevance to a study. Species studied were selected based on their widespread distribution along Portuguese estuaries (as well as in numerous European estuaries), their high abundance and ecological and economical interest, in addition to the significant previous research on biochemical responses in these species (e.g. Gravato and Santos 2003, Fonseca et al. 2006; Fernandes et al. 2007; Monteiro et al. 2007; Costa et al. 2009; Vasconcelos et al. 2009; Vieira et al. 2009).

In the context of biomarker responses to contaminant induced stress, the applied markers encompassed most criteria for what constitutes a reliable biomarker as suggested by van der Oost et al. (2003) and Stegeman et al. (1992), namely sensitivity to pollutant exposure/effects; knowledge on baseline level considering natural variability; higher ecological effects; and in some cases low costs and easy to perform methodologies.

The applied multi-biomarker approach proved to be essential to produce a holistic characterization of environmental quality. Both lack of specificity or high specificity of biochemical responses to stressors can contribute to a narrow outcome if biomarkers are used individually. Therefore a successful habitat quality assessment requires the measure of multiple biomarker responses, correctly integrated in the environmental context.

In the present water resources management context, it could be beneficial to integrate biomarker responses into recently implemented monitoring plans for Water Framework Directive (and future Marine Strategy Framework Directive). Whilst multi-metric assessment tools resolve ecological quality assessment at the community level alterations, these sub-individual responses can be key in detecting early anthropogenic disturbances. These responses can also be of added value in monitoring plans for specific impacts derived from new or existing infrastructures with potential effects on fish (e.g. industries and port developments). In spite of the sensivity of the biomarkers of exposure to and effects of contaminants, habitat quality assessment should always consider some measure of natural variability due to its possible relation with some markers at different scales, and to its strong influence on growth and condition patterns of fish in estuarine environments.

Future perspectives

In the present thesis variability patterns in fish growth, condition indices and biomarker responses in the estuarine environment were described at different temporal and spatial scales, namely from days and weeks to inter-annual and from intra to inter-estuarine variability, respectively.

Nevertheless, other important scales, which have been found to influence sub-individual fish responses in estuarine systems, still need to be further investigated. Marked seasonality is widely recognized in estuarine systems, although to a lesser degree in tropical areas. In addition, significant seasonal variation in condition indices and biomarker responses have been described in different fish species (Ramírez et al. 2004; Monteiro et al. 2007; Kopecka and Pempkowiak 2008), and research directed at identifying seasonal sources of variation remains essential for the validation of biomarkers, as suggested by Guilhermino (2007).

Habitat diversity and patchiness is a conspicuous feature of estuaries (Pihl et al. 2002), with species exhibiting complex habitat use patterns (França et al. 2009). Differential environmental settings (natural and anthropogenic) in these habitats may be reflected in different fish species health and biomarkers.

A decrease in genetic variability as well as putative selection of genotypes considered as more tolerant to environmental pollutants and stresses in fish populations from contaminated estuaries (Marchand et al. 2003), motivates further research into this topic with links to disturbance induced-selection of genetic structure.

Novel approaches, specifically “omics” technology can assist in the determination of the modes of action of chemicals on biological systems, particularly when used to assess the dose- and time-dependency of responses (Hamadeh et al., 2002; Moggs, 2005; Williams et al. 2008).

Life-history studies provide a global framework for comparison of fish species responses and trade-offs facing ecological and environmental constrains at variable spatial and temporal scales (Winemiller 2005). In this context, further development can prove useful in predicting population responses to environmental changes (natural or human induced).

The ecophysiological framework (Neill et al. 2004) tested in chapter 5, offered promising results in integrating the effects of time-varying environment on fish metabolism and growth, yet further developments should improve model performance and ecological significance with the inclusion of modules for biotic interactions (e.g. predation, competition, parasitism) and of pollutants effects.

A strong concern with the ecological relevance of biomarkers used in environmental quality assessment (Stegeman et al. 1992; van der Oost et al. 2003; Guilhermino 2007) motivated the focus given in the present thesis to their relationship with key biological features, in this case growth. In addition, relationship with other biologically and ecologically relevant features such as reproductive and endocrine effects and swimming capacity should be further investigated.

Notwithstanding the increasingly recognized value of fish biomarker responses, currently legislative and management efforts are focusing on ecosystem-based approaches, considering the assessment of habitat quality for the various biological compartments or biological quality elements (WFD, 2000/60/EC; MSD, 2008/56/EC). Hence measuring biota biomarker responses across several compartments of the estuarine fauna should be investigated in order to test for signal propagation.

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