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RESEARCH ARTICLE

Short-term toxic effects of naphthalene and pyrene on the common prawn (*Palaemon serratus*) assessed by a multi-parameter laboratorial approach: mechanisms of toxicity and impairment of individual fitness

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Abstract

The short-term (96 h) toxic effects of two polycyclic aromatic hydrocarbons (PAHs), naphthalene (NAP) and pyrene (PYR), on the common prawn (*Palaemon serratus*) were investigated in laboratory bioassays, including a fitness related assay based on the post-exposure swimming velocity. Other effect criteria were biomarkers of neurotoxicity, oxidative stress and bioenergetics, and mortality. In the range of concentrations tested (NAP: 0.13–8 mg/L; PYR: 0.006–0.4 mg/L), both PAHs impaired the swimming velocity, induced oxidative stress and damage, and changed the activity of lactate dehydrogenase and isocitrate dehydrogenase. NAP also caused mortality (96 h-LC50 = 3.5 mg/L). Thus, both PAHs were able to cause toxic effects on *P. serratus* after a short period of exposure through the water, including the reduction of individual fitness. PYR was five folds more effective in reducing the swimming velocity of *P. serratus* than NAP. These findings are of interest for the marine ecological risk assessment of oil spills.

Keywords: Acute toxicity, swimming behaviour, biomarkers, PAHs, ecological risk assessment, marine invertebrates

Introduction

In the last decades, the exploration, transformation and transport of oil by sea has been increasing and projects for the exploration of new areas in the next future exist. Thus, the risk of oil spills in the marine environment and the environmental consequences potentially resulting from these events, as recently shown by the incident occurred in the Gulf of Mexico in 2010, continues to be a matter of high concern despite the advances in preventive measures that have been made (Martínez-Gomez et al. 2010).

The ecological risk assessment of oil spills in the marine environment is challenging for several reasons. One of them is the limited knowledge on the toxic effects of oils on marine organisms that becomes obvious if one compares the high diversity of species playing important ecological roles in different types of marine ecosystems with the relatively small number of marine species that have been used as models to investigate the toxic effects of oils and their components. Another important reason is the scarcity of information on the relationship between the characteristics of exposure and the ecological relevance of the effects induced. Thus, more knowledge on the mechanisms of toxicity leading to individual and population fitness decrease is needed to improve the ecological risk assessment of oil spills in the marine environment.

Among oil components, polycyclic aromatic hydrocarbons (PAHs) deserve special attention since several of them are able to cause acute and chronic toxicity in marine organisms, including neurotoxicity, oxidative stress and damage, genetic alterations, endocrine

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disruption, carcinogenicity, teratogenicity, swimming performance reduction and mortality, among other deleterious effects (Bravo et al. 2011; Calbet et al. 2007; Kennedy and Farrell, 2006; Lewis et al. 2010; Mastral and Callen 2000; Pollino et al. 2009; Vieira et al. 2008). The mechanisms of toxicity of PAHs in several marine invertebrates commonly found in estuaries and coastal areas, including several keystone species of these ecosystems, are still unknown. In addition, lack of knowledge on how sub-individual toxic effects may decrease the individual fitness exists. Furthermore, the cost-effective technology available to assess the effects of chemicals on the individual fitness of marine invertebrates is limited. Therefore, the objective of the present study was to investigate the short-term (96 h) toxic effects of two PAHs, naphthalene and pyrene, on the common prawn *Palaemon serratus* in laboratory bioassays, including a fitness related post-exposure assay. Swimming velocity indicative of individual fitness, sub-individual neurotoxicity, oxidative stress and damage, and bioenergetics biomarkers, and mortality were used as effect criteria.

P. serratus was selected as test organism for this study because it is a keystone species in a high number of estuaries and coastal areas of Europe and North Africa which are potentially exposed to oil spills due to their proximity to main oil-tanker routes, it is a convenient test organism for use in laboratory bioassays (Frasco et al. 2008), has several characteristics suitable for being used as a sentinel species, and is included in the diet of human populations in some regions.

Pyrene and naphthalene were selected as test substances because they are important components of oils, produced waters, and other petrochemical mixtures; they are common contaminants in coastal areas and estuaries; and they are toxic to marine organisms after acute and chronic exposure (Calbet et al. 2007; Dissanayake et al. 2008; Gesto et al. 2006; Ahmad et al. 2003; Pollino et al. 2009; Santos et al. 2006; Sun et al. 2008; Teles et al. 2003; Tintos et al. 2007; Vijayavel et al. 2004). Naphathalene is a low molecular PAH evaporating easily (Diez et al. 2007). Nevertheless, naphthalene and its derivatives are frequently the most common PAHs present in the seawater immediately after oil spills (Albaigés and Bayona, 2003; González et al. 2006; Guitart et al. 2008). Pyrene is a more heavy PAH especially related with combustion (Pastor et al. 2001). Despite these differences, both naphthalene and pyrene have been found in water, sediments and several species after oil spills (Diez et al. 2007; Elordi-Zapatarietxe et al. 2010; Guitart et al. 2008; Lemkau et al. 2010; Lewis et al. 2010; Pastor et al. 2001; Tronczyński et al. 2004; Viñas et al. 2009), in produced waters (Utvik, 1999) and in corresponding matrixes of areas historically contaminated by PAHs (Lima et al. 2007; Vídal-Martínez et al. 2006).

The acute effects of naphthalene have been studied in marine crustaceans such the crab *Scylla serrata* (Vijayavel and Balasubramanian, 2006, 2008; Vijayavel et al. 2004) and the copepods *Calanus finmarchius, Paracartia grani* and *Oithona davisae* (Barata et al. 2005; Calbet et al. 2007; Hansen et al. 2008, Saiz et al. 2009), while the acute effects of pyrene were investigated, for example, in the amphipods *Gammarus aequicauda, G. locusta, Corophium multisetosum* (Sanz-Lázaro et al. 2008) and in the copepod *Oithona davisae* (Barata et al. 2005). To the best of our knowledge the mechanisms of toxicity of naphthalene and pyrene and their effects on individual fitness were not investigated before in *P. serratus*.

Material and methods

Sampling and acclimatization of animals to laboratory conditions

Animals were collected with a hand operated net at low tide in a rocky shore area of the NW Portuguese coast (41°41′40.85″N, 8°51′00.67″W). They were transported immediately to the laboratory in local water with continuous air supply. At the laboratory, they were transferred to aquaria with 250 L of filtered sea water (Filter Eheim 2217, 1000 L/h) (300 animals per aquarium), and maintained in a temperature (20 ± 1°C) and photoperiod (16 h L: 8 h D) controlled room for a minimum of 3 weeks with artificial air supply. Water was changed two times a week, *ad libidum* food (A. Coelho and Castro, Lda, sea bass food, 4.5 mm milled) was provided, water quality was monitored once per week (ammonium, nitrates and nitrites) using Palintest Ltd kits (Theta), and abiotic parameters in water were routinely measured (temperature, salinity, dissolved oxygen, pH).

Bioassays

Stock solutions of naphthalene (1600 mg/L) and pyrene (800 mg/L) were prepared in acetone. Tested concentrations of naphathalene (0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/L) and pyrene (0.006, 0.01, 0.03, 0.05, 0.1, 0.2, 0.4 mg/L) were prepared by dilution of the stock solution in filtered sea water (33%). In independent bioassays, prawns (mean ± SD: 0.6 ± 0.2 g weight, 3.6 ± 0.3 cm long for naphthalene; 0.9 ± 0.2 g weight, 3.5 ± 0.5 cm long for pyrene) were individually exposed to the toxicant in 650 mL of test media in glass beakers of 1000 mL. In each bioassay, a filtered sea water control and a filtered sea water control with acetone (0.5 mL/L), hereafter indicated as acetone-control were included in the experimental design. All the beakers were closed to prevent volatilization of the tested substances. The bioassays were carried out in semi-static conditions to maintain the concentrations of test substances above 80% of the initial ones, with medium renewal at intervals of 24 h for naphthalene and 12 h for pyrene since previous studies indicated a decay of these compounds in saltwater lower than 20% under experimental conditions comparable to those used in the present work (Calbet et al. 2007; Almeida et al. 2012). Nine animals were used per treatment. Mortality, water dissolved oxygen, conductivity, salinity and temperature were monitored every 24 h. After 96 h of exposure,

the swimming performance was individually assessed by determining the swimming velocity while swimming against a water flow of 8 cm/s, using an assay previously developed for fish (Gravato and Guilhermino, 2009) with minor adaptations. Briefly, each animal was placed in a particular point of a swimming race device (a tube open in the top) and induced to swim to avoid being dragged by the water flow. The distance that it was able to cover while swimming against the water current, and the time it spent to cover that distance were used to calculate the swimming velocity (swimming velocity = distance covered by the prawn/time spent). After the swimming velocity assessment, each animal was put back in its original test solution and left resting for 2 h. Then, animals were sacrificed by decapitation and several organs/tissues were isolated on ice and stored at –80°C for biomarkers analysis.

Biomarkers determination

Biomarkers were only determined in animals that survived until the end of the test and individual determinations for each animal were made. A Ystral GmbH d-7801 Dottingen homogenizer was used for all the homogenizations. A Bio Tek Power Wave 340 microplate reader was used for analysis of sample protein content and determination of the activity of the enzymes acetylcholinesterase (AChE), cholinesterase (ChE), lactate dehydrogenase (LDH), isocitrate dehydrogenase (IDH) and glutathione S-transferase (GST). A Jasco V-630 spectrophotometer was used for determination of lipid peroxidation (LPO) levels and the remanding enzymatic analysis. The two eyes of each animal were put in 0.5 mL of ice cold phosphate buffer (0.1 M, pH = 7.2), homogenized on ice for 1 min and centrifuged at 6000*g* for 3 min at 4°C. A similar procedure was done for a piece of dorsal muscle. The supernatants were recovered, and used to determine the ChE activity (ChE) by the Ellman's technique (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996) after standardization of the protein content to 0.5 mg/mL and 1.0 mg/mL for eye and muscle supernatants, respectively. Briefly, 0.25 mL of the reaction solution [30 mL of phosphate buffer, 1 mL of the reagent dithiobisnitrobenzoate 10 mM (acid dithiobisnitrobenzoate and sodium hydrogen carbonate in phosphate buffer) and 0.2 mL of acetylcholine iodide 0.075 M] were added to 0.05 mL of the supernatant in a 96-well microplate. The optical density was measured at 412 nm for 5 min at 25°C. Acetylthiocholine was used as substrate in all the assays. *P. serratus* eye contains only one ChE with properties of typical AChE (Frasco et al. 2006) and thus the activity measured in this tissue will be further indicated as AChE activity. *P. serratus* muscle may contain both AChE and pseudocholinesterase and the activity measured in this tissue will be hereafter indicated as ChE activity. Another piece of dorsal muscle was isolated on ice, homogenized in ice cold tris (hydroxymethyl)-aminomethane buffer (pH = 7.8, 50 mM), and centrifuged at 3300*g* for 3 min at 4°C. The supernatant was recovered and used for IDH determination after standardization of the protein content to 0.5 mg/mL. The activity of IDH was determined according to Ellis and Goldberg (1971) adapted to microplate (Lima et al. 2007). Briefly, 0.2 mL of the reaction solution containing 40 mL of tris buffer, 15 mL of 2 mM manganese (II) chloride and 15 mL of 7 mM DL-isocitric acid in ultrapure water was added to 0.05 mL of muscle supernatant plus 0.05 mL of 0.5 mM NADP+). The enzymatic activity was determined at 340 nm and 25°C. Another portion of dorsal muscle was homogenized in 1 mL of ice-cold tris/NaCl buffer (tris 81.3 mM; NaCl 203.3 mM, pH 7.2) on ice, submitted to three frozen/unfrozen cycles to damage cell membranes and release the cytoplasmatic enzyme, and centrifuged at 6000*g* for 3 min at 4°C. Supernatants were collected, their protein content was standardized to 0.25 mg/mL, and used for LDH activity determination by the method of Vassault (1983) adapted to microplate (Diamantino et al. 2001). Briefly, 0.25 mL of a 0.25 mM NADH solution in tris/NaCl buffer and 0.04 mL of a 12 mM pyruvate solution in Tris/NaCl buffer were added to 0.01 mL of muscle supernatant. The activity of LDH was determined by measuring the amount of pyruvate consumed due to NADH oxidation at 340 nm and 25° C. The digestive gland of each animal was isolated on ice and homogenized (1:15 g wt/v) in phosphate buffer (0.1 M, pH = 7.4). Part of this homogenate was used to determine the extent of endogenous LPO by measuring the thiobarbituric acid reactive substances (TBARS), according to Ohkawa (1979) and Bird and Draper (1984), preventing artifactual lipid oxidation by adding 0.2 mM of butylhydroxytoluene (Torres et al. 2002). Briefly, 1 mL of 12% trichloroacetic acid, 0.8 mL of 60 mM tris–HCl (pH = 7.4) with 0.1 mM DTPA, and 1 mL of 0.73% thiobarbituric acid were added to 0.2 ml of digestive gland supernatant. After an incubation at 100°C for 60 min, the solution was centrifuged at 12000*g* for 5 min. LPO levels were determined at 535 nm and expressed in nmol of TBARS per gram of weight. The remaining digestive gland homogenate was centrifuged at 10000*g* for 20 min at 4°C. The supernatant was used for determination of GST, glutathione peroxidase (GPx) and catalase (CAT) activities. GST activity was determined after standardization of the protein content to 0.5 mg/mL by the technique of Habig et al. (1974), with adaptations (Frasco and Guilhermino, 2002), using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Briefly, 0.2 mL of the reaction solution [75 mL of 0.2 M phosphate buffer (pH = 6.5), 2.34 mL of 60 mM CDNB in ethanol, and 13.5 mL of 10 mM GSH in ultra-pure water] were added to 0.1 mL of the digestive glad supernatant, and the enzymatic activity was determined at 340 nm and 25°C. GPx activity was determined according to Flohé and Günzler (1984). Briefly, 0.840 mL of phosphate buffer (0.05 mM, pH = 7.0) with 1 mM of EDTA, 1 mM of sodium azide and 1 U/mL of GR were added to 0.05 mL of 4 mM GSH, 0.05 mL of 4 mM GSH, 0.05 mL of 0.8 mM NADPH, 0.01 mL of 0.5 mM H_2O_2, and 0.04 mL of digestive gland supernatant. The activity of the enzyme was determined by

measuring the decrease of NADPH at 340 nm using H_2O_2 as substrate. For CAT activity determination (Clairborne, 1985), 0.950 mL of phosphate buffer 0.05 M (pH 7.0) and 0.5 mL of H_2O_2 (30 mM) were added to 0.005 mL of digestive gland supernatant in a cuvette. The consumption of H_2O_2 was measured at 240 nm for 30 s at 25°C. At the end of the enzymatic analysis, the protein content of each supernatant was determined again by the Bradford technique (Bradford, 1976) adapted to microplate (Frasco et al. 2002), and enzymatic activities were expressed as nmol of substrate consumed per min per mg of protein.

Chemicals

Naphathalene (CAS n° 91-20-3, 98% purity) and pyrene (CAS n° 129-00-0, 99% purity) were purchased from Sigma–Aldrich Chemical Corporation (St Louis, MO, USA), acetone (pro analysis) was from Merck (Darmstatt, Germany), and the Bradford reagent was from Bio-Rad (Munich, Germany). All the other chemicals were of analytical grade and were obtained from Sigma-Aldrich (USA) and Merck (Germany).

Statistical analysis

The median lethal concentrations (LC_{50}), 10% lethal concentrations (LC_{10}), 20% lethal concentrations (LC_{20}) and 90% lethal concentrations (LC_{90}) of naphthalene were calculated from the concentration/toxicity curves after log transformation of the exposure concentrations and probit transformation (Finney, 1971) of the percentages of mortality. A similar procedure was carried out to calculate the 10%, 20%, 50% and 90% effect concentrations (EC_{10}, EC_{20}, EC_{50} and EC_{90}, respectively) for swimming velocity decrease. Swimming velocity data of the pyrene bioassay and biomarkers data of both bioassays were checked for homogeneity of variances and normality of distribution, and transformed if necessary. For each parameter, different treatments were compared by one-way analysis of variance (Zar, 1996). No observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) were determined by the Dunnett's test relatively to control values. The significance level was 0.05. The SPSS statistical package was used for all the statistical analysis.

Results and discussion

In both bioassays, no mortality was recorded in any of the control treatments, the pH variation was less than 1 pH unit, temperature variation was less than 1°C, oxygen dissolved in water was always higher than 8 mg/L, and salinity variation was less than 0.05‰. In the test with naphthalene, 1 animal of the group exposed to 0.5 mg/L was lost during the experiment due to manipulation.

Lethal effects

Naphthalene caused lethal effects on *P. serratus* at concentrations higher than 1.0 mg/L, namely: 22% at 2.0 mg/L, 44% at 4.0 mg/L and 100% at 8.0 mg/L. The 96 h-LC_x values are indicated in the Table 1. The LC_{50} of naphthalene to *P. serratus* determined in the present study (3.5 mg/L) compare to corresponding values that have been reported for other crustaceans. For example, a 24 h-LC50 of 4.422 mg/L was found for *Oithona davisae* nauplii (Saiz et al. 2009), a 48 h-LC50 of 2.535 mg/L was determined for *Paracartia grani* (Calbet et al. 2007) and a 96 h-LC50 of 18 mg/L to *S. serrata* (Vijayavel and Balasubramanian, 2006). The concentrations of pyrene tested (up to 0.4 mg/L) did not cause lethal effects on *P. serratus*, suggesting that this species may be less sensitive to pyrene than *Gammarus aequicauda, G. locusta* and *Corophium multisetosum* for which 48 h-LC50 values of 0.073, 0.061 and 0.029 mg/L, respectively, were found (Sanz-Lázaro et al. 2008).

Table 1. Lethal and swimming velocity effects of naphthalene to *Palaemon serratus* after 96 h of exposure through water at concentrations ranging from 0.125 to 8 mg/L: 10%, 20% 50% and 90% lethal concentrations (LC_{10}, LC_{20}, LC_{50} and LC_{90}, respectively) and 10%, 20% 50% and 90% effect concentrations for swimming velocity inhibition (EC_{10}, EC_{20}, EC_{50} and EC_{90}, respectively) with 95% confidence limits (95% CL) within brackets.

Mortality	Naphthalene (mg/L)	Swimming velocity	Naphthalene (mg/L)
LC_{10} 95% CL	1.8 (0.76–2.5)	EC_{10} 95% CL	0.06 (0.03–0.10)
LC_{20} 95% CL	2.3 (1.21–3.05)	EC_{20} 95% CL	0.2 (0.10–0.24)
LC_{50} 95% CL	3.5 (2.52–5.09)	EC_{50} 95% CL	1.7 (1.26–2.34)
LC_{90} 95% CL	6.8 (4.77–17.97)	EC_{90} 95% CL	25.8 (13.2–72.1)

Swimming velocity

Both PAHs significantly decreased the swimming velocity of *P. serratus* (naphthalene: $F_{7,57} = 7.473$, $p < 0.05$; pyrene: $F_{8,62} = 3.103$, $p < 0.05$) at concentrations equal or higher than 2.0 mg/L for naphthalene and 0.4 mg/L for pyrene (Figure 1). The 96 h EC_{10}, EC_{20} and EC_{50} of naphthalene to *P. serratus* were 0.06 mg/L, 0.2 mg/L and 1.7 mg/L, respectively (Table 1). It was not possible to calculate EC_x values for pyrene since a significant reduction occurred only at the highest concentration tested. However, based on the LOEC values for the swimming velocity decrease (Table 1), pyrene was considerably more toxic to *P. serratus* than naphthalene (5 folds). These results indicate that naphthalene and pyrene have the capability of reduce the swimming performance of *P. serratus* after a short-term exposure through the water. These findings are in good agreement with the failure to swim induced by exposure to naphthalene and pyrene observed in freshwater amphipods (Landrum et al. 2003), and the avoidance of pyrene spiked sediments by freshwater invertebrates (De Lange et al. 2006). Impairment of the swimming behaviour was previously reported for fish exposed PAHs, including the common goby (*Pomatoshistus microps*) and the European seabass (*Dicenthrarchus labrax*) exposed to pyrene (Almeida et al. 2012; Gravato and Guilhermino, 2009; Oliveira et al. 2011), the Pacific herring (*Clupea pallasi*) exposed to the water-soluble fraction of a crude oil (Kennedy and Farrell, 2006), and the gilthead seabream (*Sparus aurata*) exposed to

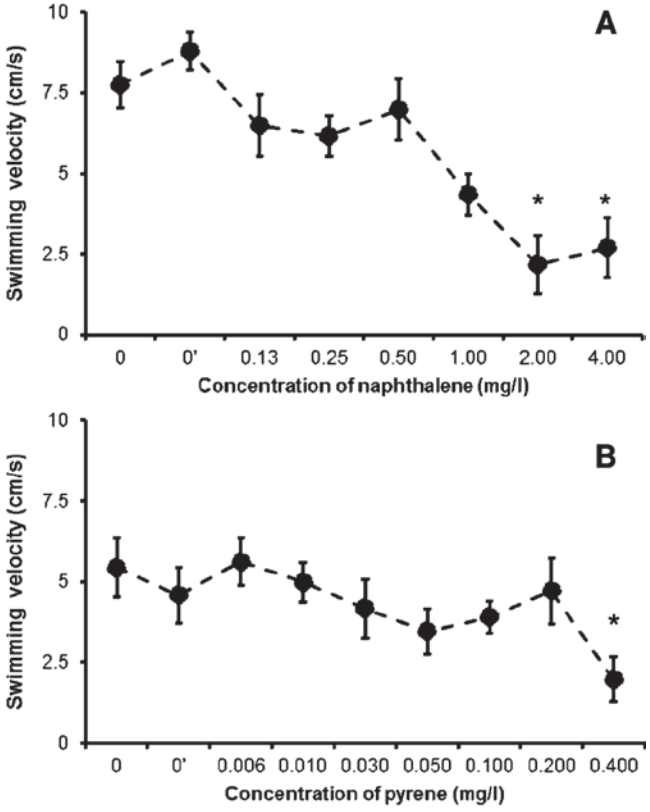

Figure 1. Effects of naphthalene (A) and pyrene (B) on the swimming velocity of *Palaemon serratus*. Values are the mean of 4 to 9 animals with corresponding S.E.M. bars. The mean of the swimming velocity of the control groups were 7.8 cm/s ± 0.7 S.E.M. and 5.4 cm/s ± 0.9 S.E.M. EC50 for naphthalene and pyerene bioassays, respectively. *indicates statistically significant differences relatively to the control group as indicated by one-way ANOVA and the Dunnett's test ($p < 0.05$). 0 – control group; 0′– solvent control group.

naphthalene and pyrene (Gonçalves et al. 2008). The impairment of marine organisms swimming performance after both acute and chronic exposure to PAHs deserves further attention since it indicates a decrease of individual fitness with potential negative effects at population level. Thus, although the concentrations of PAHs tested in our work and in some of the studies mentioned above are higher than the corresponding concentrations generally found in marine waters after oil spills (Diez et al. 2007; Elordui-Zapatarietxe et al. 2010; Guitart et al. 2008; Lemkau et al. 2010) these findings may be of interest for the ecological risk assessment of oil and other petrochemical spills in the marine environment.

Biomarkers

Exposure to naphthalene through test medium caused a significant increase of LPO levels ($F_{7,52} = 2.953$, $p < 0.05$) in the digestive gland of animals exposed to 2 and 4 mg/L of this PAH (Figure 2A). These results indicate that naphthalene causes lipid peroxidation in *P. serratus*. Naphathalene was also found to significantly increase (≈ 10 folds) the activity of CAT in the digestive gland of *P. serratus* ($F_{7,57} = 2.518$, $p < 0.05$; NOEC = 1.0) at the highest

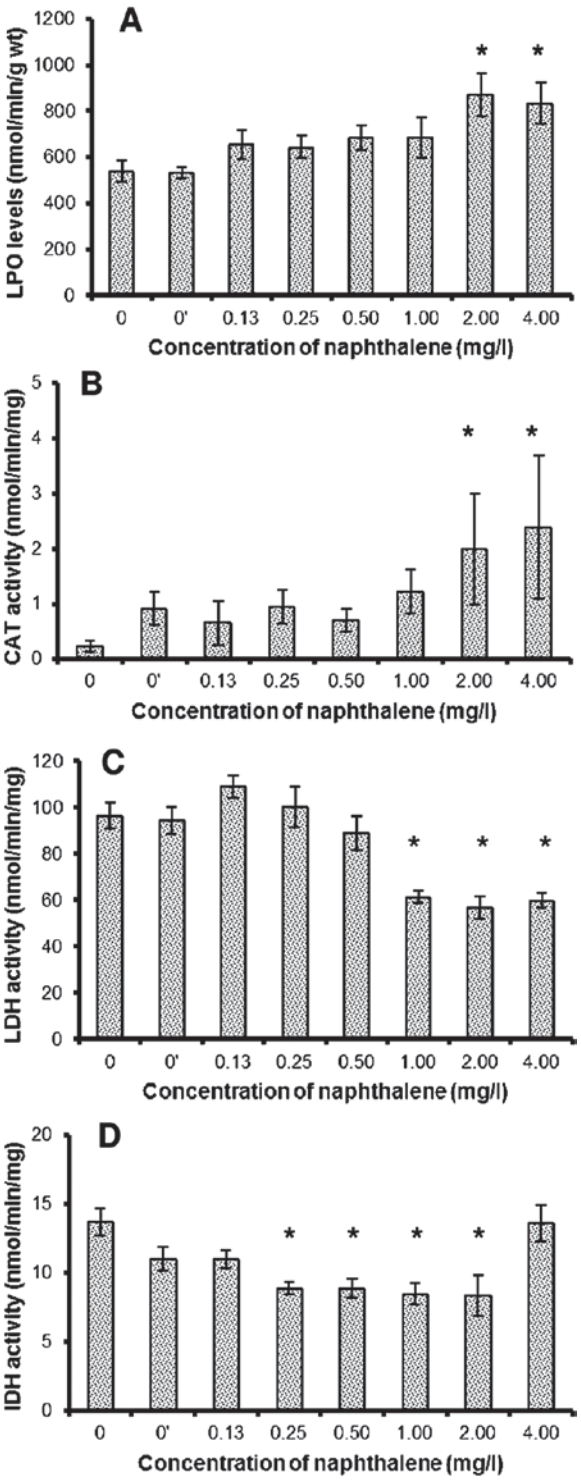

Figure 2. Biomarkers individually determined in *P. serratus* that survived to 96 h of exposure to naphthalene and showing significant differences among distinct treatments. Values are the mean of 4 to 9 animals with corresponding S.E.M. bars. *Statistically significant differences relatively to the control group (one-way ANOVA and Dunnett's test, $p < 0.05$). 0– control group; 0′– solvent control group; LPO– lipid peroxidation levels in the digestive gland (control group: 539 nmol TBARS/g wet weight ± 45 S.E.M.); CAT– catalase activity in the digestive gland (control group: 0.2 nmol/min/mg protein ± 0.10 S.E.M.); LDH– muscle lactate dehydrogenase activity (control group: 96 nmol/min/mg protein ± 6 S.E.M.); IDH– muscle NADH isocitrate dehydrogenase activity (control group: 13.7 nmol/min/mg protein ± 1.00 S.E.M.).

concentrations tested (LOEC = 2.0 mg/L) (Figure 2B). No significant changes of GST ($F_{7,57} = 1.302$, $p > 0.05$; mean ± S.E.M in the control group: 5.7 ± 0.7 nmol/min/mg protein; corresponding values at 4.0 mg/L of naphthalene: 9.2 ± 1.5) and GPx activities ($F_{7,57} = 0.363$, $p > 0.05$; mean ± S.E.M in the control group: 2.7 ± 0.5 nmol/min/mg protein; corresponding values at 4.0 mg/L of naphthalene: 3.5 ± 1.0) were found. These findings suggest that CAT induction is the main defence against naphthalene-induced oxidative stress in *P. serratus* with GST and GPx playing only minor roles if any. Thus, considering the major role that GST generally has in preventing lipid peroxidation (Hansen et al. 2008) and the lack of significant induction in *P. serratus* exposed to naphthalene found in the present study (despite the increase of LPO levels) it seems likely that naphthalene induces general cellular oxidative stress and damage and not only lipid peroxidation in this species. In the copepod *Calanus finmarchicus* acutely exposed to naphthalene (0.031–5.5 mg/L), significant inductions of GST mRNA with no significant increases of superoxide dismutase and CAT mRNA levels were found suggesting that in this species lipid peroxidation is the main mode of oxidative damage (Hansen et al. 2008). Although the comparison of the results obtained in the two studies is difficult due to the different approaches used and to the variability of response (with time and naphthalene concentrations) that anti-oxidant enzymatic activities and gene transcription have (Hansen et al. 2008; Teles et al. 2003), these findings deserve further investigation since they may suggest differences in the oxidative stress induced by naphthalene between distinct species of crustaceans. Oxidative stress induced by naphthalene has been found in several other marine organisms including microalgae (Kong et al. 2010), crustaceans (Vijayavel et al. 2004) and fish (Ahmad et al. 2003; Hansen et al. 2008). Oxidative damage owing naphthalene exposure include lipid peroxidation (Ahmad et al. 2003; Hansen et al. 2008), oxidatively generated DNA damage (Vijayavel and Balasubramanian, 2008), and protein damage (Hansen et al. 2008). The lack of significant induction of GST activity by naphthalene found in our study also suggests that this enzyme does not play a significant role in the biotransformation of this PAH in *P. serratus*. In previous studies with the crab *S. serrata* (Vijayavel and Balasubramanian, 2008) and the shrimp *Penaeus aztecus* (Rongzhong and Zou, 2008), significant increases of GST activity were found after exposure to naphthalene, suggesting the involvement of this enzyme in the conjugation of metabolites resulting from the phase I of biotransformation with glutathione. Thus, differences in the phase II of naphthalene biotransformation may exist among marine crustaceans. Although, it should be work noted that the GST activity of marine animals has been showing different types of responses after exposure to individual PAHs, namely: (i) induction (Vieira et al. 2008) that has been attributed to the role of GST in catalysing the conjugation of metabolites generated in the phase I of PAHs biotransformation with glutathione; (ii) a bell shape response with induction at relatively low concentrations and return to normal values at high concentrations (Gravato and Guilhermino, 2009; Sun et al. 2008); and (iii) inhibition of GST activity (Almeida et al. 2012; Vieira et al. 2008), possibly due to the direct binding of the enzyme to some PAHs/metabolites or to the inhibition of its synthesis. Four main factors seem to contribute for the differences of GST response: the PAH tested since compounds with distinct properties have been found to be metabolized differently; the tested species since differences in the mechanisms of PAHs biotransformation have been found in distinct organisms; the tested concentrations; and the exposure time. Additionally, experimental conditions, such as temperature, light, type of exposure, tissue/organ analysed, among others, may also contribute to the differences found. Interestingly, despite these differences in response, GST activity works particularly well as a field biomarker in relation to oil spills and to historical petrochemical pollution, where induction in several species of vertebrates and invertebrates in good agreement with contamination patterns has been reported (e.g. Lima et al. 2007; Martinez-Gomez et al. 2006; Moreira et al. 2004; Tim-Tim et al. 2009). Considering now the enzymes involved in bioenergetics, significant differences in both LDH ($F_{7,57} = 14.866$, $p < 0.05$) and IDH activities ($F_{7,57} = 9.024$, $p < 0.05$) were found among prawns exposed to different treatments. Naphthalene caused a significant decrease of LDH activity (Figure 2C), the enzyme catalysing the reversible reduction of pyruvate to lactate, with NOEC and LOEC values of 0.5 and 1.0 mg/L, respectively. These results suggest a decrease of the energy obtained through the anaerobic conversion of pyruvate to lactate at high concentrations of naphthalene. A significant reduction of IDH activity was also observed at concentrations of naphthalene between 0.25 and 2.0 mg/L but not at 4.0 mg/L (Figure 2D). IDH is an enzyme of the citric acid cycle that catalyses the oxidative descarboxilation of isocitrate to α-ketoglutarate regenerating NADH in the process (Vijayavel and Balasubramanian, 2006). Thus, the reduction of the IDH activity found suggests a decrease of the use of this aerobic pathway of energy production. However, considering this hypotheses, it is difficult to understand why there was no IDH activity reduction at the highest concentration of naphthalene tested. Therefore, these findings remain without a clear explanation. Reduction of LDH and IDH activities was also found in the mud crab *S. serrata* (Vijayavel and Balasubramanian, 2006) exposed to higher concentrations of naphthalene (8–10 mg/L); although, contrary to our findings, no return to normal IDH values were found in the mud crab up to 10 mg/L of naphthalene. Regarding neurotoxicity, no significant effects of naphthalene on eye AChE activity were observed ($F_{7,57} = 1.121$, $p > 0.05$; mean ± S.E.M. in the control group: 32.7 ± 3.8 nmol/min/mg protein; corresponding values at the highest concentration of naphthalene tested: 36.7 ± 3.2

nmol/min/mg protein) indicating that cholinergic neurotransmission of *P. serratus* is not affected by exposure to this PAH up to 4 mg/L, in good agreement with previous findings in *S. aurata* (Kopecka-Pilarczyk and Correia, 2011). Neurotoxic effects of naphthalene that have been described in aquatic species include narcosis and changes in catecholaminergic and indoleaminergic systems (Barata et al. 2005; Gesto et al. 2006; Santos et al. 2006). No significant effects were observed in muscle ChE activity ($F_{7,57} = 0.808$, $p > 0.05$; mean ± S.E.M. in the control group: 5.1 ± 0.8 nmol/min/mg protein; corresponding values at the highest concentration of naphthalene tested: 5.7 ± 30.6 nmol/min/mg protein) confirming the lack of anti-cholinesterase effect of naphthalene in *P. serratus*.

In the bioassay with pyrene (Figure 3), significant differences in LPO levels among the groups of animals exposed to different concentrations were found ($F_{8,68} = 3.067$, $p < 0.05$). The highest LPO levels were recorded at intermediate concentrations (0.013 and 0.025 mg/L), whereas at the highest concentrations of pyrene LPO levels were similar to those determined in the control group (Figure 3A). These findings suggest that somehow animals were able to prevent oxidative damage at concentrations equal or higher than 0.05 mg/L. *P. serratus* exposed to the highest concentration of pyrene tested had significantly ($F_{8,69} = 2.399$, $p < 0.05$) increased GST activity (≈ 3 folds) (Figure 3B). These results suggest the involvement of GST in the biotransformation of pyrene by catalysing the conjugation of metabolite(s) generated in the phase I with glutathione and/or the increase of GST activity in response to pyrene-induced oxidative stress. Previous studies on the biotransformation of pyrene by aquatic invertebrates indicated that sulfation and glucuronidation are the main conjugation reactions involved (Beach et al. 2009, 2010; Ikenaka et al. 2006; Jorgensen et al. 2008; Simpson et al. 2002). Therefore, it seems likely to associate the GST activity increase found in *P. serratus* exposed to the highest concentration of pyrene (Figure 3B) mainly to oxidative stress. Animals exposed to pyrene also had a significant increase of CAT ($F_{8,66} = 3.332$, $p < 0.05$) (≈ 1.5 folds at 0.4 mg/L of pyrene) (Figure 3B) but it was not possible to discriminate the concentrations causing effects significantly different from the control group. The induction of the activity of these enzymes, especially GST, may have contributed to decrease the LPO levels at the highest concentration of pyrene tested. However, the reduction of LPO levels at concentrations between 0.05 and 0.20 mg/L remains without explanation since no significant induction of GST and CAT activities (Figure 3B), nor significant changes of GPx activity ($F_{8,72} = 0.754$, $p > 0.05$; mean ± S.E.M. in the control group: 1.6 ± 0.4 nmol/min/mg protein; corresponding values at the highest concentration of pyrene tested: 2.5 ± 0.2 nmol/min/mg protein) activities were recorded at these concentrations. Considering the lack of LPO increase in animals exposed to the highest concentrations of pyrene relatively to the

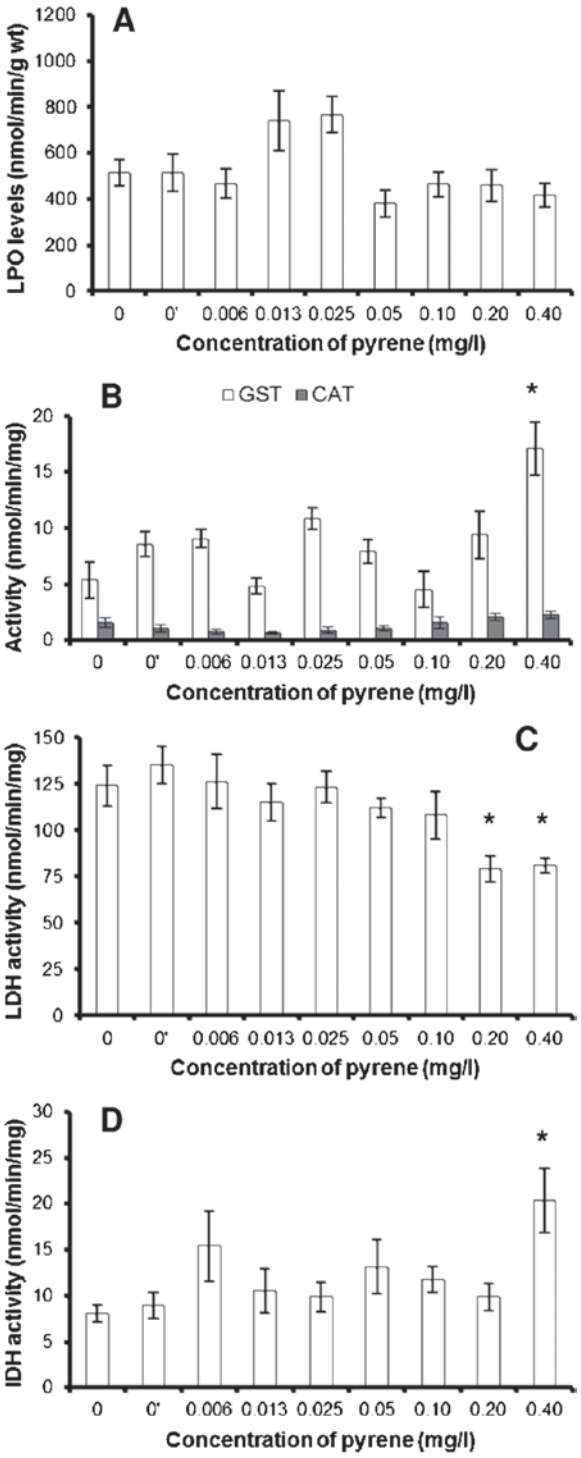

Figure 3. Biomarkers individually determined in *P. serratus* that survived to 96 h of exposure to pyrene and showing significant differences among distinct treatments. Values are the mean of 6 to 9 animals with corresponding S.E.M. bars. *Statistically significant differences relatively to the control group (one-way ANOVA and Dunnett's test, $p < 0.05$). 0 – control group; 0′ – solvent control group; LPO – lipid peroxidation levels in the digestive gland (control group: 516 nmol TBARS/g wet weight ± 56 S.E.M.); CAT – catalase activity in the digestive gland (control group: 1.6 nmol/min/mg protein ± 0.39 S.E.M.); GST – glutathione S-transferases activity in gills (control group: 5.4 nmol/min/mg protein ± 1.9 S.E.M.); LDH – muscle lactate dehydrogenase activity (control group: 124 nmol/min/mg protein ± 11 S.E.M.); IDH – muscle NADH isocitrate dehydrogenase activity (control group: 8.1 nmol/min/mg protein ± 0.9 S.E.M.).

control group, it seems likely that pyrene induces general cellular oxidative stress rather than lipid peroxidation only. Evidences from previous studies with juveniles of *P. microps* and *Dicentrarchus labrax* exposed to pyrene (Almeida et al. 2012; Oliveira et al. 2011) also suggest general oxidative stress in addition to lipid peroxidation. Regarding the results of the bioenergetics enzymes in *P. serratus* exposed to pyrene (Figures 3C and D), a significant reduction of LDH activity was found ($F_{8,69} = 4.975$, $p < 0.05$; NOEC = 0.2 mg/L, LOEC = 0.4 mg/L). Thus, similarly to naphthalene, pyrene exposure seems to decrease the energy obtained through the pyruvate reduction pathway. Animals exposed to the highest concentration of pyrene also had a significant increase of IDH activity (≈2.5) relatively to the control group ($F_{8,65} = 2.393$, $p < 0.05$) (Figure 3D). The induction of IDH activity may be included in a general increase of the citric acid cycle function in response to a demand of energy for detoxication at high concentrations of pyrene, may be a bioenergetics response to compensate the decrease of the anaerobic production of energy function due to LDH inhibition, or may be a response to an increased oxidative stress requiring additional regeneration of NADPH. Unfortunately, the approach used does not allow going further in this question. As found for naphthalene, no significant effects of pyrene on eye AChE activity $F_{8,65} = 0.931$, $p > 0.05$; mean ± S.E.M. in the control group: 47.6 ± 8.8 nmol/min/mg protein; corresponding values at the highest concentration of naphthalene tested: 37.2 ± 2.3 nmol/min/mg protein) were found indicating that cholinergic neurotransmission of *P. serratus* is not affected by exposure to pyrene up to 0.4 mg/L. Also, no anti-cholinesterase effect of pyrene on muscle ChE activity was found ($F_{8,59} = 0.669$, $p > 0.05$; mean ± S.E.M. in the control group: 2.8 ± 0.6 nmol/min/mg protein; corresponding values at the highest concentration of naphthalene tested: 3.0 ± 0.5 nmol/min/mg protein). These results are in good agreement with previous studies with juveniles of *S. aurata* and *D. labrax* exposed to concentrations of pyrene up to 0.050 mg/L and 10 mg/L, respectively (Almeida et al. 2012; Kopecka-Pilarczyk and Correia, 2011). However, in a bioassay with the common goby (*P. microps*) exposed for 96 h to pyrene, a significant reduction of ChE activity in both head and muscle was found at 0.5–1.0 mg/L (Oliveira et al. 2011). Therefore, a distinct sensitivity of ChE enzymes from different marine animals to pyrene may exist.

Mechanisms of toxicity potentially contributing to individual fitness decrease

The prevalent mechanism of PAH toxicity in invertebrates is nonpolar narcosis (Burgess, 2009). This is a reversible and physical phenomena, due to the accumulation of the toxic agent in the lipid bilayer of cell membranes, resulting in alterations of membrane properties (e.g. membrane fluidity, membrane surface tension) and function; it may also be due to the binding of the toxic agent to an hydrophobic region of membrane proteins resulting in alterations of membrane structure and function (Schultz, 1989). This phenomenon may impair behavioural responses in aquatic organisms and result in mild intoxication or mortality depending upon exposure characteristics (Barata et al. 2005; Burgess, 2009; Schultz, 1989). Since both naphthalene and pyrene were found to cause nonpolar narcosis in marine crustaceans (Barata et al. 2005), the mortality of *P. serratus* observed in the naphnthalene bioassay and the swimming velocity impairment induced by both PAHs may have been due at least in part to nonpolar narcosis.

Not excluding nonpolar narcosis as main mode of toxic action of the PAHs tested, the findings of the present study suggest the contribution of two other mechanisms of toxicity to the impairment of the swimming velocity observed under PAH exposure, namely the reduction of LDH activity and oxidative stress. Prawns exposed to 2–4 mg/L of naphthalene and those exposed to 0.4 mg/L of pyrene had a significant reduction of LDH activity (35–41% relatively to the control group), suggesting a decrease of the energy obtained through the pyruvate reduction pathway that may have contributed to the significant impairment of the swimming velocity observed in these animals (64–72% of reduction relatively to the control group). In general, animals increase the use of the LDH bioenergetics pathway to obtain energy rapidly in response to a sudden energy demand, such as an intensive physical effort or an acute exposure to chemical stress, among other situations including hypoxia (Diamantino et al. 2001; Wu and Lam, 1997). Therefore, prawns exposed to the highest concentrations of PAHs, which had a depression of LDH activity (37–41% of inhibition relatively to the control group) (Figures 2C and C), may have not been able to get additional energy rapidly to respond adequately to the sudden stimulus of water flow tending to drag them way resulting in a low swimming performance (64–72% of impairment relatively to control groups, Figure 1). A lower severity of intoxication in animals exposed to 1.0 mg/L of naphthalene or to 0.2 mg/L of pyrene, potentially causing a lower dependency from the LDH bioenergetics pathway, may explain why these animals had 44% of swimming velocity impairment only in the naphthalene assay and no impairment in the pyrene assay despite having the LDH activity depressed by about 35%. Two additional evidences seem to provide support to this hypotheses: (i) the concentration dependent mortality recorded in the naphthalene bioassay (0% at 1.0 mg/L, 22% at 2.0 mg/L, 44% at 4.0 mg/L and 100% at 8.0 mg/L; LC50 = 3.5 mg/L), suggesting a severe intoxication in the animals that survived to 96 h of exposure to 2.0 and 4.0 mg/L of naphthalene and a less-severe intoxication in prawns exposed to 1.0 mg/L of the toxicant; (ii) the increase of IDH in animals exposed to 0.4 mg/L indicating an increase of citric acid cycle activity. Both naphthalene and pyrene caused oxidative stress and damage in *P. serratus* (Figures 2 and 3). Oxidative metabolism of PAHs, such as naphthalene, may lead to multiple organ damage (Şehirli et al. 2008). Oxidative stress and lipid peroxidation damage in brain tissue may be possible

mechanisms of neural toxicity caused by PAHs (Bagchi et al. 2002). Among other effects, damage of neuronal membranes by lipid peroxidation may lead to changes of neurotransmitters levels, an effect that has been reported in fish exposed to PAHs and that may cause alterations in several physiological and biological functions (Fingerman and Short, 1983; Gesto et al. 2006), including behaviour changes and activity decrease. Therefore, oxidative stress and damage in the nervous system of *P. serratus* may have also contributed to the reduction of the swimming velocity observed in animals exposed to high concentrations of naphthalene or pyrene. More research is needed on the effects of PAHs on the nervous system of marine crustaceans to elucidate the mechanisms of neurotoxicity and how they can affect behavioural responses in these animals.

Conclusions

Both naphthalene and pyrene induced oxidative stress and lipid peroxidation in *Palaemon serratus* juveniles. The main anti-oxidant response to naphthalene was the induction of catalase activity (≈10 folds at 4 mg/L), while the corresponding response to pyrene was the induction of GST activity (≈3 folds at 0.4 mg/L). Both PAHs significantly reduced the activity of the bioenergetics enzyme LDH and changed the activity of IDH distinctly: pyrene significantly induced the activity of the enzyme at the highest concentration tested (0.4 mg/L), while naphthalene decreased IDH activity at concentrations between 0.25 and 2.0 mg/L but not at 4.0 mg/L. No significant alterations were found in any of the other biomarkers assessed. The swimming velocity of the common prawn was significantly inhibited by both naphthalene and pyrene, with pyrene being more effective (≈ 5 folds) in reducing the individual fitness of this species than naphthalene. Naphthalene caused mortality in the range of concentrations tested (96 h-LC_{20} = 2.3 mg/L; 96 h-LC_{50} = 3.5 mg/L). Nonpolar narcosis, reduction of LDH activity and general cellular oxidative stress and damage may have contributed to the impairment of the swimming velocity in animals exposed to both PAHs and to the mortality recorded under naphthalene exposure. Although the concentrations tested here are higher than those generally found in marine waters, the test species, the findings and methods of the present study are of interest for the ecological risk assessment of oil spills in the marine environment.

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Declaration of interest

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