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Monitoring PAH contamination in the field (South west Iberian Peninsula):
biomonitoring using fluorescence spectrophotometry and physiological assessments
in the shore crab *Carcinus maenas* (L.) (Crustacea: Decapoda)

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants of the marine environment, arising predominantly from petrochemical contamination and pyrogenic sources. A biomarker of PAH exposure was employed in a field study (South West, Spain) in both captured (indigenous) and deployed (caged) shore crabs (*Carcinus maenas*) in the chronic PAH-exposed Bays of Algeciras and Gibraltar (from associated harbour and boating activity) compared to a relatively 'clean' site (Cadiz). Metabolite fluorescence was attributed to the following key priority PAH groups; naphthalenes (NAPs), pyrenes (PYRs) and benzo[a]pyrenes (BAPs). Temporal variability was assessed using deployed populations over an eight week period. Petrogenic and pyrogenic PAH contamination (as an indicator of the PAH type) was demonstrated using a ratio between $FF_{BAP+PYR} / FF_{NAP}$. Physiological assessments from deployed crabs demonstrated both physiological and cellular alterations as shown by reduced heart rates (at rest) and increased cellular stress in crabs from the PAH contaminated sites.

Key words: Polycyclic aromatic hydrocarbon (PAH), *Carcinus maenas*, fluorescence spectrophotometry, Gibraltar.

1. INTRODUCTION

Coastal marine waters and sediments receiving anthropogenic inputs can contain a large variety of contaminants that are potentially toxic to aquatic organisms (Krahn et al., 1984) (Kennish, 2002). One group of chemicals of particular concern are the highly lipophilic polycyclic aromatic hydrocarbons (PAHs), which are ubiquitous pollutants of the marine environment. These compounds arrive in coastal waters from a diverse array of sources including petrochemical pollution, incomplete combustion processes (Livingstone, 1992; Clarke et al., 2001), metal smelting (Naes et al., 1995; Beyer et al., 1996) and electrolytic production of aluminium using anode technology (Beyer et al., 1998; Aas et al., 2000b; MSC-E, 2001). Concern over the fate and effect of PAHs in the environment is related to their persistence, bioaccumulation potential and acute & chronic toxicity to marine organisms. Three PAHs, naphthalenes, pyrenes & benzo[a]pyrenes, are included in the USEPA priority pollutant list (MSC-E, 2001).

Current monitoring techniques employed to determine environmental quality include the chemical analyses of sediment and water samples. PAHs are sparingly soluble in water and are difficult to detect and although they show a much greater association with sediments, chemical analysis of this material is laborious and costly due to the lengthy extraction methodology needed. Furthermore, sediment/water-based measurements provide little information about contaminant bioavailability and toxic potential. Routine chemical monitoring usually involves determination of the concentration of parent compounds present in the environmental phase of interest. This approach may overlook the risks posed by the potentially much more toxic daughter compounds produced by many organisms as a result of metabolism and biotransformation of the parent chemicals (Livingstone, 1998). Metabolites arising from biotransformation processes may be concentrated in body fluids, tissues or

excreta and the analysis of such biological compartments provides us with an
30 opportunity to detect and measure exposure of organisms to bioavailable
contaminants (Fillmann et al., 2002).

The detection of PAH metabolites is made possible due to the fluorescence
properties attributed to their delocalised π -electrons. This property was first
35 developed for environmental monitoring purposes as a rapid, inexpensive screening
method to detect PAH metabolites in fish bile and used HPLC in combination with a
fluorescence detector to process test samples. This technique has subsequently
been implemented as a monitoring tool in several environmental monitoring
programmes (Krahn et al., 1984; Stagg, 1998; Aas et al., 2000a, 2001) and has been
40 further developed for use in analysing the presence or absence of PAH metabolites
in crustacean urine by (Watson et al. 2002). Metabolites in shore crab (*Carcinus
maenas*) urine were analysed at specific fluorescence wavelengths for naphthalene-,
pyrene- and benzo[a]pyrene-type compounds. The use of crab urine to monitor
environmental contamination has proved to be a useful technique as it provides a
45 measure of contemporary levels of contamination (Watson et al. 2004b). Fixed
wavelength fluorescence (FF) method used to detect PAH metabolites has the
advantage that it is less expensive than the previously-used HPLC technique and
demonstrates a dose-response relationship. The aim of this study was to evaluate
the use of a PAH exposure biomarker (by detection of PAH metabolites) using the
50 decapod crustacean species *Carcinus maenas* to assess the extent of PAH exposure
in this region, and to determine if this technique provides a cost-effective surrogate
for other chemical analytical techniques. A specific objective of the study was to
investigate the field application of the PAH exposure biomarker in crabs collected
from both local natural populations and also deployed (caged) crabs. A second
55 objective was to investigate the relationship between the presence of PAH
metabolites in urine and selected physiological traits (cardiac activity assessed by

heart rate measurements and monitoring cellular impact by lysosomal stability measurements) in crabs that in combination could be used to support biomonitoring programmes. Physiological assessments of invertebrates using cardiac activity
60 (Fossi et al. 2000; Camus et al. 2002) and lysosomal stability (Lowe et al. 1994; Castro et al. 2004) measurements have been widely used in both laboratory and field scenarios (Brown et al. 2004; Galloway et al. 2004) as general “health” indicators. Lysosomal damage arises from the accumulation of PAHs, the resultant formation of oxyradicals leads to increased membrane permeability and subsequent release of
65 acid hydrolases into the cytoplasm, causing cellular damage and/or ultimately, cell death (Lowe et al., 1995). The resulting toxic effect of PAHs is expressed through the hierarchical levels of biological organisation i.e. cellular and physiological levels (Fossi et al. 1994).

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2. MATERIALS AND METHODS

2.1 *Study sites*

The study sites were situated in the Southwest Iberian Peninsula in the Bays of
75 Algeciras and Gibraltar and further west in the Bay of Cadiz (Fig 1). Gibraltar is a small peninsula situated at the mouth of the Mediterranean Sea with a 30km coastline. There is a heavy industrial presence from within the Bays of Algeciras and Gibraltar and an apparent lack of data concerning environmental quality with regard to specific types of contaminants within the region. The area has proximity to
80 chemical plants, refineries, thermal power plants, ironworks, paper mills, shipyards and two major ports which serve the economically important sea route of the Straits of Gibraltar (Truver, 1978; Mavor, 1980). The Bay of Algeciras receives input from two major rivers; The Palmones and the Guadarranque, which carry sewage from urban and industrialised activities corresponding to populations of 15000 and 2500

85 respectively, (Carballo et al., 1996). Polyaromatic hydrocarbons in the Bay of Algeciras indicate chronic contamination in this area ($21.4 \mu\text{g kg}^{-1}$ sediment) originating from accidental spills, bunkering activities and deliberate discharges from commercial shipping (Jimenez-Tenorio et al., 2008). The Bay of Cadiz is characterised by urban and harbour activities with low sediment contamination
90 (Cesar et al., 2007), however, the area outside the Bay of Cadiz (San Fernando) was used as the reference site as it is situated in a highly productive protected natural site where there is no urban activity (Perez et al., 2004). These particular sites were chosen due to the highly industrialised nature of the surrounding area, including the Bay of Gibraltar, which is highly populated (31,000 in 6 sq Km) (United Nations,
95 2009) and includes two major harbours; The Port of Algeciras (Spain) and The Port of Gibraltar (Gibraltar), within a naturally enclosed area situated within the Straits of Gibraltar. There is an apparent lack of data concerning environmental quality with reference to specific types of contaminants in the Bay of Gibraltar. Based on this lack of knowledge, sites were ranked based on the degree of boating activity and
100 industrial presence; Cadiz (low: no boating activity), Palmones Estuary [intermediate: minor boating activity; strong industrial presence ($36^{\circ} 10'28$ N, $5^{\circ} 26' 11$ W)], Gibraltar Harbour (middle): [intermediate boating activity ($36^{\circ} 08'13$ N, $5^{\circ} 21' 22$ W)], Gibraltar Harbour [inner ($36^{\circ} 07' 55$ N, $5^{\circ} 21' 18$ W)] (high: major boating activity) (Fig.1).

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2.2 Shore crab deployment and collection

Carcinus maenas individuals (green intermoult males; carapace width 50-75 mm) sampled from the reference site [San Fernando, Cadiz, South-west Spain ($36^{\circ} 27'53$ N, $6^{\circ} 11' 50$ W)] and were used for the deployment study (June - August 2001) using
110 cages at selected field sites within the Bay of Gibraltar, Southwest Iberian Peninsula (Fig. 1). Prior to deployment, adult individuals (> 60 mm) (Crothers, 1967) were

measured and labelled (labels attached to carapace with cyanoacrylate glue) and allowed to depurate for 14 days, fed (throughout this study with squid mantle tissue) and maintained in the laboratory in flow-through holding tanks (350 l) with filtered (10
115 μm carbon-filtered) seawater at similar conditions to that found at each site [salinity: 36_{PSU} and at ambient temperature ($22\text{ }^{\circ}\text{C}$)]. Prior to deployment, crabs were fed to satiation. Crabs were deployed sub-tidally (approx. 4m) in plastic cages ($n=40$ cage⁻¹) (cage measurements: 47 x 34.6 x 28.7cm). The entrance of each cage was covered by mesh (1cm) in order to exclude predators. Crabs were held in cages at
120 the selected field sites (Palmones Estuary and Gibraltar Harbour sites) and fed once a week with squid. Caged shore crabs ($n=8$) were sampled at random (June-August 2001) from each cage each week over several weeks (1, 2, 4, 6 and 8). Prior to caging experiments, a preliminary test for the effects of caging was performed at the reference site (Cadiz) where shore crab physiological responses (see 2.5) were
125 measured after one week and no significant differences were observed (heart rates: $F_{1,14} = 0.6$, $P = 0.45$; lysosomal stability (neutral red retention time): $F_{1,14} = 1.24$, $P = 0.28$). Local indigenous populations were collected using squid-baited traps or hand-collected at the various field sites in the month of June. During transportation (to field sites or the laboratory) all crabs were placed in cool boxes with icepacks and damp
130 absorbent paper.

2.3 *Urine Extraction and Analysis*

Urine from each crab was extracted and analysed using the techniques described by
135 Watson et al. (2004b). In summary, crabs were physically restrained prior to urine extraction by using elastic bands to hold them securely onto to small wooden boards with their ventral surface presented uppermost. The third maxillipeds were moved aside and restrained using further elastic bands to allow access to the epistome and

antennal gland opercula. The epistome was dried in order to prevent seawater
140 diluting the urine. Lifting of the antennal gland opercula, with the aid of a fine hooked
seeker, triggers the release of urine from restrained crabs. Extracted urine (20 -
1000 μ l) was transferred to siliconised micro-centrifuge tubes. All urine samples were
stored at -80°C until subsequent analysis.

145 2.4 *Spectrophotometric analysis*

Fixed fluorescence (FF) spectrometry analysis was performed on diluted urine
samples (1:20) whereby there are no significant inner filter effects (Hitachi F-4500
fluorescence spectrophotometer). All FF analyses were carried out in a 3.5 ml quartz
fluorescence cuvette and performed with excitation and emission slit widths of 2.5
150 nm. Fluorescence contributions can be attributed to three PAH groups; 2 ring, 4 ring
& 5/6 ringed PAHs typified by naphthalene-type (NAP-type), pyrene-type (PYR-type)
and benzo[a]pyrene-type (BAP-type) metabolites respectively as their behaviour is
similar to other compounds of similar ring number (Reeves et al. 2004).
Fluorescence analyses were not undertaken to quantify individual PAH compounds,
155 but to discriminate between sites of varying PAH exposure (Dissanayake and
Galloway, 2004). The assigned wavelength pairs and peak area measurements
were used to quantify the metabolite groups as follows; FF_{290/335} (315-375), FF_{341/383}
(380-390nm) & FF_{380/430} (400-490nm) for 2&3-rings (naphthalene-type PAHs), 4-rings
(pyrene-type PAHs) and 5-6 ringed PAHs respectively (benzo[a]pyrene-type PAHs).
160 Pyrene-type metabolites only were quantified by using 1-OH pyrene standards
(Sigma, U.K). The ratio between the sum of BAP & PYRs over NAPs (FF_{BAP+PYR} /
FF_{NAP}) was calculated in order to determine PAH contamination origin [i.e petrogenic
(NAPs) or pyrogenic (PYR and BAP)].

165 2.5 *Shore crab physiological assessment*

A combination of physiological (heart rate measurements) and cellular endpoints were used to determine potential physiological impact. Following urine extraction, heart rate measurements were performed on shore crabs by use of the non-invasive CAPMON system (Depledge and Andersen, 1990). Heart rate measurements were performed on individuals in 2 L aquaria (using water collected from each site); sensors were glued to the carapace above the heart and, after 30 min acclimation, cardiac activity (at rest) was recorded for 30 min as (as employed previously by Dissanayake et al., 2008b). Lysosomal stability (using neutral red dye) assesses the lysosomal compartment of cells where toxins (e.g. PAHs) are accumulated prior to detoxication. If the lysosomes are overloaded, damage may lead to cell injury, tissue dysfunction, and reduction in animal "health status" (Moore et al., 2004). Haemolymph was extracted by inserting a needle through the arthodial membrane at the base of the third walking leg and extracted into a syringe with 0.5 ml of crustacean physiological saline (0.5 M NaCl, 11 mM KCl, 12 mM CaCl₂·6H₂O, 26 mM MgCl₂·6H₂O, 45 mM Na₂HPO₄·12H₂O, 45 mM Trisma-Base, 1M HCl; pH 7.4). Cysteine (50 mg ml⁻¹) was added as an anti-coagulant (Smith and Ratcliffe, 1978). Haemolymph (40 µl) was transferred onto a glass slide cells were allowed to adhere in a humidity chamber (15 mins) before incubating (15 min) with the neutral red dye. Cells were observed under a high powered microscope over time (15, 30, 60, 75, 90, 120, 150, 190 min) and the retention of the dye within the haemocyte lysosomes was recorded (as employed by Brown et al., 2004).

2.6 Statistical analysis

Analysis of variance (ANOVA) tests were performed for the individual PAHs (NAP, PYR and BAP) physiological (heart rates) and cellular (NRR) endpoints (transformed where necessary to meet parametric normality assumptions) (GMAV 5 for

Windows®) (Underwood, 2005). Student-Newman-Keuls (SNK) tests were used to isolate differences between sites or time (weeks).

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3. RESULTS

Fluorescence spectrophotometry of diluted crab urine revealed significant PAH exposure characterised by strong fluorescence signals for both petrogenic and pyrogenic PAHs from both indigenous and deployed (caged crabs).

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No differences were observed between crabs caught from the different sites with regard to Naphthalene-type metabolites (Fig. 2A), however, significant differences were observed between sites with both PYR and BAP-type metabolites significantly higher in urine from Gibraltar Harbour crabs than Palmones Estuary or Cadiz (reference) crabs ($F_{2, 21} = 49.77$, $P < 0.001$ and log-transformed $F_{2, 21} = 32.89$, $P < 0.001$, for PYR- and BAP-type metabolites, respectively) (Fig. 2B and C). No differences in levels in any of the three PAH metabolite groups were observed between Palmones Estuary or Cadiz collected crabs. Physiological assessment of shore crabs collected from each site revealed that both Palmones Estuary and Gibraltar Harbour crabs were impacted as demonstrated by lower basal heart rates (log-transformed; $F_{2, 21} = 35.73$, $P < 0.001$) (Fig. 3A) and lower neutral red retention times ($F_{2, 21} = 450.19$, $P < 0.001$) (Fig. 3B) than Cadiz (reference) crabs.

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The investigation of the Bay of Algeciras (Palmones Estuary) and Gibraltar Harbour sites using caged crabs demonstrated the presence of PAHs (NAPs and BAPs) as observed in shore crab urine (Table 1). High levels were recorded for all PAH metabolite types, especially PYR as significantly high concentrations (approx 125 – 160 $\mu\text{g l}^{-1}$) were observed in Palmones Estuary crabs (Fig. 4A) over the 8 week period ($F_{5, 42} = 3.70$, $P < 0.01$). Significantly high PYR concentrations (153-221 $\mu\text{g l}^{-1}$)

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were also observed continuously in Gibraltar Harbour crabs (Coaling Island) ($F_{5, 42} = 4.01$, $P < 0.01$). (Fig. 4B), however, in the inner harbour site high levels were observed initially with a subsequent return to background levels (Fig. 4C) (log-transformed; $F_{5, 42} = 4.41$, $P < 0.01$). A ratio between 4 & 5 ring PAHs & 2-3 ring PAHs ($FF_{\text{BAP+PYR}} / FF_{\text{NAP}}$) was calculated in order to determine PAH contamination source (Table 2). Over the 8 week monitoring period, PAH contamination shifted from pyrogenic origin (due to high levels of PYR and BAP metabolites) (week 1) to petrogenic as observed in Gibraltar middle harbour site (week 8). The high levels of PYR and BAP metabolites also found in Palmones Estuary crabs indicate PAHs from a pyrogenic source (weeks 4) (Table 2). The observed variations in PAH levels in urine were also associated with significant physiological and cellular alterations. Physiological assessments revealed a significant acute reduction (approx. 50%) in heart rates in crabs from all sites (at rest) at week 1 compared with controls (week 0) (two-way ANOVA; $F_{10, 126} = 2.51$, $P < 0.001$) (Fig. 5A). Significant impacts at the cellular level were observed with significant reductions in neutral red retention times with the biggest reduction between week 0 and 1. Although, reductions in neutral red retention times were observed in all sites over the 8 week monitoring period, significant differences were also observed between sites with Palmones Estuary crabs displaying significantly higher NRR times compared to both Gibraltar Harbour sites (weeks 1-4) (two-way ANOVA; $F_{10, 126} = 6.20$, $P < 0.001$) (Fig. 5B).

4. DISCUSSION

Results from fluorescence spectroscopy indicates the presence of PAH metabolites at the sample sites characterised by strong PAH-associated fluorescence responses at specified wavelengths. Shore crabs caught from each site revealed inter-site variation with significant differences occurring between both Cadiz and Palmones

Estuary crabs and Gibraltar harbour crabs, where high levels of urinary PAH were
250 observed. The observed high levels of PAH metabolites verify the fact that Gibraltar
Harbour has a greater amount of boating and harbour activity due in large part the
presence of the Naval Base and dockyard when compared to San Fernando, Cadiz,
where there is relatively less boating activity. Cadiz was chosen as a reference site
even though a low level of metabolites was detected. Pyrene metabolite
255 concentrations in urine are approximately 30 % higher than that of the parent
compound in seawater (when exposed continuously) (Dissanayake et al., 2008a) and
reflect short-term exposure (upto 4 days) rather than bioaccumulation (Watson et al.,
2004b). Mean concentrations of pyrene found here (Cadiz, $20 \mu\text{g l}^{-1}$; Palmones, 13
 $\mu\text{g l}^{-1}$ and Gibraltar Harbour $51 \mu\text{g l}^{-1}$) are consistent with values found in relatively
260 clean coastal sites ($17 - 20 \mu\text{g l}^{-1}$) and PAH contaminated sites ($40 - 90 \mu\text{g l}^{-1}$)
(Watson et al., 2004a; Dissanayake et al., 2010).

The mobility of PAHs due to both aqueous and aerial transport makes it difficult also
to locate completely 'pristine' locations, although the Bay of Cadiz has been classed
265 as a 'clean' area due to undetectable levels of PAHs in sediments (Riba et al., 2004).
Other studies have also shown low metabolite levels present in various crustacean
and fish species taken from areas assumed to be 'clean' (Klumpp et al., 2002;
Eickhoff et al., 2003b; Ruddock et al., 2003; Dissanayake and Galloway, 2004). The
similarity of metabolite levels found in indigenous populations of crabs from the
270 reference site (Cadiz) and the intermediate Palmones Estuary may only reflect the
bio-available water-borne PAH levels, however, previous studies have shown that the
Palmones Estuary has high PAHs levels in sediments ($641-725 \text{ mg kg}^{-1}$) (Morales-
Caselles et al., 2008; Ramos-Gómez et al., 2009). High levels of all PAH metabolites
were observed in the Palmones Estuary over the 8 week monitoring period, which
275 was unexpected and may be largely attributed to a reported minor oil spill in the area
over the background of a low level boating activity (inferred by the levels of NAP

metabolites and low calculated pyrene concentrations in seawater). The presence of 4-ringed PAHs can also be inferred due to the elevated pyrene concentrations. The high $FF_{\text{BAP+PYR}} / FF_{\text{NAP}}$ ratios, especially at week 4 (13.02) compared to control levels (4.87), indicates that the source of the PAHs may have been pyrogenic through the dominance of 4- and 5- ringed PAHs (Table 2b). Indigenous sampled crabs, however, demonstrated that Palmones Estuary-caught crabs indicated PAHs of a petrogenic origin (Table 2a), thereby indicating a change in the PAH origin over the study period. It could be argued that the pattern observed here (of increasing and decreasing levels PAH metabolites levels) may occur due to possible down-regulation dose-dependent effects (thus reflecting environmental levels) or saturation processes of the biotransformation system. A previous study has shown that in *Carcinus*, when exposed to a set concentration similar metabolite levels are observed after 7 and 14 days exposure to a sustained dose ($200 \mu\text{g l}^{-1}$ for 7 and 14 days exposure; see Dissanayake et al. 2008a). However, saturation processes of the biotransformation (mixed function oxidase) system may occur with high doses as observed by bell-shaped dose response curves as shown in vertebrate systems (Bosveld et al. 2002; Lu et al. 2009). In shore crabs, however, there is conflicting evidence for saturation of the biotransformation system as shown by Fossi et al. (2000) evaluated by measuring both benzo(a)pyrene monooxygenase activity and ethoxyresoru@n-O-deethylase activity (EROD) in both hepatopancreas and gills and remains to be tested. In this study, however, the pattern observed of increasing pyrene metabolite levels (weeks 1 -8) as seen in the Palmones Estuary deployed crabs (Fig. 4a) does not suggest saturation of the biotransformation system and thus may reflect exposure levels.

The results from this study indicate the effectiveness of using fluorescence spectrophotometry as a rapid, cost-effective technique for the detection of PAH exposure in field situations in tandem with physiological assessments to assess

305 biological impact, especially in large coastal bays. By extrapolating the pyrene
concentrations from the metabolite concentrations, the use of the FF method when
used on urine represents a very useful tool indeed as it facilitates PAH temporal
monitoring. External control sites have therefore limited importance benchmarking
environmental concentrations as each site or location can be used as an 'internal'
310 control. The objective of rapid screening techniques is to be able to discriminate
between sites of varying contamination, thereby providing a rapid assessment of
pollution (Galloway et al., 2002). The $FF_{\text{BAP+PYR}} / FF_{\text{NAP}}$ ratio was used in order to
indicate the source of PAH contamination (Aas et al., 2000a). The rationale for using
this technique is based on the fact that PAHs occur in combination and PAH profiles
315 (e.g. ratio of high to low molecular weight PAHs) can indicate sources of PAHs (Law
and Biscaya, 1994; Wang et al., 2000). The FF technique is regarded as a measure
of groups of PAH metabolites due to the incorporation of fluorescence contributions
of other PAH compounds, such as chrysene, which fluoresces at both naphthalene &
pyrene wavelength pairs (Aas et al., 2000a). It is recognised that $FF_{290/335}$ comprises
320 mainly 2 & 3 ringed PAHs and 4 & 5 ring PAHs for wavelength pairs $FF_{341/383}$ &
 $FF_{380/430}$ respectively. Any monitoring programme which includes FF as a rapid
screening technique for PAH exposure will, therefore, highlight the most frequent and
prioritised PAH compounds associated with environmental monitoring (Aas et al.,
2000a; MSC-E, 2001). The differences observed in PAH sources (pyrogenic and
325 petrogenic) among the three deployment sites over time may also occur either by
minor oil spills or from bunkering activities, characterised by high molecular weight
PAHs (i.e. 4-5 ringed), or light engine fuels (low molecular weight PAHs; 2-3 ringed)
and /or the atmospheric emissions of an oil refinery in the vicinity, which remains to
be tested. PAH emissions are distributed via the atmosphere over a large area and
330 its fate is dictated by and dependant on wind direction (Eickhoff et al., 2003a). Inputs
from non-point sources such as atmospheric deposition and from roads would be
diffuse and pollutants would be quickly dispersed in the water column and local

currents and/or dredging activities may have an effect on suspended matter levels (Zhou et al. 1998; Rogers, 2002; King et al., 2004). The atmosphere can be classed
335 as a secondary dominant source of hydrocarbons in areas where the marine input is the primary dominant source (Zhou et al., 1996). In the context of environmental monitoring, it is therefore imperative that all modes of PAH inputs are evaluated, not only oil spills. PAH spectrofluorimetric analysis in crustacean urine and associated physiological assessments are therefore an integrated approach which represents a
340 realistic, relatively-inexpensive means of monitoring the bioavailability of PAHs (Dissanayake and Galloway, 2004; Galloway et al., 2004; Watson et al., 2004a; 2004b). The potential for PAH-induced biological impact, or specifically, cellular stress, as observed here by reduced lysosomal dye retention times corroborates previous evidence of reduced retention times with contaminant exposure (Nicholson
345 et al. 1999; Brown et al. 2004). Previous evidence of PAH-induced physiological effects includes tachycardia (increased heart rates) (Bamber & Depledge 1997; Fossi et al. 2000; Camus et al. 2002; Dissanayake et al. 2008b) with acute exposure. In this study, however, only bradycardia (depressed heart rates) was observed and is indicative of chronic PAH exposure (Hagger et al. 2009).

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A key facet of the urinary PAH metabolite technique is its ability to identify PAH 'hotspots' within relatively large coastal areas impacted by PAH contamination which promotes its use in environmental management and regulation procedures. The PAH metabolite technique has been used successfully in various decapod
355 crustaceans (Watson et al. 2004a; Eickhoff 2003a; Koenig et al. 2008). Implementation of the PAH metabolite technique, when used especially in deployed (caged) crabs, and in conjunction with other biological techniques, allows for contaminant-induced physiological impact to be monitored temporally and therefore can be used as important tools in environmental monitoring schemes (Galloway et al.
360 2002, 2004). In summary, this study highlights the use of fluorescence

spectrophotometry as a rapid, cost-effective biomonitoring tool of environmental contamination. Rapid screening techniques should therefore be incorporated with other physiological techniques (as shown here) as a means of primary modes of investigation within a framework of detailed investigative monitoring procedures, prior to mitigatory action being undertaken (Galloway et al., 2002; 2004).

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Table 1. Naphthalene-and benzo[a]pyrene-type metabolite fluorescence contributions (peak area mean \pm SE) from urine of deployed crabs (caged) over an 8 week monitoring period from Palmones Estuary and Gibraltar Harbour (middle and inner sites) ($n = 8$) (different letters denote statistical difference between weeks; same letters (e.g. ab) denote no statistical difference to either groups a or b).

Site	Palmones Estuary		Gibraltar Harbour (middle)		Gibraltar Harbour (inner)	
Metabolite type	Week	Peak area	Week	Peak area	Week	Peak area
Naphthalene-type metabolites (NAP)	0	2784 \pm 362 ^a	0	2784 \pm 362 ^a	0	2784 \pm 362 ^a
	1	4101 \pm 576 ^c	1	5659 \pm 1175 ^b	1	4635 \pm 500 ^b
	2	4817 \pm 716 ^c	2	4480 \pm 286 ^b	2	4627 \pm 337 ^b
	4	1742 \pm 86 ^b	4	4020 \pm 287 ^b	4	2897 \pm 182 ^a
	6	4016 \pm 202 ^c	6	2109 \pm 345 ^a	6	3027 \pm 185 ^a
	8	4488 \pm 322 ^c	8	4625 \pm 528 ^b	8	3469 \pm 367 ^{ab}
Statistical difference	$F_{5,42} = 13.82, P < 0.001$		log-transformed; $F_{5,42} = 4.82, P < 0.01$		log-transformed; $F_{5,42} = 6.25, P < 0.001$	
Benzo[a]pyrene -type metabolites (PYR)	0	4781 \pm 955 ^a	0	4781 \pm 955 ^a	0	4781 \pm 955 ^a
	1	10236 \pm 1739 ^b	1	11570.19 \pm 2015 ^c	1	9863 \pm 2065 ^b
	2	12110 \pm 1651 ^b	2	12309 \pm 2614 ^c	2	14766 \pm 2816 ^{bc}
	4	3668 \pm 761 ^a	4	7679 \pm 1426 ^b	4	6401 \pm 837 ^a
	6	11821 \pm 1310 ^b	6	3183 \pm 977 ^a	6	3760 \pm 839 ^a
	8	14093 \pm 2904 ^b	8	10153 \pm 1454 ^{bc}	8	8919 \pm 1713 ^b
Statistical difference	log-transformed; $F_{5,42} = 10.64, P < 0.001$		log-transformed; $F_{5,42} = 7.22, P < 0.001$		log-transformed; $F_{5,42} = 45.47, P < 0.001$	

Table 2. $FF_{\text{BAP+PYR}} / FF_{\text{NAP}}$ ratio as an indicator of PAH contamination in a) indigenous sampled populations and in b) deployed shore crabs ($n = 8$). PETRO (predominance of 2-3 ringed PAHs) indicates petrogenic contamination and PYRO (predominance of 4- and 5-ringed PAHs) indicates pyrogenic contamination (i.e. ratio values of < 4.87 and > 4.87 , respectively, relative to that of the control site). Only the lowest (i.e. petrogenic) and highest (pyrogenic) ratios are indicated here.

2a

Site		
Cadiz (control)	Palmones Estuary	Gibraltar Harbour
4.87	3.95 PETRO	36.14 PYRO

2b

Week	Site		
	Palmones Estuary	Gibraltar Harbour (middle)	Gibraltar Harbour (inner)
0	4.87	4.87	4.87
1	5.87	7.42	5.90
2	6.32	6.75	7.94
4	13.02 PYRO	7.54	7.99
6	8.36	9.32	5.10
8	7.62	8.81	6.05

List of Figures

- Fig. 1. Map showing the location of sites (see insets) within the Southwest Iberian Peninsula. Insets (1.) denote Cadiz (C) (relatively 'clean') (2) within the Bay of Algeciras (Palmones Estuary; P) and within Bay of Gibraltar (2a); Gibraltar Harbour inner (I) and middle (m).
- Fig. 2. Relative fluorescence contributions (peak area mean \pm SE) of each of the PAH groups (A; NAP; naphthalene-type, B; PYR; pyrene-type and C; BAP; benzo[a]pyrene type in crabs caught from Cadiz, Palmones Estuary and Gibraltar Harbour ($n = 8$) (different letters denote statistical difference between sites; same letters denote no statistical difference).
- Fig. 3. Shore crab physiological assessment (mean \pm SE): A; heart rate (at rest) and B; neutral red retention time of crabs caught from Cadiz, Palmones Estuary and Gibraltar Harbour ($n = 8$) (different letters denote statistical difference between sites; same letters denote no statistical difference).
- Fig. 4. Pyrene-type metabolite concentrations ($\mu\text{g l}^{-1}$) (mean \pm SE) from urine of deployed (caged) crabs over an 8 week monitoring period from A; Palmones Estuary, B; Gibraltar Harbour middle and C; Gibraltar Harbour inner sites ($n = 8$) (different letters denote statistical difference between weeks; same letters denote no statistical difference).
- Fig.5. Shore crab physiological assessment (mean mean \pm SE): A; heart rate (at rest) and B; neutral red retention time of deployed (caged) crabs over an 8 week monitoring period from Palmones Estuary and Gibraltar Harbour (middle and inner sites) ($n = 8$) (different letters denote statistical difference between sites within each week; same letters denote no statistical difference).

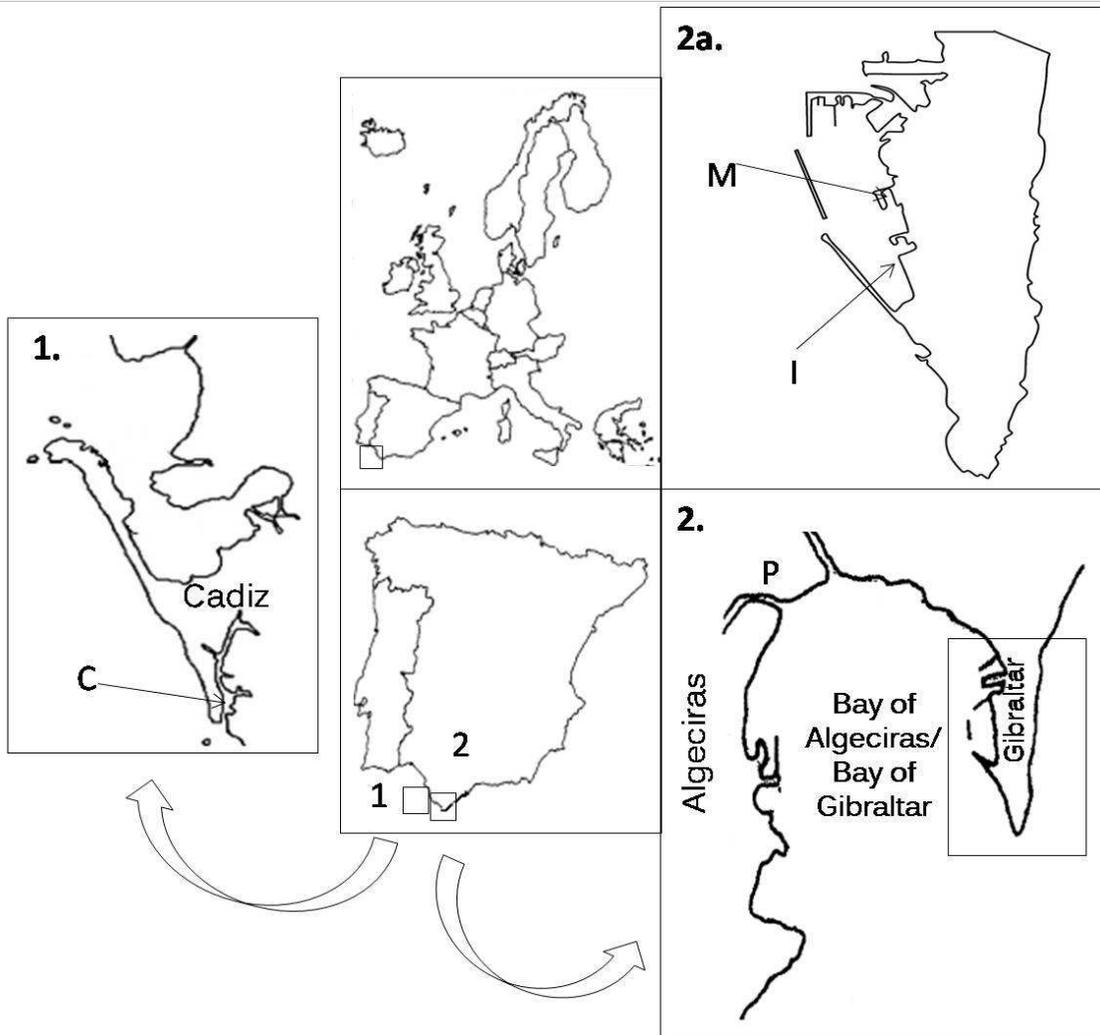


Fig. 1

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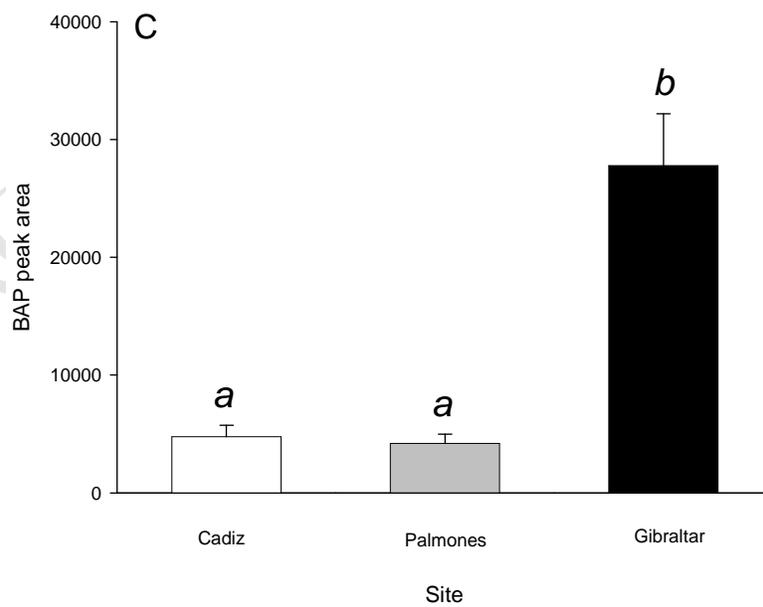
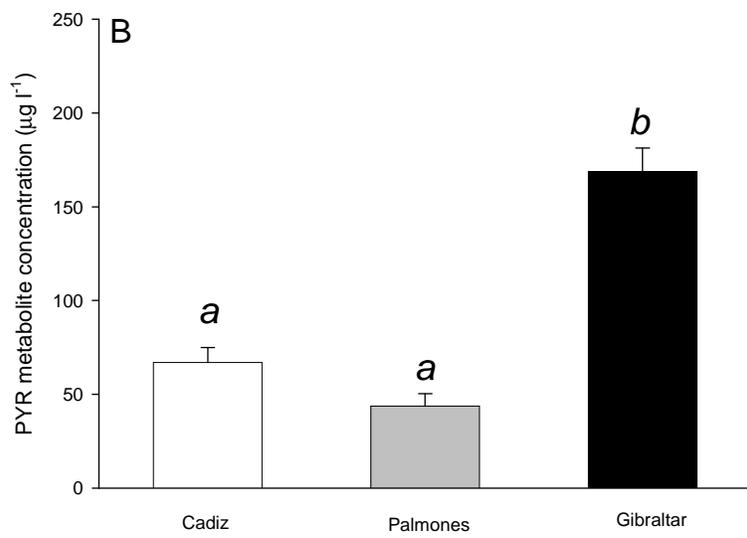
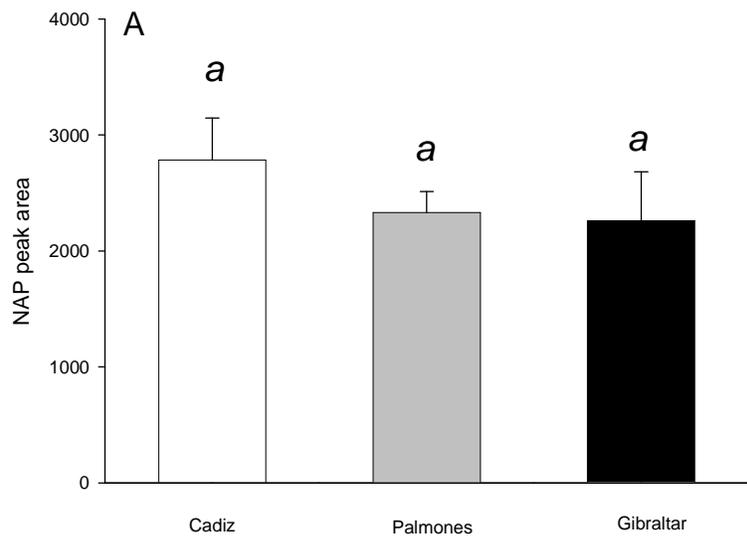


Fig. 2.

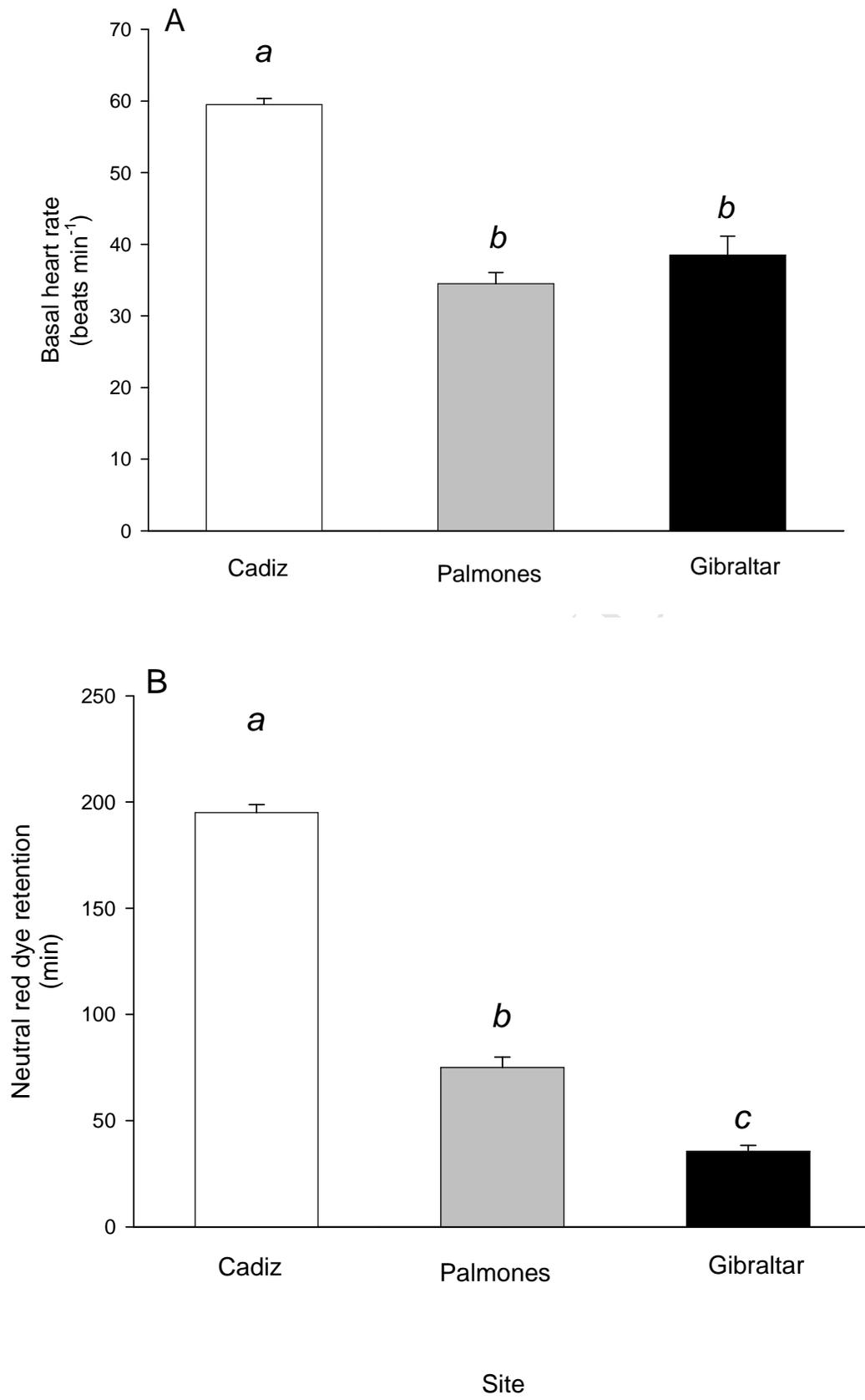


Fig. 3.

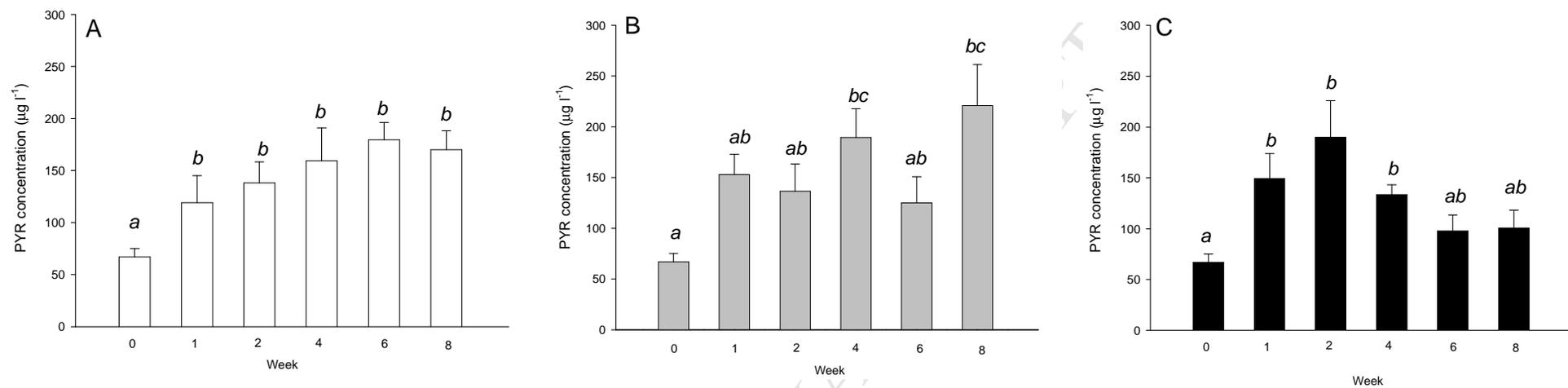


Fig. 4.

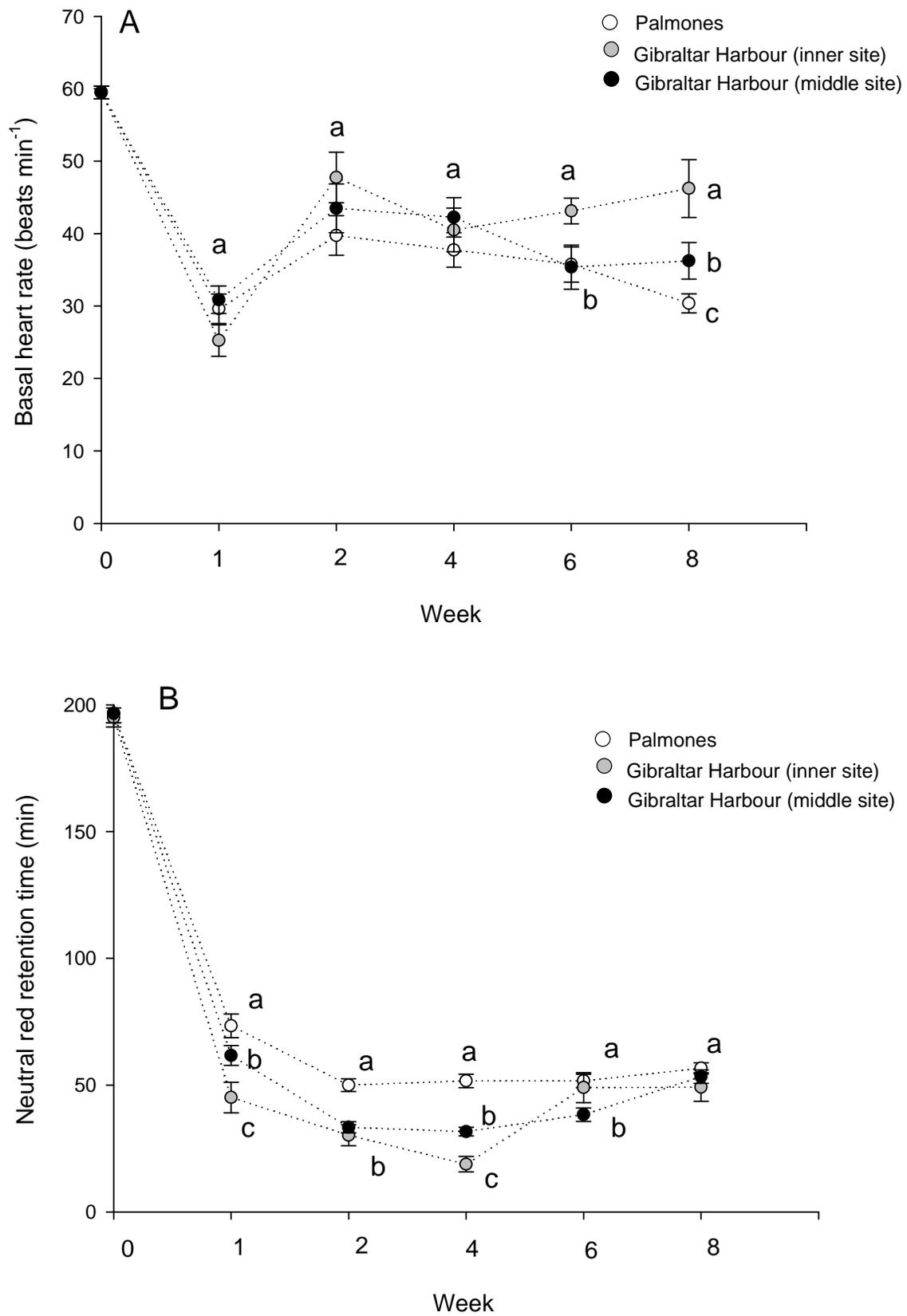


Fig. 5.