1 **Freshwater shrimp (*Palaemonetes australis*) as a potential bioindicator of crustacean health**

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11

12 **Abstract** *Palaemonetes australis* is a euryhaline shrimp found in south-western Australian estuaries. To

13 determine if *P. australis* is a suitable bioindicator species for monitoring the health of estuarine biota, they

14 were exposed to measured concentrations of the polycyclic aromatic hydrocarbon, benzo[*a*]pyrene (B[*a*]P) at

15 0.01, 0.1 or 1 ppm for 14 days under laboratory conditions. At the end of exposure the shrimp were sacrificed

16 for biomarker [ethoxycoumarin *O*-deethylase (ECOD), 8-oxo-dG concentration, and sorbitol dehydrogenase

17 (SDH) activity] analyses. Gender did not appear to influence biomarker responses of the shrimp in this study.

18 ECOD activity was induced in the treatment groups in a linear fashion from 3 (0.01 ppm) times to 12 (1 ppm)

19 times the negative controls. 8-oxo-dG concentration was reduced 3 times in treatment groups below the

20 controls suggesting impaired DNA repair pathways. There was no increase in SDH, signifying

21 hepatopancreatic cell damage had not occurred in any treatment group. The response of *P australis* to B[*a*]P

22 exposure indicates that this crustacean is suitable bioindicator species for both laboratory studies and field

23 monitoring. A combination of ECOD and SDH activities and 8-oxo-dG concentration represent a suitable

24 suite of biomarkers for environmental monitoring of the sublethal effects of organic pollution to crustaceans

25 from an estuarine environment.

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28

29 **Keywords** Biomonitoring – ECOD – Hepatopancreas – Oxidative damage – SDH – 8-oxo-dG

30

Introduction

Crustaceans are important inhabitants in aquatic ecosystems providing many ecological and economic benefits and occupying diverse niches. For example, grass shrimp (*Palaemonetes pugio*) function to repackage detritus into protein rich products that are used within different trophic levels in an ecosystem. Welsh (1975) has shown that *P. pugio* plays an active role in breaking down detritus, enabling accelerated growth rates of diatoms and bacteria on substrata in tidal march ecosystems.

Recently endocrine disruption has been reported in several decapod species (crabs, shrimps, prawns, etc; (LeBlanc 2007). Many are dioecious with strong sexual dimorphism making decapods ideal field monitoring candidates for the presence of endocrine disrupting chemicals. Urban and agricultural use of land adjacent to estuaries and their tributaries have been shown to increase the potential for contaminant influx to the environment through non point-source runoff. Pesticide usage near estuarine systems, polycyclic aromatic hydrocarbons (PAHs) and heavy metals in stormwater inputs have been shown to impact on grass shrimp (*Palaemonetes* spp.) growth, size, reproductive capacity and survival (Oberdorster et al. 2000b; Leight et al. 2005).

The hepatopancreas is the main organ for digestion and xenobiotic detoxification in aquatic invertebrates. This organ is very responsive to environmental changes (Sousa and Petriella 2007; Snyder 2000). The PAH, pyrene, has been shown to significantly induce CYP1A-like protein activity in *P. pugio* as measured by ethoxycoumarin-O-deethylase (ECOD) in crude hepatopancreatic extracts. Studies by Lee et al. (2008; 2004) have shown a relationship between reproductive abnormalities and increased DNA strand breaks when *P. pugio* were exposed to estuarine sediments receiving highway runoff.

There is the potential that similar factors are affecting the health of crustaceans in the Swan-Canning Estuary, south-west Western Australia. A three year program to study contaminant levels in both the Swan and Canning Rivers, and their catchment drains, by the Western Australian Department of Water has shown that organochlorine pesticides, PAHs, herbicides, and heavy metals are present in stormwater drains and tributaries that discharge to the estuary (Foulsham et al. 2009; Nice et al. 2009; Nice 2009). For example,

60 17% of sediment samples measured contained organochlorines, with chlordane and dieldrin most frequently
61 reported, in the range of 0.005 to 0.05 mg/kg.
62
63 The freshwater shrimp *Palaemonetes australis* is a euryhaline crustacean found in relative abundance
64 throughout the estuary and its catchment drains and tributaries (Boulton and Knott 1984). This species has
65 not previously been assessed for its potential use as a bioindicator species in environmental monitoring
66 programs using biochemical markers of health. The objective of this study is to evaluate a selection of
67 biochemical markers of health in *P. australis* [ethoxycoumarin-*O*-deethylase activity (ECOD); sorbitol
68 dehydrogenase activity (SDH); and DNA nucleoside base concentration (8-hydroxy-2'-deoxyguanosine)] by
69 exposing the shrimp to a known genotoxicant, benzo[a]pyrene, under laboratory conditions. The value of *P.*
70 *australis* as a suitable bioindicator species to understand the impact of organic chemicals on crustaceans
71 within estuarine environments such as the Swan-Canning Estuary will be assessed.
72
73 **Materials and methods**
74
75 Shrimp Collection and Maintenance
76
77 Adult *Palaemonetes australis* were collected by dip nets in April 2009 from the Canning River, Western
78 Australia (N = 100). The shrimp were carefully placed in 20 litre buckets with water from the collection site
79 for transport to the laboratory. Gentle aeration was provided using battery operated aerators with airstones
80 attached. The shrimp were randomly allocated to one of twenty, 3 L Pyrex beakers to give a final count of 5
81 shrimp per beaker. The beakers had been filled with 2 litre of river water separately collected at the same
82 time as the shrimp and aerated gently. The shrimp were acclimated to laboratory conditions for 10 days
83 during which time up to 50% water changes were performed daily to remove wastes using Millipore filtered
84 water adjusted to 3 ppt salinity. Ammonia levels were monitored daily and the shrimp were fed with frozen
85 *Artemia* until sated.
86
87 Exposure and Sample Collection
88

89 At the end of the acclimation period the beakers were allocated to one of 5 treatment groups using a
90 randomised block design. Ten mg of benzo[*a*]pyrene (B[*a*]P) was dissolved in 10 mL of dimethyl sulphoxide
91 (DMSO), then 2 mL of this solution was diluted in 198 mL 50% DMSO/H_2O. Three subsets of 4 beakers
92 were treated with the diluted solution to give concentrations of 0.01 ppm, 0.1 ppm and 1 ppm of B[*a*]P
93 respectively. A 50% solution of DMSO/H_2O was added to a fourth subset of 4 beakers to give a final
94 concentration of 0.0005% (solvent control group). The final subset of 4 beakers was left untreated as the
95 negative control group. The exposure concentrations of B[*a*]P used in this study were chosen to ensure a
96 measurable response from the shrimp and do not reflect the level of this contaminant in the field.
97
98 Shrimp were continuously exposed for a period of 14 days during which time the daily 50% water change
99 regime was continued with chemical levels within each exposure group maintained by replacement. At the
100 end of the exposure period the shrimp were anaesthetised in an ice and water slurry. The tail fan, legs and
101 exoskeleton were removed from each shrimp before the abdomen was separated from the cephalothorax. The
102 abdominal muscle was placed in a cryovial for sorbitol dehydrogenase determination and the cephalothorax
103 was placed in a separate cryovial for ethoxycoumarin *O*-deethylase and 8-hydroxy-2'-deoxyguanosine
104 assays. Each cryovial was immediately immersed in liquid nitrogen, then later transferred to a freezer and
105 held at –80 ^{o}C until analysis.
106
107 Supernatant Preparation
108
109 A homogenisation buffer was prepared containing 0.1M Trizma (tris hydroxymethyl aminomethane) base,
110 25mM phenylmethanesulphonyl fluoride (PMSF) and 1.3 mM ethylenediaminetetraacetic acid (EDTA) with
111 pH adjusted to pH 7.4. Samples were thawed on ice and homogenised in the buffer (shrimp tail 1:2 w/v;
112 cephalothorax 1:4 w/v) using a Heidolph DIAX 900 homogeniser. The homogenate was centrifuged (Jouan
113 CR3i centrifuge) at 9000xg for 20 mins at 4°C and the supernatant collected for immediate use. Protein
114 content of the cephalothorax supernatant was measured using the method of Lowry et al. (1951).
115
116 Ethoxycoumarin-O-deethylase (ECOD) Assay
117

118 ECOD activity was assessed using the method of Webb et al. (2005), optimised for invertebrates. The
119 reaction mixture containing 0.1M Tris buffer pH 7.4, KCl, $MgCl_2$, NADPH (ß-nicotinamide adenine
120 dinucleotide phosphate, reduced form) solution, and cephalothorax supernatant, was incubated for 2 minutes
121 in a water bath at 35°C. The reaction was initiated by adding 2mM ethoxycoumarin, incubated for a further
122 10 minutes at 35°C and then terminated by the addition of 5% $ZnSO_4$ and saturated $Ba(OH)_2$. Umbelliferone
123 ($C_9H_6O_3$; 7-hydroxycoumarin) standards (0.000 to 0.093 nM) and samples were centrifuged to precipitate
124 proteins and 1 mL of the supernatant was transferred to a test tube. 500 µL of 0.5 M glycine-NaOH buffer pH
125 10.4 was added to each tube and the fluorescence of the buffered supernatant was read on a Perkin-Elmer LS-
126 45 Luminescence Spectrometer at excitation/emission wavelengths of 380/452 nm . ECOD activity was
127 expressed as femtomoles of 7-hydroxycoumarin produced, per mg of total protein, per minute (fmol H mg Pr^{-}
128 ^{1} min^{-1}).
129
130 Sorbitol Dehydrogenase (SDH) Assay
131
132 The SDH assay was adapted for abdominal muscle supernatant from Webb and Gagnon (2007) methods. A
133 50 µL aliquot of supernatant was placed in a cuvette with 450 µL of ß-NADH (ß-nicotinamide adenine
134 dinucleotide, reduced form) - Tris Buffer, pH 7.5, solution. This was then incubated at room temperature for
135 10 minutes to allow for the reaction of keto acids in the serum. Following incubation, 100 µL of D-Fructose
136 solution was added to commence the reaction and the decrease in the rate of absorbance (ΔA) over one
137 minute was immediately read on a Pharmacia UV-Visible Spectrophotometer at 340 nm. The SDH activity
138 was expressed as milli-International Units (mU) in the abdominal supernatant of the shrimp.
139
140 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) Quantification
141
142 The 8-oxo-dG assay was performed on clarified cephalothorax supernatant using an enzyme immunoassay
143 (EIA) kit (Trevigen® Cat. No. 4370-096-K; purchased from BioScientific Pty Ltd, NSW, Australia). Sample
144 preparation was performed as recommended for saliva samples then diluted 1:10 v/v in sample diluent.
145 Leaving the first two wells blank, 50 µL of 8-oxo-dG Standard (0 to 60 ng/mL), and diluted samples were
146 added to the appropriate wells of the immunoassay plate in duplicate. 50 µL of anti-8-oxo-dG antibody was
147 added to each well (except the blanks) then incubated at room temperature for 1 hour. The wells were

148 aspirated and washed 6 times using a Immunowash model 1575 (Bio-Rad Laboratories Pty Ltd, NSW,
149 Australia), patted dry, then 100 µL Anti-Mouse IdG:HSP conjugate antibody added to each well (blanks
150 excluded) and incubated for 1 hour at room temperature. Following a further 6 washes, 100 µL TMB
151 Substrate (stabilised tetramethylbenzidine) was added to all wells, incubated in the dark for 15 minutes, then
152 100 µL Stop Solution added to each well. Absorbance of the wells was measured at 450 nm using a Bio-Rad
153 iMark Microplate Reader (ISO 9001 registered). The average of the absorbance of the duplicate blank wells
154 was deducted from the average of the standard and the sample wells. Plotting the standard curve with log
155 concentrations on the x-axis and absorbance measurements for the respective standards on the y-axis gave a
156 2nd order polynominal equation $y = a + bx + cx^2$. The 8-oxo-dG concentration in the supernatant was
157 expressed as nanograms of 8-hydroxy-2'-deoxyguanosine, per mg of total protein (ng 8-oxo-dG mg Pr^{-1}).
158
159 Statistical Analysis
160
161 For each biomarker, the data were tested for normality and homoscedasticity and, where necessary, $\log_{10}$-
162 transformed to achieve normality. Statistical analysis was undertaken using the SPSS statistical package
163 (Version 17; SPSS GmbH, Germany). Student t tests found no gender difference for each biomarker ($p >$
164 0.05), so data for was pooled. A two-way analysis of variance (ANOVA) was run to investigate if the data
165 was affected by beaker/replicate interactions. As no interactions were found in the data sets, main effects
166 were analysed using one-way ANOVAs. Where significant differences between treatments were found ($p <$
167 0.05), a Dunnett's (2 sided) test was run to compare the treatment groups with the negative control group.
168 Data are presented as mean ± standard error (SEM).
169

170 **Results**

171
172 Ethoxycoumarin-O-deethylase Activity
173
174 Significant induction of ECOD activity occurred in all treatment groups ($p \leq 0.001$). There was no significant
175 difference between the negative control and solvent control groups ($p = 0.22$). ECOD activity in the 0.01ppm
176 treatment group was 220% higher than the level of the negative control group while the 0.1 ppm and 1 ppm

177 treatment groups were induced 5 times (460%) and 12 times (1100%) respectively over the negative control
178 (Fig. 1).
179
180 Sorbitol Dehydrogenase Activity
181
182 No significant differences were detected in SDH activity in *P. australis* between any treatment group (p =
183 0.29; Fig. 2).
184
185 8-hydroxy-2'-deoxyguanosine Concentration
186
187 The concentration of 8-oxo-dG measured in the freshwater shrimp was significantly different between
188 treatment groups (p = 0.003). There was no significant difference between the negative control and solvent
189 control groups (p = 0.80). The three treatment groups each had 66% lower concentration of 8-oxo-dG than
190 the negative control treatment groups and 54% lower than the solvent control group (Fig.3).
191
192 **Discussion**
193
194 Shrimps, such as *P. australis*, are key links in the estuarine detritus food web. The Swan and Canning Rivers
195 and their catchment drains are known to receive anthropogenic contaminants (Foulsham et al. 2009; Nice
196 2009; Nice et al. 2009) that may adversely affect this ecologically important animal. Although the life cycle
197 of *P. australis* has been poorly studied in estuaries of south-west Western Australia, this study found it to be
198 easily maintained in the laboratory, making it an ideal candidate for the study of the impact of contaminant
199 exposure on the health of this estuarine decapod species.
200
201 PAHs have been shown to significantly induce CYP1A-like protein (P450) activity in grass shrimp (*P.*
202 *pugio*) as measured by ECOD in hepatopancreatic extracts (Oberdorster et al. 2000a). Investigations
203 elsewhere have indicated that blue crabs (*Callinectes sapidus*) were unable to metabolise and eliminate PAHs
204 the closer they were to moulting, resulting in a higher body burden (Mothershead and Hale 1992). PAHs
205 have also been shown to delay moulting by *P. pugio* males (Oberdorster et al. 2000a), and the blue crab
206 (Mothershead and Hale 1992). This is believed to be due to competition for substrates by cytochrome P450s

207 needed to metabolise ecdysone for the moult (Mothershead and Hale 1992; Oberdorster et al. 2000a). In our
208 study, *P. australis* exposed to 0.01, 0.1 and 1 ppm B[*a*]P, had a significantly elevated ECOD activity which
209 increased with each concentration of B[*a*]P. This clearly demonstrated that moulting was not a confounding
210 factor in the interpretation of the response of cytochrome P450 detoxification in the shrimp to B[a]P exposure
211 in this study. The shrimp clearly demonstrated increasing ECOD activity induction with increasing exposure
212 concentrations.
213
214 Gender differences have been found to occur in the uptake and elimination of contaminants in a range of
215 invertebrate species due to competition between moulting, reproduction, vitellin levels and P450 activity
216 (McClellan-Green et al. 2007). Gender specific induction of ECOD was detected by Oberdorster et al.
217 (2000a) in *P. pugio* when exposed to pyrene at 63 ppb. In that study, pyrene induced ECOD activity in males
218 only. It was hypothesised that the lack of induction of ECOD in the female shrimp was due to the maternal
219 transfer of the pyrene, bound to egg yolk proteins, to oocytes (Oberdorster et al. 2000a). Gender differences
220 in ECOD activity induction was not evident in *P. australis* exposed to B[*a*]P in this study, however this
221 confounding factor needs to be considered when measuring ECOD activity in field captured *P. australis* by
222 ensuring sufficient numbers of each sex are collected for analysis and taking into account their stage in the
223 reproductive cycle.
224
225 Elevated sorbitol dehydrogenase (SDH) activity in abdominal muscle homogenate is an indicator that the
226 cellular integrity of the hepatopancreas has been compromised (Battison 2006). Sousa and Petriella (2007)
227 found important alterations in the hepatopancreas of *P. argentinus* exposed to high levels of organochlorine
228 pesticides including necrotic desquamation, lesions in the tubules, nuclear retraction, and lysis of the
229 chromatin and cytoplasm. A hepatopancreas with cellular injuries is less capable of detoxifying and
230 eliminating contaminants than a non-injured hepatopancreas and represents a further confounding factor
231 when interpreting ECOD activity supporting the parallel use of this marker of hepatopancreatic damage.
232 Furthermore, SDH activity precedes the detection of histological damage in the hepatopancreas and as such
233 monitoring changes in SDH activity can be a sensitive indicator of damage. The lack of inter-treatment
234 differences in SDH activity in this study indicates there is no bias in the ECOD activity measured related to
235 hepatopancreatic tissue damage. From the results of the laboratory exposure to B[*a*] P in the negative control

236 shrimp, a baseline level of 50 mU mL^{-1} SDH in the abdominal muscle homogenate of *P. australis* is
237 established which can be used in field studies to determine whether hepatopancreatic damage has occurred.
238
239 The concentration of 8-oxo-dG indicates DNA damage by hydroxyl radicals and has been shown to be
240 induced by a range of environmental contaminants (Evans et al. 2004; Valavanidis et al. 2009). Oxidative
241 damage has been reported in both humans and fish species in response to heavy metals, peroxides,
242 antibiotics, PAHs, asbestos fibres, and tobacco smoke (Valavanidis et al. 2009; Livingstone 2001). Exposure
243 to B[*a*]P resulted in a significant decrease in 8-oxo-dG concentrations in *P. australis* which appears to
244 contradict other studies in rat, hamster and human cancers (Kasai 1997), and aquatic organisms exposed to
245 heavy metals (Cd, Cu, Fe), paraquat, peroxides and PAHs (Livingstone 2001). However a similar decrease
246 has been found in barramundi exposed to B[*a*]P injections in our laboratory (Rawson in preparation). There
247 is some evidence to suggest there are two pathways for the removal of 8-oxo-dG adducts. Bases suffering
248 oxidative damage are preferentially repaired by enzymes of the BER (base excision repair) pathway (Loft et
249 al. 2008). If the BER pathway is compromised a NER (nucleotide excision repair) pathway exists for the
250 removal of the 8-oxo-dG adduct resulting in an observed reduction the amount of free 8-oxo-dG in the
251 haemolymph. A study undertaken by Hook and Lee (2004) found that exposure of *P. pugio* embryos to B[*a*]P
252 produced complex lesions. The DNA strand breaks persisted and their level increased as these lesions were
253 repaired by the NER pathway, whereas repairs by the BER pathway resulted in reduction of strand breaks
254 when the embryos were exposed to cadmium. This suggests that B[*a*]P causes persistent lesions with the
255 potential to increase transcriptional errors, mutagenesis and cell death (Mitchelmore and Chipman 1998).
256 From the results of the laboratory exposures a baseline level of 5 ng 8-oxo-dG mg Pr^{-1} is determined, which
257 can be used to evaluate field results.
258
259 DMSO is commonly used as a polar solvent of low toxicity and to preserve cells in tissue culture, however it
260 has been shown to produce adverse changes in rat hepatocytes (Shilkin et al. 1966), and to potentiate the
261 lethal effects of aromatic hydrocarbons such as benzene (Kocsis et al. 1975). DMSO has also been shown to
262 produce apoptotic degeneration in the developing CNS of mice (Hanslick et al. 2009). No change was
263 identified in SDH activity when compared to the negative controls suggesting that exposure to the low
264 concentration of DMSO (0.0005%) used in this study has not damaged the hepatopancreas of the shrimp. The
265 shrimp appear to have had a slight response to DMSO, with ECOD activity induction marginally higher in

266 the solvent control group compared to the negative control group and 8-oxo-dG concentration was lower.
267 However, this apparent reaction to DMSO is not sufficient to influence interpretation of biomarker responses
268 of the shrimp to the PAH, B[*a*]P.
269
270 *P. australis* has been shown to be responsive to organic contaminants and is a suitable bioindicator species
271 for laboratory studies and/or field monitoring to aid our understanding of the health of biota in the Swan-
272 Canning Estuary. Further investigation is required to assess the suitability of other biomarkers of health (e.g.
273 DNA strand breakage, ecdysteroid production, embryo survival and development, incidence of intersex and
274 others) in this crustacean.
275
276
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281

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283

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359
360
361

362 **List of Figures**

363

364 **Fig. 1** Ethoxycoumarin-O-deethylase activity induction in fmol H mg Pr^{-1} min^{-1} (mean ± SE) in the
365 freshwater shrimp (*Palaemonetes australis*) following exposure to benzo[*a*]pyrene. Treatment groups
366 significantly different from the negative control ($p < 0.05$) are denoted by an asterisk (*)

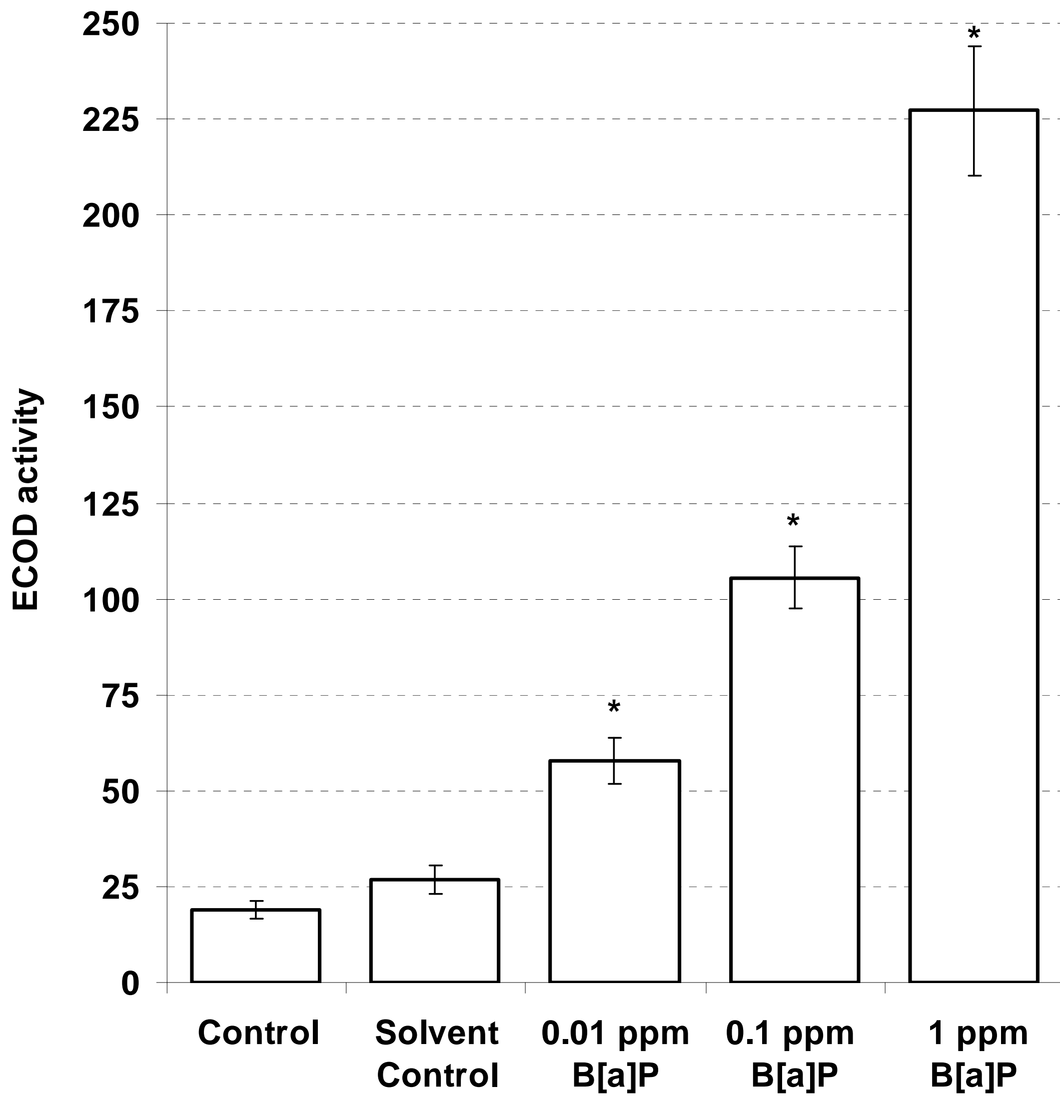
367

368 **Fig. 2** SDH activity (mU mL^{-1}; mean ± SEM) in the abdominal muscle supernatant of the freshwater shrimp
369 (*Palaemonetes australis*) following exposure to benzo[*a*]pyrene. Treatment groups significantly different
370 from the negative control ($p < 0.05$) are denoted by *

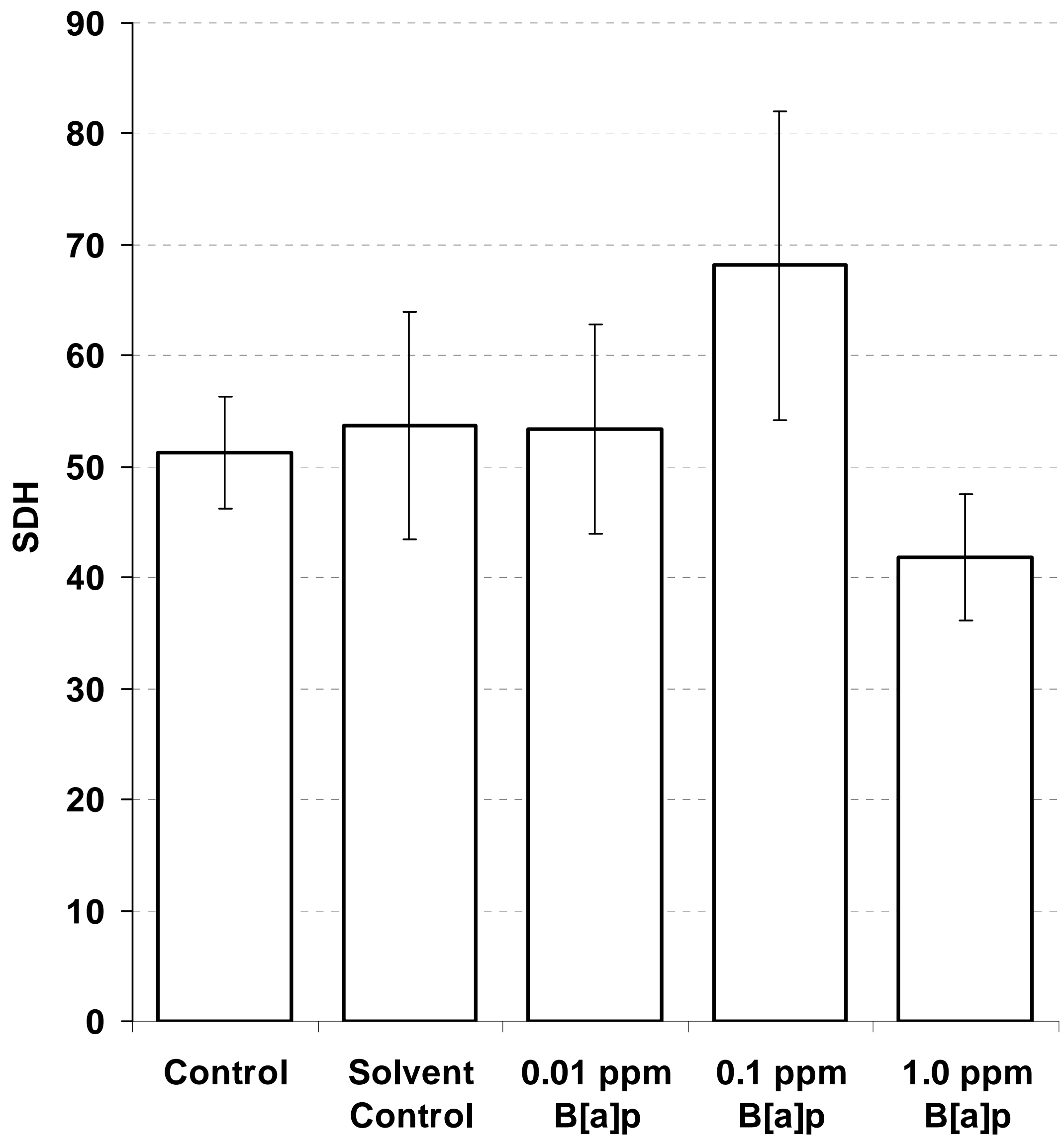
371

372 **Fig. 3** 8-hydroxy-2'-deoxyguanosine concentration in ng 8-oxo-dG mg Pr^{-1} (mean ± SE) in the freshwater
373 shrimp (*Palaemonetes australis*) following exposure to benzo[*a*]pyrene. Treatment groups significantly
374 different from the negative control ($p < 0.05$) are denoted by *

375



376



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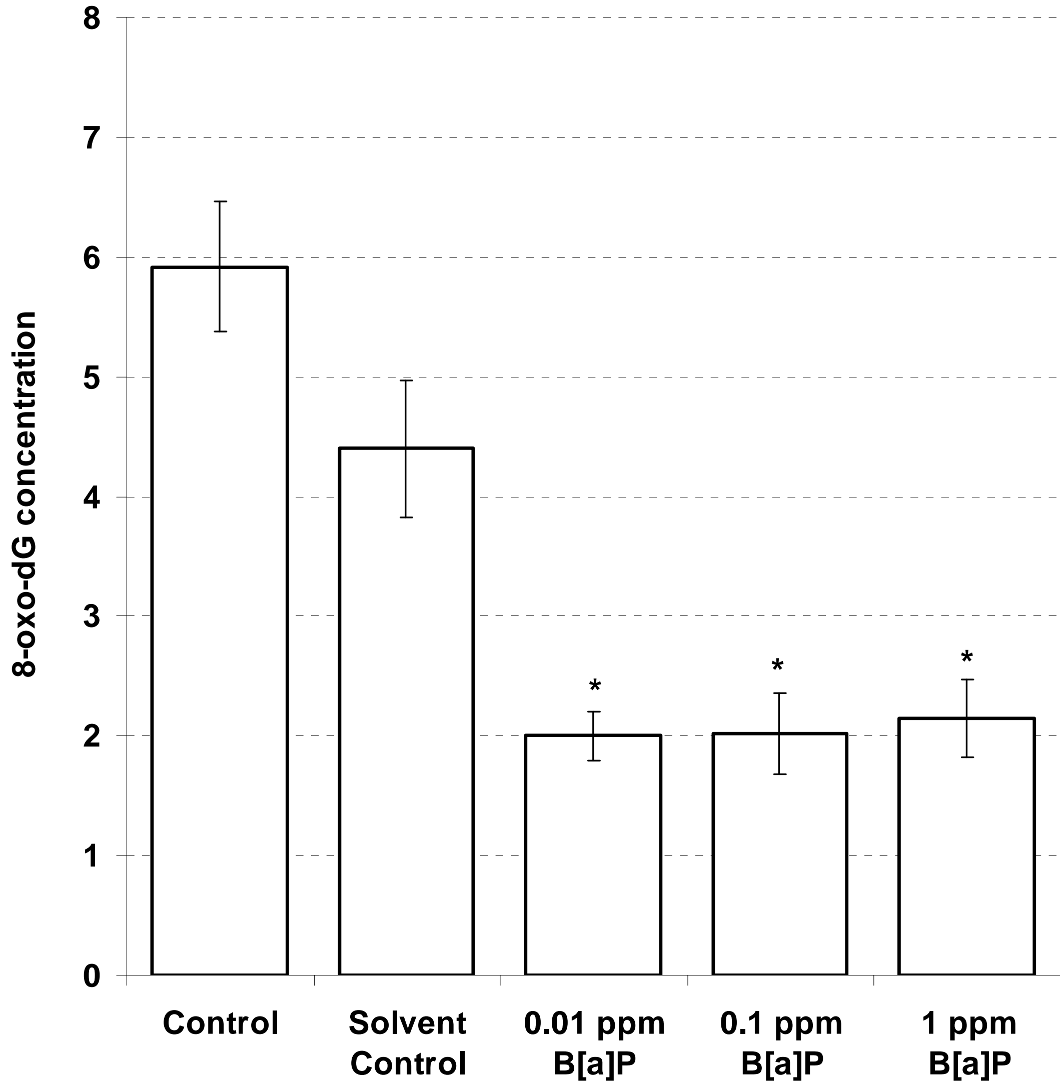

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