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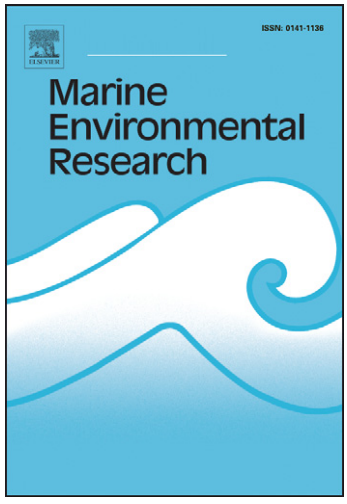

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Physiological responses of juvenile and adult shore crabs *Carcinus maenas* (Crustacea: Decapoda) to pyrene exposure

Awantha Dissanayake*[1], Tamara S. Galloway[2] and Malcolm B. Jones[1]

[1]School of Biological Sciences, University of Plymouth, PL4 8AA

[2]School of Biosciences, University of Exeter, EX4 4PS.

*corresponding author adissanayake@plymouth.ac.uk

Tel: +44 1752 23 2940

Fax: +44 1752 23 2970

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Abstract

This study measured aspects of the physiology of juvenile [(< 35 mm carapace width (CW)] and adult (> 60 mm CW) *Carcinus maenas* to test the hypothesis that these different life-history stages exhibit different sensitivities to environmental contamination. Newly-collected juveniles had significantly ($P < 0.05$) lower immunocompetence (phagocytosis and cellular integrity), lower metabolic energy (haemolymph glucose) and increased scope for growth compared with adults. Seven-day exposure to a sub-lethal concentration (200 µg L^{-1}) of pyrene significantly ($P < 0.01$) reduced immunocompetence, elevated basal heart rate and decreased respiration (at rest) for juveniles but had no overall impact on adult crab physiology. Results confirm that physiological differences exist between juvenile and adult shore crabs, and cause juveniles to be more susceptible to the effects of pyrene exposure than adults. Such differences in sensitivity to contamination between life-cycle stages of the same species have to be taken into account during the risk assessment process.

Keywords: *Carcinus maenas, physiology, pyrene, ontogenetic differences*

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INTRODUCTION

The shore crab *Carcinus maenas* (Decapoda: Brachyura) is a common inhabitant of various coastal habitats, including estuaries (Crothers, 1968), throughout Europe (Hayward and Ryland, 1995). It is used widely as a test organism in ecotoxicology (Bamber and Depledge, 1997; Fossi et al., 2000) but there has been no attempt previously to distinguish between the different benthic life-history stages (e.g. juveniles or adults) when utilising *C. maenas* as an indicator species of environmental contamination. However, *C. maenas* is a good model organism to use in this context as UK populations have relatively low genetic variability (Brian et al., 2006), reducing the problem of inter-population variability masking differences between life-cycle stages. It is well established that some aspects of shore crab physiology vary with developmental stage; for example, there is greater variation in heart rate of juvenile *C. maenas* compared to adults during the tidal cycle (Depledge, 1992; Newell et al., 1972). As the physiological tolerance of each life-cycle stage determines the survival potential of individuals (Hebel et al., 1997), it is important to establish the variability of response for all stages. Currently, there is a lack of information regarding the physiology of juvenile *C. maenas.* In this study, we compared aspects of the physiology of adult male and juvenile *C. maenas,* and tested the hypothesis that differences in physiology influence the ability to tolerate contaminant-induced stress (in the form of exposure to a sublethal concentration of pyrene exposure). Pyrene, a polyaromatic hydrocarbon (PAH), is one of a group of highly lipophilic organic compounds that originate from petrochemical pollution and from incomplete combustion processes (Clarke et al., 2001). PAHs are ubiquitous and persistent pollutants of the marine environment (Dissanayake and Galloway, 2004), and there is concern over their acute and chronic toxicity to marine organisms. The primary aim of this study was to establish if juvenile and adult *C. maenas* responded similarly to sublethal exposure of the PAH pyrene (200 µg L^{-1} for seven days). To achieve this aim, the physiological condition (assessed using a suite of end points at different levels of biological response) of juvenile and adult crabs was measured directly from the field (within 48 of collection), and after pyrene exposure.

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MATERIAL AND METHODS

Shore crab collection and maintenance

In November 2004, *Carcinus maenas* was collected from the Avon Estuary, Bantham, South-west England (grid reference: SX 6623 4380) using mackerel-baited traps. Previous studies have shown that the Avon Estuary has low levels of contamination (Hagger et al., 2008) and is categorised as 'not at risk' from both point source and diffuse pollution (Environment Agency, 2006). In the laboratory, male (green) intermoult individuals were measured [carapace width (CW) (mm)] and separated into juvenile (< 35 mm carapace width) and adult (> 60mm CW) (Crothers, 1967) size groups (*n* = 18/group); each was weighed (wet weight g) and labelled (labels attached to carapace with cyanoacrylate glue). Prior to measurements, newly-collected crabs were maintained for 48h in static holding tanks containing filtered (10 µm carbon filtered), aerated seawater (salinity of 34, 15 ± 1 ºC) under a 12h light: 12h dark photoperiod and not fed. During pyrene exposure (7 days), crabs were fed twice weekly with γ-irradiated cockle (*Cerastoderma edule*) (Gamma foods, Tropical Marine Centre, Bristol, U.K.) and water was changed within 18 h of each feeding.

Physiological assessment of newly-collected crabs

To gain a holistic assessment of shore crab physiology, within 48h of collection, a suite of measurements was taken at different levels of biological organisation, including biochemical and cellular (based on end points taken from the haemolymph), and physiological; the latter (components of scope for growth) were measured prior to haemolymph sampling. Haemolymph samples (100 µl) were extracted by puncture of the arthrodial membrane at the base of the 4th walking leg using a pre-chilled 1 ml syringe and 21 gauge needle (Sigma-Aldrich, U.K); samples were snap-frozen using liquid nitrogen and stored at -80 ºC for all subsequent analysis.

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Biochemical techniques

Total antioxidant status was determined by measuring the combined reducing power of the electron-donating antioxidants present (i.e. ferric reducing antioxidant potential) (Benzie and Strain, 1996; Rickwood and Galloway, 2004). A stoichiometric excess of the oxidant ferric
130 tripyridyltriazine (Fe^{III} -TPTZ) was added to each 10 µl of haemolymph sample (300 µl of 10 mM in 300 mM sodium acetate, pH 3.6) and its reduction to the ferrous form (Fe^{II}) was measured spectrophotometrically after 10 min at 593 nm (Griffin and Bhagooli, 2004). Glucose concentrations were assayed according to the method of Kunst et al. (1981). Haemolymph samples were deproteinated by addition of perchloric acid (0.6 M), neutralised
135 using potassium bicarbonate (2 M) and centrifuged (10,000 *g* for 20 min). The resulting supernatant was assayed for glucose concentrations using a glucose (Hexokinase) kit (Sigma, U.K). Glucose (rather than glycogen) was chosen as it represents an end point of the readily-available (free circulating) energy (Hames and Hooper, 2000) whereas glycogen measurements would represent any stored energy within the haemocytes (Johnstone et al.,
140 1973).

Cellular techniques

Cellular viability was determined *in vitro* using the microplate neutral red dye uptake method of Babich and Borenfreund (1991), whereby, incorporation of the dye within the lysosomes of
145 cells indicates viable cells, whereas decreased uptake and dye retention indicates dead or damaged cells. Two hundred microlitres of neutral red solution (0.004 %) were added to haemolymph samples (30 µl) in microtitreplates; after 3 h incubation (10 °C), cells were washed using phosphate buffered saline (phosphate buffer 0.01M, KCl 0.0027M, NaCl 0.137 M) and lysed with 1 % acidified ethanol solution (1 % acetic acid/50 % ethanol).
150 Residual dye was determined spectrophotometrically at 550 nm (Galloway et al., 2004). Cell functionality/integrity (i.e. dead or viable) was determined using a dye exclusion method; 2 mg ml^{-1} Eosin Y was added to haemolymph samples (20 µl) at a ratio of 1:10. Following 10min incubation and washing cycle, the absorbance of the residual dye was measured

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spectrometrically at 518 nm. Viable cells stain light green (due to dye exclusion) whereas
155 dead and moribund cells stain red. The phagocytic capability of haemocytes was evaluated by measuring the active uptake of neutral red-stained zymosan particles (from *Saccharomyces cerevisiae*, Sigma, U.K) *in vitro.* Haemolymph samples (30 µl) were incubated (10 °C for 30 min) with dye-stained particles (1 x 10^8 particles ml^{-1}) and washed using phosphate buffered saline to remove excess particles. Cells were lysed with 1 %
160 acidified ethanol solution and the amount of particles taken up was determined spectrophotometrically at 550 nm as described by Parry and Pipe (2004), and employed by Galloway et al. (2004).

Physiological techniques

165 Scope for growth (SFG), an indicator of the energy status of an organism, incorporates fundamental physiological processes (feeding, food absorption, respiration and excretion) (Bayne, 1984) and these components were measured from individual crabs. The overall net energy gain (*P*) through these physiological processes [energy absorbed (*A*) compared to energy respired (*R*)] was calculated using equation 1:

170 $$P = A - R \quad (1)$$

Where: $P = SFG$ (J mg dry wt^{-1} h^{-1}), A = energy absorbed (J mg dry wt^{-1} h^{-1}) and R = energy respired (J mg dry wt^{-1} h^{-1}) (Widdows et al., 1995).

Egestion rates (faecal production), measured as a surrogate for ingestion rates (as egestion
175 correlates with ingestion), were based on the method described by Roast et al. (1999). Pre-weighed food [γ-irradiated cockle tissue (Gamma foods, TMC, Bristol, U.K) 1 g ± 0.01 mg] was introduced to individual crabs (1 crab per 2 L tank) and faeces were collected 18h after feeding using a plastic Pasteur pipette. Faeces were rinsed with distilled water, stored in pre-weighed centrifuge tubes at -20 ºC for analysis and expressed as mg g dry $weight^{-1}$ h^{-1}
180 (see below). Crab absorption efficiency was estimated by the ratio method based on the proportion of the organic material (ash-free material) in the food and the faeces (Conover, 1966) and calculated using equation 2:

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(2) *Absorption efficiency* $= (F - E) / [(1 - E) \times F]$

185 Where:
F = ash free dry weight: dry weight ratio of food , E =ash free dry weight: dry weight ratio of faeces

Oven-dried (60ºC) material (food/faeces) (± 0.01mg) (dry weight) was ashed at 450ºC for 2h)
190 to ascertain the ash content of the organic material (ash free dry weight).

Oxygen consumption measurements were performed using 1 L perspex respiration chambers (with sealed o-rings) using recirculating system (15 ± 1 ℃ at a salinity of 34). Individual crabs were placed in each chamber (30 min acclimation) with recirculating sea
195 water (open chamber); water samples were taken at the start (t_0) and following 30 min (t_{30}) during static water (closed chamber) to determine dissolved oxygen concentrations (i.e. $t_0 - t_{30}$) (mg L^{-1}). Concomitant heart rate measurements demonstrated that 30 min acclimation was adequate for heart rate levels to decrease from 'active' (i.e. handling stress) to 'resting' levels. Oxygen concentrations were measured using a Cellox 325-3 oxygen probe (Multi
200 3401/SET WTW, Germany). After oxygen measurements were taken, crabs were removed from the test chambers and blotted dry using absorbent paper and wet-weighed (± 0.01 mg) using a balance (Sartorius B3100P) to allow for weight-specific expression of oxygen consumption rates (expressed as mg O_2 L^{-1} g wet wt^{-1} h^{-1}).

205 SFG was calculated by converting oxygen consumption and ingestion rates into energy equivalents, and calculating the net energy gain/loss using equation 3:

(3) $P = A - (R + U)$

Where:
P = SFG (J mg dry wt^{-1} h^{-1}), A = energy absorbed (J mg dry wt^{-1} h^{-1}), R = energy
210 excreted (J mg dry wt^{-1} h^{-1}).

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Components of equation 3 (A and R) were calculated using equations 4 and 5 and transformed into energy equivalents:

Energy absorbed:

215 (4) $A = C \times Absorption\ efficiency$

Where:
A = energy absorbed (J mg dry wt^{-1} h^{-1}), C = energy consumed (J mg dry wt^{-1} h^{-1})

220 Energy respired:

(5) $R = oxygen\ consumption\ (mg\ O_2\ g\ dry\ wt^{-1}\ h^{-1}) \times 0.02008$

R = energy respired (J mg dry wt^{-1} h^{-1}) oxygen consumption rates were converted to
225 dry-weight specific rates (*ca.* 20% for decapod crustaceans) (Roast et al., 1999), 0.02008 J(µl O_2^{-1}) = heat equivalent of oxygen uptake) (Gnaiger, 1983).

As ammonia excretion contributes < 5% of the metabolic energy expenditure (Roast et al.,
230 1999; Widdows and Salkeld, 1993; Widdows and Staff, 1997), it was not measured here; thus SFG was calculated from equation 1 ($P = A - R$).

Exposure to contaminant and post-exposure assessment

Juvenile and adult crabs (6 individuals per treatment) were exposed to 200 µg L^{-1} pyrene
235 (nominal concentration); sea water (control) and sea water with acetone (0.005 %) (solvent control) for 7 days; crabs were fed individually on 1 g of *γ*-irradiated cockle every two days and water was changed within 18h of feeding. After 7 days, the physiological end points described previously were measured together with the additional measurement of heart rate (30min recordings) using the CAPMON system (Depledge and Andersen, 1990). Heart rate
240 measurements were performed on individuals in 1 L respiration chambers (see above); sensors were glued to the carapace above the heart and, after 30 min acclimation, cardiac activity was recorded for 30 min.

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It was not possible to measure directly the pyrene concentration in the experimental tanks, however, to validate that crabs were exposed to the chemical, fluorescence analysis for 1-hydroxylpyrene-type metabolites (1-OH) was performed on diluted urine samples (10 µl in 50 % ethanol, ratio 1:20). Crabs were restrained onto a backboard and urine was collected using a 1 ml syringe with a 10 µl micropipette tip attached by prising open each opercula of the antennal gland. Pyrene toxicity results from membrane damage, and/or enzyme inactivation or damage by reactive oxygen species produced by pyrene metabolism (Livingstone, 1991). Exposure, and subsequent metabolism of PAH compounds by organisms, results in metabolites of the parent compound being concentrated in body fluids, tissues and excreta (Dissanayake and Galloway, 2004). The presence of such metabolites in crab urine is used here as a surrogate measurement for detecting exposure to bioavailable contaminants (Fillmann et al., 2002). Compared to controls, concentrations of 1-hydroxypyrene-type metabolites in the urine were significantly higher in juvenile and adult pyrene-exposed individuals (*Kruskal-Wallis,* TS = 13.86, $P < 0.05$), confirming exposure to the parent compound (pyrene) (Fig. 1).

Statistical analysis

Two methods of statistical analyses were used to isolate significant differences in physiology between newly-collected juvenile and adult *C. maenas,* and to identify differences in response to contaminant exposure. Univariate (*ANOVA or* Kruskal Wallis) analyses were performed to test for differences for a single parameter between treatments [using GMAV5 for Windows ® or StatGraphics® Plus v5 (Statistical Graphics Corporation) dependent upon homoscedasticity of data (Kruskal and Wallis, 1952)]. To obtain an integrated summary of contaminant impact, the PRIMER© v6 (Plymouth Routines in Multivariate Ecological Research, PRIMER-E Ltd) was used; multivariate analysis [ANOSIM (ANalysis Of SIMilarities)] tested for differences between the treatments using the total set of biological

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270 responses measured; ANOSIM *R* values are expressed between 0 and 1, (high values indicate discrimination between treatments) (Clarke and Warwick, 2001).

RESULTS

Physiological assessment of newly-collected crabs

There were significant differences in several biological responses between juvenile and 275 adult *C. maenas.* Univariate analyses identified that juvenile crabs had significantly higher cell viability (*ANOVA,* $F_{1,34}$ = 16.90, P < 0.01) (Fig. 2a), significantly elevated respiration rates (*ANOVA,* $F_{1,34}$ = 13, P < 0.01) (Fig. 2b) and significantly higher SFG (*ANOVA,* $F_{1,29}$ = 46.44, P < 0.001) (Fig. 2c) than adults, and significantly lower haemolymph glucose concentrations (*ANOVA,* $F_{1,34}$ = 5.62, P < 0.05) (Fig. 2d) and significantly reduced 280 phagocytic capability (*ANOVA,* $F_{1,34}$ = 7.07, P < 0.05) (Fig. 2e) than adults. Multivariate analyses of the entire set of end points confirmed that juveniles had a significantly different physiological status compared with adult crabs [R = 0.915, P (%) = 0.1].

Impact of pyrene on crab physiology

285 For juvenile crabs, univariate analyses identified that six out of the seven endpoints were significantly affected by pyrene exposure; pyrene-exposed juveniles had lower cellular integrity (*ANOVA,* SQRT-transformed $F_{5,30}$ = 2.54, P < 0.05) (Fig. 3a), elevated cellular viability (*ANOVA,* $F_{5,29}$ = 3.02, P < 0.05) (Fig. 3b), lower haemocyte phagocytic capability (50 %) (*ANOVA,* $F_{5,30}$ = 4.74, P < 0.01) (Fig. 3c), elevated basal heart rates (*ANOVA,* $F_{5,30}$ = 290 4.74, P < 0.01) (Fig. 3d), decreased respiration rates (*ANOVA,* $F_{5,30}$ = 4.39, P < 0.01) (Fig. 3e) and decreased energy consumption (*Kruskal-Wallis,* TS = 10.74, P < 0.05) (Fig. 3g) compared to unexposed (control) juveniles. There was no significant difference in SFG between newly-collected and post-exposure control juveniles ($F_{1, 19}$ = 2.28, P = 0.148). For adults, however, only two out of the seven endpoints were affected significantly; pyrene-295 exposed individuals displayed elevated phagocytic capability (*ANOVA,* $F_{5,30}$ = 4.74, P < 0.05) (Fig. 3c) and decreased scope for growth (*Kruskal-Wallis,* TS = 17.86, P < 0.01) (Fig. 3f) compared to unexposed (control) adults. Multivariate analyses confirmed that pyrene

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exposure significantly affected juvenile shore crab physiological condition compared with unexposed juveniles [R = 0.563, P (%) = 0.2]; however, no differences were observed between pyrene exposed and unexposed adult crabs [R = -0.036, P (%) = 60.8].

DISCUSSION

Measurements taken within 48h of collection from the field established that there were significant differences in several physiological end points between juvenile and adult *C. maenas*. Juveniles showed significantly elevated weight-specific respiration rates and significantly higher scope for growth, but had significantly lower immune function (phagocytic index) and reduced metabolic energy (in the form of glucose) compared with adults. The different respiration rates recorded here are consistent with those reported previously for juvenile (6.48 mg O_2 g h^{-1}) and adult (1.8 mg O_2 g h^{-1}) *C. maenas* (Arudpragasm and Naylor, 1964), and may be explained by the fact that weight-specific respiration rates decrease with increasing biomass (Dawirs, 1983). Higher scope for growth observed in juveniles is not surprising as juveniles grow faster than adults and, therefore, would be expected to channel more energy into somatic growth than adults (Styrishave et al., 2000). Adult crabs had higher haemolymph glucose concentrations than juveniles and these were similar to those reported previously for crabs at rest (0-1 mmol L^{-1}) (Sneddon et al., 1998; 1999; Thorpe et al., 1995). The total energy pool available to an individual is the sum of the circulating glucose and stored glycogen (Briffa and Elwood, 2004). As the latter was not measured, it is difficult to make too much of these differences in haemolymph levels of glucose except to say that higher concentrations in adults are consistent with behavioural differences between these two benthic stages; adults are more likely to become involved in energy-demanding activities (tidal migration and fighting behaviour) than juveniles (Briffa and Elwood, 2004; Sneddon et al., 1999; Thorpe et al., 1995). The lower immune capability of juvenile crabs (demonstrated *in vitro* by the capacity of haemocytes to ingest foreign particles) suggests

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that they are less able to combat infection of foreign particles such as bacteria compared to adults (Burnett et al., 2006); however, this remains to be tested.

On exposure to the same concentration of pyrene, significantly more biological endpoints were impacted for juvenile than adult shore crabs, with juveniles showing significantly decreased immunocompetence and cellular integrity compared with adults. Increased heart rate, and concomitant decrease in respiration rates, observed in juvenile crabs following pyrene exposure, are in accord with Spicer and Weber (1991) who reported a similar response in crustaceans exposed to heavy metals (copper and zinc at a sublethal concentration of 0.4 mg L^{-1}). Camus et al. (2004) reported increased cardiac activity for *C. maenas* exposed to copper (0.5 mg L^{-1} for three days) and interpreted this as an increase in oxygen demand to compensate for physiological malfunctions. Multivariate analyses, using the suite of biological end points, confirmed that juveniles were more susceptible to the contaminant than adults. In conclusion, significant physiological differences were measured between recently-collected adult and juvenile *C. maenas*, and these differences were manifested in different susceptibilities to pyrene exposure. Juveniles displayed increased sensitivity to contaminant-imposed toxicity compared to the adult form. The implications of present findings for environmental risk assessment are that basing 'safe' concentrations of discharge of chemicals on the tolerances of adult organisms may not be sufficient to protect other, more sensitive, life-cycle stages; to understand the total potential impact of contaminant exposure, all life-cycle stages of organisms need to be studied. In addition, environmental contamination must be avoided in ecological 'nursery' areas, such as estuaries, where the most sensitive life-stages of several species are found (Epifanio et al., 2003; Haywood et al., 1998; Moksnes et al., 2003).

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List of Figures

Fig. 1 Concentrations of 1-OH pyrene-type metabolites from urine of juvenile and adult *Carcinus maenas* ($\lambda_{340/420}$) (µg L^{-1}; mean ± 1 SE), * signify exposures are significantly different from controls $P < 0.05$ (n = 6 $group^{-1}$, however, numbers in brackets indicate crabs per treatment where urine was successfully sampled).

Fig. 2 Assessment of newly-collected juveniles and adult *Carcinus maenas* (n = 18):
a) Cell viability (O.D. mg protein-1; mean ± 1 SE);
b) Respiration rate (mg O_2 g^{-1} L^{-1} hr^{-1}); mean ± 1 SE);
c) Scope for growth (J mg^{-1} dry wt^{-1} h^{-1}); mean ± 1 SE);
d) Glucose concentrations (µg L^{-1}; mean ± 1 SE);
e) Phagocytosis (zymosan particles 10^5 mg $protein^{-1}$; mean ± 1 SE).

Fig. 3 Post-exposure (200 µg L^{-1} pyrene) assessment of juvenile and adult *Carcinus maenas* physiology (*n* = 6) based on changes in biological responses compared to controls:
a) Cellular integrity (%) (mean ± 1 SE);
b) Cellular viability (O.D. mg $protein^{-1}$) mean ± 1 SE) (n = 6);
c) Phagocytosis (zymosan particles 10^5 mg $protein^{-1}$); mean ± 1 SE) (n = 6);
d) Heart rate (beats min^{-1}; mean ± 1 SE) (n = 6);
e) Respiration rates (mg O_2 g dry wt^{-1} L^{-1} hr^{-1}); mean ± 1 SE) (n = 6);
f) Scope for growth (J mg^{-1} dry wt^{-1} h^{-1}); mean ± 1 SE) (n =6);
g) Energy consumed (%) (mean ± 1 SE) (n = 6).
* and** signify exposures are significantly different from controls $P < 0.05$ and $P < 0.01$, respectively).

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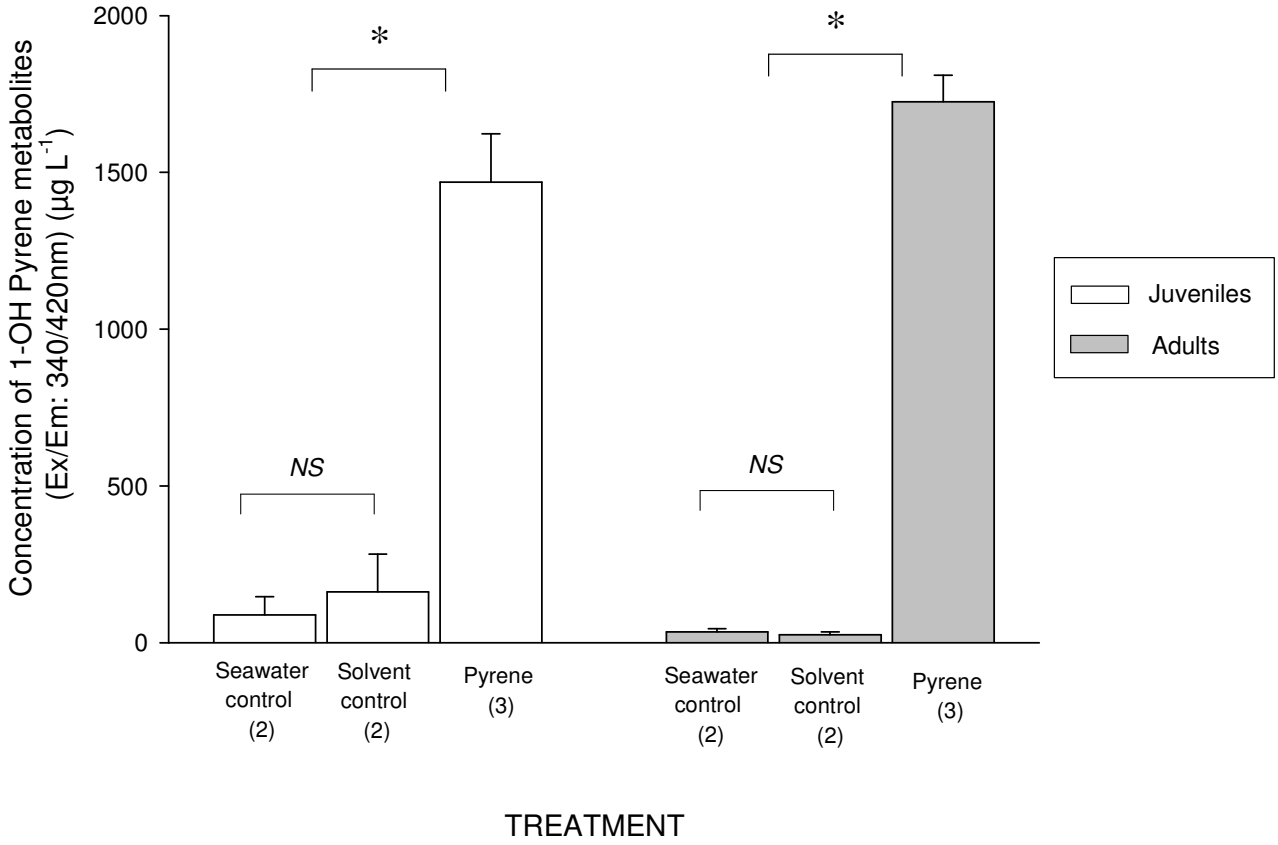

Fig. 1

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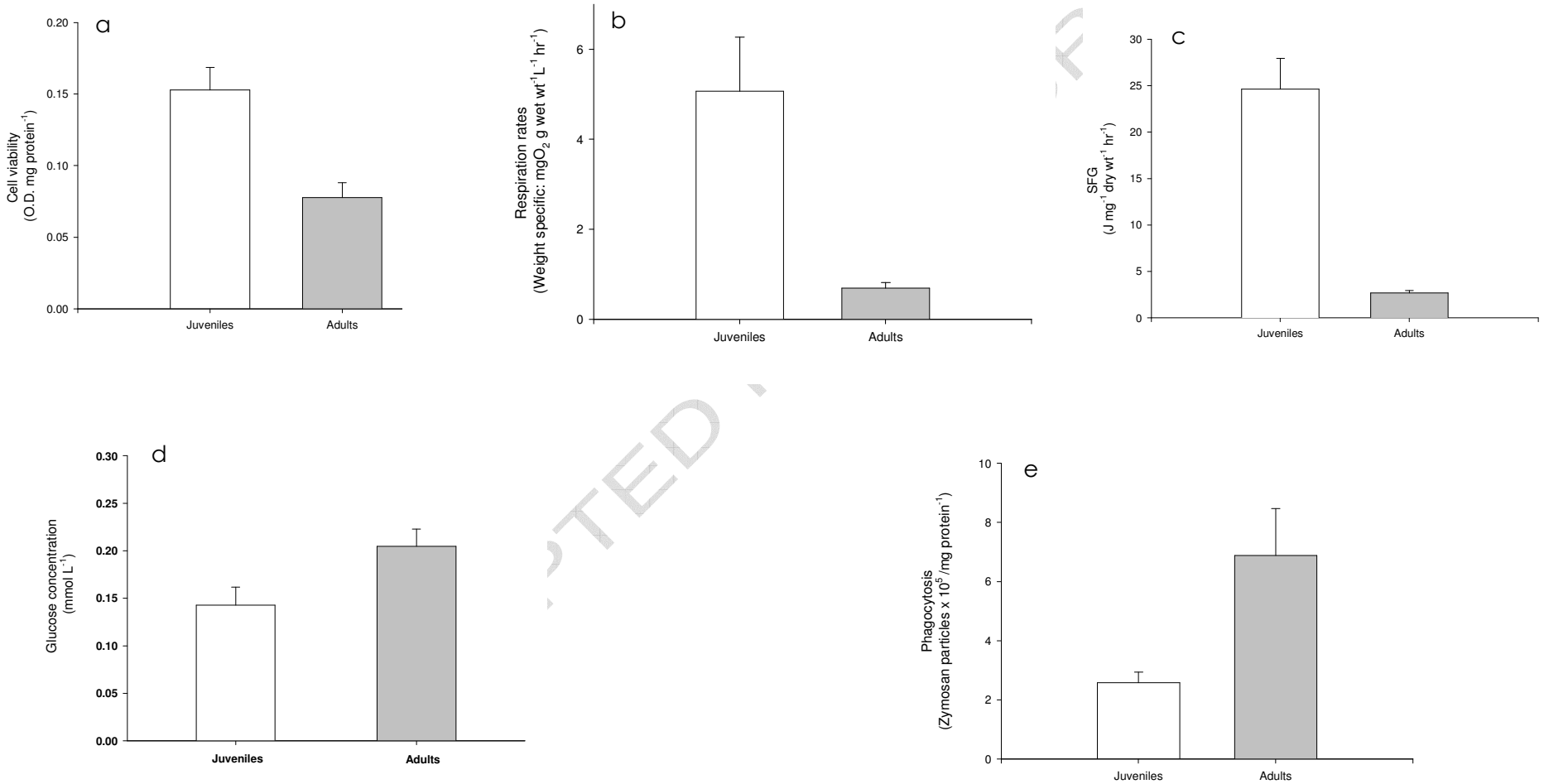
a
Cell viability
(O.D. mg protein^{-1})
0.20
0.15
0.10
0.05
0.00
Juveniles
Adults
b
Respiration rates
(Weight specific: mgO$_2$ g wet wt^{-1} L^{-1} hr^{-1})
6
4
2
0
Juveniles
Adults
c
SFG
(J mg^{-1} dry wt^{-1} hr^{-1})
30
25
20
15
10
5
0
Juveniles
Adults
d
Glucose concentration
(mmol L^{-1})
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Juveniles
Adults
e
Phagocytosis
(Zymosan particles x 10^5 /mg protein^{-1})
10
8
6
4
2
0
Juveniles
Adults

Fig. 2

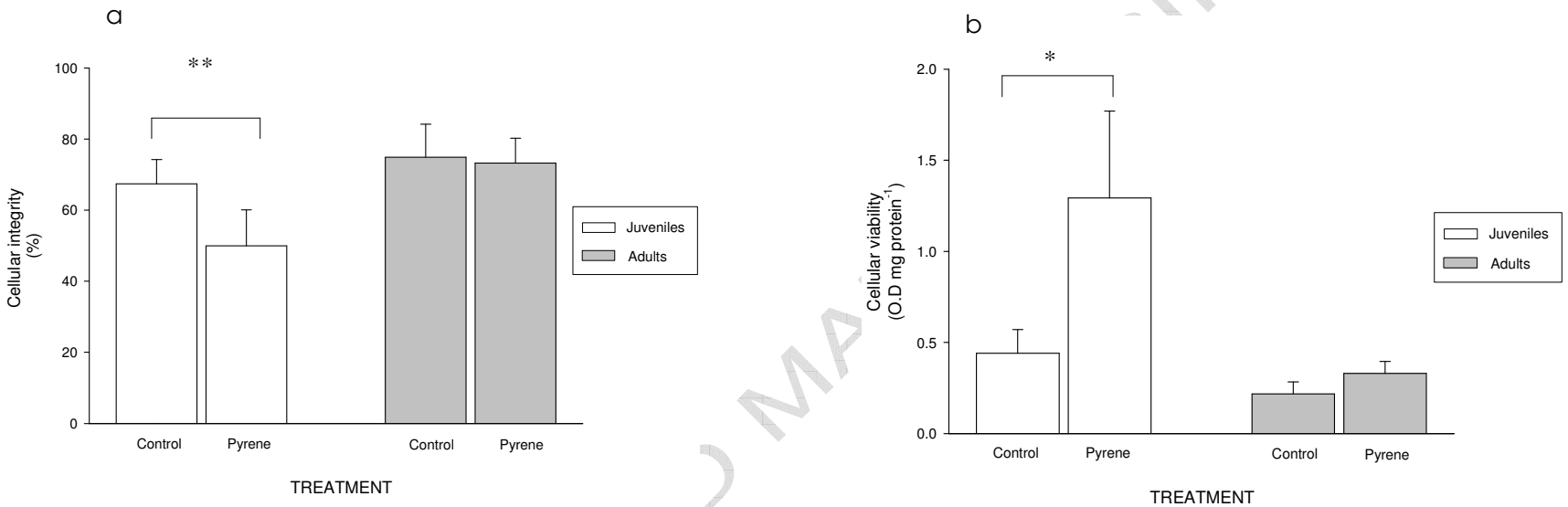

Fig. 3

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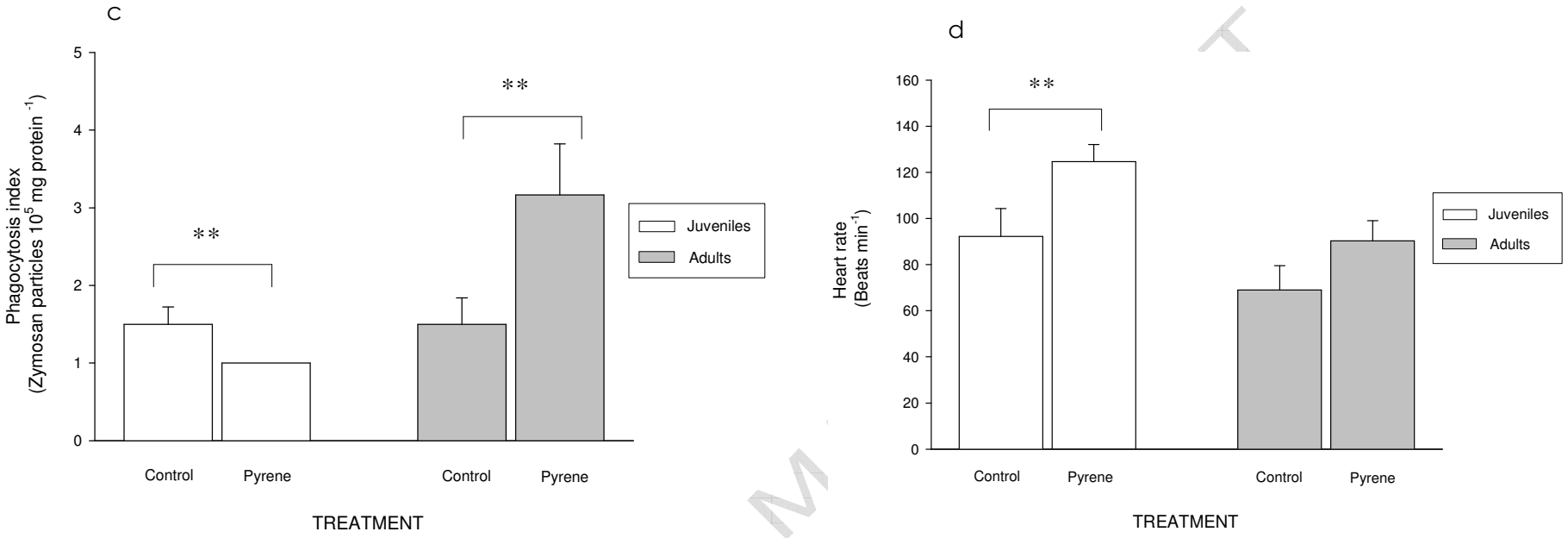

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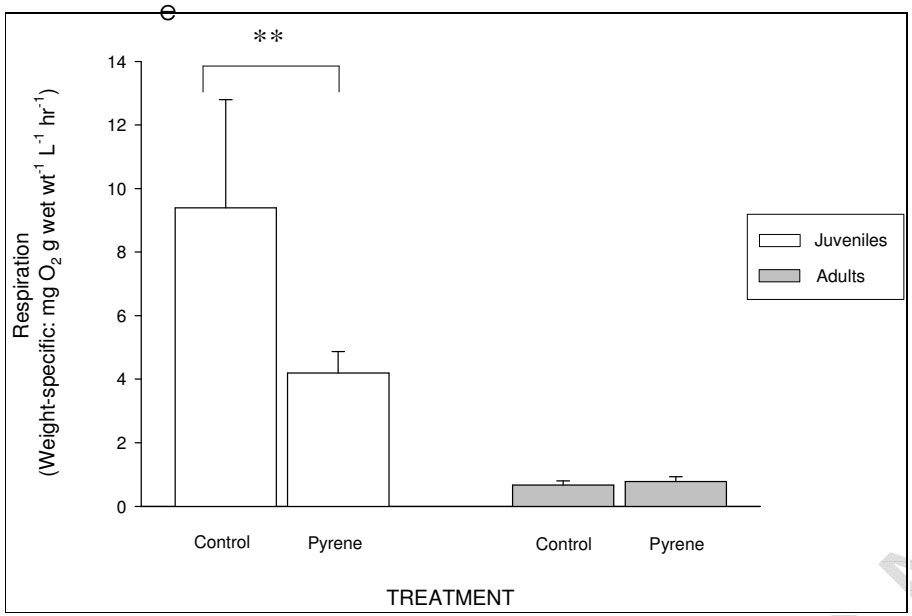
Fig. 3



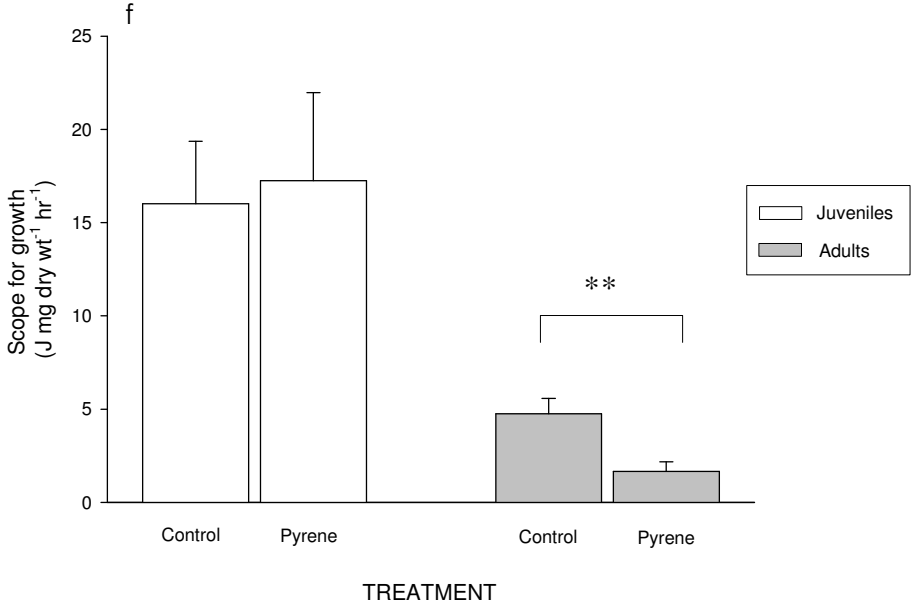


Fig. 3

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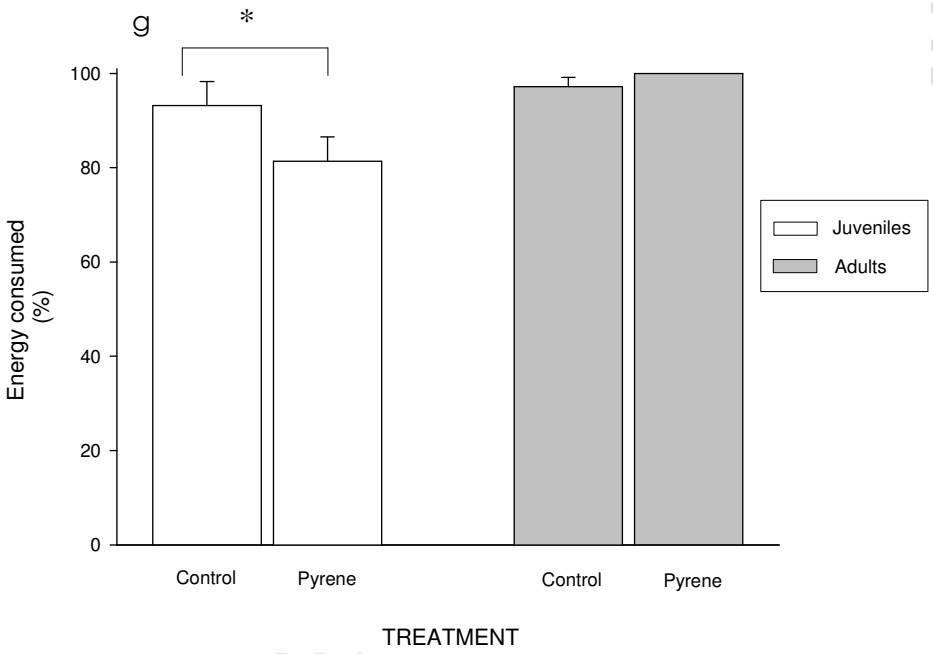

Fig. 3

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