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Prolonged phenanthrene exposure reduces cardiac function but fails to mount a significant oxidative stress response in the signal crayfish (*Pacifastacus leniusculus*)

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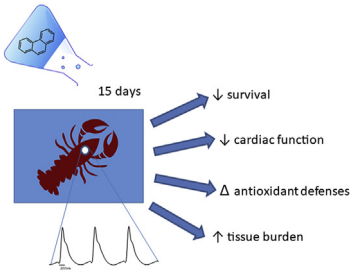
HIGHLIGHTS

- The 3 ring PAH phenanthrene is common in freshwater systems but its impact on the hearts of crustaceans is unknown.
- 15-day phenanthrene exposure impairs maximum heart rate and impacts the electrocardiogram of the signal crayfish.
- Phenanthrene induces a limited and tissue-specific response in antioxidant enzymes activity.
- Phenanthrene bioaccumulates and decreases survival, possibly due to reduced cardiac function and limited antioxidant defense.

GRAPHICAL ABSTRACT



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ABSTRACT

Crustaceans are important ecosystem bio-indicators but their response to pollutants such as poly-aromatic hydrocarbons (PAHs) remains understudied, particularly in freshwater habitats. Here we investigated the effect of phenanthrene (at 0.5, 1.0 and 1.5 mg L^{-1}), a 3-ringed PAH associated with petroleum-based aquatic pollution on survival, in vivo and in situ cardiac performance, the oxidative stress response and the tissue burden in the signal crayfish (*Pacifastacus leniusculus*). Non-invasive sensors were used to monitor heart rate during exposure. Phenanthrene reduced maximum attainable heart rate in the latter half (days 8–15) of the exposure period but had no impact on routine heart rate. At the end of the 15-day exposure period, the electrical activity of the semi-isolated in situ crayfish heart was assessed and significant prolongation of the QT interval of the electrocardiogram was observed. Enzyme pathways associated with oxidative stress (superoxide dismutase and total oxyradical scavenging capacity) were also assessed after 15 days of phenanthrene exposure in gill, hepatopancreas and skeletal muscle; the results suggest limited induction of protective antioxidant pathways. Lastly, we report that 15 days exposure caused a dose-dependent increase in phenanthrene in hepatopancreas and

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Action potential
Electrocardiogram (ECG)
Bioaccumulation

heart tissues which was associated with reduced survivability. To our knowledge, this study is the first to provide such a thorough understanding of the impact of phenanthrene on a crustacean.

1. Introduction

Freshwater ecosystems are under increasing threat due to geomorphological alterations, land use changes, water abstraction, nutrient overloading, invasive species, and pollution (Dudgeon et al., 2005). The latter includes crude oil spills, which are a major source of poly aromatic hydrocarbon (PAH) pollution in aquatic environments (Piazza et al., 2016). Other significant sources of PAHs include transportation, atmospheric deposition from incomplete combustion, and wastewater discharges from municipal and industrial sources (Wei et al., 2015; Piazza et al., 2016). Previous studies have reported developmental deformities in marine fish and the freshwater zebrafish exposed to PAHs, including pericardial and yolk sac oedema (Incardona et al., 2006, 2012; Carls et al., 2008; Li et al., 2011; Sorhus et al., 2016), which have been used as indicators of crude oil toxicity (Incardona et al., 2012). Some of the most abundant fractions of PAHs in crude oil are the lower molecular weight PAHs (containing 2 or 3 fused aromatic rings) which have been shown to be cardiotoxic to a range of fish species during development and at adult life stages (Incardona et al., 2009, 2012, 2015; Zhang et al., 2013; Brette et al., 2014, 2017; Edmunds et al., 2015; Nelson et al., 2016; Heuer et al., 2019; Ainerua et al., 2020). Our recent work (Brette et al., 2017; Ainerua et al., 2020) and that of others (Incardona et al., 2004; Carls et al., 2008; Vehniäinen et al., 2019) implicate phenanthrene ($C_{14}H_{10}$), a 3-ringed low molecular weight PAH, as *the* cardiotoxic component of crude oil (Brette et al., 2017).

Phenanthrene is a ubiquitous, persistent, bioavailable pollutant that can readily penetrate membranes and bioaccumulate over time (Mumtaz et al., 1995; Abdel-Shafy and Mansour, 2016) (Yin et al., 2007). Phenanthrene disrupts fish heart function in two key ways; (1) it inhibits the cycling of calcium to and from the myofilaments in the heart cells (cardiomyocytes) thus depressing cardiac contractility (Brette et al., 2014, 2017; Ainerua et al., 2020), and (2) it affects the electrical activity of the heart prolonging the action potential duration (APD) and the QT interval of the electrocardiogram (ECG) (Brette et al., 2017; Ainerua et al., 2020). QT and AP prolongation are due, at least in part, to phenanthrene's inhibitory action on erg (ether-a-go-go) ion channels which carry the repolarising delayed rectifier potassium current (I_{Kr}) (Brette et al., 2017; Ainerua et al., 2020). Compounds that block this channel are known to be pro-arrhythmogenic, indeed, QT prolongation has been linked to human cardiac failure (Etheridge et al., 2019) and thus assessment of inhibitory activity on erg channel activity (or I_{Kr} current) leading to delayed ventricular repolarization is a key component of any environmental/pharmacological safety strategy. Therefore phenanthrene has been included in the list of priority pollutants by the United State Environmental Protection Agency (US, EPA) (Piazza et al., 2016) for monitoring and regulatory purposes. Despite numerous reports of PAH cardiotoxicity in fish and other vertebrates, there are no studies specifically investigating the impact of PAHs on heart function of an invertebrate. This is surprising, given that invertebrates play an important role in the food web (Cock et al., 2012) and make up 70% of the animals commonly used as pollutant indicator species (Siddig et al., 2016).

In addition to being cardiotoxic, PAHs also induce cellular toxicity by increasing the production of reactive oxygen species (ROS), leading to oxidative stress (Yilmaz et al., 2007; Hannam et al., 2010; Piazza et al., 2016). For example, phenanthrene can absorb UV light which excites its upper energy state, leading to the formation of photodegradation products, such as phenanthrenequinone (PHQ). These by-products undergo redox cycling with cellular electron donors to generate superoxide and other ROS such as hydrogen peroxide (Bolton et al., 2000). Furthermore, quinones bind with cellular nucleophiles leading to the depletion of glutathione, an important endogenous antioxidant (Rodriguez et al., 2004). The combination of a compromised antioxidant system and the overproduction of ROS is predicted to cause cell toxicity. Indeed, PAH's and their photo-modified products have been reported to cause oxidative stress in a wide range of organisms, including invertebrates (Hasspieler and Digiulio, 1994; Xie et al., 2006). To counteract oxidative stress, organisms can increase the concentration and/or activity of antioxidants, such as catalase (CAT) and superoxide dismutase (SOD), which help to limit cellular damage caused by ROS production (Ye and Zou, 2008; Koenig et al., 2009; Silva et al., 2013; Bhagat et al., 2016). Hence, a change in the activity of antioxidant defence systems has been used as an indicator of toxicant exposure (Ye and Zou, 2008; Koenig et al., 2009; Silva et al., 2013; Bhagat et al., 2016).

The aim of this study was to perform the first investigation of the impact of phenanthrene on cardiac function and antioxidant capacity in an invertebrate species. We chose the signal crayfish (*Pacifastacus leniusculus*) because it is a highly invasive species that has caused major disruption to European freshwater ecosystems. For example, *P. leniusculus* has contributed directly (via competition) and indirectly (as a carrier of the crayfish plague (*Aphanomyces astaci*)) to the demise of the native white-clawed crayfish (Austropotamobius pallipes) in the United Kingdom. The signal crayfish is also an important aquaculture species for human consumption. The hearts of decapod crustaceans such as the signal crayfish have cardiac contraction mechanisms similar to vertebrates such as mammals and fish and employ similar ionic conductance for myocardial contraction (Yazawa et al., 1999; Shinozaki et al., 2002; Guadagnoli et al., 2007). Both crustacean and vertebrate cardiomyocytes undergo excitation-contraction coupling (Guadagnoli et al., 2007) linking the action potential to calcium influx which is propagated from cell to cell to result in electrical and contractile coordination of the heart (Lindeman, 1928)· (Hernandez and Mendez, 1992). However, one striking difference from vertebrates is that the decapod crustacean heart is neurogenic which means that electrical excitation (and thus cardiac contraction) is initiated by nerve cells housed in the cardiac ganglia inside the dorsal portion of the heart muscle (McMahon, 1999), not a pace-maker contained within a sino-atrial node (myogenic). The intersection of neuronal and cardiac systems for maintenance of heart function in the crayfish make it a particularly interesting model for studying PAH cardiotoxicity. Therefore, this study employed the signal crayfish as a model invertebrate to investigate the impact of 15 days of phenanthrene exposure on survival, cardiac function, bioaccumulation and antioxidant capacity. We hypothesized that phenanthrene exposure would cause bioaccumulation, disruption of electrical coupling and contractility of the heart, and activation of tissue antioxidant defence pathways.

2. Materials and methods

2.1. Crayfish collection and exposure

Signal adult crayfish (sex not determined) were purchased from a licenced supplier (Continental Crayfish, Oxford, UK), and upon arrival at the University of Manchester, were held for 2 weeks at 10 °C in a large tank (100 L) with approximately 80 L of aerated dechlorinated tap water and contained plastic pipes to serve as refuges (Guan, 2010). CaCl (80 mg L^{-1}) was included in the water to enhance exoskeleton health and all animals were in good health. At the start of the experiment, crayfish (N = 40, mean mass 62.05 ± 2.1 g, mean carapace length 5.79 ± 0.1 cm, mean total length 11.73 ± 0.1 cm) were tagged for individual recognition, weighed and measured and then transferred in pairs to treatment tanks (glass, 40 × 20 × 20 cm, containing in 4 L water and opaque glass beakers as shelters to minimise stress. The crayfish were held in these tanks to acclimate to the study conditions for 10 days before exposure began. During this time, baseline heart rate was measured (as described below). Throughout this acclimation period, all animals were healthy, and no signs of distress were observed. The tanks were then assigned to 1 of 4 treatments: control (i.e. no exposure), low, mid and high phenanthrene exposure (see below for concentrations). Each treatment group was made up of 5 identical replicate tanks per treatment with two crayfish per tank (n = 10 per treatment). Water temperature was maintained at 10 °C through the acclimation and study period and water quality was monitored daily using a YSI multimeter for dissolved oxygen (always >80% air saturation) and pH (always ~ 7.4) and nitrate, nitrite and NH_3/NH_4 concentrations that were <12.5 mg L^{-1}, <0.3 mg L^{-1} and <0.25 mg L^{-1}, respectively, and did not differ between treatments. Crayfish were offered food (sliced carrots) every second day throughout the study period. Any small amount of uneaten food was removed from the tank during the water changes that occurred every 48 h.

Phenanthrene exposure was achieved by creating stock solutions made by dissolving 1, 2 or 3 g phenanthrene (98% purity Sigma-Aldrich, UK) each in 10 ml acetone. To reach the final test concentrations of 0.5, 1.0 and 1.5 mg L^{-1} (low, mid and high, respectively), 200 μL of the appropriate stock solution was added to the 4 L exposure tanks whilst the control tanks received 200 μL acetone only. These exposure concentrations were based on published values from invertebrate phenanthrene toxicity (Sese et al., 2009) including those of the fresh water crustacean, *Daphnia magna* where an EC_{50} of 5.33 μM (~1 mgL^{-1}) was observed (Xie et al., 2006). Moreover, the exposure ranges were chosen to reflect the range of PAH levels found in polluted freshwater environments such as rivers and creeks in Ogoniland (Nigeria) where surface levels reach as high as 7.4 mg L^{-1} (Lindén and Pålsson, 2013). PAHs also accumulate in sediments where crayfish build burrows with samples from the Mersey estuary (UK) having PAH concentrations of 3.8 mg L^{-1} (dry weight) and phenanthrene concentrations between 0.05 and 0.25 mg L^{-1} (dry weight). Thus, while we acknowledge the exposure concentrations used here occur in a natural setting, we also acknowledge they are relatively high, but our intent is to demonstrate the potential mechanism(s) of toxic effect and thus we feel they are appropriate. Preliminary experiments used UV absorbance (measured at 251 nm) to assess changes in phenanthrene concentration in the aerated tanks over time (Rivera-Figueroa et al., 2004; Falconer et al., 2015). In comparison to newly spiked water, absorbance declined by ~80–85% after 24 h and to >90% of after 48hrs (range depending on dose). Thus, a semi-static renewal method was employed whereby the water in each tank was spiked every 24 h and 100% of the water in each tank was replaced every 48hrs to maintain adequate exposure, water quality and to minimise disturbance. Shelters were replaced when water was changed. Exposures were maintained for 15 days and in vivo heart rate (HR) was again measured at regular intervals throughout (see below). Effect of phenanthrene exposure on crayfish survival was determined by recording the number of deaths at the various concentrations throughout the 15-day period. At the end of exposure period, crayfish were prepared for cardiac electrical recording after which the animals were euthanized by ice-chilling followed by decapitation (Gunderson et al., 2018) and their gills, skeletal muscle, hepatopancreas and heart were dissected out and snap frozen and stored immediately at −80 °C. All procedures were in accordance with Animal Welfare and Ethical Review Board of the University of Manchester, UK.

2.2. Non-invasive heart rate recording during exposure

During the 15-day exposure period, in vivo HRs were measured using a non-invasive cardiac activity monitoring system (Pautsina et al., 2014) based on the methods developed by Ern et al. (2014) (Ern et al., 2014). In brief, a reflective infra-red LED sensor (AMP03, Newshift Ltd, Leiria, Portugal) was fitted into a plastic tube which was glued to the crayfish carapace just above the heart holding the sensor in place even if the crayfish moved. The sensors monitor the scatter of the incident light from the LED which varies when heart (located just below the sensor) is filled and emptied of haemolymph. The output of the sensors were connected to an amplifier linked to a Power Lab 4/35 system (ADInstruments, Ltd, UK), allowing the measurement of cyclic changes in light scattering which corresponded to cyclic filling and emptying of haemolymph in the crayfish heart thus providing a in vivo measure of crayfish HR. Output signals were filtered and recorded using LabChat 8.0 software (ADInstruments Ltd, UK).

Preliminary studies indicated that routine HR (i.e. HR in resting undisturbed crayfish) was quite variable in crayfish. Thus, a protocol was designed whereby maximum and routine HR would be established for each crayfish every 48hrs. Briefly, a crayfish was removed from its treatment tank and placed in a recording tank containing the same water conditions as its treatment tank. Maximum HR was achieved by touching the crayfish's antennae and to incite aggressive behaviour for 30 s (duration determined in preliminary trails). The HR sensor was then attached to the carapace, the room was darkened, and the crayfish was left for 60 min during which time HR fell from its maximum level to a routine level. Preliminary studies showed longer recording times (up to 3 h) did not result in significantly lower routine HRs. Maximum HRs were recorded in the first few minutes after connecting the crayfish to the HR sensors and fell to routine values during 60 min providing a HR recovery curve for each animal every 48 h during the 15-day exposure. By measuring the maximum HR and its recovery to routine levels, we hoped to reduce some of the variability in routine HR measurements and be able to assess any effects of phenanthrene exposure on in vivo heart function.

2.3. In situ electrical recording using a semi-isolated heart preparation

After the 15-day exposure period and before tissue extraction for enzymatic analysis and toxicity burden, the crayfish where anaesthetized in ice and the carapace above the heart cut away to reveal the pericardium containing the heart and the cardiac ganglia. The preparation was immediately transferred into a 1 L testing chamber containing aerated crayfish saline [(mmol l^{-1}): 205.1 NaCl, 5.4 KCl, 13.5 $CaC1_2$, 2.6 $MgCl_2$, 2.2 $NaHCO_3$, pH 7.72, at 10 °C]. Note that the in situ heart activity recordings were made in

the absence of additional phenanthrene doses and thus the measurements represent the effect of the 15-day phenanthrene exposure period on cardiac electrical activity. Monophasic action potentials (MAPs) and electrocardiograms (ECGs) were recorded from the semi-isolated heart using surface electrodes connected to a PowerLab system running LabChart 8.0 (ADInstruments, Ltd, UK). The QT duration was extracted from the ECG data and because measurements were made using intrinsic HR (due to the inability to pace the heart), the QT duration was subsequently corrected using Bazett's formula; QTc = QT/SQRT (60/HR) (Bazett, 1920; Tischer et al., 2016). Monophasic action potential duration (MAPD) was calculated at $MAPD_{90}$ (90% of repolarization) while triangulation (a measure of dispersion) was calculated as $MAPD_{90}$-$MAPD_{30}$. The MAP data was also corrected for spontaneous HR using Bazett's formula ($cMAPD_{90}$-B); $cMAPD_{90} = (MADP_{90}/(CL)^{1/2})$ (Doss et al., 2012), where CL (cycle length) = 60/HR.

2.4. Enzymatic assessment of oxidative stress

The effect of phenanthrene on the activity of antioxidants was assessed by measuring the activity of SOD and the total oxyradical scavenging capacity (TOSC) in the gills, muscle and hepatopancreas. Snap frozen tissues were first prepared by homogenization of 50 mg of tissue in ice-cold 50 mM phosphate buffer (pH 7.8) using a FastPrep-24™ 5G homogeniser. The homogenates were centrifuged at 1600×*g* for 5 min at 4 °C and the supernatants were collected and immediately frozen at −80 °C. The protein content of the homogenate was measured with the Bradford assay kit (Bio-Rad Laboratories, Inc, Quick Start™).

SOD was measured according to Weydert et al. (Weydert and Cullen, 2009) by the reduction of nitroblue tetrazolium (NBT) to water-insoluble blue formazan. The final concentration of the reaction mixture contained 50 mM phosphate buffer (pH 7.8), 1 mM diethylenetriaminepentaacetic acid (DETAPAC), 56 μM NBT, 100 μM Xanthine, 50 μM bathocuproine disulphonic acid (BCDA), 0.13 mg ml^{-1} bovine serum albumin (BSA), 1 U catalase, and xanthine oxidase which was diluted to a concentration that led to an optical density range between 0.02 and 0.05 in the absence of material. The rate of NBT reduction was monitored at 560 nm at 25 °C with a Biotek Synergy HTX multimode reader. Different dilutions of the same material were carried out and one unit of SOD was defined as the amount of protein that resulted in 50% inhibition of the rate of NBT reduction.

The TOSC assay tests the total capacity of tissue antioxidants to reduce hydrogen peroxide to water, including ascorbate, vitamin E, uric acid, metallothionein, catalase, glutathione peroxidase and glutamate cysteine ligase (Sroka and Cisowski, 2003). Five mg/ml of tissue homogenate was added to the final assay solution, containing 50 mM sodium citrate, 10 mM amplex red and 1U/ml horse radish peroxidase (HRP). The reaction was started by the addition of 40 μM hydrogen peroxide and the absorbance was monitored at 570 nm for 10 min. Hydrogen peroxide clearance was expressed relative to units of catalase activity; 1 unit is defined as the quantity of protein necessary to decompose 1.0 μmole H_2O_2 per minute.

2.5. Crayfish tissue phenanthrene burden

To assess the potential for crayfish to bioaccumulate and/or detoxify phenanthrene, hepatopancreas and pooled heart tissues were analysed offsite by Concept Life Sciences (Manchester, UK (https://www.conceptlifesciences.com/) using the USEPA method 8270C for semi volatile compounds by gas chromatography mass spectrometry (GCMS) (EPA, 1996). Briefly, the tissues were prepared using automated soxhlet extraction (ASE) according to the EPA SW846 method 3541 (EPA, 1994). Tissue samples were weighed; hepatopancreas (2.3 ± 0.3, 2.9 ± 0.5, 3.1 ± 0.6, 2.5 ± 0.6 g, n = 4 for control, low, mid and high treatment groups, respectively) and heart tissues (1, 0.4 and 0.6 g, n = 6–9, of pooled weight, respectively for control, low, and mid treatment groups, respectively) and homogenized. Each of the homogenized samples were spiked with 10 μL of deuterated phenanthrene as internal standard from a 10 ng/g phenanthrene d_{10} stock solution. The samples were extracted in 50 ml of a mixture of dichloromethane and hexane; 1:1 (v/v). The extracts were concentrated to 200 μL by blowing down using nitrogen and cleaned using the gel permeation chromatography (GPC) clean up technique (Hassan and Farahani, 2011). After cleaning, 1 ml of the extracts were transferred into auto sampler vials and stored at 4 °C for GCMS analysis. Quality control used a multi-point calibration with authentic standards. Here, a 3-point calibration of 0.01, 0.05 and 0.1 mg kg^{-1} was prepared. A control sample was analysed within each batch alongside analysis of reagents. Recovery of samples at each time was 99.99%. 1–2 μL of the extracted sample was injected into the GC-MS system with the limit of detection set at 0.001 mg kg^{-1} and range of application set between 0.001 and 0.1 mg kg^{-1}.

2.6. Data analysis

Data are reported as mean ± standard error of the mean (S.E.M) except the tissue burden data from heart tissues which are reported as a single concentration for each exposure as cardiac tissue from 3 animals needed to be pooled to gain enough tissues for a single test. HR and enzyme assay data were analysed using a two-way ANOVA followed by a Fisher's LSD post-hoc test; percentage enzyme synthesis relative to control was analysed using non-parametric Kruskal-Wallis test followed by a Dunn's test. MAP, ECG and tissue burden was analysed using a one-way ANOVA followed by a Fisher's LSD post-hoc test, whilst a Log-rank (Mantel-Cox) test was performed on the percentage survival data. Statistical analyses and figures were produced in GraphPad Prism 8.0 and significance was accepted if $p < 0.05$. Details for each test including n-values are contained in the figure legends.

3. Results and discussion

3.1. Effect of phenanthrene on survival

Phenanthrene exposure impacted the survival of signal crayfish. At the end of the experiment, no deaths were recorded in the control tanks, but 20, 40 and 30% mortality were recorded for the 0.5, 1.0 and 1.5 mg L^{-1} concentrations, respectively (Fig. 1). Mortality was only recorded in the early exposure phase (day 1–7) at the lowest concentration while the mid and highest concentrations recorded mortality during the late phase (day 8–15) of exposure (Fig. 1).

3.2. Effect of phenanthrene on crayfish in vivo heart rate

This set of experiments investigated whether phenanthrene exposure affected (1) maximum in vivo heart, (2) routine in vivo heart, and (3) the time course of recovery to routine HR after maximal elevation. The mean maximum and routine HR observed for each treatment group over the 15-day exposure is given in Table 1. To ease presentation and interpretation of this large in vivo data set, in vivo HR data are grouped into HR recovery curves (maximum HR and the decline toward routine level) for 3 time points: (1) baseline, before exposure, (2) early exposure, HR recovery curves averaged across days 1–7, and (3) late exposure, HR recovery curves averaged across days 8–15. Analyses show that in control (i.e. unexposed, 0 mg L^{-1}) animals, maximum, routine and

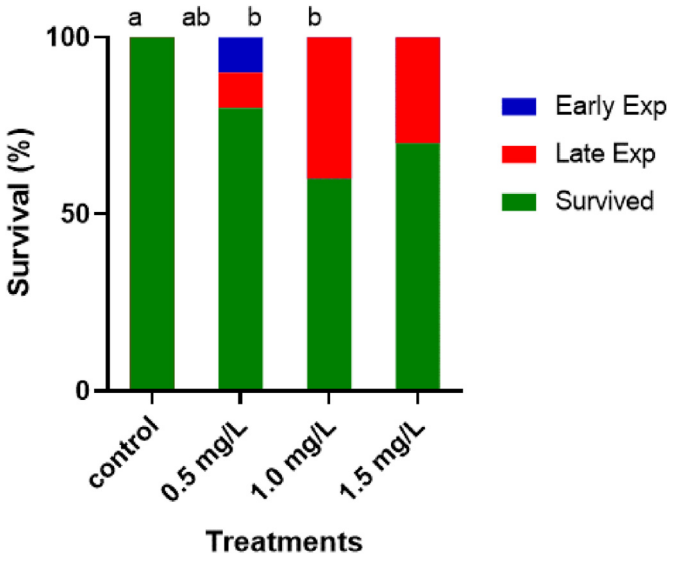

Fig. 1. Percent survival of signal crayfish exposed to varying concentrations of phenanthrene. Early Exp, early exposure phase (over days 1–7), Late Exp, late exposure phase (over days 8–15). Statistical differences are revealed by a Log-rank (Mantel-Cox) test denoted by dissimilar letters when $p < 0.05$. n = 10, per treatment.

HR recovery was consistent over the 15-day study (Fig. 2A). In the low exposure group (0.5 mg L^{-1}, Fig. 2B), a prolonged HR recovery response was observed in the early exposure period. At 15 and 30 min into recovery, mean HRs were significantly elevated compared with baseline; 62.1 ± 1.6 vs 67.5 ± 1.9 and 59.2 ± 2 vs 62.7 ± 2 BPM, respectively. At the mid exposure concentration (1.0 mg L^{-1}, Fig. 2C), phenanthrene caused a more pronounced prolongation of HR recovery during the early exposure phase. Compared to baseline, phenanthrene significantly elevated mean HRs 15 min (58.9 ± 1.7 vs 71.4 ± 1.6 BPM), 30 min (55.5 ± 2.2 vs 65.2 ± 1.8 BPM), and 45 min (54.1 ± 2 vs 60.7 ± 1.6 BPM) into the recovery period. After 60 min of recovery, mean maximum HRs were significantly decreased from 69.9 ± 1.5 to 61.6 ± 1.2 BPM (Fig. 1C, Table 1). Exposure to the highest concentrations of phenanthrene (1.5 mg L^{-1}, Fig. 2D) significantly reduced the mean HR 30 min into the recovery period at both early and late exposure indicating faster recovery from maximal HR elevation compared with baseline; 65.4 ± 2 vs 59.9 ± 1.3 and 65.4 ± 2 vs 60.2 ± 1.4 BPM, respectively. Phenanthrene also significantly reduced the mean maximum HR during the late exposure from 74.9 ± 1.8 to 69.6 ± 1.3 BPM (Fig. 1D, Table 1).

The ability of signal crayfish to maximally elevate HR was significantly reduced in the 1.0 and 1.5 mg L^{-1} exposed groups (Fig. 2C&D, Table 1) and the ability to recover HR to routine levels was impaired in the 0.5 and 1.0 mg L^{-1} exposed groups (Fig. 2B&C, Table 1). This could compromise fitness as the ability to elevate HR maximally has been associated aerobic scope which can impact exercise, digestion, and mating in fishes (Auer et al., 2015; Rosewarne et al., 2016). Similarly, exposure to the water soluble fractions of crude oil elevated HR in *C. maenas* at medium but not low and high doses (Depledge, 1984) and 15 days of exposure to PAHs in crude oil increased HR of the decapod Arctic spider crab *Hyas araneus* (Camus et al., 2002). In both studies, the authors accounted for increased HR with respiratory stress. In summary, phenanthrene affected maximum HR and recovery from HR elevation with the greatest effects observed at 1.0 mg L^{-1} exposure concentration. Higher mortality rates in the 1.0 and 1.5 mg L-1 exposure (Fig. 1) could skew the surviving population and explain why lower phenanthrene exposure levels elicited larger differences

Table 1
Maximum and routine in vivo heart rates of Signal Crayfish during exposure to phenanthrene.

	Baseline		Day 1		Day 3		Day 5		Day 7		Day 9		Day 11		Day 13		Day 15	
	Max	Rest	Max	Rest	Max	Rest	Max	Rest	Max	Rest	Max	Rest	Max	Rest	Max	Rest	Max	Rest
Control	69 ± 1.4	46 ± 2.3	72 ± 2.8	48 ± 3.5	71 ± 3.1	44 ± 2.8	71 ± 2.6	44 ± 3.2	72 ± 4	43 ± 3.5	64 ± 3.6	47 ± 4.6	69 ± 3.4	48 ± 3.6	71 ± 3.7	44 ± 3.7	64 ± 4.4	46 ± 3.3
0.5 mg L^{-1}	74 ± 1.5	53 ± 1.6	77 ± 3.8	56 ± 1.9	76 ± 2	50 ± 3.6	72 ± 1.5	54 ± 1.8	76 ± 1.9	47 ± 3.7	75 ± 2	52 ± 3.1	76 ± 2.5	51 ± 5.2	74 ± 2	48 ± 4.4	73 ± 4.1	48 ± 3.6
1.0 mg L^{-1}	**70 ± 1.5***	**52 ± 1.7^**	78 ± 4	**62 ± 2.7^**	72 ± 1.9	55 ± 3	70 ± 1.8	50 ± 2.3	70 ± 1.9	54 ± 3.1	64 ± 2.2	53 ± 3.6	65 ± 1.1	50 ± 1.8	**59 ± 2.4***	47 ± 1.7	**57 ± 3.3***	46 ± 1
1.5 mg L^{-1}	**75 ± 1.8***	51 ± 2.2	74 ± 1.9	56 ± 4.4	73 ± 2	50 ± 2.3	71 ± 2	50 ± 2.7	70 ± 2.4	51 ± 2.7	73 ± 3.2	57 ± 3	70 ± 2.2	55 ± 2.8	69 ± 2.2	52 ± 2.3	**66 ± 2.5***	51 ± 3.4

Heart rate (HR) was recorded in each individual every 48 h. Values are mean ± S.E.M, from n = 6–10 animals. Maximum and routine HR did not change significantly overtime in the control (unexposed) and low concentration exposure groups. In the mid concentration exposure group, max HR was significantly reduced on day 13 and 15 and routine HR was significantly elevated on day 1. In the high concentration exposure group, phenanthrene significantly reduced max HR on day 15. Statistical differences (bolded) are revealed by a two-way ANOVA, followed by a Fischer's LSD post-hoc test. A (*) denotes differences in max HR compared to baseline while (^) denotes difference in routine HR compared to baseline when $p < 0.05$.

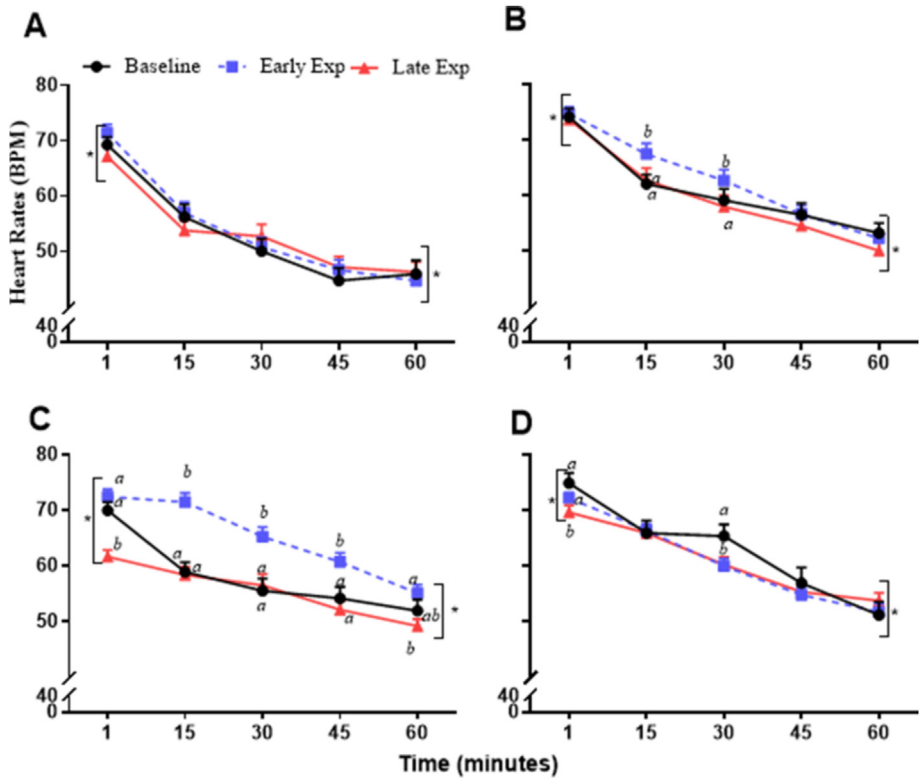

Fig. 2. The effect of phenanthrene on in vivo heart rate (HR) in the signal crayfish in beats per minute (BPM). Graphs show maximum HR (t = 1 min) and its decline to routine HR over the course of 1 h. Panels show control (no exposure, acetone only) (A), and phenanthrene concentrations of (B) 0.5 mg L-1, (C) 1.0 mg L-1 and (D) 1.5 mg L-1. Values are mean ± S.E.M of n = 6–10 crayfish for each time point. Baseline is mean HR measured before the exposure period began. Early Exp, early exposure phase is the mean HR for each crayfish averaged over days 1–7. Late Exp, late exposure phase is the mean HR for each crayfish averaged over days 8–15. Statistical differences are revealed by a two-way ANOVA, followed by a Fischer's LSD post-hoc test, $p < 0.05$. Dissimilar letters denote differences between groups at a given time point and an asterisk (*) denotes a difference between maximum (1 min) and resting (60 min) heart rate within a group.

compared to the high values. We attempted to test for this by removing animals that died during the exposure from the data presented in Fig. 2 and found no impact on the findings (not shown). We suggest future studies include respirometry for a greater understanding of the in vivo cardiorespiratory consequences of phenanthrene exposure in the signal crayfish.

3.3. Effect of phenanthrene on in situ cardiac electrical activity

After 15 days of exposure to varying concentrations of phenanthrene, crayfish heart electrical activities were measured from a semi-isolated in situ heart preparation using surface electrodes for monophasic action potentials (MAP) and electrocardiograms (ECG). Note that phenanthrene was not included in the recording saline, and thus the measurements show the effect of the 15-day exposure period on cardiac electrical activity rather than an acute effect. A representative trace of a crayfish MAP and ECG is shown in Fig. 3A. Phenanthrene caused a significant ($p < 0.05$) dose-dependent prolongation of the corrected QT interval of the ECG (Fig. 3B). Phenanthrene prolonged QTc from 336.6 ± 8.9 ms in unexposed animals to 413.8 ± 10.4, 432.8 ± 49.9, and 471 ± 52 ms in 0.5, 1.0 and 1.5 mg L^{-1}, respectively. A similar trend that did not reach statistical significance was observed in the corrected MAP duration (cMAPD) and in MAPD triangulation (measured as $MAPD_{90} - MAPD_{30}$) which is an index arrhythmic potential (Fig. 3C&D).

The ability of phenanthrene to interrupt the electrical activity of the heart has been previously reported in marine and freshwater fish species, and thus, cardiac dysfunction is a key concern for aquatic animals exposed to petroleum-based pollution. Phenanthrene causes action potential prolongation in fishes by slowing repolarization of the AP following inhibition of the delayed rectifier K^+ channels (i.e. ether-a-go go, erg channels) (Brette et al., 2014, 2017; Ainerua et al., 2020; Marris et al., 2020). Phenanthrene disrupts calcium dynamics which underlie contractility by inhibiting Ca^{2+} flux in the cardiac myocytes of a number of fish species (Brette et al., 2014, 2017; Sorhus et al., 2016; Vehniäinen et al., 2019; Ainerua et al., 2020; Marris et al., 2020). However, our study is the first to report electrical dysfunction in the heart of a crustacean in response to phenanthrene exposure. We show a concentration dependent prolongation in QTc in the crayfish heart, most likely explained by the trend towards a prolongation of the MAP (Fig. 3). Of particular interest is the fact that the crayfish heart is neurogenic and coordinated by the cardiac ganglion – a collection of cardio-accelerating and cardio-inhibitory nerve activities and neurohormones (Field and Larimer, 1975; Florey and Rathmayer, 1978; Skerrett et al., 1995; Listerman et al., 2000; Harper and Reiber, 2001; Chiba and Inase, 2016). Thus, the altered cardiac electrical activity we observed may be due to either cardiac-specific or combined cardio-neuronal effects. Indeed, phenanthrene may cause AP prolongation by influencing neuronal and/or cardiac K^+-channels. Isoforms of these channels have been reported for the decapod myocardium and they appear to respond to physiological blockers in a similar way as those in vertebrates Van Der Kloot (1970); (Shinozaki et al., 2002; Ransdell et al., 2013). Hernandez and Mendez (1996) showed that tetradotoxin (TTX) impairs Na^+ channels, and that Cd^{2+} and Ni^{2+} impairs Ca^{2+} channels, and that AP repolarization in crayfish skeletal muscle was prolonged when treated with tetraethylammonium (TEA) suggesting the presence of

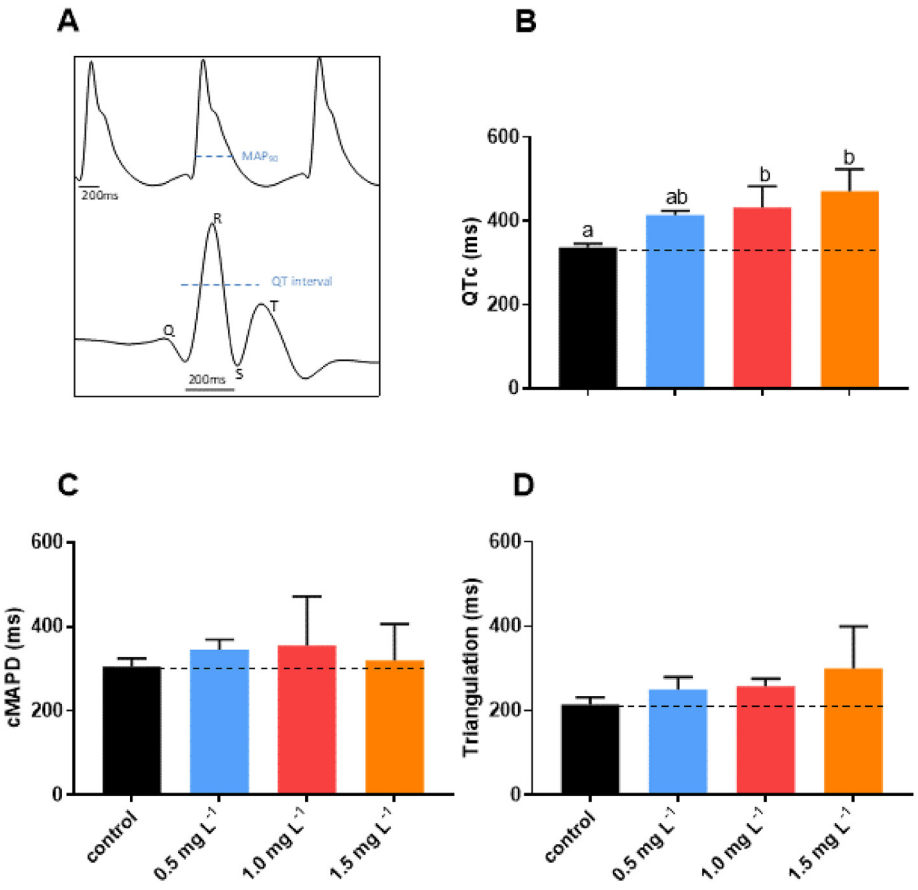

Fig. 3. Effect of 15 days of phenanthrene exposure on the electrical properties of the signal crayfish semi-isolated in situ heart preparation. (A) Representative trace of a Monophasic Action Potential (MAP) and Electrocardiogram (ECG) recorded simultaneously from a crayfish with an intrinsic heart rate of 38 BPM. Notice the alignment between the QT interval and the duration of the MAP. Phenanthrene exposure caused QTc prolongation (B), but did not affect cMAPD90 (C) or triangulation (MAPD90 − MAPD30)(D). Statistical differences are revealed by a one-way ANOVA, followed by a Fischer's LSD post-hoc test which are denoted by dissimilar letters when $p < 0.05$. Values are mean ± SEM n = 3–9 for each treatment. Mean HR for MAPD data; control (27 ± 2.9 BPM), 0.5 mg l^{-1} (35.5 ± 3.8 BPM), 1.0 mg l^{-1} (23.26 ± 7.4 BPM), 1.5 mg l^{-1} (34.85 ± 3.77 BPM).

the delayed rectifier potassium channel current, I_{Kr}. Moreover, naphthalene, another low weight molecular PAH, was found to cause hyperpolarization of crayfish skeletal muscle resting potential likely due to its action on the Na^+ pump (Nelson and Mangel, 1979). Thus, although the data is limited, and mostly related to studies with skeletal muscle, they suggest that phenanthrene could influence cardiac performance in crayfish by inhibiting ion channels in a manner analogous to vertebrates (Marris et al., 2020), which is supported by the data presented here. Future work should attempt to separate neural- and cardiac-specific ion channel sensitivity to PAHs.

Although not measured directly in this study, phenanthrene may influence the activities of neurohormones such as proctolin, serotonin and octopamine which could also impact contractility and heart rhythm. Octopamine, for instance, is known to enhance myocardial contraction by increasing HR and stroke volume in decapod crustaceans (Cooke, 1966; Battelle and Kravitz, 1978; Benson, 1984; Wilkens et al., 1985; Saver and Wilkens, 1998; Harper and Reiber, 2001) while proctolin acts directly on the myocardium resulting in increased Ca^{2+} influx (Adams et al., 1989; Saver et al., 1998; Harper and Reiber, 2001) with a corresponding increase in HR and stroke volume (McGaw et al., 1994; Saver and Wilkens, 1998; Harper and Reiber, 2001). Whether altered hormone release, is involved in the changes in maximal HRs observed in the late phase of our study is an interesting topic for future investigations.

3.4. Phenanthrene effect on enzymes of oxidative stress: SOD and TOSC

At the end of the 15-day exposure and following the in situ heart recordings, hepatopancreas, gill and skeletal muscle tissues were extracted and frozen for subsequent analysis of antioxidant capacity. Changes in antioxidant capacity in response to environmental contaminants have been reported previously in invertebrates, including crayfish (Jehosheba and Babu, 2003; Borković et al., 2008; Ye and Zou, 2008; Koenig et al., 2009; Wu et al., 2011; Silva et al., 2013; Stará et al., 2014; Bhagat et al., 2016; Gunderson et al., 2018), and they are increasingly being used as biomarkers for oxidative damage. During exposure to toxicants, SOD is the first and primary defence system induced to reduce superoxide radicals (O_2^-) to hydrogen peroxide (Wu et al., 2011; Stará et al., 2014). Therefore, we analysed the effects of phenanthrene on SOD activity in signal crayfish gills, hepatopancreas and skeletal muscle.

Mean SOD activity (U mg ml^{-1}) under control conditions (Fig. 4A) was similar among tissues (gills 2.4 ± 0.5, muscle 1.83 ± 1, and hepatopancreas 1.79 ± 0.6, n = 6–10). Phenanthrene exposure had no effect on gill or muscle SOD activity (Fig. 4A), which suggests superoxide scavenging capacity remain unaltered in these tissues. The lack of a compensatory increase in SOD in these tissues may also indicate that phenanthrene does not increase superoxide production in this tissue. There are mixed reports in the literature

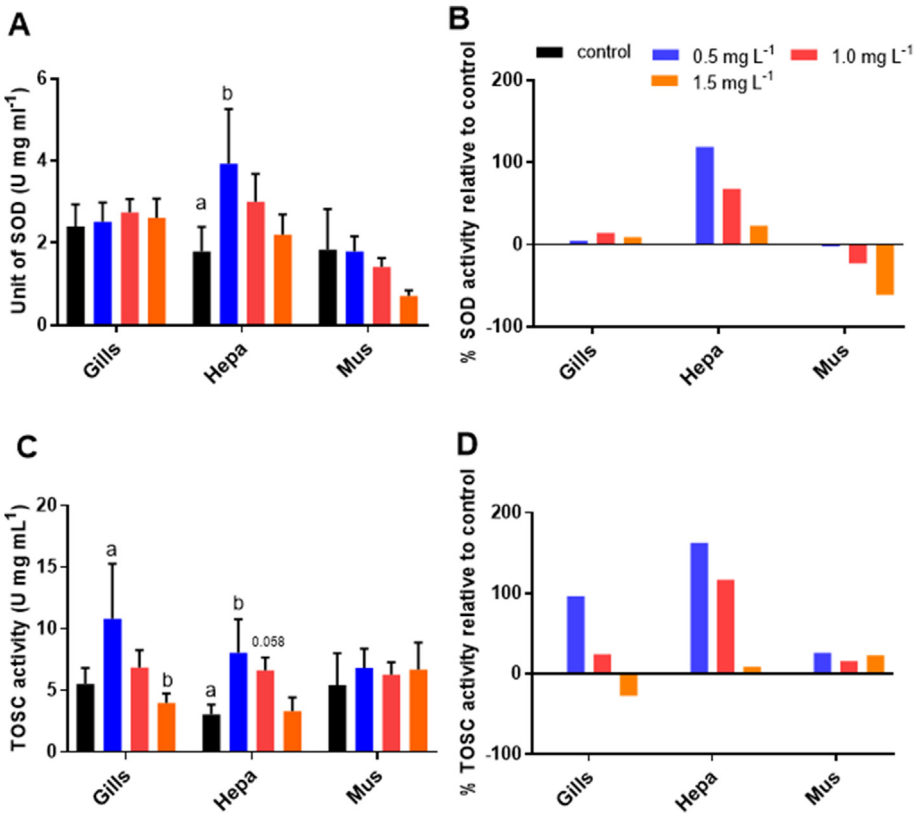

Fig. 4. The effect of phenanthrene on superoxide dismutase (SOD) and total oxyradical scavenging capacity (TOSC) in gills, hepatopancreas and skeletal muscle samples from crayfish exposed to phenanthrene for 15 days. (A) SOD activities in various samples tissues (B) Percentage SOD synthesis relative to control after exposure (C) TOSC in various tissue samples (D) Percentage TOSC relative to control after exposure. Values are mean ± S.E.M (n = 6–10). Statistical differences are revealed by a two-way ANOVA, followed by a Fischer's LSD post-hoc test ($p < 0.05$). Dissimilar letters denote significant effect of dose within a given tissue. Percentage data were analysed with Kruskal-Wallis test followed by a Dunn's test ($p < 0.05$).

of the impact of toxicant exposure and SOD activation in invertebrates (see (Bhagat et al., 2016) for a review in gastropods). However, decreased muscle SOD activities have been reported in Red Swamp Crayfish (*Procambarus clarkii*) exposed to prometryne (Stará et al., 2014). Therefore, the effects of PAH on SOD activity in invertebrates appear to be variable, perhpas suggesting SOD is not a suitable biomarker for crayfish muscle and gill toxicity. In contrast to gill and muscle, hepatopancreas SOD activity was significantly increased by phenanthrene, but only at the lowest concentration (118.9% increase at 0.5 mgL^{-1}) suggesting phenanthrene increased superoxide production in this tissue, leading to a compensatory upregulation of SOD. Higher concentrations of phenanthrene may overwhelm the SOD defence system in this tissue.

Once SOD and other antioxidants have reduced superoxide to hydrogen peroxide, there are numerous other compounds that detoxify hydrogen peroxide into water; these include ascorbate, vitamin E, uric acid, metallothionein, catalase, glutathione peroxidase and glutamate cysteine ligase. The detoxification of hydrogen peroxide plays a vital role in maintaining low levels of reactive oxygen species and avoiding oxidative stress. Therefore, we used the TOSC assay to measure the total capacity of crayfish tissues to reduce hydrogen peroxide to water. Mean TOSC activities (U mg ml^{-1}) under control conditions were comparable in the gills (5.51 ± 1.3) and muscles (5.43 ± 2.6) but lower in the hepatopancreas (3.06 ± 0.8; Fig. 4C). Phenanthrene exposure had no effect on TOSC activity in crayfish muscle at any exposure concentration. In gill, TOSC activity was similar to control values at all phenanthrene exposure concentrations, but TOSC activity at 0.5 mgL^{-1} phenanethrene was significantly elevated compared to 1.5 mgL^{-1} phenanthrene.

In contrast to gill and muscle, hepatopancreas TOSC activity was significantly increased by phenanthrene, but only at the lowest concentration (Fig. 4C–D). Like SOD activity in this tissue, our results may indicate low concentrations of phenanthrene increase H_20_2 production in the crayfish hepatopancreas, leading to a compensatory upregulation of H_20_2 antioxidant systems. However, high concentrations of phenanthrene may overwhelm the H_20_2 defence system. A similar pattern has been observed with blood glutathione peroxidase levels in humans exposed to PAH's (Wang et al., 2020) and also liver catalase levels in fish exposed to pyrene (Sun et al., 2008). Therefore, low concentrations of Phe may lead to a compensatory upregulation of endogenous antioxidants to detoxify H2o2, but high concentrations of Phe may consume or down-regulate antioxidants due to excessive oxidative stress. Exposure to the low molecular weight PAH, naphthalene, increased glutethione S-transferase (GST) activity in the hepatopancreas of the brown shrimp (*Penaeus aztecus*), but only when it was applied in combination with hypoxia (Ye and Zou, 2008). TOSC activity was not affected by PAH exposure in Arctic spider crab (*Hyas araneu*) even though heart rate was elevated (Camus et al., 2002), and the higher molecular weight PAH, benzo(a)pyrene, had no effect on catalase or GST in the common prawn *(Palaemon serratus)*, while glutathione peroxidase was reduced (Silva et al., 2013). Clearly, the effects of PAH exposure on the invertebrate H_20_2 defence system are very variable, being species and tissue dependent.

In summary our analysis of two commonly employed assays for oxidative stress (SOD and TOSC) gave mixed results that were tissue- and dose-specific. The lack of activation of either SOD or TOSC

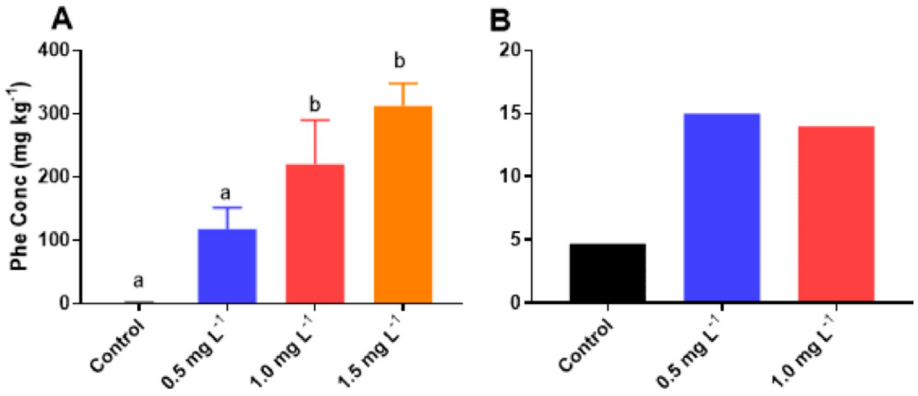

Fig. 5. Accumulation of phenanthrene in crayfish (A) hepatopancreas and (B) heart tissues following 15 days of exposure at varying doses. Values for hepatopancreas are mean ± SEM (n = 4), while 4 heart samples from each treatment were pooled to provide sufficient tissue mass for a single analyses, thus these data are presented without SEM. Statistical differences are revealed by a one-way ANOVA, followed by a Fischer's LSD post-hoc test which are denoted by dissimilar letters when $p < 0.05$ for hepatopancreas. Statistics were not performed on heart tissue.

at the highest phenanthrene dose may indicate that the oxyradical scavenging system is overwhelmed at this concentration. In this regard, it would have been interesting to assess the antioxidants at other time points within the 15-days exposure. Additionally, the higher mortality rates in the 1.0 and 1.5 mg L-1 exposure (Fig. 1) could skew the surviving population and may also account for the lower phenanthrene exposure levels eliciting larger differences compared to the medium and high values. Future studies should assess enzymatic responses across the exposure time course, as well as expanding the investigation to include other antioxidant biomarkers such as glutathione S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx) and thiobarbituric acid reactive substance (TBARS) to provide a fuller view of induction of oxidative stress and response systems.

3.5. Tissue burden

At the end of the 15-day exposure and following the in situ heart recordings, hepatopancreas and heart tissues were extracted and analysed for phenanthrene concentration. Fig. 5 shows hepatopancreas accumulated more phenanthrene than heart tissues. The control groups showed a small tissue concentration of 0.6 ± 0.2 mg kg^{-1} for the hepatopancreas compared and 4.7 mg kg^{-1} in the heart, probably due to instrument carry-over during sampling or analysis. The phenanthrene-exposed groups had a markedly higher tissue burden ranging from 117.5 ± 34 to 312.5 ± 36 mg kg^{-1} for the hepatopancreas (Fig. 5. A), and 14–15 mg kg^{-1} for the heart (Fig. 5B). Higher values recorded in the hepatopancreas compared to the heart is due to the high lipophilicity ($logK_{ow}$ equals to 4.53) of the hepatopancreas (Solbakken et al., 1982) and to the fact that it is the main detoxifying organ in arthropods. A comparable dose-dependent pattern of tissue phenanthrene accumulation was previously observed in the lions-paw scallop (*Nodipecten nodosus*) hepatopancreas (Piazza et al., 2016) and fish liver has been shown to accumulate phenanthrene in a dose-dependent manner (Yin et al., 2007). Our results suggest a limited ability to effectively detoxify and eliminate phenanthrene in the signal crayfish. This, in combination with inability to effectively deploy the antioxidant defence system in coping with oxidative stress, may underlie the mortality rates (30–40%) of crayfish in the mid to high concentrations tested in this study.

4. Conclusions

We have demonstrated that phenanthrene impairs maximum HR, prolongs QTc duration of the ECG and induces tissue-specific sensitivity in the synthesis of antioxidant enzymes. Together, these responses culminate in reduced survival of the signal crayfish during 15 days of phenanthrene exposure. Although the biomarkers employed here provide some indication of toxicant exposure, more research is needed to uncover the exact mechanisms of toxicity impacting survival. Nevertheless, this study emphasises the importance of monitoring freshwater ecosystems to help reduce the overall environmental presence of PAHs.

Author contributions

Martins O Ainerua conducted the in vivo exposures and heart rate study with help from Rory Murphy, and conducted the semi-isolated in situ heart study. Martins O Ainerua performed the corresponding data analysis, prepared figures, and drafted the manuscript. Jake Tinwell conduct the oxidative stress study under the guidance of Gina LJ Galli and they both performed the data analysis, prepared the figure, and contributed to writing. Holly A Shiels, Bart E. van Dongen and Keith N White conceptualized the study and were involved in data analysis and figure design. Holly A Shiels was responsible for writing, reviewing, and editing the final version of the manuscript. All authors approved final version of the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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