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# An integrated approach to assess the impacts of zinc pyrithione at different levels of biological organization in marine mussels.

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3 An integrated approach to assess the impacts  
4 of zinc pyrithione at different levels of biological  
5 organisation in marine mussels

6 Lorna J. Dallas,<sup>†</sup> Andrew Turner,<sup>\*,¶</sup> Tim P. Bean,<sup>‡</sup> Brett P. Lyons,<sup>‡</sup> and  
7 Awadhesh N. Jha<sup>†</sup>

8 <sup>†</sup>*School of Biological and Marine Sciences, University of Plymouth, Plymouth,*  
9 *UK*

10 <sup>‡</sup>*Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK*

11 <sup>¶</sup>*School of Geography, Earth & Environmental Sciences, University of*  
12 *Plymouth, Plymouth, UK*

13  
14 E-mail: [aturner@plymouth.ac.uk](mailto:aturner@plymouth.ac.uk)  
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## Abstract

The mechanisms of sublethal toxicity of the antifouling biocide, zinc pyrithione (ZnPT), have not been well-studied. This investigation demonstrates that 14-d sublethal exposure to ZnPT (0.2 or 2  $\mu\text{M}$ , alongside inorganic Zn and sea water controls) is genotoxic to mussel haemocytes but suggests that this is not caused by oxidative DNA damage as no significant induction of oxidised purines was detected by Fpg-modified comet assay. More ecologically relevant endpoints, including decreased clearance rate (CR), cessation of attachment and decreased tolerance of stress on stress (SoS), also showed significant response to ZnPT exposure. Our integrated approach was underpinned by molecular analyses (qRT-PCR of stress-related genes, 2D gel electrophoresis of proteins) that indicated ZnPT causes a decrease in phosphoenolpyruvate carboxykinase (PEPCK) expression in mussel digestive glands, and that metallothionein genes are upregulated; PEPCK downregulation suggests that altered energy metabolism may also be related to the effects of ZnPT. Significant relationships were found between % tail DNA (comet assay) and all higher level responses (CR, attachment, SoS) in addition to PEPCK expression. Principal component analyses suggested that expression of selected genes described more variability within groups whereas % tail DNA reflected different ZnPT concentrations.

Keywords: zinc pyrithione; sublethal toxicity; marine mussels; genotoxicity; DNA damage

## 70 1. Introduction

71 Zinc pyrithione (ZnPT,  $317.70 \text{ g mol}^{-1}$ ), an organic complex with two pyrithione  
72 ligands chelated to  $\text{Zn}^{2+}$ , is used as a booster biocide in many copper-based  
73 antifouling paints (AFPs). Its role in such products is to increase broad-spectrum  
74 efficacy via antifungal action (Thomas et al., 2000), which also makes it ideal for use  
75 in outdoor paints for resistance to mould. ZnPT also has a variety of household and  
76 medical uses (for example, it is used in the treatment of psoriasis and is the only  
77 active ingredient in medicated anti-dandruff shampoos; Reeder et al., 2011a)  
78 resulting in multiple, additional sources from which ZnPT can enter the environment.

79

80 Although ZnPT is moderately hydrophobic ( $\log K_{\text{OW}} = 0.93$  at  $25^\circ\text{C}$ , solubility in

81 water = 8 mg L<sup>-1</sup> at 20°C) and has the potential to accumulate in sediments, it is  
82 favoured over many other booster biocides because it can degrade relatively  
83 quickly when exposed to UV light (Price and Readman, 2013). Although there are  
84 uncertainties about the kinetics of ZnPT photo-degradation in sea water, its  
85 degradation products and the mechanisms by which it adsorbs to sediments (Maraldo  
86 and Dahllöf, 2004; Turley et al., 2005; Bones et al., 2006), it is clear that the compound has  
87 potential to cause detrimental effects in aquatic species at concentrations typically  
88 encountered in environments where boating activities occur (up to 100 nM; Mackie  
89 et al., 2004; Madsen et al., 2000). For example, EC50 values for developmental  
90 abnormalities in zebrafish and medaka are 28 and 15 nM, respectively (Goka,  
91 1999), and embryo toxicity is also reported in sea urchins (EC50 = 7.7 nM) and  
92 mussels (*M. edulis*, EC50 = 8 nM) (Bellas et al., 2005). Growth of diatoms is  
93 significantly reduced by as little as 5.9 nM ZnPT over 96 h (Bao et al., 2008), while  
94 cultured ascidian haemocytes show compromised immunity after exposure to ≤ 0.5  
95 μM ZnPT (Cima and Ballarin, 2015) and treatment of paddy fields with ZnPT anti-  
96 dandruff shampoo decreased growth in juvenile medaka (Sánchez-Bayo and Goka,  
97 2005). Recently, Marcheselli et al. (2013) demonstrated that ZnPT induced  
98 genotoxicity in marine mussels using the lethal-effect TUNEL assay. Thus, after 7-d  
99 exposure, adult *Mytilus galloprovincialis* showed increased DNA fragmentation and  
100 induced heat shock protein expression at ZnPT concentrations of 0.2 and 0.4 μM,  
101 with the higher concentration also causing a reduction in anoxia tolerance.

102

103 Despite these observations, there are still many unanswered mechanistic questions  
104 regarding ZnPT toxicity to aquatic biota, including mussels as ecologically  
105 important bioindicator species (Dallas et al., 2016). Furthermore, no study has  
106 attempted to connect alterations in genomic integrity with changes in protein  
107 expression by looking at intermediate processes such as transcriptional expression  
108 of relevant genes. In this context, we exposed adult *M. galloprovincialis* to ZnPT  
109 over a two-week time period to examine the effects of ZnPT across several levels  
110 of biological organization in the mussel. This included assessing the potential  
111 induction of oxidative DNA damage (Fpg-modified comet assay) and linking  
112 changes in genomic integrity throughout the mussel with transcriptional and  
113 protein expression (qRT-PCR and 2D gel electrophoresis, respectively) and with

114 changes to higher level, more ecologically relevant responses, including clearance  
115 rate [CR] and attachment.. Where appropriate, observed biological responses were  
116 analysed using Principal Component Analysis (PCA) and Cluster analyses which  
117 effectively integrate multidimensional biomarker data into a more readily  
118 interpretable two dimensional format.

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120

## 121 **2. Methods and experimental design**

122

### 123 *2.1. Reagents and mussel collection and maintenance*

124

125 Adult *M. galloprovincialis* ( $48.03 \pm 2.43$  mm) were collected from Trebarwith Strand  
126 ( $50^{\circ} 38' 40''$  N,  $4^{\circ} 45' 44''$  W), a reference site remote from significant boating activity,  
127 and maintained in the laboratory as previously described (Dallas et al., 2013; Banni  
128 et al., 2017). Sea water used in the exposures was sourced from Plymouth Sound,  
129 stored on site and filtered on line ( $< 10 \mu\text{m}$ ). Measured Cu concentrations ( $< 10 \mu\text{g}$   
130  $\text{L}^{-1}$ ) were considerably less than added Zn concentrations (see below), ensuring that  
131 significant Cu(II)-Zn transchelation of the pyrrithione ion ( $\text{PT}^{-}$ ) was unlikely (Holmes  
132 and Turner, 2009).

133

134 Unless otherwise stated, all reagents used in the exposures and for sample  
135 processing and analysis were purchased from Sigma-Aldrich Ltd (Gillingham, UK).  
136 Ultrapure water was obtained using a Milli-Q RG (Merck Millipore, Billerica, MA,  
137 USA) or Elga Purelab Option system (Elga LabWater, Marlow, UK) and had a  
138 resistivity of  $> 18.2 \text{ M}\Omega \text{ cm}^{-1}$  at  $25^{\circ}\text{C}$ .

139

### 140 *2.2. Exposure scenario and determination of higher level responses*

141

142 Concentrations used were based on acute range-finding exposures that compared  
143 ZnPT to  $\text{ZnCl}_2$  and the pyrrithione ion (as NaPT) at equimolar concentrations of Zn or  
144 PT and between  $0.20$  and  $16.20 \mu\text{M}$ . These produced a 96-h LC50 for *M.*  
145 *galloprovincialis* of  $14.50 \pm 1.45 \mu\text{M}$  for PT as ZnPT (50 % mortality not achieved for  
146 the other treatments), 7-d LC50s of  $24.58 \pm 1.57 \mu\text{M}$  for PT as NaPT and  $8.94 \pm 1.30$   
147  $\mu\text{M}$  for PT as ZnPT, and 14-d LC50s of  $2.54 \pm 1.32$  (as NaPT) and  $2.97 \pm 1.36 \mu\text{M}$

148 (as ZnPT), with 50 % mortality not achieved for inorganic Zn (Fig. S1). Degradation  
149 of ZnPT into inorganic Zn was also assessed under our experimental conditions by  
150 retention and subsequent 3 ml min<sup>-1</sup> methanol elution of Zn on conditioned octadecyl  
151 silane (C18) columns (15 mL; Chromabond, Macherey Nagel GmbH, Düren,  
152 Germany) (Holmes and Turner, 2009) (Fig. S2 A, S2 B).

153

154 Depurated (28 d) and acclimatized (48 h) *M. galloprovincialis* ( $n = 16$ ) were exposed  
155 for 14 d and under a 12:12 h photoperiod to 0.2  $\mu\text{M}$  ZnPT (low), 2  $\mu\text{M}$  ZnPT (high), 2  
156  $\mu\text{M}$  ZnCl<sub>2</sub> (inorganic) and no added Zn (control) in triplicate and in pre-washed  
157 plastic tanks containing 20 L of sea water (salinity  $31.82 \pm 0.34$ , pH  $8.16 \pm 0.21$ ,  
158 dissolved oxygen  $90.02 \pm 2.84$  % and temperature  $15.88 \pm 0.52$  °C). ZnPT  
159 concentrations were either chronic (< 10% of 14-d LC50) or acute (67% of 14-d  
160 LC50) as determined by the range-finding experiment described above. As the  
161 degradation of ZnPT approached 80% at 48 h (Fig. S2 C), full water changes were  
162 performed every 2 d with replenishment of ZnPT. Mussels were fed 2 h prior to water  
163 changes with *Isochrysis galbana* ( $\sim 1.0^5 \times 10^6$  cells mL<sup>-1</sup>), and during changes on  
164 days 4, 6 and 12 (i.e. those closest to the sampling days), mussel attachment was  
165 recorded where adherence to the interior surface or to other mussels was evident  
166 when the emptied tank was tilted 45°. Clearance rate was determined for nine  
167 mussels per treatment (and three from each replicate) on day 14, as previously  
168 described (Devos et al., 2015), and after this time period the stress on stress (SoS)  
169 test was performed in continuous anoxic conditions for a further 20 d (Viarengo et  
170 al., 1995). Thus, tanks were drained and remaining mussels blotted dry before being  
171 transferred to open petri dishes where they were maintained without water but in a  
172 humid environment at 15 °C. Mortality, defined as persistent opening of the valves  
173 and failure to respond when tapped, was recorded daily and subsequently used to  
174 calculate LT50 values (median lethal time for 50 % mortality).

175

### 176 2.3. Genotoxicity in circulating haemocytes

177

178 After 0, 4, 7 and 14 d, haemolymph was extracted from nine mussels per treatment,  
179 and stored on ice pending assay, whilst 5 mm<sup>2</sup> sections of gill and digestive gland  
180 were removed and stored at -80 °C pending analysis. The enzyme-modified comet

181 assay was performed using the haemocytes according to methods previously  
182 validated using H<sub>2</sub>O<sub>2</sub> as a positive control (Dallas et al., 2013), with two slides per  
183 sample: one control containing buffer only (40 mM HEPES, 0.1 M KCl, 0.5 mM  
184 EDTA, 0.20 mg mL<sup>-1</sup> BSA, pH 8.0) and one with the bacterial enzyme,  
185 formamidopyrimidine glycosylase (Fpg), to detect oxidised purine and pyrimidine  
186 bases. Prior to the performance of the comet assay, cell viability was checked using  
187 Eosin Y stain and was found to be > 80 % for all treatments (data not shown). The  
188 micronucleus (MN) assay was performed on haemocytes as also described  
189 previously (Dallas et al., 2013) with 1000 cells scored on each coded and  
190 randomized slide.

191

#### 192 *2.4. Alterations in transcriptional expression of candidate genes*

193

194 Total RNA was isolated from gill and digestive gland using the RNeasy mini kit  
195 (Qiagen Ltd, UK). Two µg from samples with OD<sub>260</sub>:OD<sub>280</sub> > 1.95 and crisp bands  
196 was used for reverse transcription with M-MLV reverse transcriptase and random  
197 primers (Promega Corporation, USA). Real-time- (q-)PCR was performed on  
198 samples in duplicate (Applied Biosystems Step-One Plus RT-PCR system, StepOne  
199 Software v2.2.2) in reactions containing 7.5 µL SYBR Green Jumpstart Taq  
200 ReadyMix, 0.2 µM forward and reverse primers (as detailed in Table S1), 4.44 µL  
201 ultrapure water and 3 µL template cDNA. Initial denaturation was at 95 °C for 2 min,  
202 followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, plus a melt curve to  
203 verify PCR-product purity. Relative expression ratio (RER) of 3 genes (*mt10*, *mt20*  
204 and *hsp70*) was quantified using REST 2009 (v2.0.13; Qiagen Ltd) from PCR  
205 efficiency (measured using LinRegPCR) and threshold cycle (C<sub>q</sub>), relative to the  
206 reference genes *atub* (alpha tubulin) and *ef1* (elongation factor 1) with control  
207 samples as calibrators (Pfaffl et al., 2002; Ramakers et al., 2003). Both reference  
208 genes showed low variability across samples (C<sub>q</sub> *atub* = 20.45 ± 1.26, *ef1* = 18.01 ±  
209 1.01).

210

#### 211 *2.5. Alterations in protein expression*

212

213 Digestive glands from three mussels per exposure tank were pooled and  
214 homogenised with four parts homogenisation buffer (10 mM Tris-HCl, 0.5 M sucrose,

215 0.15 M KCl, 1 mM EDTA, 1 mM PMSF) to provide sufficient protein and reduce inter-  
216 individual variation (Karp and Lilley, 2005). Samples were prepared for 2D gel  
217 electrophoresis (2DGE) using methods of Schmidt et al. (2003). Briefly, total protein  
218 was extracted with TCA-acetone, rehydrated and loaded onto a 7 cm IPG strip  
219 overnight (pH 3-10; GE Healthcare). Iso-electric focusing was performed using a  
220 Protean IEF cell (Bio-Rad) at 300 V for 3 h, 1000 V for 6 h and 8000 V for 3 h,  
221 followed by 8000 V for 20 kVh and hold at 500 V. Strips were equilibrated firstly with  
222 2% DTT and secondly with 2.5% iodoacetamide. After transfer of strips to 12%  
223 polyacrylamide gels (Mini-PROTEAN TGX, Bio-Rad), electrophoresis was carried  
224 out at 90 V for 1 h, followed by 120 V for 30 min (in 0.25 M Tris, 1.92 M glycine, 0.03  
225 M SDS). Gels were stained with 0.2% Coomassie brilliant blue (R-250 in 30%  
226 methanol, 10% acetic acid) for 1 h, destained overnight (40% methanol, 10% acetic  
227 acid) and visualised using a Gel Doc XR+ (Bio-Rad).

228

229 Spots of interest (i.e. those not evident in the control) were excised using sterile  
230 razor blades, dried and sent for identification (Mass Spectrometry Unit, Instituto de  
231 Tecnologia Quimica e Biologica, Universidade Nova de Lisboa). After in-gel tryptic  
232 digestion, extracted peptides were loaded onto a R2 micro column (RP-C18  
233 equivalent), desalted, concentrated, and eluted directly onto a MALDI plate using  $\alpha$ -  
234 cyano-4-hydroxycinnamic acid as the matrix solution in 50% acetonitrile and 5%  
235 formic acid. Mass spectra were acquired in positive reflectron MS and MS/MS  
236 modes using a 4800plus MALDI TOF/TOF analyser with an exclusion list of trypsin  
237 autolysis peaks (842.51, 1045.56, 2211.11 and 2225). Resulting spectra were  
238 analysed in combined mode using the Mascot search engine and NCBI database  
239 (restricted to 50 ppm peptide mass tolerance with no taxonomy restrictions).

240

## 241 *2.6. Statistical analysis*

242

243 Statistics were performed in R (i386, v2.15.2; [www.R-project.org](http://www.R-project.org)) and 2DGE data  
244 were analysed using Progenesis Samespots software (v4.5.4325.32621; Nonlinear  
245 Dynamics Ltd, UK). Two-way ANOVA was undertaken on micronuclei data, after  
246 square root transformation, and comet assay data (as medians for each slide), with  
247 LC50 values determined by probit analysis and survival curves and lethal time to  
248 50% mortality (LT50) generated according to Kaplan-Meier and compared by log-

249 rank test. PCA ('prcomp' in R) and Cluster Analysis was performed for the six  
250 biomarkers common to samples from days 4 and 14 (i.e.  $C_q$  for *hsp70*, *mt20* and  
251 *mt10*; % tail DNA [buffer]; % tail DNA [Fpg]; MN/1000 cells). Proteomic results and  
252 higher level responses were excluded from PCA due to pooling/non-paired samples,  
253 but relationships between these parameters were examined by generating Pearson's  
254 moment correlation coefficients.

255

### 256 **3. Results and discussion**

257

#### 258 *3.1. Higher level responses*

259 Equimolar concentrations of PT in both ZnPT and NaPT induced similar mortality to  
260 adult *M. galloprovincialis* after exposure for 14 d (Fig. S1), suggesting that  $PT^-$  ions  
261 are largely responsible for the effect. However, the fact that ZnPT was notably more  
262 toxic than NaPT after 4-d exposure suggests the complex as a whole has a higher  
263 acute toxicity, possibly due to its greater hydrophobicity and propensity to  
264 accumulate than the  $PT^-$  ion alone.

265

266 Control mussels showed increasing attachment with time, peaking at 100% for the  
267 sea water control and ~ 98% for the inorganic Zn treatment (Fig. 1A). In contrast,  
268 exposure to both low and high concentrations of ZnPT significantly reduced  
269 attachment at all time points compared to the control or inorganic Zn ( $p < 0.0001$ ),  
270 and never exceeded 5 % throughout the time course. This effect may be the result of  
271 a generalised stress response that causes a reduction in the capacity for byssus  
272 production (Babarro et al., 2008) or a specific chemical interaction effected between  
273 ZnPT or  $PT^-$  and byssus proteins. Regardless of the cause, these observations  
274 suggest that ZnPT is an effective repellent in antifouling formulations for hard-fouling  
275 organisms.

276

277 Clearance rate was also affected by ZnPT, with a reduction from  $> 1.75 \text{ L h}^{-1}$  in the  
278 control and inorganic Zn exposure to  $< 0.3 \text{ L h}^{-1}$  at both concentrations of the  
279 complex tested (Fig. 1B). The magnitude of this decrease is comparable to that  
280 observed for mussels exposed to  $18 - 56 \mu\text{g L}^{-1}$  Cu for 5 d (Al-Subiai et al., 2011),  $32$   
281  $\text{mg L}^{-1}$  methyl methanesulfonate for up to 7 d (Canty et al., 2009) and  $56 \mu\text{g L}^{-1}$   
282 benzo( $\alpha$ )pyrene for 12 d (Di et al., 2011), and is in excess of that reported for 14-d

283 exposure to branched alkyl benzenes from crude oil (Scarlett et al., 2008) and 3-d  
284 exposure to C60 fullerene nanoparticles (Al-Subiai et al., 2012). Reduced filtration in  
285 mussels causes both decreased gas exchange at the gills and reduced food intake  
286 (Bayne, 1976), and either of these parameters could have significant consequences  
287 for the energy stores that result in weakened anoxia tolerance (SoS).

288

289 During the SoS test, mussels exposed to the control or inorganic Zn declined  
290 steadily after 8 - 10 d (Fig. 1C), with similar LT50 values defining the two curves  
291 ( $14.00 \pm 0.58$  and  $14.00 \pm 0.55$ , respectively). In contrast, mussels treated with either  
292 concentration of ZnPT declined rapidly between 3 and 6 d and reached total  
293 mortality earlier, with LT50 values for 0.2 and 2  $\mu\text{M}$  ZnPT that were significantly  
294 lower than the controls ( $6.00 \pm 0.40$  and  $6.00 \pm 0.34$ , respectively;  $p < 0.0001$ ). Our  
295 LT50 values are higher than those reported by Marcheselli et al. for *M.*  
296 *galloprovincialis* exposed to 0.4  $\mu\text{M}$  ZnPT for 7 d (Marcheselli et al., 2011), indicating  
297 that mussels exposed to the higher concentration of ZnPT (2  $\mu\text{M}$ ) in the current  
298 experiment survived longer under anoxic conditions. However, it must be borne in  
299 mind that control, harbour mussels in the earlier study were defined by an LT50 of  
300 10.70 d, suggesting that either these mussels were stressed before the exposures or  
301 there is an inherent difference between the anoxia tolerance of *M. galloprovincialis*  
302 collected for the two studies. The latter could be an artefact of the mussels' biological  
303 situation, such as current reproductive status (Bignell et al, 2008). Significant  
304 variation in LT50 values for mussels sampled from different sites and in different  
305 seasons supports this assertion (Hellou and Law, 2003; Koukouzika and Dimitriadis,  
306 2005) and until natural variability is fully characterised, SoS should be reported as  
307 impact relative to control organisms. On this basis, mussels used here showed a  
308 greater decrease (-62.50%) than those of Marcheselli et al. (2011) (-49.53%),  
309 presumably because of the longer exposure to ZnPT employed in the present study.

310

### 311 3.2. Genotoxicity in circulating haemocytes

312

313 There was no significant effect of Fpg for any treatment and concurrent validation  
314 with in vitro exposure to hydrogen peroxide (Dallas et al., 2013) showed positive  
315 results. This confirmed that the Fpg enzyme had no significant effect on the  
316 chemicals tested (data not shown); for brevity, therefore, only buffer-treated data are

317 shown and discussed. Concentrations of 2  $\mu$ M ZnPT induced significantly elevated  
318 DNA strand breaks in haemocytes at all time points compared with the control and  
319 inorganic Zn ( $p < 0.001$ ), with the lower concentration of ZnPT exhibiting elevated  
320 strand breaks on day 14 only (Fig. 2). Control mussel haemocytes showed normal  
321 levels of micronuclei ( $\sim 3$  per 1000 cells) whereas those treated with 2  $\mu$ M inorganic  
322 Zn exhibited a non-significant ( $p > 0.05$ ) increase. In contrast, ZnPT caused a  
323 concentration- and time-dependent increase in micronuclei (MN;  $p < 0.0001$ ), with a  
324 maximum of 22.42 MN per 1000 cells in the 2  $\mu$ M ZnPT exposure on day 14 (Fig. 2).

325

326 These results provide independent confirmation that ZnPT is genotoxic to mussels,  
327 as reported by Marcheselli et al. (2011) in gills and digestive gland using the TUNEL  
328 assay. This is, however, the first report of ZnPT-induced sublethal genotoxicity in  
329 mussel haemocytes, which does not appear to be caused by purine oxidation (based  
330 on the enzyme-modified comet assay results). Similar results were obtained when  
331 potential genotoxicity in haemocytes were determined following exposure of mussels  
332 to nickel (Dallas et al., 2013). These studies suggest that mussel haemocytes might  
333 have some inherent limitations to express metal- or organometallic-induced oxidative  
334 damage to DNA. In this context, enzymatic repair of DNA oxidation induced by  
335 chromium (VI) in mussel gill cells have suggested that DNA repair processes could  
336 mask the oxidative damage and this could be influenced as a function of sampling  
337 time (Emmanouil et al., 2006). Results from this and earlier studies (Dallas et al.,  
338 2013) suggest that mussel haemocytes are able to maintain homeostasis with  
339 respect to DNA oxidation but further studies involving repeated sampling would be  
340 required to shed light on the persistence of DNA oxidation. Furthermore, our results  
341 suggest good correlations between induction of DNA strand breaks (as determined  
342 by the comet assay) and cytogenetic damage (as determined by the MN assay). This  
343 is in line with many in vivo and in vitro studies reported previously (Canty et al.,  
344 2009; Dallas et al., 2013)., strengthening the case for the concomitant use of these  
345 genotoxicity endpoints.

346

347 Although Nunes et al. (2015) studied oxidative stress in the freshwater fish,  
348 *Gambusia holbrooki*, after exposure to ZnPT, to our knowledge the present  
349 investigation is the first to examine such effects in a marine organism., with both  
350 studies finding no increase in catalase or glutathione-s-transferase activity effected

351 by the chemical. The current comet assay results are also in agreement with in vitro  
352 data from rat cardiomyocytes, where ZnPT reduced superoxide generation and  
353 oxidative injury (as both LDH release and cell survival) after reperfusion (Kasi et al.,  
354 2011). However, the mammalian literature is somewhat contradictory in that ZnPT  
355 causes increased susceptibility to ROS-induced damage in rat thymocytes co-  
356 exposed to hydrogen peroxide (Oyama et al. 2012). Furthermore, a study on human  
357 skin cells reported upregulation of metallothionein genes, commonly associated with  
358 oxidative stress (Lamore and Wondrak, 2011). The latter observation may be  
359 explained by the known metal binding capacity of metallothionein proteins, i.e. the  
360 effect may be induced by Zn itself rather than ZnPT. Further research to directly  
361 quantify ROS in mussel cells (of different tissues) is recommended to elucidate the  
362 mechanisms behind the genotoxic effects of ZnPT.

363

### 364 3.3. Alterations in transcriptional expression of key genes

365

366 Whilst many studies have been carried out to determine the toxicity of metals to  
367 mussels (Lemoine et al., 2000; Banni et al., 2007; Al-Subiai et al., 2011; Dallas et al.,  
368 2013; Varotto et al., 2013), there is limited information on their response to ZnPT or  
369 other pyrithione compounds. Although the anti-dandruff characteristics of ZnPT may  
370 result from indirect effects on skin cells (e.g. suppressed DNA synthesis; Imokawa et  
371 al., 1983), in yeast the complex exhibits specific anti-fungal activity via iron starvation  
372 or decreased iron-containing protein activity as a result of elevated intracellular Cu  
373 (Yasokawa et al., 2010; Reeder et al., 2011b). There are also several reports of  
374 genotoxicity and stress response induction (especially HSPs and increased p53  
375 expression) in human skin cells exposed to ZnPT (Lamore and Wondrak, 2011;  
376 Rudolf and Cervinka, 2011; Lamore et al., 2010). The molecular approaches used  
377 here were designed to elucidate which - if any - of these potential mechanisms  
378 contribute to ZnPT-induced toxicity in marine mussels.

379

380 In the present study, qPCR efficiencies were *hsp70* 1.750/1.798, *mt10* 1.791/1.830  
381 and *mt20* 1.838/1.846 for gill/digestive gland of *M. galloprovincialis*. Significant  
382 changes in expression were tissue-specific and only seen in digestive gland at day 4,  
383 where all three target genes showed upregulation at the low ZnPT concentration but  
384 only *hsp70* and *mt20* showed upregulation at the high concentration (Fig. 3); in gill,

385 upregulation of *hsp70* and *mt20* occurred for 2  $\mu$ M ZnPT at day 14 only (Fig. 3).  
386 Temporal differences in *hsp70* and *mt20* expression observed between the gill and  
387 digestive gland may be linked to time-dependent variations in the location of ZnPT  
388 accumulation by mussels; for example, Marcheselli et al. (2010b) report increased  
389 accumulation in digestive gland compared with gill of *M. galloprovincialis* after  $\geq 2$  d  
390 exposure to 1.5  $\mu$ M ZnPT. Regarding HSPs, both the present study and that of  
391 Marcheselli et al.(2011) have identified these genes as components of the ZnPT  
392 response in *M. galloprovincialis*. Furthermore, studies in human skin cells have  
393 reported upregulation of HSPs and metallothionein genes after ZnPT treatment,  
394 suggesting that these genes and their toxic responses might be highly conserved  
395 (Lamore and Wondrak, 2011; Lamore et al., 2010).

396

397 PCA and Cluster Analysis of the six common biomarkers showed that the first  
398 component (PC1) explained 44.1 % and 59.0 % of total variance at day 4 and 14,  
399 respectively (Fig. 4B and 4C), and was mostly related to *mt20* activity at day 4 but  
400 with no defining biomarker at day 14. Treatment-specific clusters were evident at  
401 both time points, but were more diffuse by day 14. However, the treatment-related  
402 clusters were more clearly separated from each other at day 14, whereas there was  
403 considerable overlap between treatments at day 4. Gene expression described more  
404 variability within groups, while genotoxic parameters distinguished between  
405 treatments. This suggests that either intra-individual variability is masking treatment-  
406 specific effects on these genes, or that investigation of other genes (e.g. p53 and  
407 those for other DNA repair proteins) may provide a better understanding of the  
408 mechanistic aspects of the genotoxicity of ZnPT.

409

410 It is well accepted that the toxicity of a chemical or environmental stressor is a cell-  
411 or tissue-specific phenomenon (Jha, 2008; 2004; Di et al., 2011). In the present  
412 study, DNA damage using the comet assay was determined in the circulating  
413 lymphocytes whereas the expression of genes were determined in different tissues.  
414 While assessing the biological responses at the cellular and tissue level due to  
415 technical and tissue-specific inherent limitations, it is not always possible to apply the  
416 same assay across the biological samples obtained. For example, circulating  
417 lymphocytes which could be obtained in small amounts from mussels are ideal for  
418 the analysis of DNA damage using the comet assay whilst other tissues are ideal for

419 gene expression analyses. For the application of comet assay, it is a prerequisite to  
420 obtain a single cell suspension, which is not necessary for circulating haemocytes.  
421 On the other hand, application of the comet assay on solid tissues (e.g. digestive  
422 glands, gills etc.) require mechanical and enzymatic treatments, which could induce  
423 DNA damage in their own right (Jha, 2008). It is also important to point out that gene  
424 expression results could give different results in different tissues (Di et al., 2011). In  
425 addition, since different tissues have different turnover rates (cell cycle durations)  
426 and metabolic properties and the fact that contaminants could induce cell cycle stage  
427 specific changes for gene expression (Di et al., 2011), it is difficult to justify selection  
428 of a particular cell or tissue type for different biological assays. In this study,  
429 therefore, we used a range of cell types and the observed biological responses were  
430 analysed using PCA and Cluster analysis to obtain a holistic picture.

431

#### 432 3.4. Alterations in protein expression

433

434 Digestive gland proteins were well-separated on the gel, with minimal streaking or  
435 smearing (Fig. S3). Only spots showing significant fold change ( $\geq 1.5$  and  $p < 0.05$ )  
436 relative to both the sea water control and inorganic Zn exposure were considered as  
437 ZnPT-specific effects (5 spots and all on day 4, Table 1). Two such spots were  
438 positively identified by mass spectroscopy; specifically, spot 355 was significantly  
439 homologous to a predicted protein sequence for phosphoenolpyruvate  
440 carboxykinase (PEPCK) in barley (GenBank BAK02183.1), and spot 550 was a  
441 small HSP from *M. galloprovincialis* (GenBank AEP02968.1). Of the remaining spots,  
442 1 could not be identified due to low protein content (607) and the other 2 had no  
443 matching sequences, possibly due to extensive post-translational modification.

444

445 Although several studies have examined PEPCK levels in mussels in response to  
446 physical stressors like temperature and salinity (Anestis et al., 2010; Lockwood and  
447 Somero, 2011; Tomanek and Zuzow, 2010), our investigation appears to be the first  
448 to demonstrate a negative impact on PEPCK by a contaminant in a model marine  
449 organism. If decreased PEPCK protein after 4-d ZnPT exposure corresponds to  
450 decreased PEPCK activity, this could compromise the ability of mussels to cope with  
451 anoxic conditions, as seen in the SoS assay. In contrast, Widdows et al. (1982)  
452 reported increased PEPCK activity in *M. galloprovincialis* after 140 d exposure to 30

453  $\mu\text{g L}^{-1}$  water-accommodated hydrocarbons. The discrepancies between these  
454 two responses may be attributed to a number of factors. Thus, firstly our study did  
455 not measure enzyme activity but determined expression of protein, and post-  
456 translational modification to activate or suppress PEPCK may result in differences  
457 between expression and activity. Secondly, given that changes in PEPCK  
458 expression appeared herein at day 4 but were not evident by day 14, it is possible  
459 that temporal shifts in expression-activity patterns may have occurred in our  
460 experiment, suggesting we are observing baseline variation rather than biological  
461 effect. Thirdly, responses are often contaminant- or tissue-specific; for instance,  
462 kinetic studies of glycolytic enzymes in *M. edulis* have shown significant variation in  
463 activity between adductor muscle (catabolic tissue) and mantle (anabolic tissue)  
464 (Churchill and Livingstone, 1989).

465

466

### 467 3.5. Environmental risk assessment and future work

468

469 Given the detrimental effects of ZnPT on *M. galloprovincialis* at all levels of biological  
470 organization, it is perhaps surprising that mussels are found in abundance in many  
471 marinas where the biocide has the potential to reach levels considerably higher than  
472 in pristine waters through leaching from boat hulls and from spent antifouling waste  
473 (Holmes and Turner, 2009). It is possible, however, that mussels from environments  
474 impacted by boating have adapted to elevated ZnPT concentrations through some  
475 resistance mechanism or compensatory response. For instance, it is known that *M.*  
476 *edulis* exhibit a high adaptive capacity to the impact of a variety of metallic and  
477 lipophilic contaminants (Bakhmet et al., 2009). To this end, a useful extension to the  
478 current work would be a direct comparison of the condition and response of mussels  
479 from a reference site with those from a region where boat storage or maintenance is  
480 significant.

481

482 There are several issues that need to be addressed before the general  
483 environmental risk from ZnPT can be more accurately estimated. Regarding  
484 environmental concentrations, there exist only limited data, with a reported range in  
485 the aqueous phase (as PT) between about 2 and 100 nM for marinas and navigation  
486 channels (Mackie et al., 2004; Madsen et al., 2000). While this range is lower than

487 concentrations employed in the present study, environmental concentrations may be  
488 elevated locally by point sources and more generally in turbid or shaded sea water  
489 (e.g. under pontoons and boats) where the biocide is more persistent (Marcheselli et  
490 al., 2010b). ZnPT induces considerable physiological and behavioural effects in  
491 mussels in addition to its genotoxicity, and emphasizing the need for integrated  
492 studies that examine a wide range of effects. Also, as mussels appear to be more  
493 tolerant of ZnPT than crustaceans and polychaetes (Bao et al., 2008; Mochida et al.,  
494 2006; Marcheselli et al., 2010b), the wide-ranging effects demonstrated here also  
495 suggest a greater potential risk to other marine biota. Studying the effects of this  
496 compound in a wider range of aquatic invertebrate species is, therefore, essential.  
497 Indirectly, ZnPT has additional impacts through transchelation of  $PT^-$  ions with  $Cu^{2+}$   
498 (the primary ion formed during the ablation of most contemporary antifouling  
499 formulations) (Holmes and Turner, 2009; Grunnet and Dahllof, 2005). Studies  
500 suggest that CuPT is more toxic than its Zn counterpart (Bao et al., 2011) and that  
501 mixtures exhibit synergistic effects (Bao et al., 2014), but more experimental studies  
502 would be required to explore these effects in a broader context.

503

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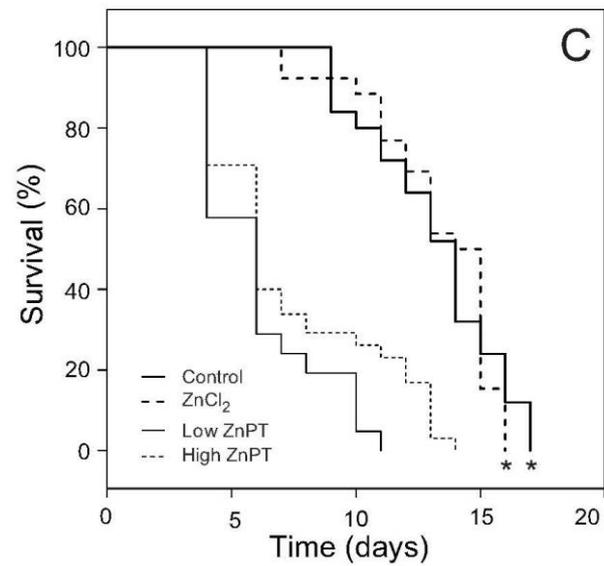
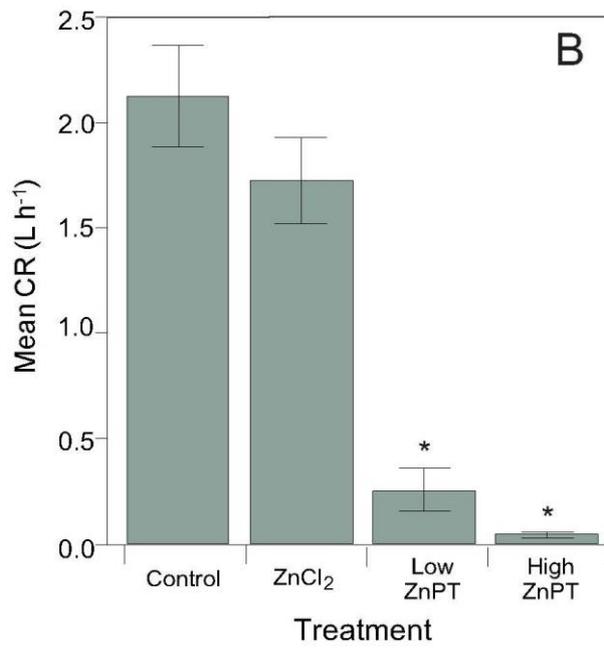
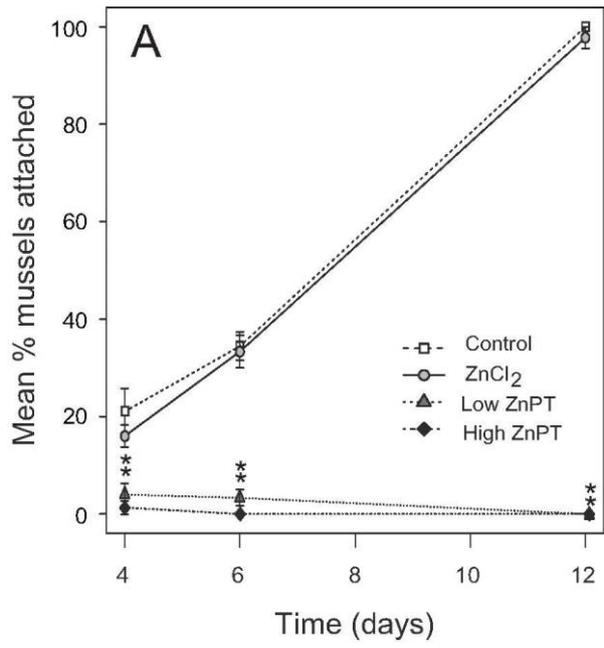
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798 Figure 1: Higher level responses of *M. galloprovincialis* exposed to inorganic Zn or  
799 ZnPT. (A) Percentage of mussels showing attachment during 14 d exposure; (B)  
800 clearance rate (CR) after 14 d exposure; (C) survival (SoS;  $n = 25$ ) in anoxic  
801 conditions during 20 d after exposure. Error bars are  $\pm$  one SE and asterisks indicate  
802 significant differences ( $p < 0.05$ ) from both sea water and inorganic Zn treatments.  
803

804 Figure 2: Genotoxicity in *M. galloprovincialis* haemocytes after exposure to inorganic  
 805 Zn or ZnPT for (A) 0, (B) 4, (C) 7 or (D) 14 d, and as determined by the enzyme-  
 806 modified comet assay (% tail DNA; bars) and micronuclei assay (per 1000 cells;  
 807 black circles). Error bars are  $\pm$  one SE and asterisks indicate significant differences  
 808 ( $p < 0.05$ ) from both sea water and inorganic Zn treatments on the same day,  
 809 whereas daggers indicate differences from day 0.

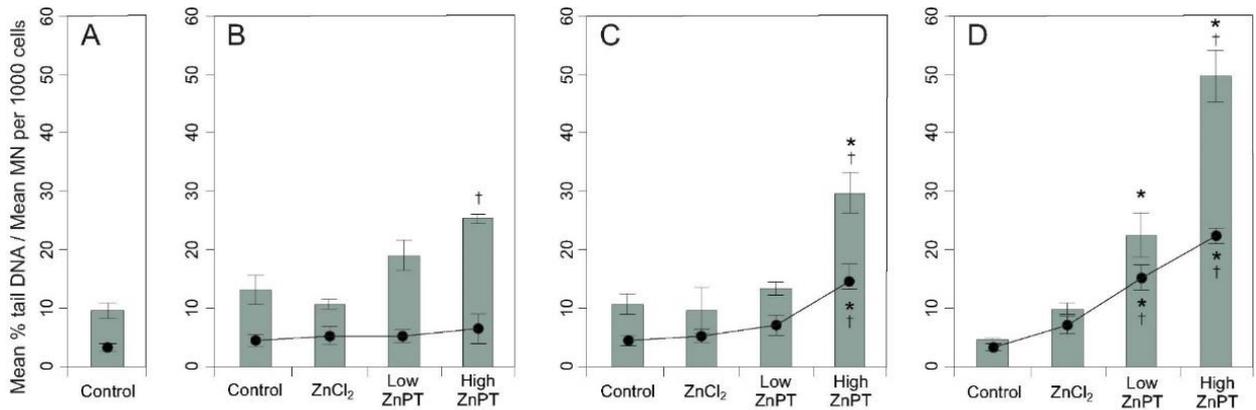
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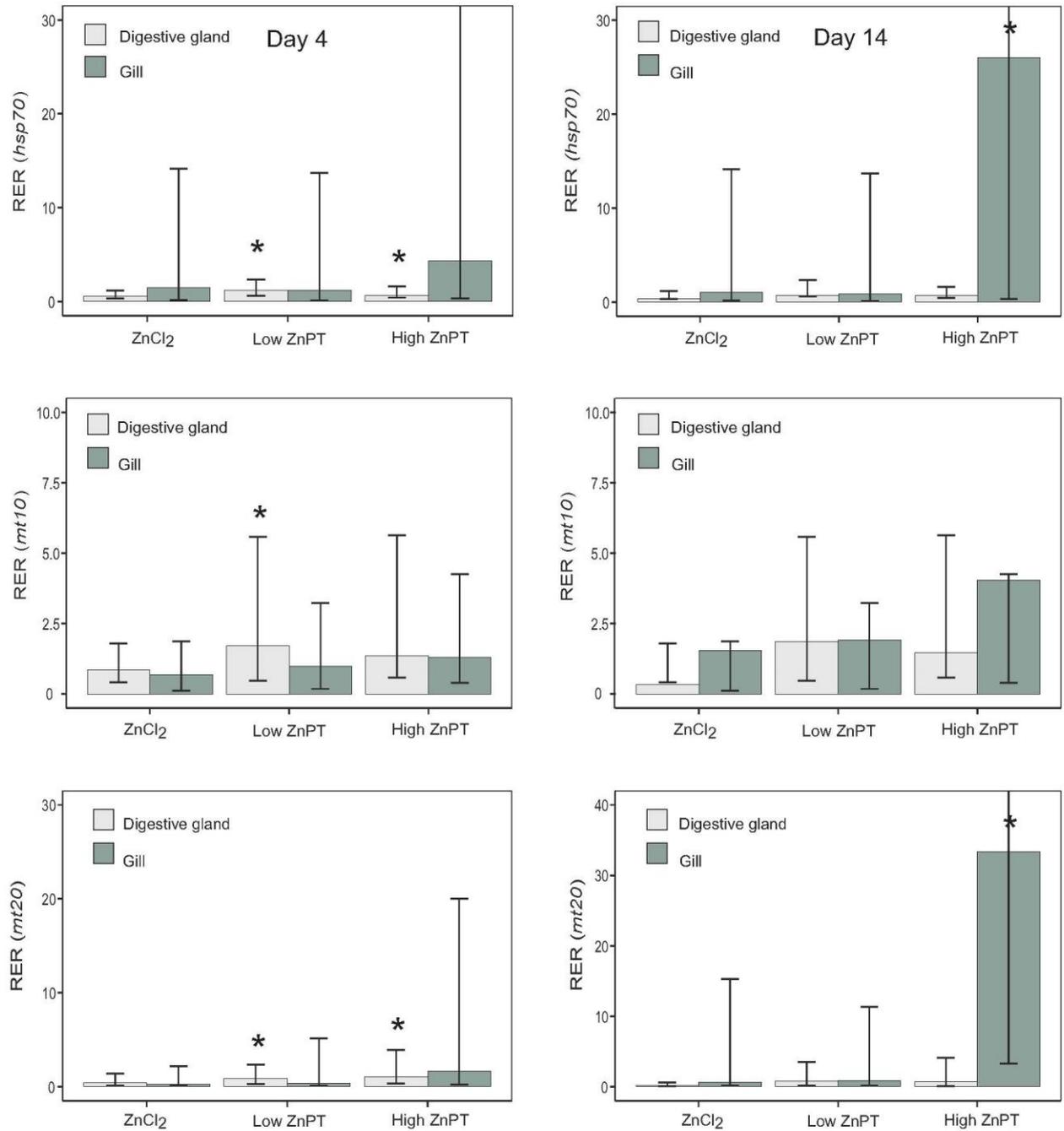
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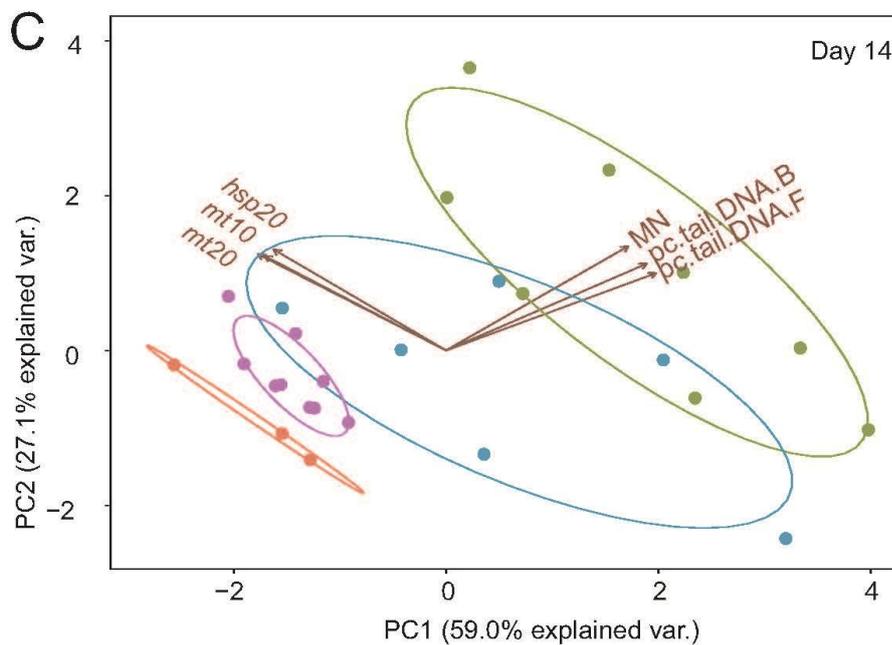
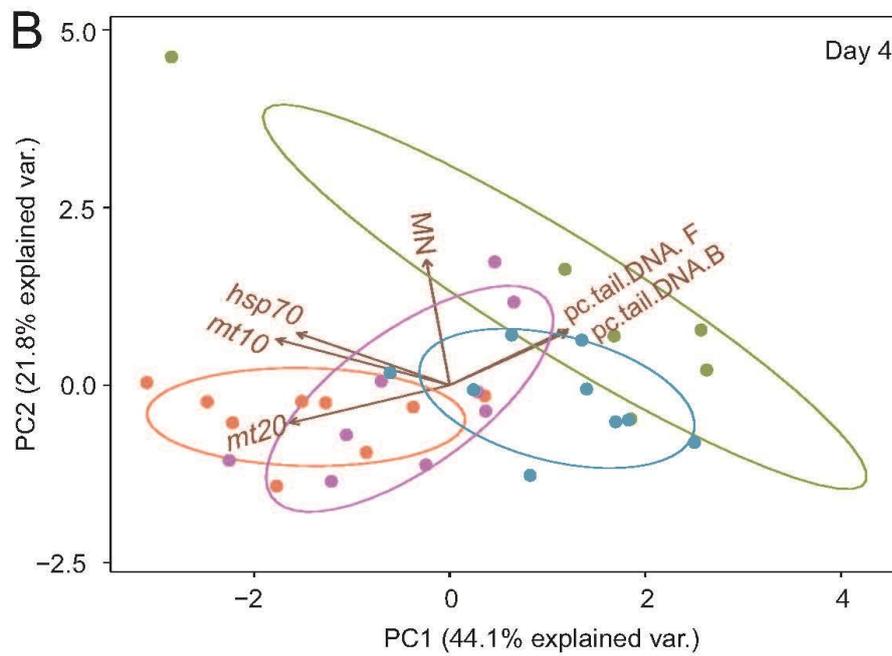
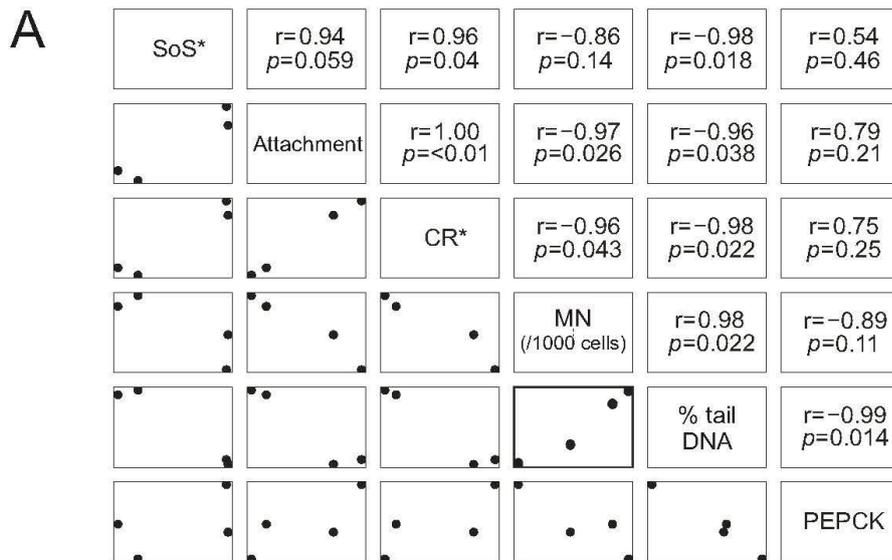
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816 Figure 3: Relative expression ratios (RER) of three genes (*hsp70*, *mt10* and *mt20*) in  
 817 digestive gland and gill tissue of *M. galloprovincialis* after exposure to inorganic Zn or  
 818 ZnPT. Data are normalised for two reference genes (*atub* and *ef1*) and the sea water  
 819 control treatment and are shown with ± 95% confidence intervals. Values above 1  
 820 indicate upregulation and those below downregulation. Significant differences  
 821 (PFRRT, *p* < 0.05) from the corresponding tissue control at day 0 are indicated by  
 822 asterisks.



824 Figure 4: Links between responses at different levels of biological organisation in *M.*  
825 *galloprovincialis* across all treatments. (A) Correlation matrix showing relationships  
826 between higher level responses, genotoxicity and expression of PEPCK. Data were  
827 averaged for each treatment and parameters were measured from day 14 onwards  
828 except where noted with an asterisk, with lower left panels showing data plots and  
829 upper diagonal panels showing corresponding  $r$  and  $p$  values. (B) and (C) Principal  
830 component analysis of 6 genotoxicity and gene expression parameters ( $\Delta C_q$  values  
831 for *hsp70*, *mt20* and *mt10*, % tail DNA [buffer], % tail DNA [Fpg] and MN/1000 cells)  
832 for 4 and 14 d, respectively.

833 Table 1: Differentially expressed proteins in the digestive gland of *M.*  
 834 *galloprovincialis* exposed to 0.2 or 2  $\mu$ M ZnPT for 4 d. Non-significant fold changes  
 835 ( $p > 0.05$ ) have been omitted.

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837	Spot id	protein id	Fold change relative to		Fold change relative to	
838			sea water control		inorganic Zn	
839			0.2 $\mu$ M	2 $\mu$ M	0.2 $\mu$ M	2 $\mu$ M
841	239	unidentified	+2.10	+3.91		+1.89
842	355	phosphoenolpyruvate		-2.91		-1.94
843		carboxykinase				
844	412	unidentified	-1.68	-2.04	-1.73	-2.10
845	550	small heat shock 24.1	+1.78		+1.90	
846	607	unidentified		+1.59		+2.00

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