Stress gene responses in amphipods to cadmium

1 **Title:** Investigation of cellular stress response related heat shock protein *hsp70*/Hsp70 and

2 multixenobiotic transporter *abcb1* in Siberian freshwater amphipods upon cadmium exposure

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4 **Authors:** Marina V. Protopopova[1*], Vasiliy V. Pavlichenko[1**], Till Luckenbach[2***]

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6 **Affiliations:** [1] Siberian Institute of Plant Physiology and Biochemistry SB RAS, Lermontov

7 str., 132, 664033 Irkutsk, Russia; [2] Department of Bioanalytical Ecotoxicology, UFZ-

8 Helmholtz Centre for Environmental Research (UFZ), Permoserstraße 15, D-04318 Leipzig,

9 Germany

10

11 **Contact   data**:   *marina.v.protopopova@gmail.com**;   **vpavlichenko@gmail.com;

12 ***till.luckenbach@ufz.de (corresponding author)

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21   **Abstract:** Induction of stress response genes *hsp70* and *abcb1* and Hsp70 protein by

22   cadmium chloride ($CdCl_2$) was explored in amphipod species with different stress adaptation

23   strategies from the Lake Baikal area. Based on lethal concentrations (LC) the sensitivities to

24   $CdCl_2$ were ranked (24 hr LC50 – mg/L $CdCl_2$): *Gammarus lacustris* (1.7) <

25   *Eulimnogammarus cyaneus* (2.9) < *E. verrucosus* (8.3) < *E. vittatus* (18.2). Conjugated dienes

26   indicating lipid peroxidation were significantly increased by 5 mg/L $CdCl_2$ (24 hr exposure)

27   only in *G. lacustris* and *E. cyaneus*. Upon treatment with 0.54 – 5.8 mg/L $CdCl_2$ *hsp70*

28   transcript levels were more increased in the toxicologically more sensitive species. Relating

29   the exposure concentrations to LCx values revealed that across the species the increases of

30   *hsp70* transcript levels were comparatively low (up to 2.6-fold) up to LC50; at higher LCx

31   values *hsp70* induction was more pronounced (up to a 9.1-fold by 5 mg/L $CdCl_2$ (≙LC70) in

32   *E. cyaneus*). In contrast, *abcb1* inductions did not correspond with $CdCl_2$ LCx values across

33   species; *abcb1* induction was highest (4.7-fold) in *E. verrucosus* by 5.0 mg/L $CdCl_2$ (≙LC45,

34   24 hr exposure). Induction of stress gene responses by lethal $CdCl_2$ concentrations indicates

35   that in the amphipods they are rather insensitive.

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39   **Key words:** conjugated dienes, heavy metal, Lake Baikal, P-glycoprotein, stress protein

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Stress gene responses in amphipods to cadmium

42 **Introduction**

43   Cadmium is a non-essential heavy-metal that reaches the environment *via* various

44 anthropogenic and natural sources and at low concentrations causes poisoning in humans and

45 wildlife [1]. Toxic cadmium effects have often been attributed with increased levels of

46 reactive oxygen species (ROS) and reactive nitrogen species (RNS) causing damages of

47 biological macromolecules such as proteins [2]. Exposure to cadmium was found to lead to

48 the induction of cellular stress response genes *hsp70* [3-12] and *abcb1* [13, 14] in a range of

49 aquatic organisms. This induction of *hsp70* and *abcb1* by cadmium can be related to the

50 increased abundance of damaged cellular macromolecules such as cellular membrane

51 fragments or misfolded proteins [15, 16].

52   Lake Baikal in Eastern Siberia, the oldest, deepest and by volume largest lake in the

53 world, is a biodiversity hotspot with high degrees of endemicity of species [17, 18]. Baikal´s

54 water is generally highly pristine, however, the risk of water contamination by heavy metals

55 is increasing especially through the Selenga river, which is the largest tributary of Lake

56 Baikal and comprises almost half of the riverine inflow into the lake [19, 20]. Amphipods

57 are a dominant taxon of the benthic communities of Lake Baikal and the more than 350

58 endemic species and subspecies represent 45.3% of all freshwater amphipod species of the

59 world [21, 22]. The numerous phylogenetically closely related species featuring a range of

60 adaptation strategies are interesting models for comparative studies [23]. In the here studied

61 amphipod species constitutive expression levels of cellular stress response genes vary within

62 an order of magnitude which could be related with differences across species regarding stress

63 tolerance. Thus, constitutive *hsp70* levels relate to the species-specific differences in

64 thermotolerance [24-26]. Furthermore, species-specific degrees of gene responses of *hsp70*

65   and *abcb1* to organic compounds as humic substances [25] and phenanthrene [27] were

66   found.

67   It is stated in many studies on the effects of cadmium on *hsp70*/Hsp70 levels that the

68   test organisms were exposed to sublethal cadmium levels but the published molecular effect

69   data are generally not related to the general level of stress caused by the cadmium

70   concentration applied, such as lethal cadmium concentrations. It is thus unclear how sensitive

71   the observed gene response to cadmium is.

72   We therefore here addressed the question at which stress levels cellular stress response

73   genes *hsp70* and *abcb1* are induced upon exposure to $CdCl_2$ in amphipod species with

74   different cellular stress response capacities. The lethal concentrations (LCx 24h) of $CdCl_2$

75   served as a measure for the cadmium-related stress levels. Transcript levels of *hsp70* and

76   *abcb1* were quantified *via* quantitative polymerase chain reaction (qPCR) in RNA extracted

77   from tissue of amphipods upon exposures of up to 24 hrs to different $CdCl_2$ concentrations.

78   The study was performed with four amphipod species that differ with regard to their

79   ecological preferences and habitats and accordingly with their physiological and cellular

80   adaptations to environmental conditions. Three of the species are littoral amphipods from

81   Lake Baikal belonging to the *Eulinogammarus* genus; the Holarctic species *Gammarus*

82   *lacustris* that occurs in waters connected to Lake Baikal but not in areas of the lake inhabited

83   by endemic fauna was also examined.

84   **Materials and Methods**

85   *Studied species and animal sampling*

86   The experimental species were *Eulimnogammarus cyaneus* (Dyb., 1874),

87   *Eulimnogammarus verrucosus* (Gerstf., 1858) and *Eulimnogammarus vittatus* (Dyb., 1874),

88   endemic to Lake Baikal, and *Gammarus lacustris* (Sars, 1863) common in surface waters

89   across the Holarctic. *Eulimnogammarus cyaneus* is most abundant along the shallow shoreline

90   (mainly up to 1 m water depth), few individuals occur at water depths up to 20 m [28] at

91   temperatures between 5-13°C [22]. Habitats of *E. vittatus* are at water depths of up to 30 m,

92   the abundance peak is at 2–3 m depth [29]; the water temperatures in the habitat range

93   between 9-13°C [22]. *Eulimnogammarus verrucosus* commonly occurs close to shore across

94   Lake Baikal at water depths of less than 1 m to up to 10–15 m [29] at temperatures of 5-13°C

95   [22]. *Gammarus lacustris* is common in shallow, eutrophic lakes with seasonal temperature

96   fluctuations in the Lake Baikal region at water depths of 0-7m but the species is not found in

97   the open Baikal [30]. Constitutive transcript/protein levels of heat shock protein 70

98   (*hsp70*/Hsp70) and transcript levels of ATP binding cassette (ABC) transporter 1 (*abcb1*)

99   indicate the cellular stress response capacities of the species in the order: *Eulimnogammarus*

100   *vittatus ≈ Eulimnogammarus verrucosus < < Eulimnogammarus cyaneus < Gammarus*

101   *lacustris.* Indeed, the species-specific constitutive/induced *hsp70*/Hsp70 levels correlate with

102   higher degrees of thermotolerance of *E. cyaneus* and *G. lacustris* compared to *E. verrucosus*

103   [24-26, 31].

104   *Eulimnogammarus* specimens for the experiments were sampled at the Baikal shoreline

105   close to Irkutsk State University's biological station at the Bolshie Koty settlement (Southern

106   Baikal) and *G. lacustris* was collected from a small shallow artificial lake close to Bolshie

107   Koty ("Lake 14", for specifications of the sampling points refer to [25]). Body lengths and

108   weights of the animals used in the experiments were: *E. verrucosus* – 20-25 mm, 403 ± 95

109   mg; *E. vittatus* – 18-20 mm, 121 ± 16 mg; *E. cyaneus* – 9-14 mm, 17 ± 3 mg; *G. lacustris* –

110   14-18 mm, 80 ± 16 mg. Upon sampling the animals were brought to the lab in a cold box with

111   water from the sampling sites. Animals were acclimated to lab conditions in aerated water in

112   2 L tanks at 6.5-7°C for 1-3 days prior to the experiments. The water used for maintaining the

113   animals in the tanks and for the experiments was withdrawn from Lake Baikal with buckets

114   from the pier next to the Biological Station in Bolshie Koty. Lake Baikal water instead of

115   water from the pond where *G. lacustris* was collected was also used for *G. lacustris* exposures

116   to avoid that differences in water contents (minerals, organic matter) affected the toxic effects

117   of cadmium.

118   *3.2. Acute toxicities of CdCl$_2$*

119   Acute toxicity of CdCl$_2$ to amphipods was determined in 24 hr exposure experiments.

120   Aqueous CdCl$_2$ solutions for the experiments were set up in glass tanks with 2 L of well-

121   aerated water along a control with clean water. The water temperature in the tanks was

122   maintained at 6.5-7.0°C during the exposures. Twenty amphipods of each species were placed

123   in separate tanks with eight different CdCl$_2$ concentrations and a control. The CdCl$_2$

124   concentration ranges were 0.5 – 8 mg/L for *G. lacustris* and *E. cyaneus* and 0.625-30 mg/L

125   for *E. verrucosus* and *E. vittatus*. During the experiments, tanks were regularly checked for

126   dead animals that were removed and recorded. After 24 hrs alive and dead animals in all tanks

127   were counted. Exposure experiments were repeated two to three times with different CdCl$_2$

128   concentrations that were chosen depending on the mortality rates determined for the already

129   tested concentrations. Cadmium concentrations in water from a control and a CdCl$_2$ exposure

130   were determined with atomic absorption spectroscopy (AAS) at the Analytical Chemistry

131   Department at the UFZ. The actual deviated from the nominal Cd concentration by 15 %

132   (nominal: 10 $CdCl_2$ mg/L; actual: 8.5 $CdCl_2$ mg/L) and all nominal $CdCl_2$ concentration

133   values were adapted accordingly.

134   *3.3. Measurements of conjugated diene (CD) levels*

135   Levels of conjugated dienes (CD) were measured in amphipods upon exposure to 5

136   mg/L $CdCl_2$ for 1, 6 and 24 hrs according to [32] with some modifications. Several entire

137   animals were pooled to weights of 150 to 800 mg depending on the species and homogenized

138   in a heptane/isopropyl alcohol mixture (1:1, v/v) using a Potter-Elvehjem tissue homogenizer,

139   the extract was then filled in a glass tube and brought up to a volume of 4.5 ml with a

140   heptane/isopropyl alcohol mixture. One ml of distilled water was added, the mix was

141   vigorously shaken and incubated at 25°C for 30 min for phase separation. 0.5 ml of the

142   heptane phase were mixed with ethanol in a 1:3 ratio (v/v) and the absorbance at 233 nm was

143   measured on a SmartSpec Plus spectrophotometer (Bio-Rad) with extraction blanks used as

144   references. An extinction coefficient of $2.52 \times 10^4\,M^{-1}cm^{-1}$ [33] was used to determine the

145   amount of CD present. The data are reported as nmol g^{-1} wet weight.

146   *3.4. $CdCl_2$ exposures for investigating cellular stress responses*

147   Two sets of exposure experiments were performed for examining cellular responses of

148   the different amphipod species to $CdCl_2$ exposure: 1) $CdCl_2$ exposure concentrations were

149   scaled to species-specific LC10 and LC50 values enabling to compare responses at equal

150   stress levels. Respective $CdCl_2$ exposure concentrations (LC10/LC50) were: *E. cyaneus* −

151   0.68/2.89 mg/L, *E. verrucosus* – 0.54/5.75 mg/L, *G. lacustris* – 0.59/1.7 mg/L; *E. vittatus* was

152   only exposed to 5 mg/L corresponding to LC10. 2) Exposures were with all species at 1.7

153   (corresponding to the LC50 for *G. lacustris*) and 5.0 mg/L $CdCl_2$ (corresponding to the LC90

154   for *G. lacustris*) enabling to determine species-specific responses to equal concentrations.

155   CdCl$_2$ exposures were performed in aerated 2 L tanks (n=5/concentration) with Baikal water

156   at 7°C. Specimens were sampled after 1, 6, and 24 hr exposures, frozen and stored in liquid

157   nitrogen for extraction of total protein and diene conjugates or placed in QIAzol Lysis

158   Reagent (Qiagen, Hilden, Germany) and frozen in liquid nitrogen for RNA isolation. Control

159   animals were kept in CdCl$_2$ uncontaminated water but otherwise at equal temperature and

160   aeration conditions as CdCl$_2$ exposed animals. One whole specimen of *E. verrucosus* or a

161   pool of several whole animals of the other species equalling 200 to 500 mg of tissue were

162   used for the further analyses. All exposure experiments were repeated at least five times and

163   all samples were assessed in triplicate.

164   *3.5. Isolation of total RNA and cDNA synthesis*

165   Isolation of total RNA and cDNA synthesis were performed as described in [27].

166   Tissue preserved in QIAzol Lysis Reagent was homogenized with a MM400 homogenizer

167   (Retsch, Haan, Germany) followed by a phenol-guanidine-chloroform based extraction of

168   total RNA. To improve the separation of the organic and aqueous phases MaXtract gel

169   (Qiagen) was used according to the manufacturer. Total RNA was purified from the obtained

170   aqueous phase with the miRNeasy kit with DNase treatment using a QIAcube instrument

171   (Qiagen). One µg of total RNA was reverse-transcribed to single-stranded cDNA using

172   Oligo(dT)18 primer (Fermentas, USA, #SO131) and H Minus Reverse Transcriptase system

173   (Fermentas, USA, #EP0452) following the manufacturer's instructions.

174   *3.6. Quantitative real-time PCR analysis*

175   Gene expression levels were analysed with quantitative polymerase chain reaction

176   (qPCR) using a StepOnePlus Real-Time PCR System (Applied Biosystems) as described

177   previously [25]. The amplification was performed in a final volume of 12.5 μl using the

178   SensiMix SYBR Low-ROX Kit (Bioline) with each primer at 200 nM. Primer sequences for

179   qPCR of *hsp70* and *abcb1* and housekeeping genes *β-actin, gapdh* and *ef1-a* were from [25];

180   qPCR conditions were according to [25, 27]: 94 °C for 4 min; 35 (for *hsp70* and references

181   genes) and 45 cycles (for *abcb1*) with 95 °C for 15 sec, 60-62 °C for 15 sec and 72 °C for 15

182   sec followed to fluorescent measurement step with 78-79°C (close to the melting temperature

183   of the PCR products) for 10 sec. The extra step for fluorescence measurement was added to

184   avoid detection of unspecific amplification products and primer dimers. Relative expression

185   levels of *hsp70* and *abcb1* genes were calculated with the comparative ΔΔCt method [34]

186   using efficiency corrected calculation models and Best Keeper levels based on the geometric

187   mean of the Ct values of the housekeeping genes [35-37].

188   *3.7. Western-blotting*

189   Isolation of total protein from amphipod tissue and Western-blotting were performed

190   as described in [25]. Tissues were homogenized in 0.1 M Tris buffer (pH=7.6) containing 1.5

191   mg phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail (Amresco,

192   USA); proteins were then subjected to denaturation in Tris-HCl buffer (0.25 M Tris-HCl (pH

193   = 6.8), 5% sodium dodecyl sulphate (SDS), 0.5 mM ethylenediamine tetraacetic acid

194   (EDTA), 10% glycerol, 5% ß-mercaptoethanol and 0.03% Bromophenol Blue) at 95 °C for 5

195   min. Total protein concentrations were determined following [38]. Equal amounts of total

196   protein per sample were separated by SDS gel electrophoresis on a 12.5% polyacrylamide gel

197   [39] using a Mini-PROTEAN II Electrophoretic Cell (Bio-Rad, USA). Wet-transfer of the

198   proteins from the gel to a polyvinylidene difluoride transfer membrane (GE Healthcare, UK)

199   was according to [40] with minor modifications. Hsp70 protein was labelled with anti-HSP70

9

200   (Sigma-Aldrich, # H9776) at 1:3000, which detects both the inducible and constitutive forms

201   of HSP70, and with secondary antibodies conjugated with alkaline phosphatase (Anti-Mouse

202   IgG:AP Conj., Stressgen # SAB-101) at 1:1000. Anti-actin antibody (Sigma-Aldrich, #

203   A2668) dissolved 1:400 and secondary antibodies (Anti-Rabbit IgG, Sigma, # A9919)

204   dissolved 1:1000 were used for actin labelling. Sixty ng of bovine Hsp70 (Sigma-Aldrich, #

205   H9776) and 60 ng of bovine actin (Sigma-Aldrich, A3653) were used as positive controls.

206   Bands were visualized with 0.4 mM BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 0.4

207   mM nBT (nitroblue tetrazolium). The Gel Explorer software package (DNAtechnology,

208   Russia) was used for semi-quantitative measurements of Hsp70 and actin levels on the

209   membranes and Hsp70 levels were normalized relative to actin for each sample.

210   *3.8. Data analysis and statistics*

211   Regressions of concentration-mortality relationships were calculated by fitting

212   mortalities at the respective concentrations to the non-linear HILL model:

213    $$Y(c_{CdCl_2}) = \min + \frac{(\max - \min)}{1 + 10^{(LogLC50 - X) * P}} \quad (eq.1)$$

214   where

215   **Y** is the mortality at the CdCl$_2$ concentration *c*

216   **X** is the logarithm of the CdCl$_2$ concentration

217   **min** is the minimum percentage mortality (control, constrained to 0)

218   **max** is the maximal percentage mortality (constrained to 100)

219   **P** is the shape parameter

220   **LC50** is the constant describing the CdCl$_2$ concentration causing 50% mortality

221

222   Regressions of *hsp70* transcript/Hsp70 protein levels at respective CdCl$_2$

223   concentrations expressed as LCx values were calculated by fitting the observations at the

224   respective concentrations to the Gaussian distribution model:

225$$Y_{(LCx)} = ampl * \exp\left(-0.5 * \left(\frac{X - mean}{SD}\right)^2\right) \text{ (eq. 2)}$$

226   where

227   **Y** is the gene expression level at LCx (lethal concentration for x% of individuals)

228   **X** is LCx

229   **ampl** (amplitude) is the height of the center of the distribution in Y units

230   **SD** is a measure of the width of the distribution (in LCx)

231

232   Data for qPCR, Western-blot and lipid peroxidation analyses were found to satisfy the

233   assumptions of equal variance and normality and parametric statistics were used. For pairwise

234   comparisons of *abcb1* and *hsp70*/Hsp70 levels in treatments and respective controls at each

235   time point the t-test was applied. Multiple comparisons of several treatments with one

236   respective control were done with two-way ANOVA and Dunnett's test. To analyse the

237   significance of observed changes in each time-point, pair-wise comparisons with the

238   respective control were done. The results of multiple pair-wise comparisons were done using

239   Steel-Dwass method for all pairs' comparison. Differences were regarded as significant if $P <$

240   0.05. Regressions were calculated with Graphpad Prism version 7. Statistical analyses were

241   performed with JMP version 10.0 (SAS Institute, Cary, NC).

242

243 **Results**

244   *4.1. Lethal CdCl$_2$ concentrations*

245   Acutely toxic CdCl$_2$ concentrations were across the different amphipod species from < 1

246 to < 100 mg/L (Fig. 1, Table 1). The range of LCx values calculated from CdCl$_2$ concentration

247 − lethality relationships were over an order of magnitude across the species. LCx values were

248 highest for *E. vittatus* indicating the lowest sensitivity of this species to CdCl$_2$. The order of

249 the species with regard to their sensitivities to CdCl$_2$ was: *G. lacustris* < *E. cyaneus* < *E.*

250 *verrucosus* < *E. vittatus* (Fig. 1, Table 1).

251   *4.2. Conjugated diene (CD) levels*

252   After 1 and 6 hr exposures to 5 mg/L CdCl$_2$ no significant changes in CD levels were

253 seen in any of the examined species. Significant increases of CD levels by about 58% and

254 47% occurred only in *E. cyaneus* and *G. lacustris*, respectively, after 24 hr exposures (Fig. 2).

255 The CdCl$_2$ concentration of 5 mg/L corresponds to LC70 and LC90 for *E. cyaneus* and *G.*

256 *lacustris*, respectively. For *E. vittatus* and *E. verrucosus* this CdCl$_2$ concentration is equivalent

257 to LC10 and LC45, respectively (Fig. 1, Table 1).

258   *4.3. Heat shock protein 70 transcript/protein levels*

259   *Hsp70* transcript levels across the amphipod species were upon exposures to CdCl$_2$

260 significantly ($p < 0.05$) increased by between 1.4 - (*E. cyaneus* at LC30 for 24 hrs) to up to

261 9.1-fold (*E. cyaneus* at LC70 for 24 hrs) (Fig. 3a, b). With longer exposure times to CdCl$_2$

262 changes in *hsp70* transcript levels were in all amphipod species generally more pronounced:

263 After 1 hr exposures *hsp70* levels were significantly ($p < 0.05$) increased in two cases (*E.*

264   *cyaneus* at LC30, *G. lacustris* at LC90), after 6 hr exposures in four cases (*E. cyaneus* at

265   LC30, *E. verrucosus* and *G. lacustris* at LC50, *G. lacustris* at LC90) and after 24 hr exposures

266   in nine cases (*E. vittatus* at LC10, *G. lacustris* at LC10, LC50 and LC90, *E. verrucosus* at

267   LC25, LC45 and LC50, *E. cyaneus* at LC30 and LC70; Fig. 3a). Although significant changes

268   in *hsp70* transcript levels were also seen with $CdCl_2$ at lower LCx, changes were more

269   pronounced at higher LCx levels and could be described with a regression curve over all data

270   following a Gaussian shape with an overall maximum *hsp70* transcript level at LC70 followed

271   by a decrease (Fig. 3b).

272   Hsp70 protein levels across amphipod species from $CdCl_2$ treatments also followed a

273   Gaussian function when expression data were related to the respective LCx values (Fig. 4).

274   Significantly ($p < 0.05$) increased Hsp70 protein levels were seen in *E. cyaneus* at 1.8- and

275   1.7-fold, respectively, after 1 and 6 hr exposures to 5 mg/l $CdCl_2$ corresponding to the

276   species-specific LC70; and in *E. verrucosus* after 24 hrs exposure with a 2.6-fold increased

277   protein level above the control. For this species 5 mg/l $CdCl_2$ corresponded to the LC45. In *E.*

278   *vittatus* and *G. lacustris* exposed to the species-specific LC10 and LC90, respectively, Hsp70

279   protein levels were not significantly different from the respective controls (Fig. 4).

280   *4.4. Abcb1 transcript levels*

281   Upon exposures of amphipods to $CdCl_2$ *abcb1* transcript levels were significantly ($p <$

282   0.05) increased by between 2.2- (*E. cyaneus* at LC50 for 24 hrs) to up to 4.7-fold (*E.*

283   *verrucosus* at LC45 for 24 hrs). Over the different exposure times the number of cases of

284   *abcb1* increases only slightly changed. Thus, *abcb1* transcript levels were increased in three

285   cases at 1 hr exposures (*E. verrucosus* at LC25, *E. cyaneus* at LC70, *G. lacustris* at LC90), in

286   two cases at 6 hr exposures (*E. cyaneus* at LC50, *G. lacustris* at LC90) and in four cases at 24

287   hr exposures (*E. verrucosus* at LC45, *E. cyaneus* at LC50, *G. lacustris* at LC90, *E. vittatus* at

288   LC10). Significant *abcb1* transcript increases at all three time points occurred only in *G.*

289   *lacustris* at LC90; in *E. cyaneus* at LC50 *abcb1* transcripts were increased at two time points.

290   In the other cases, significant *abcb1* transcript increases were seen only at one time point.

291   Changes in *abcb1* transcript levels did not seem to depend on $CdCl_2$ concentration / LCx

292   value; the significant changes were seen across different LCx values and were not generally

293   induced at higher or lower LCx values (Fig. 5).

294

295   **Discussion**

296   The four examined amphipod species showed clear differences regarding toxic

297   sensitivities to $CdCl_2$ that became evident by LC50 values ranging over one order of

298   magnitude (Table 1). Along those lines the levels of conjugated dienes, indicating cadmium

299   effects on the molecular level, were at equal $CdCl_2$ exposure concentrations only increased in

300   the toxicologically more sensitive species *E. cyaneus* and *G. lacustris* (Fig. 2). Conjugated

301   dienes are primary products of lipid peroxidation resulting from oxidative stress [41, 42], a

302   main cause of the toxic action of cadmium [43, 44]. This effect of $CdCl_2$ on gammarids was

303   indicated in a previous study where it was found that $CdCl_2$ exposure leads to the inhibition of

304   common antioxidant enzymes in gammarids [45]. Differences in internal cadmium levels as a

305   consequence of different cadmium uptake rates can, to a certain extent, be seen as a reason for

306   the differences in toxicological sensitivities of the different species. Body size has previously

307   been shown to be an important determinant of cadmium uptake by aquatic insects [46]. Along

308   those lines, cadmium uptake rates during $CdCl_2$ exposures were higher in the concurrently

309   toxicologically more sensitive *E. cyaneus* than in *E. verrucosus* that differed by 10- to 50-fold

14

310 in freshweight [47]. In the present study, *G. lacustris* individuals that were similar in size to

311 *E. cyaneus* individuals showed toxic sensitivities that were accordingly in the same range as

312 for *E. cyaneus* (Table 1). *Eulimnogammarus vittatus*, however, was from the examined

313 species least sensitive to $CdCl_2$ although the body sizes of the individuals were clearly below

314 those of *E. verrucosus* and it can be assumed that the cadmium uptake rate was accordingly

315 higher. It was previously found that the occurrence of toxic effects by $CdCl_2$ in *E. verrucosus*

316 was in addition to a in comparison to *E. cyaneus* lower cadmium uptake rate further depressed

317 by a reaction to $CdCl_2$ exposure with metabolic depression [47]. This was not examined here

318 but it seems conceivable that the in comparison low toxic sensitivity of *E. vittatus* to $CdCl_2$

319 may be due to particularly pronounced metabolic depression as a reaction to $CdCl_2$ exposure.

320 Although metabolic depression by cadmium may have decreased activity on a

321 physiological level it appeared to have no effect on the transcription activity. Thus, *hsp70* was

322 significantly induced in *E. verrucosus* that previously showed a metabolic depression

323 response to $CdCl_2$ exposure but not in *E. cyaneus* that does not respond to $CdCl_2$ with

324 metabolic depression [47] in the 6 and 24 hr $CdCl_2$ treatments at LC50 (Fig. 3). Furthermore,

325 a significant *abcb1* increase was seen in *E. verrucosus* at LC25 but not in *E. cyaneus* at LC30

326 after 1 hr exposure to $CdCl_2$ and in *E. vittatus* but no other species at LC10 after 24 hrs

327 exposure to $CdCl_2$ (Fig 5).

328 The $CdCl_2$-dependent *hsp70* transcript and Hsp70 protein response levels could across

329 the species be related to the respective $CdCl_2$ –related stress levels. When plotting all *hsp70*

330 transcript or Hsp70 protein levels across species against the respective species-specific LCx

331 values the values showed an increase to a maximum followed by a decrease at higher LCx

332 values (Figs. 3, 4). When assuming that the increasing LCx values represent increasing

333   degrees of stress this course represents stress-level dependent expression degrees that for

334   Hsp70 has been described before: The Hsp70 titre increase in the "compensation phase" is

335   followed by a Hsp70 titre decrease in the "non-compensation phase" at higher toxicity levels

336   when the ability of the cells to react to the toxicant is increasingly degraded [48]. The applied

337    $CdCl_2$ concentration represented different degrees of stress for the different amphipod

338   species; at this stage it is assumed but it cannot be definitely asserted that the *hsp70*

339   transcript/Hsp70 protein responses are equal in the different amphipod species at

340   corresponding stress levels.

341   Provided that across the gammarid species equal internal, toxicologically relevant

342   cadmium concentrations cause equal levels of toxic effects, as was indicated earlier [47], the

343   LCx values can be regarded as approximate measures of the respective internal cadmium

344   concentrations. Hence, across the species the degrees of *hsp70* transcript and Hsp70 protein

345   induction appear to be approximately equal at corresponding internal cadmium levels.

346   Induction of *abcb1* transcripts tends to depend on LCx levels and time of exposure, i.e.,

347   internal cadmium levels. Increased *abcb1* levels were more seen at higher $CdCl_2$ LCx values

348   and occurred at lower LCx levels after longer times of exposure (Fig. 5). However, across

349   species *abcb1* induction levels are not uniform at equal LCx levels. Thus, after 24 hrs

350   exposure to $CdCl_2$ at LC10 *abcb1* levels were only significantly increased in *E. vittatus* but

351   not in *E. verrucosus*, *E. cyaneus* and *G. lacustris*; after 6 and 24 hrs exposure to $CdCl_2$ at

352   LC50 significant *abcb1* increases were only seen in *E. cyaneus* but not in *E. verrucosus* and

353   *G. lacustris* (Fig. 5).

354   The differences in sensitivity of the *hsp70* and *abcb1* transcript responses among the

355   gammarid species may confirm that induction of the two genes is *via* different pathways.

16

356   Misfolded proteins that are damaged e.g. by the impact of cadmium-induced ROS [15, 16]

357   trigger the induction of the *hsp70* gene as, according to the chaperone titration model [49],

358   heat shock factor (Hst1) is released when Hsps bind to the misfolded proteins thus initiating

359   *hsp* transcription. Induction of *abcb1* appears to be triggered *via* another pathway involving

360   the transcription factor NF-κB [15, 16].

361   Both Hsp70 and Abcb1 proteins act as protective proteins against cadmium toxicity:

362   Hsp70 as chaperone preserves the structure of intact proteins and re-folds damaged proteins;

363   Abcb1, a cellular efflux transporter, appears to efflux cell membrane components, such as

364   ceramides, that result from membrane damage by ROS and can induce apoptosis [15, 16]. It

365   was found earlier that *E. cyaneus* that shows higher constitutive and induced levels of Hsp70

366   than *E. verrucosus* [24, 25] detoxifies a higher proportion of cadmium by binding it to

367   proteins of the biologically detoxified heat stable protein fraction (BDF) [47]. The in

368   comparison high constitutive Hsp70 titres in *Gammarus lacustris* [25] may indicate that also

369   in this species a comparatively high level of the internal cadmium is bound to the BDF.

370   However, when considering that the toxicologically more sensitive gammarid species have

371   higher titers of cadmium detoxifying proteins and that the transcripts of both here examined

372   proteins are induced by cadmium only at comparatively high stress levels, it seems obvious

373   that overall the detoxifying effect by those cellular stress response proteins on cadmium is

374   only minor. Major determinants of the cadmium sensitivity of the species appear to be body

375   size and in relation to that the cadmium uptake rate and one additional parameter that could be

376   the degree of metabolic depression caused by cadmium.

377   **Summary and Conclusions**

17

378   In this study cadmium induced *hsp70*/Hsp70 and *abcb1* levels in gammarids exposed to

379     $CdCl_2$ were for the first time phenotypically anchored, i.e., exposure concentrations of

380   treatments for transcript measurements were equated with the respective lethal concentrations

381   (LCx). When gene/protein responses were related to LCx values the four gammarid species

382   examined showed a relatively uniform *hsp70*/Hsp70 response with pronounced responses in

383     $CdCl_2$ treatments at higher LCx and in lower LCx treatments after longer times of exposure to

384     $CdCl_2$ (Figs. 3, 4). For *abcb1*, responses to $CdCl_2$ exposure when related to LCx levels were

385   not uniform across the species; however, *abcb1* was overall also induced by $CdCl_2$ in the

386   lethal concentration range (Fig. 5). Induction of *hsp70*/Hsp70 and *abcb1* responses by $CdCl_2$

387   in the lethal concentration range in the gammarids indicates that changes in transcript levels

388   of those genes are rather insensitive markers for cadmium stress.

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References

408 1.   Pinot F, Kreps SE, Bachelet M, Hainaut P, Bakonyi M, Polla BS. Cadmium in the

409 environment: sources, mechanisms of biotoxicity, and biomarkers. Rev Environ Health.

410 2000;15(3):299-323. Epub 2000/10/26. PubMed PMID: 11048333.

411 2.   Nemmiche S. Oxidative signaling response to cadmium exposure. Toxicol Sci.

412 2017;156(1):4-10. Epub 2016/11/03. doi: 10.1093/toxsci/kfw222. PubMed PMID: 27803385.

413 3.   Kim BM, Rhee JS, Jeong CB, Seo JS, Park GS, Lee YM, et al. Heavy metals induce

414 oxidative stress and trigger oxidative stress-mediated heat shock protein (hsp) modulation in

415 the intertidal copepod *Tigriopus japonicus*. Comp Biochem Physiol C Toxicol Pharmacol.

416 2014;166:65-74. Epub 2014/07/25. doi: 10.1016/j.cbpc.2014.07.005. PubMed PMID:

417 25058597.

418 4.   Haap T, Köhler HR. Cadmium tolerance in seven *Daphnia magna* clones is associated

419 with reduced hsp70 baseline levels and induction. Aquat Toxicol. 2009;94(2):131-7. Epub

420 2009/07/17. doi: 10.1016/j.aquatox.2009.06.006. PubMed PMID: 19604586.

421 5.   Blechinger SR, Warren JT, Jr., Kuwada JY, Krone PH. Developmental toxicology of

422 cadmium in living embryos of a stable transgenic zebrafish line. Environ Health Perspect.

423 2002;110(10):1041-6. Epub 2002/10/04. doi: 10.1289/ehp.021101041. PubMed PMID:

424 12361930; PubMed Central PMCID: PMCPMC1241031.

425 6.   Schill RO, Görlitz H, Köhler HR. Laboratory simulation of a mining accident: acute

426 toxicity, hsc/hsp70 response, and recovery from stress in *Gammarus fossarum* (Crustacea,

427   Amphipoda) exposed to a pulse of cadmium. Biometals. 2003;16(3):391-401. Epub

428   2003/04/12. PubMed PMID: 12680701.

429   7.   da Silva Cantinha R, Borrely SI, Oguiura N, de Braganca Pereira CA, Rigolon MM,

430   Nakano E. HSP70 expression in *Biomphalaria glabrata* snails exposed to cadmium.

431   Ecotoxicol   Environ   Saf.   2017;140:18-23.   Epub   2017/02/24.   doi:

432   10.1016/j.ecoenv.2017.02.026. PubMed PMID: 28231501.

433   8.   Eufemia NA, Epel D. Induction of the multixenobiotic defense mechanism (MXR), P-

434   glycoprotein, in the mussel *Mytilus californianus* as a general cellular response to

435   environmental stresses. Aquat Toxicol. 2000;49(1-2):89-100. Epub 2000/05/18. PubMed

436   PMID: 10814809.

437   9.   Werner I, Nagel R. Stress proteins HSP60 and HSP70 in three species of amphipods

438   exposed to cadmium, diazinon, dieldrin and fluoranthene. Environ Toxicol Chem.

439   1997;16(11):2393-403.

440   10.   Lee SM, Lee SB, Park CH, Choi J. Expression of heat shock protein and hemoglobin

441   genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various

442   environmental pollutants: a potential biomarker of freshwater monitoring. Chemosphere.

443   2006;65(6):1074-81. Epub 2006/04/21. doi: 10.1016/j.chemosphere.2006.02.042. PubMed

444   PMID: 16624371.

445   11.   Piano A, Valbonesi P, Fabbri E. Expression of cytoprotective proteins, heat shock

446   protein 70 and metallothioneins, in tissues of *Ostrea edulis* exposed to heat and heavy metals.

447   Cell Stress Chaperones. 2004;9(2):134-42. Epub 2004/10/23. PubMed PMID: 15497500;

448   PubMed Central PMCID: PMCPMC1065293.

449   12.   Singer C, Zimmermann S, Sures B. Induction of heat shock proteins (hsp70) in the

450   zebra mussel (*Dreissena polymorpha*) following exposure to platinum group metals

451   (platinum, palladium and rhodium): comparison with lead and cadmium exposures. Aquat

452   Toxicol. 2005;75(1):65-75. Epub 2005/08/23. doi: 10.1016/j.aquatox.2005.07.004. PubMed

453   PMID: 16111776.

454   13.   Ivanina AV, Sokolova IM. Effects of cadmium exposure on expression and activity of

455   P-glycoprotein   in   eastern   oysters,   *Crassostrea   virginica*   Gmelin.   Aquat   Toxicol.

456   2008;88(1):19-28. Epub 2008/05/03. doi: 10.1016/j.aquatox.2008.02.014. PubMed PMID:
457   18453012.

458   14.   Zucchi S, Corsi I, Luckenbach T, Bard SM, Regoli F, Focardi S. Identification of five
459   partial ABC genes in the liver of the Antarctic fish *Trematomus bernacchii* and sensitivity of
460   ABCB1 and ABCC2 to Cd exposure. Environ Pollut. 2010;158(8):2746-56. Epub
461   2010/07/16. doi: 10.1016/j.envpol.2010.04.012. PubMed PMID: 20627496.

462   15.   Beyersmann D, Hechtenberg S. Cadmium, gene regulation, and cellular signalling in
463   mammalian cells. Toxicol Appl Pharmacol. 1997;144(2):247-61. Epub 1997/06/01. doi:
464   10.1006/taap.1997.8125. PubMed PMID: 9194408.

465   16.   Thévenod F, Friedmann JM, Katsen AD, Hauser IA. Up-regulation of multidrug
466   resistance P-glycoprotein via nuclear factor-kappaB activation protects kidney proximal
467   tubule cells from cadmium- and reactive oxygen species-induced apoptosis. J Biol Chem.
468   2000;275(3):1887-96. Epub 2000/01/15. PubMed PMID: 10636889.

469   17.   Timoshkin OAe. Index of animal species inhabiting Lake Baikal and its catchment
470   area. Novosibirsk: Nauka; 2001. 832 p.

471   18.   Kozhova OM, Izmest'eva LRe. Lake Baikal - Evolution and Biodiversity. Leiden:
472   Backhuys Publishers; 1998. 447 p.

473   19.   Ciesielski TM, Pastukhov MV, Leeves SA, Farkas J, Lierhagen S, Poletaeva VI, et al.
474   Differential bioaccumulation of potentially toxic elements in benthic and pelagic food chains
475   in Lake Baikal. Environ Sci Pollut Res Int. 2016;23(15):15593-604. Epub 2016/05/01. doi:
476   10.1007/s11356-016-6634-0. PubMed PMID: 27130338.

477   20.   Kulikova NN, Mekhanikova IV, Chebykin EP, Vodneva EV, Timoshkin OA, Suturin
478   AN. Chemical element composition and amphipod concentration function in Baikal littoral
479   zone. Water Resourc. 2017;44(3):497-511. doi: 10.1134/S0097807817030125. PubMed
480   PMID: WOS:000405126000014.

481   21.   Bedulina DS, Takhteev VV, Pogrebnyak SG, Govorukhina EB, Madyarova EV,
482   Lubyaga YA, et al. On *Eulimnogammarus messerschmidtii*, sp. n. (Amphipoda: Gammaridea)
483   from Lake Baikal, Siberia, with redescription of *E. cyanoides* (Sowinsky) and remarks on

484   taxonomy of the genus *Eulimnogammarus*. Zootaxa. 2014;3838(5):518-44. Epub 2014/08/02.

485   doi: 10.11646/zootaxa.3838.5.2. PubMed PMID: 25081795.

486   22.   Takhteev VV. Essays on the amphipods of Lake Baikal: systematics, comparative

487   ecology, evolution. Irkutsk, Russia [in Russian]: Irkutsk State University Press; 2000.

488   23.   Luckenbach T, Bedulina D, Timofeyev M. Is the endemic fauna of Lake Baikal

489   affected by global change? In: Kappas M, Karthe D, Chalov S, Kasimov N, editors. Water

490   and Environment in the Selenga- Baikal Basin: International Research Cooperation for an

491   Ecoregion of Global Relevance. 23. Stuttgart, Germany: ibidem-Verlag/ibidem Press; 2015.

492   p. 219-35.

493   24.   Bedulina DS, Evgen'ev MB, Timofeyev MA, Protopopova MV, Garbuz DG,

494   Pavlichenko VV, et al. Expression patterns and organization of the hsp70 genes correlate with

495   thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and

496   *E. verrucosus*) from Lake Baikal. Mol Ecol. 2013;22(5):1416-30. Epub 2013/01/22. doi:

497   10.1111/mec.12136. PubMed PMID: 23331571.

498   25.   Protopopova MV, Pavlichenko VV, Menzel R, Putschew A, Luckenbach T, Steinberg

499   CE. Contrasting cellular stress responses of Baikalian and Palearctic amphipods upon

500   exposure to humic substances: environmental implications. Environ Sci Pollut Res Int.

501   2014;21(24):14124-37. Epub 2014/07/24. doi: 10.1007/s11356-014-3323-8. PubMed PMID:

502   25053285.

503   26.   Axenov-Gribanov D, Bedulina D, Shatilina Z, Jakob L, Vereshchagina K, Lubyaga Y,

504   et al. Thermal Preference Ranges Correlate with Stable Signals of Universal Stress Markers in

505   Lake Baikal Endemic and Holarctic Amphipods. PLoS One. 2016;11(10):e0164226. Epub

506   2016/10/06. doi: 10.1371/journal.pone.0164226. PubMed PMID: 27706227; PubMed Central

507   PMCID: PMCPMC5051968.

508   27.   Pavlichenko VV, Protopopova MV, Timofeyev M, Luckenbach T. Identification of a

509   putatively multixenobiotic resistance related Abcb1 transporter in amphipod species endemic

510   to the highly pristine Lake Baikal. Environ Sci Pollut Res Int. 2015;22(7):5453-68. Epub

511   2014/11/05. doi: 10.1007/s11356-014-3758-y. PubMed PMID: 25367643.

512   28.   Weinberg IV, Kamaltynov RM. Macrozoobenthic communities of rocky beach of

513   Lake Baikal. 1. Fauna Zoologicheskii Zhurnal. 1998;77:259- 65 [In Russian].

514   29.   Bazikalova A. Amphipods of Baikal. Moscow: [Department of Commerce of the USA
515   translation of the original]; 1945.

516   30.   Bekman MY. Biologiya *Gammarus lacustris* Sars v pribaikal'skikh ozerakh [Biology
517   of *Gammarus lacustris* Sars from water bodies near Lake Baikal]. Trudy Baikal'skoy
518   limnologicheskoi Stancii [Proceedings of the Baikal Limnological Station]. 1954;14:268-311
519   [in Russian].

520   31.   Jakob L, Axenov-Gribanov DV, Gurkov AN, Ginzburg M, Bedulina DS, Timofeyev
521   MA, et al. Lake Baikal amphipods under climate change: thermalconstraintsand ecological
522   consequences. Ecosphere. 2016;7(3). doi: ARTN e01308

523   10.1002/ecs2.1308. PubMed PMID: WOS:000374896800038.

524   32.   Stalnaya ID. The method for determining diene conjugation of unsaturated fatty acids.
525   In: Orehovich VN, editor. The modern methods in biochemistry. Moscow, Russia: Izdatelstvo
526   Meditsina; 1977. p. 63-4 [in Russian].

527   33.   Low PA, Nickander KK. Oxygen free radical effects in sciatic nerve in experimental
528   diabetes. Diabetes. 1991;40(7):873-7. Epub 1991/07/01. PubMed PMID: 2060723.

529   34.   Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
530   quantitative PCR and the 2(T)(-Delta Delta C) method. Methods. 2001;25(4):402-8. doi:
531   10.1006/meth.2001.1262. PubMed PMID: WOS:000173949500003.

532   35.   Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable
533   housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper -
534   Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509-15. doi: Doi
535   10.1023/B:Bile.0000019559.84305.47. PubMed PMID: WOS:000220217900008.

536   36.   Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al.
537   Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of
538   multiple internal control genes. Gen Biol. 2002;3(7). doi: ARTN 0034.1

539   10.1186/gb-2002-3-7-research0034. PubMed PMID: WOS:000207581200010.

540   37.   Pfaffl M. Relative quantification. In: Dorak T, editor. Real-time PCR: International
541   University Line 2006. p. 63-82.

542   38.   Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin
543   phenol reagent. J Biol Chem. 1951;193(1):265-75. Epub 1951/11/01. PubMed PMID:
544   14907713.

545   39.   Laemmli UK. Cleavage of structural proteins during the assembly of the head of
546   bacteriophage T4. Nature. 1970;227(5259):680-5. Epub 1970/08/15. PubMed PMID:
547   5432063.

548   40.   Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from
549   polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl
550   Acad Sci U S A. 1979;76(9):4350-4. Epub 1979/09/01. PubMed PMID: 388439; PubMed
551   Central PMCID: PMCPMC411572.

552   41.   Jozwik M, Wolczynski S, Jozwik M, Szamatowicz M. Oxidative stress markers in
553   preovulatory follicular fluid in humans. Mol Hum Reprod. 1999;5(5):409-13. Epub
554   1999/05/25. PubMed PMID: 10338363.

555   42.   Chang D, Sha Q, Zhang X, Liu P, Rong S, Han T, et al. The evaluation of the
556   oxidative stress parameters in patients with primary angle-closure glaucoma. PLoS One.
557   2011;6(11):e27218. Epub 2011/11/19. doi: 10.1371/journal.pone.0027218. PubMed PMID:
558   22096540; PubMed Central PMCID: PMCPMC3214032.

559   43.   Dally H, Hartwig A. Induction and repair inhibition of oxidative DNA damage by
560   nickel(II) and cadmium(II) in mammalian cells. Carcinogenesis. 1997;18(5):1021-6. Epub
561   1997/05/01. PubMed PMID: 9163690.

562   44.   Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic
563   Biol Med. 1995;18(2):321-36. Epub 1995/02/01. PubMed PMID: 7744317.

564   45.   Timofeyev MA, Shatilina ZM, Bedulina DS, Protopopova MV, Pavlichenko VV,
565   Grabelnych OI, et al. Evaluation of biochemical responses in Palearctic and Lake Baikal
566   endemic amphipod species exposed to $CdCl_2$. Ecotoxicol Environ Saf. 2008;70(1):99-105.
567   Epub 2007/10/09. doi: 10.1016/j.ecoenv.2007.06.014. PubMed PMID: 17920682.

568   46.   Buchwalter DB, Cain DJ, Martin CA, Xie L, Luoma SN, Garland T, Jr. Aquatic insect
569   ecophysiological traits reveal phylogenetically based differences in dissolved cadmium
570   susceptibility. Proc Natl Acad Sci U S A. 2008;105(24):8321-6. Epub 2008/06/19. doi:

24

Stress gene responses in amphipods to cadmium

571 10.1073/pnas.0801686105. PubMed PMID: 18559853; PubMed Central PMCID:
572 PMCPMC2448835.

573 47.   Jakob L, Bedulina DS, Axenov-Gribanov DV, Ginzburg M, Shatilina ZM, Lubyaga
574 YA, et al. Uptake kinetics and subcellular compartmentalization explain lethal but not
575 sublethal effects of cadmium in two closely related amphipod species. Environ Sci Technol.
576 2017;51(12):7208-18. Epub 2017/05/12. doi: 10.1021/acs.est.6b06613. PubMed PMID:
577 28493692.

578 48.   Eckwert H, Alberti G, Köhler HR. The induction of stress proteins (hsp) in *Oniscus*
579 *asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and
580 toxicological assessment. Ecotoxicology. 1997;6:249-62.

581 49.   Richter K, Haslbeck M, Buchner J. The heat shock response: life on the verge of
582 death. Mol Cell. 2010;40(2):253-66. Epub 2010/10/23. doi: 10.1016/j.molcel.2010.10.006.
583 PubMed PMID: 20965420.

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586   **Tables**

587   **Table 1.** Lethal concentration values (LCx; x: % lethality) for $CdCl_2$ (mg/L) and curve

588   parameters for the different amphipod species determined with the HILL model (eq. 1, see

589   Fig. 1). CI: confidence interval, R^2: curve fit

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		E. vittatus	*E. verrucosus*	*E. cyaneus*	*G. lacustris*
LCx	10	4.47	0.54	0.67	0.59
	25	8.98	1.76	1.38	1.01
	30	10.63	2.31	1.64	1.14
	45	16.07	4.65	2.52	1.55
	50	18.05	5.72	2.89	1.71
	95% CI	12.4-26.6	3.8-8.7	2.1-4.0	1.3-2.4
	70	31.05	14.31	5.05	2.59
	75	36.74	18.76	5.96	2.91
	90	73.81	60.81	12.22	4.98
HillSlope		0.34	0.20	0.34	0.53
95% CI		0.7 - 2.4	0.5 - 1.4	0.7 - 2.3	0.8 - 3.3
R^2		0.88	0.83	0.87	0.88

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Stress gene responses in amphipods to cadmium

600   **Figure captions**

601   **Figure 1**. Concentration-mortality relationships for $CdCl_2$ in the different amphipod

602   species. Symbols represent the observed % mortality at a certain $CdCl_2$ concentration in a

603   single treatment. Solid lines represent the regression curves fitted with the HILL model (eq. 1)

604   and dashed lines the 95% confidence intervals.

605   **Figure 2.** Levels of conjugated dienes (CD; nmol g^{-1} wet weight) in the different

606   amphipod species upon exposure to 5 mg/L $CdCl_2$ that correspond to the species-specific LCx

607   values given under each species name. Depicted are means (symbols) and standard deviations

608   (bars) in the $CdCl_2$ exposed animals. The control levels are shown as dotted (mean) and

609   dashed (standard deviations) lines. Significant differences between treatments and respective

610   controls are indicated by * ($p< 0.05$).

611   **Figure 3.** *Hsp70* transcript levels in tissue of the different amphipod species upon

612   exposure to $CdCl_2$ for 1, 6 and 24 hrs. $CdCl_2$ concentrations were scaled to the species-

613   specific LCx values (refer to Table 1 for the LCx equivalences in mg/L). a) Depicted are

614   mean *hsp 70* expression levels (symbols) and standard deviations (bars) in $CdCl_2$ exposed

615   animals. Expression levels in controls are shown as dotted (mean) and dashed (standard

616   deviations) lines. Significant differences between treatments and respective controls are

617   indicated by * ($p< 0.05$). b) The *hsp 70* expression level data of all species were combined

618   and are depicted in relation to the correspondent species-specific LCx value. The regression

619   line was fitted over all data with the Gaussian distribution model (eq. 2).

620   **Figure 4.** Hsp70 protein levels in tissue of the different amphipod species upon

621   exposure to 5 mg/L $CdCl_2$ for 1, 6 and 24 hrs. $CdCl_2$ concentrations were scaled to the

622   species-specific LCx values. Depicted are mean Hsp70 expression levels (symbols) and

27

623   standard deviations (bars) in $CdCl_2$ exposed animals. Expression levels in controls are shown

624   as dotted (mean) and dashed (standard deviations) lines. Significant differences between

625   treatments and respective controls are indicated by * (p< 0.05). The regression line was fitted

626   with the Gaussian distribution model (eq. 2).

627   **Figure 5.** Relative *abcb1* levels in amphipods upon exposure to $CdCl_2$ for 1, 6 and 24

628   hrs. $CdCl_2$ concentrations were scaled to the species-specific LCx values. Depicted are mean

629   *abcb1* levels (symbols) and standard deviations (bars) in $CdCl_2$ exposed animals. Expression

630   levels in controls are shown as dotted (mean) and dashed (standard deviations) lines.

631   Significant differences between treatments and respective controls are indicated by * (p<

632   0.05).

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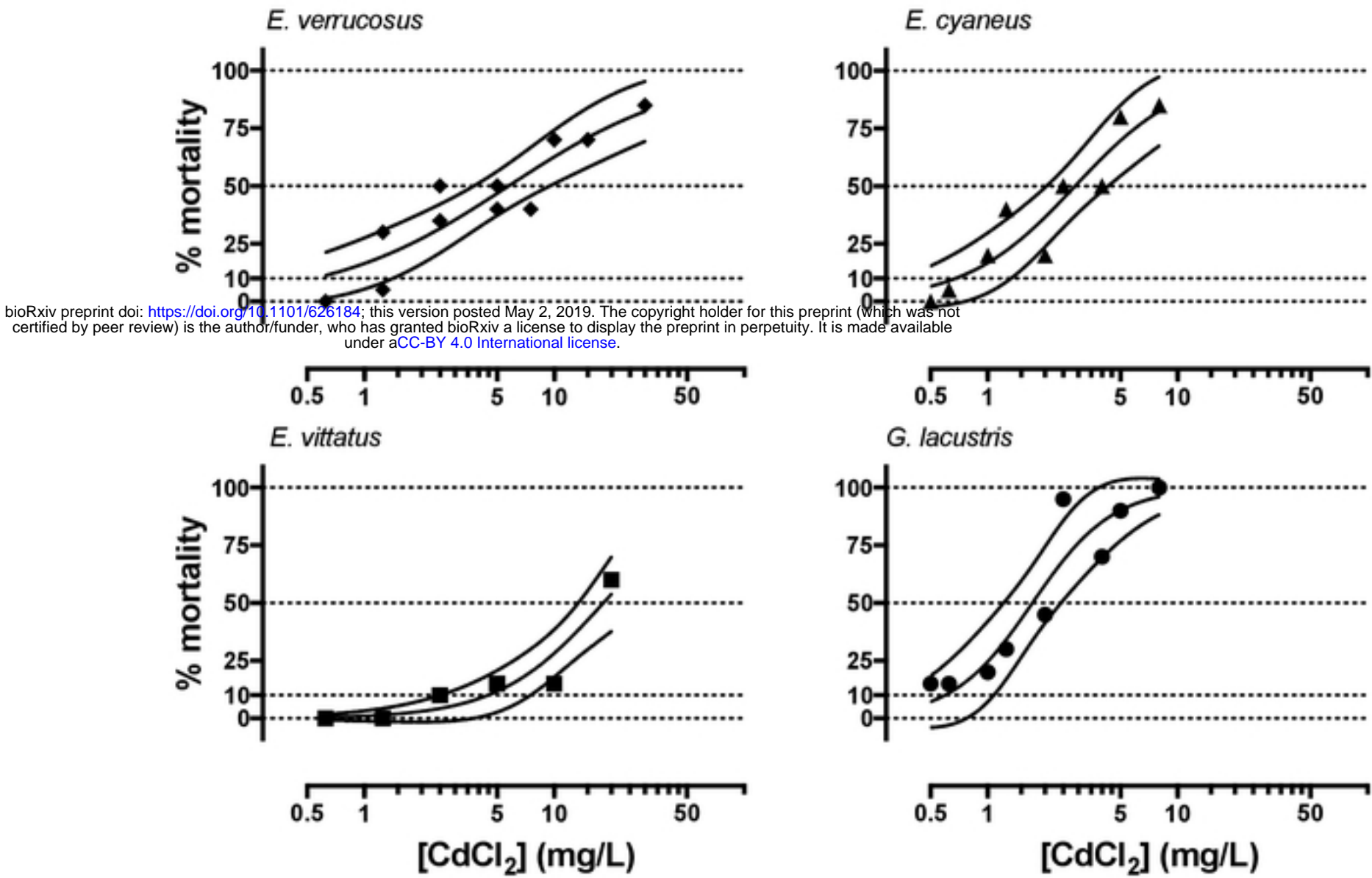634   **Data accessibility statement:** All raw data can be obtained from the authors upon request.
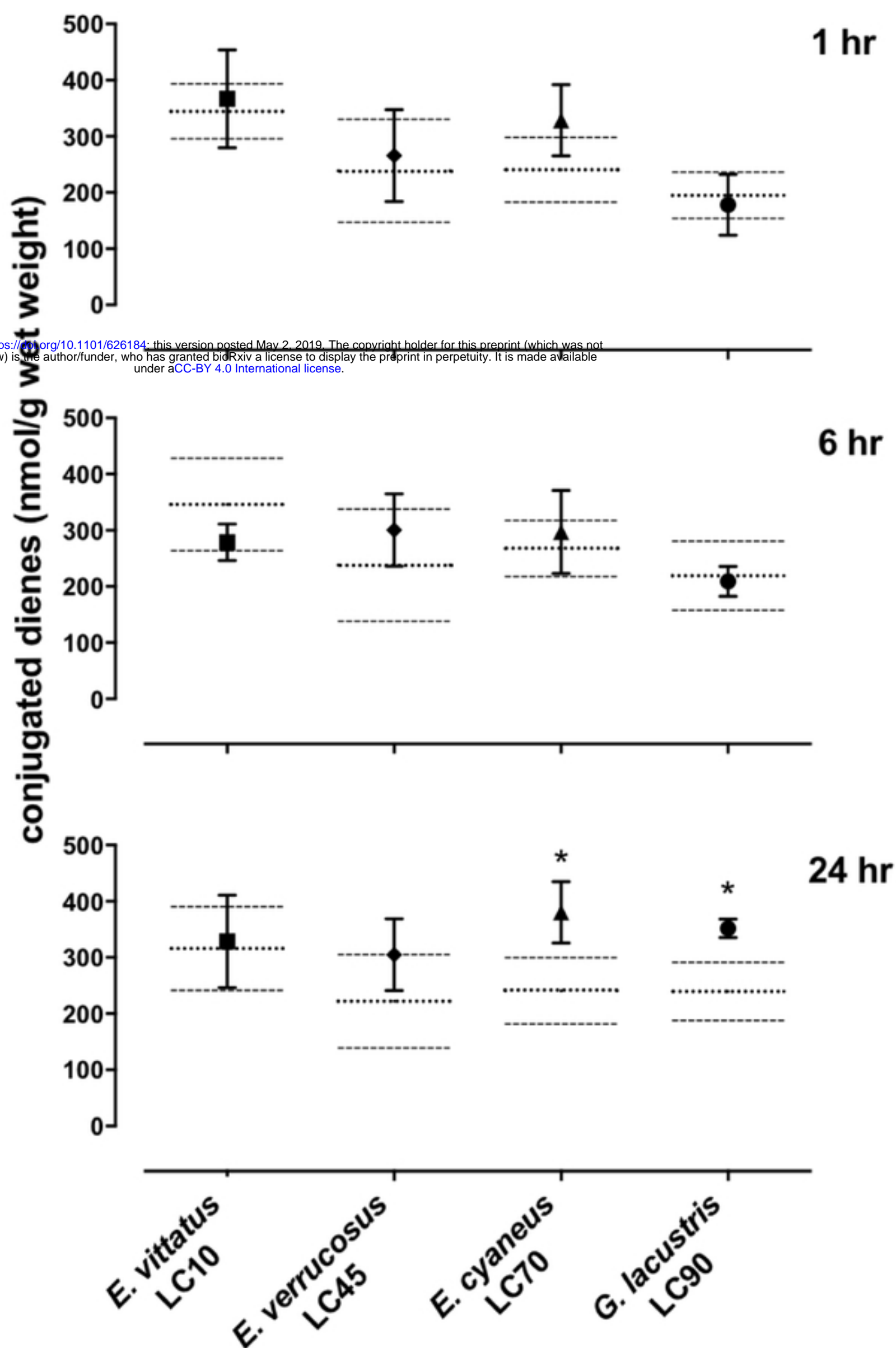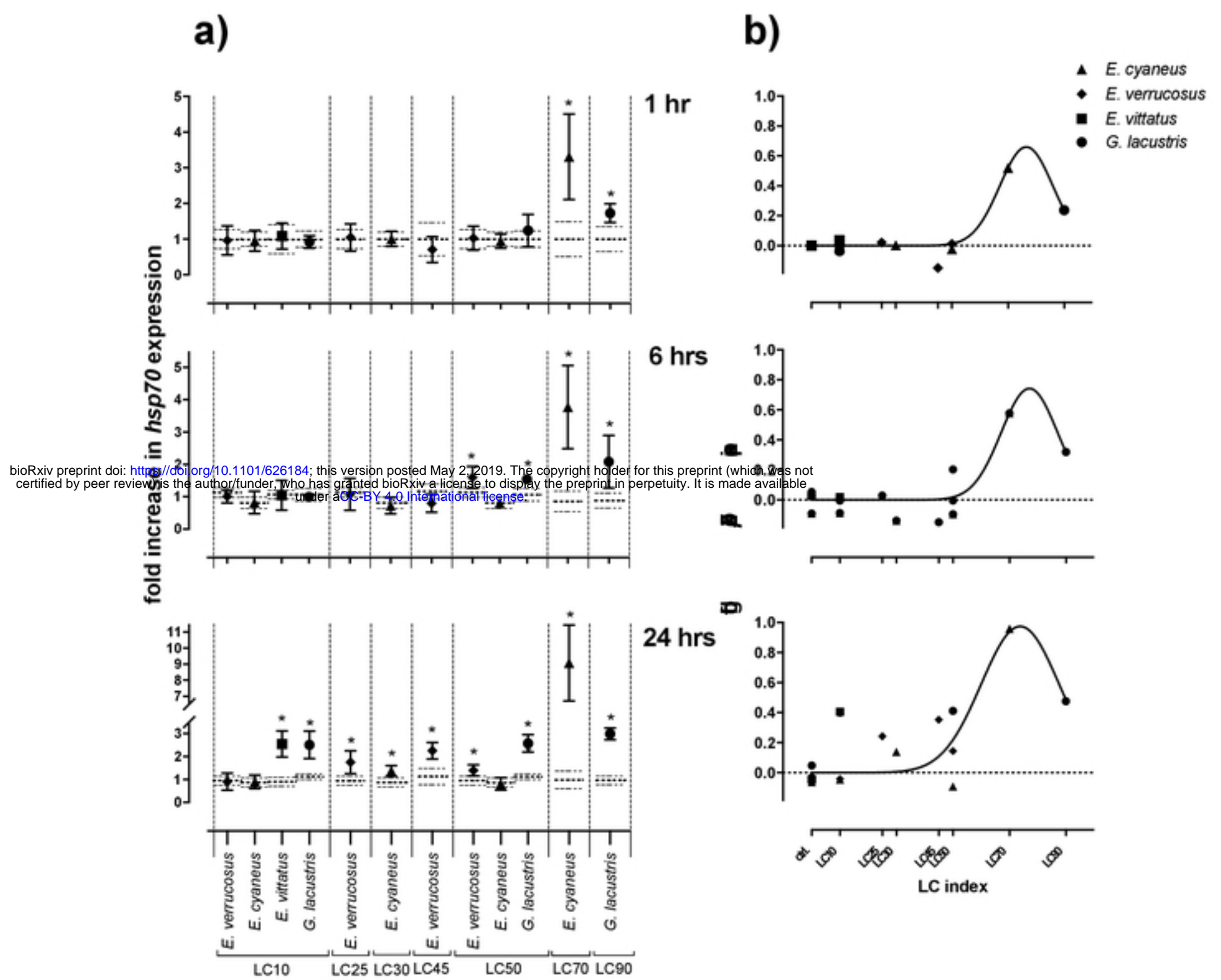
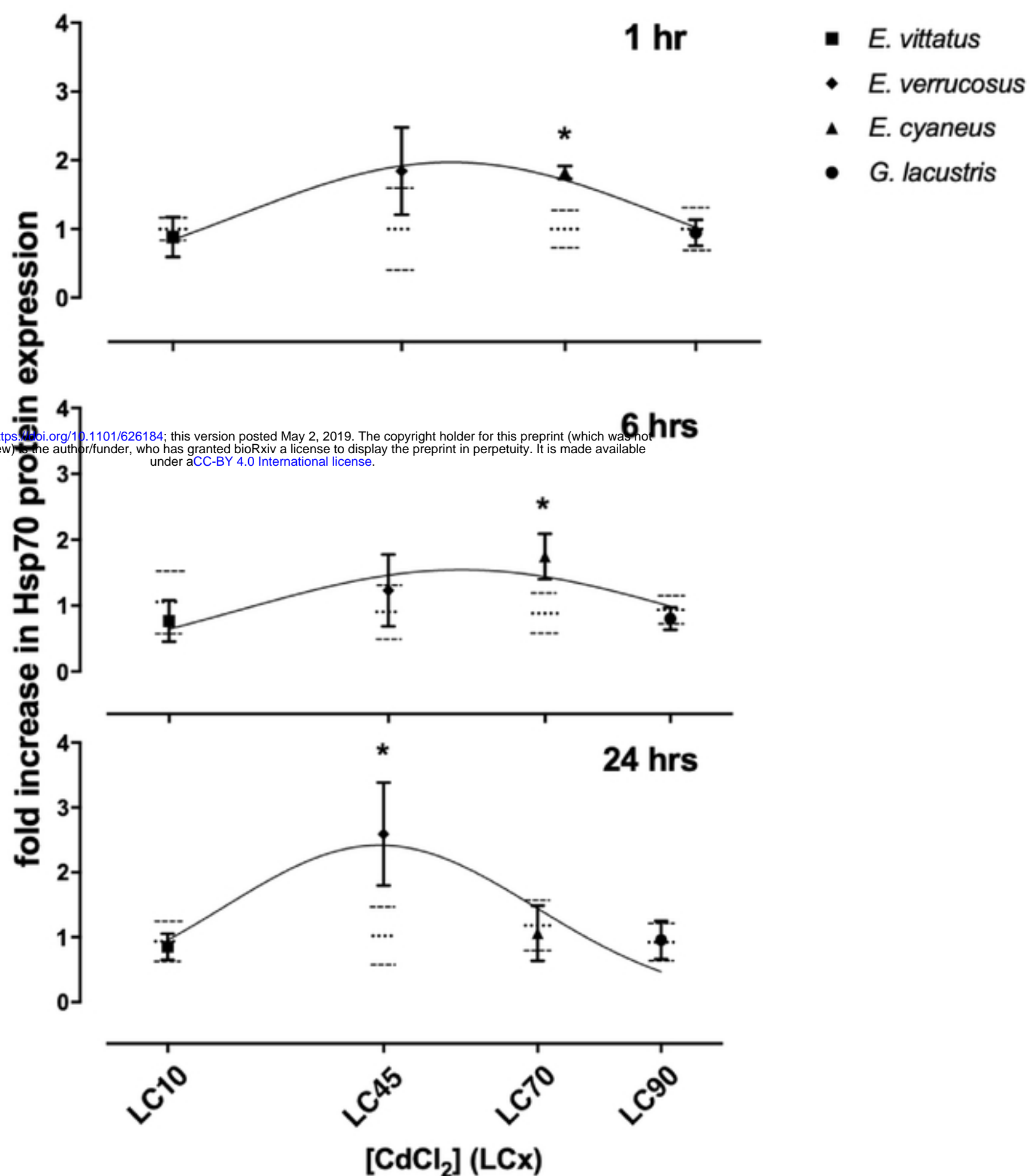**Figures**

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.