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1 <Ecotoxicology and Environmental Safety>

2 **Multigenerational study of life history traits, bioaccumulation, and molecular**
3 **responses of *Pseudodiaptomus annandalei* to cadmium**

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21

22 **Abstract**

23 Metal pollution provide a substantial challenge for environmental health. This study investigated
24 the multigeneration effects of cadmium on populations of the copepod species *Pseudodiaptomus*
25 *annandalei*, exposed to a sublethal concentration, 40 µg/L of cadmium (Cd), over 10
26 generations. At the end of each generation, copepod individuals were collected to estimate
27 fecundity, bioaccumulation, and real time qPCR quantification of selected differentially
28 expressed genes to evaluate Cd effects and sex-specific responses of copepods across multiple
29 generations. Our results revealed a sex-specific accumulation of Cd integrating 10 successive
30 generations. The concentration of Cd was significantly higher ($p < 0.05$) in males than in females.
31 We also observed a generational increase in Cd accumulation. Fecundity increased, with the
32 exception of the first generation, possibly as a compensation for a decrease of copepod
33 population size under Cd exposure. Protein expression of copepods exposed to Cd occurred in a
34 sex-specific manner. Hemerythrin was mostly up-regulated in both copepod sexes exposed to Cd
35 with males having the highest expression levels, while heat shock protein 70 was mostly up-
36 regulated in males and down-regulated in female copepods, both exposed to Cd. Although
37 copepods are known to develop adaptive mechanisms to tolerate toxic chemicals, continuous
38 exposure to metals could lead to the bioaccumulation of metals in their offspring through
39 maternal transfer and direct uptake from the medium over several generations. As a
40 consequence, increased metal concentrations in copepods could result in physiological damage,
41 reducing their fitness, and possibly compromise copepod population structures. This study
42 showed that mortality, life history traits and molecular responses of a copepod species provided
43 important toxicological endpoints and bio-markers for environmental risk assessments.

44 Environmental pressure resulting from continuous exposure to persistent pollutants like Cd,
45 could have evolutionary significance. The tendency for copepods to selectively adapt to a toxic
46 environment through modifications, could increase their chance of survival over a long term.

47 **Keywords:** Cadmium toxicity, multigeneration, calanoid copepod, sex-specific toxicity,
48 bioaccumulation.

49 **1. Introduction**

50 Copepods are important model species in ecotoxicology, partly because of the intermediary
51 position between producers and consumers they occupy in aquatic ecosystems (Das et al., 2020;
52 Reid and Williamson, 2010; Williams et al., 1994). The biological characteristics of copepods
53 including dominance, abundance, cultivability, size and responsiveness to stress (Dayras et al.,
54 2020; Dinh et al., 2020a; Souissi et al., 2010; Wang et al., 2014) qualifies them as suitable test
55 models in toxicology. Since *Pseudodiaptomus annandalei* is sexually dimorphic (Beyrend-Dur et
56 al., 2011), it is also possible to conduct studies on the sex specific toxicity of chemicals. The
57 rapid development and short life cycle of copepods allow for toxicity testing considering male
58 and female specificity, life history traits, and multigenerational approaches (Dahms et al., 2016;
59 Hussain et al., 2020).

60 Cadmium (Cd) is highly toxic to copepods and its toxicity is species- and sex-specific
61 (Kadiene et al., 2017). Cadmium is known to cause oxidative stress or DNA damage in different
62 tissues, possibly leading to a loss of membrane function (Huang et al., 2020; Liu et al., 2020).
63 Cadmium toxicity also elicits other biochemical responses in fish (Almeida et al., 2009; Jia et al.,
64 2011) as well as in copepods (Ensibi and Yahia, 2017). Acute exposure to Cd resulting in stress

65 to copepods has more striking effects than chronic exposure due to induced adaptation
66 mechanisms under chronic exposure (Patra et al., 2011). The concept of ecotoxicology is to
67 provide insight to the ecological consequences associated with pollutants in the environment and
68 develop measures that could address the problem (Jian et al., 2020).

69 Ecotoxicogenomics describes the studies involved in the analysis of environmental or
70 environmentally relevant organism responses to toxic exposure at the molecular level (Li et al.,
71 2017). It uses quantitative polymerase chain reaction (qPCR) or complementary DNA
72 microarrays as a tool to analyse sequenced target genes responding to environmental
73 contaminants. The resulting endpoints provide increasingly important biomarkers for sublethal
74 effects of environmental pollutants. The analysis of transcriptional changes in copepods through
75 the use of genetic tools can give a robust knowledge on pollutant's mechanism of action (Dahms
76 et al., 2016; Sørensen et al., 2020).

77 Multigenerational effects of heavy metals were shown to include physiological acclimation
78 and genetic adaptation (Kwok et al., 2009; Lilley et al., 2012; Sun et al., 2014; Tsui and Wang,
79 2005; Vidal and Horne, 2003). Organisms could transmit information about their environmental
80 condition to their offspring, which may influence their adaptability in the next generation
81 (Alekseev and Lampert, 2001). Adaptation to continual Cd exposure reduces the production of
82 reactive oxygen species (ROS) but acquired tolerance to Cd with abnormal gene expression
83 contributing significantly to chronic Cd toxicity (Patra et al., 2011) and may vary with
84 generations. Very few reports are available on transcriptomic studies of *P. annandalei*. Jiang et
85 al. (2013) investigated the differential gene expression of *P. annandalei* to nickel exposure.
86 Wang et al. (2017), investigated the molecular response of *P. annandalei* copepods exposed to

87 mercury chloride (HgCl₂) (Wang et al., 2017). A recent study was carried out on differential
88 gene expression profiles of male and female *P. annandalei* copepods after exposure to cadmium
89 (Kadiene et al., 2020). However, the molecular responses of male and female *P. annandalei*
90 copepods when exposed to cadmium over multiple generations have not yet been studied. A
91 more recent analysis carried out by Wang et al. showed that the proteome response of *P.*
92 *annandalei* to mercury (Hg) exposure, differed with generation (Wang et al., 2021). Variations in
93 chemical tolerance between copepod populations are influenced by acclimation and adaptation
94 among other factors linked to exposure history and its changes across several generations of
95 exposure (Sun et al., 2014). Short-term multigenerational studies with smaller population sizes
96 have shown full plasticity when copepods were exposed to copper (Cu) (Kwok et al., 2009).
97 However, other environmental factors may affect their pattern or rate of response (Sun et al.,
98 2014). The degree of how these conditions affect their biological responses may depend on the
99 fitness of each generation. Acclimation response to plastics, a selective tolerance that could be
100 switched on and off to cope with changing environmental conditions can be highly energy
101 demanding (Sun et al., 2014), thereby exerting pressure on a population by potentially reducing
102 its growth performance and causing genetic evolution and affecting the physiology and
103 development of individual organisms (Lin et al., 2017).

104 Recent developments of molecular biomarkers in ecotoxicology involve real-time
105 measurements of changes in messenger RNA relating to proteins involved in the metabolism
106 and/or detoxification of metals, or generally responding to chemical stress. This may provide a
107 specific chemical “fingerprint” for the type and concentration of the exposure chemical (Koski et
108 al., 2021). This study was performed to investigate the multigenerational effect of Cd in

109 copepods which is important to understand the response of copepods to persistent metal pollution
110 in their environment. We aimed to compare the responses of male and female *P. annandalei*
111 copepods exposed to Cd for 10 generations at the molecular level. Our approach demonstrates
112 the different molecular response pattern to Cd toxicity in two aspects; sex-specific response
113 pattern and generational response pattern. This will provide the base of further research on
114 factors that could influence results on toxicity testing on copepods or aquatic animals and
115 provide comprehensive data that could guide environmental risk assessments.

116 **2. Materials and Methods**

117 **2.1. Experimental design**

118 This study was designed following Kadiene et al. (2020). Gravid females were randomly
119 selected from the stock culture and incubated for naupliar production. The nauplii were further
120 exposed to 40 µg/L Cd, a sublethal concentration corresponding to LC10 of *P. annandalei*
121 copepods, estimated from a previous study (Kadiene et al., 2017). The protocol for the
122 multigenerational study used here followed Souissi et al. (2016), with major modifications.
123 Polypropylene beakers (5 L) were cleaned and pre-contaminated with Cd for about 3 days (to
124 prevent further absorption of the metal to the walls of the beakers, which may reduce Cd
125 availability) (Clason and Zauke, 2000). When Cd or metals quite generally are introduced to
126 water, they tend to precipitate or adsorb to suspended particulate matter and colloidal or
127 dissolved organic materials (Salomons and Foster, 1984). The copepods were fed once every 2
128 days with uncontaminated phytoplankton (*Isochrysis galbana* and *Nannochloropsis oculata*, at
129 the exponential phase containing ~ 5000 cells/mL), and the culture water was changed when they

130 got to copepodid stage. After the first generation we observed that many of the females were
131 carrying eggs after 13-16 days of the experiment (Kadiene et al., 2020), 60 of the gravid or
132 ovigerous females were then selected randomly and transferred to another container with Cd
133 contaminated water to produce the next generation. This process was repeated for up to 10
134 generations. At the end of each generation, ovigerous females were preserved to estimate
135 fecundity, some male and females (without eggs) were selected separately for RNA extraction
136 (300 copepods) and bioaccumulation analysis (200 copepods). The remaining copepods were
137 preserved to estimate the size of the copepod population. Selected differentially expressed genes
138 provided by Kadiene et al., (2020) were analysed using real time qPCR in male and non-
139 ovigerous females of *P. annandalei* to evaluate treatment effects (control *versus* Cd) and sex-
140 specific effects (male *versus* female) of multiple generations (F1-F4 and F9).

141 **2.2. Bioaccumulation analysis of cadmium in copepod samples**

142 Copepod samples were collected and filtered through a membrane filter paper which was
143 prior rinsed with ultra-pure water to remove salt. Copepod samples were cautiously dried to
144 constant weight at 60 °C. Dried and weighted samples were carefully folded and placed at the
145 bottom of Teflon tubes. Mixtures of ultrapure HNO₃ and HCl at a ratio of 1:3 were added, tightly
146 covered and then placed in a hot block at 120 °C for 3 hours mineralization. The mineralized
147 samples were diluted with milli-Q water to 10 mL at room temperature and analysed using
148 Inductively Coupled Plasma Optical Emission Spectrometry (Agilent 5100 SVDV ICP-OES).
149 The quality of the analysed data was validated using Certified Reference Materials (CRM)
150 PACS-3 and HISS-1.

151 **2.3. RNA extraction and Real-time qPCR**

152 RNA extraction and Real-time qPCR was carried out on the copepods following the same
 153 protocol as in Kadiene et al., (2020). Three genes (**Table 1**) were selected from a number of
 154 differentially expressed genes provided by Kadiene et al. (2020) because of the role they play in
 155 Cd metabolism. They were used as target genes in the qPCR analysis of male and female
 156 copepod samples from F1-F4 and F9, to estimate differences in gene expression across
 157 generations. The internal reference gene used was the elongation factor 1-alpha (EF1a). Primers
 158 were designed using *Primer3* Software (**Table 1**). Beacon Designer™ (free edition) was used to
 159 check for primer secondary structures. qRT-PCR analysis was performed following the same
 160 protocol as in Kadiene et al., (2020) for the genes of *myohemerythrin isoforms and heat shock*
 161 *protein 70 kDa*. $2^{-\Delta\Delta C_t}$ relative response method was used to extrapolate the relative expression
 162 of “Differentially Expressed Genes” (DEGs) (Livak and Schmittgen, 2001).

163 **Table 1.** Target genes of interest for qPCR analysis of *Pseudodiaptomus annandalei* exposed to
 164 cadmium.

Protein Code	Protein name	F and R Primers
MHEMT1	Myohemerythrin-1	>> TGTGCTCCTCGTCAAGATT << TGAGTTTGCTGTGTTTGTGAG
MHEMT	Myohemerythrin	>> GATTATAGCCTAACACCTCTCAAT << GGTTACAGGGTTTACAGTCTATT
HSP70	Heat shock protein 70	>> ACTCTCTGGCTGAAGGTATTG << TCGAGTGTGCCCTTGAAC
EF1a	Elongation factor 1-alpha (Reference gene)	>> TTCAAGTACGCCTGGGTTT << AAACCTCCAGAGAGCAATGTC

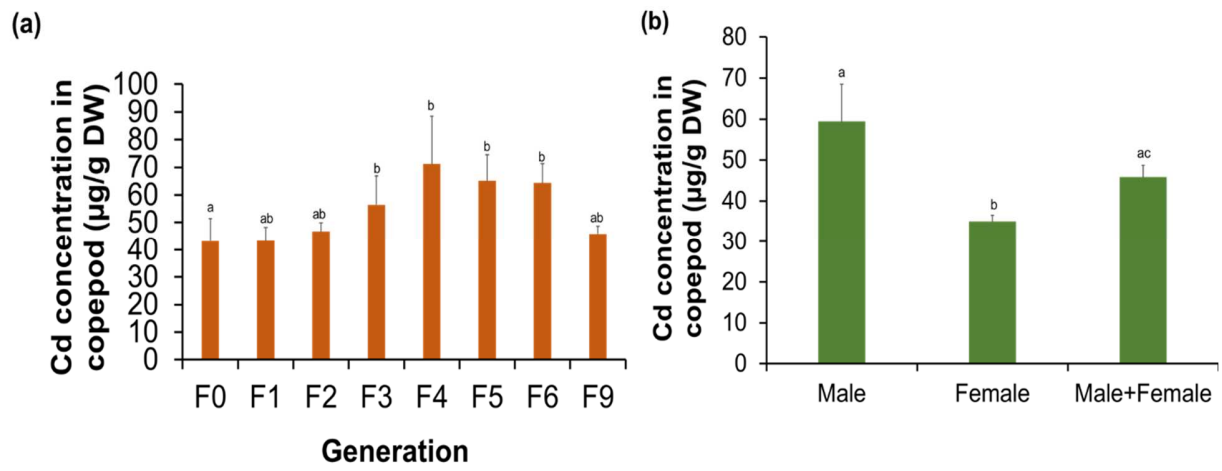
165 2.4. Statistical analysis

166 One-way Analysis of Variance (ANOVA) and independent sample t-test was used to
167 compare differences between groups, then Tukey's test was carried out to identify statistically
168 distinct groups. Significant differences were accepted at $p < 0.05$. These analyses were carried
169 out using SPSS software, version 18 and Microsoft excel 2016. The experiments were carried out
170 in triplicate.

171 3. Results

172 3.1. Bioaccumulation of Cd in *P. annandalei*

173 **Figure 1a** shows the bioaccumulation of cadmium (Cd) in *P. annandalei* (mixture of males
174 and females) from the 1st generation (F0) to the 7th generation (F6) and the 10th generation (F9).
175 The average Cd concentration in copepods was 43 $\mu\text{g/g}$ in F0 which increased to 71 $\mu\text{g/g}$ Cd in
176 F4, followed by a slight decrease from F5 to F9 generation. **Figure 1b** shows the sex specific
177 accumulation after 10 generations (F9). The Cd concentrations accumulated in males were
178 significantly ($p < 0.05$) higher than those in females, but not significantly different ($p > 0.05$) from
179 the concentration of Cd accumulated from the combination of male and female copepods (the
180 number of females was higher than those of males).

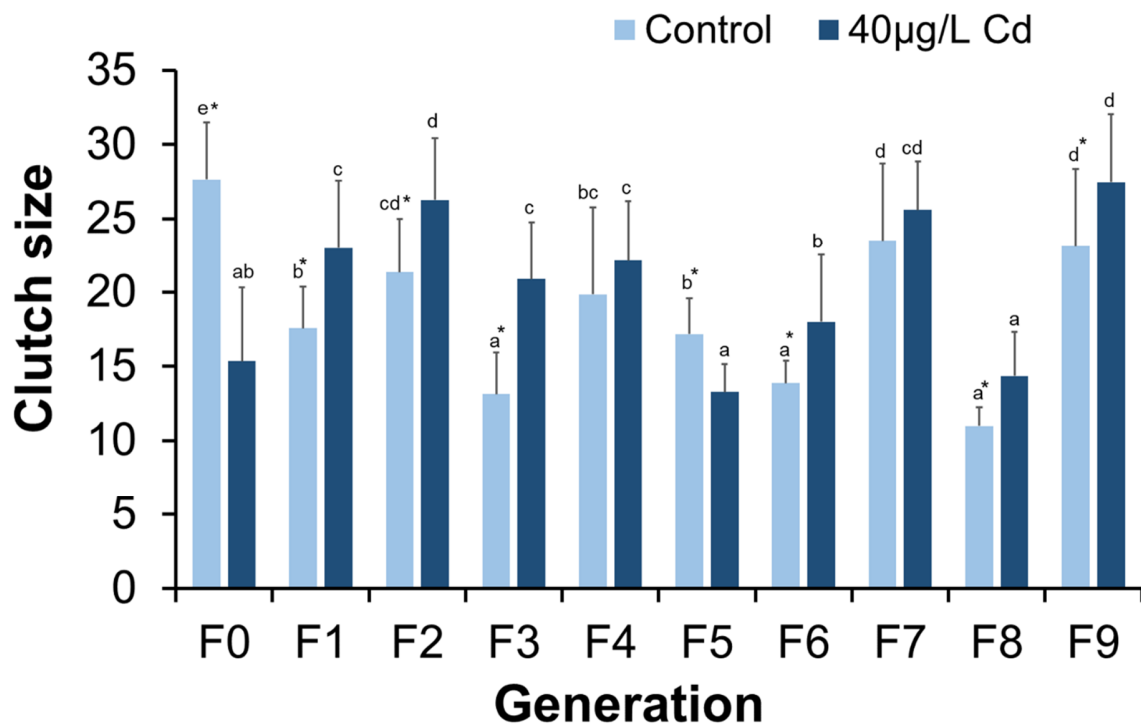


181

182 **Fig. 1.** Cadmium accumulation in *P. annandalei* adults at the end of each generation **(a)** and
 183 sex-specific Cd accumulation of *P. annandalei* at the end of F9 **(b)** [n = 3, mean ± SD].

184 **3.2. Fecundity and population density**

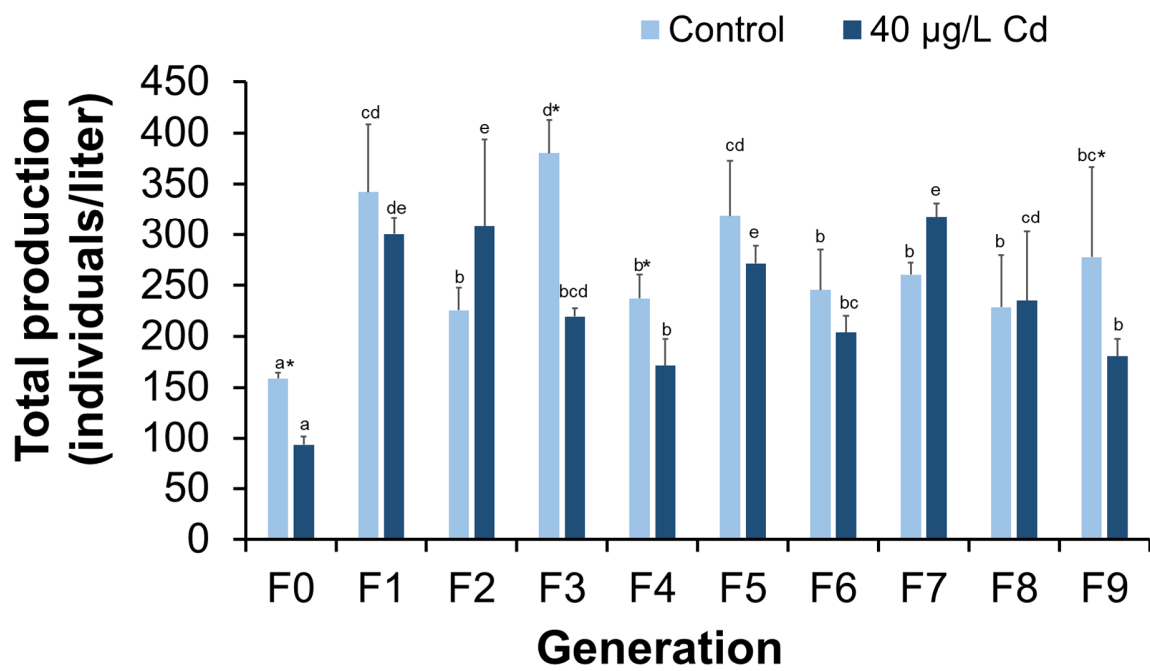
185 Clutch size (number of eggs in a pair of egg sacs) of copepods exposed to Cd was
186 significantly lower than in the control of F0 (**Fig. 2**). However, clutch size increased significantly
187 in subsequent generations to more than in the control groups except in F5. Moreover, the
188 population density of Cd treated copepods was lower than those in controls except in F2, F7, and
189 F8 (**Fig. 3**). In addition, a decrease in the population density of exposed copepods was
190 statistically significant ($p < 0.05$) in F0, F3, F4 and F9.



191

192 **Fig. 2.** Clutch size (number of eggs per female) of *P. annandalei* in the control and cadmium
193 exposed treatment at the end of each generation. Mean ± SD.

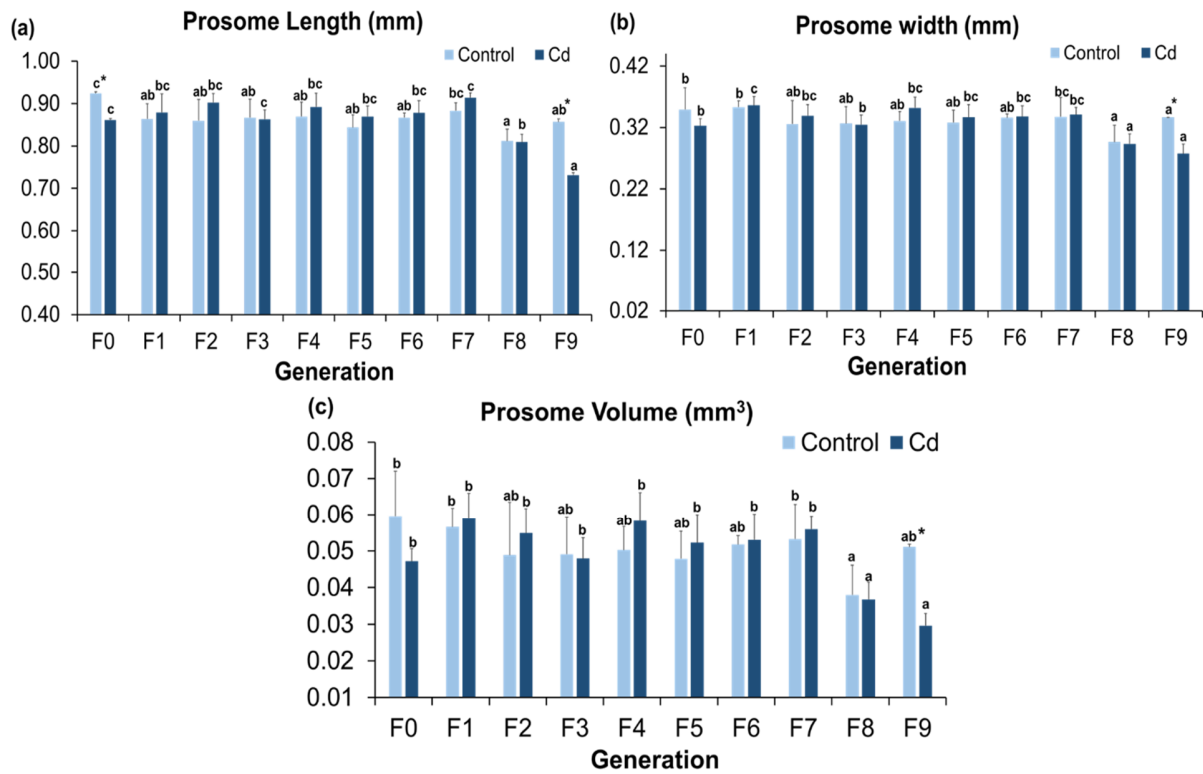
194



195

196 **Fig. 3.** Total population density of *P. annandalei* in the control and cadmium exposed treatment
 197 at the end of each generation. Mean \pm SD.

198 In the control, female prosome length (PL) of copepods in F0 was significantly ($p < 0.05$)
199 higher than those in F1 – F9 and that in F8 was significantly ($p < 0.05$) lower. Female prosome
200 lengths (PL) of copepods exposed to Cd was significantly ($p < 0.05$) lower in F9. Moreover, PL of
201 females in F0 were not significantly different ($p > 0.05$) from those in F1 - F7. However, they
202 were significantly higher than those in F8 and F9. Student t-test analysis showed statistical
203 significance ($p < 0.05$) in PL, prosome width (PW) and prosome volume (PV) between control
204 females and those exposed to Cd in F0 and F9. In the control, PV of females in F0, F1 and F7
205 were significantly different from those in F8. However, PV of females exposed to Cd in F8 and
206 F9 were significantly lower than those in F0 – F7 (**Fig. 4**).



207

208 **Fig. 4.** Prosome length (a), prosome width (b), prosome volume (c) of *P. annandalei* female
 209 copepods in the control and cadmium exposed treatment at the end of each generation. Mean \pm
 210 SD. Alphabets represent statistical significance ($p < 0.05$) between generations of each treatment
 211 and asterisks (*) represent statistical significance ($p < 0.05$) between control and Cd.

212 **Table 2** shows the Pearson correlation (R) between population (Pop) vs. prosome length
213 (PL), population (Pop) vs. clutch size (CS) and prosome length (PL) vs. clutch size (CS) of *P.*
214 *annandalei* at cadmium (Cd) treatments and control. A linear relationship between each
215 comparison of control and Cd treatment was carried out first among the whole data set
216 (generations F0 – F9), then for the first 3 generations (F0 – F2), the intermediate generations (F3
217 –F6), and the last 3 generations (F7 – F9). The same holds for the first 5 generations (F0 – F4)
218 and the last 5 generations (F5 –F9). A segmented linear relationship is explained by
219 intergenerational differences.

220 **Table 2.** Relationship (R) between population (Pop) vs. prosome length (PL), population (Pop) vs. clutch size (CS) and
 221 prosome length (PL) vs. clutch size (CS) of *P. annandalei* at control and cadmium (Cd) treatments.

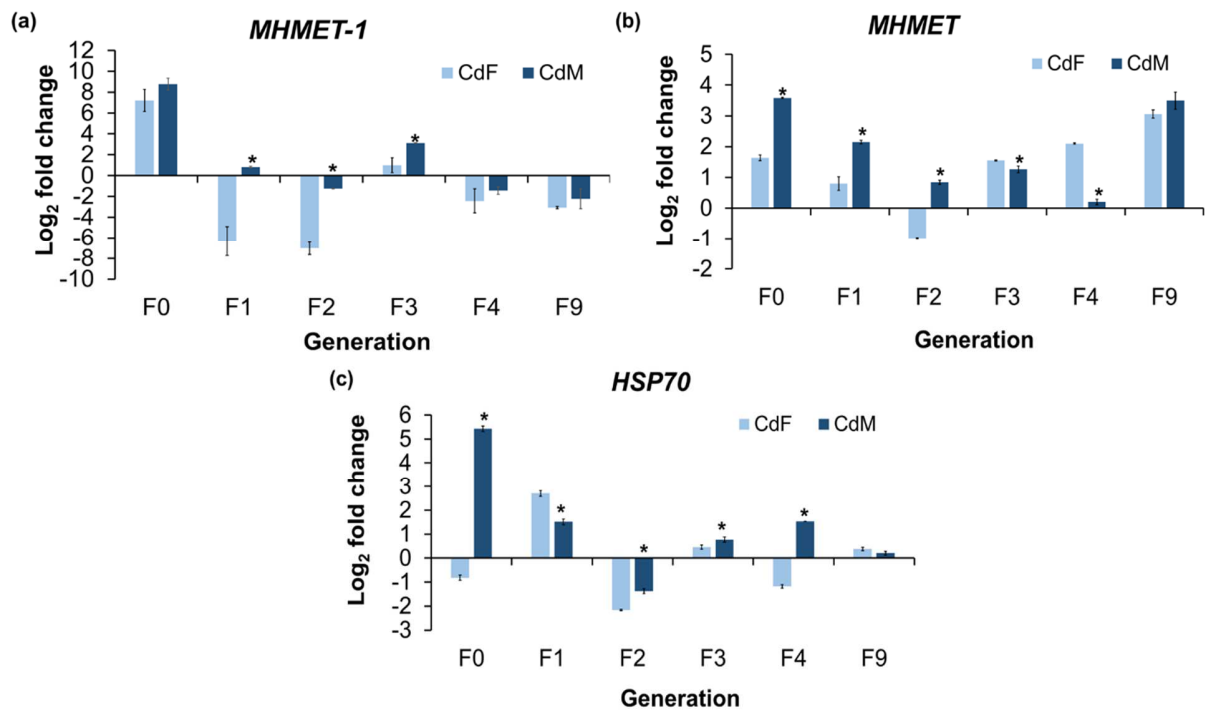
		F0-F9		F0-F2		F3-F6		F7-F9		F0-F4		F5-F9	
		Control	Cd	Control	Cd	Control	Cd	Control	Cd	Control	Cd	Control	Cd
Pop vs. PL	R	-0.236	0.239	-0.496	0.44	-0.127	-0.519	0.213	0.803**	-0.483	0.255	0.212	0.527*
	p-value	0.202	0.195	0.174	0.236	0.664	0.069	0.612	0.009	0.068	0.359	0.449	0.043
Pop vs. CS	R	-0.361*	0.168	-0.764*	0.871**	-0.319	-0.706**	0.401	-0.121	-0.793**	0.780**	0.258	-0.248
	p-value	0.046	0.366	0.017	0.002	0.266	0.007	0.325	0.756	<0.001	0.001	0.354	0.372
PL vs. CS	R	0.619**	0.061	0.815**	0.769*	0.196	0.209	0.777*	-0.011	0.636*	0.666**	0.569*	-0.218
	p-value	<0.001	0.746	0.007	0.015	0.501	0.494	0.023	0.977	0.011	0.007	0.027	0.435

222 *: Correlation is significant at the 0.05 level (2-tailed); **: Correlation is significant at the 0.01 level (2-tailed)

223 **3.3. Multi-generation expression of target genes in *P. annandalei* treated with**
224 **Cd: *female vs male***

225 Two isoforms of myohemerythrin proteins (MHMET-1 and MHMET) were significantly
226 expressed in males (CdM) and females (CdF) exposed to cadmium (Cd). MHMET-1 expression
227 in male and female copepods exposed to Cd was 434- and 146-fold change relative to their
228 control in F0 respectively (**Fig. 5a**). However, in F1, the expression level of MHMET-1 was
229 significantly reduced in CdM and downregulated in CdF. In F2, MHMET-1 was downregulated
230 in both CdM and CdF. In F3, MHMET-1 was upregulated in both CdM and CdF; however,
231 MHMET-1 was significantly ($p<0.05$) more expressed in CdM than in CdF. In F4 and F9,
232 MHMET-1 was downregulated in both CdM and CdF. MHMET isoform expression in male and
233 female copepods exposed to Cd was 12- and 3-fold change relative to their control, respectively,
234 in F0 (**Fig. 5b**). However, in F1, the expression level of MHMET decreased in CdM and CdF. In
235 F2, MHMET expression was further reduced in CdM; however, it was downregulated in CdF. In
236 addition, MHMET was upregulated in both CdM and CdF from F3 to F9. Upregulation of
237 MHMET was significantly ($p<0.05$) higher in CdM than in CdF in F0 and F1. However, it was
238 significantly ($p<0.05$) lower in CdM than CdF in F3 and F4.

239 Heat shock protein 70 (hsp70) was downregulated in CdF in F0 and was upregulated in F1.
240 However, it was downregulated in F2 and then upregulated in F3, and downregulated in F4 and
241 again upregulated in F9 (**Fig. 5c**). In F0, hsp70 was upregulated about 43-fold in CdM, then the
242 expression level was reduced in F1. In F2, hsp70 was downregulated in CdM and then in F3 it
243 was upregulated with further increase in expression levels in F4. However, in F9, hsp70 levels in
244 CdM decreased (**Fig. 5c**).



245

246 **Fig. 5.** Myohemerythrin-1 (a), Myohemerythrin (b) and Heat shock 70 kDa protein (c) expressed
 247 (Log₂ FC) in female and male of *P. annandalei* treated with cadmium relative to the control after
 248 each generation. n = 3, mean ± SD. CdF: female exposed to cadmium; CdM: male exposed to
 249 cadmium.

250 **Figure 6** shows muscle deformation of a *P. annandalei* female copepod when continuously
251 exposed to Cd for 10 generations. In addition, slow swimming speed of male and female
252 copepods after 10 generations of exposure to Cd was observed microscopically.

253



254

255 **Fig. 6.** Normal *P. annandalei* female copepod (a). Muscle deformation (b and c), curved-like
256 shape (red arrows) in *P. annandalei* female copepod exposed to cadmium in the 10th generation.

257 **4. Discussion**

258 It was discovered that toxicity effects can be sex-specific (Kadiene et al., 2017). The question
259 remains why this could be so? Toxicity is linked to body loads or concentrations of the toxicant
260 and/or pathway and mechanism of action which also depends on the organism's physiology. A
261 follow-up study carried out by Kadiene et al., showed that body load or toxicant accumulation

262 was also sex-specific (Kadiene et al., 2019; 2020). This led to investigate the mechanism of
263 action of the studied toxicant in copepods at the molecular level (Kadiene et al., 2020). Further
264 investigations carried out in the present study showed subsequent response pattern under
265 exposure to multiple generations that will ascertain possible adaptive tolerance ability or reveal
266 sex-specific plasticity across generations.

267 Multigenerational studies aim to understand the cascading effect of toxic substances on the
268 next generation after exposure to the previous generation. Parental exposure to a pollutant can
269 either increase or decrease the fitness of the resulting offspring, depending on the type of species,
270 type of pollutant, concentration and duration of exposure (Donelson et al., 2012; Krause et al.,
271 2017). In the present study, we found that parental exposure to Cd resulted in lower performance
272 of *P. annandalei* offspring such as reduced clutch size and survival. This is in agreement with
273 findings from recent studies on a range of aquatic animals like mosquitoes exposed to the
274 pesticide chlorpyrifos and warming (Tran et al., 2018), the cladoceran *Moina dubia* exposed to
275 Pb (Pham et al., 2021), *P. annandalei* exposed to Cu (Dinh et al., 2020b), rotifers and
276 cladocerans exposed to algal toxins (Beyer and Hambright, 2017; Dao et al., 2018), showing
277 decreased fitness of their offspring through several generations. As observed in the present study,
278 exposure to stressors, may reduce the quantity and quality of the produced eggs, which
279 consequently affect their survival (Doan et al., 2019; Dinh et al., 2020b) and an overall decrease
280 of reproductive output (Corrales et al., 2014).

281 Prior exposure of an organism to a stressor, can create a memory that affects its response to
282 the present or subsequent stressor of the same type or of different origin (Hughes et al., 2019;
283 Jackson et al., 2021). Our study provides strong evidence for how transgenerational effects of
284 parental exposures to Cd may change the magnitude of the response of offspring exposure to the

285 same stressor. Indeed, Cd-exposed parents already produced low performance offspring and a
286 direct exposure of the offspring (F2) to Cd further reduced their performance.

287 Our study indicated relatively high concentrations of cadmium (Cd) bioaccumulated in *P.*
288 *annandalei* in all generations. Zidour et al. (2019), reported an unsaturated increase of Cd in *E.*
289 *affinis* over time. High body accumulation of Cd could indicate a low ability of the copepods to
290 excrete trace metals. In this study, we observed increasing accumulation of Cd from F1 to F4.
291 This could imply that Cd was accumulated from one generation to the next through maternal
292 transfer in addition to direct uptake from the media, corresponding to similar observations in
293 previous reports (Kadiene et al., 2020; Li et al., 2015; Wang et al., 2018). In addition, we
294 analysed the amount of Cd accumulated in copepod sexes separately in F9 and observed that, Cd
295 concentration in males was higher than those in females. This could imply that female copepods
296 were able to eliminate Cd faster or more efficiently than males did (Kadiene et al., 2019). This
297 also corresponds to what was observed in our previous study, where *P. annandalei* male
298 copepods showed more sensitivity towards Cd than females (Kadiene et al., 2017). It is generally
299 known that metals become toxic when the bioaccumulated amount exceeds the concentrations
300 which the given organism can tolerate. However, several studies showed that toxicity changes of
301 a chemical are not consistently related to bioaccumulation. The toxicity of the bioaccumulated
302 xenobiotic depends on the organism's ability to excrete, store, or metabolize a xenobiotic
303 (Arunakumara et al., 2008; Gomez et al., 2010; Bai et al., 2020).

304 Bioaccumulation of Cd significantly affected egg production in the first generation of the
305 present study. Another study on the calanoid *Pseudodiaptomus sinensis* showed that metal
306 pollutants do not affect egg production (Zhou et al., 2016). However, in our study, we observed
307 an increased clutch size in copepods exposed to Cd in subsequent generations compared to the

308 control. Higher clutch size in copepods exposed to Cd, could indicate an adaptive trait in *P.*
309 *annandalei* females as a way to ensure continuity under unfavourable conditions. Although
310 increased egg production was observed after the first generation, it is possible that *P. annandalei*
311 females optimize a trade-off between increasing egg production and the survival of their
312 offspring. Upon certain environmental stress exposure, females of some copepods produce
313 diapause or resting eggs (Hairston and Olds, 1984; Djebbi et al., 2021). *P. annandalei* females
314 are egg carriers and are not known to produce diapause or resting eggs. The increase in clutch
315 size in the present study could be an adaptation to long-term Cd exposure. Population density
316 follows a similar oscillating pattern as female fecundity and its regulation also depends on
317 individual or within-generation plasticity of copepods. Similarly, significant toxic effects of Cd
318 were observed in the total population at the end of the first generation as in clutch size. However,
319 in subsequent generations, population density increased and decreased with fluctuations.
320 Moreover, total population density was generally reduced. This may be related to a decrease in
321 overall hatching and high naupliar or early copepodid mortality with increased Cd accumulation.
322 The lower egg production seen in F5 is similar to that seen in F0 relative to the control. This may
323 be a result of high Cd accumulation from maternal transfer in combination with direct
324 accumulation of Cd from water. Generally, an increase in population density (Pop) is inversely
325 correlated with prosome length (PL) and clutch size (CS) in copepods (Maly, 1973; Yi et al.,
326 2019). However, prosome length correlates positively with clutch size (Souissi et al., 2016). This
327 agrees with our correlation results for all generations (see **Table 1.2**). However, under Cd
328 treatment, Pop positively correlates with PL and CS. Therefore, the larger clutch sizes observed
329 in copepods exposed to Cd compared to the control may not be due to low density, but may be a
330 response strategy to Cd exposure. Within the first 3 generations, the same positive correlation

331 between Pop and PL, Pop and CS was observed. At the same time, a positive relationship
332 between Pop and CS was observed also in the first 3 generations. In the following 4 generations,
333 Pop and PL, Pop and CS tend to return to normal as in the control. However, in the last 3
334 generations, a correlation between Pop and PL was positive as in the first 3 generations. A strong
335 positive correlation was generally observed between PL and CS for copepods exposed to Cd. In
336 this study, the normal biological processes in copepods show an up and down regulatory pattern
337 alternating between each generation, which could indicate a mechanism of biological fitness
338 adjustment. However, an interesting pattern was observed in copepods exposed to Cd, whereby
339 there was a stepwise increase or decrease in 2, mostly 3 consecutive generations at the beginning
340 and towards the end of the 10th generation. Moreover, the intermediate generations (F3/F4 – F6)
341 tend to up and down regulate more frequently than the control, in a bid to recover or restore
342 normal processes. The toxicity of Cd was particularly observed at earlier and/or later generations
343 as indicated by the endpoints: total population size, clutch size, and female morphology.
344 Metallothionein-like and non-metallothionein-like proteins are major types of low-molecular-
345 mass Cd-binding proteins (Cd-BP) known from invertebrates (Stone and Overnell, 1985; Slice et
346 al., 1990; Hussain et al., 2021). Hemerythrin (HMET) is a non-metallothionein cadmium-binding
347 protein, a non-heme iron protein consisting of a di-iron oxygen binding site joined to a protein.
348 Myohemerythrin (MHMET) is a multi-functional protein. This participates in respiration, oxygen
349 transfer and/or storage and may act as a buffer to control the concentration and accordingly the
350 toxicity of cadmium. Aspects of innate immunity and their functions have effects on oxidation-
351 reduction regulation (Stenkamp, 1994; Terwilliger, 1998; Bailly et al., 2008; Meyer and Lieb,
352 2010; Yang et al., 2012; Liu et al., 2013; Coates and Decker, 2017; Genchi, et al., 2020). The
353 physiological response of copepods to metal exposure was fast, since we observed it in the first

354 generation. This followed a decrease in the production of both MHMET isoforms (MHMET-1
355 and MHMET) after exposure to Cd for multiple generations. MHMET-1 tends to decrease in the
356 2 generations after F0, increased again in F3, followed by downregulation in subsequent
357 generations. However, MHMET production increased in later generations after a significant
358 decrease in one generation. The decrease in expression level or downregulation of one gene
359 responding to Cd and the increase or upregulation of another gene similar in function could be
360 supplementary or complementary as observed in this study.

361 Heat shock proteins (HSPs) may have different functions, including ensuring proper protein
362 folding, thereby preventing misfolding, and protein repair. HSPs are expressed when an
363 organism is exposed to stress (Kim et al., 2014; Semmouri et al., 2019). Hsp70 is usually
364 expressed when organisms are exposed to basic environmental stressors as a protective response
365 (Rhee et al., 2009; Kim et al., 2014). Under unstressed conditions, heat shock proteins have
366 constitutive functions that are essential in various aspects of protein metabolism (Han et al.,
367 2020; Nikinmaa and Rytönen, 2011). In this study, hsp70 was upregulated in control females.
368 However, when the females were exposed to Cd (CdF), hsp70 was downregulated, but
369 upregulated in males exposed to Cd (CdM) in F0. Whereas, hsp70 in CdF became upregulated in
370 F1, and in subsequent generations, hsp70 was regulated in an up or down manner. For males
371 exposed to Cd, hsp70 was consistently upregulated in all generations, except in F2 where
372 downregulation occurred. However, the expression levels in subsequent generations were lower
373 than in F0. A similar pattern of expression was observed in *Calanus finmarchicus*, a calanoid
374 species exposed to stressors (Hansen et al., 2008). Both up- and downregulation of stress related
375 genes were reported as responses to xenobiotics (Jeong et al., 2020). Downregulation of these
376 genes could indicate tolerance in one generation with further upregulation or increase in

377 expression level when plasticity becomes costly. It could also indicate a change in sensitivity in
378 another generation. The energetic costs for egg production in female copepod and general protein
379 biosynthesis which also produced reactive oxygen species (ROS) can be high (Sokolova et al.,
380 2012; Tomanek, 2014; Han et al., 2020). Therefore, downregulating protein synthesis under
381 extended exposure to Cd could be a strategy for conserving energy and minimizing oxidative
382 damage from excess ROS production (Bailey et al., 2017). The low production or
383 downregulation of proteins is generally also a strategy to conserve energy. Energy conservation
384 could also explain the slow swimming speed of copepods which was observed at later
385 generations.

386 In our previous study (Kadiene et al., 2017), we reported that metal toxicity to calanoid
387 copepods is sex-specific. We observed corresponding molecular responses between males and
388 females in the present study. Moreover, gene expression in response to Cd such as MHMET and
389 hsp70 were more significantly expressed in males than in females. The multigenerational
390 regulation of expressed genes may indicate plasticity or differences in individual fitness. Most
391 genes that were highly upregulated in the F0 generation were either downregulated or their
392 expression levels were reduced in subsequent generations. This could be attributed to tolerance
393 or suppression, when they become overwhelmed, since the amount of accumulated Cd increased
394 with each generation. Kimberly and Salice (2015) reported that chronic exposure to cadmium
395 alters tolerance levels in a non-monotonic way. As a result of the effect observed after one-
396 generation removal from Cd, their results indicated that multigenerational exposure may result
397 from a transgenerational effect. The fluctuations in activated genes responding to metal exposure
398 are usually linked to a variation in normal protein metabolism rather than to changes in metal
399 exposure (Legras et al., 2000; Wong et al., 2020). Vandegheuchte et al. (2010), attributed the

400 differential gene regulation between subsequent control generations after exposure to zinc to
401 possible differences in synchronization of the molting and reproductive cycle of cladocerans
402 during multiple generations. Furthermore, high variations and fluctuations in response to
403 multigenerational studies are often explained by different sampling time per generation, sex
404 specific sensitivity to contaminants, within and trans-generational plasticity, and influence of
405 non-contaminant stressors, such as handling stressors at different generations. Based on the type
406 of test carried out, these factors cannot easily be predicted and controlled, underscoring the need
407 to use multiple toxicity methods.

408 In this study, female copepods exposed to Cd showed a lower cadmium uptake or rather
409 faster excretion rate than males, and secondly, the clutch sizes were mostly higher under Cd
410 treatment when compared to those of the control, however, population density was lower, either
411 caused by low hatching success or mortality. Further studies are required here to understand this
412 generational plasticity. Physiological aspects could be compromised, such as the observed
413 deformation of muscular structure. Transmission electron micrographs of the muscle of
414 *Pseudodiaptomus annandalei* after 12 days of Cd exposure showed evidence of damage of the
415 myofibril ultrastructure attributed to ROS overproduction caused by oxidative damage (Ibrahim
416 et al., 2015). In a study of Chen (2016) the same phenomenon was observed without stating
417 whether in males and/or in females. This is consistent with the external muscle contortion or
418 deformation observed in our study (**Fig. 6**). The disparate physiological changes or molecular
419 responses observed from multiple generations continuously exposed to Cd may represent
420 decreased and/ or increased sensitivity or plasticity (Bellas and Gil, 2020) and could be regulated
421 by each generation's plasticity.

422 **5. Conclusion**

423 An increased egg production and slight improvement in the total population density of
424 copepods exposed to Cd, the observed slow swimming speed in both sexes, and muscle
425 deformation observed in most of the females, indicate specific responses of the copepod *P.*
426 *annandalei*. Copepods were able to adapt to HM-stressed environmental conditions. In terms of
427 reproduction, *P. annandalei* showed adaptations and fitness-related adjustments to Cd toxicity
428 after several generations. The existing data have the potential to be used in the development of
429 stable water quality criteria and to estimate long-term effects of persistent pollutants in aquatic
430 ecosystems. Environmental pressure resulting from continuous exposure to persistent pollutants
431 like Cd will have evolutionary impacts. Bioaccumulation of Cd in copepods over several
432 generations through maternal transfer in addition to direct uptake from the media may either
433 increase or decrease their adaptability or level of tolerance. Genetic regulations and
434 physiological adjustments of copepods observed in this study could lead to adaptive outcomes.
435 The tendency of copepods to adapt to a toxic environment through epigenetic and phenological
436 modifications could increase their chance of survival over a long term. From the results of this
437 study, we hypothesize that *P. annandalei* females produce more eggs, to either compensate for
438 low survival of their offspring that are constantly faced with toxicants and/or to eliminate toxic
439 compounds from their bodies.

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456 **Data availability statement**

457 The authors confirm that the data supporting the findings of this study are available within the
458 article. Any further information needed on this study is available from the corresponding author
459 Sami Souissi upon reasonable request.

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