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1 Sex and heavy metals: study of sexual dimorphism in response
2 to soil pollution

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22 **Short title:** Sexual dimorphism in response to heavy metal pollution

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25

26 **Abstract**

27 In dioecious plants, males and females often show distinct morphological, physiological and
28 life history traits as result of their different demands for reproduction. Such sexual
29 dimorphism is likely to be accentuated under stressful conditions, such as that imposed by
30 exposure to heavy metals. However little is known about the response of dioecious plants to
31 stress by heavy metals. Here we use the dioecious herb *Silene latifolia* to investigate the
32 growth and reproduction of males and females growing in soil polluted with either Cu or Cd.
33 We also examined whether the sexes differed in the patterns of metal accumulation in their
34 tissues. Patterns of biomass allocation to reproduction, roots, leaves and shoots (stem and
35 leaves) were compared in male and female *S. latifolia* plants that were harvested after
36 growing for 14 weeks in different soil conditions (non-polluted soil, Cu-polluted soil and Cd-
37 polluted soil). In addition, patterns of metal accumulation between the sexes were also
38 compared post-harvest by analysing the metal content in their tissues. Overall, metals
39 decreased plants' total dry mass allocated to leaves and to roots- particularly in males.
40 Females accumulated more Cu in their tissues. However, this did not seem to correspond with
41 females performing worse (in terms of growth and reproduction) than males when growing in
42 soil polluted with this metal. Despite males and females having similar levels of Cd in their
43 tissues, males seemed to have lower tolerance to this metal, as indicated by a lower total and
44 leaf dry mass than females, and also by a lower number of flowers when growing in Cd-
45 polluted soil. We also found contrasting differences in female seed production due to heavy
46 metals, with number of seeds (but not mass) decreasing with Cu and increasing with Cd. Our
47 results indicate the presence of sexual dimorphism in response to heavy metals, with the sexes
48 differing in both patterns of accumulation and tolerance.

49

50 **Keywords:** heavy metals, dioecy, cost of reproduction, sexual dimorphism, *Silene latifolia*

51 **1. Introduction**

52 Dioecy, where male and female functions are housed in different individuals, is a rare sexual
53 system in flowering plants (~ 6- 7%; Renner and Ricklefs 2008). Despite its rarity, it has
54 evolved repeatedly among flowering plants, occurring in almost half of all angiosperm
55 families (Heilbuth, 2000) probably in response to selection either for inbreeding avoidance
56 (Charlesworth and Charlesworth, 1978; Freeman et al., 1997) or sexual specialization
57 (Charnov et al., 1976; Freeman et al., 1997). Once unisexuality has evolved, selection may
58 favour further divergence between the sexes as consequence of their different reproductive
59 roles (production of seeds vs. pollen). Sexual dimorphism in plants is quite common (Barrett
60 and Hough, 2013; Willson, 1991) and males and females usually differ in their vegetative
61 morphology (Dawson and Geber, 1999; Obeso, 2002; Sánchez-Vilas and Retuerto, 2009;
62 Sánchez-Vilas et al., 2012), phenology (Bullock and Bawa, 1981; Delph, 1990), physiology
63 (Case and Ashman, 2005; Dawson and Geber, 1999; Sánchez-Vilas and Retuerto, 2011,
64 2009), life history (Geber et al., 1999), competitive abilities and allocation to defence
65 (Cornelissen and Stiling, 2005; Sánchez-Vilas et al., 2011).

66
67 Sexual dimorphism has been commonly attributed to the different cost of reproduction in
68 males vs. females, namely, as the result of trade-offs between allocation to reproduction and
69 to other functions (e.g., to growth and/or defence). Such trade-offs are likely to be accentuated
70 under more stressful conditions, such as under nutrient-deficient soil, strong competition from
71 other plants, or herbivory. In fact, several studies have found that the differences between
72 males and females are affected by the environmental context, particularly by resource
73 availability (Dawson and Ehleringer, 1993; Eppley, 2001; Geber et al., 1999). Female
74 function is usually associated with a greater demand for resources, needed to produce both
75 flowers and fruits (Agren, 1988; Korpelainen, 1992), and male-biased sex ratios are

76 commonly found in more stressful environments (see review in Field *et al.* 2013). However,
77 there is not yet a clear pattern in the responses of the sexes to environmental stress, and their
78 differences in tolerance seem to vary depending on the species considered and type of stress,
79 demanding a greater range of studies (Juvany and Munné-Bosch, 2015).

80 Soil pollution by heavy metals, as consequence of human activities (e.g., mining, waste
81 disposal, industrial activities), represents a dramatic case of environmental stress for living
82 organisms. High concentration of heavy metals are considered environmental pollutants due
83 to their strong toxic effects; in plants, they may interfere with growth by disturbing nutrient
84 uptake, altering photosynthesis and other metabolic processes (Flemming and Trevors 1989;
85 Pålsson 1989; Tyler *et al.* 1989; Fernandes and Henriques 1991). Since they interfere with
86 resource uptake, patterns of allocation to growth and reproduction may be also expected to
87 change. However, despite the widespread contamination of soils by heavy metals, few studies
88 have considered how they affect to the cost of reproduction (but see, Saikkonen *et al.* 1998;
89 Koivunen *et al.* 2004) and we are only aware of a handful of studies that have examined how
90 males and females of the same species (in particular, trees of the genus *Populus*) respond to
91 their presence in the soil (Chen *et al.*, 2011; Chen *et al.*, 2013; Han *et al.*, 2013; Jiang *et al.*,
92 2013).

93 Here, we aim to increase our knowledge of responses to stress in dioecious plants, by
94 investigating the effect of heavy metals in growth and reproductive traits of males and
95 females. Specifically, we explore how soil contaminated with two different heavy metals, Cu
96 and Cd, affects the extent of sexual dimorphism in males and females of the dioecious herb
97 *Silene latifolia* Poir. Considering that female function demands a greater amount of resources
98 and that heavy metals may interfere with resource uptake, we expect a poorer performance of
99 females relative to males in terms of growth and reproduction in heavy metal contaminated
100 soils.

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105 **2. Materials and Methods**

106 *2.1. Study species*

107 *S. latifolia* (Caryophyllaceae), the white campion, is a short-lived perennial and dioecious
108 plant native to most of Europe, Western Asia and Northern Africa. It is commonly found
109 growing in disturbed or cultivated ground (Tutin et al., 1993) and has also been found
110 growing in heavily contaminated mine sites in Southern France (Escarré et al., 2010). Sexual
111 dimorphism in *S. latifolia* has been extensively studied (Bernasconi et al., 2009), with males
112 having less allocation of biomass to vegetative growth and reproduction, but also producing
113 more flowers (smaller) than females (see Delph *et al.* 2005, and references therein).

114

115 *2.2. Experimental design*

116 In summer 2011, seeds were collected from five natural populations of *S. latifolia* growing in
117 Galicia, Northwest Spain (see map in Fig. 1) by sampling approx. 20 individuals per
118 population (8-10 capsules per individual). Seeds were separated from capsules, discarding the
119 damaged ones, and stored at room temperature.

120 In March 2012, seeds from these five populations were randomly sown in 40-cell germination
121 trays in a greenhouse at the Faculty of Biology, University of Santiago de Compostela
122 (Spain). We transplanted a total of 240 seedlings (evenly and randomly chosen across
123 populations) to individual 1.6 L pots, filled with garden soil, when the second pair of true
124 leaves started to grow (approx. 2 months after germination). Pots were distributed spatially on
125 the greenhouse benches and their position re-arranged weekly in order to avoid position

126 effects. One week after transplanting, seedlings were randomly assigned to one of three
127 treatments: Control (no metal), Cadmium contaminated soil (10 ppm Cd) and Copper
128 contaminated soil (125 ppm Cu); 80 seedlings per treatment. Cu was added as sulphate
129 (copper sulphate 5-hydrate: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ Panreac, Barcelona, Spain) and Cd was added as
130 chloride (cadmium chloride anhydrous: CdCl_2 , Merck, Munich, Germany) in a unique dose at
131 the start of the experiment. Copper is an essential micronutrient for plant growth; higher
132 levels than 100 ppm are often toxic (Ross and Kaye, 1994) affecting key processes such as
133 photosynthesis, respiration or nitrogen fixation (Fernandes and Henriques, 1991; Maksymiec,
134 1997). Cadmium is a non-essential element that can be highly phytotoxic inhibiting
135 photosynthesis (Baszynki et al., 1980), transpiration (Bazzaz et al., 1974) and metabolic
136 activities (Moya et al., 1993; Sharma et al., 1998). Cd concentrations in European non-
137 polluted soils range from 0.06 to 0.50 ppm depending on the nature of the parent material
138 (Jensen and Bro-Rasmussen, 1992). Seedlings of *S. latifolia* were assigned to the
139 experimental treatments before the sex was known, which allowed to expose the plants to the
140 effects of metal contamination well before reproduction started. The final number of males
141 and females for which traits were measured depended on the number of individuals that set
142 flower and fruits and these can be found in legends to figures 2, 3 and 4. Both ends of the
143 greenhouse were kept open over the duration of the experiment to allow local pollinators
144 access to the flowers.

145 2.3. *Growth and biomass allocation*

146 After 14 weeks of growth under experimental conditions, we harvested all the plants that had
147 flowered (and therefore sex could be identified) and dissected them into reproductive organs,
148 roots, stems and leaves. At this time, plants were not showing any signs of being pot-bound,
149 and upon harvest, it was observed that some of the soil volume still remained unoccupied by
150 roots. Plant tissues were then oven dried at 40 °C to constant weight and their dry mass

151 recorded. Roots, stems and leaves were cleaned and washed in distilled water for 15 min with
152 shaking before drying.

153

154 *2.4. Reproduction*

155 Reproductive effort (RE) was calculated by dividing the dry mass allocated to reproduction by
156 vegetative dry mass (roots plus stems and leaves). Root:shoot ratio was calculated by dividing
157 the root by the shoot (stem and leaf) dry mass. Flowering time was measured by recording the
158 number of days since the start of the experiment (when treatments were applied) to the first
159 flower. At harvest, we also recorded the total number of flowers produced and the number of
160 seeds produced by females. The number of seeds was obtained by counting all the seeds in all
161 the capsules of a plant and the total dry mass of seeds per plant measured.

162

163 *2.5 Leaf spectral reflectance*

164 At the end of July 2012, we measured spectral reflectance on a new fully formed and healthy
165 leaf that had developed during the experiment for each plant, using a portable spectrometer
166 (UniSpec Spectral Analysis System, PP Systems, Haverhill, MA). Reflectance spectra
167 (wavelengths 306-1136) were calculated as the spectral radiance of the leaf divided by the
168 radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere, North
169 Sutton, NH). The following spectral reflectance indices were calculated: (1) PRI, the
170 photochemical reflectance index, was calculated as $(R_{539} - R_{570}) / (R_{539} + R_{570})$, where R_{539} is
171 reflectance at 539 nm (affected by processes of dissipation of excess radiation as the
172 epoxidation state of the xanthophylls, thylakoid Δ pH formation and chloroplasts
173 conformational changes; Demmig-Adams, 1990) and R_{570} is reflectance at 570 nm (a

174 reference wavelength unaffected by these processes). PRI has been shown to be strongly
175 correlated with leaf-scale photosynthetic radiation-use efficiency ($\text{mol CO}_2 \text{ mol}^{-1} \text{ photons}$)
176 (Filella et al., 1996; Gamon et al., 1997; Peñuelas et al., 2011, 1995). (2) CHL, the
177 chlorophyll content index, was calculated as R_{750}/R_{700} and is correlated to the chlorophyll
178 content of leaves (Lichtenthaler et al., 1996).

179

180 *2.6. Metal content in plant tissues*

181 At the end of the experiment, 10 dried plant samples (roots plus stems and leaves) randomly
182 selected for each combination of sex and metal treatment were homogenized and ground
183 ($<120 \mu\text{m}$) using an ultra-centrifugal mill Retsch ZM 100 (Retsh GmbH, Haan, Germany).
184 When necessary, samples included more than a single individual to achieve enough sample
185 size to determine Cu and Cd. A representative sample of 0.5 g was digested with 8 ml of
186 NO_3H and 2ml of H_2O_2 in Teflon bombs in a microwave oven and subsequently diluted in
187 deionized water to a final volume of 25 ml. Quality control of the digestion process was
188 carried out by parallel analysis of three samples of certified reference materials (NIST MR
189 apple leaves 1515). Concentration of Cu and Cd were then determined by means of
190 inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x). Each analysis was
191 carried out three times and all samples were run in batches that included blanks. Final
192 concentrations in plant tissues were expressed referring to dry weight.

193

194 *2.7. Data analysis*

195 Analyses of growth and biomass allocation (including reproductive effort and total dry mass
196 of seeds) and leaf spectral reflectance indices were carried out using linear mixed-effects

197 models using the R ‘lme’ function from the ‘nlme’ package (Pinheiro et al., 2010). Analyses
198 of number of flowers, number of seeds and flowering time were fitted using a generalized
199 linear mixed model (GLMM) with poisson errors and log link function by means of the
200 ‘glmer’ function in the lme4 package (Bates et al., 2011). In these models, metal treatment
201 and sex were fitted as fixed factors and population was fitted as random ‘blocking’ effect to
202 account for population-to-population variation. *P*-values were determined by comparing
203 models with and without the factor being tested. Model comparison was performed by means
204 of likelihood ratio tests by calling the function ‘anova’ to compare models that, for fixed
205 effects, were fitted using maximum likelihood (Crawley, 2007; Pinheiro and Bates, 2000).
206 Little explained variance was associated with the random effect of population, which was
207 non-significant for most variables (except for stem dry mass).

208 To test for differences in metal content in plant tissues, we used a two-way ANOVA (‘aov’
209 function in R), with sex, treatment and the interaction as fixed factors, and Cu and Cd
210 contents (mg/Kg) as response variables. Root dry mass, leaf dry mass, root:shoot ratio, and
211 the Cu and Cd contents were log₋₁₀ transformed and total dry mass of seeds was square-root
212 transformed to meet the assumptions of the analysis of variance. When significant differences,
213 post-hoc tests were performed to examine the mean differences among groups using Tukey’s
214 HSD tests by means of the ‘lsmeans’ function in R.

215

216 **3. Results**

217 *3.1. Metal content in plant tissues*

218 The presence of the metal in the soil significantly increased the content of the same metal in
219 the plant tissues (treatment: Cu: $F_{2,57} = 1549$, $P < 0.0001$; Cd $F_{2,57} = 1836$, $P < 0.0001$).

220 Females accumulated more Cu than males (sex: $F_{1,57} = 12.92$, $P < 0.001$; Fig. 2A), but no

221 differences between the sexes were found for Cd (sex: $F_{1,57} = 0.156$, $P = 0.694$; Fig. 2B).

222

223 3.2. Growth and biomass allocation

224 Plants growing in soil contaminated with heavy metals had lower total dry mass (TDM) than
225 those growing in non-contaminated soil. Interestingly, the response of the sexes differed
226 depending on the metal present in the soil (TDM, sex \times treatment: Log-likelihood ratio (L-
227 ratio) = 6.18, d.f. = 2, $P = 0.046$): males and females had similar decrease in biomass when
228 growing in Cu rich soil, but males had a greater decrease in biomass than females when
229 growing in soil contaminated with Cd (Fig. 3A).

230 The amount of biomass allocated to leaves (LDM) decreased in males when growing in the
231 presence of metals (more in Cd than in Cu), but remained constant in females (LDM, sex \times
232 treatment: L-ratio = 5.96, d.f. = 2, $P = 0.051$; Fig. 3B). This created differences between the
233 sexes in the dry mass of leaves that depended on the treatment; males growing in non-polluted
234 soil tended to have greater leaf dry mass than females, whilst the opposite was true in soil
235 containing Cd (Fig. 3B).

236 The root dry mass (RDM) of plants decreased similarly when growing in presence of both
237 metals (RDM, treatment: L-ratio = 30.1, d.f. = 2, $P < 0.001$; Fig. 3C). However, the stem dry
238 mass (SDM) decreased only when plants were growing in soil polluted with Cd and not with
239 Cu (SDM, treatment: L-ratio = 7.08, d.f. = 2, $P = 0.029$; Fig. 3D), and consequently, plants
240 growing in Cu had the lower root:shoot ratio (below-: above-ground dry mass ratio)
241 (treatment: L-ratio = 11.5, d.f. = 2, $P = 0.003$; Fig. 3E). Males and females did not differ in
242 their biomass allocated to roots (RDM, L-ratio = 2.03, d.f. = 1, sex: $P = 0.154$), shoots (SDM,
243 sex: L-ratio = 0.415, d.f. = 1, $P = 0.512$), or in their root:shoot ratio (sex: L-ratio = 1.53, d.f. =
244 1, $P = 0.215$), and these traits were not differently affected in response to growth under metal-
245 polluted soil (RDM, sex \times treatment: L-ratio = 1.99, d.f. = 2, $P = 0.370$, Fig. 3C; SDM, sex \times

246 treatment: L-ratio = 0.826, d.f. = 2, $P = 0.661$, Fig. 3C; Root:shoot, sex \times treatment: L-ratio =
247 0.581, d.f. = 2, $P = 0.748$, Fig. 3E).

248

249 *3.3. Reproduction*

250 Total reproductive dry mass was greater in females than males (sex: L-ratio = 30.5, d.f. = 1, P
251 < 0.0001), but no differences were found among treatments (treatment: L-ratio = 2.28, d.f. =
252 2, $P = 0.319$; sex \times treatment: L-ratio = 4.94, d.f. = 2, $P = 0.084$).

253 Reproductive effort was not affected by the metal treatments that plants were exposed to
254 (treatment: L-ratio = 2.42, d.f. = 2, $P = 0.298$). Overall, females had a greater reproductive
255 effort than males (1.7 times greater; sex: L-ratio = 36.1, d.f. = 1, $P < 0.0001$; Fig. 4A),
256 regardless of the treatment they were growing in (sex \times treatment: L-ratio = 3.10, d.f. = 2, $P =$
257 0.212; Fig. 4A). However, males produced a greater number of flowers than females, and
258 male flower production, but not female, decreased significantly when plants were growing in
259 soil polluted with Cd (sex \times treatment: $\chi^2_2 = 12.46$, $P = 0.002$; Fig. 4B). The number of seeds
260 produced by females differed across treatments, decreasing under Cu but increasing under Cd
261 (treatment: $\chi^2_2 = 802$, $P < 0.0001$; control: 293 ± 32 , Cu: 240 ± 30 , Cd: 363 ± 36 ; all Tukey
262 HSD post-hoc comparisons $P < 0.001$), but the total dry mass of seeds produced was similar
263 across treatments (treatment: L-ratio = 3.27, d.f. = 2, $P = 0.195$; control (mean \pm S.E.): $0.21 \pm$
264 0.024 , Cu: 0.18 ± 0.023 , Cd: 0.23 ± 0.019). The average number of days to first flowering
265 since the application of the experimental treatments (overall mean \pm S.E. = 57 ± 0.96) was not
266 affected by sex, treatment or the interaction (sex: $\chi^2_1 = 0.842$, $P = 0.359$; treatment: $\chi^2_2 =$
267 0.695 , $P = 0.706$; sex \times treatment: $\chi^2_2 = 2.25$, $P = 0.325$).

268

269 *3.4. Leaf spectral reflectance*

270 PRI, the photochemical reflectance index, was similar in both males and females (sex: L-ratio
271 =0.127 d.f. = 1, $P = 0.722$) and no differences were found among treatments (treatment: L-
272 ratio = 4.78, d.f. = 2, $P = 0.092$; sex \times treatment: L-ratio = 1.52, d.f. = 2, $P = 0.466$).

273
274 Males had slightly lower values of CHL, the chlorophyll index, than females (mean \pm S.E.:
275 2.25 \pm 0.045, 2.34 \pm 0.056, respectively), but this difference was not statistically significant
276 (sex: L-ratio =0.340 d.f. = 1, $P = 0.065$). CHL was also not affected by the different metal
277 treatments (treatment: L-ratio = 0.70, d.f. = 2, $P = 0.704$; sex \times treatment: L-ratio = 0.234, d.f.
278 = 2, $P = 0.890$).

279

280 **4. Discussion**

281

282 Our experiment shows evidences of sexually dimorphic responses to growth in metal polluted
283 soil. On one hand, we found sexual dimorphism in metal accumulation patterns, with females
284 accumulating more Cu than males in their tissues. On the other hand, the metals affected the
285 sexes differently in terms of growth and reproduction, particularly, males had lower total and
286 leaf dry mass than females when growing in soil polluted with Cd. Males also experienced a
287 reduction in the number of flowers when growing in Cd.

288

289 Overall, both metals inhibited growth (below- and above-ground), confirming the toxicity of
290 Cd and Cu at the levels applied. Although the mechanisms of metal toxicity are still not fully
291 understood, an excess of heavy metals can hamper nutrient uptake by affecting the availability
292 of nutrients in the soil, or by reducing the microbes in the soil (Ke et al., 2007; Moreno et al.,
293 1999). Additionally, the transport and use of water and essential nutrients can also be reduced
294 by the presence of metals (Bouazizi et al., 2010; Das et al., 1997). At cellular level, an excess

295 of heavy metals can cause damage by leading to the formation of reactive oxygen radicals or
296 by the interaction with proteins impairing key cellular processes (e.g., photosynthesis),
297 inactivating enzymes and disturbing protein structure (Assche and Clijsters, 1990; Dietz et al.,
298 1999).

299

300 In non-polluted soil, males and females did not differ in their total biomass, although males
301 allocated slightly more resources to leaves than females. This lack of differences in growth
302 between the sexes of *S. latifolia* has been reported before, particularly when considering
303 plants at the onset of flowering – before the investment in reproduction (Delph and Meagher,
304 1995). In our experiment, plants are harvested after the onset of reproduction, and despite
305 females having greater allocation to reproduction there is no trade-off between growth and
306 reproduction. This apparent lack of trade-off has been considered in detail by Delph and
307 Meagher (1995) pointing out to differences in the source/sink ratio between the sexes as a
308 plausible explanation. Reproductive organs are net consumers of carbon, acting as sinks and
309 potentially regulating photosynthesis (Neales and Incoll, 1968; Sweet and Wareing, 1966).
310 Therefore, a greater allocation to reproductive organs in females could increase the sink
311 strength, causing the rate of carbon fixation to be increased and therefore masking any
312 potential trade-off (Delph and Meagher, 1995; Laporte and Delph, 1996). In dioecious plants,
313 Retuerto *et al.* (2006) have suggested that the specific sink demands associated with male and
314 female reproduction may be most easily met by the physiological specialization of both sexes.

315

316 The excess of Cu in the soil inhibited growth similarly in both sexes, although females
317 accumulate more of this metal in their tissues. In addition, the presence of Cd in the soil had a
318 greater detrimental effect – reducing growth and investment in reproduction- in males than in
319 females, despite no differences in accumulation of Cd in their tissues. As mentioned above,

320 females of *S. latifolia* allocate more of their resources to reproduction (seed production) than
321 males, and therefore we could expect greater trade-offs between growth and reproduction in
322 females than males, particularly under stressful conditions. However, no trade-offs (or even
323 the opposite pattern) have been found in our study, indicating a greater tolerance to metals in
324 females than in males. Interestingly, in the dioecious tree *Populus yunnanensis* the opposite
325 (and expected) pattern is found: males accumulate in their tissues and tolerate greater levels of
326 Cu and Cd than females (Chen et al., 2011; L. Chen et al., 2013). In *S. latifolia*, the greater
327 sink-strength of females, as pointed above, could be one plausible hypothesis to explain this
328 result. If reproductive females experience an increased photosynthetic rate (in regards to
329 males), it could mask any potential detrimental effect of exposure to excess of metals.

330 However, our results on spectral reflectance (PRI) do not support sex-differential
331 photosynthetic light use efficiency. Additionally, the differences in nutritional status between
332 the sexes, particularly nitrogen content, can also be playing a role in shaping the sexual
333 differences in response to metals. For example, optimal nitrogen nutrition has been found to
334 decrease the inhibitory effects of Cd (Pankovic et al., 2000). High levels of heavy metals, and
335 particularly Cd, promote the synthesis of phytochelatins capable of binding metal ions and
336 playing a crucial role in metal-detoxification (Grill et al., 1987; Vatamaniuk et al., 2000).
337 Nutrient status may affect the synthesis of these phytochelatins, because they are peptides and
338 therefore rich in nitrogen (Finkemeier et al., 2003). In dioecious plants, Chen *et al.* (2011)
339 found that sexual differences in Cd sensitivity decreased under N deposition in the tree
340 *Populus yunnanensis*. In *S. latifolia* males had a greater investment in flower production than
341 females, so we can expect greater costs in terms of nitrogen associated with pollen
342 production. Greater costs of reproduction in terms of nitrogen have been reported in other
343 dioecious herbs, see for example *Mercurialis annua* (Harris and Pannell, 2008). Here, our
344 results on spectral reflectance indicate that males tend to have less chlorophyll content than

345 males (measured at the end of the growing season), which could be related to lower nitrogen
346 content (Evans, 1989), but further direct measurements are required to verify this assumption.
347 Males could thus experience a less optimal nitrogen status than females, which in turn would
348 result in greater sensitivity to the same Cd levels (e.g. due to less detoxifying compounds),
349 such as we observe in this study. Sex-specific strategies in response to heavy metals have
350 been found before in dioecious trees (*Populus* spp). Particularly, in *P. yunnanensis*, males
351 synthesized and accumulated greater amount of protective biochemical molecules, showing a
352 stronger defence capacity to respond to an excess of Zn and acid rain than females (Jiang et
353 al., 2013). In *P. cathayana*, variation in protein expression patterns between the sexes appears
354 to be the underlying mechanisms regulating the physiological differences in response to heavy
355 metal stress (Chen et al., 2013). Therefore, further studies at the molecular level could help to
356 elucidate both the magnitude and underlying mechanisms of sex-related differences in
357 response to stress in *S. latifolia*.

358
359 The effects of heavy metals on seed production revealed an interesting pattern. On one hand,
360 females of *S. latifolia* had similar total mass of seeds regardless of the presence or absence of
361 metals in the soil. However, the number of seeds produced was affected by the presence of
362 metals in contrasting ways: the number of seeds decreased with Cu and increased with Cd.
363 Little is known about the effect of heavy metals on seed traits, particularly on how they affect
364 the number of seeds produced. Due to its toxic nature, a reduction in seed production as result
365 of exposure to heavy metals can be expected and has been reported before (Ernst et al., 2008;
366 Malan and Farrant, 1998). However, it is surprising to note that Cd enhanced seed production.
367 Differences in seed production could be attributed to differences in the phenological status of
368 the plants, i.e., different developmental status of flowers and seeds in the different treatments.
369 However, we have ruled out this possibility as no differences in flowering time were found in

370 this experiment. Increased seed production can be considered a strategy that allows the
371 maintenance of a population in adverse conditions, such as heavy metal stress. In support of
372 this argument, Gan *et al.* (2013) found that populations of *Kummerowia stipulacea* have
373 adapted to heavy metal polluted sites by investing more resources in reproduction (both in
374 seed size and seed number) than those growing in non-polluted sites.

375

376 **5. Conclusion**

377 In recent years, there has been an increasing interest on understanding how males and females
378 of dioecious species respond to environmental stress, but results to date depend on the type of
379 stress (e.g., abiotic vs biotic) and on the species without any general pattern, highlighting the
380 need for further studies (Juvany and Munné-Bosch, 2015). Heavy metal toxicity is potentially
381 a major limiting factor in plant growth; however, the study of the responses of dioecious
382 plants to such stress has only begun to emerge and has been focused only on two woody
383 species (*Populus* spp.). Our study provides further evidence of sexual dimorphism in response
384 to heavy metals in *S. latifolia*, a herbaceous plant. However, our results mostly contrast with
385 those found previously in *Populus* spp., highlighting the importance of considering different
386 species in order to draw more clear conclusions on the effects of stress in the sexes of
387 dioecious plants. In short, we identified sexual dimorphism both in terms of accumulation
388 patterns, with females accumulating more Cu in their tissues than males, and in terms of
389 tolerance, with males experiencing greater negative effects in growth and reproductive traits.
390 A greater allocation to reproduction by females did not seem to hamper their ability to better
391 tolerate heavy metals, but rather the opposite. The greater allocation of carbon to developing
392 fruits may cause photosynthetic rate to increase (greater sink-strength for photosynthesis) and
393 mask any potential detrimental effect of exposure to excess of metals. In addition, differences
394 in the nutritional status (particularly in nitrogen content) between the sexes can also be

395 playing a role in explaining the differences in their response to metals. Finally, we observed
396 contrasting differences in female seed production due to heavy metals, with number of seeds
397 (but not mass) decreasing with Cu and increasing with Cd. Little is known on the effect of
398 heavy metals on seed traits, but a reduction in number could be expected due to metal toxicity
399 and therefore we found surprising the increase observed in females from Cd-polluted soil.

400

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406

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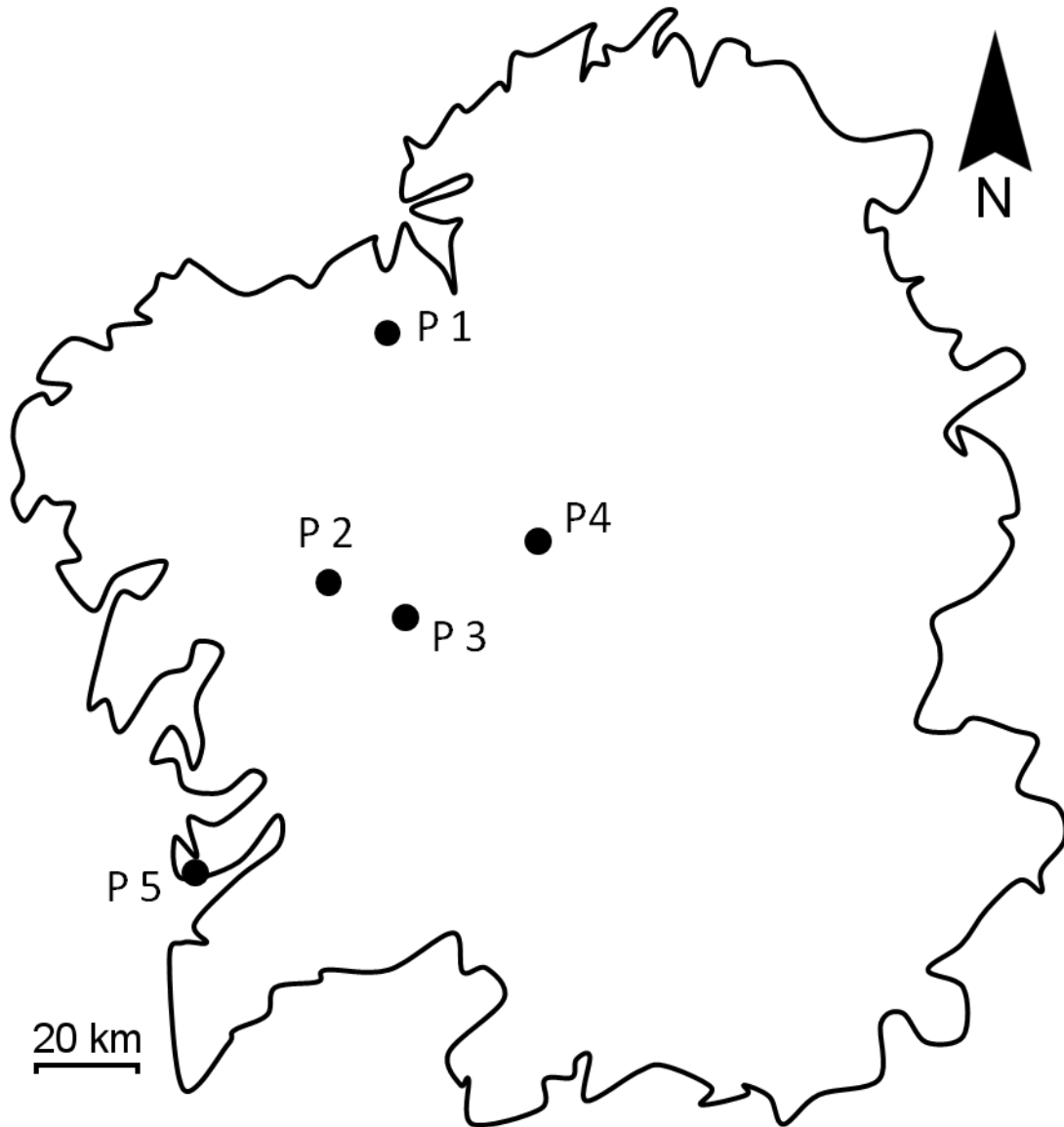
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610 **Figure legends**

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612 **Figure 1.** Map showing the location of the five populations (P1-P5) of *S. latifolia* sampled in

613 Galicia (NW Spain) and used in this study.



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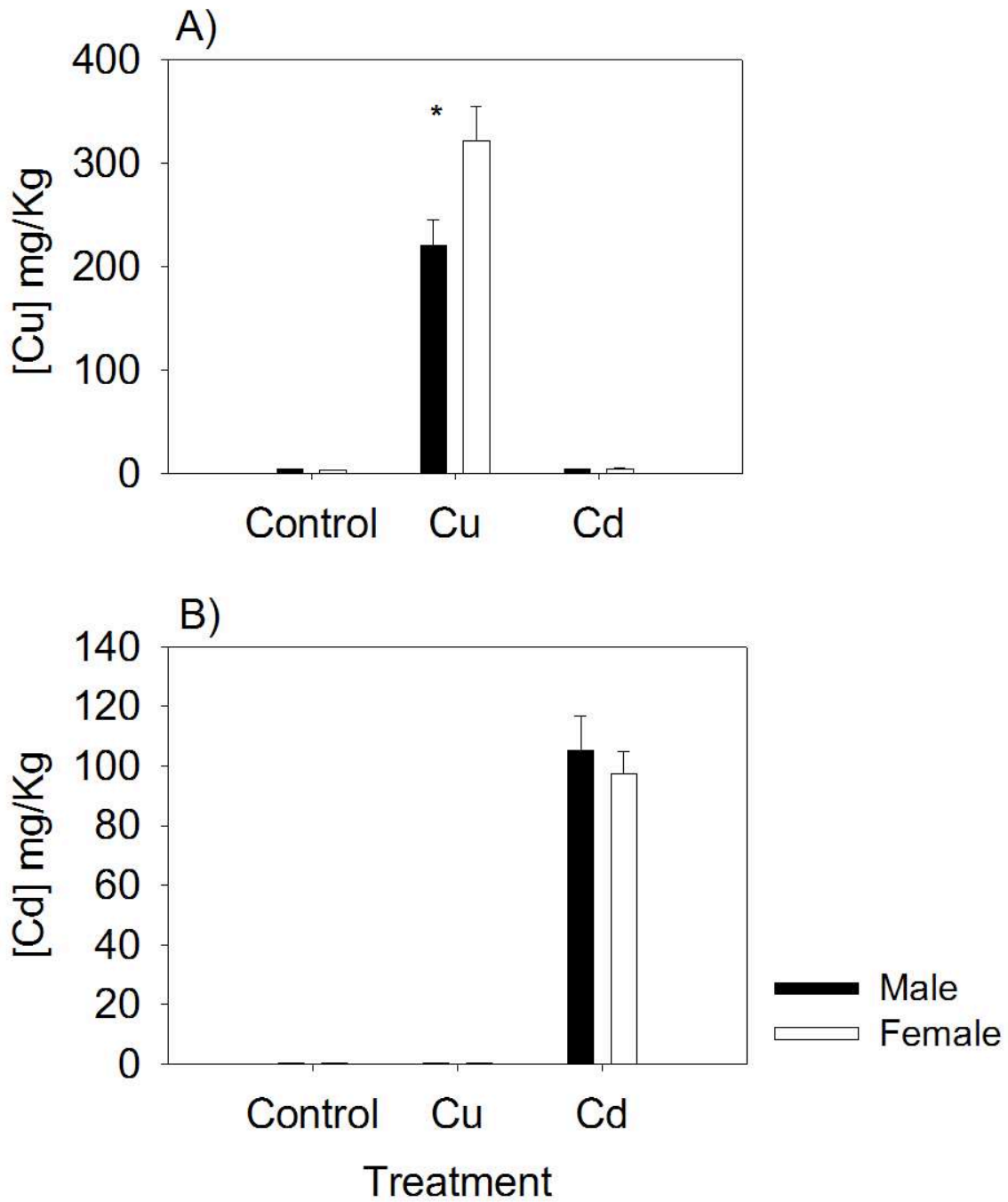
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620 **Figure 2.** Mean values (± 1 S.E.) of (A) Cu concentration and (B) Cd concentration per plant
621 dry mass in males and females (as indicated) of *S. latifolia* growing under different treatments
622 (non-polluted soil (Control), soil polluted with Cu, and soil polluted with Cd; N = 10).
623 Asterisks indicate significant differences ($P < 0.05$) between the sexes within a given
624 treatment.

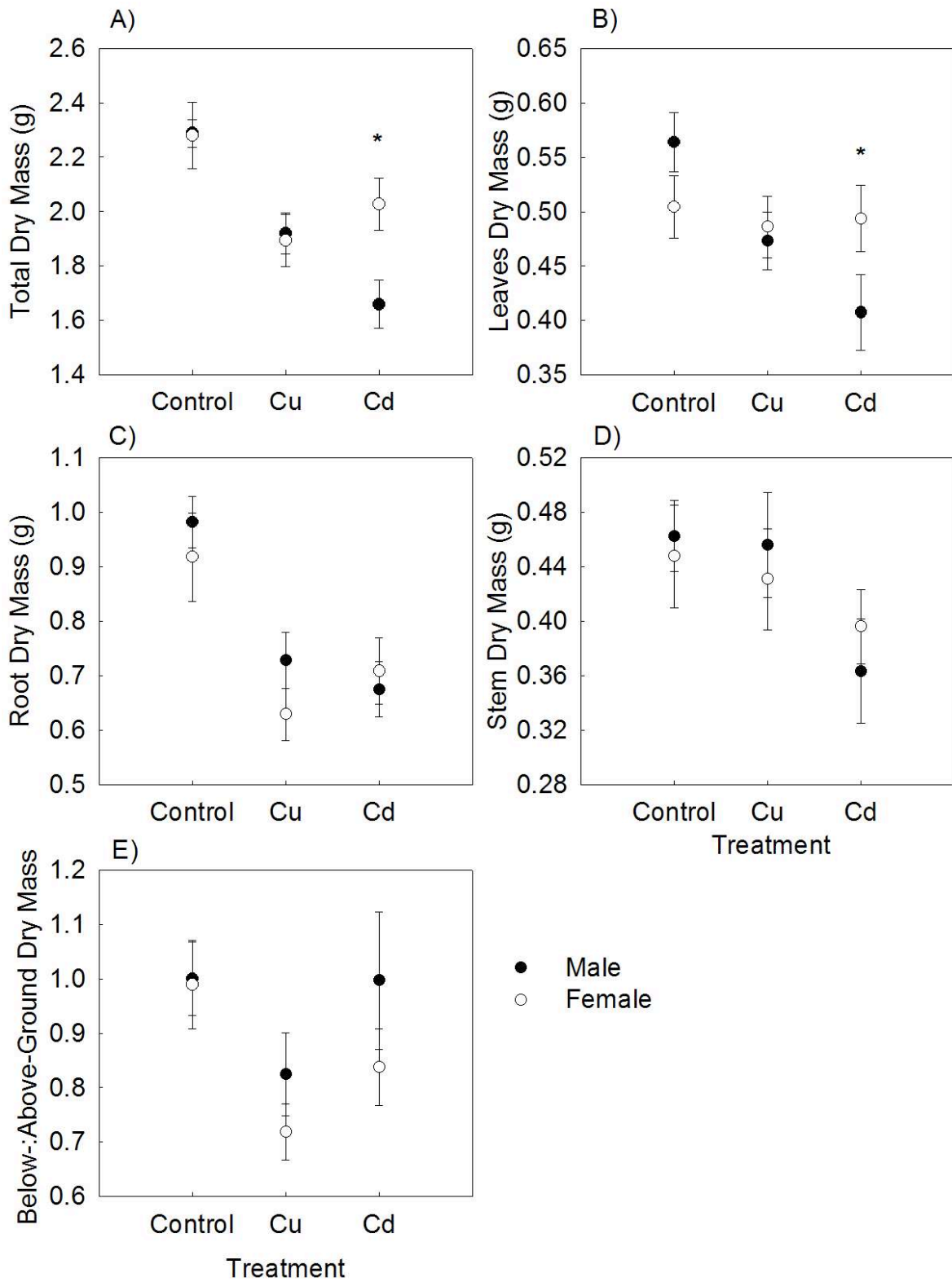


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628 **Figure 3.** Mean values (± 1 S.E.) of (A) total dry mass, (B) leaves dry mass, (C) root dry
629 mass, (D) stem dry mass and (E) below-:above-ground (root:shoot) dry mass for males and
630 females (as indicated) of *S. latifolia* in response to the treatments (Control (non-polluted soil),
631 N = 31 males and 22 females; soil polluted with Cu, N = 21 males and 25 females; and soil
632 polluted with Cd, N = 19 males and 27 females). Asterisks indicate significant differences (P
633 < 0.05) between the sexes within a given treatment.

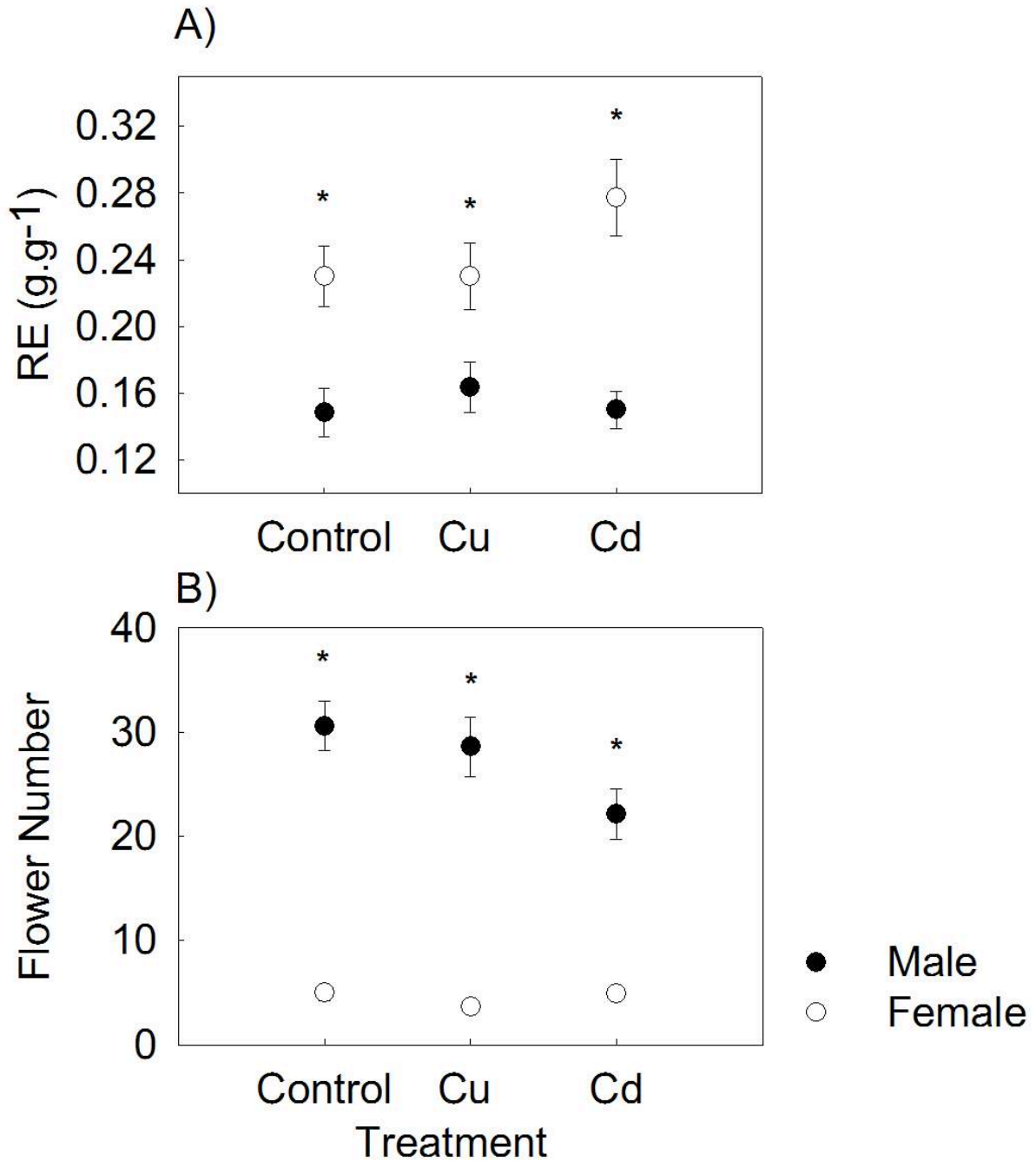


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637 **Figure 4.** Mean values (± 1 S.E.) of (A) reproductive effort (RE) and (B) flower number for
 638 males and females (as indicated) of *S. latifolia* in response to the treatments (Control (non-
 639 polluted soil), soil polluted with Cu, and soil polluted with Cd). N as in Figure 2. Asterisks
 640 indicate significant differences ($P < 0.05$) between the sexes within a given treatment.



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