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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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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.

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

1. Introduction

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

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

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

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

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

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2. Materials and Methods

2.1. Study species

S. latifolia (Caryophyllaceae), the white campion, is a short-lived perennial and dioecious

plant native to most of Europe, Western Asia and Northern Africa. It is commonly found

growing in disturbed or cultivated ground (Tutin et al., 1993) and has also been found

growing in heavily contaminated mine sites in Southern France (Escarré et al., 2010). Sexual

dimorphism in *S. latifolia* has been extensively studied (Bernasconi et al., 2009), with males

having less allocation of biomass to vegetative growth and reproduction, but also producing

more flowers (smaller) than females (see Delph *et al.* 2005, and references therein).

2.2. Experimental design

In summer 2011, seeds were collected from five natural populations of *S. latifolia* growing in

Galicia, Northwest Spain (see map in Fig. 1) by sampling approx. 20 individuals per

population (8-10 capsules per individual). Seeds were separated from capsules, discarding the

damaged ones, and stored at room temperature.

In March 2012, seeds from these five populations were randomly sown in 40-cell germination

trays in a greenhouse at the Faculty of Biology, University of Santiago de Compostela

(Spain). We transplanted a total of 240 seedlings (evenly and randomly chosen across

populations) to individual 1.6 L pots, filled with garden soil, when the second pair of true

leaves started to grow (approx. 2 months after germination). Pots were distributed spatially on

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: $CuSO_4 \cdot 5H_2O$ 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 ºC 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.

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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.

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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 Δph formation and chloroplasts

173 conformational changes; Demmig-Adams, 1990) and R_{570} is reflectance at 570 nm (a

reference wavelength unaffected by these processes). PRI has been shown to be strongly correlated with leaf-scale photosynthetic radiation-use efficiency (mol CO2 mol^{-1} photons) (Filella et al., 1996; Gamon et al., 1997; Peñuelas et al., 2011, 1995). (2) CHL, the chlorophyll content index, was calculated as R_{750}/R_{700} and is correlated to the chlorophyll content of leaves (Lichtenthaler et al., 1996).

2.6. Metal content in plant tissues

At the end of the experiment, 10 dried plant samples (roots plus stems and leaves) randomly selected for each combination of sex and metal treatment were homogenized and ground (<120 μm) using an ultra-centrifugal mill Retsch ZM 100 (Retsh GmbH, Haan, Germany). When necessary, samples included more than a single individual to achieve enough sample size to determine Cu and Cd. A representative sample of 0.5 g was digested with 8 ml of NO$_3$H and 2ml of H$_2$O$_2$ in Teflon bombs in a microwave oven and subsequently diluted in deionized water to a final volume of 25 ml. Quality control of the digestion process was carried out by parallel analysis of three samples of certified reference materials (NIST MR apple leaves 1515). Concentration of Cu and Cd were then determined by means of inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x). Each analysis was carried out three times and all samples were run in batches that included blanks. Final concentrations in plant tissues were expressed referring to dry weight.

2.7. Data analysis

Analyses of growth and biomass allocation (including reproductive effort and total dry mass 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 × 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 ×

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 × treatment: L-ratio = 1.99, d.f. = 2, $P = 0.370$, Fig. 3C; SDM, sex ×

246   treatment: L-ratio = 0.826, d.f. = 2, P = 0.661, Fig. 3C; Root:shoot, sex × 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 × 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 × 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 × treatment: χ^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: χ^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 ± S.E.): 0.21 ±

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 ± S.E. = 57 ± 0.96) was not

266   affected by sex, treatment or the interaction (sex: χ^2_1= 0.842, P = 0.359; treatment: χ^2_2 =

267   0.695, P = 0.706; sex × treatment: χ^2_2 = 2.25, P = 0.325).

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269   *3.4. Leaf spectral reflectance*

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

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

4. Discussion

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

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

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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

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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 synthetized 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 photsynthesis) 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.

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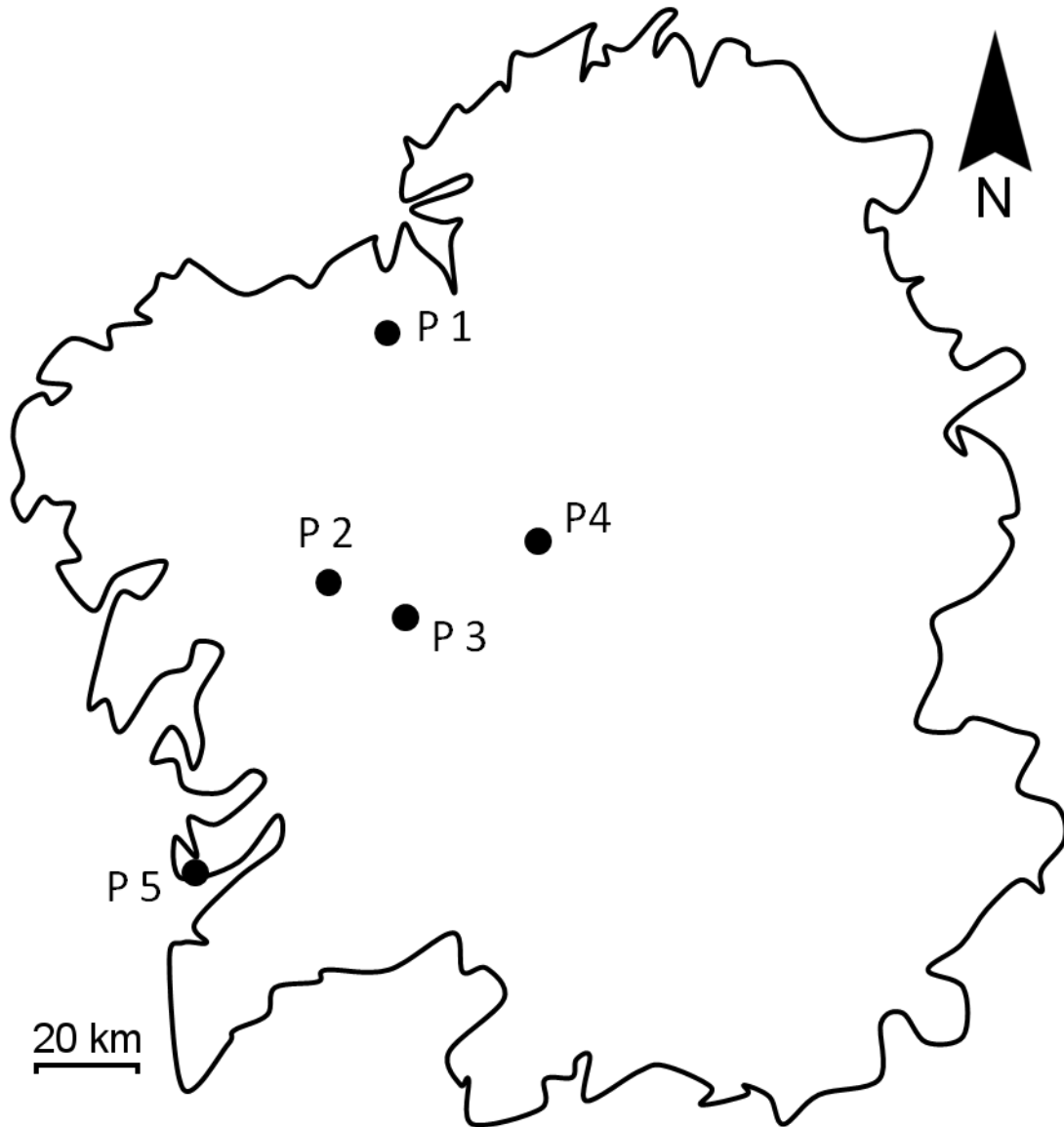606   69–87.

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Figure legends

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

Galicia (NW Spain) and used in this study.

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
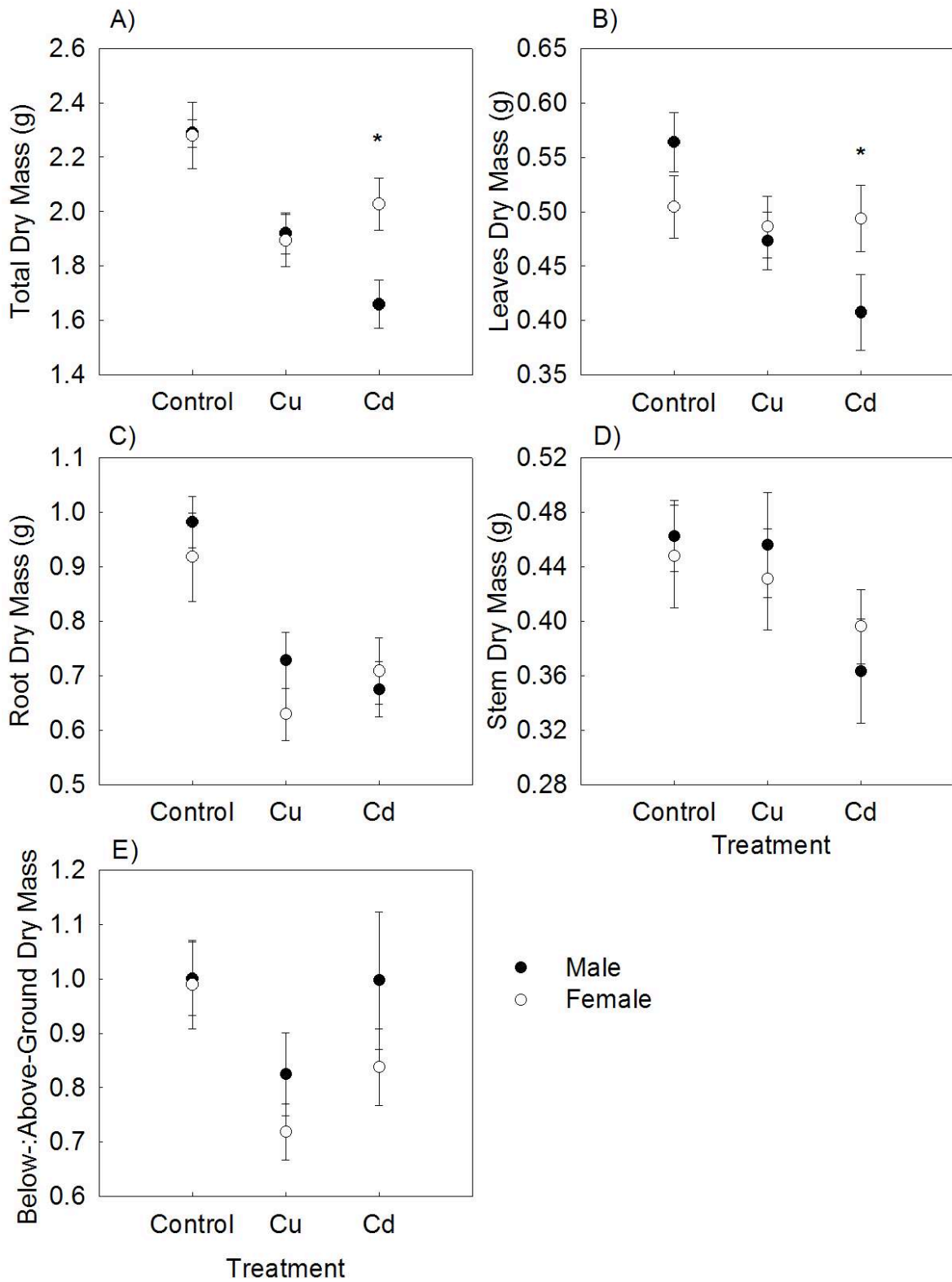
624   treatment.
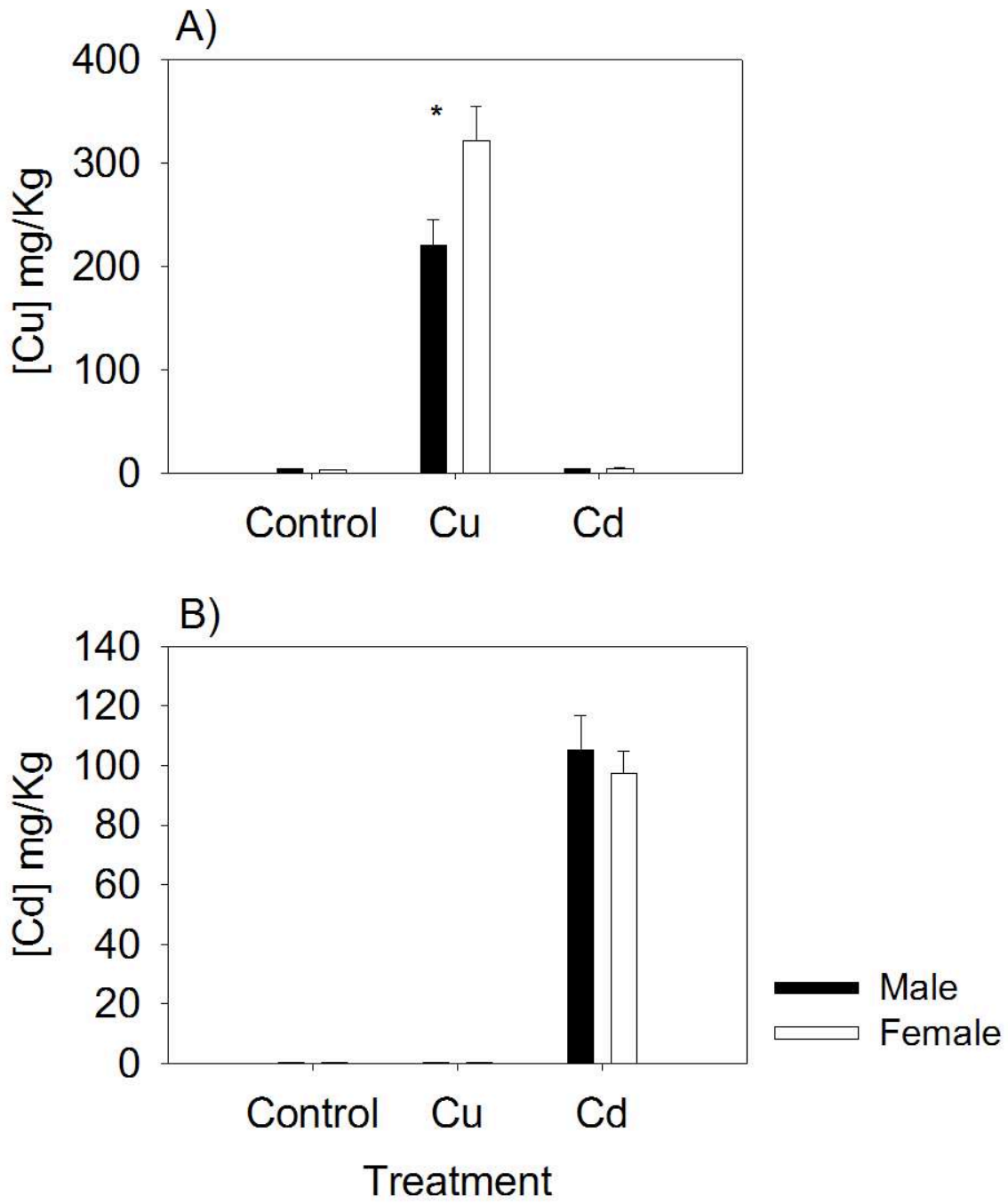
628 **Figure 3.** Mean values (± 1 S.E.) of (A) total dry mass, (B) leaves dry mass, (C) root dry

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

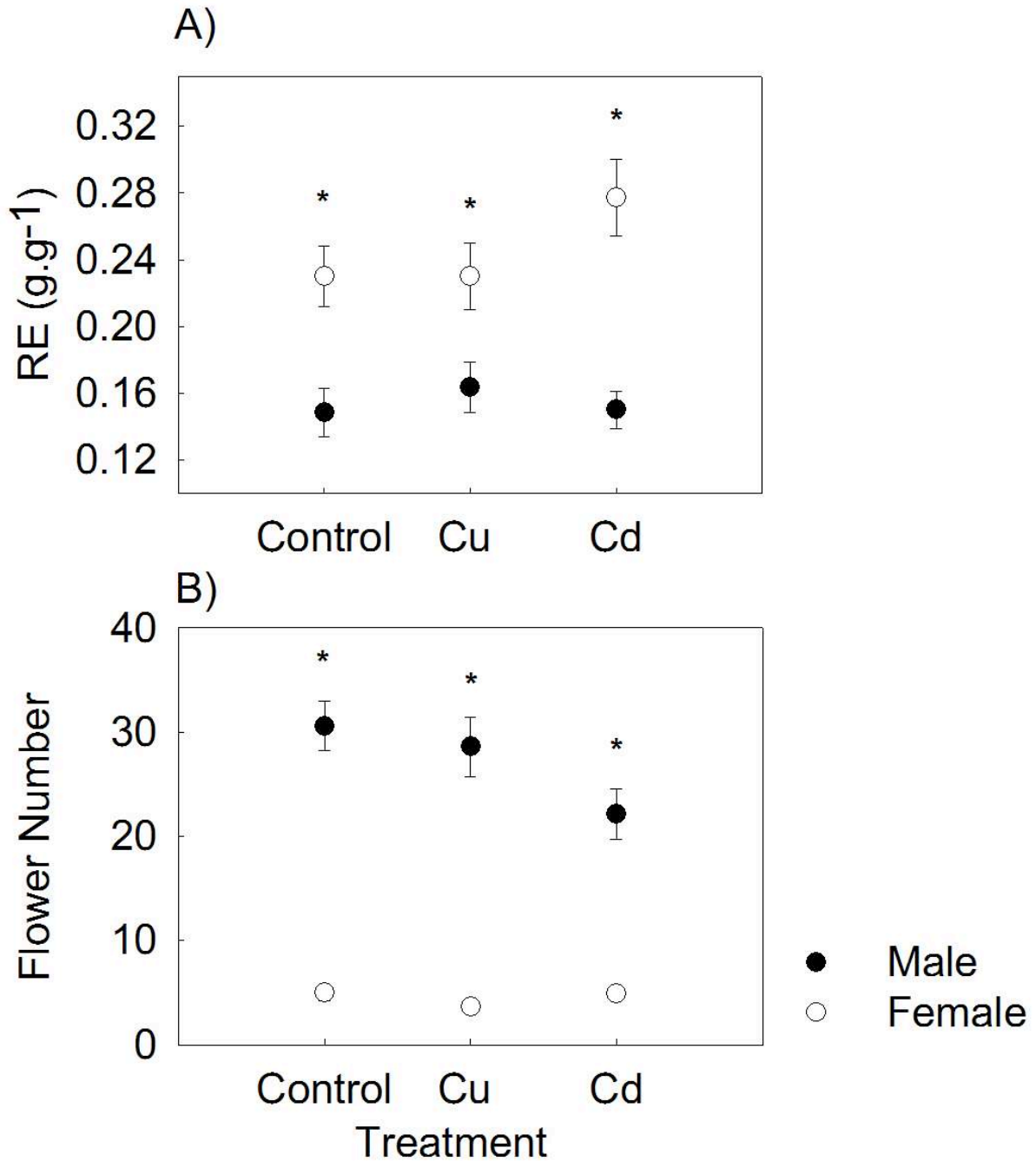633 < 0.05) between the sexes within a given treatment.

Figure 4. Mean values (± 1 S.E.) of (A) reproductive effort (RE) and (B) flower number for

males and females (as indicated) of *S. latifolia* in response to the treatments (Control (non-

polluted soil), soil polluted with Cu, and soil polluted with Cd). N as in Figure 2. Asterisks

indicate significant differences ($P < 0.05$) between the sexes within a given treatment.