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

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Short title: Sexual dimorphism in response to heavy metal pollution

Abstract

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

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.

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 effects. One week after transplanting, seedlings were randomly assigned to one of three treatments: Control (no metal), Cadmium contaminated soil (10 ppm Cd) and Copper contaminated soil (125 ppm Cu); 80 seedlings per treatment. Cu was added as sulphate (copper sulphate 5-hydrate: $CuSO_4 \cdot 5H_2O$ Panreac, Barcelona, Spain) and Cd was added as chloride (cadmium chloride anhydrous: $CdCl_2$, Merck, Munich, Germany) in a unique dose at the start of the experiment. Copper is an essential micronutrient for plant growth; higher levels than 100 ppm are often toxic (Ross and Kaye, 1994) affecting key processes such as photosynthesis, respiration or nitrogen fixation (Fernandes and Henriques, 1991; Maksymiec, 1997). Cadmium is a non-essential element that can be highly phytotoxic inhibiting photosynthesis (Baszynki et al., 1980), transpiration (Bazzaz et al., 1974) and metabolic activities (Moya et al., 1993; Sharma et al., 1998). Cd concentrations in European non-polluted soils range from 0.06 to 0.50 ppm depending on the nature of the parent material (Jensen and Bro-Rasmussen, 1992). Seedlings of *S. latifolia* were assigned to the experimental treatments before the sex was known, which allowed to expose the plants to the effects of metal contamination well before reproduction started. The final number of males and females for which traits were measured depended on the number of individuals that set flower and fruits and these can be found in legends to figures 2, 3 and 4. Both ends of the greenhouse were kept open over the duration of the experiment to allow local pollinators access to the flowers.

2.3. Growth and biomass allocation

After 14 weeks of growth under experimental conditions, we harvested all the plants that had flowered (and therefore sex could be identified) and dissected them into reproductive organs, roots, stems and leaves. At this time, plants were not showing any signs of being pot-bound, and upon harvest, it was observed that some of the soil volume still remained unoccupied by roots. Plant tissues were then oven dried at 40 ºC to constant weight and their dry mass recorded. Roots, stems and leaves were cleaned and washed in distilled water for 15 min with shaking before drying.

2.4. Reproduction

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

2.5 Leaf spectral reflectance

At the end of July 2012, we measured spectral reflectance on a new fully formed and healthy leaf that had developed during the experiment for each plant, using a portable spectrometer (UniSpec Spectral Analysis System, PP Systems, Haverhill, MA). Reflectance spectra (wavelengths 306-1136) were calculated as the spectral radiance of the leaf divided by the radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere, North Sutton, NH). The following spectral reflectance indices were calculated: (1) PRI, the photochemical reflectance index, was calculated as $(R_{539} - R_{570})/(R_{539} + R_{570})$, where R_{539} is reflectance at 539 nm (affected by processes of dissipation of excess radiation as the epoxidation state of the xanthophylls, thylakoid Δph formation and chloroplasts 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_3H and 2ml of H_2O_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 models using the R ‘lme’ function from the ‘nlme’ package (Pinheiro et al., 2010). Analyses of number of flowers, number of seeds and flowering time were fitted using a generalized linear mixed model (GLMM) with poisson errors and log link function by means of the ‘glmer’ function in the lme4 package (Bates et al., 2011). In these models, metal treatment and sex were fitted as fixed factors and population was fitted as random ‘blocking’ effect to account for population-to-population variation. *P*-values were determined by comparing models with and without the factor being tested. Model comparison was performed by means of likelihood ratio tests by calling the function ‘anova’ to compare models that, for fixed effects, were fitted using maximum likelihood (Crawley, 2007; Pinheiro and Bates, 2000). Little explained variance was associated with the random effect of population, which was non-significant for most variables (except for stem dry mass).

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

3. Results

3.1. Metal content in plant tissues

The presence of the metal in the soil significantly increased the content of the same metal in the plant tissues (treatment: Cu: $F_{2,57} = 1549$, $P < 0.0001$; Cd $F_{2,57} = 1836$, $P < 0.0001$). Females accumulated more Cu than males (sex: $F_{1,57} = 12.92$, $P < 0.001$; Fig. 2A), but no differences between the sexes were found for Cd (sex: $F_{1,57} = 0.156$, $P = 0.694$; Fig. 2B).

3.2. Growth and biomass allocation

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

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

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

treatment: L-ratio = 0.826, d.f. = 2, P = 0.661, Fig. 3C; Root:shoot, sex × treatment: L-ratio = 0.581, d.f. = 2, *P* = 0.748, Fig. 3E).

3.3. Reproduction

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

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

3.4. Leaf spectral reflectance

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

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

The excess of Cu in the soil inhibited growth similarly in both sexes, although females accumulate more of this metal in their tissues. In addition, the presence of Cd in the soil had a greater detrimental effect – reducing growth and investment in reproduction- in males than in females, despite no differences in accumulation of Cd in their tissues. As mentioned above, females of *S. latifolia* allocate more of their resources to reproduction (seed production) than males, and therefore we could expect greater trade-offs between growth and reproduction in females than males, particularly under stressful conditions. However, no trade-offs (or even the opposite pattern) have been found in our study, indicating a greater tolerance to metals in females than in males. Interestingly, in the dioecious tree *Populus yunnanensis* the opposite (and expected) pattern is found: males accumulate in their tissues and tolerate greater levels of Cu and Cd than females (Chen et al., 2011; L. Chen et al., 2013). In *S. latifolia*, the greater sink-strength of females, as pointed above, could be one plausible hypothesis to explain this result. If reproductive females experience an increased photosynthetic rate (in regards to males), it could mask any potential detrimental effect of exposure to excess of metals. However, our results on spectral reflectance (PRI) do not support sex-differential photosynthetic light use efficiency. Additionally, the differences in nutritional status between the sexes, particularly nitrogen content, can also be playing a role in shaping the sexual differences in response to metals. For example, optimal nitrogen nutrition has been found to decrease the inhibitory effects of Cd (Pankovic et al., 2000). High levels of heavy metals, and particularly Cd, promote the synthesis of phytochelatins capable of binding metal ions and playing a crucial role in metal-detoxification (Grill et al., 1987; Vatamaniuk et al., 2000). Nutrient status may affect the synthesis of these phytochelatins, because they are peptides and therefore rich in nitrogen (Finkemeier et al., 2003). In dioecious plants, Chen *et al.* (2011) found that sexual differences in Cd sensitivity decreased under N deposition in the tree *Populus yunnanensis*. In *S. latifolia* males had a greater investment in flower production than females, so we can expect greater costs in terms of nitrogen associated with pollen production. Greater costs of reproduction in terms of nitrogen have been reported in other dioecious herbs, see for example *Mercurialis annua* (Harris and Pannell, 2008). Here, our results on spectral reflectance indicate that males tend to have less chlorophyll content than males (measured at the end of the growing season), which could be related to lower nitrogen content (Evans, 1989), but further direct measurements are required to verify this assumption. Males could thus experience a less optimal nitrogen status than females, which in turn would result in greater sensitivity to the same Cd levels (e.g. due to less detoxifying compounds), such as we observe in this study. Sex-specific strategies in response to heavy metals have been found before in dioecious trees (*Populus* spp). Particularly, in *P. yunnanensis*, males synthetized and accumulated greater amount of protective biochemical molecules, showing a stronger defence capacity to respond to an excess of Zn and acid rain than females (Jiang et al., 2013). In *P. cathayana*, variation in protein expression patterns between the sexes appears to be the underlying mechanisms regulating the physiological differences in response to heavy metal stress (Chen et al., 2013). Therefore, further studies at the molecular level could help to elucidate both the magnitude and underlying mechanisms of sex-related differences in response to stress in *S. latifolia*.

The effects of heavy metals on seed production revealed an interesting pattern. On one hand, females of *S. latifolia* had similar total mass of seeds regardless of the presence or absence of metals in the soil. However, the number of seeds produced was affected by the presence of metals in contrasting ways: the number of seeds decreased with Cu and increased with Cd. Little is known about the effect of heavy metals on seed traits, particularly on how they affect the number of seeds produced. Due to its toxic nature, a reduction in seed production as result of exposure to heavy metals can be expected and has been reported before (Ernst et al., 2008; Malan and Farrant, 1998). However, it is surprising to note that Cd enhanced seed production. Differences in seed production could be attributed to differences in the phenological status of the plants, i.e., different developmental status of flowers and seeds in the different treatments. However, we have ruled out this possibility as no differences in flowering time were found in this experiment. Increased seed production can be considered a strategy that allows the maintenance of a population in adverse conditions, such as heavy metal stress. In support of this argument, Gan *et al.* (2013) found that populations of *Kummerowia stipulacea* have adapted to heavy metal polluted sites by investing more resources in reproduction (both in seed size and seed number) than those growing in non-polluted sites.

5. Conclusion

In recent years, there has been an increasing interest on understanding how males and females of dioecious species respond to environmental stress, but results to date depend on the type of stress (e.g., abiotic vs biotic) and on the species without any general pattern, highlighting the need for further studies (Juvany and Munné-Bosch, 2015). Heavy metal toxicity is potentially a major limiting factor in plant growth; however, the study of the responses of dioecious plants to such stress has only begun to emerge and has been focused only on two woody species (*Populus* spp.). Our study provides further evidence of sexual dimorphism in response to heavy metals in *S. latifolia*, a herbaceous plant. However, our results mostly contrast with those found previously in *Populus* spp., highlighting the importance of considering different species in order to draw more clear conclusions on the effects of stress in the sexes of dioecious plants. In short, we identified sexual dimorphism both in terms of accumulation patterns, with females accumulating more Cu in their tissues than males, and in terms of tolerance, with males experiencing greater negative effects in growth and reproductive traits. A greater allocation to reproduction by females did not seem to hamper their ability to better tolerate heavy metals, but rather the opposite. The greater allocation of carbon to developing fruits may cause photosynthetic rate to increase (greater sink-strength for photsynthesis) and mask any potential detrimental effect of exposure to excess of metals. In addition, differences in the nutritional status (particularly in nitrogen content) between the sexes can also be playing a role in explaining the differences in their response to metals. Finally, we observed contrasting differences in female seed production due to heavy metals, with number of seeds (but not mass) decreasing with Cu and increasing with Cd. Little is known on the effect of heavy metals on seed traits, but a reduction in number could be expected due to metal toxicity and therefore we found surprising the increase observed in females from Cd-polluted soil.

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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.

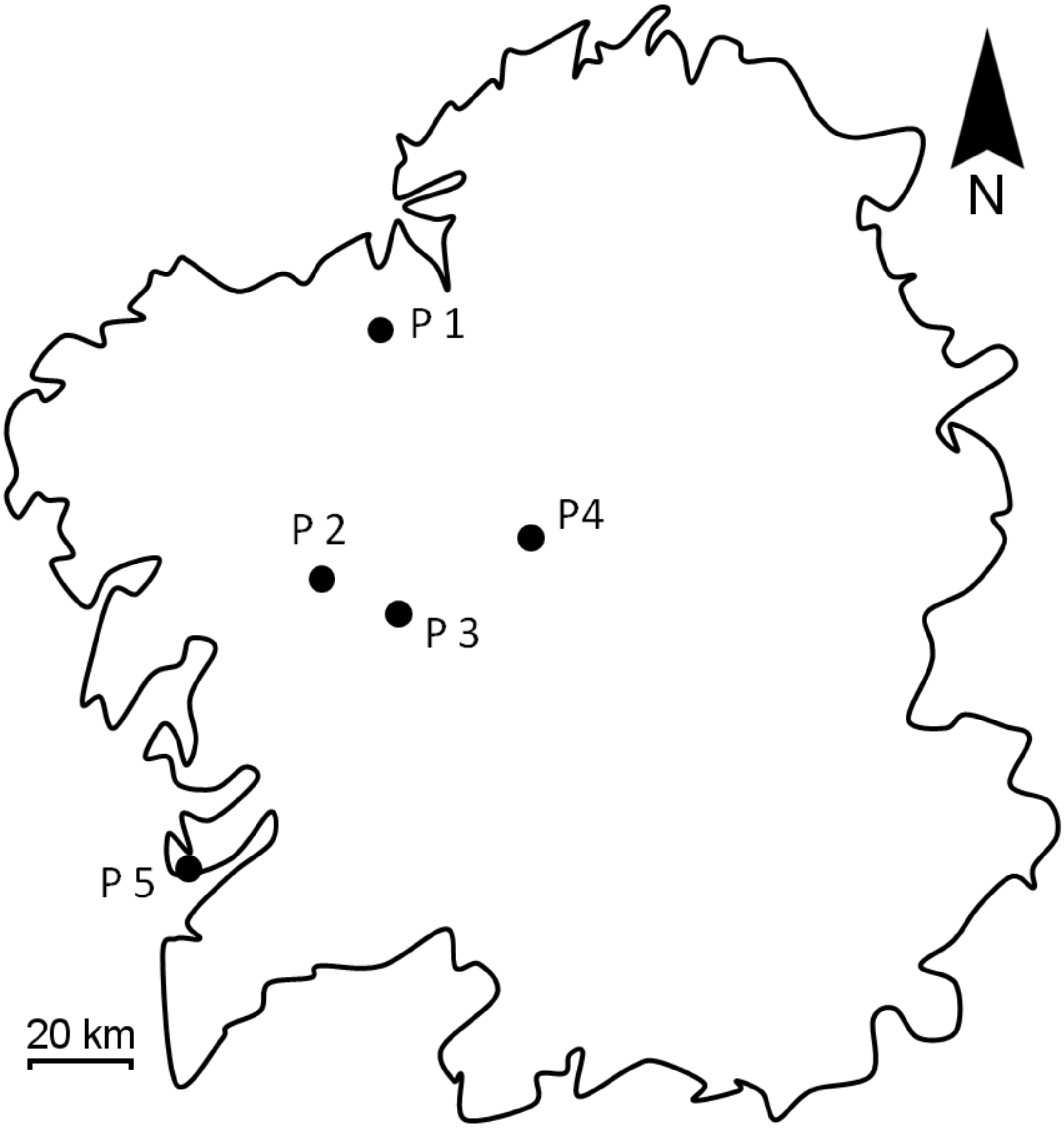

Figure 2. Mean values (± 1 S.E.) of (A) Cu concentration and (B) Cd concentration per plant dry mass in males and females (as indicated) of *S. latifolia* growing under different treatments (non-polluted soil (Control), soil polluted with Cu, and soil polluted with Cd; N = 10). Asterisks indicate significant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

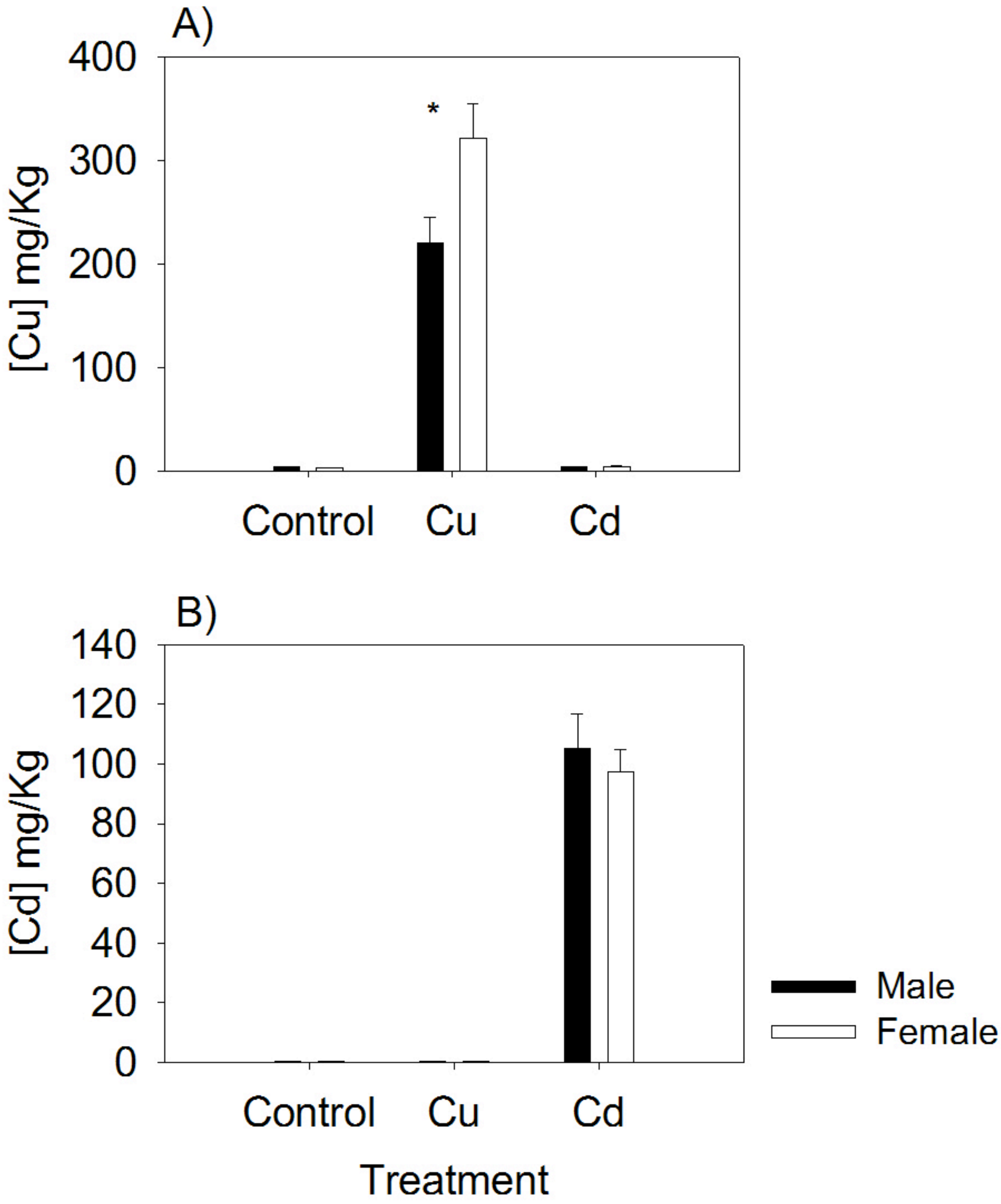
A)
400
300
200
100
0
[Cu] mg/Kg
*
Control
Cu
Cd
B)
140
120
100
80
60
40
20
0
[Cd] mg/Kg
Control
Cu
Cd
Treatment
Male
Female

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

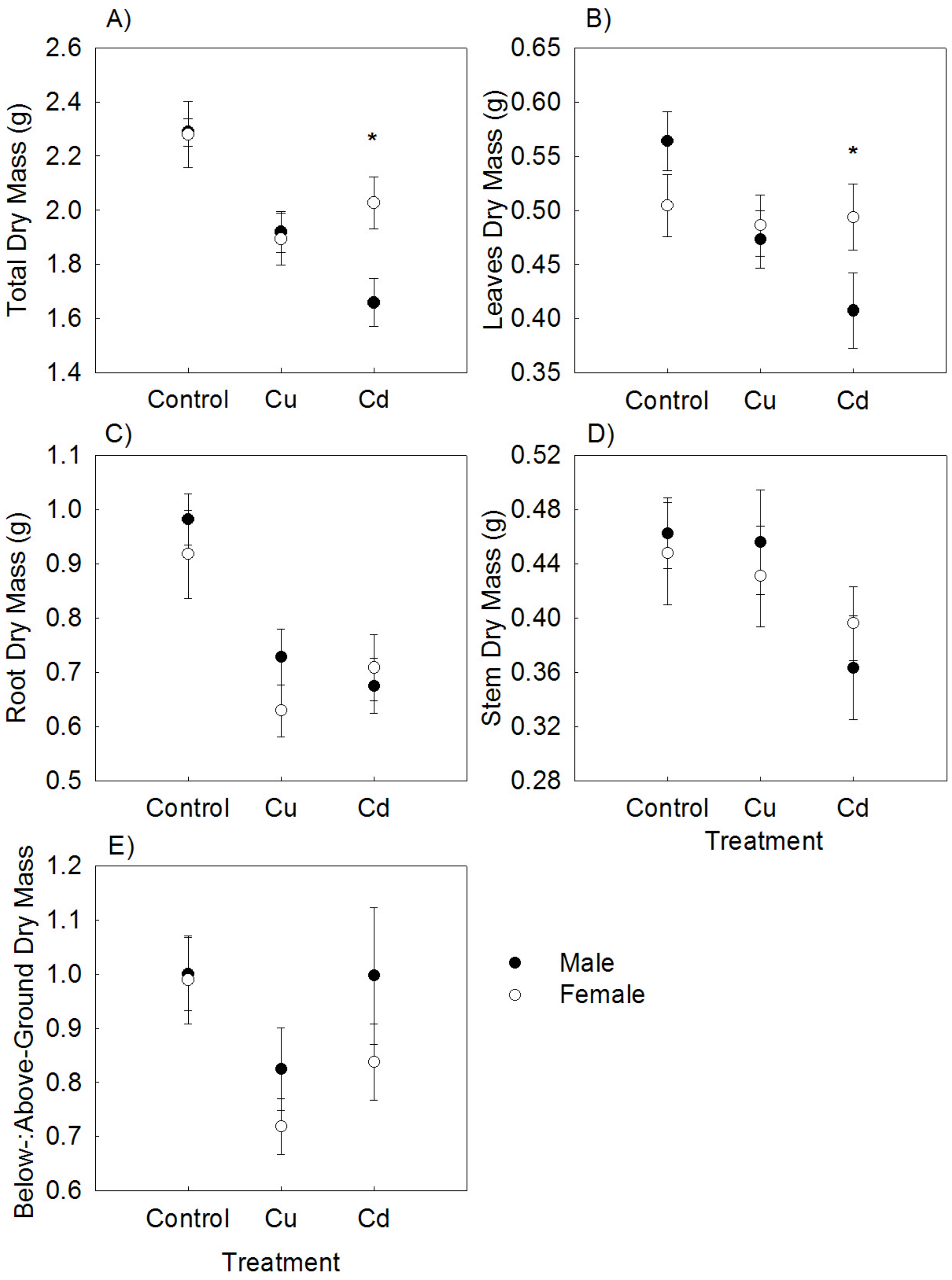
A)
Total Dry Mass (g)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
*
Control
Cu
Cd
B)
Leaves Dry Mass (g)
0.65
0.60
0.55
0.50
0.45
0.40
0.35
*
Control
Cu
Cd
C)
Root Dry Mass (g)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Control
Cu
Cd
D)
Stem Dry Mass (g)
0.52
0.48
0.44
0.40
0.36
0.32
0.28
Control
Cu
Cd
Treatment
E)
Below-:Above-Ground Dry Mass
1.2
1.1
1.0
0.9
0.8
0.7
0.6
Control
Cu
Cd
Treatment
Male
Female

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.