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RESEARCH ARTICLE

Sexual dimorphism at different life stages: early life sexual differences in root growth in *Silene latifolia*

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ABSTRACT

- Male and female dioecious plants often show sexual dimorphism, differing in morphological, physiological and life-history traits. Most previous studies have focused on differences between males and females during or after reproduction, paying little attention to the pre-reproductive stages of the individuals.
- Here we assessed the response of male and female individuals of the dioecious plant *Silene latifolia* to abiotic stress at different life stages, including pre-reproductive (i.e. seedlings and young plants) and reproductive individuals. We measured growth, resource allocation and discrimination against ^{13}C under nutrient deficiency, water stress, as well as their interaction.
- We observed sexual dimorphism in root growth, with female seedlings having longer main roots than male plants. Pre-reproductive male and female plants also responded differently, in terms of root allocation, to nutrient and water availability. At reproduction, females grew more roots than males when water was not limiting. These differences could help explain the female-skewed sex ratios found in natural populations of *S. latifolia*. We found no evidence of sexual dimorphism in aboveground dry mass, although females had longer leaves than males at the seedling stage.
- We conclude that sexual dimorphism in *S. latifolia* may occur not as a consequence of reproduction, but well before it.

INTRODUCTION

Dioecy, i.e., separate male and female individuals, is a rare reproductive system in plants occurring in only 6–7% of all angiosperms (Renner & Ricklefs 2008). However, dioecy has evolved multiple times and is widespread, being represented in almost half of all angiosperm families (Barrett 2013). Male and female plants of dioecious species usually differ not only in "primary" traits (i.e. reproductive organs) but also in "secondary" traits, such as size, morphology, physiology or life history (Barrett & Hough 2013). This is known as sexual dimorphism and has been commonly attributed to the different costs of reproduction associated with male and female functions, particularly, as a result of trade-offs between allocation to reproduction and growth and/or defence (Obeso 2002). Given that plants have access to a limited amount of resources, the allocation of resources to one function (e.g. reproduction) can constrain the allocation to other functions (e.g., growth and defence) (Obeso 2002). In particular, it has been reported that females commonly have a higher investment in nutrients for reproduction at the expense of vegetative growth, and therefore females usually show lower productivity than males (Gross & Soule 1981; Ågren 1988; Korpelainen 1992; Cipollini & Whigham 1994). Sexual dimorphism may also reflect different strategies for the acquisition of limiting resources associated with reproduction (Sánchez & Pannell 2010, 2011). Males demand high amounts of nitrogen during pollen production that may be met by increasing allocation to roots, whilst the demands of fruiting in females might be met by increasing the capacity for carbon fixation above ground (Sánchez & Pannell 2011).

Sexual dimorphism in dioecious species can not be only the result of the cost of reproduction but also reflect innate differences between male and female plants (Nicotra 1999). Such innate differences are predicted to evolve in response to sexual selection or selection for ecological divergence (Meagher 1984). To date, most studies on sexual dimorphism have focused on reproductive individuals, where it is impossible to disentangle the costs of reproduction from any innate differences. However, there is little empirical evidence to demonstrate sex-related innate differences due to the difficulty in identifying the sex before flowering (but see Nicotra 1999; Zluvova *et al*. 2010). The few studies that have looked at sexual dimorphism in pre-reproductive individuals show that differences can occur at the seed/seedling stage and before any investment in flower and/or fruit production. For example, in *Spinacia oleracea* and *Rumex nivalis* male seeds are heavier and germinate earlier than female seeds (Freeman *et al*. 1994; Stehlik & Barrett 2005). In the tropical shrub *Siparuna grandiflora*, pre-reproductive females grow faster than males (Nicotra 1999). In *Silene latifolia*, genetic correlations between sexes could cause coupled evolutionary responses of males and females (Steven *et al.* 2007; Delph *et al.* 2010), and sex-differential gene expression has already been detected at the seedling stage (Zluvova *et al.* 2010).

In general, sex-related differences in vegetative traits appear to be accentuated under stressful environmental conditions (Medina-Gavilán *et al.* 2008; Retuerto *et al.* 2018; Yu *et al.* 2023). It has been postulated that different physiological mechanisms may give males and females different tolerances to environmental stress, although empirical evidence is limited (Juvany & Munné-Bosch 2015; Retuerto *et al.* 2018). For example, females often present compensatory mechanisms to sustain a higher investment in reproduction (Case & Ashman 2005; Díaz-Barradas *et al.* 2014), such as higher photosynthesis rates through increasing stomatal conductance, thus implying extra costs and making them more dependent on available water resources (Dawson & Ehleringer 1993). As a result, females may perform worse than males under certain abiotic stresses, such as water deficit or nutrient deficit, as commonly found in the literature (Liu *et al.* 2021). However, exceptions exist and there is not yet a clear pattern in the responses of the sexes to environmental stress (reviewed by Obeso 2002), and their differences in tolerance seem to vary depending on the species considered and type of stress, demanding a wider range of studies (Juvany & Munné-Bosch 2015; Retuerto *et al.* 2018). Most studies to date have focused on the response of the sexes to single stress factors, but little is known about the sex-specific physiological and ecological responses of dioecious species to multiple interacting stresses.

In this study, we explore the sexual dimorphism in pre-reproductive and reproductive individuals in response to multiple interacting stresses using the dioecious plant *Silene latifolia* (Caryophyllaceae) as study species. *S. latifolia* is a model species in the study of sexual dimorphism (Delph 2007), with females generally allocating more to reproduction and to total biomass than males (Gross & Soule 1981; Doust *et al.* 1987; Gehring & Linhart 1993; Lyons *et al.* 1994; Delph & Meagher 1995) and having better water-use efficiency (Gehring & Monson 1994; Delph 2019); whereas males have higher photosynthesis rates (Gehring & Monson 1994; Laporte & Delph 1996; Delph *et al.* 2005) and, frequently, have larger specific leaf areas (Delph *et al.* 2002, 2005). *S. latifolia* has sex chromosomes, with heterogametic males (XY) and homogametic females (XX), and the sex can be genetically identified using molecular tools (Desfeux *et al.* 1996; Negrutiu *et al.* 2001; Charlesworth 2002; Filatov 2005; Ming *et al.* 2011; Hewett *et al.* 2024), making it an ideal system to study sexual dimorphism in pre-reproductive individuals. In addition, previous studies indicate that sex-related innate differences may occur in this species (e.g. Zluvova *et al.* 2010).

The specific objectives of our study were to: (i) investigate the existence of sexual dimorphism at different life stages, from pre-reproductive (i.e. seedlings and young plants) to reproductive individuals, in *Silene latifolia*; and (ii) examine whether the degree of sexual dimorphism is influenced by environmental factors. In particular, the response of male and female plants to different resource availability (nutrients and water) was examined by measuring growth, biomass and resource (C and N) allocation and their physiological performance (carbon isotope discrimination). In addition, (iii) to achieve these aims, sex-linked markers were used to identify the sex of seedlings and non-flowering individuals.

MATERIAL AND METHODS

Study species

Silene latifolia Poir. (Caryophyllaceae), the white campion, is a short-lived dioecious perennial plant. It has a tap root system, characterized by a primary/main root with lateral roots branching out from it. It is commonly found growing in disturbed or cultivated ground and is native in most of Europe, Western Asia and North Africa (Tutin *et al.* 1993). *S. latifolia* is a broadly studied dioecious species and not only varies in primary sexual traits but also secondary traits, such as flower number, flower size, transpiration and photosynthesis, biomass allocation and leaf size, among others (Delph 2007).

Experimental design

To assess the magnitude of sexual dimorphism in *S. latifolia* at different life stages we established two different complementary experiments in the Plant Growth Facilities of Cardiff University (UK, Talybont, 51°30′03.0″ N 3°12′05.6″ W). At the seedling stage, we established a glasshouse experiment (experiment I) to study sex-specific patterns of root growth in response to different nutrient levels (with and without added fertilizer). At the young and adult stages, we established a glasshouse experiment (experiment II) to study differences between the sexes in growth, morphological and physiological traits in response to water and nutrient availability.

Experiment I: Sexual dimorphism in seedlings

A seed batch containing 250 seeds of *S. latifolia* was purchased from Chiltern Seeds (Wallingford, UK) and sown into 100-ml pots (1 seed pot^{-1}) filled with Westland™ Horticulture sand on 15 May 2018. Seedlings started to germinate 10 days after sowing, with most germination on the 27 and 28 May 2018. On 31 May we chose 114 seedlings that germinated between these two dates and randomly assigned them to two different nutrient levels: low nutrients (LN) and high nutrients (HN). Seedlings allocated to low nutrients were watered with tapwater every 4 days, whilst those allocated to high nutrients were watered at the same time with a solution of 0.5 g·l^{-1} Phostrogen All Purpose Plant Food (16:10:24 NPK; Bayer CropScience, Cambridge, UK). On 11 June 2018, i.e. when seedlings were approximately 2 weeks old, we harvested all the seedlings; two died in the LN treatment, so n = 57 for HN, and n = 55 for LN. Roots were washed and scanned using an EPSON perfection V700 Pro scanner set at a 600-dpi resolution. Primary and lateral root growth and root area were measured using the program RootNav® (Pound *et al.* 2013) following the protocol at 23.622047 pixels·mm^{-1}. The aboveground parts of each experimental seedling, consisting of two cotyledons, were used for sex identification using molecular markers (Hewett *et al.* 2024), before measuring their length (leaf length hereinafter). Briefly, two primer pairs which target length polymorphisms between the X- and Y-linked copy of the spermidine synthase gene were used to amplify sections of this gene, and fragment length profiles on agarose gels enabled us to determine sex from plant material even at pre-flowering stages (Hewett *et al.* 2024).

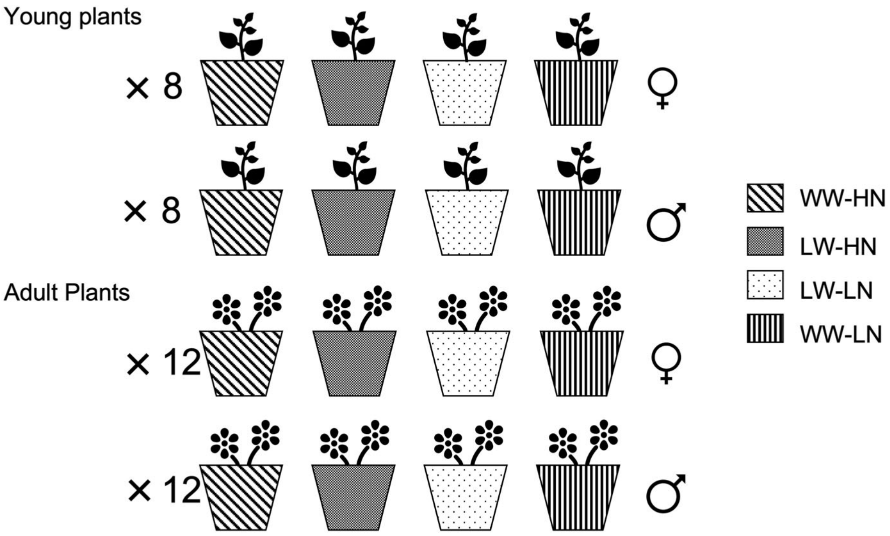

Fig. 1. Schematic representation of experimental design for Experiment II. Plants were grown in different combinations of nutrients and water availability: well-watered and high nutrients (WW-HN), low-watered and high nutrients (LW-HN), low-watered and low nutrients (LW-LN); well-watered and low nutrients (WW-LN). n = 8 replicates per condition were harvested in young plants (before flowering) and n = 12 in adult plants (when flowering).

Experiment II: Sexual dimorphism in young and adult individuals

Seeds (approx. 280) of *S. latifolia* purchased from Chiltern Seeds were sown in germination trays filled with 1:2 Verve™ Multipurpose compost (UK) and Westland™ Horticulture sand (UK) on 27 January 2017. Two weeks later, 200 germinated seedlings were transplanted into 0.3-l pots filled with the same mix of compost and sand as above. Plants were randomly allocated to the following experimental conditions: low water and low nutrients (LW-LN); well- watered and low nutrients (WW-LN); low water and high nutrients (LW-HN) and well-watered and high nutrients (WW-HN). No fertilizer was applied to the low nutrient treatment, while an organic fertilizer (Westland Fish, Blood and Bone, 4% N, 7% P_2O_5, 4% K_2O) was applied at a rate of 3.9 $g \cdot l^{-1}$ soil to the high nutrient treatment. Note that for simplicity, we used the terms 'low nutrients' and 'high nutrients' to compare the two treatments with distinct nutrient levels, therefore these are relative (lower and higher nutrients) and not absolute indicators of nutrient content. Plants were allowed to establish by watering *ad libitum* for a further 2 weeks before imposing different levels of water availability. Well-watered plants continued to be watered *ad libitum* with tapwater to field capacity; plants under water restriction were watered to field capacity only when showing signs of wilting. Throughout the whole experiment, plants in the low water treatment received ~50% of the water received by plants without water stress.

Approximately 1 month after imposing the experimental treatments, a pre-reproductive harvest was carried out by randomly taking a sample of non-flowering plants across all the different treatments for growth and physiological measurements; at this point, it was not possible to identify sex before harvest, so leaf samples from 85 randomly chosen plants from all treatments were sexed using molecular markers (Hewett *et al.* 2024). Among these 85 plants, sex was biased, and we harvested 8 male and 8 female plants from each water and nutrient combination, making a total of 64 non-flowering plants (hereinafter 'young plants'; see Fig. 1).

One month later, a second harvest (reproductive harvest) was carried out on reproductive individuals (hereinafter 'adult plants') when most individuals were flowering. A total of 96 flowering plants (12 female and 12 male per condition) were randomly chosen (Fig. 1) and their growth and biomass allocation measured. Leaf nitrogen (N) and carbon (C) content, as well as carbon isotopic discrimination were also assessed for a subsample of these individuals (see below).

Growth and biomass allocation

At harvest, plants were separated into above- and belowground organs. For adult plants, the aboveground dry mass was split into vegetative dry mass and reproductive dry mass (flowers and fruits, when present). Plant organs were oven-dried at 70 °C then weighed on a precision balance. The root:shoot ratio was calculated as belowground dry mass divided by the aboveground vegetative dry mass. Reproductive Effort (RE) was calculated as the ratio of the reproductive dry mass to aboveground vegetative dry mass.

Content of N and C, and carbon isotope discrimination

Leaf samples were taken from healthy fully expanded leaves of individuals growing under the experimental treatments (n = 5; except n = 6 for young males growing in LW-HN and adult females growing in WW-HN; n = 4 for adult males and adult females growing in LW-HN). Leaf samples were oven-dried at 70 °C and subsequently ground using an ultra-centrifuge mill (TissueLyser II; Qiagen, USA). The concentrations of total C and N, the C/N ratio, and $^{13}C/^{12}C$ ratios were determined in subsamples (two per sample of ca. 1 mg dry weight per plant) using an isotope ratio mass spectrometer (Thermo Delta V Advantage IRMS; Thermo Fisher Scientific, Bremen, Germany) at Cardiff University. The standards used for C and N were an in-house analytical grade caffeine and commercial gelatine calibrated via IAEA-CH6 and IAEA-600. Leaf isotope values ($\delta^{13}C$) were calibrated against a standard, the Vienna Pee Dee Belemnite calcium carbonate (VPDB). $\delta^{13}C$ values (‰) were calculated as $\delta^{13}C = ([R_{sam} / R_{std}]-1)$, where R is $^{13}C/^{12}C$ ratio in the plant sample (R_{sam}) and standard (R_{std}), respectively. $^{13}C/^{12}C$ discrimination ($\Delta^{13}C$) was calculated as: $\Delta^{13}C = (\delta^{13}C_{air} - (\delta^{13}C_{plant})/(1 + [\delta^{13}C_{plant}/1000])$. We assumed a $\delta^{13}C$ value for atmospheric CO_2 of 7.8‰ (Farquhar *et al.* 1989).

Carbon isotope composition ($\delta^{13}C$), commonly expressed as discrimination ($\Delta^{13}C$) against a source (i.e. atmospheric) CO_2, has been used in plant research as a long-term integrator of ecophysiological processes such as leaf conductance, photosynthetic capacity and water-use efficiency (WUE) (Farquhar *et al.* 1989; Ehleringer 1993). WUE is negatively correlated with discrimination against ^{13}C ($\Delta^{13}C$), meaning that plants with better efficiency in use of water will discriminate less against ^{13}C. In C_3 plants, $\Delta^{13}C$ values are around ~15‰ and 25‰ for plants with high and low WUE, respectively (Dawson *et al.* 2002).

Data analysis

All analyses were conducted in R v. 4.2.2 (R Core Team 2022). Differences between sex, nutrient and water availability, and their interaction (added as fixed factors) were evaluated using linear models by means of the 'lm' function. *P*-values were calculated with the 'ANOVA' function from the 'car' package, using type III sum of squares. If interactions between factors were significant, Tukey's post-hoc tests were then carried out to determine differences between groups ($P < 0.05$) using the 'lsmeans' package (Lenth 2016). Shapiro–Wilk test for normality of residuals was performed using the 'shapiro.test' function, while Levene's test was carried out to test for homogeneity of variances among treatments using the 'leveneTest' function from the 'car' package (Fox 2009). To meet the assumptions of the ANOVA, the following variables were transformed: leaf length of seedlings was square-root transformed, length of the main root of seedlings was $\log_{10}$ transformed, belowground dry mass and root:shoot ratio of young individuals were square-root transformed and $\log_{10}$ transformed, respectively, aboveground dry mass, belowground dry mass, reproductive dry mass, and RE of adult individuals were also square-root transformed, and the root:shoot ratio of adult individuals was $\log_{10}$ transformed.

RESULTS

Experiment I: Root growth in seedlings

The total root length of *S. latifolia* was not affected by sex or availability of nutrients in the soil (Table 1, Fig. 2a). However, length of the main root was significantly affected by both sex and nutrients (Table 1). In particular, females had longer main roots than males; and seedlings growing without added nutrients also had longer main roots, regardless of sex (Fig. 2b). Finally, leaf length was also significantly affected by sex and nutrients (Table 1): females had longer leaves than males, and the addition of fertilizer significantly increased the leaf length (Fig. 2c).

Table 1. Results of the ANOVA for total root length, length of the main root, and the leaf length (average of longest length of the two largest leaves per seedling) of seedlings of different sexes (male, female) of *Silene latifolia* exposed to different nutrient availability.

source of variance		total root length		length of the main root		leaf length	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Sex	1	3.84	0.053	6.149	**0.032**	4.151	**0.044**
Nutrients	1	1.24	0.267	4.784	**0.006**	26.929	**<0.001**
Sex × nutrients	1	1.54	0.217	0.755	0.160	0.317	0.575
Error	108						

Significant differences ($P < 0.05$) are shown in bold. n = 27 for males in LN and HN conditions, n = 30 for females in HN conditions, and n = 28 for females in LN conditions.

Experiment II: Sexual dimorphism in young and adult individuals

Growth and biomass allocation

Young plants. Young male and female plants had similar aboveground dry mass (Table 2). Both water and nutrient availability significantly affected aboveground dry mass of young individuals of *S. latifolia* (Table 2). In particular, plants growing under high nutrients grew more than those growing under low nutrients (HN (mean ± SE) = 3.26 ± 0.18 g, LN = 2.52 ± 0.11 g). Similarly, plants growing under well-watered conditions had a larger dry mass than those with limited water (WW = 3.16 ± 0.19 g, LW = 2.63 ± 0.12 g).

There were differences between the sexes depending on water and nutrient treatment, as indicated by a significant third-order interaction between Sex × Nutrients × Water in belowground dry mass and the root:shoot ratio (Table 2). Females had higher belowground dry mass than males when growing under low nutrients and well-watered conditions (Fig. 3a; WW-LN); however, the post-hoc test was not significant (Tukey test, $P = 0.157$). Differences in root:shoot ratio appeared when comparing the most extreme conditions: females growing under WW-HN had a significantly lower allocation to roots than males growing under LW-LN conditions (Fig. 3b).

Adult plants. Adult male and female plants did not differ in aboveground dry mass (Table 3). Nutrient availability did not significantly affect the adult aboveground dry mass (Table 3); however, plants that were well-watered during the experiment had larger aboveground dry mass than those with restricted watering (Table 3; WW = 5.26 ± 0.34 g, LW = 3.51 ± 0.18 g).

Belowground dry mass was not affected by nutrient availability; however, there were differences between the sexes in the response to water availability (Table 3). In particular, well-watered females had the largest belowground dry mass (Fig. 3c).

The root:shoot ratio was significantly affected by nutrient availability, regardless of sex or water treatment (Table 3), with plants growing with low nutrients having a larger allocation to roots than those growing with high nutrients (Fig. 3d).

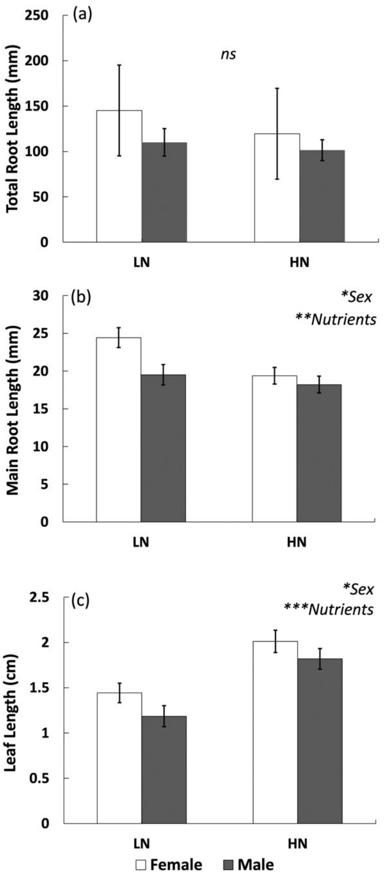

Fig. 2. Total root length (a), length of main root (b), and leaf length (c) of male and female seedlings of *S. latifolia* growing under two different levels of nutrient availability: low (LN) and high (HN). n = 27 for males in LN and HN conditions, n = 30 for females in HN conditions, and n = 28 for females in LN conditions. Bars represent mean ± SE. For each plot, significant effects are indicated with asterisks (ns = non-significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$).

There were no differences in reproductive dry mass of males and females, or in responses to the experimental treatments (Table 3). However, the sexes did allocate resources differently to reproduction (RE) depending on nutrient availability (Table 3), with females growing with low nutrients showing greater reproductive effort than those growing with high nutrients (Fig. 4a). The reproductive effort was also affected by water availability, but this effect depended on the level of nutrients (Table 3), with those plants growing in the less stressful conditions (well-watered and high nutrients) having the lowest reproductive effort (Fig. 4b).

Content of N and C, and carbon isotope discrimination

Young plants. Nitrogen content and C/N ratio in leaves of young male and female plants were significantly affected by nutrients and water (Table 4; Sex × Nutrients × Water; Figure S1a,b). However, the post-hoc test was not significant (Tukey test, $P > 0.05$) and did not identify differences among the groups.

The %C in leaves of young plants was not significantly affected by any of the experimental treatments or their interactions (Table 4).

Values of Δ^{13}C for young male and female plants were not significantly affected by sex, water, nutrients or any of their interactions (Table 4, Figure S1c).

Adult plants. The %N in leaves of adult plants was significantly affected by water availability (Table 4): well-watered plants had a lower N content than plants with restricted water (Figure S1d). Neither sex nor nutrients affected the %N of adult plants (Figure S1d).

The %C and C/N ratio in adult plants was not affected by sex, water, nutrients or any of their interactions (Table 4, Figure S1e).

Adult plant Δ^{13}C was significantly affected by nutrient and by water availability (Table 4, Figure S1f); well-watered plants and those with no added fertilizer had higher Δ^{13}C than water-restricted plants (WW = 21.42 ± 0.31, LW = 20.61 ± 0.23) or those that received fertilizer (HN = 20.51 ± 0.30, LN = 21.55 ± 0.25). Sex did not affect the values of Δ^{13}C in reproductive individuals (Table 4).

DISCUSSION

Growth and biomass allocation

The results of this study show, for the first time, that male and female plants of *S. latifolia* differ in their root growth already before initiation of flowering. We here document such differences to be present at the seedling stage, with females having longer main roots than males. This finding could have implications for the female-biased sex ratios found in natural populations of *S. latifolia* (Carroll & Mulcahy 1993). Unlike other organisms that usually maintain a balanced sex ratio (1:1), in some dioecious populations, including *S. latifolia*, sex ratio biases (both female- and male-biased) are common (Field *et al.* 2013). Sex-linked mortality as a consequence of both genetic and ecological differences between sexes has been invoked as a possible cause to explain this bias (Eppley 2001). The differences seen here in root growth could contribute to generating ecological differences between the sexes. As such, if each sex uses the resources from the soil differently this could potentially lead to differences in competitive ability, particularly in resource-limited environments (Sánchez & Pannell 2010). Larger root growth in females could also help to explain the faster rate of emergence of female than male plants in *S. latifolia* after germination (Purrington & Schmitt 1995, 1998). Emergence time can have a strong effect on survivorship and reproduction, therefore this could influence the establishment of male and female plants and ultimately the sex ratio of the population (Purrington & Schmitt 1998). Later, young male and female plants also showed different allocation to roots in response to the interaction of nutrient and water availability. Males growing under more limited resources (low nutrient and water-restricted) had higher allocation to roots than females growing with high nutrients and well-watered. Differences, therefore, occurred when comparing two different environmental conditions (i.e., sexes had similar responses to growing in a particular environment). Interestingly, we also found sex-specific responses to water availability at reproduction, with

Table 2. Results of the ANOVA for aboveground dry mass, belowground dry mass and root:shoot ratio for pre-reproductive individuals of different sexes (male, female) of *Silene latifolia* exposed to different nutrient and water availability.

	aboveground dry mass			belowground dry mass		root:shoot	
source of variance	df	*F*	*P*	*F*	*P*	*F*	*P*
Sex	1	1.213	0.275	0.786	0.379	0.133	0.717
Nutrients	1	7.028	**0.010**	1.738	0.193	8.776	**0.004**
Water	1	13.72	**<0.001**	0.0006	0.980	6.232	**0.015**
Sex × nutrients	1	0.609	0.438	0.710	0.403	1.375	0.246
Sex × water	1	3.302	0.074	2.424	0.125	0.467	0.497
Nutrients × water	1	2.139	0.149	0.613	0.437	0.140	0.710
Sex × nutrients × water	1	0.548	0.462	4.378	**0.041**	5.536	**0.022**
Error	56						

Significant differences ($P < 0.05$) are shown in bold. n = 8.

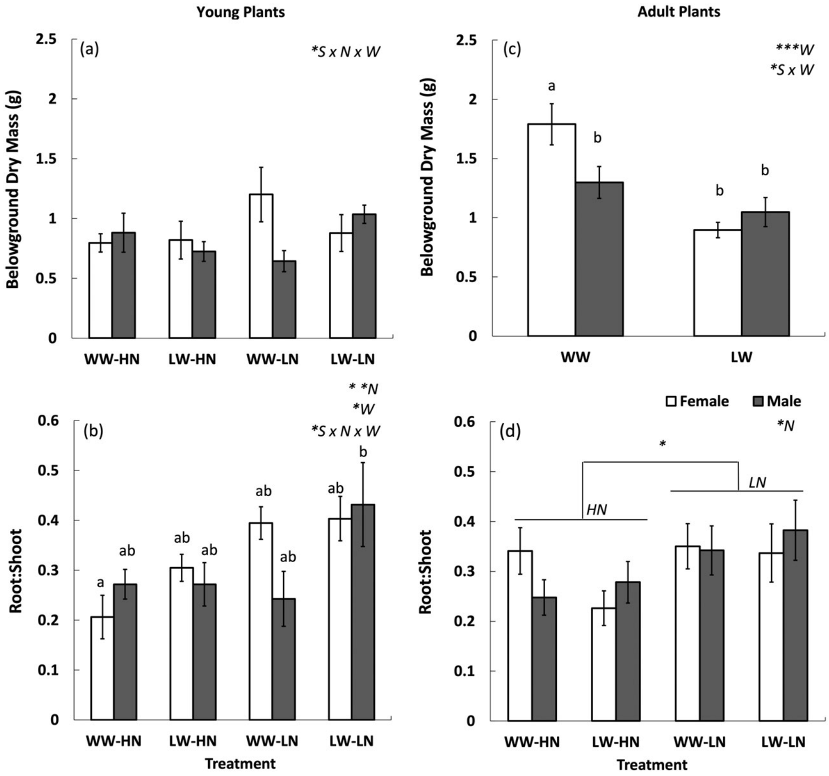

Fig. 3. Belowground dry mass and root:shoot ratio of male and female young (a and b, respectively) and adult (c and d, respectively) plants of *S. latifolia* growing under different levels of nutrient availability: low (LN) and high (HN) and water: well-watered (WW) and low-watered (LW). n = 8 for young plants, and n = 12 for adult plants, except for (c) n = 24. Bars represent means ± SE. For each plot, letters and asterisks indicate the significant effects (S = Sex, N = Nutrients, W = Water; $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Different letters indicate significant differences ($P \leq 0.05$) among groups based on Tukey HSD (for (a) post-hoc tests fell short of significance – see text for details).

adult females having larger belowground dry mass than adult males when growing in well-watered conditions. These sex-specific responses to environmental factors could also have implications in explaining skewed sex ratios, particularly in heterogeneous or fluctuating environments (Retuerto *et al*. 2018).

Differences in root growth early in life were consistent with differences found aboveground: seedlings with longer main roots also had longer leaves. Females of *S. latifolia* are usually larger (in terms of biomass) than males (Gehring 1993; Delph & Meagher 1995). Such sexual size dimorphism in *S. latifolia* has been attributed to different compensatory mechanisms to overcome the costs of reproduction (Gehring & Delph 2006). In particular, females of *S. latifolia* may compensate for the usually higher cost of reproduction (in terms of C) by increasing the size of the organ that takes up C (i.e. leaves) (Gehring & Delph 2006). In addition, the larger size of females has also been attributed to a better use of resources for reproduction

Table 3. Results of the ANOVA for aboveground dry mass, belowground dry mass, root:shoot ratio, reproductive dry mass and reproductive effort (RE) for adult individuals of different sexes (male, female) of *Silene latifolia* exposed to different nutrient and water availability.

		aboveground dry mass		belowground dry mass		root:shoot		reproductive dry mass		RE	
source of variance	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sex	1	1.927	0.168	1.63	0.205	0.008	0.929	1.482	0.227	0.716	0.400
Nutrients	1	3.345	0.071	0.33	0.564	6.627	**0.012**	0.509	0.478	4.897	**0.029**
Water	1	19.596	**<0.0001**	19.32	**<0.0001**	0.277	0.600	0.746	0.390	6.233	**0.014**
Sex × nutrients	1	1.353	0.248	2.81	0.097	0.271	0.604	0.719	0.399	4.759	**0.032**
Sex × water	1	0.073	0.788	5.15	**0.026**	2.802	0.098	0.116	0.734	0.0001	0.994
Nutrients × water	1	0.0041	0.949	0.65	0.423	0.635	0.428	3.769	0.055	5.373	**0.023**
Sex × nutrients × water	1	0.0063	0.937	1.01	0.318	0.718	0.399	0.005	0.944	0.456	0.501
Error	88										

Significant differences ($P < 0.05$) are shown in bold. n = 12.

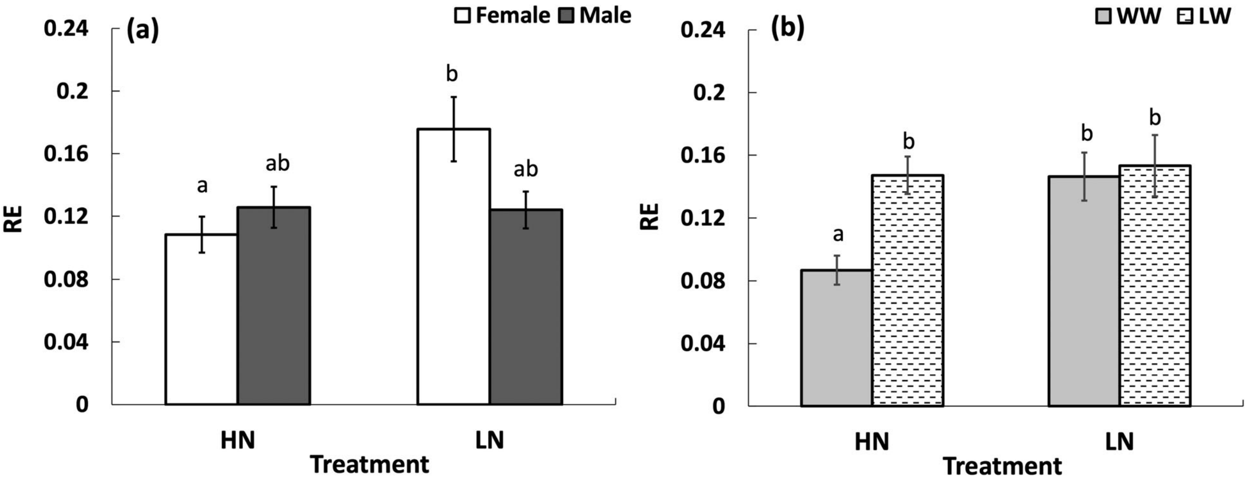

Fig. 4. (a) Reproductive Effort (RE) of male and female plants of *S. latifolia* growing under different levels of nutrient availability: low (LN) and high (HN); (b) Reproductive Effort (RE) of plants of *S. latifolia* growing under different levels of nutrient availability: low (LN) and high (HN) and water availability: well-watered (WW) and low-watered (LW). n = 24. Bars represent mean ± SE. Different letters above bars indicate significant differences ($P \leq 0.05$) among treatments based on Tukey HSD.

(Gehring & Linhart 1993). One mechanism through which female plants could have a more efficient reallocation to reproduction and, thus, not constraining allocation to e.g. growth, could be via photosynthesis by the calyx of immature fruits (Bazzaz *et al.* 1979; Bazzaz & Carlson 1979; Garrido *et al.* 2023). In our study, however, we did not find differences in aboveground dry mass between males and females at later life stages (young and adult plants). Previous studies have found that differences between the sexes in aboveground dry mass occurred during fruit maturation only (Gehring 1993). Interestingly, this study failed to show sexual dimorphism in reproductive effort. Higher reproductive effort in females than in males of *S. latifolia*, as well as in many other dioecious species, is expected because they produce not only flowers but also seeds and fruits (Obeso 2002; Delph 2007). Note, however, that some exceptions exist, and in wind-pollinated plants, males can match or exceed the reproductive expenditure of females (Delph *et al.* 1993; Harris & Pannell 2008; Barrett & Hough 2013). The lack of differences in allocation to reproduction in this study could be a consequence of a low overall investment in reproduction; the experiment was undertaken in an indoor glasshouse, and therefore pollination and seed production were rare due to the absence of pollinators. There were, however, differences in how the sexes responded to experimental treatments in their allocation to reproduction. In particular, females decreased allocation to reproduction when growing with high nutrients (compared to allocation in low nutrient conditionw). Overall, plants, regardless of sex, growing under less stressful conditions, i.e. well-watered and high nutrients, had the lowest RE. This may appear counterintuitive, as one would expect that abundant resources would lead to increased investment in reproduction (Obeso 2002). However, it may also be a consequence of plants growing larger (as suggested by our data) under the most favourable conditions – well-watered and with high nutrients. It is possible that our experimental treatments influenced plant development and flowering so that at the time of the reproductive harvest, individuals in the different environments were at distinct stages of their life cycle (Sherrard & Maherali 2006; Franks *et al.* 2007; Bernal *et al.* 2011; Franks 2011). The presence of our

Table 4. Results of the ANOVA for nitrogen and carbon content (%N and %C), carbon/nitrogen ratio (C/N ratio), and discrimination against ^{13}C (Δ^{13}C) for young and adult female and male plants of *Silene latifolia* exposed to different nutrient and water regimes.

	young plants									adult plants								
	%N			%C		C/N ratio		Δ^{13}C		%N			%C		C/N ratio		Δ^{13}C	
source of variation	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sex	1	0.234	0.632	2.944	0.096	0.280	0.600	0.471	0.497	1	0.125	0.726	0.006	0.940	0.029	0.865	0.242	0.626
Nutrients	1	1.295	0.263	0.009	0.924	2.183	0.149	3.055	0.090	1	1.895	0.179	0.011	0.917	1.414	0.243	8.214	**0.007**
Water	1	0.422	0.520	2.825	0.102	0.533	0.471	0.231	0.634	1	5.153	**0.030**	1.914	0.176	3.733	0.062	5.746	**0.023**
Sex × nutrients	1	0.059	0.809	0.766	0.388	0.024	0.878	3.466	0.071	1	1.096	0.303	0.876	0.357	0.887	0.354	0.060	0.808
Sex × water	1	3.516	0.070	0.484	0.492	3.946	0.055	0.002	0.960	1	0.036	0.851	1.656	0.208	0.101	0.753	0.050	0.825
Nutrients × water	1	0.737	0.396	0.937	0.340	0.749	0.393	0.632	0.432	1	0.669	0.420	0.016	0.901	0.793	0.380	0.829	0.369
Sex × nutrients × water	1	6.174	**0.018**	0.635	0.431	6.151	**0.018**	3.526	0.069	1	0.994	0.327	3.924	0.057	0.290	0.594	2.177	0.150
Error	33									31								

Significant differences ($P \leq 0.05$) are shown in bold. For young plants, n = 5 for all treatments, except for males in the LW-HN treatment where n = 6. For adult plants, n = 5 except for males in WW-HN where n = 6, and for males and females in LW-HN, where n = 4.

environmental stressors (nutrient deficiency and drought) might have changed timings in the life cycle of our plants. In particular, plants under the less optimal conditions may have flowered earlier, accumulating more reproductive biomass per unit of vegetative biomass at the time of harvest, whereas plants in the well-watered and high nutrient conditions were measured at flowering.

Content of N and C, and carbon isotope discrimination

Contrary to our expectations, neither water availability nor nutrient availability influenced the C isotope discrimination of pre-reproductive plants. This may be related to plants only growing for a short period under these conditions. However, as the study progressed, availability of water imposed differences in C isotope discrimination of mature plants, with lower values in water-restricted plants, as expected. Δ^{13}C integrates the overall metabolic performance during the entire plant life cycle, providing information on the long-term transpiration efficiency and, thus, can be used as an indicator of WUE (Farquhar & Richards 1984; Farquhar *et al.* 1989; Ellsworth & Cousins 2016). Water stress has been shown to influence WUE and Δ^{13}C at a given N level (Fu *et al.* 1993). Similarly, nutrient availability also led to differences in C isotope discrimination of mature plants, with larger Δ^{13}C values in plants growing with low nutrients, which is consistent with previous studies — e.g. Fu *et al.* (1993) also found that Δ^{13}C was higher in *Phaseolus vulgaris* L. grown with low N. Negative relationships between Δ^{13}C and N content may occur at lower N concentrations as this decreases photosynthesis and increases intercellular CO_2 thereby increasing Rubisco discrimination against the heavier C isotope (^{13}C). In the present study, leaf N content of mature individuals was lower when well-watered, which could explain the larger Δ^{13}C values of well-watered individuals. However, surprisingly, there were no differences in leaf N content in response to nutrient availability in mature individuals. Our results may be a consequence of stressed plants photosynthesising less and therefore growing less in comparison to well-watered plants, while maintaining the N uptake, and therefore, concentrating more N in tissues, as reported in wheat and millet (Clarke *et al.* 1990; Diouf *et al.* 2004).

There were differences in N content and the C/N ratio between young male and female plants in response to the interaction of nutrient and water availability. Such differences may be related to males having a higher %N and lower C/N ratio than females when growing in well-watered conditions (WW-LN). However, the post-hoc tests did not identify significant differences between groups, perhaps as a consequence of the low sample size at the level of the third-order interaction, and therefore our interpretation here should be considered with caution. There were no differences between sexes in C isotope discrimination, suggesting that plants consumed a similar amount of water per unit of C fixed. There is evidence of sexual dimorphism in C isotope discrimination in several dioecious species, but results are inconsistent: males have been found with lower (Dawson & Ehleringer 1993; Marshall *et al.* 1993; Ward *et al.* 2002), higher (Dawson & Bliss 1989; Houle 1996; Jones *et al.* 1999; Sánchez & Retuerto 2017) or similar values (Kohorn *et al.* 1994; Leigh & Nicotra 2003; Hultine *et al.* 2013) to females.

CONCLUSIONS

To our knowledge, this is the first study to reveal sexual dimorphism in *S. latifolia* in pre-reproductive individuals, particularly in early root growth patterns. These observed differences early in plant life may explain the skewed sex ratios (predominantly female-biased) found in natural populations of this species. Males and females of *S. latifolia* also responded differently to changes in nutrient and water availability. It appears that moist environments favour root production in females, which could provide an advantage to this sex in terms of survival and competition for resources, and might also contribute to female-biased sex ratios in the wild. In contrast, there was no sexual dimorphism in aboveground dry mass, neither in pre-reproductive individuals nor at reproduction — although female seedlings had longer leaves. Finally, we note the paucity of previous studies on the effect of the interaction of multiple abiotic stresses on dioecious plants. Given the few existing studies, our results reveal complex and somewhat inconsistent patterns, emphasizing the need for further research on a broader range of species, and investigations in both indoor as well as natural conditions.

AUTHORS CONTRIBUTIONS

MP-L: Data collection, data curation, data analysis, writing original draft and editing. AH: Data collection, writing – editing. APP: Data collection, writing – editing. FH: Supervision, resources, writing – editing. JSV: Conceptualization, supervision, resources, data collection, data curation, data analysis, writing – original draft and editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. % N, C/N ratio and Δ^{13}C of male and female young (a–c respectively) and adult (d–f respectively) plants of *S. latifolia* growing under different levels of nutrient availability: low (LN) and high (HN) and water: well-watered (WW) and low-watered (LW). For young plants, n = 5 for all treatments, except for males in the LW-HN treatment where n = 6. For adult plants, n = 5 except for males in WW-HN where n = 6, and for males and females in LW-HN, where n = 4. Symbols represent mean ± SE. For each plot, letters and asterisks indicate significant effects (S = Sex, N = Nutrients, W = Water; ns = non-significant, $*P \leq 0.05$, $**P \leq 0.01$).

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