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Environmental variation in sex ratios and sexual dimorphism in three wind-pollinated dioecious plant species

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Variation in plant sex ratios is often attributable to sex-specific mortality in heterogeneous environments that differentially limit male and female plant reproduction. Yet sexual dimorphism and plastic responses to environmental heterogeneity are common and may co-vary with variation in sex ratios. Here, we show that the sex ratio and the degree of sexual dimorphism for a number of plant traits varied along climatic and elevation gradients in three wind-pollinated dioecious species, *Rumex lunaria*, *Urtica dioica* and *Salix helvetica*. Some of the observed sex-specific responses to climatic variation are consistent with greater sensitivity of females to water scarcity, but most responses rather point to the greater sensitivity of males to ecological stress, consistent with larger male reproductive effort, as has been commonly reported for wind-pollinated plants. In contrast, we found no evidence for variation in either sex ratios or sexual dimorphism expected under sexual selection. Interestingly, sex ratios and sexual dimorphism varied both along distinct and the same ecological axes of variation, suggesting that the evolution of sexual dimorphism in the measured traits was not sufficient to prevent sex-specific mortality.

Keywords: cost of reproduction, dioecy, environmental gradients, sex ratio, sexual dimorphism, sexual selection

Introduction

Approximately 6% of flowering plant species are dioecious (Renner 2014). Although we should expect sex ratios in dioecious populations of these species to be roughly equal (Fisher 1930), a recent meta-analysis indicated that only half of the 243 surveyed dioecious plant species displayed an even sex ratio, and that male-biased sex ratios were twice as common as female biases (Field et al. 2013b). Sex ratio bias may occur for a number of reasons. Primary sex ratios may be biased by the presence of genetic elements distorting sex ratios (Alström-Rapaport et al. 1997, Taylor 1999), by a sex-specific ability to compete for maternal resources (Field et al. 2013a) or by the sex-determination system itself (Alström-Rapaport et al. 1997). Biased secondary sex ratios may result from differences in the rate of germination between male and female seeds

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(Alström-Rapaport et al. 1997) or sex differences in adult mortality (Stehlik and Barrett 2005). Stochastic events have also been reported to skew sex ratios in small isolated populations (Engen et al. 2003, Vandepitte et al. 2010). In addition to skews in the actual number of males and females in a population at a given point in time, populations of mature individuals may also show skewed 'operational sex ratios' as a result, for example, of gender differences in flowering time or in the age at maturity (Allen and Antos 1993).

Both secondary and operational sex ratio biases may ultimately be attributable to differences between males and females in their nutritive needs, driven by their divergent functions and costs of reproduction. Females may often bear greater reproductive costs than males because of the requirement for developing seeds and fruits (Lloyd and Webb 1977, Antos and Allen 1990). However, males have been reported to sometimes bear the greater cost of reproduction in wind-pollinated herbs because of the large amounts of pollen they produce (Harris and Pannell 2008, Tonnabel et al. 2017). In addition to potentially different absolute costs of reproduction between the sexes, components of male and female reproduction will often be limited by different resource currencies, with seed and fruit production drawing heavily on carbon and water (Lloyd and Webb 1977, Antos and Allen 1990, Ishida et al. 2005) and pollen production requiring large amounts of nitrogen (Ishida et al. 2005, Harris and Pannell 2008, but see Dudley 2006). Because the sex investing more of a particular resource to reproduction should be more vulnerable to its depletion (Lloyd 1973, Stehlik and Barrett 2005), sex-specific environmental stress may result in spatial segregation of the sexes or biased sex ratios as a result of gender-specific mortality (Lloyd 1973).

Several lines of evidence point to sex differences in reproductive costs as an important basis of variation in sex ratios. In several plant species, females are more susceptible to stressful conditions than males, including those that decrease resource availability (Dudley 2006, Hultine et al. 2018). Confirming a link with the cost of reproduction, Antos and Allen (1990) documented greater mortality for female plants after flowering. More generally, based on their meta-analysis, Field et al. (2013a) found ample evidence that variation in sex ratios can often be attributed to differences in reproductive costs: male-biased sex ratios were associated with costly fleshy fruits and biotic seed dispersal, while female-biased sex ratios were more often found in herbaceous species with abiotic pollen dispersal (which generally implies large pollen production; Harris and Pannell 2008). Less is known about the relationship between sex ratio variation and ecological conditions along environmental gradients, but a meta-analysis of such data suggests that sex ratios become more male-biased in more abiotically stressful environments, such as at higher altitudes or at more xeric sites (Eppley 2001, Field et al. 2013a, Petry et al. 2016, Hultine et al. 2018). In some species, apparently male-biased sex ratios can be attributed to delayed, shorter or less frequent flowering in females caused by their larger reproductive costs (Lloyd 1973, Allen and Antos 1993, reviewed by Field et al. 2013a).

The expression of sexual dimorphism may mitigate against differential mortality between males and females, to the extent that dimorphism reflects differences that may reduce the burden of reproduction on the costlier sex. Understanding sex ratio bias ought therefore to be considered along-side assessment of sexual dimorphism. Indeed, dioecious plants commonly show sexual dimorphism in their susceptibility to stress, as well as in several vegetative characters that might have implications for survival (Harris and Pannell 2008, Barrett and Hough 2013, Tonnabel et al. 2017). A growing number of studies confirm that differences in reproductive ecology that may be related to differential costs of reproduction are linked to sexual dimorphism (Lloyd and Webb 1977, Delph 1999). For example, female plants often display 'carbon-harvesting' morphologies and physiologies that allow greater production of photosynthetic vegetative biomass than males (e.g. increased leaf number or leaf area), or increased rates of photosynthesis with correspondingly greater water requirements (Dawson and Geber 1999). In contrast, males may invest more heavily in root growth, plausibly to increase nitrogen harvesting from the soil (Harris and Pannell 2008).

An important corollary of potential links between sexual dimorphism and costs of reproduction is that sexual dimorphism should vary in space and time in response to variation in resource availability. Few studies have investigated the link between plant sexual dimorphism and ecological factors, but they converge in documenting climate-related variation in sexual dimorphism (Dudley 2006, Puixeu et al. 2019). Furthermore, Delph et al. (2011) showed that differences between sexes in selection on leaf area depended on water availability. In general, the joint assessment of variation in sex ratios and sexual dimorphism may help to understand the causes of sex-specific mortality as well as how males and females might mitigate against mortality (Petry et al. 2016, Lei et al. 2017).

Here, we investigate variation in both the sex ratio and sexual dimorphism along environmental gradients of three wind-pollinated dioecious species, *Rumex lunaria* (Polygonaceae), *Urtica dioica* (Urticaceae) and *Salix helvetica* (Salicaceae). For each of these species separately, we tested 1) whether sex ratios of flowering individuals and sexual dimorphism in plant architecture and leaf morphology varied along particular axes of climatic variation; 2) whether sex ratios were correlated with the degree of sexual dimorphism, which may occur if a constant sex ratio bias through time elicits selection related to competition for resources or mates; and 3) whether more male-biased sex ratios were associated with an increased proportion of non-flowering plants, as should be expected if a large cost of reproduction in females delays their flowering.

Relationships between climatic conditions and sex ratio or sexual dimorphism are to be expected when males and females diverge in their reproductive needs. For instance, males could be more frequent at sites with reduced water availability (e.g. due to lower precipitation and/or higher ambient temperatures) if females are subject to greater sensitivity to water scarcity. Similarly, if female reproduction requires more water

than male reproduction, females may show decreased leaf area and/or plant size compared to males at sites at which survival might be compromised by water deficits. This hypothesis relies on the assumption that the production of greater photosynthetic surfaces may compromise plant survival less at wet and/or cold sites. Greater competition for accessing mates through more male-biased sex ratios could select for increased male plant sizes, given that pollen release height has been reported as affecting pollen dispersal to more mates at least in one wind-pollinated species (Klinkhamer et al. 1997, Tonnabel et al. 2019a, b). Alternatively, sex ratio biases may foster competition for accessing nutritive resources, thus enhancing selection in males or females for developing vegetative organs that harvest resources most needed for their reproduction.

Material and methods

Study species

We studied three dioecious species: *Rumex lunaria*, *Urtica dioica* and *Salix helvetica*. These three species were chosen for their wind-pollination habit and their accessibility for field work. The analyses were performed separately for each study species as they displayed different growth habit and ecology.

Rumex lunaria (Polygonaceae) is a wind-pollinated, perennial, endemic shrub to the Canary Islands (Méndez et al. 2003). This species is a pioneer of volcanic soils and presents adaptations to semi-arid conditions, including a shrub habit with tough leaves (Méndez et al. 2003). *Rumex lunaria* has been described as gynodioecious (Mariotti et al. 2006), but it was strictly dioecious in the populations we visited. Sex ratios in *Rumex* species vary from male- to female-biased, with some populations and species having no significant bias (Harris 1968, Stehlik and Barrett 2005, Pickup and Barrett 2013). *Rumex* species typically flower between March and September, with males flowering earlier than females in *R. hastatulus* (Conn 1981, Matsuhisa and Ushimaru 2019).

Urtica dioica (Urticaceae) is a wind-pollinated perennial herbaceous dioecious herb that shows some sex inconstancy (leakiness in sex expression), i.e. some individuals with a low proportion of flowers of the opposite sex (Glawe and de Jong 2005, Taylor 2009). Sex determination in *U. dioica* appears to be under the control of multiple genes (Shannon and Holsinger 2007, Glawe and de Jong 2009), and primary sex ratios can differ from 1:1 and be male- or female-biased (Glawe and de Jong 2009). *Urtica dioica* is clonal and forms dense unisexual patches connected by rhizomes (Glawe and de Jong 2005). Both sexes flower usually between May and September, with males flowering earlier than females (Taylor 2009).

Salix helvetica (Salicaceae) is a perennial, dioecious and wind-pollinated shrub, distributed in the Alps and in northern Europe. Many *Salix* species have female-biased sex ratios (Pucholt et al. 2017, Hroneš et al. 2019) and have homomorphic or no sex chromosomes, with females often the heterogametic sex (Hou et al. 2015, Chen et al. 2016, Pucholt et al. 2017). Flowering usually occurs in early summer, with males flowering first (Choudhary et al. 2011).

Sex ratio variation

To investigate sex ratio variation and variation in sexual dimorphism, we counted males and females and measured plant traits at 14 naturally occurring populations of *R. lunaria* on the island of Tenerife (Spain) in April 2017, at eleven populations of *U. dioica* in 'Le Plateau' area (Switzerland) in September 2017, and at ten populations of *S. helvetica* in the Alps (Switzerland) in July 2017 (see Fig. 1 for maps of the study populations; Supporting information). All data collected in this study are available at <https://doi.org/10.5061/dryad.z612jm6df>. In all three species, plants were distributed sporadically in dense vegetation, preventing us from using transects to count and sample individuals. First, to ensure an unbiased sample, we recorded the sex of every individual identified, moving through the populations in only one direction to avoid sampling any individual more than once. This protocol was slightly adapted in *U. dioica*, because its clonal habit rendered the identification of individuals more complex. Following Glawe and de Jong (2005), we assigned two unisexual patches of the same sex in *U. dioica* to two distinct individuals only if at least 2 m separated them, and if no conspecific individual of the same sex was located in between. In *R. lunaria* and *S. helvetica*, multiple shoots were recorded as a single plant when shoots were connected above ground. Both elevation and GPS coordinates were recorded at the centre of the populations (Supporting information). For each species, we counted all individuals up to a maximum of 150 per population.

Among the 35 sampled populations across the three species, the number of individuals for which sex was identified varied between 50 and 150, with an average of 121 individuals (Supporting information). This yielded to a total of 1736 males and 2493 females sampled across all populations (Supporting information). We did not record the position of every individual within populations, but we saw no obvious evidence of spatial segregation of the sexes within populations. For all three study species, we also counted non-flowering individuals to test the hypothesis that females could be subject to a delay in flowering, eliciting a relationship between more male-biased sex ratios and larger proportions of non-flowering plants. For *S. helvetica*, we observed the presence of shorter plants (< 0.5 m), which were assigned to a 'non-mature' category (and thus removed from the 'non-flowering' category), because they also presented juvenile traits such as leaf hairs; the number of juveniles present was not used further in the analyses but is reported in the Supporting information.

Sex-specific leaf and architectural traits

Plant height, defined as the distance from the ground surface to the highest point of the plant canopy, was recorded for all sampled mature flowering individuals in *R. lunaria* and *S. helvetica*. In these two species, we also recorded the canopy

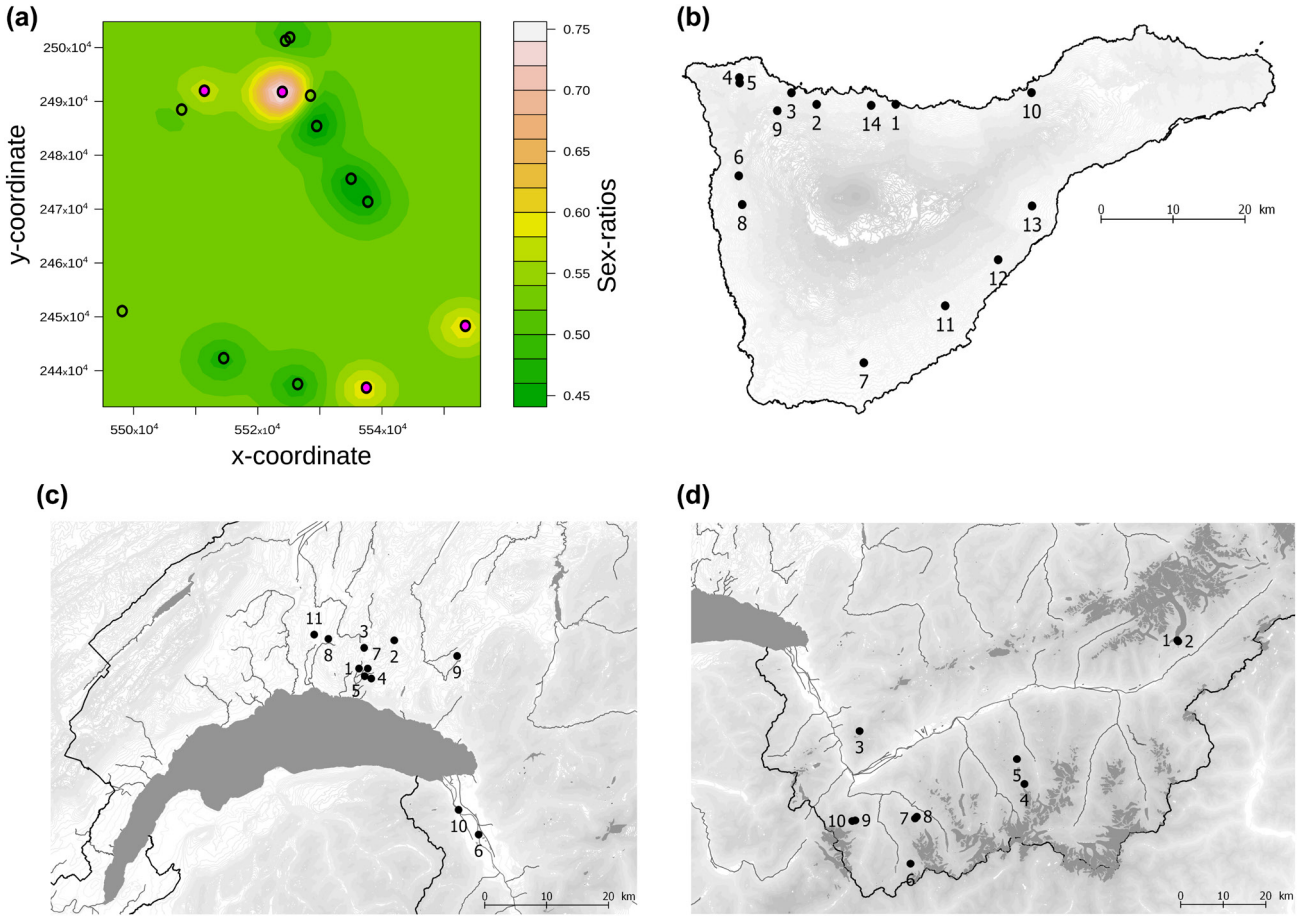

Figure 1. Spatial prediction of sex ratios along x- and y-coordinates, as predicted by a generalized linear mixed-effect model including a spatially autocorrelated random effect for *R. lunaria* (a) and geographical distribution maps (© OpenStreetMap contributors) of *R. lunaria* (b), *U. dioica* (c) and *S. helvetica* (d).

width, defined as the largest horizontal distance between two extremities of the canopy for all mature flowering individuals. We report only results for plant height, as canopy height was significantly correlated with canopy width in both species, as revealed by comparing linear models that did or did not explain plant height by canopy width (and including population and sex nested within population as random factors) using likelihood ratio tests (*R. lunaria*: $\chi_i^2 = 1116$, df = 1, $p < 0.0001$ and *S. helvetica*: $\rho = 276.2$, df = 1, $p < 0.0001$) performed in R ver. 3.5.2 (<www.r-project.org>). However, most of our results were robust to the use of these other plant morphological traits. For *U. dioica*, we estimated plant cover for roughly half of the mature individuals identified (see the Supporting information for a summary of the number of individuals measured), using a visual criterion to determine the shape best approximating the plant cover per individual (e.g. circle, ellipse or rectangle), and we further measured the parameters of the projected shape to assess their cover. Hereafter, we refer to plant height and canopy cover as 'plant size' for uniformity across the three study species.

To study sexual leaf dimorphism, we sampled shoots (not displaying fruit or flowers) in each sampled population for ten females and ten males, randomly chosen among the mature flowering individuals. We took pictures of all leaves from the fifth node to the tip of the sampled shoot, which were later analysed to measure leaf surface with the software Image J (Schneider et al. 2012). We further calculated the mean leaf area for each sampled individual. Among all measured populations, we analysed an average of 13.6 ± 5.3 leaves (mean ± standard error of the mean) per individual, giving a total of 8603 leaves measured overall (see the Supporting information for details per population).

Deviation from equal sex ratios

For each population, we calculated the operational sex ratio (SR) as the proportion of females divided by the total number of flowering individuals in a population. Note that for the few leaky individuals in *U. dioica* (Pop. 1: 0.04%, Pop. 2 and 7: 0.03%, Pop. 5, 8 and 10: 0.01%, Pop. 3, 4, 6, 9 and 11: 0%) we determined sex on the basis of their dominant sex expression. For each population separately, we performed a G-test to identify significant departures from 1:1 (following Field et al. 2013a), using the DescTools package

(Signorell et al. 2018) in R ver. 3.5.2 (<www.r-project.org>), and adjusting p-values for multiple testing (several populations per species) via a Bonferroni correction. We observed only a few dead individuals (in only two populations of *R. lunaria*); these were not considered in the sex ratio calculation, as we were specifically interested in testing whether variation in ecological conditions could lead to gender-specific mortality. Our observation of a few dead individuals, however, confirms that adult mortality has occurred in the visited populations. Finally, and for each study species separately, we performed an overall G-test that included all populations to test for differences in sex ratios between populations.

Sexual dimorphism in leaf and plant size

We tested whether plant size and leaf area (i.e. leaf area was treated as the average per sampled individual) differed significantly between the sexes within each population using Wilcoxon rank sum tests, as the Normality assumption for parametrical tests was not met for both traits in several populations. We further calculated, for each population, two sexual dimorphism indices, one for leaf area and one for plant size, hereafter termed SLD and SSD, respectively. The SLD and SSD were calculated as the log of the ratio of the mean female to the mean male leaf area or plant size, respectively (Smith 1999), with a null expectation of 0 for no difference. Separately for males and females, we also tested for the correlation between plant size and leaf area using Pearson correlation tests.

Ecological conditions at the population level

To investigate the effect of climate on the sex ratio and sex-specific trait values, we extracted for each population nine bioclimatic variables at a spatial resolution of 1 km^2 from WorldClim global climate layers (Fick and Hijmans 2017) using the R package raster (Hijmans et al. 2018) in R ver. 3.5.2 (<www.r-project.org>). We chose these nine bioclimatic variables in the WorldClim database as those most likely related to our hypotheses: in particular, we included variables expressing stress caused by 1) high temperature, 2) low precipitation and 3) the temporal variability of temperature and precipitation (see Table 1 for a list of the chosen variables). As commonly observed for climate data, high multicollinearity was present between the climatic variables in all three study species, as revealed by Pearson correlation tests (Supporting information). These high levels of multicollinearity prevented us from including several climate variables simultaneously in multivariate analyses. We addressed multicollinearity by also performing principal component analysis (PCA), as suggested by Chong et al. (2018). We chose to perform separate PCA for each species (and therefore separate subsequent analyses), because correlations between individual climatic variables differed between species, as shown by pairwise Pearson correlation tests (Supporting information). For each species, we retained the first three principal components (PC), each of which explained more than 10% of the variation in climate (Table 1). The contribution of our nine bioclimatic variables to the PCs are provided in Table 1.

We also characterized climatic variation along altitude gradients separately for each species, because altitude is often used as a proxy for stress in studies on plant sex ratio variation (reviewed by Field et al. 2013a), and may capture variance in ecological conditions beyond climatic variables included in our analysis. We used the R package MASS (Venables and Riplex 2002) to select climatic variables that best predicted variation in elevation among our nine climatic variables of interest, using the stepAIC function and both backward and forward variable selection. We then fitted a linear model predicting elevation against all climate variables selected. We used likelihood ratio tests (LRTs) to assess the significance of each of these climate variables by comparing models with and without them.

Spatial structure for plant traits and sex ratios

We used linear mixed models (LMM) to test for spatial structure in plant traits and generalized linear mixed models (GLMM) to test for variation in sex ratios (modelled as a binomial variable), using the Matérn correlation function for both traits. The Matérn function models spatial auto-correlation as a function of distance between populations, using

Table 1. Climatic variation summarized by principal component analysis. Contribution of each of our nine bioclimatic variables to each of the three studied PCs as performed separately in each species: *Rumex lunaria* (RL), *Urtica dioica* (UD) and *Salix helvetica* (SH) and proportion of variance explained by each axis.

	PC1			PC2			PC3		
	RL	UD	SH	RL	UD	SH	RL	UD	SH
Annual mean temperature	0.45	0.42	−0.40	0.13	0.31	−0.02	0.10	0.7	−0.12
Mean diurnal range	−0.18	0.26	−0.15	0.54	−0.55	0.50	0.11	−0.17	0.37
Isothermality	−0.22	0.30	−0.24	0.50	−0.43	0.14	−0.09	−0.34	0.79
Temperature seasonality	0.34	−0.17	0.02	0.05	−0.14	0.53	0.51	0.61	−0.34
Maximum temperature of the warmest month	0.42	0.46	−0.34	0.24	0.16	0.30	0.19	0.16	−0.26
Temperature annual range	−0.14	0.07	−0.01	0.53	−0.59	0.55	0.26	0.28	−0.13
Annual precipitation	−0.37	−0.45	−0.46	−0.17	−0.15	−0.15	0.48	0.08	−0.06
Precipitation of the wettest month	−0.31	0.09	−0.46	−0.24	−0.05	−0.14	0.57	0.56	−0.06
Precipitation seasonality	0.41	0.46	−0.47	−0.09	0.04	−0.12	0.23	0.23	−0.13
Proportion of variance	0.59	0.46	0.43	0.32	0.25	0.33	0.14	0.19	0.13

a random effect that includes three parameters (see Rousset and Ferdy 2014 for further details). We regressed plant traits (either plant size or leaf area) and sex ratios as a function of a random Matérn spatial effect, including the three selected climate PCs as covariates. The effect of the random spatial structure was tested using LRTs to compare models with and without it. Models were fitted by restricted maximum likelihood, as we were investigating random effects. The spatial random effect was retained in all subsequent analyses only when significant. This allowed us to account for the non-independence of populations sampled in close proximity. Given that we measured several leaves per individual, leading to the inclusion of several measures per plant in LMMs (treating leaf area as response variable), we also included a random effect for individuals in these models to avoid any pseudo-replication; this random variable structure was retained for all LMMs, treating leaf area as a response variable below. We fitted these models, and all following ones, using the R package spaMM ver. 2.6.39 (Rousset and Ferdy 2014) in R ver. 3.5.2 (<www.r-project.org>).

The relationship between ecological variation and sexual dimorphism and sex ratios

We tested for a possible relationship between climate and elevation and both sexual dimorphism in plant traits and sex ratios. First, we tested for differences in sexual dimorphism between populations by comparing LMMs, modelling plant traits as the response variable and either including or excluding the interaction between sex and population as a fixed effect using LRTs (see below for the structure of random effects). We also used LRTs to test for the effect of climatic variation on sexual dimorphism. Here, we constructed multivariate null models that included plant traits (i.e. plant size or leaf area) as response variables, the three selected first climate PCs as explanatory variables, and the interaction with sex and these three explanatory variables. First, we tested whether variation in plant traits varied between sexes accordingly to overall climatic variation by comparing the null model described above with a model in which we kept sex as a covariate but removed its interaction with all PCs. Second, we tested for an effect of a particular climate variable on sexual dimorphism by comparing the null models to models in which we removed the interaction with sex, one PC at a time. Only when the interaction between sex and a PC was significant did we proceed to fit a multivariate model, including all selected climate PCs for each sex separately, and tested the effect of the particular PC by removing it from the model and using LRTs. As we did not expect that the selected climatic variables alone would affect variance in morphology between populations, we also included one random effect for each sex to account for variance between populations in all models predicting plant traits.

To test for the effect of climate and elevation gradient on sex ratios, we modelled sex ratios using GLMM when a random spatial effect was significant, or using generalized mixed models (GLM), treating sex ratio as a binomial variable. We built multivariate null models that included sex ratio as a response variable and the three selected first climatic PCs as explanatory variables. We further used LRTs to compare the null model with simpler models in which we removed the effect of each PC at a time. Using univariate GLMM or GLM to explain sex ratios against the proportion of plants in flower, we used LRTs to compare models with and without the proportion of flowering plants. Finally, because variation in elevation might represent ecological variation not accounted for in our climatic analysis, we also fitted univariate models and used LRTs, as described above, to test for the effect of elevation (on sex ratio) and of its interaction with sex (on plant traits). We used univariate models, including only elevation as a response variable (and its interaction with sex), to avoid problems of collinearity, not least because we found a significant correlation between elevation and PCs summarizing climate variation for several populations (Table 2); these correlations indicate whether the effect of elevation can be interpreted independently of that of PCs or not. All models described in this section were fitted by maximum likelihood, as we were testing for fixed effects.

Results

Variation in sex ratios

Sex ratios varied significantly among populations in both *R. lunaria* ($G=76.2$, $df=13$, $p < 0.001$) and *S. helvetica* ($G=32.5$, $df=9$, $p < 0.001$), but not in *U. dioica* ($G=10.8$, $df=10$, $p=0.38$). Sex ratios ranged from male-biased to female-biased in *R. lunaria* and *U. dioica* (i.e. from 0.43 to 0.79, mean across populations of 0.53 and 0.46–0.65, mean of 0.56, respectively) and included strictly female-biased sex ratios in *S. helvetica* (i.e. from 0.62 to 0.83, mean of 0.74; Fig. 2, 3, 4, Supporting information). Within populations, we found a significant female-biased sex ratio in one of the 14 *R. lunaria* populations, in one of the eleven *U. dioica* populations, and in eight of the ten *S. helvetica* populations (Fig. 2, 3, 4, Supporting information). None of these biases were associated with the proportion of non-flowering plants (Supporting information). We detected spatial structure in sex ratios only in *R. lunaria* (Fig. 1, Supporting information).

Variation in the strength and direction of sexual dimorphism

Sexual dimorphism in leaf area varied significantly among populations of *R. lunaria*, as revealed by a significant interaction between sex and population (Supporting information). In *S. helvetica*, we found only marginally significant differences in leaf-area sexual dimorphism between populations, and no differences in *U. dioica* (Supporting information). Within populations, several populations of all three species showed significant differences in leaf area between males and females (five over twelve populations, three over ten and three over nine, respectively, for *R. lunaria*, *U. dioica* and *S. helvetica*), including both female-biased and male-biased sexual

Table 2. Species specific statistical analysis of the relationship between ecological factors and both leaf area and plant size sexual dimorphism and sex ratios. Sex-specific responses in log(leaf area) and log(plant size) to ecological factors were tested by comparing models including or not the interaction between sex and PCs or elevation. Relationships between sex ratio and ecological factors were evaluated by comparing models including or not PCs and elevation. Multivariate models were performed to test for the effect of climatic factors (PCs) and univariate ones to evaluate the effect of elevation. Correlations between elevation and each PCs were assessed using Pearson correlation tests. Significance was evaluated using likelihood ratio tests: · $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Results with associated p-value below 0.05 are highlighted in bold.

	Multivariate models								Univariate models			
	Intercept	Sex (♀)	PC1	Sex × PC1	PC2	Sex × PC2	PC3	Sex × PC3	Intercept	Elevation	Sex (♀)	Sex × elevation
R. lunaria												
Leaf area	7.03	−0.0765	−0.0596	0.00800	−0.00113	0.0109	−0.0783	0.0745	7.04	-2.64×10^{-5}	-4.18×10^{-2}	-7.09×10^{-5}
Plant size	5.01	−0.0131	−0.0724	−0.00338	−0.0489	**0.0286***	0.0244	0.0140	5.06	-1.32×10^{-4}	-1.81×10^{-2}	1.16×10^{-5}
Sex-ratio	0.116	–	−0.0553	–	−0.0469	–	−0.00745	–	−0.428	**0.00130****	–	–
Pearson correlation–elevation			−0.250		0.0851		−0.335					
U. dioica												
Leaf area	6.76	−0.353	−0.00361	0.0769	−0.0122	**0.173****	0.0470	−0.087	6.75	9.93×10^{-6}	6.49×10^{-1}	$\mathbf{-1.46 \times 10^{-3}}$**
Plant size	10.7	−0.207	0.0963	−0.108	0.109	0.0618	0.0197	−0.00812	11.2	7.65×10^{-4}	−0.667	6.94×10^{-4}
Sex-ratio	0.236	–	**0.0784***	–	−0.0634	–	−0.0558	–	0.582	5.16×10^{-4}		
Pearson correlation–elevation			**−0.851****		−0.446		−0.070					
S. helvetica												
Leaf area	5.68	−0.00930	0.0181	**−0.0579****	0.0289	−0.0259	−0.0298	−0.0601	5.10	2.64×10^{-4}	0.910	-4.21×10^{-4}
Plant size	3.94	0.00786	−0.117	−0.0248	−0.101	−0.0164	−0.0588	−0.0168	5.47	-7.20×10^{-4}	0.760	$\mathbf{-3.56 \times 10^{-4}}$*
Sex-ratio	1.13	–	**0.0811***	–	−0.0313	–	**−0.197****		−0.899	$\mathbf{9.47 \times 10^{-4}}$**		
Pearson correlation–elevation			**0.599***		−0.00620		−0.0490					

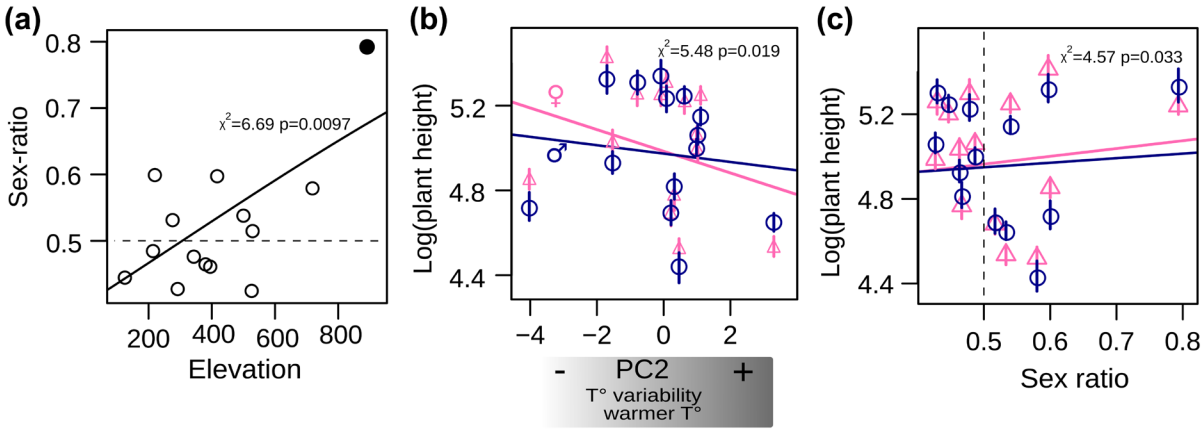

Figure 2. Significant sex ratio response to elevation gradients: (a) sex-specific log(leaf area) responses to climatic variation; and (b) sex-specific log(plant size) to sex ratio in *R. lunaria*. The description of climatic variation occurring along the PC2 axis is shown in the grey zone (Table 1). Results of the likelihood ratio tests performed to test the interactions between sex and the climatic axis or the sex ratio for plant height and the relationship between sex ratio and elevation are displayed in each panel. Black circles represent values of sex ratio, and blue circles and pink triangles represent plant height for males and females, respectively. Filled symbol indicates a significant deviation from a 1:1 sex ratio. The dashed line indicates a 1:1 sex ratio.

dimorphism, except for *U. dioica*, which only showed female-biased sexual dimorphism (Fig. 2, 3, 4, Supporting information). Sexual dimorphism in plant size differed between populations only in *R. lunaria*, as revealed by a significant interaction between sex and population (Supporting information). In contrast with leaf area, there was no sign of within-population sexual size dimorphism for any population of the three species (Supporting information). Neither leaves nor plant size displayed a spatial structure, as models predicting traits with climatic variables including a spatial auto-correlation random effect did not perform better than models without such a random effect (Supporting information). Plant size and leaf area were not correlated within males or females in any of the three species (Supporting information).

Variation of sex ratios and sexual dimorphism along environmental gradients

In general, sexual dimorphism in either leaf area or plant size and/or sex ratios varied with climatic or elevation gradients (Table 2). In both *R. lunaria* and *U. dioica*, sexual dimorphism and sex ratios varied along different axes of environmental variability, while in *S. helvetica* they jointly varied along both elevation and climatic gradients (Table 2).

In *R. lunaria*, the proportion of females increased with elevation (Table 2, Supporting information, Fig. 2a). Altitude in *R. lunaria* was significantly positively correlated with maximum temperature of the warmest month and precipitation of the wettest month, and negatively correlated with annual precipitation and minimum temperature of the coldest month (see Supporting information for details and correlations with variables expressing climate temporal variability). In this species, plant size decreased more steeply for females than for males in locations with increased temperature variability and warmer temperatures (i.e. higher values along the PC2 axis, Table 2, Fig. 2b). However, neither female nor male plant size significantly decreased along the PC2 axis (Supporting information). Both male and female plant sizes significantly increased in populations with an increased proportion of females, but this increase was steeper in females than in males, as revealed by a significant effect of the interaction between sex ratio and sex (Table 2, Fig. 2c, Supporting information).

In *U. dioica*, the proportion of females increased in locations with warmer temperatures and lower precipitation, as revealed by a significant effect of PC1 in models predicting sex ratio (i.e. higher values along PC1 axis, Table 2, Fig. 3a, Supporting information). In this species, male leaf area increased at locations with decreased temperature variability and warmer temperatures, while female leaf area was not affected by such climatic variation (i.e. higher values along PC2 axis, Table 2, Fig. 3b, Supporting information). In *U. dioica*, male leaf area decreased with increasing elevation, while female leaf area was not affected by elevation (Table 2, Fig. 3c, Supporting information). Altitude in *U. dioica* was significantly positively associated with the maximal temperature of the warmest month and the precipitation of the wettest month, and negatively correlated with annual precipitation and the mean annual temperature (Supporting information). Finally, male and female leaf area showed different responses to variation in the sex ratio: leaf area decreased significantly with the proportion of females for males but not for females (Table 2, Fig. 3d, Supporting information).

In S. *helvetica*, the proportion of females increased in locations with higher temperature and both lower precipitation and its temporal variability, as revealed by a significant effect of PC1 in models predicting sex ratio (i.e. higher values along PC1 axis; Table 2, Fig. 4d, Supporting information). In parallel, sexual dimorphism in leaf area also varied along this axis of climatic variation, as revealed by a significant sex by PC1 effect (Table 2, Fig. 4a, Supporting information). While male leaf area decreased marginally significantly along PC1 climatic gradient, the increase in female leaf area along PC1 was

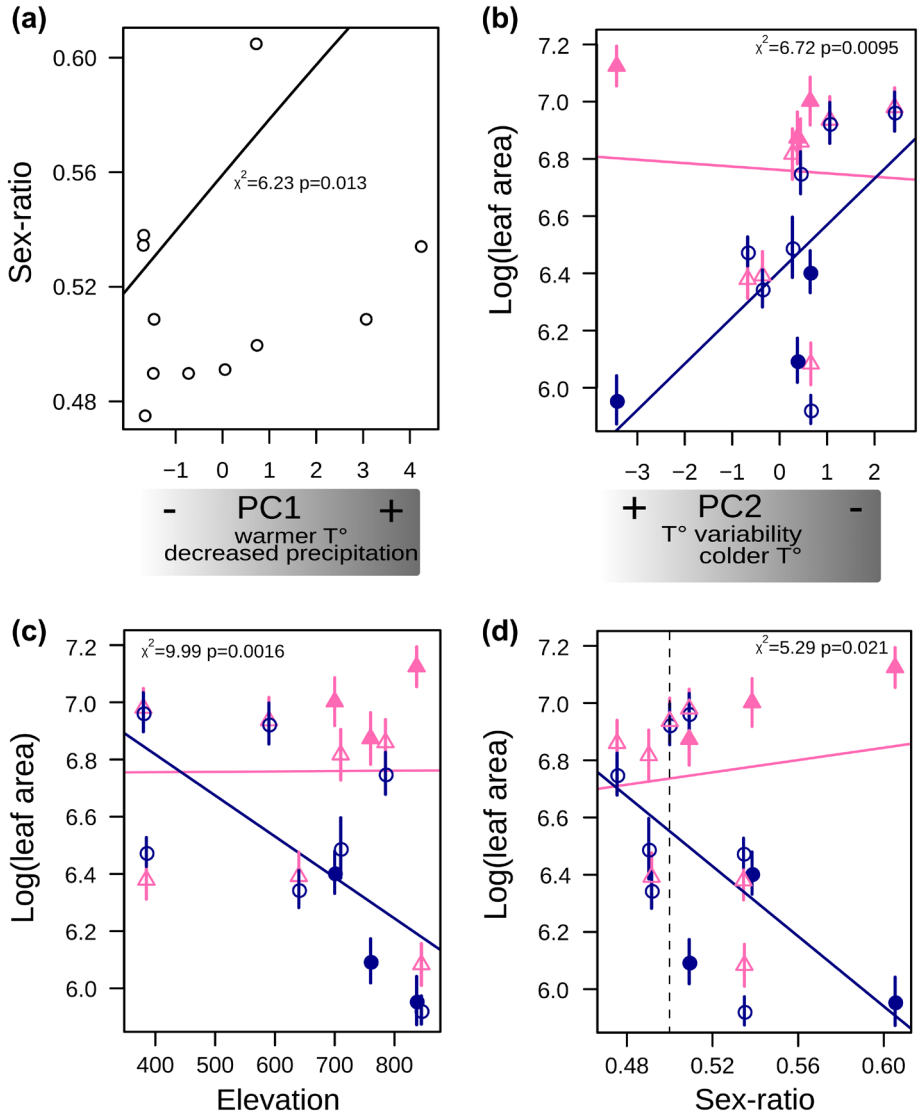

Figure 3. Significant sex ratio response to climatic variation displayed in *U. dioica* on (a) the PC1 axis; (b) sex-specific log(leaf area) responses to climatic variation, PC2; (c) elevation; and (d) sex ratio. The description of climatic variation occurring along the PC axes are shown in the grey zones (Table 1). Statistics of the likelihood ratio tests performed to test the interaction between sex and ecological factors or sex ratio for leaf area and of the effect of PC1 on sex ratio are displayed in each panel. Black circles represent values of sex ratio, and blue circles and pink triangles represent leaf area for males and females, respectively. Filled symbols indicate a significant sexual dimorphism in leaf area. Dashed lines indicate a 1:1 sex ratio.

not significant (Supporting information). The proportion of females also decreased in locations with milder temperature, increased isothermality and temperature diurnal range and decreased temperature seasonality, as revealed by a significant effect of PC3 in models predicting sex ratios (Table 2, Fig. 4e, Supporting information). Finally, the proportion of females increased with elevation (Table 2, Fig. 4c, Supporting information). Also, male plant size showed a steeper decrease with altitude than female size, as revealed by a significant effect of the interaction between sex and elevation (Table 2, Fig. 4b, Supporting information). The male decrease in plant size with elevation was marginally significant, while the decrease in female plant size was non-significant (Supporting information); there was no significant sexual dimorphism in any population. In *S. helvetica*, altitude was positively correlated with the maximal temperature of the wettest month, the minimum temperature of the coldest month, annual precipitation and its seasonality, and was negatively correlated with mean annual temperature and the precipitation of the wettest month (Supporting information).

Discussion

We found that both sex ratios and sexual dimorphism in morphology varied along environmental gradients for all

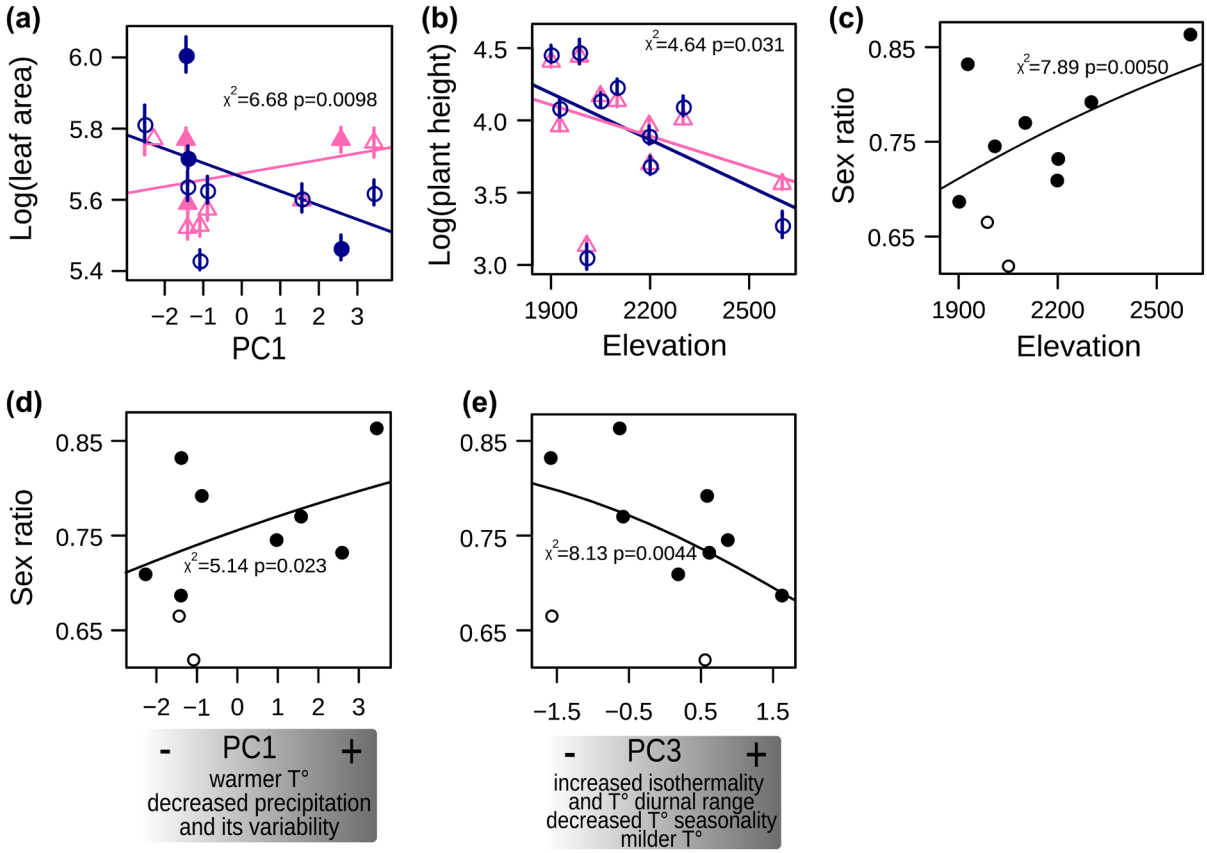

Figure 4. Significant sex-specific log(leaf area) and log(plant height) responses and sex ratio responses to ecological factors (i.e. climate and elevation) and mean population plant height found in *S. helvetica*. Sex-specific relationship between log(leaf area) and PC1 and between log(plant height) and elevation are respectively displayed in panels (a) and (b). Significant relationship between sex ratios and elevation, PC1, PC3 and mean population plant height are respectively shown in panels (c), (d), (e) and (f). The description of climatic variation occurring on PCs is shown in the grey zones. Statistics of the likelihood ratio tests performed to test the effect of interaction between sex and ecological factors for plant traits, and the relationship between sex ratio and ecological factors are displayed in each panel. Black circles represent values of sex ratio, and blue circles and pink triangles represent values of plant traits (either leaf area or plant height) for males and females, respectively. Filled symbols indicate a significant sexual dimorphism in leaf area or a significant deviation from a 1:1 sex ratio.

three species studied, indicating that both the strength and direction of sexual dimorphism can display strong intra-specific variation. This result is similar to observations made for a limited number of dioecious plant species (Delph et al. 2002, Dudley 2006, Puixeu et al. 2019). Most populations in our study showed female-biased sex ratios, which is typical for species with abiotic pollen dispersal, as in our three study species (reviewed by Field et al. 2013a). Female-biased sex ratios have previously been reported for several *Rumex*, *Urtica* and *Salix* species (De Jong et al. 2005, Stehlik and Barrett 2005, Myers-Smith and Hik 2012), and attributed to male-biased mortality caused by large male reproductive costs in these wind-pollinated species (Field et al. 2013a). Our results highlight the diversity of sex-specific responses to ecological variation, both in terms of morphology and of sex ratio variation. Importantly, we also found patterns of joint variation in sexual dimorphism and sex ratios along environmental gradients in one species. Such a pattern suggests that the sex-specific morphological response to ecological changes in the measured traits is insufficient to prevent gender-specific mortality, assuming that the observed variation in sex ratios are indeed caused by gender specific mortality. Our joint evaluation of the variation of sexual dimorphism and sex ratios points to the need for future studies of this kind to determine whether sex-specific changes in morphologies can sometimes prevent gender-specific mortality to be caused by ecological variation or not.

In one of the three study species, *R. lunaria*, we observed significant changes in both female morphology and the proportion of female plants along climatic gradients that are congruent with expectations based on a higher cost of reproduction in females compared to males. In *R. lunaria*, female plant size showed a steeper reduction compared to that of males at sites with both warmer and more variable temperatures. While sex ratios were not affected by an axis of climatic variation, the observed increase in the proportion of females in populations of *R. lunaria* with elevation may reflect lower female mortality at higher altitude, i.e. where precipitation in the wettest month was greater (a climatic variable that may impact plant water reserves; Gavilán 2005). Yet higher

altitudes were also characterized by warmer temperatures in the warm season and lower annual temperatures for *R. lunaria*, so we ought to exercise caution when interpreting patterns of sex-specific variation along elevation gradients. These caveats notwithstanding, our evaluation of the ecological correlates of sex ratios and sexual dimorphism in *R. lunaria* are congruent with the general expectation that the substantial water needs for female reproduction, reported both for insect- and wind-pollinated plants, are a primary cause of variation in sex ratios and sexual dimorphism in plants (Dudley 2006, Harris and Pannell 2008, Field et al. 2013a, Hultine et al. 2016, Puixeu et al. 2019).

Similar caution is warranted when interpreting the observed increase in the proportion of females in *U. dioica* in locations with warmer temperatures and decreased precipitation, which may reflect increased female mortality at drier sites. We also emphasise that our sampling protocol (which involved counting individuals that were more than 2 m apart), may have introduced some sex-specific detection bias, especially if sex ratios varied with density. Nor can we distinguish between gender-specific mortality and initial biases within seeds, which might play a role in sex ratio variation in *U. dioica* (Glawe and de Jong 2009). Finally, clonal species such as *U. dioica* need longer to reach an equilibrium sex ratio (Field et al. 2013b), so that the patterns shown by *U. dioica* may also reflect a transitory state.

Apart from our analysis of climatic variation, other correlations we observed provided mixed support for the influence of the cost of reproduction in females on variation in sexual dimorphism and sex ratios. On the one hand, we found that females of *R. lunaria* were taller in populations with a greater female frequency, which may reflect increased competition for light with increased female-biased sex ratios. On the other hand, the female cost of reproduction may bring about delayed and/or shorter reproduction in females, which might also contribute to explaining the high frequency of protandry in plants (Forrest 2014). However, we found no association between the sex ratio and the proportion of flowering plants in any of the three species studied, suggesting that any putative female cost of reproduction did not impact flowering duration. We cannot however rule out the possibility that some individuals characterized as non-flowering were simply too young to flower.

Contrary to the patterns found in *R. lunaria*, most sex-specific changes observed in the two other study species are actually congruent with a higher cost of reproduction in males rather than females. In both *U. dioica* and *S. helvetica*, variation in sexual dimorphism along environmental gradients was clearly caused by a change in male morphology, with female morphology remaining unchanged. Because of the nutrient-rich content of pollen, variation in sex ratios and sexual dimorphism in wind-pollinated species might be mainly caused by environmental factors affecting males more than females. We observed variation in male allocation to vegetative aerial parts along the climatic and altitudinal gradients in both *U. dioica* and *S. helvetica*, which are congruent with tradeoffs between allocation to shoots versus nutrient-harvesting roots. Both climate and altitude are known to affect plant growth, with an impact on the spatial patterns of nutrient availability (Fisher et al. 2013), although it is not possible here to predict soil richness directly from climate data. Taken together, these results suggest that both male and female costs of reproduction contribute to the sex-specific correlative patterns observed and point to a complex interplay between these effects.

In contrast with our expectations based on competition among males for accessing mates, we found a decrease in male height with more male-biased sex ratios in *R. lunaria*. We hypothesized that competition among male plants to sire ovules on females, enhanced by more male-biased sex ratios, might contribute to shaping sexual dimorphism by selecting traits that enhance competitive ability and pollen dispersal (Tonnabel et al. 2019a, b). At least in one wind-pollinated species in which the number of sexual partners has been estimated, selection for mate acquisition was identified as a strong determinant of male reproductive success (Tonnabel et al. 2019a). Under such circumstances, we might expect male-biased sex ratios to be associated with larger males, given that pollen is dispersed better from greater heights (Klinkhamer et al. 1997, Tonnabel et al. 2019a, b). However, this hypothesis was not supported in any of our study species. Instead, we found a decrease in male plant height with more male-biased sex ratios in *R. lunaria*, thus rather pointing towards greater competition for nutrients having caused the evolution of male morphologies, with investment into root development coming at the expense of above-ground development. Within-population studies of fitness variation have accordingly reported greater sensitivities to plant density in males compared to females (Tonnabel et al. 2021). Regarding sexual selection, we cannot, however, rule out the possibility of variation in the intensity of competition for mates along climatic and elevation gradients, e.g. as a result of variation in wind conditions or plant density. Future studies specifically addressing the relationship between plant density, sex ratio and sexual dimorphism along environmental gradients might provide more insights on the specific effects of biotic and abiotic stress on females and males in dioecious plants.

By jointly documenting variation in both sexual dimorphism and the sex ratio, our study suggests that sex-specific changes in the measured traits are insufficient to buffer against gender-specific mortality, assuming that the biases in sex ratios observed are the result of gender-specific mortality. Measuring sexual dimorphism in common gardens in plants originating from populations sampled across the species range should allow us to disentangle evolutionary from plastic responses (Puixeu et al. 2019). Common gardens in particular could help to identify associations between sex ratios and plant morphologies that simply result from spatial variation in resource availability in natural populations with joint effects on mortality and access to resources. Fully understanding the interplay between ecology and sexual dimorphism also requires integrating a temporal dimension, because plant size differences can change direction with plant age or status (Teitel et al. 2016). Variation of sex ratios along

climatic or elevation gradients may generally result from differences in phenology, especially given that males tend to flower earlier than females in angiosperms (Forrest 2014). Yet the variation in sex ratio observed along both elevation and climatic gradients in our study were not strongly affected by differences in phenology, given that we found no association between sex ratios and the proportion of flowering plants.

Hultine et al. (2016) drew attention to the potential risk of climate change in causing male-biased sex ratios, based on the assumption of higher costs of reproduction for females. However, the variation we have observed in sex ratios and sexual dimorphism along environmental gradients in three dioecious plant species highlight the potential complexity of effects of differential costs of reproduction between the sexes, at least in wind-pollinated plants in which flowering implies heavy costs on males, too. In all three studied species, the sex-specific variation in morphology and sex ratios cannot be seen through the lens of the cost of reproduction for one sex. Given that the evolution of dioecy tends to be more frequent in clades with abiotic pollen dispersal (Renner and Ricklefs 1995), a high cost of reproduction may be important in both sexes in many dioecious species, and is likely to vary with environment. Future studies should monitor temporal change in sex ratio in relation with ongoing ecological changes, and investigate whether plant traits associated with reproductive female or male costs (e.g. wind-pollination, fleshy fruits, biotic fruit dispersal) mediate sex-specific plant responses to climate change.

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Author contributions

John R. Pannell and **Jeanne Toonnabel** are co-senior authors. **Sarah Bürli**: Conceptualization (supporting); Formal analysis (supporting); Investigation (lead); Writing – original draft (equal); Writing – review and editing (supporting). **John R. Pannell**: Conceptualization (equal); Funding acquisition (lead); Supervision (equal); Writing – review and editing (lead). **Jeanne Tonnabel**: Conceptualization (equal); Formal analysis (lead); Investigation (supporting); Supervision (equal); Visualization (lead); Writing – original draft (equal); Writing – review and editing (lead).

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.z612jm6df> (Bürli et al. 2022).

Supporting information

The Supporting information associated with this article is available with the online version.

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