

# Negative effects of high-temperature stress on gametophyte performance and their consequences for seed reproduction in wild plants

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## ABSTRACT

(1) Plant regeneration by seeds is highly sensitive to temperature stress, particularly in the gametophyte stage. However, most of the existing research has focused on one single stage of gametophyte development and/or conducted using cultivated or a very few model species. Thus, it is unclear whether the results of such studies can be applied to natural populations. (2) To fill this gap, we investigated a) the effects of chronic heat stress (CHS; 17 days) at 35/30 °C (moderate stress) and 40/35 °C (severe stress) on gametophyte performance, and b) how these effects translated into seed quantity and quality. We measured six traits related to male (anther length, pollen production and size) and female (ovary length, ovule production, and size) gametophyte performance and leaf chlorophyll fluorescence (Fv/Fm) in four wild *Silene* species. The ripe seeds of the treated plants were used to measure seed mass and seed production; the seed germination was characterized in terms of germination percentage, speed, and synchrony. (3) Fv/Fm decreased significantly in both heat treatments, confirming a negative effect of CHS on overall plant performance. All male gametophyte traits decreased significantly in both CHS treatments compared to the control. The length and size of the ovary were significantly smaller in the 40/35 °C treatment than in the 35/30 °C treatment and the control, while ovule production decreased significantly in both CHS treatments compared to the control. The negative effects on gametophyte performance translated into significantly fewer seeds in the 35/30 °C and 40/35 °C treatments compared to the control. CHS treatments did not affect the seed mass. The final germination percentage differed weakly significantly between the severe treatment and the control but did not show any negative impacts by heat stress, whereas seed germination was significantly faster in the treated plants, both moderate and severe. Germination synchrony was not affected by heat treatments. (4) The high sensitivity of gametophytes in vascular plants to high-temperature stress implies that climate change-associated heat waves can significantly impact seed reproduction in wild plants. The altered seed quantity could have potential consequences for the long-term survival of the wild plant populations and the performance of the granivores.

## 1. Introduction

The close relationship between the environment and the success of plant reproduction has long been known. Numerous studies have shown that exposure of plants at various plant reproductive phases to (a)biotic stress almost always results in altered seed quantity and quality (Wahid et al., 2007; Hedhly, 2011; Fahad et al., 2017). In the last two decades, interest in the possible effects of high temperatures on plant performance has increased considerably, due to recent climate change (Gitay et al., in press; Solomon et al., 2007; Hedhly et al., 2009). To begin with,

some published evidence has indicated that global warming can have positive effects on sexual reproduction in certain plants (Hedhly et al., 2009; Raza et al., 2019). These effects include longer and warmer flowering periods (e.g., 0.6-day longer flowering per 1 °C of warming, Miller-Rushing et al., 2007; Zhou et al., 2022), along with elevated carbon dioxide levels (e.g., a 2.3-day boost in spring wheat growth at 550 ppm, Streck, 2005). These changes are expected to enhance photosynthesis, potentially increasing plant growth and seed production (Reyes-Fox et al., 2014; Dusenge et al., 2019; Bhargava and Mitra, 2021). However, any potential benefits may be offset by other negative

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impacts of climate change (Orsenigo et al., 2014; Hatfield and Prueger, 2015). For example, the accelerated rise of air temperatures has also resulted in the increased severity, frequency, and duration of extreme temperature fluctuations (eg, cold snaps and heat waves; Orth et al., 2016; Ummenhofer and Meehl, 2017; Cardell et al., 2020). These extreme weather events are known to have negative effects on flower bud formation, flowering, fruit ripening, and seed germination, resulting in lower seed yields and seed quality (Hatfield and Prueger, 2015; Raza et al., 2019; Yadav et al., 2022). In turn, altered rates of viable seed production could have profound ecological consequences. For instance, low seed input can lead to a decline in the abundance of seed plant populations and ultimately to the extinction of the species (Turnbull et al., 2000; Willis et al., 2008). Furthermore, in natural plant populations, fluctuations in fruit and seed production can affect other trophic levels, given the complex interactions between plant reproduction and the diets of many animal species (Lewis et al., 2014; Bogdziewicz et al., 2016). Finally, a low seed yield in crop production could have negative consequences for food security (for example, estimated crop yields are expected to decline 30% by 2050; Bapna et al., 2019).

In general, plants can maintain their vegetative growth and development over a relatively wide range of temperatures, approximately between  $-10$  and  $+60$  °C (Luo, 2011; Nievola et al., 2017). However, a remarkable temperature sensitivity has been attributed to the gametophytic phase (that is, the stages from gametophyte formation to fertilization) that has a much narrower range of optimal temperatures for functioning between  $10$  and  $30$  °C (Luo, 2011; Żróbek-Sokolnik, 2012; Nievola et al., 2017). Experimental and observational studies have shown that even slight increases in temperature above the optimum during the gametophytic phase can alter plant reproductive ability by negatively affecting micro and macrosporogenesis, reducing the number and viability of pollen grains deposited on the stigma, subsequent fertilization of the ovules, and increasing rates of embryo abortion (Hedhly et al., 2009; Zinn et al., 2010; Hedhly, 2011; Arshad et al., 2017). Importantly, among gametophytic organs and tissues of angiosperms, pistil-protected ovules are more tolerant to abiotic stress than pollen grains and anthers (Zinn et al., 2010; Hedhly, 2011; Raja et al., 2019). The increased sensitivity of pollen compared to ovules is due to its comparatively small size, haploid set of chromosomes, lack of protective tissue, and direct exposure to the environment at anthesis (Bedinger, 1992; Pacini and Dolferus, 2016; Lohani et al., 2020).

Despite the large body of research on the effects of high-temperature stress on angiosperm gametophyte performance, there remain several gaps and biases. To begin with, the emphasis is often on the effects of individual gametophyte stages, either on microgametogenesis (see, e.g., Elshookie et al., 2021), microsporogenesis (see, e.g., Porch and Jahn, 2001; Iovane and Aronne, 2022), megasporogenesis (see, e.g., Shi et al., 2022); or gamete fusion (see, e.g., Dupuis and Dumas, 1990; Snider and Oosterhuis, 2012). Although convenient in terms of labor and time costs, such studies often do not provide a comprehensive picture of high-temperature effects on overall regeneration by seed. First, different stages of gametophytes differ in their thermotolerance (Hedhly, 2011; Raja et al., 2019); therefore, the negative effects of temperature on one stage may be amplified (Wang et al., 2017) or overcompensated (Raja et al., 2019) on the others. Second, not every study has considered the downstream consequences of temperature stress-altered performance of gametophytes on seed quality and quantity (eg, Morrison and Stewart, 2002; Prasad and Djanaguiraman, 2014; Ye et al., 2015; Mácová et al., 2021). Due to several compensatory mechanisms (eg increased production of heat shock proteins, increased antioxidant capacity, changes in gene expression; Hasanuzzaman et al., 2013; Chaturvedi et al., 2021), pollen and ovule performance altered by heat stress are not necessarily translated into lower seed quality and quantity (Cross et al., 2003; Jiang et al., 2020; Choudhary et al., 2022).

Finally, the commonly accepted high sensitivity of gametophytes to temperature stress is based mainly on the experimental work carried out with cultivated species (eg, rice, cotton, tomato; Lohani et al., 2020) or a

few model species cultivated indoors (eg, *Arabidopsis thaliana*; Bac-Molenaar et al., 2015). The experimental populations used in such studies might be either preselected to specific temperature conditions (i.e., the cultivars of cultivated species; Xu et al., 2021; Alsamir et al., 2021) or lack specific adaptations to the environmental conditions of the growing sites/chambers (e.g., mutants in model species; Stephenson and Bertin, 1983; Lyndon, 1992). On the contrary, studies on gametophyte performance under temperature stress in wild species are extremely scarce and are limited to plants grown under conditions with unclear levels of stress exposure to extreme temperatures (eg, Steinacher and Wagner, 2012; Wagner et al., 2016; Rosbakh and Poschlod, 2016). Thus, it is unclear whether the results of such studies can be applied to natural populations.

Here, we address these knowledge gaps by assessing how exposure of wild herbaceous plants in the reproductive stage to heat stress affects their gametophyte performance at various stages and how these effects are translated into seed quantity and quality. Specifically, in a fully controlled experiment, we exposed individuals of four wild *Silene* species (*S. coeli-rosa*, *S. gallica*, *S. laeta*, and *S. noctiflora*) to chronic heat stress (CHS) treatments at  $35/30$  °C (moderate CHS) and  $40/35$  °C (severe CHS) for 17 days. To estimate the effects of CHS on *Silene* reproduction by seed, we measured six plant traits related to the performance of male gametophytes (anther length, pollen production, and size) and female gametophytes (ovary length, ovule production, and size). We also measured leaf chlorophyll fluorescence and flowering phenology to assess the effects of treatments on overall plant performance. The ripe seeds of the treated plants were used to measure seed mass and seed production, and to study their germination performance. The experimental data set was analyzed using linear mixed-effect models, to answer the following questions:

- 1) Are both the male and female gametophytes in wild plants sensitive to high-temperature stress and, if so, to what extent? Similarly to previous research on cultivated species (see, e.g., Lohani et al., 2020), we anticipated that high temperature stress in wild plants during the reproductive phase will negatively affect both male and female gametophyte performance. Existing studies have indicated that heat stress can cause abnormalities in pollen/ovule development, resulting in decreased fertilization success. However, natural plant populations might show differences in response to heat stress because they react differently to environmental stressors due to natural selection pressure than cultivated species that undergo selective breeding (Lippmann et al., 2019). Furthermore, we expected that the negative effects of heat stress would be stronger in the traits of male gametophytes than their female counterparts, due to the lack of protective tissue and direct exposure to the environment at anthesis in pollen (Bedinger, 1992; Pacini and Dolferus, 2016).
- 2) Are the negative effects on gametophyte performance translated into lower seed quality and quantity? Since heat stress negatively affects plant growth and development, with particularly strong effects on sporogenesis (Djanaguiraman et al., 2018), we expected stressed plants to produce fewer ovules and pollen of lower quality, which would ultimately lead to reduced seed mass and production and altered germination patterns.

## 2. Material and methods

### 2.1. Study species and experimental setup

We selected four herbaceous species of the genus *Silene* (campion or catchfly, Caryophyllaceae) that commonly occur in a range of different climates and non-forest habitats in Europe (Table 1). The genus *Silene* has been traditionally used in ecological studies (Alatalo and Totland, 1997; Ferrarini et al., 2019; Kahl et al., 2019), because champions are easy to breed and have short life cycles, making experimental studies convenient (Bernasconi et al., 2009). The selected *Silene* species all have hermaphroditic flowers (i.e., both male and female reproductive organs

**Table 1**

The characteristics of the study species based on (Brickell, 1996; Phillips and Rix, 2002; Pilkington, 2007; Farris et al., 2013).

Species	Distribution	Ecology	Origin of the seed material
<i>Silene coeli-rosa</i> (L.) Godr.	Southwestern Europe, North Africa, and the Canary Islands	Occurs in wastelands, railway yards, and in damp, grassy places such as riverbeds	Saint Florent, Corsica, France
<i>Silene gallica</i> L.	Native to Europe, North Africa and Western Asia	Occurs in dry, open habitats, often along roadsides and wastelands	Rocher de Roquebrune, France
<i>Silene laeta</i> (Aiton) Godr.	Native to North Africa and Southwestern Europe	On wet soils, sometimes in stagnant water	Compomoro Corsica, France
<i>Silene noctiflora</i> L.	Native to Eurasia	Arable land on dry, sandy and calcareous soils	Blaufelden, Germany

in the same flower) with a short period to reach sexual maturity from seed of about seven to nine weeks. Only *Silene laeta* is a short-lived perennial, while the other *Silene* species (*S. coeli-rosa*, *S. gallica*, and *S. noctiflora*) are annual.

The study was carried out in the greenhouse of the University of Regensburg, Germany, from November 2021 to July 2022, under fully controlled conditions. The experimental plants were propagated from seeds collected in the natural habitats of the species (*Silene coeli-rosa*, *Silene gallica*, and *Silene laeta*) or cultivation conditions similar to those of the natural (*Silene noctiflora*). Several hundreds of seeds of each *Silene* species were initially seeded in trays (60 cm × 40 cm) filled with substrate (three parts of low-nutrient planting soil [CL Pikier- Einheitserde], three parts of coarse sand [Geser GmbH, Germany], and one part of dry compost soil [CL Topf Einheitserde]). The juvenile plants (approximately two weeks after germination) were transplanted into 9 cm pots, three plants per pot, filled with the same soil substrate. After a growing period of about one month, the plants were again separated and repotted into individual 9 cm pots (i.e. one plant per pot). At each repotting step, we selected only healthy plants and those in a similar phenological state, to ensure similar treatment effects on all individuals tested (see below). The pots were randomly rearranged and regularly watered within the greenhouse during germination and cultivation. All plants were grown in similar greenhouse conditions with day/night temperatures of 20/15 °C and natural illumination supported with additional lighting (Osram Plantastar 400 W, Osram, China) to achieve a photoperiod of 12 h in winter months (November – April). To promote blooming in *Silene laeta* we applied growth and flowering fertilizer ('Wuxal Super', rWuxal, Germany).

The experimental setup consisted of six similar grow chambers (Homebox Vista Medium, rHOMEbox, Germany; Supplementary Fig. S2): two chambers with day/night temperatures of 35/30 °C (moderate chronic heat stress [CHS]), two chambers with 40/35 °C (severe CHS) and two used as a control (30/25 °C). The chambers were equipped with two heating mats ('Fyto heat Deluxe', Schilling Phyto-technik GmbH, Italy, and 'Heating mat aluminium', rBio Green, Germany), to heat the chambers to test temperatures, and three photosynthesis-powered LED lamps ('Sanlight Flex 20 W', SANlight, Austria) for illumination. Thermostats ('Thermo2', rBio Green, Germany) in each box and a control panel ('dnt RoomLogg Pro', dnt, China) were used to measure and maintain the temperature at a constant level.

For heat treatments, we selected a total of 36 plants per species in a similar phenological stage (first visible flower bud). The plants were randomly divided into three groups of 12 individuals and grown under the three temperature conditions in a random block design for 17 days. The duration of treatment was selected to ensure that pollen and flowers of different stages of development received stress treatment (Mesihovic et al., 2016). Further, it corresponds with the observed and predicted

heat waves that European plants experience or will experience during the sexual reproduction process (Lin et al., 2022; Lhotka and Kysely, 2022).

To avoid the drought stress associated with high temperatures, a steady state of soil humidity was maintained during application of heat stress by regular watering. Subsequently, all plants were returned to the greenhouse at constant temperatures of 20/15 °C, and natural illumination was supported by additional lights (Osram Plantastar 400 W, Osram, China) to allow seed ripening.

## 2.2. Plant trait measurements

To evaluate the effects of treatments on overall plant performance, we measured leaf chlorophyll fluorescence ('Pocket PEA', Hansatech, Germany), on the last day (17) of treatments. Leaf clips were applied to ten mature leaves of ten different individuals per heat treatment and species to allow dark adaptation. After an adaptation period of about 20 min (Maxwell and Johnson, 2000), the photosynthetic rate was measured and the maximum quantum yield (efficiency) of PS II photochemistry (Fv/Fm) was calculated by the tool.

## 2.3. Flowering phenology

To estimate the effects of heat treatment on flower development during the experiment, we also made regular (every three or four days, i.e. a total of six observation days) phenological observations of all treated plants. On each observational day, we recorded the number of individuals at the peak of flowering (i.e., 50% of all flowers on an individual plant are open).

## 2.4. Gametophyte traits

To estimate the effects of heat treatment on sexual reproduction, we measured six traits related to male (anther length, pollen production, and size) and female (ovary length, ovule production, and size) gametophyte performance. For measurements, we selected from ten to twelve open flowers in full anthesis per species per treatment and control on day 17 of the heat treatment and preserved them in 70% ethanol for further analysis. In the laboratory, the flowers were dissected under a stereomicroscope to randomly select eight ovaries and eight anthers to be photographed for measurements. Scaled images of ovaries and anthers were used to measure their length using ImageJ software (Schneider et al., 2012). Anther length is defined as the average of the lengths of both thecae (Vries, 1974) while the ovary length is defined as the sum of proximal length, placenta length and distal length (Damodharan et al., 2016).

The ovaries were further dissected under the stereomicroscope, ovule images were taken, and the number of ovules was counted using ImageJ software (Schneider et al., 2012). The same images were used to measure the size of the ovules with the help of ImageJ software. To determine the size and number of pollen, a whole anther per replicate was placed in 100 µl of deionized water in an Eppendorf tube. The anther was then crushed and vortexed to achieve uniform pollen dispersion, and 5 µl of the solution was pipetted onto a microscope slide. Scaled images of the solution were taken under a microscope (Nikon Eclipse TS100); the images were analyzed for pollen size and number in ImageJ software. Pollen count in 5 µl was used to estimate the number of pollen grains released per anther of stock solution (100 µl).

## 2.5. Seed mass and production

To estimate the downstream consequences of the effects of heat stress on gametophyte performance, we also measured seed mass and seed production (number of seeds produced per individual plant) in treated plants. To achieve this, the treated plants were left in the greenhouse for about three months until full seed maturation; the plants

were bagged with organza bags to prevent seed loss. For the measurements, we collected all seeds from ten plants per species and treatment, which were consequently counted and weighed. The average weight of an individual seed was calculated as the total weight of seeds divided by the number of seeds per individual. After the measurements, the seeds were stored at 4 °C prior to the seed germination experiments to preserve their viability.

## 2.6. Seed germination traits

*Silene* seeds germinated in climate chambers (Panasonic, MIR-254) at 22/14°C (photoperiod 14/10 h of light/dark regime), in five replicates with 20 seeds each. Germination was scored every other day for 15 days (most seeds germinated during the first week of the trial). The viability of the non-germinated seeds was tested using the ‘cut’ test: seeds with a white, firm embryo and endosperm were considered viable (Ooi et al., 2004). Non-viable seeds were excluded from the analysis.

Seed germination was characterized by (1) final germination percentage (FGP; seed ability to complete the germination process), (2) mean germination time (MGT; a proxy for germination speed with lower values indicating faster germination), and (3) germination synchrony. Germination synchrony was estimated by calculating the Z synchronization index (Lozano-Isla et al., 2019), which varies from 0 (events of seed germination were evenly spread throughout the entire incubation period) to 1 (all seeds germinated at the same time). The three traits were calculated for each replicate (Petri dish) in each treatment.

## 2.7. Statistical analysis

All data analyses were performed with R software version 4.2.0 (R Core Team, 2023).

The effects of heat treatments on plant performance traits were analyzed using generalized linear mixed effect models conducted with the help of the *lme4* and *lmerTest* packages in R (Bates et al., 2015; Kuznetsova et al., 2017). In each model, the trait of interest (Fv/Fm, gametophyte, or seed trait) was included as a response variable, heat as fixed effects, and species identity and experimental block (tray) as random factors. Differences among treatment effects were estimated with the help of the post hoc Tukey test ( $p < 0.05$ ), implemented in the packages *emmeans* and *multcomp* (Hothorn et al., 2008; Lenth, 2023). GLM for FGP data included family ‘binomial’ (logistic regression), while the remaining traits were analyzed using family ‘Gaussian’ (‘simple’ linear mixed effect models).

Trait data of anther length, pollen number, pollen size, and seed mass were first log-transformed as  $\log(x + 1)$  to improve the normality of residuals. All the model assumptions were met in all the cases.

## 3. Results

### 3.1. Leaf chlorophyll fluorescence

CHS treatments had a significant negative effect on overall plant performance (measured as photosynthesis capacity via leaf chlorophyll fluorescence) in experimental plants, with the strongest effects in severe CHS (mean Fv/Fm values: control = 0.81, 35/30°C = 0.74, 40/35°C = 0.67; Fig. 1, Table 2).

### 3.2. Flowering phenology

In general, most of the individuals flowered during the 17-day heat treatments, except for a few species x treatment combinations where flowering was delayed. Specifically, *S. gallica* and *S. laeta* plants did not show differences in flowering phenology in all three treatments (Fig. 2). In both species, flowering began immediately, increasing exponentially in *S. laeta* and gradually in *S. gallica* throughout the observation period.

In *Silene coeli-rosa*, the individuals in all the treatments started to flower approximately on the sixth day. Subsequently, flowering gradually increased in the 30/25 °C and 35/30 °C treatments, while it was generally low in the 40/35 °C treatment. In *Silene noctiflora*, flowering increased exponentially in the control (30/25 °C), gradually increased at 35/30 °C, while flowering generally ceased in the treatment at 40/35 °C (Fig. 2).

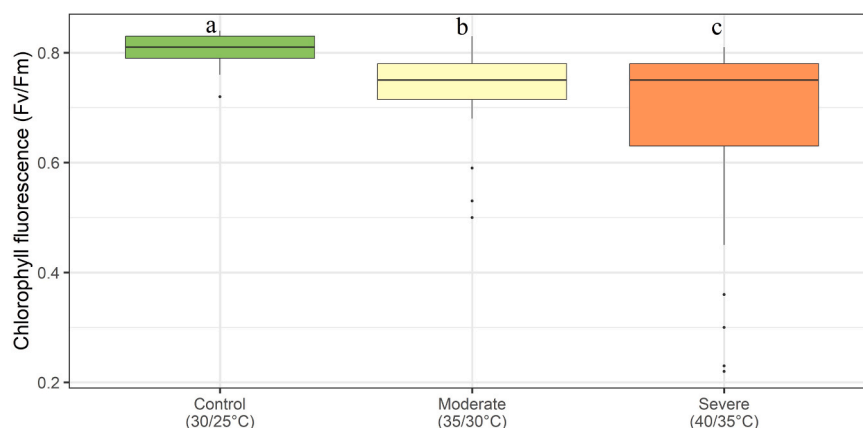
### 3.3. Gametophyte traits

#### 3.3.1. Female gametophyte traits

The effects of CHS treatments on ovary length exhibited a significant decrease in the 40/35 °C treatment (mean ovary length = 0.87 mm) compared to the 35/30 °C treatment (1.22 mm) and the control (1.38 mm) (Fig. 3A, Table 2). Similar effects were also detected for the mean ovule size, with significantly smaller ovules produced in the 40/35 °C treatment (control: 94 μm, 35/30 °C: 95 μm, 40/35 °C: 84 μm; Fig. 3C, Table 2). Similarly, ovule production was strongly negatively affected by CHS treatments, being the lowest in the 40/35 °C treatment (control: 111 ovules, 35/30 °C: 81, and 40/35 °C: 50; Fig. 3B, Table 2).

#### 3.3.2. Male gametophyte traits

The three male gametophyte traits showed a significant negative response to heat stress treatments, with significantly larger effect sizes in the 40/35 °C treatment compared to the control and the 35/30 °C treatment (Fig. 4, Table 2). Specifically, mean anther length (0.14 mm), pollen number (50) and pollen size (23.58 μm) were significantly lower in the 40/35 °C treatment than in the 35/30 °C treatment (0.39 mm,



**Fig. 1.** Effects of chronic heat stress treatments on leaf chlorophyll fluorescence (Fv/Fm) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30°C and severe – 40/35°C) as deduced from generalized linear mixed effect models and post-hoc Tukey test ( $p < 0.05$ ).



**Table 2**  
Results of the linear mixed effects models and post-hoc Tukey tests for the chronic heat stress effects on leaf chlorophyll fluorescence (Fv/Fm), female and male gametophyte, and seed traits. Bold values indicate significant treatment effects ( $p < 0.05$ ). Different letters indicate significant differences between the control and two treatments as induced by the Tukey Post-hoc test ( $p < 0.05$ ). SE – standard error.

Treatment	Fv/Fm			Ovary length (mm)			Ovule count			Ovule size ( $\mu\text{m}$ )			Anther length (mm)			Pollen count			Pollen size ( $\mu\text{m}$ )			Seed count			Seed mass (mg)			Final germination percentage (%)			Mean germination time (Days)			Germination synchrony		
	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$	Mean	SE $\pm$				
30/25 °C (control)	0.81a	0.02	1.38a	0.33	111a	22	93.86a	10.5	0.66a	0.09	1782a	788	44.69a	2.85	1368a	348	0.44a	0.22	91a	6	5.3a	0.3	0.54a	0.06												
35/30 °C (moderate heat stress)	<b>0.74b</b>	0.02	1.22a	0.10	<b>81b</b>	10	94.69a	3.5	<b>0.39b</b>	0.05	<b>1003b</b>	327	<b>37.68b</b>	1.11	<b>796b</b>	97	0.43a	0.02	89a	2	<b>5.0b</b>	0.3	0.62a	0.06												
40/35 °C (severe heat stress)	<b>0.67c</b>	0.02	<b>0.87b</b>	0.10	50c	10	<b>84.45b</b>	3.8	<b>0.14c</b>	0.05	50c	327	<b>23.58c</b>	1.34	<b>744b</b>	99	0.41a	0.02	<b>96b</b>	2	<b>4.9b</b>	0.3	0.59a	0.06												

1003, and 37.68  $\mu\text{m}$ ), and significantly lower in the 35/30 °C treatment than in the control (0.66 mm, 1782 and 44.69  $\mu\text{m}$ , respectively).

### 3.4. Seed traits

#### 3.4.1. Seed mass and production

Both heat treatments resulted in a significantly lower number of seeds produced (35/30 °C treatment, 796 seeds, 40/35 °C, 744) than in the control (1368) (Fig. 5A, Table 2). There were no significant differences in the mean seed mass across all species exposed to the different CHS treatments (Fig. 5B, Table 2).

#### 3.4.2. Seed germination traits

In general, most of the *Silene* seeds, regardless of species or treatment, germinated quickly and with high final percentages, as high germination was already achieved on the fifth day of incubation (Fig. 6A). The GLM revealed only a small, yet significant difference between the final germination percentage control (91%) and the severe heat treatment (96%). No clear treatment effects were detected in this trait.

The seed germination speed (Fig. 6C) was positively affected by both treatments (mean germination time for control 5.3 days, moderate stress – 5.0 days, and severe stress – 4.9 days), although the effects sizes were relatively small. We did not detect significant differences in seed germination synchrony.

## 4. Discussion

### 4.1. Heat stress negatively affects gametophyte performance in wild plants

Confirming our expectation, chronic heat stress (CHS) treatments had significant negative effects on the traits of female and male gametophytes in the four wild *Silene* species. Except for ovary length and ovule size in the moderate CHS (35/30 °C), the treated plants had smaller anthers and ovaries that contained smaller numbers of pollen grains and ovules, respectively, of smaller sizes. These negative effects were more pronounced under severe CHS (40/35 °C). Therefore, our study confirms previous findings for cultivated and model species that heat stress is an essential factor that negatively affects the gametophytic performance of vascular plants (Prasad et al., 2008; Raja et al., 2019; Hedhly et al., 2020). These findings further suggest that exposure of plants occurring in natural populations to heat stress (for example, during climate change-associated heat waves) at the flowering stage can considerably affect reproductive success (Zinn et al., 2010; Mesihovic et al., 2016; Hedhly et al., 2020).

Chlorophyll fluorescence measurements revealed that photosynthetically active vegetative tissues were also negatively affected by heat stress treatments. This reduced the overall performance of the treated *Silene* plants and could also have indirect negative effects on the anther/pollen and ovary/ovule traits. Although not measured in this study, the low Fv/Fm values measured could indicate that the photosynthetic apparatus and the PSII complex were damaged by heat stress and did not function at their maximum capacity (Sharma et al., 2015; Shanker et al., 2022). Reduced photosynthetic rates could limit the production, transport, and allocation of photosynthetic assimilates from leaves to gametophytic tissues and organs (Sharma et al., 2012; Poudyal et al., 2018). In turn, reduced carbohydrate supply to pollen grains and pistils results in low energy production (Adenosine triphosphate (ATP)) and resources required for normal growth, development, and fertilization of female and male gametophytes (Snider et al., 2009). Additionally, the heat stress could significantly increase dark respiration (Timlin et al., 2006) and photorespiration (Jiao and Grodzinski, 1996), which further depletes the carbohydrate supply for developing gametophytes.

The poor performance of the female and male gametophytes under heat stress could be due to direct and indirect high-temperature effects on plant sexual reproduction. As for the former, heat stress can directly

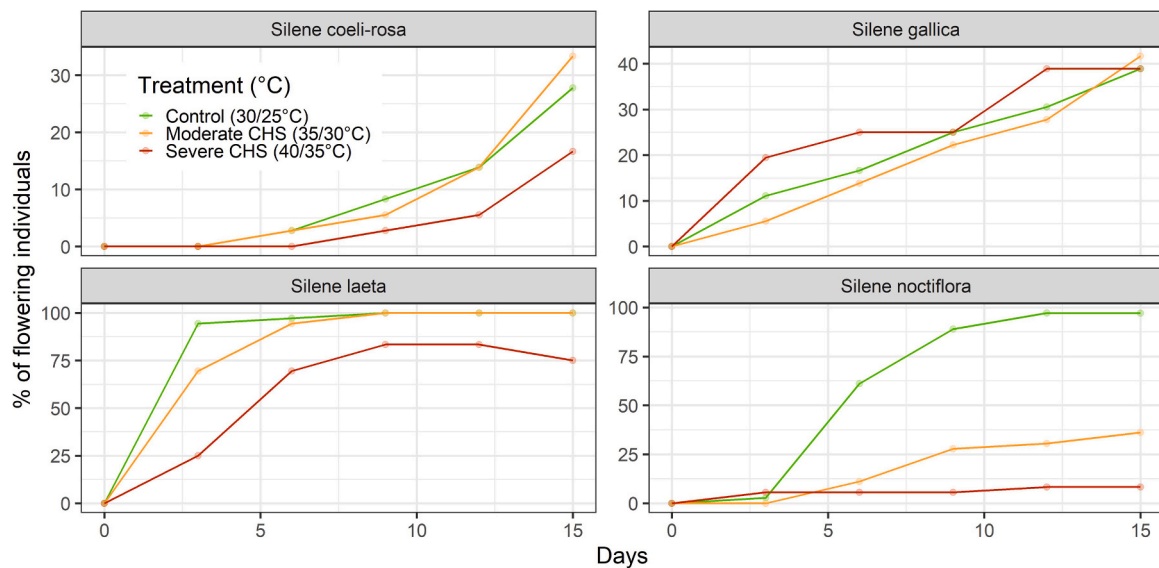


Fig. 2. Percentage of flowering individuals observed during chronic heat stress treatments in the four *Silene* species.

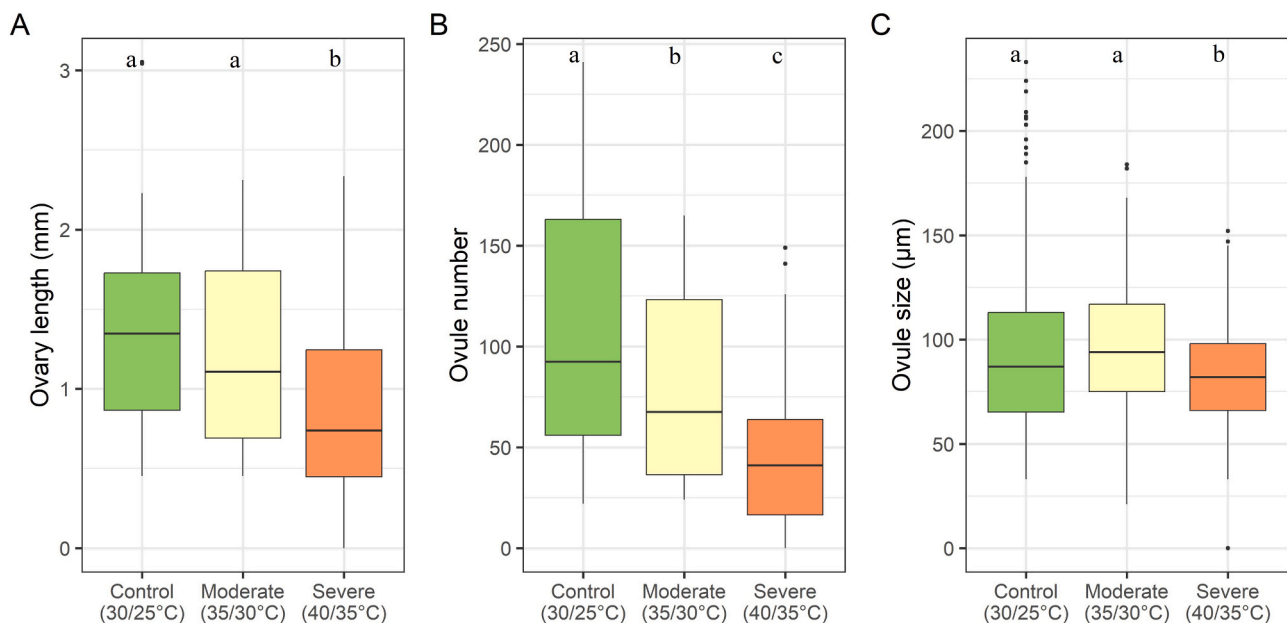
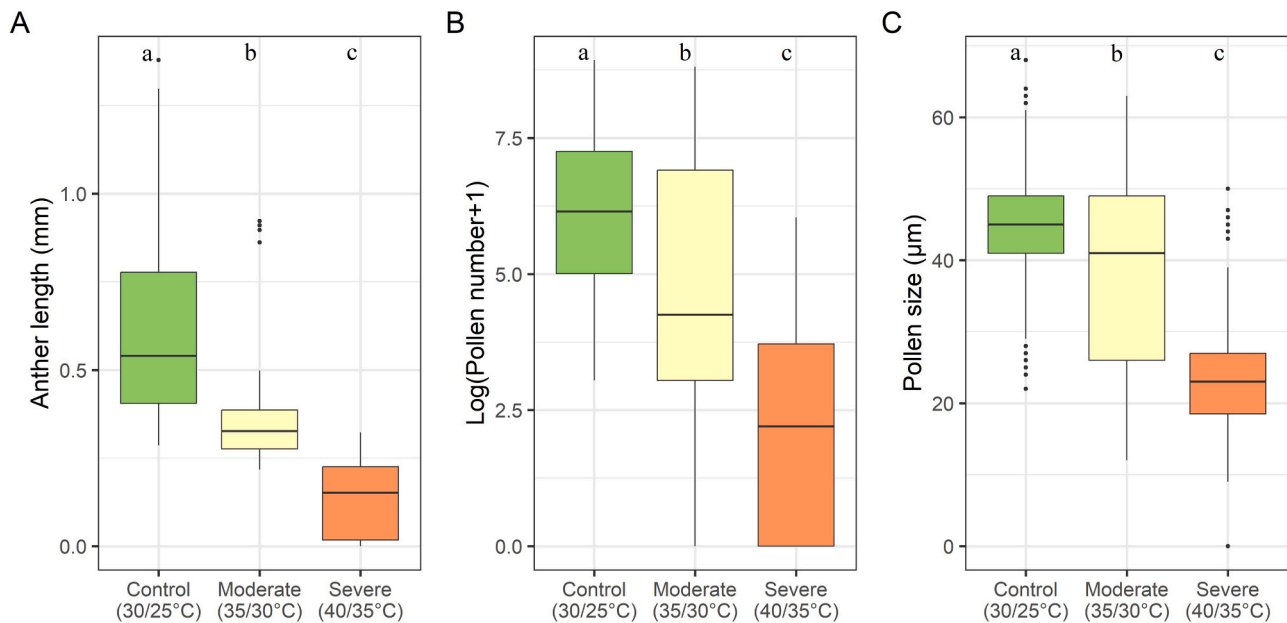


Fig. 3. Effects of chronic heat stress treatments on mean ovary length (A), ovule number (B), and ovule size (C) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30°C and severe – 40/35°C) as deduced from generalized linear mixed effect models and post-hoc Tukey test ( $p < 0.05$ ).

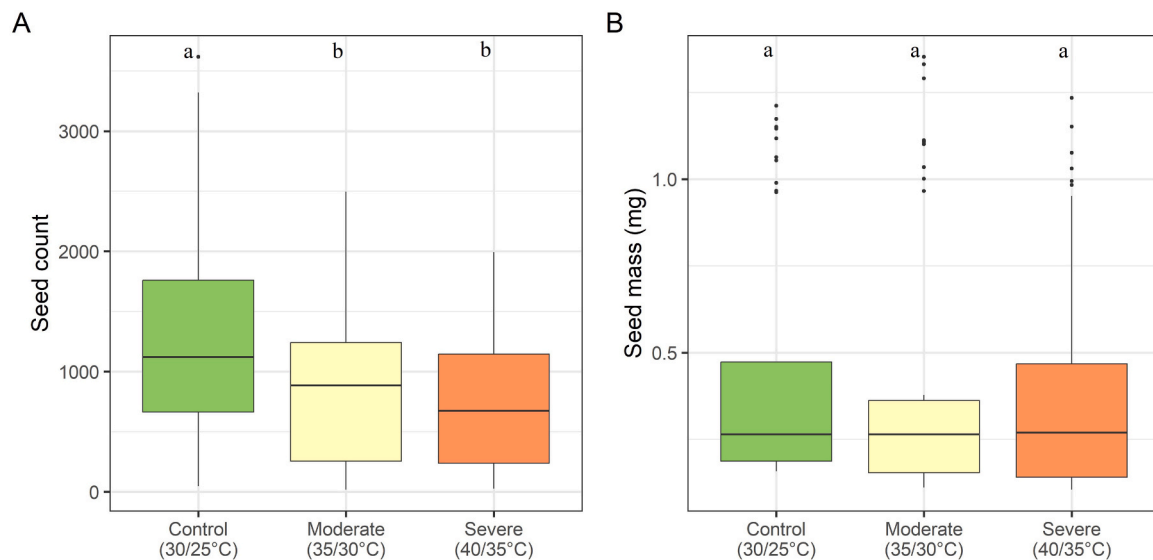
affect enzymatic activity (e.g., reduced starch and/or sugar biosynthesis), and metabolic processes (e.g., glycolysis, a main metabolic pathway primarily responsible for capturing energy in the form of ATP) required for optimal gametophyte development (Hasanuzzaman et al., 2013; Kumar et al., 2022). Disruption of these processes affects the normal production and accumulation of key components, such as carbohydrates, proteins, and lipids, required for cell growth and function (Wahid et al., 2007; Sehgal et al., 2018). Furthermore, in the female gametophyte, heat stress can cause a decline in auxin levels, a hormone that promotes cell growth and division in the ovary (Ruan et al., 2012; Wang et al., 2021; Kaur et al., 2021). This can lead to a significant decrease in ovary length (see, e.g., Wang et al., 2021), as also observed in our study with severe CHS treatment (Fig. 3A). The heat stress-induced disruption of normal ovary development appears to transfer the negative effects to the ovules, resulting in their reduced

production and size (Fig. 3B and C, Table 2). Moreover, the meiotic phase during ovule production is highly sensitive to heat stress (eg Prasad and Djanaguiraman, 2014; Shi et al., 2022; Choudhary et al., 2022) which potentially contributes to the significant reduction in ovule number at both moderate and severe CHS treatments (Fig. 3B).

Similarly, in the performance of male gametophytes, heat stress can affect the resources required during the critical stages of anther development and differentiation (Chaturvedi et al., 2021). Disrupting normal processes of anther formation can lead to premature anther maturation and dehiscence before they reach their full size (length; Snider and Oosterhuis, 2012; Raja et al., 2019). Anther length is a critical determinant in pollen production, since longer anthers typically produce more pollen grains (Harder and Barrett, 1993). The shorter anthers that produced fewer and smaller pollen grains than in the control (Fig. 4), as observed in the heat-stressed *Silene* plants, can confirm these



**Fig. 4.** Effects of chronic heat stress on mean anther length (A), pollen number (B), and pollen size (C) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30°C and severe – 40/35°C) as deduced from generalized linear mixed effect models and post-hoc Tukey test ( $p < 0.05$ ).



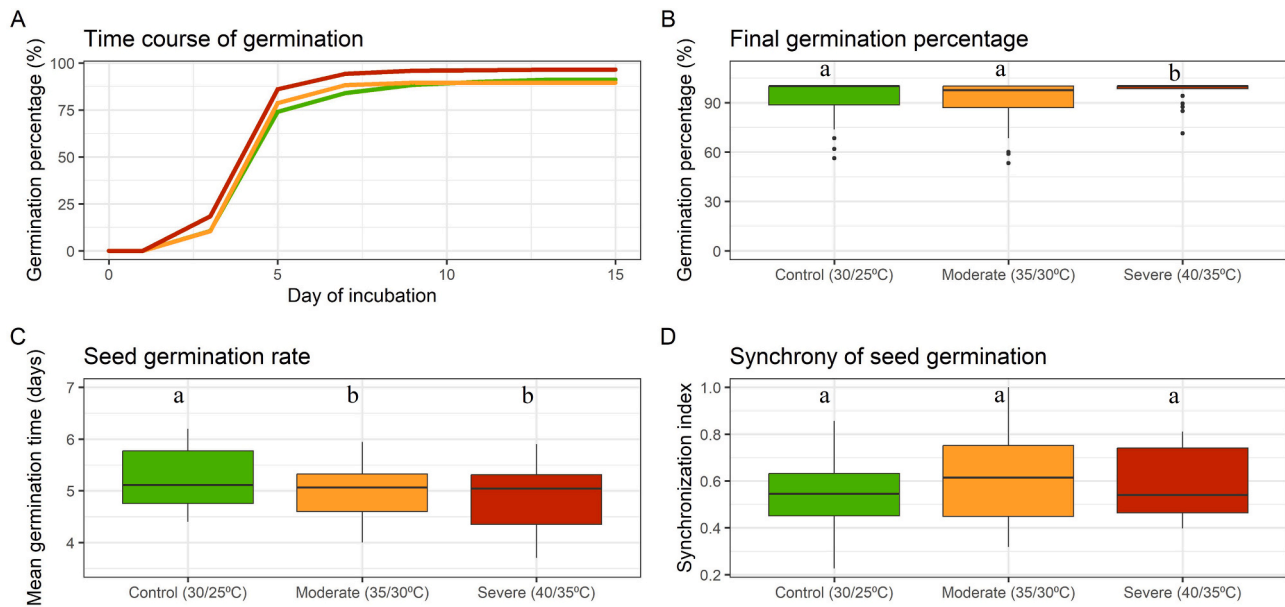
**Fig. 5.** Effects of chronic heat stress treatments on mean seed count (A) and seed mass (B) in the four *Silene* species. Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30°C and severe – 40/35°C) as deduced from generalized linear mixed effect models and post-hoc Tukey test ( $p < 0.05$ ).

observations made in cultivated species. Finally, at particularly high temperatures, heat stress can lead to rapid damage and death of gametophyte cells, especially during sporogenesis, resulting in a decrease in the overall number and size of pollen and ovules (Ahuja et al., 2010; Li et al., 2018; Iovane and Aronne, 2022), a possible explanation for the stronger negative effects of severe CHS (40/35 °C) applied to the test *Silene* species.

#### 4.2. The male gametophyte is more sensitive to heat stress compared to its female counterpart

When comparing the effects of heat stress on the characteristics of female and male gametophytes, we revealed that anthers and pollen

grains in *Silene* species were more sensitive to CHS than ovaries and ovules in the same flowers. According to our expectations, all male generative traits, anther length, pollen production, and size, were strongly negatively affected by moderate (35/30 °C) and severe (40/35 °C) CHS treatments, while female gametophyte traits, ovary length, and ovule size were significantly affected only by severe CHS treatment (40/35 °C). These findings are in line with previous studies showing that the male gametophyte is more sensitive to heat stress than their female counterparts (Zinn et al., 2010; Hedhly, 2011; Jagadish, 2020). The relatively thicker ovary tissues of the female gametophyte provide a protective environment that protects it from moderate abiotic stresses (Zinn et al., 2010; Hedhly, 2011). However, the lack of protective tissue in pollen makes them more sensitive to even mild abiotic stress



**Fig. 6.** Effects of chronic heat stress treatments on time course of germination (A), final germination percentage (B), seed germination speed (C), and germination synchrony (D). Letters indicate statistical differences between the control and two chronic heat stress treatments (moderate – 35/30°C and severe – 40/35°C) as deduced from generalized linear mixed effect models and post-hoc Tukey test ( $p < 0.05$ ).

(Bedinger, 1992; Pacini and Dolferus, 2016; Lohani et al., 2020).

#### 4.3. Heat stress effects on gametophyte performance are translated into lower seed quantity but not quality

The negative downstream consequences of gametophyte temperature stress-altered performance on seed quality and quantity have long been suggested but have been tested in a limited number of experimental studies, almost exclusively on crops or model species (eg *Arabidopsis thaliana*, Huang et al., 2014; wheat, Prasad and Djanaguiraman, 2014; rice, Ye et al., 2015; sorghum, Djanaguiraman et al., 2018). One of the most interesting results of our study is the significant reduction in seed production in wild *Silene* species (Fig. 5A) exposed to CHS. We attribute these results mainly to the fact that stressed plants produced less pollen and ovules in the 35/30 °C and 40/35 °C CHS treatments (Figs. 3B and 4B), which reduced the chances of successful pollination and fertilization. Furthermore, heat stress could impair pollen germination and tube growth (Walters and Isaacs, 2023), reduce pollen or ovule viability and/or stigma receptivity, leading to floret infertility (Nguyen et al., 2013; Prasad and Djanaguiraman, 2014). Alternatively, the lowered overall plant performance (that is, the lower photosynthetic rates in both heat treatments; Fig. 1) could have also contributed to the lower number of seeds, since the plant's ability to produce and store the necessary resources needed for seed production was reduced (see, e.g., Poudyal et al., 2018; Sommer et al., 2023).

In contrast to seed production, the average seed mass was not affected by the different CHS treatments (Fig. 5B). Several studies have shown that the environmentally induced reduction in seed production may be compensated by the increase in seed mass, due to the seed number-weight trade-off (by e.g., increasing availability of more assimilates to developing seeds; Huang et al., 2017). However, the negative effects of CHS on ovules (low number and size; Fig. 3) probably imposed a limit to the trade-off between seed mass and number (see, e.g., Lázaro and Larrinaga, 2018) resulting in the unaffected seed mass in four focal *Silene* species. Although seed numbers are generally more directly affected by reproductive processes and seed set, seed mass is a product of seed-filling rate and duration (Prasad et al., 2008). In our study, seed filling and maturation of treated *Silene* plants occurred after the CHS treatment ended leaving these states unaffected by heat stress.

In addition, seed mass is largely dependent on the availability of photosynthetic reserves (e.g., water and nutrients) during the seed-filling stage (Sehgal et al., 2018). Since water and nutrients were not limiting factors throughout our study, the lower number of seeds probably did not affect the source-sink balance of resources and thus did not have a major impact on seed mass (see, e.g., Wardlaw et al., 1980). Moreover, as an evolutionary adaptation, seed mass is generally more conservative than seed number for most species (Sadras, 2007).

Analyzing the seed germination process of the treated plants, we found no significant effects of heat treatment on the establishment of *Silene* seedlings. Although we detected significant differences in the final germination percentage (+5% Fig. 6B) and the germination speed (full germination 0.3–0.4 days earlier in both heat treatments than in the control, Fig. 6C), we assume that the detected deviations, most likely, would not have any impact on plant establishment in wild populations. We attribute the almost unchanged seed germination process in the experimental plants, similarly to the unaltered seed mass patterns, to the fact that seed filling and maturation were not affected by the heat treatments.

#### 4.4. Conclusions

In conclusion, our study adds a new important piece of evidence that heat waves associated with climate change can have significant and complex impacts on the reproductive process in wild plants. Specifically, we demonstrated that heat stress during gametogenesis can have negative cascading effects on seed quantity. These findings further imply that producing fewer seeds in a warming climate would inevitably result in a lower number of individuals that can; disperse to colonize new areas (Soons and Heil, 2002), successfully germinate and recruit in time and space (Jakobsson and Eriksson, 2000), and persist in changing environmental conditions (Long et al., 2015). This can decrease the species' ability to persist and expand its range, resulting in a more concentrated population with smaller population size and reduced genetic diversity over time (Long et al., 2015; Schierenbeck, 2017). Limited dispersal and persistent abilities alone or in combination with low genetic variation can limit the ability of a population to adapt to changing environmental conditions or to resist diseases and pests, making them more vulnerable to extinction (Aitken et al., 2008; Schierenbeck, 2017).



Finally, we admit that our findings cannot be extended to all wild plant species due to the study's limited scope (we only examined four herbaceous species within one genus, which inhabit diverse climates and non-forest habitats in Europe). Thus, caution should be exercised in applying these results to wild plants in general. Consequently, additional research should be conducted to establish a more comprehensive understanding of the impacts of heat waves on wild plants, encompassing a broader array of genotypes and/or species.

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### Authors' contributions

SR designed the study. DT, FA, EK, and SW conducted the experiment. DT analysed the data and led the writing process. All authors proofed and corrected the manuscript.

### Declaration of Competing Interest

The authors declare that they have no conflict of interest.

### Data Availability

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author/s.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2023.105532](https://doi.org/10.1016/j.envexpbot.2023.105532).

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