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Host plant and arbuscular mycorrhizal fungi show contrasting responses to temperature increase: Implications for dioecious plants

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ABSTRACT

Individual plants live in complex environments where they interact with other organisms such as herbivores, pollinators, fungi and pathogens. The influence of rising temperature on biotic interactions has begun to receive attention, and is an important research frontier currently. However, the belowground interactions with organisms such as arbuscular mycorrhizal (AM) fungi have received little attention so far. In this study, we investigated the response of the dioecious plant *Antennaria dioica* and its AM fungi to increased temperature in a controlled environment simulating the period of growth of *A. dioica* in central Finland. Specifically, we evaluated the effect of rising temperature on plant survival, growth, flowering and physiology in plants growing with or without AM fungi. Overall, increased temperature had a positive effect on plant survival, but a negative effect on the growth and flowering compared with the control temperature, while it did not affect the physiological parameters analyzed. Females suffered more of rising temperature in terms of reduced flowering, but a larger proportion of plants survived compared to males. In contrast, the rising temperature had positive effects on the frequency of AM fungal colonization in roots regardless of sex, but sex-specific differences were observed in the amount of extraradical hyphae and the number of spores produced. These findings suggest than the sexes in dioecious species and their associated fungi respond differently to increasing temperature. If rising temperature affects host plants and symbionts in a contrasting way, a potential functional mismatch might appear.

1. Introduction

Global average temperatures have increased 0.2 °C per decade since the 1970s (IPPC, 2007), and the increase is predicted to be most pronounced in cold climate. Global warming has already caused a number of changes in the natural life history of species (reviewed in Walther et al., 2002; Parmesan, 2006). Several studies have quantified the effects of global warming on the phenology and physiology of organisms as well as changes in the abundance and distribution of species (see Parmesan, 2006; Walther, 2010). Thus, direct individual responses to global change have been well characterized. However, species live in complex environments in which they interact with other organisms both above- and belowground, and studies using a more holistic approach are therefore needed. Recently, Gellesch et al. (2013) reviewed the effect of climate change on pairs of interacting organisms. Rising temperature has an effect on interactions independent from the interacting organisms (e.g. plant–plant, plant–herbivore, plant–fungus). Reports document positive (e.g. facilitative neighbor effects on survival, increased growth and reproduction, earlier developing) and negative (e.g. increases in seed predator populations, saprophagous macrofauna, grazing rates) effects (Gellesch et al., 2013). However, few studies deal with belowground interactions even though belowground organisms may alter plant responses to increased temperature (see Kivlin et al., 2013 for a recent review).

Among plant–fungus interactions, arbuscular mycorrhizal (AM) symbioses are the most abundant and widespread. AM fungi are obligate symbionts that colonize the roots of about 74% of angiosperm species and occur in almost all terrestrial ecosystems (Brundrett, 2009). Plants supply their associated AM fungi with carbohydrates that are essential for fungal survival and growth; the fungi may consume up to 30% of plant's carbohydrates (Jakobsen and Rosendahl, 1990; Drigo et al., 2010). In return, AM fungi form extensive networks of hyphae in the soil (extraradical hyphae) that forage for mineral nutrients more efficiently than the roots, and translocate them to their host plant in symbiotic-specific structures that develop inside roots (Smith and Read, 2008). Due

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to the improved nutrient acquisition, mycorrhizal symbiosis often increases plant growth and fitness, and mycorrhizal plants are better able to tolerate abiotic and biotic stresses than non-mycorrhizal plants (Smith and Read, 2008). In general, AM fungi seem to be cost-efficient for the host, and host plant may detect and preferentially reward the "best" fungal partners with more carbohydrates, and in turn, the fungal partners can increase the translocation of soil nutrients to their host plant (Kiers et al., 2011). It has been suggested that the benefit of a plant associating with fungal symbionts depends on the symbiont's identity (Johnson et al., 1997), the fungal colonization frequency (Vannette and Hunter, 2011), and biotic (e.g. herbivory, Gehring and Bennett, 2009) and abiotic (e.g. drought, salinity, Miransari, 2010) factors.

Temperature is a critical ecological factor for most biological processes and understanding the effects of global warming is therefore crucial. Rising temperatures not only directly alter the physiology and performance of plants, but it may also affect plants indirectly via their fungal symbionts (Staddon et al., 2002; Kivlin et al., 2013). Studies addressing AM symbioses have shown that high temperatures (around 15–25 °C) have generally positive effects on both plant and fungal performance, with variation among the hosts and fungal species involved. For example, in plants, greater germination percentage, larger biomass and shoot nitrogen and phosphorus concentration have been documented in response to higher temperatures (e.g. Ruotsalainen and Kytöviita, 2004; Kytöviita and Ruotsalainen, 2007; Gavito and Azcón-Aguilar, 2012). In AM fungi, greater frequency of colonization inside roots (Staddon et al., 2002; Heinemeyer and Fitter, 2004; Kytöviita and Ruotsalainen, 2007; Gavito and Azcón-Aguilar, 2012) and more prolific extraradical hyphae (Heinemeyer and Fitter, 2004; Gavito et al., 2005; Gavito and Azcón-Aguilar, 2012) have been observed at higher temperature. Both plants and fungi usually benefit from rising temperatures even though their benefit may be asymmetric. For instance, Kytöviita and Ruotsalainen (2007) showed that an increase in temperature may benefit the AM fungus an order of magnitude more than the host plants.

In dioecious plants, where the female and male sexual functions are in separate individuals, sexes often allocate different amounts of resources to growth, defense and reproduction (Geber et al., 1999; Obeso, 2002), and females usually show higher proportional investment into reproduction than males (Obeso, 2002). Over evolutionary time, this mode of resource allocation could lead to differences in morphology, physiology, life history traits (Geber et al., 1999), and intensity of biotic interactions (reviewed in Vega-Frutis et al., 2013a) between females and males of the same species. Few studies have evaluated if the relationship between dioecious plants and AM fungi is sex-specific (reviewed in Varga, 2010; Vega-Frutis et al., 2013a), and even fewer studies have examined how abiotic factors shape this relationship (but see Varga and Kytöviita, 2008, 2010 for the effects of drought and soil pH). The limited evidence suggests that females tend to have higher frequency of root AM colonization, and to obtain higher benefit from AM in terms of growth and reproduction compared to males (reviewed in Varga, 2010; Vega-Frutis et al., 2013a). Nevertheless, the opposite pattern in colonization frequency (Gehring and Whitham, 1992), or no differences between sexes have also been reported (Varga and Kytöviita, 2008, 2011, 2012). Given the crucial role of temperature, the next logical step is to evaluate the effect of rising temperature on the sex-specific symbiotic relationship between plants and AM fungi. It has been shown that in dioecious species females are more responsive and suffer more from elevated temperatures than males (Wang, 2007; Xu et al., 2008; Tognetti, 2012), but the effect for the AM fungal partner or dioecious plants in symbiosis with AM fungi has never been examined.

In the present study, we evaluated the performance of the dioecious herb *Antennaria dioica* in response to AM fungal inoculation under three different temperatures simulating the period of growth of *A. dioica* in central Finland. We chose *A. dioica* because it is declining in abundance in Fennoscandia (Öster and Eriksson, 2007), and is red listed in Finland (Kalliovirta et al., 2010), most likely because of the change in land use practices and soil acidification (Öster and Eriksson, 2007; van den Berg et al., 2008). In addition, it is a dioecious species, and global climate change may differently impact the capacity of female and male plants to reproduce successfully, with consequences for demography, evolution and long-term persistence of populations. Our aim was to quantify the effect of temperature on both the plant and the AM fungal performance, focusing on potential differences between females and males. Therefore, we examined plant growth, reproduction, and chlorophyll fluorescence as a proxy of plant photosynthetic capacity in *A. dioica* plants grown with or without AM fungi. We also evaluated the effect of temperature on the intraradical fungal structures (hyphae, vesicles and arbuscules), length of extraradical AM hyphae, and the number of spores produced. Because female plants generally obtain a greater benefit of AM fungi, but also suffer more from abiotic stress than males, our specific research hypotheses were: (1) both plants and AM fungi will have better performance at higher temperatures, but males will benefit more from warmer temperatures than females, (2) both sexes will benefit from AM symbiosis, but the benefit will be different between sexes and larger at higher temperatures, and (3) the performance of the AM fungi will also depend on the sex of the host plant and be greater at warmer temperatures.

2. Materials and methods

2.1. Study species

Antennaria dioica (L.) Gaertn (Asteraceae) is a dioecious, perennial and clonal herb that grows in nutrient-poor habitats such as heaths, dry grassland, sandy or stony places and forest margins. It is widely distributed in temperate to Arctic regions of the northern hemisphere (Tutin et al., 1976). Each individual plant (genet) can produce one to several propagules (ramets) by clonal growth of surface crawling stolons, and generally the ramets produce one flowering shoot when flowering. Female and male plants exhibit secondary sexual dimorphism: males produce more flowering shoots and inflorescences which are also heavier than those of females, even though there is variation among years and populations (Varga and Kytöviita, 2011). In Finland, flowering occurs between June and July and the frequency of reproduction is similar between sexes (Varga and Kytöviita, 2011). *Antennaria dioca* is pollinated by generalist insects (Willis and Burkill, 1903) and produces small seeds that are easily dispersed by wind (Eriksson, 1997). In addition, both sexes have been reported as mycotrophic in the field (Varga and Kytöviita, 2011, 2012; Vega-Frutis et al., 2013b), with female-biased sex ratios and without spatial segregation of the sexes (Öster and Eriksson, 2007; Varga and Kytöviita, 2011).

2.2. Experimental design

2.2.1. Plant and fungal material

In August 2011, 20 female and 20 male plants (i.e. 40 different genotypes) were collected in central Finland (62° 3′ 10″ N, 25° 32′ 48″ E). Each genotype was divided in several clonal fragments (ramets) which were propagated in individual pots filled with sterilized sand under greenhouse conditions at the University of Jyväskylä, Finland. This was done in order to replicate each individual genotype into the six different treatments (see below).

To ensure that the plant material was not colonized by AM fungi, roots were removed before potting the ramets. The ramets were allowed to grow and develop new root systems for two months. A commercial liquid fertilizer (Substral vital+plus, 7 mL per liter) was given in three occasions. The AM fungal inoculum was obtained from a site where *A. dioica* grows naturally in central Finland. Soil underneath *A. dioica* plants was collected and the AM fungi were propagated using *Plantago lanceolata* as the host. After a 5-month period, newly developed AM spores were extracted by wet sieving and decanting (Gerdemann and Nicolson, 1963) and used as AM inoculum (see below). A total of 13 different AM fungal morphotypes were identified in the inoculum: *Glomus hoi*, *Claroideoglomus claroideum*, *Funneliformis mosseae*, *Scutellospora calospora*, four different *Glomus* spp. and five *Acaulospora* spp. (see Vega-Frutis et al., 2013c for further details).

2.2.2. Experimental setup

The experiment was initiated in October 2011. We performed a factorial experiment with three factors: plant sex (female, male), AM fungal inoculation (yes, no) and temperature (16, 21 and 26 °C). We had 20 replicates of each treatment combination, in total 240 pots. The initial fresh total mass and the number of ramets were determined at the onset of the experiment. We selected plants of similar size to ensure we did not get effects due to differences in the initial plant mass. Therefore, in the beginning of the experiment the sexes did not differ in fresh weight (mean ± standard error, 0.77 ± 0.03 g for females and 0.83 ± 0.03 g for males; $F_{1,38} = 0.916$, $P = 0.345$, see data analyses) or in the number of the ramets (1.23 ± 0.04 for females and 1.36 ± 0.05 for males; $\chi^2_1 = 0.821$, $P = 0.365$, see data analyses). Each clonal fragment was transferred into individual pots filled with 720 cm^3 of a soil mixture containing autoclaved natural soil and perlite (2:1). The natural soil was collected from the site where the plants were originally collected. Soil and perlite were heat sterilized at 125 °C for 1 h to kill all AM propagules. The soil chemical parameters after sterilization were: pH 7.4 (ISO 10390; 2005), organic matter 1.4% (SFS 3008), total nitrogen < 0.1% (Kjeldahl test), total phosphorus 0.05% (HNO_3 dissolution + ICP-OES), and total potassium 0.1% (HNO_3 dissolution + ICP-OES). Soil chemical analyses were carried in the Institute for Environmental Research, a laboratory certified by FINAS (Finnish Accreditation Service) as T142 (EN ISO/IEC 17025). To improve soil fertility, we added 1.5 g L^{-1} bone meal (Äetsä Trading Co., Äetsä, Finland) to the mixture. Half of the plants were allocated to the AM inoculation treatment. AM spores were washed out of the soil with water and experimental plants received 1 mL of water containing 200 AM fungal spores (mycorrhizal plants, referred as AM plants hereafter). The other half of the plants received 1 mL of soil-water mixture that passed through a 45 μm sieve and was therefore without spores (non-mycorrhizal plants, referred as NM plants hereafter). All the plants received an additional 1 mL of a soil microbial suspension filtered through a 5.0 μm nitrocellulose Millipore filter. The bacterial suspension was prepared from unsterilized soil from the original habitat of the experimental plants and given to plants to partially return the soil microflora to the sterilized soil.

After AM inoculation, one third of the pots were randomly assigned into three separate climate chambers (Sanyo MLR-351H) set to 16, 21 and 26 °C. Temperatures were chosen as the mean (16 °C) and maximum (21 °C) average temperatures registered for July (flowering period of *A. dioca*) in Jyväskylä region for a period of 30 years (Drebs et al., 2002), and 26 °C indicates an increase in temperature (projected by the 2080s, relative to the annual mean baseline period 1961–1990) in agreement with the predicted climate change in Finland (Jylhä et al., 2004). Plants were grown under a long light period (22 h light/2 h darkness), and a constant relative air humidity of 70% in the three temperature regimes. Light intensity inside the growth chambers was 200 μmol m^{-2} s^{-1}, provided by 600W fluorescent lamps (15 lamps, FL40SS-W/37). The plants and temperature regimes were switched among the three chambers every two weeks to avoid potential chamber-specific effects. The plants were watered with tap water when needed (about three times per week).

2.3. Plant parameters

After 45 days, chlorophyll fluorescence was measured using Li-Cor 6400XT portable photosynthesis measuring system (Li-Cor, Inc., Lincoln, Nebraska, USA). After darkening the leaves for 30 min, the minimal fluorescence (F_0), maximal fluorescence (F_m) and the ratio of variable to maximal fluorescence (F_v/F_m) were recorded in three randomly chosen fully expanded leaves from different ramets per plant, and the readings were averaged. Dark-adapted values of F_v/F_m reflect the potential quantum efficiency of photosystem II (Maxwell and Johnson, 2000), with optimal values of around 0.8 (±0.05) measured in most plant species. Values lower than 0.75 are considered an indicator of damage caused by environmental stresses to the photosystem II (photoinhibition, Björkman and Demmig, 1987; Maxwell and Johnson, 2000; Baker, 2008).

Survival and flowering phenology were recorded for each individual. The experiment lasted 90 days (simulating the growth period for this species) after the AM inoculation and then the plants were harvested and the number of ramets counted. The spacer length (rhizome distance between the initial ramet and the adjacent newly produced ramets, cm) was measured and averaged per plant. Subsequently, the aboveground mass (ramets and floral shoot mass) and belowground mass (roots) were separated and oven-dried at 60 °C for 3 days, and weighed. The root/shoot ratio was calculated as dry belowground mass/dry aboveground mass.

Mycorrhizal plant benefit, defined as performance ratio between mycorrhizal and non-mycorrhizal plants (Kytöviita et al., 2003), was calculated for the number of ramets and the spacer length, below-, aboveground, and total masses, and root/shoot ratio. Because some individuals died during the experiment, mycorrhizal plant benefit was only calculated in plants where the same individual genotype with and without AM fungi survived in the given temperature. Ratios >1 indicate that symbiosis was beneficial to the plants, ratios <1 indicate that symbiosis was detrimental to the plants, and ratios close to one indicate no net mycorrhizal benefit for the plant.

2.4. Fungal parameters

We measured three fungal parameters: production of intraradical AM fungal structures (hyphae, vesicles and arbuscules), length of extraradical hyphae in soil, and the total number of spores per pot. To estimate the percentage of intraradical AM structures, we collected a subsample of fine roots from each plant before drying. The roots were processed according to the method of Koske and Gemma (1989), and stained with trypan blue (0.05%). We calculated the colonization frequency by AM fungi in 15 root fragments (each ~15 mm long) from each plant. Each root fragment was examined at three equally spaced points under a light microscope at 125× and 312.5× total magnification, using the cross-hair intersection method (McGonigle et al., 1990). The presence/absence of fungal structures was used to calculate the percentage of root colonized by AM fungi: positive counts were summed and divided by the total number of points observed.

The length of extraradical hyphae was measured in oven-dried (60 °C for 3 days) experimental soil by the filtration-gridline method (Sylvia, 1992). A 5 g sample of dry soil (per each AM

pot) was suspended in a mixture of 100 mL of de-ionized water and 15 mL of sodium hexametaphosphate (5%) for 10 min to disperse soil particles, and subsequently shaken vigorously using a blender for 30 s to homogenize the soil suspension. The sample was collected on a 45 μm sieve and washed with water. The recovered material from the sieve was then transferred to a beaker, mixed with 200 mL of water and allowed to settle for 1 min. A 10 mL aliquot was transferred on a 0.8 μm membrane filter (Schleicher & Schuell) and vacuum-filtered. After filtering off the water, the hyphae were stained with 2 drops of trypan blue (0.05%) for 5 min and then vacuum-filtered again. The membrane was transferred to a microscope slide and mounted with polyvinyl alcohol–lacto–glycerol solution. Length of extraradical hyphae was estimated using a grid in the eyepiece of light microscope at 312.5× magnification. Extraradical hyphae were identified as aseptate hyphae and stained blue by trypan blue. Each membrane was examined in 10 random points and the number of hyphae crossing the grid lines incorporated in the microscope was applied into Newman's formula (Newman, 1966) including the dilution factor: $LEH = (\pi NA/2H) \times D$, where *LEH* is the length of extraradical hyphae in centimeters, *N* is the number of intersections, *A* is the active area of membrane filter (1.77 cm^2), *H* is the total length of grids (10 grids = 8 cm), and *D* is the dilution factor (200 mL).

AM spores were extracted from 100 g of dry soil sample (per each AM pot) by wet sieving and decanting (Gerdemann and Nicolson, 1963) using 500 μm and a 45 μm sieves, followed by two centrifugation cycles: first with water at 3000 rpm for 5 min, and after that the sediment was centrifuged at 2500 rpm for 2 min in 70% sucrose solution. All spores in the 100 g sample of soil were counted at 12× magnification under a stereomicroscope.

2.5. Data analyses

All statistical analyses were conducted in R (R Development Core Team, 2012). To analyze the data, we used linear mixed-effects (LME, library nlme) or generalized linear mixed-effects models (GLMER, library lme4). The LME models were fitted by restricted maximum likelihood, and significant differences between levels of a factor (i.e. temperature) were explored using a *posteriori* contrast based on *t*-tests (Warnes, 2012). The GLMER models were fitted by Laplace approximation, and to explore any significant differences between levels of a factor (i.e. temperature) model simplification and analysis of variance between models were used (Bates et al., 2012). For all models we checked that the residuals were normally distributed and the variances homogeneous, and when necessary data transformations were used (see below).

2.5.1. Plant parameters

To test for differences in initial fresh plant mass we used a LME model with plant sex as fixed factor and plant genotype nested within plant sex as a random factor. Differences in the initial number of ramets were analyzed with a GLMER model with Poisson error distribution and log link function, and with the same fixed and random components as described above.

To test for differences in survival, spacer length, above-, belowground, total, and floral shoot masses, root/shoot ratio, onset of flowering, and chlorophyll fluorescence we used LME models. Plant sex (female and male), temperature (16, 21, and 26 °C), and mycorrhizal treatment (AM and NM) were used as fixed factors, and we included all the two-way interactions between these factors. In the random component of the models, we included the plant genotype nested within plant sex. To meet the model assumptions, the above-, belowground and floral shoot masses, root/shoot ratio, and F_0 were square root transformed, the Neperian logarithmic transformation was applied to onset of flowering, and data on F_v/F_m, and survival were average rank transformed. We used a GLMER with Poisson error distribution and log link function to test differences in the final number of ramets, and a GLMER with Binomial error distribution and logit link function to test differences in whether plants flowered or not. We used the same fixed and random components as described above.

We used LME models to explore differences in mycorrhizal plant benefit. Plant sex (female and male), temperature (16, 21, and 26 °C), and its interaction were used as fixed factors. In the random component of the models, we included the plant genotype nested within plant sex. To meet the model assumptions, logarithmic $(x+1)$ transformation was applied to all variables (spacer length and number of ramets, below-, aboveground and total masses, and root/shoot ratio) related to mycorrhizal plant benefit.

2.5.2. Fungal parameters

We used LME models to explore the differences in fungal parameters, and the same fixed and random components as described in mycorrhizal plant benefit was used. To meet the model assumptions the percentage of root length colonized by the different fungal structures (hyphae, vesicles and arbuscules) were arcsine transformed, the length of extraradical hyphae was square root transformed, and the number of spores were Neperian logarithmically transformed.

3. Results

3.1. Plant parameters

3.1.1. Survival and growth

We observed secondary sexual dimorphism in plant survival and in the spacer length. During the experiment two females and 13 males died ($F_{1,38} = 5.143$, $P = 0.029$) and these plants were excluded from further analyses. Females had on average half centimeter longer spacer lengths than males and we found no statistical evidence to support sexual dimorphism in any other growth parameter measured (Tables 1 and 2).

Eight and seven plants died at 16 and 21 °C, respectively, while all plants survived at 26 °C ($F_{2,192} = 5.048$, $P = 0.007$). The temperature treatment had a strong effect on all growth-related plant parameters analyzed regardless of the sex of the plant (Table 1). The largest growth in terms of total plant mass was observed in plants growing at 16 °C and plants grew significantly less at increased temperatures (all comparisons $P \leq 0.015$, Fig. 1A). Aboveground mass accumulation was larger in plants growing at 16 and 21 °C (comparison $P = 0.669$) compared to plants growing at 26 °C (comparisons $P \leq 0.023$, Fig. 1B), and belowground mass was significantly larger only in plants growing at the lowest temperature (comparisons $P < 0.001$, Fig. 1C). Taken together, root/shoot ratio was significantly higher in plants at 16 °C when compared to the other temperature treatments (comparisons $P < 0.001$, Fig. 1D). The pattern of mass allocation among the temperature treatments did not fully resemble that of the number of ramets produced; the highest number of ramets was observed in plants growing at 16 and 26 °C (comparison $P = 0.145$, Fig. 1E). In contrast, the lowest spacer length was observed in plants growing at 16 °C when compared with 21 and 26 °C (comparisons $P < 0.001$ and $P = 0.002$, respectively, Fig. 1F).

AM inoculation increased all plant growth-related parameters measured regardless of the sex of the plant (Tables 1 and 2) without affecting the number ($\chi^2_1 = 0.006$, $P = 0.934$) or the spacer length (Tables 1 and 2). However, the effects of AM inoculation on mass allocation varied with temperature. AM fungi increased total mass (Fig. 1A), belowground mass (Fig. 1C), and the root/shoot ratio (Fig. 1D) only in plants growing at 21 and 26 °C compared with NM plants.

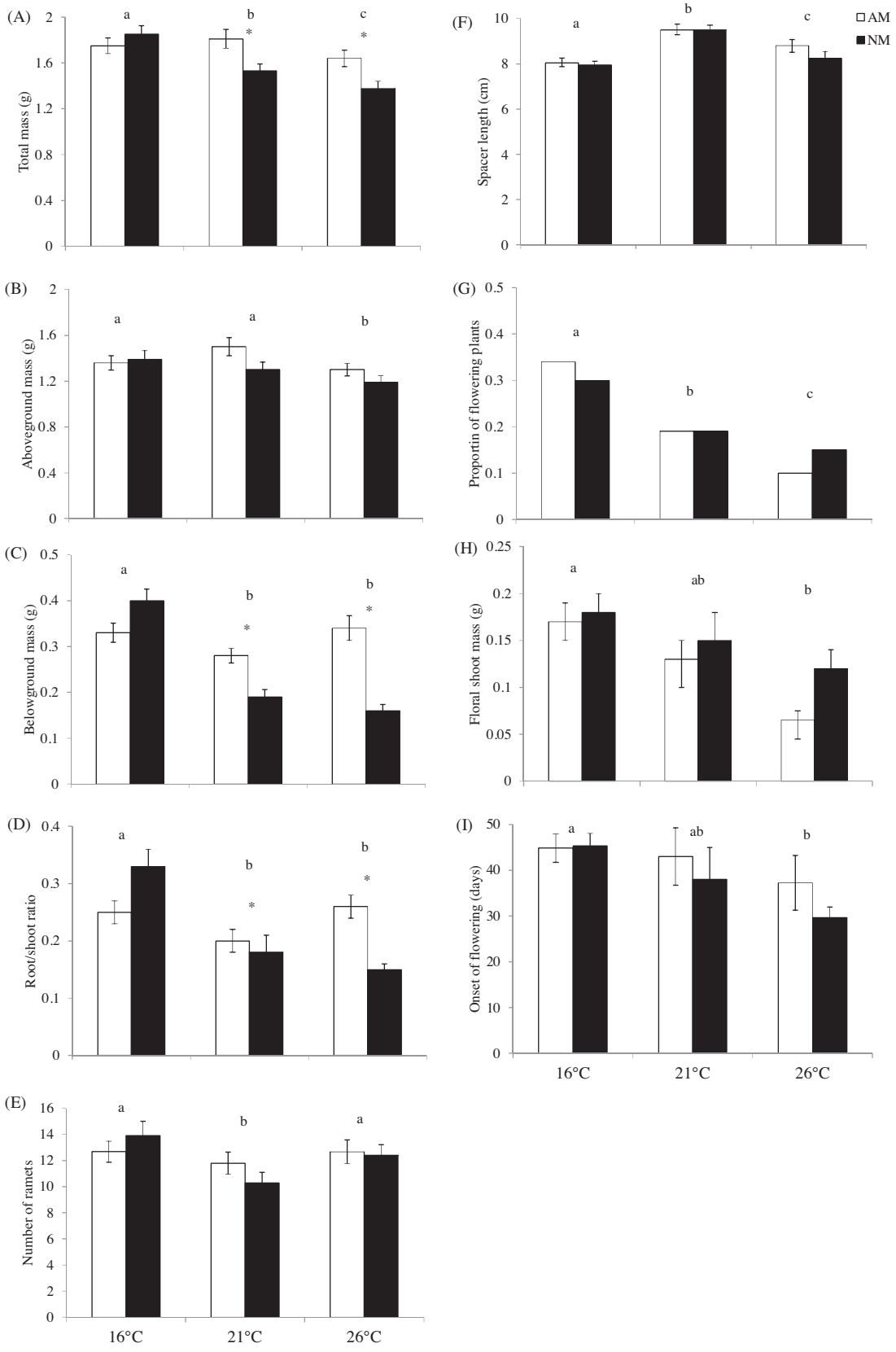

Fig. 1. Interactive effects of AM inoculation and temperature on (A) total mass, (B) aboveground mass, (C) belowground mass, (D) root/shoot ratio, (E) number of ramets, (F) spacer length, (G) proportion of flowering plants, (H) floral shoot mass, and (I) onset of flowering in *Antennaria dioica* plants inoculated (white bars) or non-inoculated (black bars) with AM fungi grown under three different temperatures. Different letters above the bars indicate statistically significant differences among temperatures according to *t*-test contrast ($P < 0.05$), and asterisks indicate statistically significant differences between AM and NM plants within the same temperature treatment. Mean ± standard errors are given without data transformation.

Table 1
Summary of statistical results of linear mixed effects models for plant growth parameters analyzed in *Antennaria dioica*. Significant values according to the linear mixed effects models are shown in bold.

Source of variation		Aboveground mass (g)		Belowground mass (g)		Total mass (g)		Root/shoot ratio		Spacer length (cm)	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sex	1,38	0.761	0.388	0.214	0.645	0.481	0.496	0.026	0.871	4.512	**0.042**
Temperature	2,196	4.406	**0.013**	25.132	**<0.001**	13.416	**<0.001**	16.536	**<0.001**	32.269	**<0.001**
Fungus	1,196	5.828	**0.016**	22.198	**<0.001**	11.738	**<0.001**	6.387	**0.012**	2.770	0.097
Sex:temperature	2,196	0.160	0.851	0.399	0.671	0.061	0.940	0.600	0.549	0.702	0.496
Sex:fungus	1,196	2.169	0.142	0.368	0.544	2.602	0.108	0.021	0.883	0.025	0.874
Temperature:fungus	2,196	2.466	0.087	20.061	**<0.001**	8.820	**<0.001**	11.266	**<0.001**	1.150	0.318

3.1.2. Flowering

During the experiment 21% of the plants flowered. Compared to females, more males flowered ($\chi^2_1 = 5.205$, $P = 0.022$, Table 2), and started flowering on average 10 days earlier than females ($F_{1,16} = 7.042$, $P = 0.017$, Table 2), with no differences in the floral shoot mass produced between sexes ($F_{1,16} = 0.519$, $P = 0.481$, Table 2). Increased temperature decreased the proportion of flowering plants ($\chi^2_2 = 16.857$, $P < 0.001$, Fig. 1G) from 49, 30 to 21% of plants flowering at 16, 21 and 26 °C, respectively. In the same way, the floral shoot mass decreased with the increasing temperature (Fig. 1H). Opposite to that, the onset of flowering was earlier at higher temperatures ($F_{2,21} = 4.602$, $P = 0.022$, Fig. 1I). There were no significant differences between AM and NM plants in terms of flowering ($\chi^2_1 = 0.073$, $P = 0.787$, Table 2), onset of flowering ($F_{1,21} = 1.599$, $P = 0.219$, Table 2), and floral shoot mass ($F_{1,21} = 1.550$, $P = 0.226$, Table 2).

3.1.3. Chlorophyll fluorescence

The sexes did not differ in any chlorophyll fluorescence parameter measured ($P \geq 0.250$ in all LME models, Table 3), and there were no significant differences between AM and NM plants in any parameter ($P \geq 0.700$ in all LME models, Table 3). However, all chlorophyll fluorescence parameters were affected by the temperature. High temperature decreased the F_0 compared to plants at 21 and 16 °C ($F_{2,161} = 59.542$, $P < 0.001$, Table 3). A similar pattern was observed in F_m ($F_{2,161} = 41.974$, $P < 0.001$, Table 3), even though plants at 21 °C had the highest value of F_m. In contrast, F_v/F_m ($F_{2,161} = 11.831$, $P < 0.001$) was lowest at 16 °C when compared to the other two temperatures (comparisons $P < 0.001$, Table 3). No significant interactions among the factors were found ($P \geq 0.137$ in all LME models).

3.2. Fungal parameters

At the end of the experiment, all NM plants remained non-mycorrhizal. The percentage of root length colonized in mycorrhizal plants by intraradical structures (hyphae: $F_{1,38} = 0.169$, $P = 0.682$, vesicles: $F_{1,38} = 0.255$, $P = 0.616$, and arbuscules: $F_{1,38} = 1.008$, $P = 0.321$), the length of extraradical hyphae ($F_{1,38} = 1.753$, $P = 0.193$), and the number of spores ($F_{1,38} = 0.364$, $P = 0.549$) produced were similar between females and males (Fig. 2A–E), but temperature affected the amount of all fungal parameters measured both at the intra- (hyphae: $F_{2,67} = 4.052$, $P = 0.021$, vesicles: $F_{2,67} = 7.922$, $P < 0.001$, arbuscules: $F_{2,67} = 11.075$, $P < 0.001$) and extraradical (extraradical hyphae: $F_{2,67} = 10.590$, $P < 0.001$, number of spores: $F_{2,67} = 41.975$, $P < 0.001$) levels. The amount of intraradical hyphae and vesicles was lowest at 16 °C (all comparisons $P \leq 0.024$ for hyphae and vesicles), with no differences between 21 and 26 °C (comparisons $P = 0.862$ and $P = 0.160$, respectively, Fig. 2A and B). The highest amount of arbuscules was observed in plants growing at 21 °C, without differences between 16 and 26 °C (comparison $P = 0.107$, Fig. 2C). Overall, the production of extraradical hyphae was lowest at 16 and 21 °C, but differences between the sexes were observed ($F_{2,67} = 3.710$, $P = 0.029$, Fig. 2D). At 16 °C, males were associated with higher extraradical hyphae production than females (comparison $P = 0.022$), but the inverse pattern was observed at 21 °C (comparison $P = 0.033$, Fig. 2D). No differences between sexes were observed at 26 °C (comparison $P = 0.560$, Fig. 2D).

The highest number of spores produced was observed at 26 °C, and there was a decrease in spore production with reduction in temperature ($P < 0.001$, in all comparisons). However, there was a significant interaction between temperature and the sex of the plants ($F_{2,67} = 4.299$, $P = 0.017$): in male pots, there were on average 2.12 more spores per gram of soil than in female pots (comparison $P = 0.022$, Fig. 2E) at 21 °C, while no differences were observed between sexes at 16 °C (comparison $P = 0.600$, Fig. 2E) nor at 26 °C (comparison $P = 0.326$, Fig. 2E).

3.3. Mycorrhizal plant benefit

Female and male plants gained a similar benefit from mycorrhizal inoculation in the growth parameters analyzed ($P \geq 0.190$ in all LME models, Fig. 3). The temperature affected total and belowground masses, and root/shoot ratio. The net effect derived in plant growth in these parameters from AM inoculation was only

Table 2
Growth and reproductive parameters analyzed in *Antennaria dioica* plants. Data shown are means ± standard errors. Different letters indicate statistically significant differences between sexes, fungal (according to linear mixed effects models) or temperature treatments (according to *t*-test contrast). The start of flowering was recorded two weeks after inoculation took place.

Variables	Sex		Fungal treatment		Temperature		
	Female	Male	AM	NM	16 °C	21 °C	26 °C
Aboveground mass (g)	1.38 ± 0.040	1.29 ± 0.036	1.39 ± 0.383a	1.29 ± 0.039b	1.38 ± 0.049a	1.40 ± 0.052a	1.24 ± 0.039b
Belowground mass (g)	0.28 ± 0.013	0.28 ± 0.015	0.31 ± 0.013a	0.25 ± 0.014b	0.36 ± 0.017a	0.23 ± 0.012b	0.25 ± 0.017b
Total mass (g)	1.68 ± 0.043	1.62 ± 0.042	1.73 ± 0.043a	1.58 ± 0.041b	1.80 ± 0.051a	1.67 ± 0.053b	1.51 ± 0.049c
Root/shoot ratio	0.23 ± 0.014	0.23 ± 0.014	0.24 ± 0.010a	0.22 ± 0.017b	0.29 ± 0.019a	0.19 ± 0.017b	0.20 ± 0.012b
Number of ramets	11.78 ± 0.513	12.88 ± 0.511	12.40 ± 0.492	12.21 ± 0.537	13.32 ± 0.681a	11.03 ± 0.592b	12.55 ± 0.598a
Spacer length (cm)	8.91 ± 1.256a	8.41 ± 1.732b	8.80 ± 1.510	8.55 ± 1.502	7.99 ± 1.324a	9.50 ± 1.652b	8.53 ± 2.007c
Proportion of flowering plants	0.10 ± 0.028a	0.33 ± 0.045b	0.21 ± 0.039	0.21 ± 0.038	0.32 ± 0.055a	0.19 ± 0.046b	0.12 ± 0.037b
Flowering start (days)	49.50 ± 3.281a	38.20 ± 2.065b	42.96 ± 2.634	39.29 ± 2.681	45.09 ± 2.031a	40.50 ± 4.534ab	32.70 ± 2.848b
Floral mass (g)	0.16 ± 0.017	0.14 ± 0.015	0.14 ± 0.017	0.16 ± 0.016	0.17 ± 0.017a	0.14 ± 0.019ab	0.10 ± 0.018b

Table 3
Summary of the effects of sex, fungal treatment and temperature on chlorophyll fluorescence parameters analyzed in *Antennaria dioica*. Data shown are means ± standard errors. Different letters indicate statistically significant differences among temperatures according to *t*-test contrast ($P < 0.05$).

Variables	Sex		Fungal treatment		Temperature		
	Female	Male	AM	NM	16 °C	21 °C	26 °C
Minimal fluorescence (F_0)	110.35 ± 4.328	104.62 ± 3.964	109.49 ± 4.328	105.78 ± 4.278	134.11 ± 4.473a	129.38 ± 3.471a	66.33 ± 2.421b
Maximal fluorescence (F_m)	539.32 ± 19.902	534.47 ± 19.451	552.05 ± 20.226	523.10 ± 19.106	587.55 ± 18.977a	689.99 ± 18.088b	357.08 ± 14.076c
Ratio of variable to maximal fluorescence (F_v/F_m)	0.79 ± 0.005	0.80 ± 0.004	0.79 ± 0.004	0.79 ± 0.004	0.76 ± 0.008a	0.81 ± 0.002b	0.81 ± 0.003b

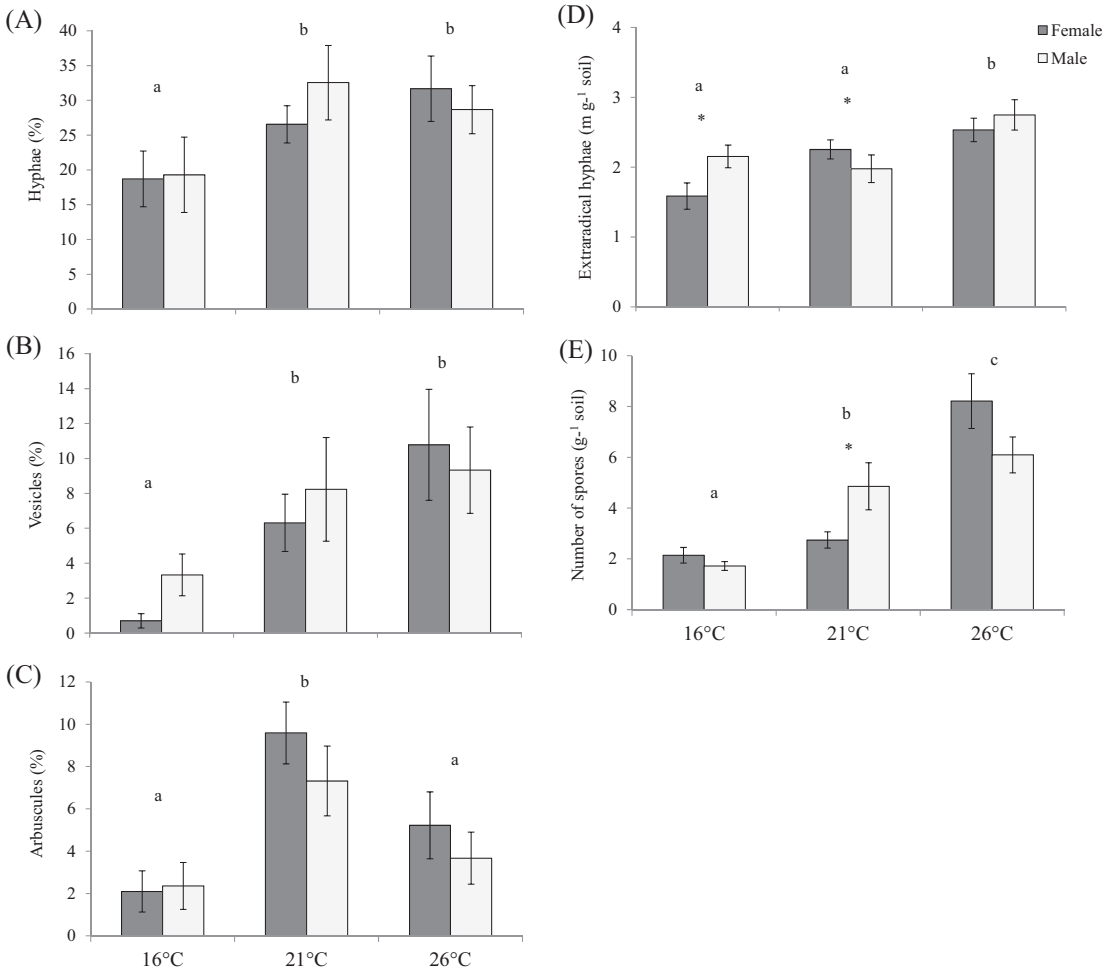

Fig. 2. Interactive effects of sex and temperature on percentage of root colonized by (A) hyphae, (B) vesicles, (C) arbuscules, (D) extraradical hyphae production (in 1 g of soil), and (E) number of spores (in 1 g of soil) in female (dark gray bars) and male (light gray bars) *Antennaria dioica* plants grown under three temperatures. Different letters above the bars indicate statistically significant differences among temperatures according to *t*-test contrast ($P < 0.05$), and the asterisks indicate statistically significant differences between female and male plants within the same temperature treatment. Values are means ± standard errors without data transformation.

beneficial at 21 and 26 °C (total mass: $F_{2,65} = 8.394$, $P < 0.001$, belowground mass: $F_{2,65} = 16.555$, $P < 0.001$, and root/shoot ratio: $F_{2,65} = 11.421$, $P < 0.001$; Fig. 3A, C and D), and only the root/shoot ratio of females at 21 °C was significantly higher than males ($F_{2,65} = 3.360$, $P = 0.041$, Fig. 3D). Although the mycorrhizal benefit for the number of ramets tended to be higher at 21 °C, no statistically significant differences among the different temperatures were observed ($F_{2,65} = 1.837$, $P < 0.167$, Fig. 3E).

The aboveground mass and spacer length did not show any mycorrhizal benefit and differences between sexes ($F_{1,19} = 1.606$, $P = 0.220$, and $F_{1,19} = 0.515$, $P = 0.481$ for aboveground mass and spacer length respectively), temperatures ($F_{2,65} = 1.540$, $P = 0.221$, and $F_{2,65} = 2.356$, $P = 0.102$ for aboveground mass and spacer length respectively) or their interactions ($F_{2,65} = 0.571$, $P = 0.567$, and $F_{2,65} = 2.822$, $P = 0.066$ for aboveground mass and spacer length respectively) were not significant (Fig. 3B and F).

4. Discussion

In this study, we investigated the single growth season response of *A. dioica* and AM fungi to increased temperature in a controlled environment. Warming is expected to increase both plant productivity (e.g. Wu et al., 2011) and fungal growth (e.g. Heinemeyer and Fitter, 2004), but we could corroborate our initial hypotheses

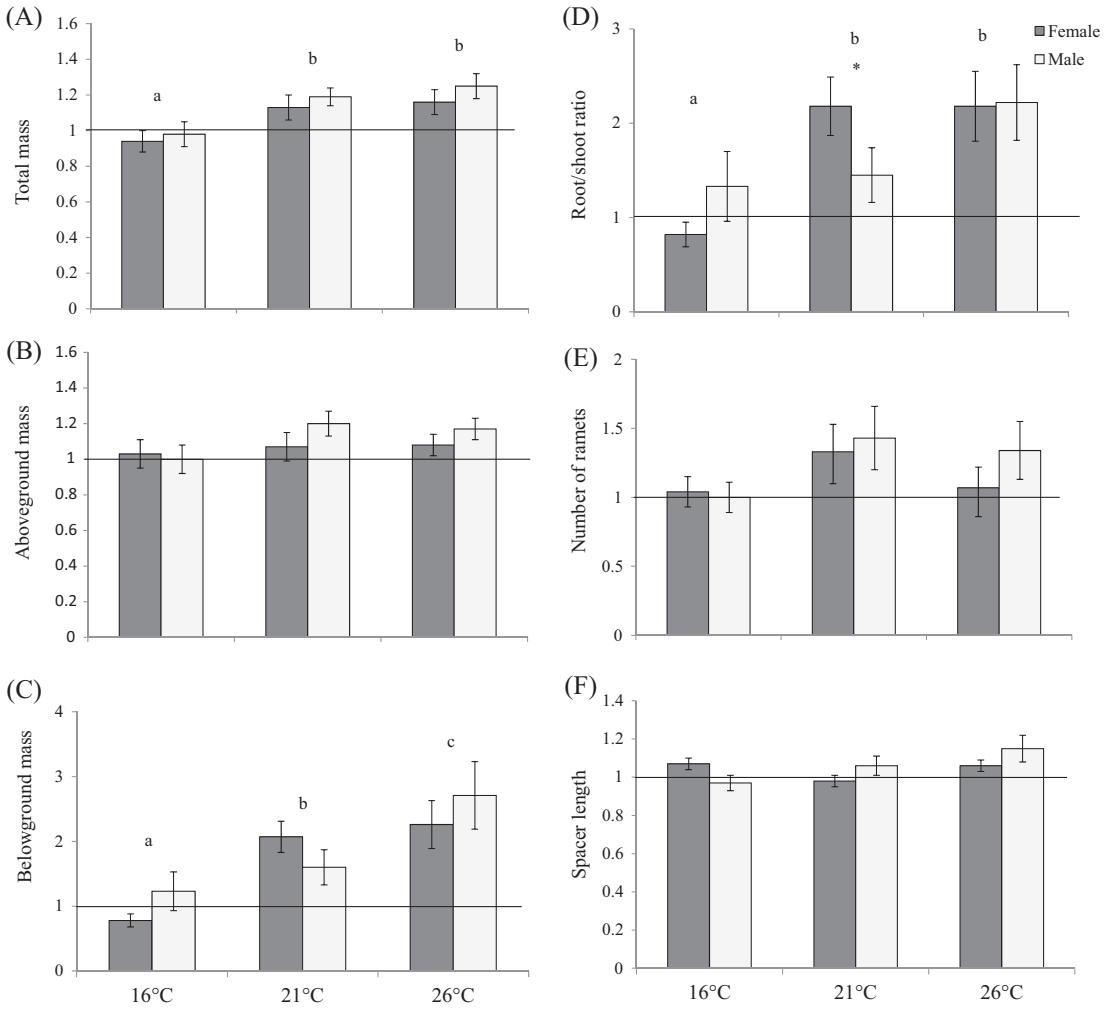

Fig. 3. Mycorrhizal plant benefit, defined as the ratio between mycorrhizal and non-mycorrhizal plants in terms of (A) total mass, (B) aboveground mass, (C) belowground mass, (D) root/shoot ratio, (E) number of ramets, and (F) spacer length in female (dark gray bars) and male (light gray bars) *Antennaria dioica* plants grown under three temperatures. Different letters above the bars indicate statistically significant differences among temperatures according to *t*-test contrast ($P < 0.05$), and the asterisks indicate statistically significant differences between female and male plants within the same temperature treatment. Values above the line denote positive mycorrhizal benefit. Means ± standard errors are given without data transformation.

of enhanced growth only for the fungal partner. The host plant *A. dioica*, overall, suffered from increased temperature. Our experiment simulated the mean and maximum temperature conditions measured during the natural growing season of the experimental organisms in central Finland. *Antennaria dioica* thrived best at the lowest temperature which represented the average local temperature indicating local adaptation to cool climate. Both sexes responded similarly in terms of growth and reproduction to increasing temperatures, and obtained a similar benefit from AM.

4.1. Plant response

Plant survival was sex-specific with higher female survival. This agrees well with earlier findings of female-biased population structure (Varga and Kytöviita, 2011). Spacer length was also greater in females, and fewer female plants produced flowers and started flowering later compared to males. These differences between females and males may be associated with the higher resource need for sexual reproduction in female plants (Obeso, 2002).

Sex-specific growth responses to elevated temperature have been examined in few dioecious species. Overall, elevated temperature promotes a greater increase in growth parameters and better adaptation to stress in males than females (Xu et al., 2008; Nybakken et al., 2012; but see Zhao et al., 2012). In the present study, even though temperature affected most plant growth parameters measured, the effect was the same in females and males. Taken together, the available results are inconclusive, but they highlight the potential for rising temperature to influence the performance of the two sexes in a different way, potentially influencing the distribution of the sexes in dioecious plants, as reported in the field for *Corema album* (Álvarez-Casino et al., 2013).

Antennaria dioica is a native cool climate species, and is more frequent in latitudes higher than presently studied. Increasing temperature was overall negative for our experimental plants as it decreased plant biomass and flowering incidence, suggesting that the *A. dioica* population under study was locally adapted and performed best at the local average temperature (16 °C). Temperature is a trigger of growth and flowering phenology in most species, and in northern hemisphere the length of the growth and reproductive period could change as a consequence of altered seasonal patterns of temperature (e.g. Hülber et al., 2010), affecting survival and flowering phenology in a short time frame as was observed in our study. Females and males did not respond in the same way to temperature, although the synchronization of flowering in female and male plants is crucial for reproductive success in dioecious

species. Flowering phenology is part of local adaptation and tight synchronization in temperature response between females and males could be expected. The observed asynchronous response by the sexes may render dioecious species particularly vulnerable to temperature fluctuations.

Temperature affected ramet production, and plants had the smallest number of ramets at 21 °C, but these plants had the longest spacer length, indicating a potential trade-off between ramet number and spacer length. Ramets are the result of vegetative reproduction and they have several ecological advantages over sexual reproduction. For instance, clonally propagating individuals may persist in habitats unfavorable for sexual reproduction, and are able to forage for resources or colonize habitats with adverse conditions (Vallejo-Marín et al., 2010). Our results suggest that rising temperature may favor the asexual reproduction, possibly as a mechanism to survive in a stressful environment. This finding, along with the fact that growth and reproduction was reduced at higher temperatures emphasizes that rising temperature has negative effects on *A. dioica* performance.

Previous studies show that extreme temperatures decrease F_v/F_m, indicating plant stress (e.g. Zhu et al., 2010, 2011). In the present study, the F_v/F_m ratios were significantly lower at 16 °C indicating lower potential photosynthetic performance compared to 26 °C, although F_v/F_m ratios fluctuated around values close to 0.8 in all temperatures, suggesting little stress. In the same way, there were no signs of photoinhibition in AM and NM plants, although other studies have shown that AM fungi lessen the damage caused to the PSII by high temperatures (e.g. Zhu et al., 2011). The values of F_v/F_m may be due to increase in F_0 or decrease in F_m. Under stress conditions, an increase in F_0 or a decrease in F_m reflects the destruction and loss of PSII reaction center or disruption of electron transport for excitation of reaction centers (Baker, 2008). At 16 °C, plants had higher F_0 indicating that the ability to absorb light for photosynthesis was lower, at least when comparing with plants grown at 26 °C. The lower ability to absorb light could be due to the conditions of light and humidity in the climate chambers in interaction with the temperature. Low-temperature damage to photosynthetic apparatus is evident under excessive light, but the interactive effect of low temperatures and moderate light on photosynthetic performance has not been totally elucidated as yet (Goh et al., 2012). In our study, changes in potential photosynthetic performance were not reflected in plant biomass, which suggests that respiratory losses determined biomass accumulation to a large extent. Females and males had similar values of F_v/F_m, F_0, and F_m, although differences between sexes have been reported in other plant species (e.g. Álvarez-Cansino et al., 2012). A possible explanation for the lack of sexual differences in our study is the young age of the ramets produced during the experiment. Oñate et al. (2011) pointed out when evaluating several physiological parameters in the dioecious *Urtica dioica*, that differences might become only apparent in the mature phase, but not in the juvenile phase.

Our study is in general agreement with a vast number of studies showing the widely recognized positive effects of AM fungi on host growth parameters (Smith and Read, 2008). We confirmed our hypothesis of beneficial AM effects, especially at elevated temperatures, but only for belowground parameters. However, contrary to our expectation, the benefits gained were similar between the sexes. We have shown previously using the same species that the benefit gained from AM fungi may be dependent on the sex of the plant and the stress conditions (Varga and Kytöviita, 2008, 2010). In general, females gain greater mycorrhizal benefit when grown without stress while under stress conditions (drought and low pH) the mycorrhizal benefit is reduced and similar in both sexes.

4.2. Fungal response to different temperatures

The frequency of colonization by intraradical and extraradical AM fungal structures and the number of spores were greater at higher temperatures. These findings are in agreement with other studies (e.g. Schenck et al., 1975; Daniels and Trappe, 1980; Staddon et al., 2002; Heinemeyer and Fitter, 2004; Gavito et al., 2005; Kytöviita and Ruotsalainen, 2007; Gavito and Azcón-Aguilar, 2012). In our case, the host plant mass was reduced at higher temperature, but the abundance of the symbiont was higher indicating opposite responses to temperature. AM fungi use photosynthates to build their own biomass, and at higher temperatures they may allocate more photosynthates in maintaining their respiration instead of allocating the photosynthates for nutrient uptake. Therefore, symbiosis with AM fungi at higher temperature may not result in larger plant biomass, but increased respiration by the root symbionts (Heinemeyer et al., 2006; Valentine and Kleinert, 2007). Our chlorophyll fluorescence data suggest that *A. dioica* probably had higher net photosynthetic rates at higher temperatures, but the plant growth was lower. Fungal growth was higher at higher temperature as indicated by higher amount of extraradical mycelium and spores at 26 °C. Since the lower amount of intraradical structures coincided with higher amount of roots at 16 °C, it is difficult to compare the net effect of temperature on intraradical structures. Altogether these results indicate that there may have been a trade-off between resource allocation to fungus and the plant.

4.3. Effects of host sex on AM fungi

Even though differences in AM colonization in response to plant sex have been reported previously in *A. dioica* under field conditions (Vega-Frutis et al., 2013b,c), and in some other dioecious species (Eppley et al., 2009; Vega-Frutis and Guevara, 2009), we found no differences in the present study. This is in line with our earlier work comparing mycorrhizal response in *A. dioica* in the greenhouse (Varga and Kytöviita, 2008). The sex-specific patterns have been linked to resource allocation to reproduction (Vega-Frutis et al., 2013b,c; Varga and Kytöviita, 2008). In the present experiment, few plants were reproducing sexually, and therefore the lack of sexual dimorphism in AM colonization could be explained by the similar resource demands in vegetatively reproducing females and males.

Even though the data are limited, dioecious plants may differ in the amount of symbiotic structures in plant roots (reviewed in Varga, 2010; Vega-Frutis et al., 2013a). These previous investigations could not resolve the net benefit the fungus derives from associating with female or male plants, given that only fungal structures inside the roots have been evaluated, and extraradical structures are also implicated in the bidirectional transfer of nutrients between the host plant and its fungal partners. In the present work, at 16 °C, fungi associated with males produced more external hyphae than those with females, but at 21 °C male associated fungi produced lower amount of external hyphae, but higher number of spores. These results demonstrate that the sexual dimorphism in AM fungi not only include the frequency and type of intraradical structures, but also extraradical structures. Altogether, this gives strong support toward the view that the benefit the fungus derives is host sex-specific.

4.4. Implications for dioecious plants

Studies using empirical data of local flora, phylogenetic analyses of species richness, and models of floral display have hypothesized that angiosperms with dioecious breeding systems may be more prone to extinction (Heilburth, 2000; Vamosi and Otto, 2002; Vamosi and Vamosi, 2005). Dioecious species are obligately outcrossed, rely on pollen vectors and some species depend on

animals to disperse seeds. These traits, along with anthropogenic disturbances and global climate change may increase the risk of extinction or limit potential for speciation (Vamosi and Vamosi, 2005). In our study, rising temperature favored asexual reproduction, and decreased the number of individuals producing flowers. In addition, increasing temperature resulted in asynchrony of the sexual activities decreasing potentially sexual reproduction, and indirectly affecting the interaction with its symbionts. In contrast, AM fungi had a better performance at the higher temperatures; this asymmetry might decrease the transfer of resources between the host plant and its fungi, therefore, mycorrhizal benefit could also decrease as was indeed shown in this study in contrast to previous studies with *A. dioica* (see Varga and Kytöviita, 2008, 2010). Kivlin et al. (2013) suggested that if climate change affects host plants and symbionts in different ways, the symbionts could disappear, plant-fungal mismatches would appear, and novel interactions with no historical analogs could arise. This study provides evidence for potential plant-fungal mismatch at increasing temperature.

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