



## Transgenerational effects of three global change drivers on an endemic Mediterranean plant

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Plant populations are subjected to changes in their natural environment as a result of the incidence of simultaneous global change drivers. Despite the fact that these changes can largely affect early fitness components, information on the effects of simultaneous drivers of global change on offspring traits and performance is particularly scant. We analyzed the combined effect of three global change drivers of critical importance in Mediterranean ecosystems (habitat fragmentation, reductions in habitat quality and water availability) on germination and seedling performance of the gypsophile shrub *Centaurea hyssopifolia*. Seedlings from 39 mother plants from eight different environments (resulting from the combination of the three global change drivers) were sown and grown in a common garden. First, germination percentage, seedling size and seedling survival were monitored. Secondly, seedling performance and ecophysiological traits were measured under well and low-watered conditions. Fragmentation showed the largest negative effect on germination and offspring performance. Seedlings of mothers from small fragments germinated more slowly, showed lower survival, died faster, and showed lower photosynthetic rates under well-watered conditions compared to seedlings of mother plants from large populations. Seedlings of different maternal origins did not differ in their response to water stress or in their ability to survive to drought. Ninety-five percent of the seedlings survived until soil water content was as low as 3%. Our study shows that global change can have not only immediate impacts on plant populations but also transgenerational effects, and highlights the importance of studies involving multiple drivers and a more integral understanding of global change.

In the last decades an increasing number of studies have reported rapid human-mediated changes in the environment and their impact on plant populations and communities (Chapin et al. 2001, Peñuelas and Filella 2001, Parmesan and Yohe 2003). Plant populations can respond to these environmental changes via plastic responses in phenotype, but long-term responses that are only observable after one or more generations may also occur. These transgenerational responses can result either from maternal environmental effects or from evolutionary responses to novel selection pressures, and are important because they may alter the ultimate ecological impact of the environmental change.

Mediterranean-type ecosystems and particularly semi-arid gypsum habitats are very vulnerable to global change (Lavorel et al. 1998, Mooney et al. 2001). In these habitats, several global change drivers can occur simultaneously, such as increased aridity (i.e. number and length of dry periods; Christensen et al. 2007) reductions in habitat quality and habitat fragmentation (Lavorel et al. 1998, Sala et al. 2000). Reductions in water availability (due to increased aridity) and habitat quality may hinder germination, seedling establishment, growth and seedling survival, thereby affecting the most critical life stages of plant cycle in these

water-limited communities (Harrington 1991, Eliason and Allen 1997). Habitat fragmentation, caused by agriculture and land exploitation, transforms the landscape into patches of natural vegetation interspersed in agriculture fields (Kosmas et al. 2002, Foley et al. 2005), and leads to the loss of genetic variability and the increase of inbreeding (reviewed by Lienert 2004).

Overall, different global change drivers interacting simultaneously create new scenarios under which the capacity of species to respond is poorly unknown, primarily because interactions among drivers may generate non-additive effects that cannot be easily predicted based on single-factor studies (Sala et al. 2000, Shaver et al. 2000, Alonso and Valladares 2008, Matesanz et al. 2009). Despite several studies address the effects of global change drivers on the reproductive output of plant species (Ågren 1996, Aguilar et al. 2006, Brys et al. 2004), information of their effects on offspring performance is far more scanty (Kéry et al. 2000, Kolb 2005, Yates et al. 2007), and is virtually inexistent for several drivers considered simultaneously (Lau et al. 2008).

Maternal environmental effects occur when the environmental conditions experienced by parents influence offspring traits and refer to the capacity of the maternal

environment to induce changes in gene expression, interact with the genotype and directly influence seed provision (Roach and Wulff 1987, Lacey 1996, 1998, Molinier et al. 2006). These maternal effects have been considered especially relevant during the early developmental life stages, such as germination and seedling survival (Guterman 1993, Weiner et al. 1997, Galloway 2001, Luzuriaga et al. 2006) although they may persist in adult life stages and even affect plant reproductive success (Wulff et al. 1994, Galloway 2001). Recent studies have revealed that plant species are able to express transgenerational phenotypic plasticity as an inherited maternal environmental effect (Donohue and Schmitt 1998, Donohue 1999, Galloway 2001, Etterson and Galloway 2002, Sultan et al. 2009). In this sense, maternal effects can result in reduced or increased plasticity to the offspring environmental conditions (Bezemer et al. 1998, Huxman et al. 2001). When maternal and offspring environments are correlated, transgenerational phenotypic plasticity may be adaptive, improving plant fitness (Mosseau and Fox 1998, Van Zandt and Mopper 2004, Galloway 2005, Molinier et al. 2006, Galloway and Etterson 2007, Sultan et al. 2009).

The main aim of this study was to compare the performance of the offspring of mothers from eight different environments (resulting from the combination of the three global change drivers: habitat fragmentation, changes in habitat quality and water availability) of the endemic gypsum-philic *Centaurea hyssopifolia* (Asteraceae). In a previous field study, mother plants were selected in natural habitats that differed in the level of habitat fragmentation (large and small fragments) and habitat quality (high and poor), and were submitted to experimental manipulation of water availability for two years (Matesanz et al. 2009). In this study, Matesanz et al. (2009) found complex and non-additive effects of these three drivers on plant survival, phenology and reproductive effort; so transgenerational effects of global change are expected in this species. We performed a common garden experiment using the offspring of these mother plants to test the following hypothesis: 1) germination, seedling survival and growth are lower in seedlings from mother plants from the most unfavourable environments and 2) the maternal environment influences seedlings' physiological plasticity to water stress and their ability to survive to drought.

## Methods

### Study species

*Centaurea hyssopifolia* is a perennial shrub, endemic to gypsum outcrops of semiarid central Spain (Luzuriaga et al. 2006). It constitutes one of the dominant species of the dwarf shrub plant communities of the Iberian gypsum habitats, which are considered among the most threatened habitats in the Mediterranean Basin. Human activities have strongly fragmented gypsum shrub communities, including *C. hyssopifolia* populations. Flowering plants produce 2–550 capitula with an average of 2.75 (range 0–11) viable seeds per capitulum (Matesanz unpubl.). Flowering period extends from mid May to mid July and seeds ripen from early June to later July. It is a self-incompatible species with generalist entomophilous pollination (Luzuriaga et al. 2006).

### Seed origin and experimental design

Seeds were collected in July 2006 from natural populations of *C. hyssopifolia* located in gypsum soils close to Belinchón (Spain, 754 m a.s.l., 40°03'N, 3°03'W). We chose five mother plants from eight different maternal environments. Each maternal environment resulted from the combination of three controlled experimental factors, each with two different levels. The three experimental factors, with their two levels, represented three different global change drivers: fragmentation (small and large fragments), habitat quality (high and poor) and water availability (mesic: watered plants and dry: non-watered plants). To select the two levels of fragmentation we identified three small (area < 1.5 ha) and three large (area > 11 ha) fragments of natural vegetation within a matrix of agricultural fields (six fragments total). Within each fragment, we randomly selected two different plots of 15 × 15 m; one of high habitat quality and one of poor habitat quality (12 plots in total), according to plant cover and soil conditions (see Matesanz et al. 2009 for a detailed characterisation of each habitat quality level). Each plot was divided into two contiguous halves containing 20 plants, and each half was randomly assigned to one water treatment. Water manipulation was conducted in the spring (May and June) of 2005 and 2006; these two years were drier than the average, so plants were carefully irrigated to simulate a normal year (see Matesanz et al. 2009 for a detailed description of the irrigation treatments). In summary, the combination of the three experimental factors rendered the following maternal environments: watered and non-watered plants of large fragments of high habitat quality, watered and non-watered plants of large fragments of poor habitat quality, watered and non-watered plants of small fragments of high habitat quality and watered and non-watered plants of small fragments of poor habitat quality. Each maternal environment was replicated three times. These maternal environments represent a severity gradient from the most favourable future scenario for plants (watered mother plants from large fragments of high habitat quality) to the least favourable scenario (non-watered plants from small fragments with poor habitat quality). Five mother plants were randomly selected within the three replicates of each maternal environment (total n = 39 mother plants due to very low germination of one of the mother plants). Plants were selected among those with higher fruit production to ensure subsequent germination and to minimize differences due to any-size dependent effect. All mature and closed capitula were collected in each mother plant at the fruiting peak in late July 2006. Capitula were transported to the laboratory in paper bags and then dissected to count the viable seeds. Viable seeds (filled, undamaged seeds) were stored dry and at room temperature until germination.

### Germination, seedling growth and morphological measurements

On 31 August 2006, simulating germination following natural dispersal, seeds were sown in an experimental glasshouse in order to determine the effects of the maternal environment on germination, seedling growth and survival. Seeds were randomly sown in 390 cavities (4 × 4 cm) filled

with gypsum soil collected from the study area. Soil was collected from a relatively small area to avoid heterogeneity in the substrate, and it was thoroughly mixed and sieved afterwards. A total of 5394 seeds were sown (2–9 per cavity and 80–300 per mother plant, depending on seed availability). Seeds were monitored twice a week during September 2006, and every two weeks during October and November. They were considered to have germinated as soon as cotyledons emerged above the soil. During this period, trays were periodically watered to ensure permanent humidity, and temperature in the experiment was controlled to match the optimum reported for this species (Escudero et al. 1997). Final germination percentage and number of days required for germination of 50% of the total number of germinated seeds ( $t_{50}$ ) were calculated for each mother plant. In the situations where several seeds germinated in the same cavity, extra seedlings were clipped immediately and the first one to emerge was kept. These clipped seedlings were not included in the survival analyses but they were considered for germination analyses. Seedling survival rate was evaluated since seedling emergence to the beginning of the water stress experiment.

On 5 December 2006, a total of 360 (25–53 per maternal environment) surviving healthy seedlings were transplanted into randomly arranged pots (500 ml each) filled with the same type of soil used for the germination trays. During this period, pots were watered twice a week to keep them moist. On 12 March 2007, number of leaves and major and minor diameter of the rosette were recorded for each seedling. Diameters were measured using a digital calliper with an accuracy of 0.01 mm. Rosette area was calculated as the area of an ellipse. These measurements were repeated after two months in early May.

### Water stress experiment and physiological measurements

An experiment simulating Mediterranean summer drought was carried out in May–June 2007 to evaluate the effect of the maternal environment on seedling performance and plasticity in response to stressful conditions mimicking field conditions. We determined the survival curve along the

drought experiment and measured maximal photochemical efficiency of photosystem II ( $F_v/F_m$ ), maximum net photosynthetic rate ( $A_{max}$ ) and instantaneous water use efficiency (iWUE) at two different soil water content. Volumetric soil water content (SWC) was determined for a random subsample of 40 pots twice each week. Probes comprised of two parallel stainless steel rods, 5 cm in length. Separation between their axes was also 5 cm. In order to obtain optimal measurements, the probes were inserted in the pots before the seedling transplant to avoid interferences with plant growth and to ensure the maximum contact between the rods and the soil (Topp and Davis 1985). Also, all pots were weighted twice a week to determine water loss. Water stress was slowly imposed adjusting soil water content of the pots by replacing part of the transpired water (Fig. 1). Measurements were taken before watering to obtain an estimate of maximum stress between two watering events.

Physiological measurements were carried out at two levels of soil water content: 1) well-watered conditions (20–25% SWC), and 2) low-watered conditions (5–6% SWC). It has been shown that effects of water stress on seedling performance of Mediterranean woody plants are noticeable for values of SWC below 10% (Valladares et al. 2005). Therefore, soil was slowly dried and SWC was maintained below this level during ten days before low-watered measurements (Fig. 1). Air temperature and available photosynthetic photon flux density (PPFD) were recorded every 5 min during the whole water stress experiment with a data logger. Mean daily temperature for this period was 25.3°C; mean maximum and minimum temperature were 39.3°C and 14.8°C respectively; and mean daily PPFD (400–700 nm) was 31.6 mol m<sup>-2</sup> s<sup>-1</sup>. These conditions were very similar to the conditions experienced by mother plants in the field (Matesanz unpubl.).

The maximal photochemical efficiency of photosystem II ( $F_v/F_m$ ) in one fully expanded leaf was determined for all seedlings (n = 360) with a portable pulse-modulated fluorometer. Measurements were taken from 8:00 to 10:00 GMT in healthy leaves dark-adapted for 30 min with leaf clips provided by the manufacturer. Minimal ( $F_o$ ) and maximal ( $F_m$ ) fluorescence were measured, and these values were used to calculate  $F_v/F_m$  as  $F_v/F_m = (F_m - F_o)/F_m$  (Maxwell

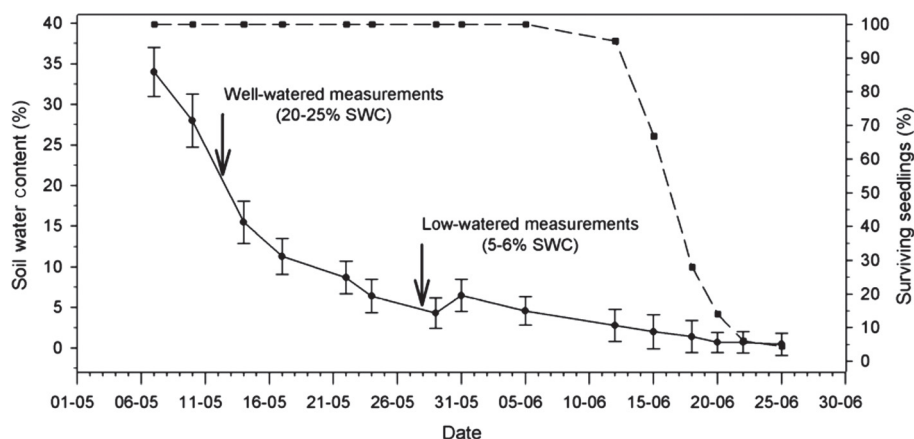


Figure 1. Temporal dynamics of soil water content in the pots during the water stress experiment. The dashed line shows the percentage of surviving seedlings at each soil water content. The arrows denote two levels of soil water content (SWC) where the physiological measurements were done.

and Johnson 2000). Maximum net photosynthetic rate ( $A_{\max}$ ) was measured in 80 randomly chosen seedlings (ten per maternal environment) in one fully-expanded leaf using a portable gas exchange system. The leaves were exposed to an atmospheric  $\text{CO}_2$  concentration of  $400 \mu\text{l l}^{-1}$  (using the built-in  $\text{CO}_2$  controller) and saturating light ( $> 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR). Seedlings' leaves were too narrow for the standard chamber, so the 'Arabidopsis-chamber' was used. This chamber lacks an internal light source, and measurements were taken using an external source of cold light. Measurements were performed at 25–26°C (i.e. growth temperature) and relative humidity of 45–50%. Instantaneous water use efficiency (iWUE) was estimated as the ratio between maximum photosynthetic rate and stomatal conductance (Gulías et al. 2002).

Watering was decreased after low-watered measurements to keep soil water content below 5%, so the effects of maternal environment on seedling survival to drought could be addressed. All pots were examined every day until seedling death, and pot weight was then recorded to calculate soil water content. We labelled as dead those seedlings presenting no photosynthetically active leaves (i.e. green and bendy leaves) and exhibited loss of flexibility in the stems. No resprouting was observed after watering in a random sample of seedlings previously labelled as dead, confirming our estimation of mortality. SWC for each pot at seedling death was estimated as:  $\text{SWC} = (\text{soil weight} - \text{dry soil weight}) / \text{soil weight}$ , where  $\text{soil weight} = \text{pot weight} - (\text{empty pot weight} + \text{TDR probes weight})$ . Soil was dried at ambient temperature during two weeks to obtain the dry soil weight measurement. We checked in a random soil sample dried in an oven at 60°C that SWC was negligible after this period.

## Statistical analysis

Analyses were carried out using SAS 9.0 (SAS Inst.). The effects of the three global change drivers (fragmentation, habitat quality and mother plant water availability, and their interactions) on germination percentage, germination rate ( $t_{50}$ ), survival rate and seedling growth of *C. hyssopifolia* were explored fitting generalized linear mixed models using the procedure GLIMMIX of SAS. We used the restricted maximum-likelihood method (REML) and type III sum of squares. Global change drivers were included in our models as fixed effects (one degree of freedom per treatment) and mother plant identity (nested within fragmentation  $\times$  habitat quality  $\times$  water availability interaction) as a random factor. GLMMs allow the analysis of response variables that are generally non-normally distributed, such as percentages, by adjusting adequate function responses and are appropriate because they allow for a distinction between fixed and random effects in the models (see Littell et al. 1996 and Bolker et al. 2008 for further details). Germination and final survival rate were modelled as binomial distributions with a logit link function, and  $t_{50}$ , number of leaves per seedling and seedling rosette size were modelled using a normal distribution with an identity link function. Effect of the mother plant identity was tested using Wald Z-statistic test, which is appropriate for large samples (Littell et al. 1996). Post-hoc tests were performed using the LSMEANS option of the GLIMMIX procedure.

Survival curves of seedlings of different treatments were estimated by means of the Kaplan–Meier product-limit

method and compared using non-parametric logrank test (Pyke and Thompson 1986). This statistical analysis was performed using STATISTICA 6.0 (Statsoft).

Effects of global change drivers on physiological measurements in the water stress experiment were explored separately for each level of seedlings' water availability (well watered and low-watered conditions) due to the complexity of the model. Differences in  $F_v/F_m$ ,  $A_{\max}$  and iWUE values among factors were explored by means of GLMMs including mother plant identity as a random factor following the same approach as used for previously described GLMMs. We assumed a binomial error distribution with a logit link function in  $F_v/F_m$  variable response and a normal error distribution with an identity link function in  $A_{\max}$  and iWUE variables.

To test treatment effect (water conditions) and treatment  $\times$  maternal environment interaction effect we used additional repeated measures analyses performed by means GLIMMIX procedure of SAS. Maternal environment (resulting from the combination of the three global change drivers: habitat fragmentation, changes in habitat quality, and water availability) was in this case a factor with eight different levels corresponding to each maternal environment. Individual seedling (for  $F_v/F_m$ ), and individual mother plant (for  $A_{\max}$  and iWUE) were considered as random factors (subject), and treatment (within-subject), and maternal environment (among-subject) were considered fixed factors. Finally, effects of global change factors on drought seedling tolerance (i.e. SWC in the day of seedling death) were explored following the same approach as used for previous GLMMs. We assumed binomial distributions with a logit link function in this case.

## Results

### Germination and survival under well-watered conditions

Germination percentages were in general low. Mean germination percentage of plants from each maternal environment ranged from 13% to 21%. We found a positive maternal effect of water availability on germination, with lower germination percentages of the seeds from non-watered mother plants (Table 1, Fig. 2a). Likewise, we found a significant three-way interaction (fragmentation  $\times$  habitat quality  $\times$  water availability) on this trait, with lower values of germination of the seeds from the least favourable maternal environment, i.e. non-watered plants from small fragments of poor habitat quality (Table 1, Fig. 2a). In addition, fragmentation also had a significant and negative effect on the number of days required for germination of 50% of the total germinated seeds ( $t_{50}$ ). Seeds of plants from large fragments germinated faster than those from small fragments (Table 1, Fig. 2b).

Percentage of surviving seedlings from each environment ranged between 32% and 60% (Appendix 1). We found a significant and negative effect of fragment size on final survival rate (Table 1): seedlings of mothers from large fragments had higher final survival. Moreover, Kaplan–Meier analysis revealed significant differences in the survival function of seedlings of different mother plants, with those from large fragments having a slower rate of mortality than those from small fragments (log rank = 2.54,  $p = 0.010$ , Fig. 3).

Table 1. Results for the fixed effects of generalized linear mixed models (GLMMs) used to test the effects of global change factors (fragmentation, habitat quality and water availability, and their interactions) on germination percentage, time at which 50% of seeds germinated ( $t_{50}$ ) and final survival rate. Germination and survival were modelled using binomial distributions, and  $t_{50}$  with normal distribution.  $p < 0.05$  are in bold.  $n = 39$  mother plants.

Source of variation	Germination			$t_{50}$			Survival		
	F	DF	p	F	DF	p	F	DF	p
Fragmentation (F)	0.62	1	0.433	4.22	1	<b>0.043</b>	4.18	1	<b>0.041</b>
Habitat quality (Q)	0.55	1	0.459	0.22	1	0.642	0.28	1	0.596
Water availability (W)	8.10	1	<b>0.005</b>	0.93	1	0.336	0.05	1	0.816
F × Q	0.54	1	0.4639	0.18	1	0.674	0.11	1	0.735
F × W	0.77	1	0.3824	1.27	1	0.262	2.07	1	0.151
Q × W	0.53	1	0.469	0.45	1	0.502	0.04	1	0.837
F × Q × W	0.12	1	<b>0.043</b>	0.57	1	0.450	2.72	1	0.099

Mother plant identity did not have any significant effect on these variables ( $Z < 1.20$ ,  $p > 0.11$ ).

### Seedling growth

There was a marginally significant and positive effect of mother plant water availability on rosette size in March measurements ( $F = 4.12$ ,  $DF = 1$ ,  $p = 0.052$ ). Seedlings from watered mother plants had significantly higher area than non-watered ones at this time (mean  $\pm$  SE:  $24.06 \pm 0.67$  and  $22.70 \pm 0.61$  cm<sup>2</sup>, respectively). However, no significant effects of global change drivers or their interactions were found on the number of leaves per seedling ( $F < 0.44$ ,  $DF = 1$ ,  $p > 0.510$  in all cases) or on rosette size ( $F < 2.65$ ,  $DF = 1$ ,  $p > 0.113$  in all cases) in May measurements. Mother plant identity did not have any significant effect on these variables ( $Z < 1.05$ ,  $p > 0.14$ ).

### Seedling performance and survival under water stress

We did not find any significant effect of global change drivers on maximal photochemical efficiency ( $F_v/F_m$ ) in either well-watered or low-watered conditions (Table 2). Repeated measures analysis showed that  $F_v/F_m$  values were significantly

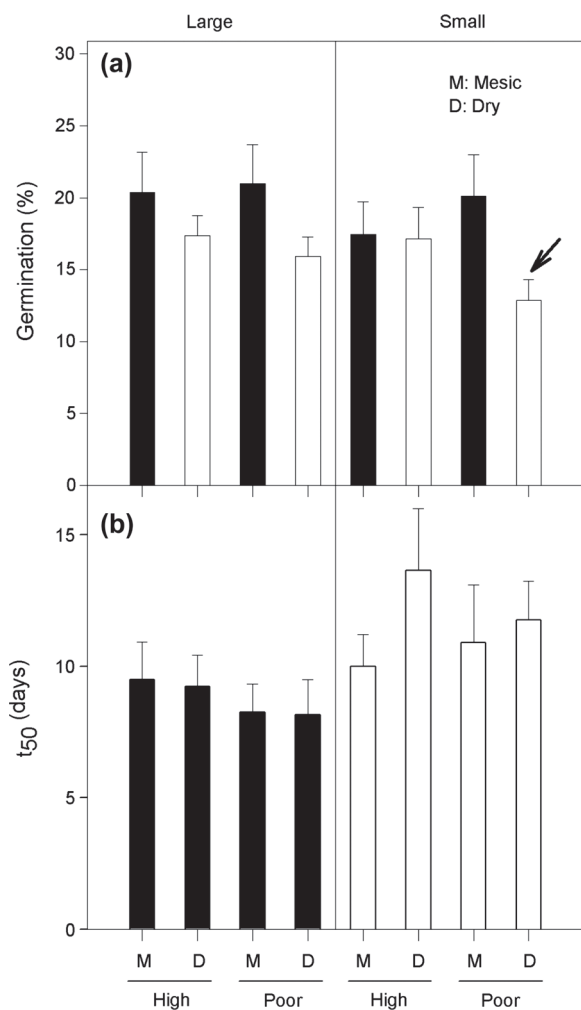


Figure 2. Mean values of (a) germination percentage and, (b) number of days required for germination of 50% of the total seed number, for the eight different maternal environments with different fragment size (small and large), habitat quality (high and poor), and water conditions (well- and low-watered). Different colours in the bars indicate significant differences at the 5% level for the main effects. A post-hoc test showed significantly lower germination percentages in seeds from less favourable environments, as indicated by the arrow.

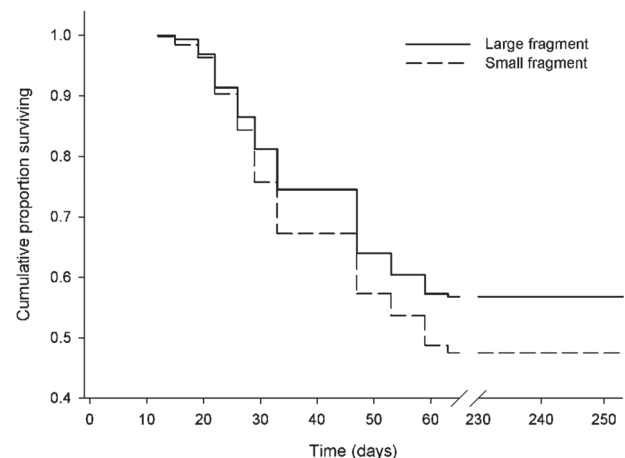


Figure 3. Kaplan–Meier survivorship curves for seedlings of mother plants from large and small fragments. Curves differed significantly at the 5% level in the log-rank test. Figure shows survival curves only for small versus large fragments because only fragmentation had an effect on final survival rate.

Table 2. Results for the fixed effects of generalized linear mixed models (GLMMs) used to test the effects of global change factors (fragmentation, habitat quality and water availability, and their interactions) on seedling performance under well-watered and low-watered conditions in the water stress experiment. Analyses were based on a GLMM with binomial error and logit link for maximal photochemical efficiency ( $F_v/F_m$ ), and with normal distributions and identity function link for maximum photosynthetic rate ( $A_{max}$ ) and water use efficiency (iWUE).  $p < 0.01$  are in bold.

Source of variation	$F_v/F_m$			$A_{max}$			iWUE		
	F	DF	p	F	DF	p	F	DF	p
a) Well-watered									
Fragmentation (F)	0.98	1	0.323	12.64	1	< <b>0.001</b>	1.23	1	0.267
Habitat quality (Q)	0.66	1	0.416	3.58	1	0.058	0.37	1	0.542
Water availability (W)	0.18	1	0.669	0.35	1	0.556	1.00	1	0.317
F × Q	0.63	1	0.427	2.26	1	0.132	3.69	1	0.054
F × W	0.02	1	0.887	2.00	1	0.157	1.37	1	0.241
Q × W	1.41	1	0.236	4.77	1	0.028	0.84	1	0.358
F × Q × W	2.32	1	0.129	6.57	1	<b>0.010</b>	1.90	1	0.167
b) Low-watered									
Fragmentation (F)	0.01	1	0.924	1.09	1	0.297	0.96	1	0.329
Habitat quality (Q)	0.62	1	0.432	0.85	1	0.359	0.47	1	0.494
Water availability (W)	0.02	1	0.902	0.00	1	0.976	0.83	1	0.355
F × Q	0.53	1	0.467	1.23	1	0.205	2.48	1	0.120
F × W	0.45	1	0.502	1.11	1	0.297	0.40	1	0.531
Q × W	1.35	1	0.246	0.30	1	0.584	0.94	1	0.336
F × Q × W	4.89	1	0.066	0.81	1	0.326	0.71	1	0.401

lower under low-watered ( $F = 3.27$ ,  $DF = 1$ ,  $p < 0.001$ ; mean  $\pm$  SE:  $0.82 \pm 0.04$  and  $0.68 \pm 0.03$ , respectively; Fig. 4), although they were still relatively high.

Maximum photosynthetic rate ( $A_{max}$ ) was negatively affected by fragmentation under well-watered conditions. Likewise, we found a significant three-way interaction (fragmentation  $\times$  habitat quality  $\times$  water availability) under well-watered conditions, indicating higher values for seedlings from non-watered mother plants from large fragments of poor habitat quality (Table 2). Repeated measures analysis showed that  $A_{max}$  was also significantly lower under low-watered conditions ( $F = 58.22$ ,  $DF = 1$ ,  $p < 0.001$ ; mean  $\pm$  SE:  $9.27 \pm 0.33$  and  $2.58 \pm 0.31$  for well-watered and low-watered conditions respectively; Fig. 4). In this case, we found a significant treatment  $\times$  maternal interaction, where seedlings of watered mother plants from small fragments of poor habitat quality showed a lower  $A_{max}$  reduction ( $F = 26.44$ ,  $DF = 7$ ,  $p < 0.001$ ).

Water use efficiency (iWUE) was significantly higher under water stress ( $F = 19.57$ ,  $DF = 1$ ,  $p < 0.001$ ; mean  $\pm$  SE:  $11.38 \pm 1.33$  and  $30.04 \pm 2.14$  for well-watered and low-watered conditions respectively; Fig. 4) and no effect of global change drivers were found for this variable (Table 2).

Likewise, there was no significant effect of the global change factors ( $F < 2.14$ ,  $DF=1$ ,  $p > 0.158$ ) or the mother plant identity ( $Z = 1.59$ ,  $p = 0.156$ ) on seedling survival to drought (i.e. SWC in the day of the seedling death). Ninety-five percent of the seedlings survived until soil water content was as low as 3%, and they tolerated SWC values below 5% for many days (Fig. 1).

## Discussion

Overall, we found a significant effect of the global change drivers on the offspring of *Centaurea hyssopifolia*. Specifically,

we found an effect of fragmentation on germination rate and seedling performance, matching the patterns found for other fragmented populations of self-incompatible species (Menges 1991, Kolb 2005). Germination under controlled conditions of the seeds from small fragments was lower and slower, and seedling mortality was higher and occurred faster. Likewise, we found a positive effect of water availability experienced by mother plants on germination. Finally, we detected a significant three-way interaction of the three global change drivers on the germination percentage; i.e. it was lowest in the least favourable environment (non-watered mother plants from small fragments of poor habitat quality). This result highlights the importance of multifactor experiments, as non-additive synergistic effects among global change drivers have been shown to affect plant responses (Sala et al. 2000).

Negative effects of fragmentation on germination and survival may be due to several non-exclusive reasons: genetic deterioration, quality of the available pollen for plants, and other maternal environment effects related to habitat fragmentation. In small fragments, pollinators abundance and behaviour may be profoundly affected, decreasing the abundance and the quality of available pollen (Aizen et al. 2002, Duncan et al. 2004, Aguilar et al. 2006, Lopes and Buzzato 2007). In self-incompatible species, as in our study plant, this fact may lead to a decrease in the amount of potential mates, and to the increase of crosses among related congeners (biparental inbreeding), which in turn may increase homozygosity and other deleterious genetic effects linked to inbreeding. Furthermore, reductions in pollen competition and gametes selectivity, before and after fertilization, may reinforce these negative effects on seed viability, germination, and also on offspring fitness (Colling et al. 2004). Kolb (2005) discussed the role of genetic effects to explain the low seedling survival of *Phyteuma spicatum* in small and fragmented populations. Several authors have tried to separate this so-called genetic

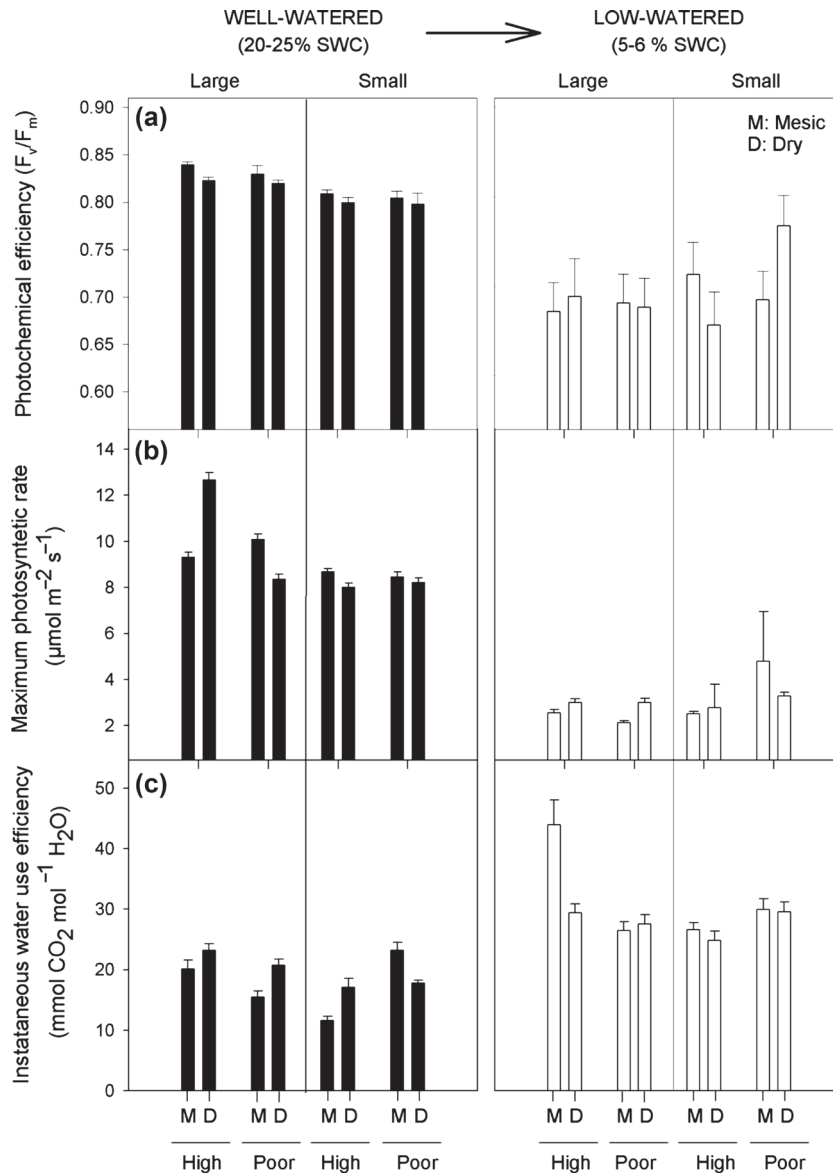


Figure 4. Mean values of (a) photochemical efficiency, (b) maximum photosynthetic rate and (c) water use efficiency of seedlings from the different maternal environments in well (left panels) and low-watered conditions (right panels). Different colours of the bars show significant differences between values in well- and low-watered conditions at the 5% significance level. Error bars represent one standard error of the mean.

effect from the maternal environment when describing transgenerational effects (Weiner et al. 1997). However, it is not possible to fully disentangle the genetic component of this response from the ecological component (maternal environment). In our study, some kind of genuine genetic effects may be responsible for the observed results. This is supported by previous findings of the same study population, where plants from small fragments had lower viable seed production and lower adult plant survival (Matesanz et al. 2009) which are traits frequently related to inbreeding (reviewed by Lienert 2004). An effect of fragmentation on subsequent life stages would support the genetic hypothesis to a greater extent (Roach and Wulff 1987). Despite our results do not show a significant effect of fragmentation on rosette size or number of leaves (as measures of plant growth), the effects of inbreeding depression may

arise under more stressful natural conditions (Ramsey and Vaughton 2000, Kolb 2005).

We also found that water shortage during mother plant growth exerted a negative effect on the germination of its seeds. The effect of water stress during mother plant life has been found to negatively affect germination in other plant species (Acosta and Kohashi 1989, Nielsen and Nelson 1998). Furthermore, in our case, water availability of mother plants had a positive effect on seedling growth, although it was only visible in the first measurement date. Transmission mechanisms can explain this genuine maternal environmental effect by: (1) affecting the quantity and quality of the resources supplied to the seeds (Weiner et al. 1997), (2) changing the structure and thickness of the seed coat (Lacey et al. 1997), (3) inducing abortion of seeds (Marshall and Ellstrand 1988) and (4) affecting the levels

of hormones, enzymes, etc during germination and early performance. Water stress during seed formation must have affected seed provisioning and enzymatic activity related to germination and initial development of the seedling, while subsequent stages of the offspring life can be expected to be less influenced (Roach and Wulff 1987).

The effect of water shortage on germination was boosted by the negative effect of both fragmentation and poor habitat quality. This finding concurs with Kolb (2005), who found that small-sized populations are more sensitive to unfavourable environmental conditions, and again highlights the importance of multifactor studies. This is particularly relevant for the Mediterranean region (Christensen et al. 2007) where more frequent and severe drought episodes are predicted to occur, leading to increased aridity. This, together with longer time for germination and an advanced mortality of seedlings from small fragments, may translate into lower water availability at the seedling stage, as seedlings would be smaller at the beginning of summer drought, which in turn can translate into higher mortality.

All the physiological variables were affected by water stress, but contrary to our expectations, seedlings' response did not differ in relation to their maternal environment. Although maximal photochemical efficiency ( $F_v/F_m$ ) values are generally considered to be more sensitive to high temperature and light intensity than to water stress (Long et al. 1994, Llorens et al. 2003), we found decreases in  $F_v/F_m$  under the optimum values (0.83 according to Maxwell and Johnson 2000). Aragón et al. (2008) found that even small deviations in  $F_v/F_m$  values of a Mediterranean chamaephyte (*Helianthemum squamatum*) had strong consequences on plant fitness, and highlighted that the timing of stressful events may be even more important than the stress intensity. On the other hand, water use efficiency increased significantly under low-watered conditions, probably as a consequence of the reduction of stomatal conductance while maintaining similar photosynthetic rates, and concurs with other studies (Liu et al. 2005, Valladares et al. 2005). Valladares and Sánchez-Gómez (2006) identified a second stage where iWUE decreased pronouncedly as a response to prolonged and severe water stress, when SWC was around 7%. This is in contrast with our study, where seedlings showed an increase in iWUE under SWC of 5–6%. In this context, it is noteworthy that 95% of the plants survived until SWC was extremely low (3%), highlighting the capacity of this species to cope with water stress, and agreeing with results for other gypsum species (Aragón et al. 2007).

In conclusion, our results show that combinations of global change drivers can have effects not only on adult plant performance in natural conditions but also transgenerational effects on the plant offspring, as shown by the significant effect of the maternal environment on early fitness components and offspring performance under controlled conditions. These results suggest that detrimental effects of global change drivers can affect demographic trends not only by constraining the fertility rates of the population but also by conditioning the performance of future generations. Our findings also highlight the importance of human-mediated changes on population persistence, as they affected not only germination and seedling survival but also the capacity to exploit spring resource pulses, a typical feature of Mediterranean environments, and suggest that these effects are a

complex sum of both genetic and ecological factors. Despite the ability of seedlings to cope with water stress found for this species, our results show high vulnerability to the predicted climate change, as shown by its effects on germination. Finally, our study revealed the importance of including not only different global change drivers but also their assessing their effects on the performance of the descendants.

*Acknowledgements*—The authors would like to thank José Margalet (URJC) and Lorena Suárez (UdeC) for their help in the greenhouse. Financial support was provided by the Spanish Ministry of Education and Science (ECOCLIM, CGL2007-66066-C04-02/BOS and EXTREM, CGL 2006-09431) and by Programa de Actividades de I+D de la Comunidad de Madrid REMEDINAL-CM (S-0505/AMB/000335).

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Appendix 1. Mean values  $\pm$  SE of the study variables in the different maternal environments.

	Large fragments						Small fragments					
	High habitat quality			Poor habitat quality			High habitat quality			Poor habitat quality		
	Mesic	Dry		Mesic	Dry		Mesic	Dry		Mesic	Dry	
<b>Morphological traits</b>												
Germination (%)	20.39 $\pm$ 2.81	17.37 $\pm$ 1.40	20.97 $\pm$ 2.75	15.94 $\pm$ 1.33	17.45 $\pm$ 2.29	17.13 $\pm$ 2.21	20.10 $\pm$ 2.90	12.87 $\pm$ 12.87				
T <sub>50</sub> (days)	9.50 $\pm$ 1.43	9.22 $\pm$ 1.19	8.25 $\pm$ 1.08	8.15 $\pm$ 1.34	10.01 $\pm$ 1.19	13.65 $\pm$ 2.32	10.90 $\pm$ 2.19	11.75 $\pm$ 1.19				
Survival (%)	59.93 $\pm$ 8.10	51.47 $\pm$ 3.6	52.49 $\pm$ 5.6	60.12 $\pm$ 6.56	32.37 $\pm$ 7.50	52.99 $\pm$ 6.14	45.47 $\pm$ 6.44	45.94 $\pm$ 5.18				
No. leaves	16.96 $\pm$ 0.39	15.11 $\pm$ 0.49	16.17 $\pm$ 0.47	16.33 $\pm$ 0.42	16.31 $\pm$ 0.58	16.14 $\pm$ 0.43	15.32 $\pm$ 0.44	16.68 $\pm$ 0.51				
Size March (cm <sup>2</sup> )	24.46 $\pm$ 1.09	23.91 $\pm$ 1.56	25.61 $\pm$ 1.14	21.24 $\pm$ 1.10	21.83 $\pm$ 1.52	22.64 $\pm$ 1.40	24.11 $\pm$ 1.33	23.06 $\pm$ 1.96				
Size May (cm <sup>2</sup> )	42.90 $\pm$ 0.09	42.23 $\pm$ 1.66	40.52 $\pm$ 0.80	45.48 $\pm$ 1.10	41.03 $\pm$ 0.70	43.20 $\pm$ 1.20	42.15 $\pm$ 1.07	42.02 $\pm$ 1.06				
<b>Physiological traits</b>												
<b>Well-watered</b>												
F <sub>v</sub> /F <sub>m</sub>	0.84 $\pm$ 0.003	0.82 $\pm$ 0.003	0.83 $\pm$ 0.008	0.82 $\pm$ 0.004	0.81 $\pm$ 0.004	0.79 $\pm$ 0.005	0.80 $\pm$ 0.007	0.78 $\pm$ 0.010				
A <sub>max</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	9.32 $\pm$ 0.21	12.67 $\pm$ 0.31	10.09 $\pm$ 0.23	8.36 $\pm$ 0.21	8.69 $\pm$ 0.12	8.01 $\pm$ 0.16	8.48 $\pm$ 0.20	8.23 $\pm$ 0.17				
iWUE (mmol CO <sub>2</sub> mol <sup>-1</sup> H <sub>2</sub> O)	20.14 $\pm$ 1.46	23.23 $\pm$ 1.09	15.46 $\pm$ 0.98	20.77 $\pm$ 1.01	11.58 $\pm$ 0.72	17.10 $\pm$ 1.49	23.19 $\pm$ 1.31	17.78 $\pm$ 0.47				
<b>Low-watered</b>												
F <sub>v</sub> /F <sub>m</sub>	0.67 $\pm$ 0.032	0.69 $\pm$ 0.042	0.68 $\pm$ 0.031	0.67 $\pm$ 0.032	0.71 $\pm$ 0.036	0.65 $\pm$ 0.037	0.68 $\pm$ 0.032	0.77 $\pm$ 0.034				
A <sub>max</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	2.13 $\pm$ 0.15	2.59 $\pm$ 1.68	1.68 $\pm$ 0.08	2.60 $\pm$ 0.17	2.08 $\pm$ 0.11	2.35 $\pm$ 1.05	4.43 $\pm$ 2.23	2.88 $\pm$ 0.18				
iWUE (mmol CO <sub>2</sub> mol <sup>-1</sup> H <sub>2</sub> O)	44.74 $\pm$ 4.16	29.94 $\pm$ 1.51	26.91 $\pm$ 1.55	27.51 $\pm$ 1.54	27.01 $\pm$ 1.18	25.33 $\pm$ 1.54	30.47 $\pm$ 1.79	30.13 $\pm$ 1.64				