

## Seed size explains within-population variability in post-fire germination of *Cistus salviifolius*

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In fire-prone environments, studying the variability of regeneration traits is important to show the potential of plant species to survive under consecutive post-fire and fire-free conditions. We studied the relationship between seed size and germination patterns in *Cistus salviifolius* (Cistaceae), which is a fire-follower species distributed throughout the Mediterranean Basin. Since fire plays an important role in the life cycle of the studied species, we made a germination experiment that included a heat-shock treatment (to simulate fire) and a control without a heat-shock (to simulate fire-free conditions) using seeds collected from individual plants to assess within-population germination responses to fire. Seed germination of most of the individuals was stimulated by heat-shock, but the germination characteristics showed substantial variation among individuals within the population. Mean seed mass (i.e. seed size) was positively correlated with germination percentage in the heat treatment, but no correlation was found between seed mass and germination percentage in the control. On the other hand, mean seed mass was negatively correlated with seed mortality both in the heat-shock treatment and in the control. The results show that even if the response of the majority of the individuals in the population follows the population-level germination response of the species, a significant variability in germination actually exists within this population (among individuals). Individuals with larger seeds may be favored in post-fire conditions, while seed size is unimportant in fire-free conditions. Together with the positive selective pressure of seed predation over small-seeded individuals, fire shapes the within-population variability of the regeneration traits such as seed size and post-fire germination in *C. salviifolius*. This variability might have evolved as a bet-hedging strategy against unpredictable conditions in the Mediterranean environment, especially the occurrence of fire and fire-free periods.

### Introduction

In many plants, seed traits such as size, shape, dormancy level, and germination ability may

show substantial variation among populations (Michaels *et al.* 1988, Wyllie-Echeverria *et al.* 2003, Moreira *et al.* 2012), among individuals of the same population (Thompson 1984, Cruz

*et al.* 2003), or even among fruits of the same individual (Tieu *et al.* 2001, Boyd *et al.* 2007, Castellanos *et al.* 2008). Excluding the effect of post-dispersal stochastic events on germination success, such as the availability of resources or safe sites, variability of seed traits among individuals may arise from (1) genetic differences among individual seeds (Keim *et al.* 1990, Silady *et al.* 2011), (2) local differences in resource availability of maternal plants (i.e., due to environmental effects; Venable 1992), or (3) genetic or phenetic differences among maternal plants (i.e., due to maternal effects; Biere 1991, Castellanos *et al.* 2008, Donohue 2009).

Seed traits are related to each other, and in most cases one trait affects another. For instance, the well-known seed size vs. seed number trade-off model predicts that the larger the seeds, the lower the number of seeds produced and *vice versa* (Venable 1992, Jakobsson & Eriksson 2000). However, recent analyses showed that plant size, longevity, juvenile survival rate and time to reproduction may also have an effect on the evolution of seed size (Moles & Westoby 2006, Guo *et al.* 2010). Moreover, size is often evaluated as one of the major indicators of seed quality, and the positive effects of larger seeds on germination and early seedling growth has been reported for many plants (Vera 1997, Jakobsson & Eriksson 2000, Moles & Westoby 2004). Therefore, we expect that seed size should be under high selection pressure (e.g., Lord and Westoby 2006, Martínez *et al.* 2007), and it is a trait reflecting the evolutionary history of species and populations.

As an important evolutionary force for plant life histories (Bond & Keeley 2005, Verdú & Pausas 2007, Pausas & Keeley 2009), fire has shaped many plant traits in fire-prone environments (Keeley *et al.* 2011, Pausas *et al.* 2012). One of the most prominent and well-studied fire-adapted traits among plants is fire-stimulated seed germination, i.e. breaking of seed dormancy by fire-related germination cues (Paula *et al.* 2009). Most of the studies have focused on the germination response of individual species to heat-shock and smoke (Keeley & Fotheringham 1998, Crosti *et al.* 2006, Dayamba *et al.* 2008, Reyes & Trabaud 2009, Moreira *et al.* 2010, and many others), but the variability of fire-related

germination among populations of a species (Tieu *et al.* 2001, Cruz *et al.* 2003, Quaderi & Cavers 2003, Pérez-García *et al.* 2006, Moreira *et al.* 2012) or among individuals of a population (Delgado *et al.* 2001, Cruz *et al.* 2003, Delgado *et al.* 2008) has been less studied. Since the plants have adapted to specific fire regimes but not to the fire itself (Keeley *et al.* 2011), among-population (local) variability of fire-related germination may be a common phenomenon (*see* Moreira *et al.* 2012). Even within the same population, individuals differ in their resource status and genetic structure, thus post-fire germination traits may vary among individuals, which would have significant ecological and evolutionary consequences after the following fire event.

Seed size is a trait shaped by local processes such as fire regime (Moreira *et al.* 2012), and there is evidence for patterns of post-fire germination of plant species depending on their seed size (Hanley *et al.* 2003). Since seed mortality in the upper soil layers is expected to be higher during a fire, large-seeded species in which the seedlings can emerge from deeper layers in the soil have an advantage over small-seeded ones, in which the seeds buried in the deeper soil layers are less likely to emerge as seedlings (Bond *et al.* 1999). However, small-seeded species in fire-prone environments have adapted to higher fire temperatures for germination than larger-seeded ones (Hanley *et al.* 2003). Fire may also act as a selective force on the seed size at individual level within a population. For example, post-fire germination is positively related to seed mass in *Cistus ladanifer* (Delgado *et al.* 2001), but lighter seeds germinate faster than heavier ones in the absence of fire temperatures (Delgado *et al.* 2008). Therefore, it is possible that recruitment from lighter seeds is favored in the lack of fire (inter-fire years), while plants with heavier seeds are more likely to recruit just after the fire in that species (Delgado *et al.* 2008). Although those studies give some insight on how post-fire germination can be related to seed size in a plant population, there is an insufficient number of similar studies on different species, so it is at present difficult to make generalizations on fire-related germination and seed size relationship within plant populations.

The aim of the present study was to investigate the relationship between seed size and ger-

mination traits in *Cistus salviifolius* (Cistaceae), a fire-follower species, at the individual plant level. We selected *C. salviifolius* not only because it is a species with a well-known germination response to heat-shock, but also because it is distributed throughout the fire-prone areas of the Mediterranean Basin (Moreira *et al.* 2012). To reach our aim, we conducted a germination experiment and correlated the results with previously measured seed mass values. Since fire plays an important role in the life cycle of *C. salviifolius*, our experiment included a heat-shock treatment (to simulate fire) and a control without a heat shock (to simulate fire-free conditions). We hypothesized that the germination percentage and seed size should be positively correlated in the heat-shock treatment but not in the control at the individual plant level. We also aimed to detect if seed size explains a significant proportion of the variability of germination among individuals in *C. salviifolius*.

## Material and methods

### Study site and climate

The study was conducted at a site 1 ha in size with a large *C. salviifolius* population (high density and cover value) located within a four-year-old burned area (burned in 2002) in the Çetibeli region, Marmaris, southwestern Turkey (36°59'N, 28°20'E, 239 m a.s.l.). The climate is a Mediterranean one including five-month-long dry summers (May–September) with only 56.8 mm of rainfall, and wet winters. The mean total precipitation is 1211.7 mm and the yearly mean temperature is 18.7 °C (data from Turkish State Meteorological Service, 1975–2006). The total precipitation and mean temperature during the six-month period before the sampling (October 2005–March 2006) were 1154 mm and 13.2 °C, respectively, whereas the rainfall during March 2006 was 218.7 mm, enough to produce a good seed yield for *C. salviifolius* in the region (Tavşanoğlu 2010).

### Seed collecting

A total of 17 *C. salviifolius* individuals 3–4

years old were randomly selected (but shaded ones were excluded) in the study site in July 2006. Fruits of the selected individuals were collected and stored in nylon bags until bringing them into the laboratory. For each individual, all the collected fruits were opened by hand and the seeds obtained. Seeds from each individual were placed separately in plastic Petri dishes and stored in a dry and dark place at room temperature until the germination experiment commenced.

### The experiment

The germination experiment was conducted between November 2009 and January 2010. Although the seed storage period before we started the experiment had been rather long (more than three years), we expected very little viability loss in the seeds, because of the long-term persistence (~20 years) of the hard-coated water-impermeable seeds of *Cistus* species in the soil seed bank (Cerabollini *et al.* 2003, Probert *et al.* 2009).

Before the experiment, four replicates of seed lots composed of 50 seeds were weighed on a digital balance to estimate the mean seed mass of each individual. We used the mean seed mass as a measure of the seed size of the individual plants. Because the numbers of seeds were limited for an individual-level germination experiment with several treatments, we only included a control and a heat-shock treatment in the experiment. We used 120 °C for 5 minutes as the heat-shock treatment; one of the temperatures in which the best germination response was obtained for the *C. salviifolius* populations of the region (Moreira *et al.* 2012).

The heat-shock treatments were applied in a temperature-controlled oven for each individual. The procedure was repeated three times for each individual (one time for one replicate) to avoid pseudoreplication (Morrison & Morris 2000). Some seeds of the separate individuals remained untreated to serve as the control for the treatment. The treated and untreated seeds were sown into Petri dishes containing agar as substrate. For each individual and treatment (i.e., the control and the heat treatment) combination,

three replicates of 25 seeds (a total of 75 seeds) were evenly spaced in Petri dishes. A total of 2550 seeds were used for the experiment. The dishes were placed in a germination cabinet and incubated at 20 °C ( $\pm 0.5$  °C) in darkness.

The seeds were checked to see if they germinated every two or three days during the first four weeks of the experiment, and then once a week until the end of the experiment. The germinated seeds were counted and removed from the Petri dishes at every check. Radicle emergence was the criterion for scoring germination. During the checks, empty seeds and the seeds showing clear indication of rotteness were removed from the dishes and recorded. The experiment was ended on the 66th day of the incubation period when no germination occurred in the heat-shock treatment and in the control for a week.

## Data analysis

We estimated the germination percentage of each treatment for each individual as the mean of three replicates of the corresponding treatment. While estimating the germination percentage, empty seeds were excluded from the total seed number and all further analyses were performed after this correction was done. Non-germinated and rotten seeds in the dishes at the end of the experiment were included in the analysis as non-germinated ones. Rotten seeds were also assumed to indicate seed mortality.

Probability of germination stimulation by heat-shock treatment was analyzed using the

analysis of deviance (GLM) with binomial error distribution. We included the germinated vs. non-germinated seeds (including rotten but not empty ones) in the analysis. For each individual, the total number of seeds that germinated in the heat treatment was compared with that in the control. To see the effect of seed size on the germination, a two-way ANOVA was conducted with seed size class (based upon mean seed mass for each individual) and treatment as fixed factors. For this analysis, the individuals were divided into almost equal three groups, as large ( $> 0.99$  mg), medium (0.84–0.98 mg) and small ( $< 0.83$  mg) (seed size class) according to their mean seed mass, and the data were log-transformed ( $\log_{10}[x + 0.01]$ ) to obtain normality and homoscedasticity. The germination rate for each individual in each treatment was estimated as  $t_{50}$  (time to reach 50% germination) using a CurveExpert Basic software (Hyams 2010). The germination curve of each replicate was fit to a logistic curve, and  $t_{50}$  was calculated as the point on the curve that intersected the line for 50% germination (Kettenring *et al.* 2006). The association of mean seed mass with germination percentage, seed mortality, and germination rate was examined by fitting linear models. The germination percentage and  $t_{50}$  data were log-transformed before the analysis for a better approximation to the normal distribution. All the analyses but the  $t_{50}$  estimation were conducted in R version 2.9.2 (R Development Core Team 2009).

## Results

The germination percentages in the control and in the heat-shock treatment varied among individuals. In total,  $\sim 41\%$  (21.5% + 19.6%) of the deviance of germination response to heat-shock was explained by the difference among individuals (Table 1). At the same time, however, there were clear trends in the germination percentages. Most of the individuals (13) had a significantly higher germination percentage in the heat-shock treatment as compared with that in the control, except for four individuals (numbers 6, 8, 11, 17 in Table 2). Most of the control germinations were lower than 8% except for two individuals (8 and 15) with  $> 20\%$  germination, and most of

**Table 1.** Analysis of germination probability in relation to heat-shock treatment and individuals (GLM). The deviance explained refers to the percentage of null model deviance explained by each factor. Akaike Information Criterion (AIC) of the model is 310.8.

Factor	Deviance ( $\chi^2$ )	Deviance explained (%)	df	$p$
Heat	280.6	46.6	1	$< 0.0001$
Individual	129.9	21.5	16	$< 0.0001$
Heat $\times$ Individual	118.5	19.6	16	$< 0.0001$
Model	529.0	87.7	33	
Residual	73.8		68	
Total	602.8		101	

the germinations in the heat-shock treatment were higher than 15% except for three individuals (6, 8 and 11), whereas some individuals reached more than 60% germination in the heat-shock treatment (Table 2). As a whole, germination varied substantially among individuals within the population (Fig. 1).

The mean seed mass of the population was 0.920 mg, but the seed mass among individuals varied (CV = 0.184, min = 0.688 mg, max = 1.308 mg). The seed mass was positively correlated with the germination percentage in the heat treatment (Fig. 2a), but no correlation was found between the seed mass and germination percentage in the control ( $r^2 = 0.02$ ,  $p = 0.26$ , not shown). Indeed, the two-way ANOVA of the germination percentage of the different seed size classes showed that both the seed size and heat-shock treatment affected the germination percentage, and their interaction was also significant (Fig. 2b and Table 3). This also shows that the difference in the germination percentage among the different seed size classes was due to the difference in germination among the heat-shock treatments but not among the controls. On the other hand, the mean seed mass was negatively correlated with seed mortality both in the heat-shock treatment and in the control (Fig. 3). There was no significant correlation between mean seed mass and germination rate ( $t_{50}$  in days) neither in the heat-shock treatment nor in the control ( $r^2 = 0.03$ ,  $p = 0.49$ ;  $r^2 = 0.23$ ,  $p = 0.08$ , respectively).

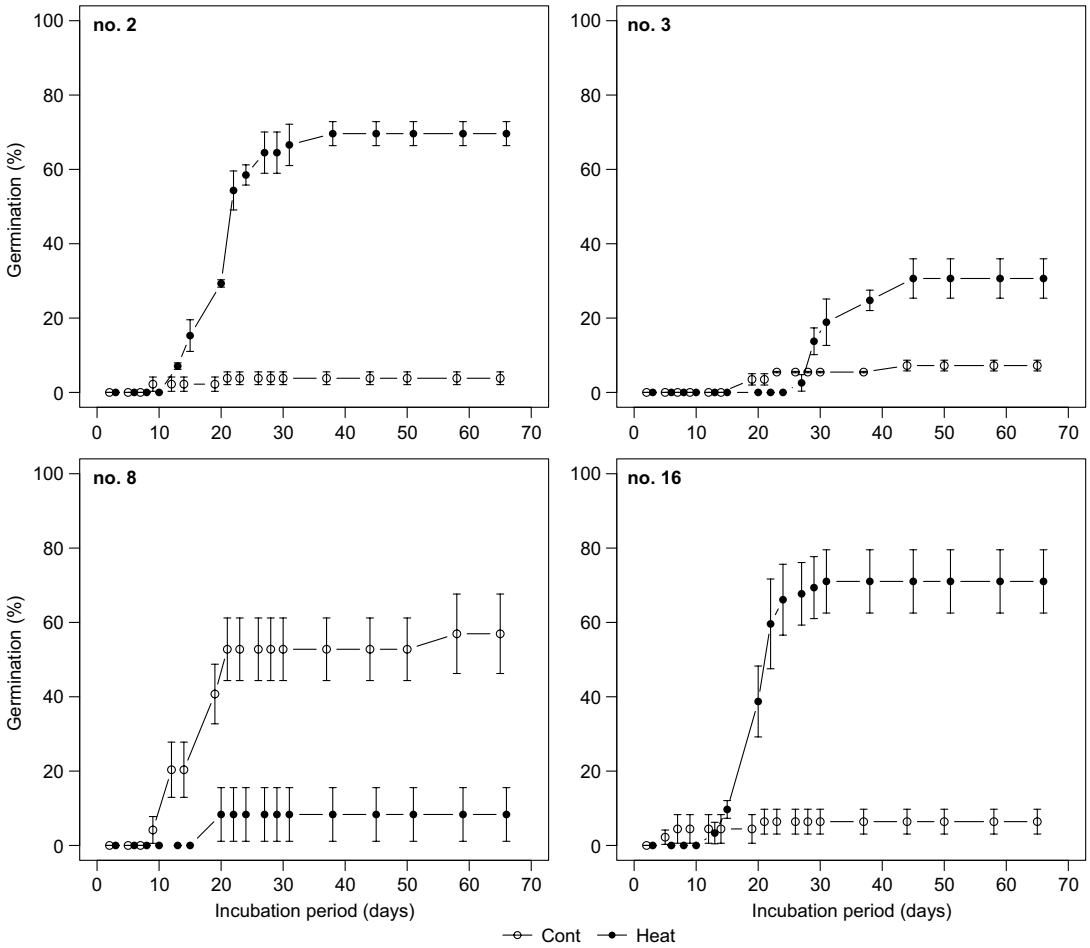
## Discussion

Most of the individuals of the *C. salviifolius* population give a positive germination response to the heat-shock treatment. This is consistent with the current literature on the presence of heat-stimulated germination in this species throughout the Mediterranean Basin (Trabaud & Oustric 1989, Corral *et al.* 1990, Roy & Sonié 1992, Scuderi *et al.* 2010, Moreira *et al.* 2012). All those previous studies were performed at a population or species level, but our study gives the first results on the individual-level (within-population) germination response to heat-shock in *C. salviifolius*. Focusing on germination at

the individual level showed that there was a considerable variation among individuals albeit the population-level trend in stimulation of germination by heat-shock could also be found. In population-level germination studies, the experiments are performed using a mixture of seeds from different individuals. Thus, in such designs, individual-level variability in the germination response is obscured. However, the result of our study suggests that even if the response of the majority of the individuals follows the population-level germination response of the species, a significant variability in seed dormancy and germination actually exists within a population (or among individuals). Moreover, it seems that the seeds of a few individuals in the population lose their dormancy, are ready for germination, and cannot tolerate fire temperatures after some years of storage (as individual no. 8 in our study), but the seeds of most individuals still remained dormant and only germinated after fire. This variability may have population-level consequences, and might be crucial to cope

**Table 2.** Mean germination percentages of individuals of *Cistus salviifolius* in the control and in the heat-shock treatment (Heat), the population mean (Mean) and the coefficient of variation (CV). Probability values set in boldface indicate significant difference between the control and heat treatment (GLM). Individuals with more than 88% seed mortality in the heat treatment are marked with an asterisk (\*).

Indiv. no.	Control	Heat	$p$
1	0.0	78.1	< <b>0.0001</b>
2	3.8	69.6	< <b>0.0001</b>
3	7.2	30.6	<b>0.0012</b>
4	3.7	21.0	<b>0.0371</b>
5	0.0	25.0	<b>0.0005</b>
6*	4.2	7.4	0.4150
7	2.8	19.7	<b>0.0175</b>
8*	56.9	8.3	<b>0.0001</b>
9	4.9	64.6	< <b>0.0001</b>
10	3.8	75.6	< <b>0.0001</b>
11*	0.0	11.4	0.0733
12	2.8	41.2	< <b>0.0001</b>
13	2.4	67.8	< <b>0.0001</b>
14	2.6	15.9	<b>0.0492</b>
15	23.7	49.9	<b>0.0007</b>
16	6.4	71.0	< <b>0.0001</b>
17	5.6	16.8	0.1361
Mean	7.7	39.6	< <b>0.0001</b>
CV	1.91	0.71	



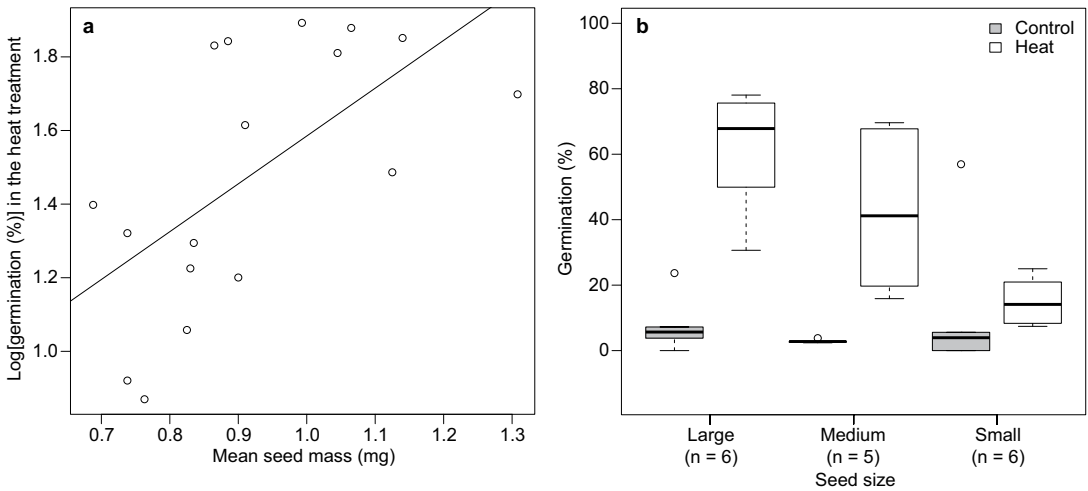
**Fig. 1.** Mean germination percentages ( $\pm$  SE) of four individuals of *Cistus salviifolius* in the control (Cont) and in the heat treatment (Heat) during the incubation period. The numbering of the individuals is the same as in Table 2. The individuals were selected to reflect different post-fire germination patterns at the individual level in the population.

with environmental stochasticity (in our case, unpredictable fire events or fire-free periods). In consequence, to investigate the trait variability within a population is important for a better understanding of how selective forces (such as fire) affect plant traits.

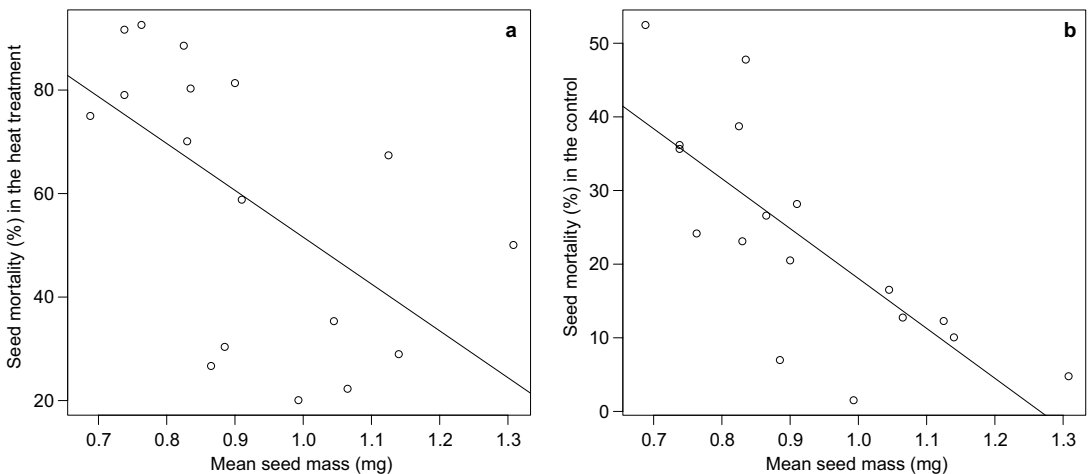
The results also indicate that mean seed mass of individual plants explains a significant proportion of individual-level variability in post-fire germination in *C. salviifolius*. However, this is not true for the germination in the control (i.e., in fire-free conditions), in which no relationship

**Table 3.** Two-way analysis of variance of the effects of treatment and seed size classes (Fig. 2b) on germination. The analysis was performed on log-transformed data.

	df	SS	MS	F	p
Seed size	2	2733.7	1366.9	5.216	0.0119
Treatment	1	8680.0	8680.0	33.127	< 0.0001
Seed size $\times$ treatment	2	4087.7	2043.8	7.800	0.0020
Residuals	28	7336.7	262.0		



**Fig. 2.** The relationships between seed size and germination in the heat-shock treatment. — **a**: The correlation between mean seed mass and germination percentage in the heat treatment ( $y = 1.300x + 0.286$ ,  $r^2 = 0.35$ ,  $p = 0.007$ ). Each circle represents one individual. — **b**: Germination percentages in the control and in the heat-shock treatment according to the different seed size classes. Large, medium and small size classes correspond to  $> 0.99$  mg,  $0.84\text{--}0.98$  mg and  $< 0.83$  mg mean seed mass, respectively.



**Fig. 3.** The relationships between seed mass and seed mortality (**a**) in the heat-shock treatment ( $y = 90.5x + 142.1$ ,  $r^2 = 0.29$ ,  $p = 0.015$ ), and (**b**) in the control ( $y = 67.8x + 85.8$ ,  $r^2 = 0.56$ ,  $p < 0.001$ ). Each circle represents one individual.

existed with mean seed mass. Seed mass-related germination success in the heat-shock treatments and lack of any significant relationship between seed mass and control germinations was demonstrated previously in *Cistus* (*C. ladanifer*; Delgado *et al.* 2001), and our results confirm those findings. Therefore, individuals with larger seeds may be favored in post-fire conditions, while seed size is unimportant in fire-free conditions.

However, individuals with larger seeds have additional advantage of surviving in both post-fire and fire-free conditions in comparison with the ones with smaller seeds, as the larger the seeds the lesser the mortality, suggesting that seed size is also a determinant of seed mortality. This creates a contradiction: if the individuals with larger seeds are more advantageous than the ones with smaller seeds in survival and in

germination after fire, then selection should lead to the elimination of individuals with smaller seeds. This suggests that another selective force may be affecting the seed size in the population balancing the disadvantage in the fitness of the individual with the smaller seeds in fire-prone environments. One possible explanation is seed predation, a positive selective force on smaller seeds for survival and persistence in soil seed bank (Hulme 1994, Moles *et al.* 2000, Cerabolini *et al.* 2003). This may allow the co-occurrence of small-seeded individuals (those not favored by fire) with large-seeded ones (those favored by fire) within a population. Therefore, the selective pressures created by fire and seed predation may be responsible for sustaining within-population variability in seed size.

The observed within-population variability in germination and seed size may give advantage to the populations of *C. salviifolius* under the unpredictable environmental conditions (e.g., drought or fire event) common in the Mediterranean Basin, and against various selective forces (especially seed predation). This plasticity may explain the seedling emergence and persistence of *Cistus* species in both post-fire (Schiller *et al.* 1997, Tavşanoğlu & Gürkan 2005) and fire-free conditions (Tarrega *et al.* 2001, Skorou 2003 as cited in Arianoutsou 2004), and further the widespread range of *C. salviifolius* in various habitat types throughout the Mediterranean Basin. A similar conclusion was reached by Cruz *et al.* (2003) for *Erica australis*, a Mediterranean facultative resprouter species. They suggested that variability in the germination among the individuals and the fruits of this species is a response to highly variable conditions in space and time in Mediterranean-type ecosystems. Plasticity of plant traits in disturbed or unpredictable habitats may be evidence for the evolution of a bet-hedging strategy (Simons & Johnston 2003), exhibiting more variability in unpredictable environments in comparison with the more predictable ones (Bonser & Aarsen 2009). For instance, Enright *et al.* (1998) showed that a *Banksia* species in fire-prone environments of Australia exhibits a similar strategy in the serotiny trait (i.e. canopy seed storage) when stochastic variability among fire intervals is present. It is suggested that if the variance in

seed size is of ecological importance and subject to natural selection, then the selection for variation in seed size, rather than towards higher or lower mean values, would be stronger (Michaels *et al.* 1988). Recent molecular studies showed that fire increases the genetic variation among plant populations (Dolan *et al.* 2008, Schrey *et al.* 2010, Segarra-Moragues & Ojeda 2010), and therefore leads to increased variability of plant traits. As seed size explains a significant proportion of the variability in germination after fire, but not in fire-free conditions in *Cistus* species (Delgado *et al.* 2001 and the present study), we suggest that fire regimes may shape the variability pattern of seed size in *Cistus*. A similar conclusion was reached by Delgado *et al.* (2008), suggesting that fire regimes have a potential of affecting within-population variability of seed size in *C. ladanifer* by selecting smaller seeds under low-frequency fire regimes and by selecting larger seeds under frequent fires. Moreira *et al.* (2012) suggested that local forces such as fire regimes are responsible for the variability in seed traits among populations of *C. salviifolius*. Thus, it can be concluded that not only the selective pressure by fire shapes the variability in seed traits among populations, but it also may act as a force increasing the within-population variability of these traits.

In conclusion, together with the positive selective pressure of seed predation over small-seeded individuals, fire shapes within-population variability of regeneration traits such as seed size and post-fire germination in *C. salviifolius*. This variability might have been evolved as a bet-hedging strategy against the unpredictable conditions of the Mediterranean environment, especially the occurrence of fire and fire-free periods. The variability in regeneration traits further bears a potential of allowing the Mediterranean plant populations to survive under the expected changes in climate and fire regimes in the Mediterranean Basin (Garzón *et al.* 2011). Further work is needed on within-population variability in the regeneration traits to understand the trait-shaping effect of fire on plant species and to assess the contribution of other selective forces such as seed predation to maintain this variability.



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