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Mediterranean Heathland as a Key Habitat for Fire Adaptations: Evidence from an Experimental Approach

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Abstract: Some fire ecology studies that have focused on *garrigue*-like vegetation suggest a weak selective pressure of fire in the Mediterranean Basin compared to other Mediterranean-type regions. However, fire-prone Mediterranean heathland from the western end of the Mediterranean Basin has been frequently ignored in the fire ecology literature despite its high proportion of pyrogenic species. Here, we explore the evolutionary ecology of seed traits in the generalist rockrose *Cistus salviifolius* L. (Cistaceae) aiming to ascertain the role of the Mediterranean heathland for fire adaptations in the Mediterranean Region. We performed a germination experiment to compare the relationship of seed size to (i) heat-stimulated germination, (ii) dormancy strength, and (iii) heat survival in plants from 'high-fire' heathland vs. 'low-fire' coastal shrubland. Germination after heat-shock treatment was higher in large seeds of both 'high-fire' and 'low-fire' habitats. However, dormancy was weaker in small seeds from 'low-fire' habitats. Finally, seed survival to heat shock was positively related to seed size. Our results support that seed size is an adaptive trait to fire in C. *salviifolius*, since larger seeds had stronger dormancy, higher heat-stimulated germination and were more resistant to heat shock. This seed size–fire relationship was tighter in 'high-fire' Mediterranean heathland than 'low-fire' coastal shrubland, indicating the existence of differential fire pressures and evolutionary trends at the landscape scale. These findings highlight the Mediterranean heathland as a relevant habitat for fire-driven evolution, thus contributing to better understand the role of fire in plant evolution within the Mediterranean region.

Keywords: *Cistus*; germination; dormancy; fire-driven evolution; Mediterranean-type ecosystems

1. Introduction

The importance of fire in the evolutionary history and diversity of plants from Mediterranean-type ecosystems is widely acknowledged [1,2]. The apparent convergence of functional traits among plant species from these distant ecosystems is explained by comparable fire regimes [3], in addition to similar climatic conditions (e.g., traits related to summer drought tolerance such as leaf sclerophylly; [4]).

Some examples of fire-related convergent traits are post-fire resprouting from lignotubers [5], fireproof thick bark [6], aerial seed-bank (i.e., serotiny; [7]), and fire-triggered germination of dormant seeds [8].

In species whose adults are killed by fire but seeds are stimulated to germinate (i.e., seeder species), seed traits are particularly important in mediating fire responses [9]. Seeder species produce dormant seeds that form a long-lived seed bank whose germination is stimulated by fire cues such as heat shock or chemicals from smoke and ashes [8,10]. It is well established that the fire regime (particularly fire intensity) shapes the heat-shock temperature threshold needed for dormancy release in species with fire-stimulated germination [11]. At the same time, seed responses to fire are ultimately mediated by seed traits, such as seed coat thickness and pubescence [12,13], seed moisture [14], and seed size or weight [15–18]. This later trait is the more frequently investigated seed trait in the fire ecology literature, but studies have shown contrasting patterns depending on the species, ecosystems, or fire regimes. For example, seed size (or weight) and post-fire germination were negatively correlated in Australian species [15,17] but positively correlated in some Mediterranean species (e.g., *Cistus* spp.; [16,18]), whereas no correlation was found for European grassland species [19]. Since seedlings from small seeds cannot emerge from deep soil depths, small seeds in fire-prone habitats need to be more resistant to fire since they are exposed to intense fire at shallow depths [17]. In some species (e.g., *Cistus* spp.), however, larger seeds might be selected by fire since they have competitive advantages in the post-fire environment related to early emergence and higher seedling vigor [18]. All in all, the evolutionary ecology of seed traits associated with post-fire germination is still poorly understood [17,20]. Since fire adaptations are species-specific (e.g., [21]), exploring how seed traits such as dormancy strength or size vary with the fire regime requires the analysis of the relationship between seed trait variability and post-fire responses at the intraspecific level [20,22,23].

Although all Mediterranean-type ecosystems are subject to frequent summer fires, fire regimes can vary even at the landscape scale, as some habitats or plant communities are more fire-prone than others. This variability is in part determined by soil fertility: plant communities on nutrient-poor, acid soils seem to be more flammable and, hence, more fire-prone than those on richer soils, thus generating differences in the frequency of adaptive traits to fire at the regional scale (e.g., [24]). For example, fire adaptations in the Mediterranean western Cape Floristic Region (South Africa), are far more frequent in *fynbos* (nutrient-poor, acid soils) than in *thicket* (richer soils) or coastal shrublands (*strandveld*), although these habitats co-occur in the landscape [25,26]. Similarly, in the western Mediterranean Basin, the proportion of species with fire-stimulated germination was higher in Mediterranean heathland (acid, nutrient-poor soils) than in *garrigue*-like shrublands (more fertile soils; [24]). Since the investigation of the role of fire in the evolution of Mediterranean species in the Mediterranean region has traditionally focused on *garrigue* vegetation (e.g., [27–30]), comparative studies have shown that fire-recruiting species are less frequent in the Mediterranean Basin than in other Mediterranean-type regions, thus concluding that fire-selective pressures would not have been so strong in the Mediterranean region [1,31]. However, the highly fire-dependent Mediterranean heathland habitat [24], although less abundant than the *garrigue*, has a non-negligible, wide range in the western Mediterranean Basin [32]. Therefore, studying the role of fire as a selective pressure in this habitat could bring new insights into the importance of fire in plant evolution in the Mediterranean region.

Here, we explored the evolutionary ecology of seed traits in relation to fire in Mediterranean heathland plants in order to ascertain the importance of this fire-prone habitat for fire adaptations. To do so, we used the Mediterranean seeder species *Cistus salviifolius* L. (Cistaceae) as a case study. This is a generalist species that occurs in fire-prone Mediterranean heathland patches [33], but is also frequent in not, or much less, fire-prone habitats, such as coastal shrublands [34] with similar climatic conditions, thus providing an opportunity to address key fire-selection hypotheses. *Cistus salviifolius* seeds have a physical dormancy that is broken by heat shock [11,16,35]. Variability in seed dormancy and heat-shock stimulation has been reported both within [16] and among populations [22]. That variability has been related to seed size at the within-population level, showing that large seeds have a higher probability

of germination after heat-shock treatment than small seeds [16]. However, those patterns have not been associated with different fire regimes.

We carried out a germination experiment in which seeds of plants from populations of 'high-fire' heathland and 'low-fire' coastal habitats had their size (estimated as surface area) recorded and were either exposed or not exposed to a heat-shock treatment. We predicted that, if fire has a more preponderant role in shaping seed traits in 'high-fire' heathland than in 'low-fire' coastal shrubland, populations from heathland sites will show stronger correlations between seed size and (i) heat-stimulated germination, (ii) dormancy strength, and (iii) survival to heat-shock, than those from coastal shrubland sites.

2. Materials and Methods

2.1. Study Species

Cistus salviifolius L. (Cistaceae) is a common rockrose species in the Iberian Peninsula with a broad geographic distribution across the Mediterranean Basin [22]. It can be found in a wide variety of ecosystems on different soil-types and under different fire regimes. It has fire-stimulated seed germination [35], but recruitment has also been reported in unburned areas [36]. Since adult plants do not resprout and are thus killed by fire, this species can be referred to as seeder [37]. It is a small shrub, 20–90 cm high, with radiate white flowers and dry capsules containing globose, reticulate seeds [38]. Flowering occurs in early spring and fruiting in summer [39].

2.2. Study Area, Populations and Seed Collection

Seeds were collected in 15 natural populations from the Strait of Gibraltar region, at the southernmost tip of Spain (Figure 1). Climate in this area is mild Mediterranean, with cool rainy winters and warm dry summers. Mean annual temperature is 17.6 °C and mean annual rainfall is 908.6 mm [40]. Nine of the 15 populations were located in fire-prone Mediterranean heathland patches ('high-fire'), which harbour a large proportion of pyrogenic species (e.g., *Cistus populifolius*, *Calluna vulgaris*, *Erica australis*, *Genista tridens*) and is considered a fire-adapted vegetation (Figure S1; [24]). The remaining six populations were located in coastal shrublands ('low-fire'), where fire pressure seems to have been lower at an evolutionary time scale, as vegetation is dominated by non-pyrogenic species (e.g., *Pistacia lentiscus*, *Halimium calycinum*, *Juniperus* spp.; [34]) (Figure S1). Although current fire frequencies are similar in both habitats (Table S1) due to anthropogenic activities [41], fire-related traits of dominant species of these two habitats indicate that fire has historically been a much more relevant selective agent in Mediterranean heathland than in coastal shrubland, as it occurs in the South African *fynbos*-coastal shrubland system [26]. In each population, we collected seeds from mature capsules of 15 randomly selected individuals, at least 5 m apart from each other. Seeds from each individual were placed in labelled paper bags and preserved in the lab at room temperature and dark conditions for approximately six months until the germination experiment commenced.

Figure 1. Site location of the sampled *Cistus salviifolius* populations from Mediterranean heathland ('high-fire') and coastal shrubland ('low-fire') sites in the Strait of Gibraltar area, at the southernmost tip of Spain. See Table S1 for additional information on sampling sites.

2.3. Seed Size Measurement and Germination Experiment

Seed surface area was used as a proxy for size (as in previous studies; e.g., [13]) and was measured under a Digital Microscope Leica© DMS1000 with LAS (Leica Application Suite) V4.4.0 software. We measured the surface area (hereafter called seed size for simplicity) of six randomly selected seeds per individual and then obtained the average value of each individual plant to be used in the analyses.

We then conducted a growth-chamber germination experiment to assess (i) the proportion of dormant seeds, (ii) the effect of heat-shock on the probability of seed germination, and (iii) the survival of seeds to heat-shock, at the individual (plant) level, and then relate these variables to (average) seed size of each plant. From each of the 15 plants per population, we allocated 12 seeds to a heat-shock treatment, and 12 were not treated (control). The number of seeds per individual and treatment was lower than 12 in some instances due to limitations in seed availability (many seeds were predated; File S1). For the heat-shock treatment, seeds of each plant were placed on small aluminum trays in a dry oven (Dry-Big JP Selecta® S.A., Barcelona) and were exposed to 120 °C during 5 min, following previous germination experiments performed in this species [11,16].

After the heat shock, seeds of each plant and treatment (heat-shock and control) were placed in labelled Petri dishes with filter paper, introduced in a growth chamber (Fitoclima S600; Aralab, Lisbon) at 20 °C constant temperature and dark conditions (*C. salviifolius* seed germination is not affected by light conditions; [35]) and watered periodically with distilled water. These conditions were applied to maximize germination based on a previous study [22]. Every two days, seeds were checked for germination, and were considered as germinated when the radicle was visible more than 1 mm. Germinated seeds were removed from the Petri dishes once they were counted. This germination assay lasted 90 days, after which viability of non-germinated seeds (from both control and treated samples) was tested with tetrazolium staining (TTC 1% in phosphate buffer, pH 7.3, and 24 h in dark). The tetrazolium was applied to seeds cut in half to expose the embryo and facilitate the staining. By doing so, the number of germinated seeds and the number of total viable seeds (germinated plus tetrazolium-positive seeds) for each individual and treatment were

calculated. Dormancy strength was then estimated as the proportion of non-germinated, viable seeds (i.e., dormant seeds) in control samples. Heat-shock survival was calculated as the number of seeds alive (germinated plus non-germinated, viable seeds) in heat-treated samples.

2.4. Statistical Analyses

To assess whether the relationship between seed size and heat-stimulated germination depended on the type of habitat, we performed three-factor generalized linear mixed models (GLMM). In these models, the proportion of germinated seeds was the dependent variable (binomial distribution) and the independent variables were seed size (continuous), heat shock treatment (heat vs. control), and habitat type ('high fire' vs. 'low-fire'). The difference between habitats in the seed size–dormancy relationship was evaluated using a two-factor GLMM, in which the dependent variable was the proportion of dormant seeds in control samples (binomial distribution) and the independent variables were seed size and habitat type. Finally, to assess the differences in the relationship between seed size and heat-shock survival in function of habitat type, we used a two-factor GLMM where the dependent variable was the proportion of total viable seeds after heat-shock (binomial distribution) and the independent variables were seed size and habitat type ('high fire' vs. 'low-fire'). In all these models, population was included as random factor to account for the dependence of the data. The effect of fixed factors was evaluated by forward model selection and likelihood ratio test (LRT) [42]. We calculated the Nakagawa's R^2 for mixed models; the marginal R^2 for fixed effects and the conditional R^2 for both fixed and random effects [43]. All analyses were performed with R software [44] using the lme4 package [45].

3. Results

The average seed viability was 85.6 ± 1.2% (mean ± SE) in control and 80.4 ± 1.5% in heat-shock treated plants. Average seed germination was 8.5 ± 0.9% and 78.2 ± 5.2% in control and heat-shock samples, respectively. Seed germination was stimulated by heat shock in plants from both 'high-fire' heathland habitats and 'low-fire' coastal shrublands, although this stimulation tended to be higher in 'high-fire' habitats (marginally significant *heat × habitat* interaction; Table 1, Figure 2, Table S2) and varied among populations (significant random effect on heat slope; $\chi^2 = 73.12$, $p < 0.001$). Besides, heat-shock effect on seed germination depended on seed size (highly significant *heat × size* interaction; Table 1), as germination after heat-shock was higher for larger seeds in both 'high-fire' and 'low-fire' habitat types. By contrast, germination of small seeds in control samples was considerably higher in plants from 'low-fire' habitats than in those from 'high-fire' (Figure 2), as also indicated by a significant *heat × size × habitat* interaction (Table 1).

Table 1. Results of likelihood ratio tests (LRTs) showing the effects of heat shock, seed size, habitat type and their interactions on seed germination of *Cistus salviifolius*. All models included population as random factor. Significant *p*-values (<0.05) are highlighted in bold.

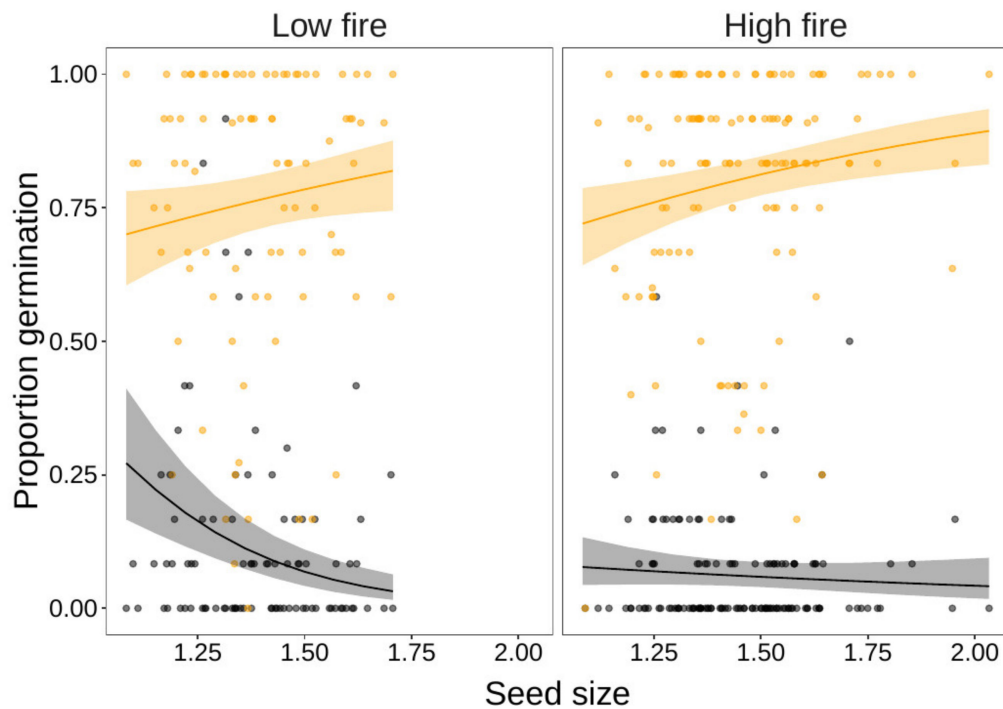| Model	No. of Parameters	Deviance	χ^2	*p*
Intercept	4	2086.7		
Heat	5	2041.8	44.91	**<0.001**
Heat + Size	6	2041.6	0.21	0.645
Heat + Habitat	6	2040.5	1.29	0.255
Heat × Size	7	2013.2	28.63	**<0.001**
Heat × Habitat	7	2036.7	5.16	0.075
Heat × Size × Habitat	11	2000.2	12.97	**0.011**

Figure 2. Relationship between germination (proportion germinated) and seed size (mm^2) in *Cistus salviifolius*, in 'low-fire' coastal shrubland and 'high-fire' Mediterranean heathland habitats. Dots represent plant individuals (grey: control; orange: heat-shock treated). Regression lines and 95% confidence intervals of predicted models are shown (seed size ranged from 1.08 to 2.03). Conditional R^2 = 0.55 and marginal R^2 = 0.52.

This corresponds to results of dormancy strength, lower in small-sized control seeds of plants from 'low-fire' habitats than in those from 'high-fire', rendering a significant *size × habitat* interaction (Table 2, Figure 3). Finally, seed survival to heat-shock treatment was higher in larger seeds (significant *size* effect; Table 2, Figure 4). Although in this variable the *size × habitat* interaction was not significant (Table 2), plants from 'high-fire' habitats tended to have a more positive relationship between seed size and heat survival compared to 'low-fire' habitats (Figure 4 and see also Table S3).

Table 2. Results of likelihood ratio tests (LRTs) showing the effects of seed size, habitat type and their interactions on dormancy strength and heat survival of *Cistus salviifolius*. All models included population as random factor. Significant *p*-values (<0.05) are highlighted in bold.

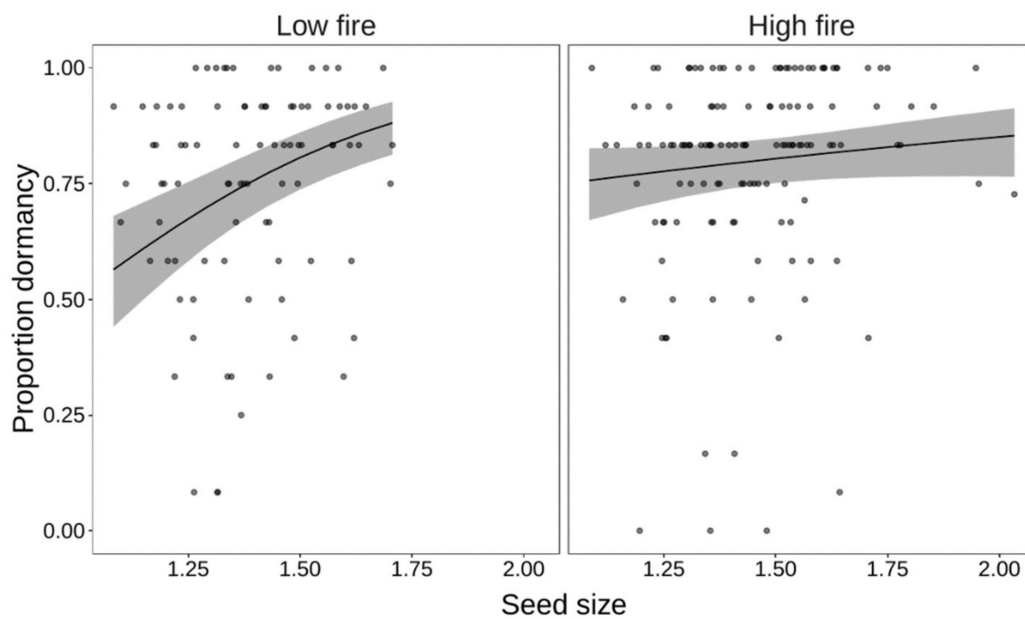|	Dormancy Strength				Heat Survival			
	No. of Parameters	Deviance	χ^2	*p*	No. of Parameters	Deviance	χ^2	*p*
Intercept	2	1182.8			2	1215.5		
Habitat	3	1164.1	1.69	0.194	3	1212.7	2.71	0.100
Size	3	1181.1	17.0	**<0.001**	3	1203.8	11.68	**<0.001**
Size × Habitat	5	1156.1	8.07	**0.005**	5	1199.0	4.80	0.090

Figure 3. Relationship between dormancy and seed size (mm^2) in *Cistus salviifolius*, in 'low-fire' coastal shrubland and 'high-fire' Mediterranean heathland habitats. Dots represent plant individuals. Regression lines and 95% confidence intervals of predicted models are shown (seed size ranged from 1.08–2.03). Conditional R^2 = 0.08 and marginal R^2 = 0.03.
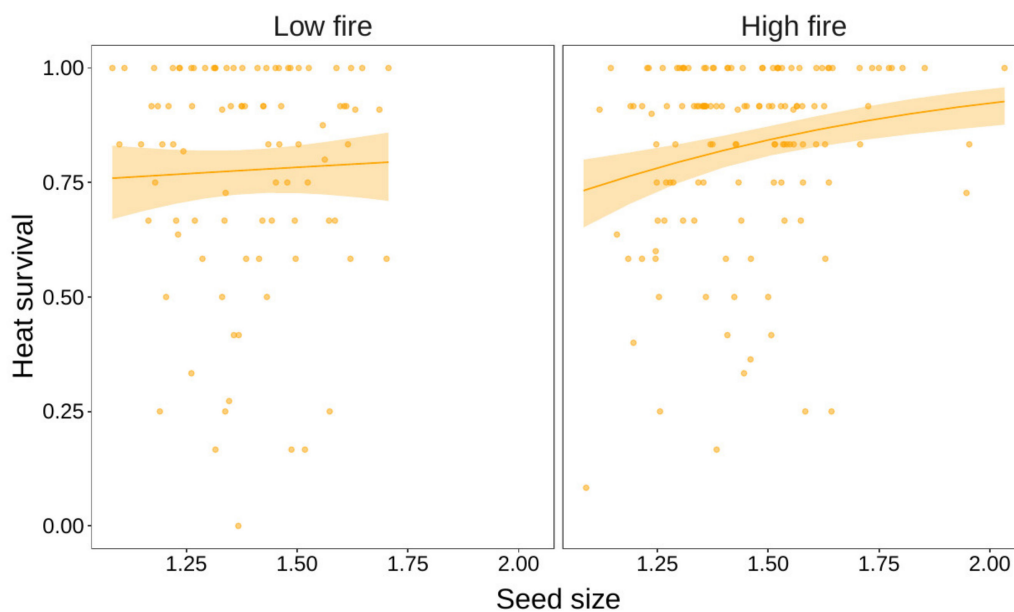

Figure 4. Relationship between seed survival to heat shock and seed size (mm^2) in *Cistus salviifolius*, in 'low-fire' coastal shrubland and 'high-fire' Mediterranean heathland habitats. Dots represent plant individuals. Regression lines and 95% confidence intervals of predicted models are shown (seed size ranged from 1.08 to 2.03). Conditional R^2 = 0.05 and marginal R^2 = 0.02.

4. Discussion

Our results showed that, in *C. salviifolius* seeds, the variation in the responses to heat shock (germination and survival) and dormancy strength relate to seed size. All these seed–trait–fire relationships were different between 'high-fire' Mediterranean heathland and 'low fire' coastal shrubland habitats, which indicates the existence of differential selective processes driven by fire at the landscape scale.

The ability of *C. salviifolius* to successfully colonize post-fire environments is well established [46], as well as its physical seed dormancy and heat-shock stimulated germination [11,16]. Tavşanoğlu and Çatav (2012) [16] also found that seed size (estimated as seed mass in that case) in this species was positively correlated with germination after heat-shock treatment within a single population, but such correlation was not found in non-treated, control seeds, most of which remained dormant regardless of size. Our results corroborate their finding across populations, but only in 'high-fire' Mediterranean heathland habitats. In 'low fire' coastal shrubland habitats, by contrast, we found weaker seed dormancy and, consequently, higher germination than in 'high-fire' habitats for small seeds of control samples. This might imply that larger seed size is more advantageous, and thus adaptive, to resist heat-shock and germinate after fire. On the other hand, in 'low fire' coastal shrubland habitats, some seed germination and seedling recruitment would occur in the absence of fire because of the germination of weakly-dormant, smaller seeds. This can be seen as a bet-hedging strategy, allowing *C. salviifolius* populations to persist in habitats where wildfires are rare events with long fire-free periods, such as coastal shrublands [16]. Furthermore, the high variability we found in the heat-shock responses among populations and individuals suggests that other selective pressures are probably interacting with fire to shape seed traits in *C. salviifolius* and might in part explain the generalist character of this species.

Interestingly, the range of seed sizes was wider in 'high-fire' than in 'low-fire' habitats, where seeds with surface area greater than 1.75 mm^2 were not found (Figure 2). This pattern, together with the facts that larger seeds tended to have (i) a higher probability of surviving heat shock, and (ii) higher heat-shock germination percentages in 'high-fire' sites, might indicate that fire is causing a directional selection on seed size in *C. salviifolius* populations from Mediterranean heathlands but not in those from coastal shrublands. In addition to that, the stronger dormancy of smaller seeds in 'high-fire' habitats supports the idea that, in the context of the Mediterranean region, fire has played a more relevant, evolutionary role in the Mediterranean heathland than in other Mediterranean shrublands [24]. This means that a strict fire suppression policy could have detrimental effects, not only on the viability of *C. salviifolius* populations from this habitat, but also on the population viability of many other Mediterranean heathland species. The Mediterranean heathland is a highly diverse, fire-prone ecosystem that harbours a great variety of singular, endemic species (e.g., *Drosophyllum lusitanicum*), including some postfire ephemerals such as *Silene gaditana* [47]. In the Mediterranean heathland, fire has driven plant diversity [48] and modulates the population dynamics of its distinctive plant species (e.g., [49]).

Although the proportion of variance explained by estimated models in the cases of heat-shock survival and dormancy strength was very low, we consider that the observed trend in these variables is biologically significant enough to suggest that seed size in *C. salviifolius* is an adaptive trait selected by fire. There are several functional reasons that could explain why larger seeds are adaptive to fire while smaller seeds are favoured in the absence of fire. First, larger seeds, producing larger seedlings, would be favoured under frequent fire in ecosystems where post-fire germination is profuse and within-species competition for recruitment is strong, as Delgado et al. (2008) [50] suggested for *Cistus ladanifer*. Second, larger seeds would protect the embryo more efficiently from elevated temperatures, thus allowing them to survive the heat pulse associated with the passage of fire ([51] and references therein). The latter explanation might be associated with the existence of a positive correlation between seed size and other traits that modulate seed dormancy and/or heat survival, such as testa thickness or hardseededness. Hardseededness certainly imposes physical dormancy, which is broken by fire (mainly due to the physical scarification generated by heat shock) in many pyrogenic species from fire-prone ecosystems [8], being a characteristic trait of the family Cistaceae [52–54]. Variation in hardseededness could explain the differences between habitats in heat shock survival, since it modulates seed moisture and the lethal temperature thresholds of seeds [14]. The correlated selection of seed traits explains the germination response to heat-shock in other species [20]. Nevertheless, fire-selection experiments are needed to test this hypothesis. On the other hand, as mentioned above, the interaction

of fire with other selective pressures (e.g., seed predation, competition, nutrient availability) in shaping seed size in *Cistus* species cannot be discarded [16].

Contrasting results have been reported in other fire-prone environments (e.g., from Australia), where species with smaller seeds are more resistant to high temperatures than species with larger seeds [14,15,17]. As seedlings from small seeds are unable to emerge from deeper in the soil, they endure higher temperature thresholds, which allows them to survive the passage of fire in the soil surface, where seedling emergence is possible [17]. This variation in the association of seed size with germination after heat-shock among species or ecosystems could be a consequence of the complex interaction among seed size (and shape), seedling emergence and soil depth, which might generate species-specific responses to fire pressure [15,55]. Cochrane et al. (2015) [21] pointed out that environmental gradients do not necessary explain trait variation among populations and selective responses can be individualistic. Therefore, seed size alone should not be used as a predictor of post-fire recruitment success across species and ecosystems [15] and a wide variety of seeds traits need to be studied to better understand the evolutionary ecology of seeds [56]. Nonetheless, it is important to highlight that, despite the variability in the direction of fire-driven selection among species, our results provide evidence to support that fire is an evolutionary agent shaping seed traits in plant species from fire-prone ecosystems [13,20]. Understanding the role of fire in plant trait evolution and the ecological effects of trait variation with changes in fire regimes contribute to the knowledge of how plant communities are structured and, therefore, help develop adequate management policies for conservation at a regional scale [57].

5. Conclusions

Seed size in *C. salviifolius* is an adaptive trait to fire, since heat-shock responses (seed germination and survival), as well as dormancy strength were higher in large seeds. In *C. salviifolius* populations from 'high-fire' Mediterranean heathland sites, dormancy of small-sized seeds was stronger than in those from 'low-fire' coastal shrublands, suggesting the existence of differential fire selective pressures at the landscape scale. We cannot ascertain whether fire trait selection has occurred from 'low-fire' to 'high-fire' habitats or vice versa (i.e., acquirement of seed dormancy in 'high-fire' habitats or relaxation of dormancy in 'low-fire' habitats). In any case, our results illustrate that the fire-prone Mediterranean heathland is a key habitat for fire adaptations within the context of the Mediterranean region. Considering that the Mediterranean heathland is a singular habitat with great plant biodiversity and conservation values at a wide regional scale [32], management plans aimed to preserve this habitat should not ignore the role of fire as a key driver of its biodiversity.

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