# Signs of local adaptation and phenotypic plastic response to elevation shifted between environmental backgrounds in Snapdragon plants

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**Multiple environmental drivers can shape the plastic and microevolutionary adaptive responses of plants. Yet experimental studies on local adaptation and phenotypic plasticity rarely investigate how different backgrounds might interact and modify the signature patterns of these mechanisms. Here, we evaluated local adaptation and plasticity in response to elevation in two Snapdragon plant subspecies (*Antirrhinum majus striatum* and *A. m. pseudomajus*) by using common garden experiments at different elevations. We tested whether the phenotypic signatures of plasticity and local adaptation recorded in an open habitat were similar between subspecies and maintained when the experiment was replicated onsite under the shade of understory vegetation. Our results showed that population genetic divergence in germination-related traits was suggestive of a pattern of local adaptation to elevation under regular sunlight in *A. m. striatum*, but not in *A. m. pseudomajus*. They also revealed potentially neutral or adaptive plastic responses to elevation for these traits. The magnitude of plastic responses was stronger than trait genetic divergence. Under understory shade, phenotypic patterns were different and suggested maladaptive or neutral responses to elevation. Our findings imply that the genetic and plastic adaptive signatures of elevation cannot be inferred without taking into account the variability of the environmental background. They also imply that selection mechanisms linked to germination vary across heterogeneous environments in Snapdragon plants. Forecasting the ability of plants to adapt to environmental changes based on common garden and reciprocal transplant experiments must account for the multivariate nature of the environment.**

Altitudinal gradient | *Antirrhinum majus* | local adaptation | quantitative genetics | phenotypic plasticity | shade-induced plasticity | subspecies divergence

## 1. Introduction

Local adaptation and adaptive phenotypic plasticity are widely recognized as important mechanisms allowing species to cope with ongoing climate change (Jump & Penuelas, 2005; Hoffmann & Sgrò, 2011; Franks *et al.*, 2014; Kelly, 2019). Local adaptation is the microevolutionary response to local selection that makes populations fitter in their own local habitat than in any other populations' local habitats (Kawecki & Ebert, 2004). Evidence for past microevolutionary responses to selection caused by climate differences does not necessarily indicate that adaptation to climate change will occur (Jump & Penuelas, 2005; Valladares *et al.*, 2014). It nevertheless provides basic information about adaptive mechanisms that shape the standing genetic variation found among populations for climate-related responses. This information can in turn help building scenarios of species adaptation to climate change. Obtaining this information in plants is usually done by conducting experimental approaches where phenotypic traits are compared between populations grown in different environmental backgrounds. These environmental backgrounds are highly complex and multidimensional, and are generally simplified to be studied. Whether the genetic background of populations and the experimental simplification of population environmental backgrounds can impact information about adaptive mechanisms and the related extrapolated adaptive scenarios remains however poorly tested (but see Anderson & Wadgymar, 2019).

Phenotypic plasticity refers to the ability of the phenotype for a given genotype to change in response to environmental conditions (Bradshaw, 1965). Trait plasticity can be adaptive or maladaptive in relation to a plant fitness (Ghalambor *et al.*, 2007, 2015). Adaptive plasticity is widely recognized as a mechanism that can allow plants to track rapidly shift in phenotypic optima, thereby increasing the likelihood of populations persistence under environmental changes. Identifying adaptive plasticity to contrasted climatic conditions can therefore help predicting the ability of populations to persist to ongoing and future climate changes (Kelly, 2019). Today, adaptive plasticity and local adaptation are well documented. A lot of research is now directed towards understanding their relative roles and their

interaction in adaptive evolution. For example, adaptive plasticity has been described as a mechanism that can precede and promote (i.e. plasticity-first hypothesis, Levis & Pfennig 2016), act synergistically with (i.e., cogradient variation) or impede adaptive evolutionary responses to selection (i.e. countergradient variation, Conover & Schultz 1995; Ghalambor *et al.* 2015). The plasticity of a trait was also found to change in different environmental backgrounds so that plasticity itself can be considered to be plastic (Roubeau Dumont *et al.*, 2019). This obviously translates the multidimensional complexity of the natural environment that organisms live in, which affects in complex ways the phenotypic expression of traits (Morel-Journel *et al.*, 2020). Whether the adaptive significance of plasticity, and therefore its interaction with local adaptation, was assessed correctly by running experiments might therefore rely on the environmental background of the given experiment.

Elevation gradients have long been used to assess climate related signatures of adaptation (Halbritter *et al.*, 2018). Several environmental factors vary along elevation gradients (e.g., temperature, humidity, air pressure, vegetation cover, see Körner 2003). Some environmental factors (e.g., shade provided by the vegetation cover) can also vary between and within populations independently from elevation below tree lines. Most studies comparing the effect of elevation on plant populations cultivated in common gardens and reciprocal transplants do not decompose the effects of onsite environmental drivers because experimental settings can only incorporate a limited number of environmental treatments. Whether replicating the experiment at a similar elevation, even in a similar location, but in a different environmental background (e.g., regular light in open habitat vs shaded by understory vegetation) might affect trait values, results and conclusions on adaptive mechanisms is rarely tested (but see Anderson & Wadgymar 2019). Anderson & Wadgymar (2019) tested the impact of environmental conditions on the effect of elevation, and found a disrupted local adaptation to elevation caused by snow removal treatments. Our identification of ecological and evolutionary mechanisms shaping the diversity of traits might therefore be biased because we neglect the complex effect of the background environmental heterogeneity of wild populations in reciprocal transplant and common garden experiments (Chevin & Lande, 2015; Westneat *et al.*, 2019). As a consequence, our understanding of plant adaptation to elevation by means of local adaptation and phenotypic plasticity might be limited because neglecting the multivariate nature of the environment may lead to incorrect assessments of adaptive responses. Since this information is also used to forecast population responses to climate change, extrapolated scenarios from these approaches on the ability of populations to cope with climate change might

also be incorrect.

Here, we evaluated the signatures of local adaptation and plasticity in response to elevation for two germination-related traits widely recognized for their role in plant adaptation (Donohue *et al.*, 2010): the seed germination rate, and the timing of seed germination. We compared the relative importance of phenotypic plasticity (i.e. environmental variation, and genotype-by-environment interaction), and genetic differentiation (between populations, or between families) on these traits. We reproduced this approach in the two parapatric yet genetically closely related subspecies of *Anthirrinum majus* (ssp. *striatum* and ssp. *pseudomajus*) that inhabit closely similar ecological niches in the south of France. This was achieved by conducting two common garden experiments at high and low-elevation using seed families from seven populations of *A. m. striatum* and eight populations of *A. m. pseudomajus* originating from different elevations. Our main aim was to test the reproducibility of these signatures between two separate environmental backgrounds: open habitat and understory. Although reciprocal transplant and common garden experiments are usually conducted in broad day light in the absence of shade, the natural habitat of many populations is heterogeneous and combines both conditions. We expect to observe the signature of local adaptation to elevation in *A. m. striatum* but not in *A. m. pseudomajus*, based on the results from a study conducted in a single common garden experiment (Marin *et al.*, 2020) and to evaluate to what extent the observed signatures differ between environmental backgrounds.

## 2. Material and methods

**Study system.** *Antirrhinum majus* L. (Plantaginaceae) is a hermaphroditic, self-incompatible, short-lived perennial species producing annual inflorescences with zygomorphic flowers. It produces small seeds dispersed by gravity a few metres apart from the plant when the fruit dehisces (Andalo *et al.*, 2010; Khimoun *et al.*, 2011). Cultivated *A. majus* horticultural varieties are known to have a poor and slow rate of seed germination (Bhargava *et al.*, 2015). Seeds germinate better on the surface of soil and at mild temperature (around 20°C, Kang & Choi 2006). While *A. majus* has been used as a model for developmental genetics for more than 80 years (Schwarz-Sommer *et al.*, 2003), knowledge on the ecology of this species in wild populations remains limited. No data on the role of the seed bank, its longevity, and its germination temporal dynamics in wild populations are yet available. Recently some authors suggested that *A. majus* has a persistent seed bank with seeds able to survive longer than one year (unpublished data in Arathoon *et al.* 2020). Yet, it is reasonable to expect that most seeds remain viable in the soil seed bank until they have an opportunity
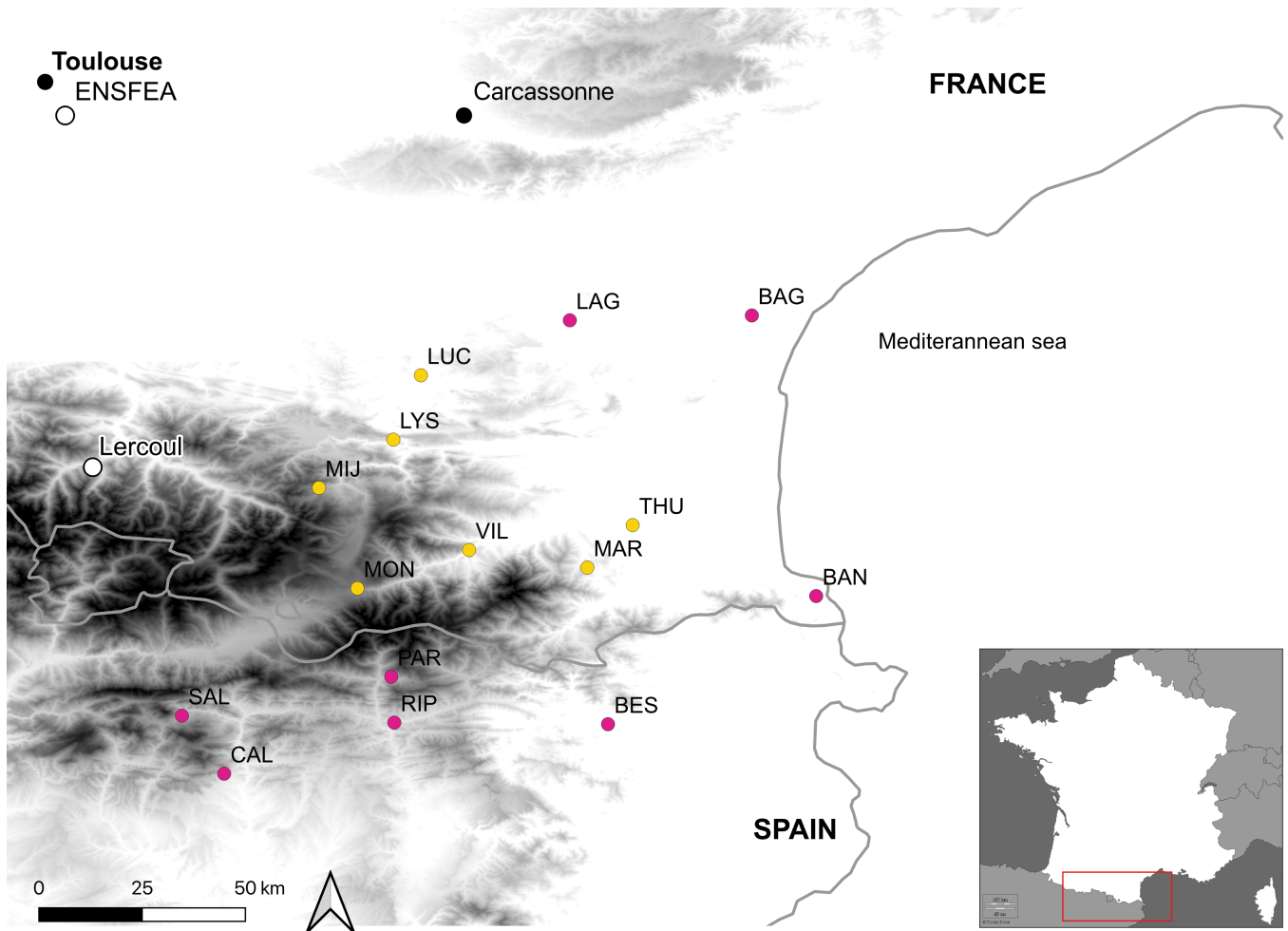
**Fig. 1.** Map of *A. majus* populations that were sampled across the geographic range of the species in Southern France. Pink dots represent *A. m. pseudomajus* populations, yellow dots represent *A. m striatum* populations.

to germinate in spring of the following year. This is coherent with results from studies on cultivated *A. majus* which generally present a unique peak of germination (Kang & Choi, 2006; Bhargava *et al.*, 2015). Geographic distribution of *A. majus* in southern Europe is centred over the Pyrenees Mountains (Khimoun *et al.*, 2013). It occurs from sea level to an altitude of 1900 m (Andalo *et al.*, 2010), on limestone or siliceous substrates and in habitats with contrasted moisture regimes (rainfall 500-1000 mm per year). *A. majus* thrives in disturbed habitats, and is especially common along roadside and railway embankments (Khimoun *et al.*, 2013). *A. majus* plants grow in a large variety of light environments, including fully open (e.g., scree), fully shaded (e.g., understory vegetation, dense grassland meadows), or heterogeneous (sparse shrubland) areas (Khimoun *et al.*, 2013).

**The subspecies level.** *A. majus* plants harbour either magenta or yellow flowers, which can be used to distinguish between the two interfertile subspecies *A. m. pseudomajus* and *A. m. striatum* respectively (Andalo *et al.*, 2010). The two subspecies are distributed parapatrically and come into contact at their geographic range margins (Khimoun *et al.*, 2011). The geographic range of *A. m. striatum* is surrounded by the range of *A. m. pseudomajus* (Khimoun *et al.*, 2013). The transition between subspecies in the contact zones can occur over a very short distance (<1 km) (Whibley, 2006).

At the genetic level, 1% genetic differentiation was found between *A. m. pseudomajus* and *A. m. striatum* on the basis of putatively neutral microsatellite loci, which was one order of magnitude lower than the 10% differentiation found amongst populations (Pujol *et al.*, 2017). There is evidence for gene exchange between subspecies in multiple populations across contact zones (Khimoun *et al.*, 2011). Genome scans across a particular contact zone in the Pyrenees also revealed little to negligible differentiation between the two subspecies, with the exception of loci underlying flower colour differences between the two subspecies that were characterised by

high differentiation (Whibley, 2006; Tavares *et al.*, 2018). At the environmental level, the separation between the geographic distribution of *A. m. pseudomajus* and *A. m. striatum* is not explained by habitat differences, as illustrated by the substantial overlap of environmental conditions between the two species (Khimoun *et al.*, 2013). Phenotypic differentiation was found between these two subspecies in a $Q_{ST}$-$F_{ST}$ approach conducted in one common garden but it was very low (c.a. 2%, excluding flower colour). The same approach however suggested a pattern of local adaptation to elevation across *A. m. striatum* populations, but not across *A. m. pseudomajus* populations (Marin *et al.*, 2020).

**Populations and seed collection.** Fifteen wild populations of *A. majus* were sampled in 2011 from low and high elevation habitats distributed across its native geographic range (between north-eastern Spain and south-western France, Fig 1). The studied populations covered most of the altitudinal range of the species (0 m to 1600 m, see Table S1 in Supporting Information). They have been chosen based on i) their location spread across the geographic range of the species, ii) their altitude to cover the elevation range of the species that can be separated in two strata (6-750 and 750-1800 meters) presenting contrasted climate conditions (Marin *et al.*, 2020), and iii) their within-population heterogeneity in vegetation cover resulting in diverse light conditions. None of these populations grows above tree lines. Populations from low and high elevation habitats are confronted to contrasted environmental conditions (Fig S1). For example, these conditions ranged from 14.8°C and 52 mm (at BAN, 61 m above sea level) to 6.1°C and 94 mm (at MON, 1564 m above sea level) based on fifty-year averages (1950-2000) of mean annual temperature and annual average rainfall extracted from the WorldClim database (resolution 1 $km^2$, www.worldclim.org, Hijmans *et al.* 2005). We used the same populations as in Marin *et al.* 2020, completed by one population for *A. m. striatum* (VIL see, Fig 1), in order to better balance the number of populations between subspecies and elevation categories in an attempt to improve our statistical comparison testing for the pattern of local adaptation to elevation across *A. m. striatum* populations, but not across *A. m. pseudomajus* populations suggested in previous studies (Marin *et al.*, 2020).

Seed families used to produce the plants grown in this experiment were not sampled directly in the wild but produced by two successive generations of parental plants that were germinated and grew in a common garden environment (Fig S2). Only the first parental generation of plants was germinated from seeds collected from field populations. These two generations of plants regenerated before our experiment are expected to have reduced maternal environmental effects that could have otherwise biased the trait values recorded during the experiments presented here. In each wild population, seeds were sampled in October 2011 and randomly collected from mature plants. Seeds sampled in the wild were sown in spring 2012 in individual pots ($9 \times 9 \times 10$ cm) filled with universal compost in a greenhouse at the CNRS Experimental Ecology Station in Moulis, France. This first generation of plants germinated and grew with no nutrient addition under an average temperature from 15 to 28°C and weekly watering. Mature plants were hand-pollinated during the summer 2012. Crosses were conducted within populations where mates were assigned randomly. The seeds produced by these crosses constitute the 2012 collection of seed families. We sowed these seed families in spring 2014 in a common garden at ENSFEA (Toulouse, France). This second generation of plants were germinated and grew in individual pots ($9 \times 9 \times 10$ cm) filled with universal compost, with no nutrient addition, under outdoor climatic conditions (average month temperatures ranging from 20.6 to 21.5°C and cumulative monthly rainfall ranging from 28.3 to 73.4 mm). Plants were supplied with water in case of prolonged drought. Mature plants were hand-pollinated during summer 2014. Crosses were conducted within populations where mates were assigned randomly. The seeds resulting from these crosses constitute the 2014 collection of seed families that were used in 2015 in the experiments presented here.

**Common garden sites at low and high elevation.** In our study, we were interested in testing whether germination participates to the local adaptation of populations to elevation. We used a "parallel" approach as described by Kawecki & Ebert (2004); several replicate populations originating from each habitat type (e.g. low- vs high-elevation habitats) were sampled and compared, here in terms of germination in each habitat type (low- vs high-elevation gardens). If germination participates directly to the local adaptation of populations to elevation, the populations originating from high-elevation habitats should outperform the populations originating from low-elevation habitats in the high-elevation garden whereas the populations originating from low-elevation habitats should outperform the populations originating from high-elevation habitats in the low-elevation garden. We therefore transplanted seeds from every population in two sites (Fig S2). One site was located at low elevation, in Toulouse, France (elevation 152 m). The other one was located at high elevation, in the Siguer valley at Lercoul, France (elevation 1100 m; see Fig 1). These two sites were chosen because their climatic conditions were respectively representative of the average climatic conditions experienced by the populations sampled in the lowest half and highest half of the elevation

range respectively. As expected, the site at higher elevation received more rainfall, was cooler, and had a less severe summer drought than the low-elevation site (See supplementary information, Fig S1).

**Shade treatment: shaded by understory vegetation.** At each site, two different common gardens were used to expose plants to two different environmental background conditions: an open environment (in full light, meadow) and a closed environment (shaded by understory vegetation). These common gardens were within 200 m of each other to keep the background climate conditions that are not affected by shade as similar as possible. Because we were interested in testing the reproducibility of the signature of local adaptation to elevation, rather than estimating the importance of light/shade factor in local adaptation, we choose to use natural understory vegetation to induce a differential environmental background based on the presence and absence of shade. Therefore, along with this "shade treatment" induced by understory vegetation, moisture and biotic interactions were undoubtedly different between treatments. These separate treatments (completely open or completely shaded) mimic conditions experienced by populations in their native habitats where individuals develop in diverse light conditions within or between populations. These two conditions (light and shade) are known to induce plastic changes in the morphology and germination in *A. majus* (Gourcilleau *et al.*, 2019). We evaluated the contrast in light conditions by measuring the photosynthetically active radiation (PAR), which was significantly reduced under shade (See supplementary information, Fig S3 and Fig S4).

**Experimental design in the common gardens.** We sowed 5360 seeds in spring 2015. Seeds germinated and plants grew outdoor in the gardens. In every garden, the constitution of the 15 study populations was the same. In every garden, seeds used for a given population came from 14 seed families (between 13 and 15 depending on the population). The same seed families were used in every garden, so that the four gardens were composed by a similar gene pool. In every garden, every seed family was represented by six individuals (Table S1, Supporting Information). Seeds were sown on the top of individual pots ($9 \times 9 \times 10$ cm) with clay universal (TS3 Argile code 404, Klasmann©) and compost universal (BP2 Kompact code 294, Klasmann©). These pots were arranged in a randomized design on a tarpaulin covered with compost universal. Plants grew in pots filled with no nutrient addition and under outdoor climatic conditions in planting sites. Plants were supplied with water in case of prolonged drought.

**Germination-related traits and fitness optimum.** Here, we focused on two germination-related traits: the seed germination rate, and the time to germination. In both common gardens, the germination date was monitored during the summer 2015 three times per week. To our knowledge, no evidence for several peaks of germination has been reported in *A. majus*. We therefore did not consider that seeds that did not germinate the first year harboured potential for germination in future years. As a result, we also did not consider that seeds that did not germinate the first year could play a role in a particular ecological strategy of delayed germination across seasons.

Seed germination and time to germination are traits of interest when considering the response of plants to elevation. The seed germination is a direct measure of plant survival; it is monotonically related to fitness (i.e. under directional selection in all populations, and all sites). The timing of seed germination is not a direct measure of plant performance but it has a strong effect on seedling survival: it influences seedling seasonal exposure to potentially lethal environmental factors and to advantageous conditions for subsequent growth and reproduction (Donohue *et al.*, 2010). The timing of seed germination is not monotonically related to fitness. Optimal times to germination may differ in different locations. Selection may favour either early or delayed germination, depending on when environmental conditions are advantageous or deleterious. Selection for increased fecundity should favour early germination. Indeed, plants germinating earlier can reach a larger size before reproduction and reproduce over a longer period (Hoyle *et al.*, 2015). At low elevation, selection for increased fecundity and summer drought mortality are expected to favour early germination. At high elevation, two contrasted hypotheses can be drawn (Schütz, 2002). Short growing seasons in sub-alpine habitats should favour early germination, to provide enough time for growth and reproduction. This is particularly true for annual plants, but would be less advantageous for short-lived perennial plants such as *A. majus*. On the other hand, the high risk of seedling mortality due to adverse spring conditions may select for delayed germination. Studies on alpine environments suggest that there is no global alpine germination strategy (Körner, 2003; Giménez-Benavides *et al.*, 2005; Wagner & Simons, 2009; Hoyle *et al.*, 2015).

Seed germination and time to germination are also traits of interest when considering the response of plants to light and shade environments. As other small-seeded species, *A. majus* requires light to germinate (seeds germinate only on or near soil surface but not buried in the soil, Leishman *et al.* 2000; Milberg *et al.* 2000). Consequently, we expect a lower germination rate under shade than under light in all populations and in both

subspecies. Additionally, earlier germination might be expected under shade because it may provide a head start in the presence of competition for light. Plants that germinate later than average may be incapable of overtopping their neighbours due to their small initial size (Weinig, 2000).

## Statistical analysis.

***Estimating environmental, genetic and G × E interactions variances.*** For each subspecies, we used GLMMs (Generalized Linear Mixed Model) with fixed and random effects to quantify the magnitude of the environmental, genetic and genetic-by-environment (G × E) interaction variances in the response of germination-related traits to site elevation and shade treatment. Fixed effects included the site elevation effect ($V_{Elevation}$), the shade treatment effect ($V_{Shade}$) and their interaction ($V_{ElevationxShade}$) effect on the phenotype. These fixed effects were therefore used to estimate environmental variances. For the germination rate, the random effects were included to estimate the between-population variance ($V_B$), the between-family effect variance ($V_F$), and the family × site elevation × shade treatment interaction variance ($V_{GXE}$). Both $V_B$ and $V_F$ refer to genetic effects, whereas $V_{GXE}$ refers to the genetic by environment interaction. The phenotypic plasticity corresponds to environmental variance ($V_{Elevation}$, $V_{Shade}$, and $V_{Elevation} \times V_{Shade}$) and is also partly included in the genetic by environment interaction (Scheiner & Goodnight, 1984; Scheiner & Lyman, 1989). Since only a subset of individuals germinated, the analysis of the time to germination was conducted on a smaller dataset than the dataset for the germination rate. We therefore simplified the random effect structure of the models for the time to germination. Random effects included the between-population variance ($V_B$), and the population x site elevation x shade treatment interaction variance ($V_{GXE}$), but not the family effect variance. The error distribution was chosen to fit each trait: (i) a binomial model (with a logit link function) was used to analyze the germination success (0 vs 1), (ii) a Poisson model (with a log link function) was used to analyze the time to germination.

We established whether the environmental effects (site elevation, shade treatment and interactions) explained significant variance in germination-related traits by comparing models with and without the environmental effects on the basis of their log-likelihood ratios (Zuur *et al.*, 2009). If the log-likelihood ratio test (LRT) returned a significant p-value, then the model including the environmental effects explained the data better than the model without the environmental effects.

***Testing for a pattern of local adaptation to elevation.*** The "local" elevation vs. "foreign" elevation (Kawecki & Ebert, 2004) was chosen to analyse the local adaptation of populations to elevation on the basis of germination-related traits. We considered that local adaptation to elevation is operating if populations originating from high-elevation habitats had higher germination rates than populations from low-elevation habitats in high-elevation site, whereas populations originating from low-elevation habitats had higher germination rates than populations from high-elevation habitats in low-elevation site. The reaction norms (i.e. phenotypic responses of same genotypes between high and low elevation sites) of populations originating from high- and low-elevation habitats should logically be crossing in the presence of adaptation to elevation for germination success at high and low elevations. For the time to germination, expectations in high-elevation sites remain unclear. As explained above, delayed or early germination of populations from high-elevation habitats can be both advantageous at higher elevation. Therefore, for this trait, crossing and non-crossing reaction norms can both reflect a pattern of local adaptation to elevation.

For each subspecies, we performed GLMMs with fixed and random effects that are closely similar to the models presented in the above section but differ to some extent to allow for specific hypotheses to be tested. The fixed effects included the site elevation effect, the shade treatment, the elevation of origin of the population (as a discrete variable, "high" vs "low"), and their interactions. The random effects included the between-population variance ($V_B$). The error distribution was chosen to fit each trait: (i) a binomial model (with a logit function) was used to analyze the germination success (0 vs 1), (ii) a Poisson model (with a log link function) was used to analyze the time to germination. We established whether the elevation of origin explained significant variance in germination-related traits by comparing models with and without the elevation of origin on the basis of their log-likelihood ratios (Zuur *et al.*, 2009). If the log-likelihood ratio test (LRT) returned a significant p-value, then the model including the elevation of origin explained the data better than the model without the environmental effects. Finally, significant differences between populations originating from high and low elevation within each site, and significant differences between high and low-elevation sites for the same populations were evaluated by using Wilcoxon tests.

**Availability of code and data.** All statistical analyses were performed using the R.3.5.0 software (R Core Team, 2018). All generalized mixed-model were implemented in R via the lme4 package (Bates *et al.*, 2015). The code and data for producing figures and results in this paper

**Table 1. Results from the generalized mixed models for germination-related traits for both subspecies of *Anthirrinum majus*. Marginal $R^2$ is the part of variance explained by fixed effects. Conditional $R^2$ is the part of variance explained by both fixed and random effects.**

| | *A. majus striatum* | | | | *A. majus pseudomajus* | | | |
|---|---|---|---|---|---|---|---|---|
| a) Germination rate (binomial) | Marginal $R^2$ = 0.10 | | Conditional $R^2$ = 0.22 | | Marginal $R^2$ = 0.14 | | Conditional $R^2$ = 0.26 | |
| **Fixed effects** | Estimates | CI 95% lower | CI 95% upper | p-value | Estimates | CI 95% lower | CI 95% upper | p-value |
| *Environmental effects* | | | | | | | | |
| Intercept | -0.77 | -1.04 | -0.50 | **0** | -0.98 | -1.22 | -0.74 | **0** |
| Site elevation | -1.64 | -2.00 | -1.28 | **0** | -1.70 | -2.06 | -1.34 | **0** |
| Shade treatment | -0.15 | -0.45 | 0.15 | 0.32 | 0.21 | -0.07 | 0.49 | 0.135 |
| Site elevation x Shade | 0.85 | 0.37 | 1.34 | **0.001** | 0.90 | 0.44 | 1.36 | **0** |
| **Random effects** | Variance | CI 95 % lower | CI 95% upper | Nb group | Variance | CI 95% lower | CI 95% upper | Nb group |
| *Genetic effects* | | | | | | | | |
| Between populations ($V_B$) | 0.04 | 0.03 | 0.05 | 7 | 0.02 | 0.01 | 0.02 | 8 |
| Family nested in populations ($V_F$) | 0.07 | 0.06 | 0.08 | 103 | 0.27 | 0.26 | 0.29 | 120 |
| *G x E effects* | | | | | | | | |
| Family x Site elevation x Shade | 0.36 | 0.34 | 0.38 | 412 | 0.32 | 0.30 | 0.34 | 479 |
| b) Time to germination (poisson) | Marginal $R^2$ = 0.46 | | Conditional $R^2$ = 0.60 | | Marginal $R^2$ = 0.51 | | Conditional $R^2$ = 0.57 | |
| **Fixed effects** | Estimates | CI 95% lower | CI 95% upper | p-value | Estimates | CI 95% lower | CI 95% upper | p-value |
| *Environmental effects* | | | | | | | | |
| Intercept | 3.46 | 3.37 | 3.55 | **0** | 3.41 | 3.35 | 3.46 | **0** |
| Site elevation | -0.68 | -0.81 | -0.55 | **0** | -0.73 | -0.84 | -0.62 | **0** |
| Shade treatment | -0.23 | -0.35 | -0.11 | **0** | -0.23 | -0.32 | -0.15 | **0** |
| Site elevation x Shade | 0.50 | 0.32 | 0.68 | **0** | 0.47 | 0.33 | 0.61 | **0** |
| **Random effects** | Variance | CI 95 % lower | CI 95% upper | Nb group | Variance | CI 95% lower | CI 95% upper | Nb group |
| *Genetic effects* | | | | | | | | |
| Between populations ($V_B$) | 0.00 | -0.00 | 0.01 | 7 | 0.00 | -0.00 | 0.00 | 7 |
| *G x E effects* | | | | | | | | |
| Population x Site elevation x Shade | 0.01 | 0.00 | 0.02 | 280 | 0.01 | 0.00 | 0.01 | 310 |

## 3. Result

**Stronger phenotypic plasticity than genetic differentiation among populations.** Phenotypic plasticity was found for both germination-related traits, as illustrated by significant environmental effects of the site elevation or shade treatment or site elevation × shade treatment interaction (Table 1). The slopes of the reaction norms were negative and drove a strong decrease between high- and low- elevation sites for both traits in both subspecies (Table 1, Fig 2 and 3). Germination rates in the high-elevation site were significantly higher (32 %) than in the low-elevation site (14 %). The time to germination was longer in the high-elevation site (28 days) than in the low-elevation site (18 days). In both subspecies, the shade treatment significantly influenced the time to germination, but not the germination rate, although it can be argued that it had an effect on the germination rate through the significant site elevation x shade treatment interaction. Seeds required on average five additional days to germinate under shade compared to light condition.

The models including the environmental effects (site elevation, shade treatment and interactions) explained the data better than the model without the environmental effects, as demonstrated by significant p-values for the log-likelihood ratio test (LRT, Table 2). The environmental variance explained approximately 10 to 50% of the variation in germination-related traits. Indeed, the marginal $R^2$ describing the proportion of trait variance explained by the fixed effects ranged from 0.1 to 0.51 (Table 1). A non-negligible yet lower amount of trait variation was explained by random effects (between- and within-population variation and G × E interactions). The proportion of variance explained by the conditional $R^2$ accounting for fixed and random effects was higher than the marginal $R^2$ accounting for fixed effects as illustrated by its increase by 6 to 14% between conditional and marginal (Table 1).

The largest component of genetic variation for germination related-traits was the genetic variation of the degree of plasticity (G × E interaction, Table 1). Genetic variation between populations and between families within populations were smaller in both subspecies. Small variation for germination rates was found among populations for both subspecies, as indicated by the low but significant variance (Table 1 a, CI 95% not overlapping zero). A larger proportion of variation for germination rates was explained by the within-population genetic variation estimated by the family effect, in particular in *A. m. pseudomajus* (Table 1 a). No variation for the time to germination was found between populations (Table 1 b,

CI 95% crossing zero). Between-family within-population variance was not estimated for this trait. A graphical representation of average population phenotypic values is available in the supplementary (Fig S5 and S6).

**Table 2. Results of the log likelihood ratio test (LRT) performed on the generalized mixed models for germination-related traits for both subspecies of *Antirrhinum majus*. If the log-likelihood ratio test (LRT) returned a significant p-value, then the model including the environmental effects (site elevation, shade treatment and their interactions) or the elevation of origin explained the data better than the models without the environmental effects. See models estimates in Table 1 and 3.**

|  | LRT | p-value |
|---|---|---|
| a) With and without environmental effects | | |
| | | |
| **Germination rates** | | |
| *A. majus. striatum* | 97.4 | **0** |
| *A. majus. pseudomajus* | 128.0 | **0** |
| **Time to germination** | | |
| *A. majus. striatum* | 43.1 | **0** |
| *A. majus. pseudomajus* | 67.8 | **0** |
| b) With and without elevation of origin | | |
| | | |
| **Germination rates** | | |
| *A. majus. striatum* | 29.1 | **0** |
| *A. majus. pseudomajus* | 18.6 | **0.001** |
| **Time to germination** | | |
| *A. majus. striatum* | 34.8 | **0** |
| *A. majus. pseudomajus* | 37.1 | **0** |

**Partial signatures of local adaptation to elevation under light for *A. m. striatum*.** The models including the elevation of origin fitted the data better than the null models (i.e. models without the elevation of origin) for all traits and all subspecies (Table 2). In *A. m. striatum*, the "local" elevation vs. "foreign" elevation criterion was partially satisfied under light conditions for both the germination rate and the time to germination. As expected, plants originating from high-elevation habitats had a significantly higher germination rate compared to plants originating from low-elevation habitats, in the high-elevation site under light condition (Fig 2 a). Yet, in the low-elevation site, differences in germination success were not significant between populations from high and low-elevation habitats (Fig 2 a). Therefore the local vs foreign criterion holds in high-elevation site but not in low elevation site for the germination success. For the time to germination, as expected, plants originating from low-elevation habitats germinated significantly earlier than plants originating from high-elevation habitats in the low-elevation site under light condition (Fig 2 c). In the high-elevation site under light, plant from high-elevation habitat germinated later than plants from low-elevation habitats (Fig 2 c). In the subspecies *A. m. pseudomajus*, the "local elevation vs. foreign elevation"
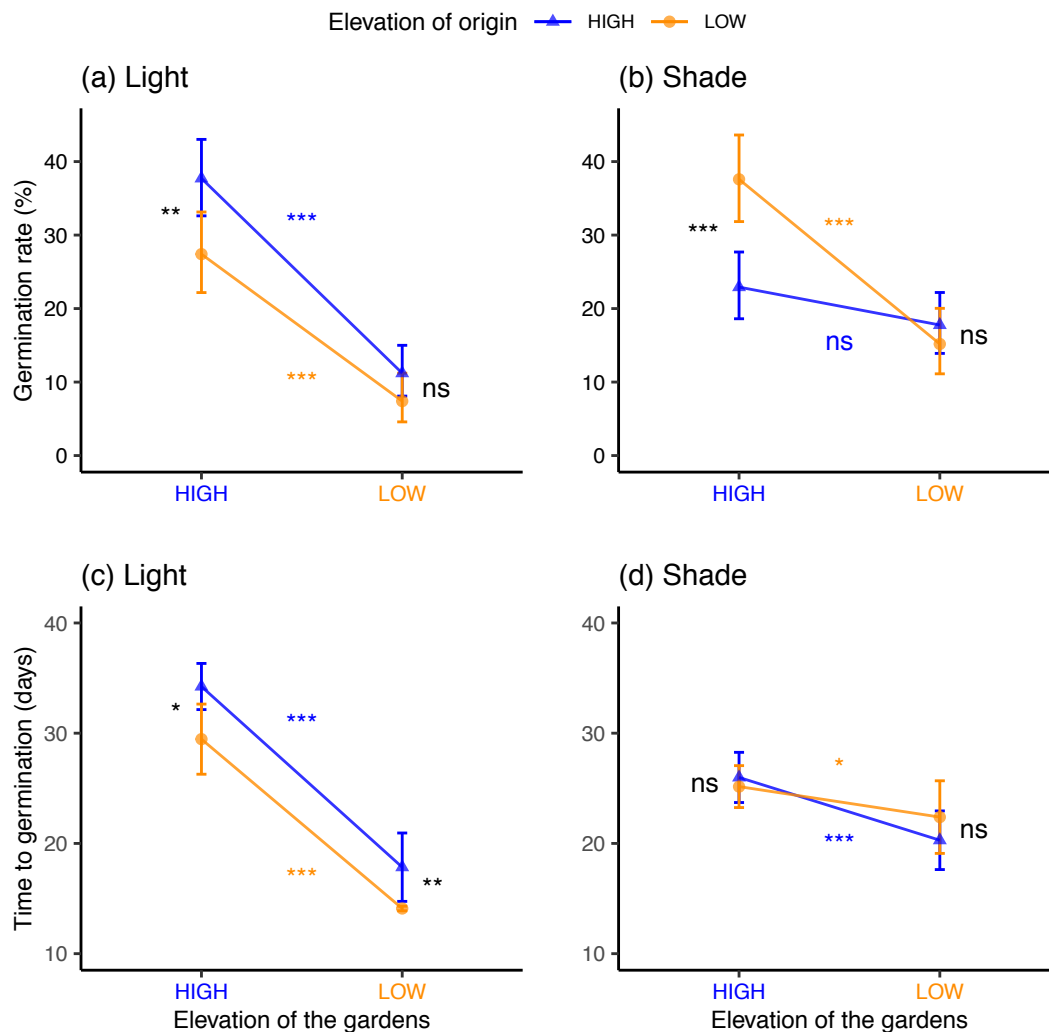
**Fig. 2.** Reaction norms of germination-related traits (mean values ± 95% CI) for seven populations of *Anthirrinum majus striatum* in the two sites (low and high elevation) and under two treatments (open light and understory shade). plots a) and b) refer to germination rate, c) and d) to time to germination, a) and c) refer to light treatment, b) and d) to shade treatment. Significant differences are indicated by asterisks. ***: p.value ≤ 0.001 , **: 0.001 < p.value ≤ 0.01, *: 0.01 < p.value ≤ 0.05, '.': 0.5 < p.value < 0.1, 'ns': p.value ≥ 0.1.

criterion was never satisfied, reflecting the lack of local adaptation for both germination-related traits. Indeed, the populations originating from high-elevation habitats never outperformed the populations originating from low-elevation habitats in the high-elevation garden, and vice-versa (Fig 3).

**Adaptive evolutionary responses disturbed by shade.**
Patterns of responses to elevation observed under light vanished or reversed under shade in both subspecies (Fig 2 and 3 b and d). Changes were suggestive of maladaptive plasticity in response to shade for *A. m. striatum*. The significant effects of the three-way interaction elevation of origin × site elevation × shade treatment and the two-way interaction site elevation × shade treatment on germination related-traits showed that the patterns that we interpret as evolutionary signatures of adaptation to elevation were different under light and under shade (Table 3). In the high-elevation site and under light, *A. m. striatum* plants from high-elevation populations had a significantly higher germination rate and a delayed germination compared to plants originating from low-elevation populations. Under shade, we obtained contrasted results in the high-elevation site; plants from high-elevation populations had lower germination rates and a similar time to germination compared to plants from low-elevation populations (Fig 2 b and d). In the low-elevation site and under light, plants from low-elevation populations had similar germination rates and germinated earlier than plants from high-elevation populations but there were no significant differences under shade (Fig 2 b and d).
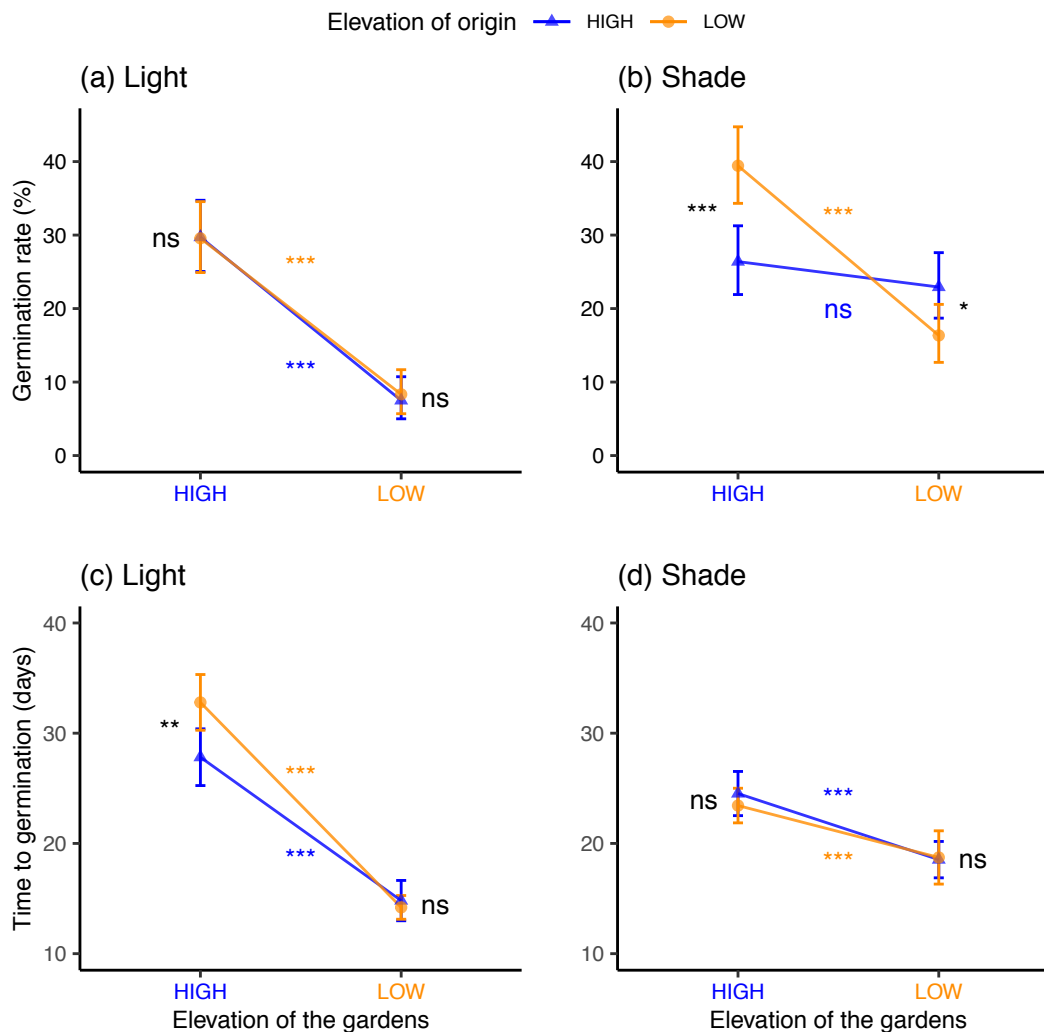
**Fig. 3.** Reaction norms of germination-related traits (mean values ± 95% CI) for eight populations of *Anthirrinum majus pseudomajus* in the two sites (low and high elevation) and under two treatments (light and shade). The legend is identical to Fig 2.

## 4. Discussion

Our findings illustrate how our understanding of local adaptation and adaptive plasticity is affected by taking into account complex environments. Our comparison between high and low elevation, open habitat and understory, of multiple *A. majus* populations which gene pool was replicated between environments, revealed an impact of the multidimensional nature of their complex environment on the experimental signature of adaptive mechanisms. In open light conditions, we found partial evidence suggesting local adaptation to elevation in *A. m. striatum*, but not in *A. m. pseudomajus* by analysing germination-related trait data from multiple populations in common garden experiments replicated at different elevations. The slightly differential genetic background of the populations representing these closely related subspecies represented in our experiment was not only associated with different signatures of adaptation

to elevation but also with differential phenotypic plasticity. Plastic responses to elevation were stronger than genetic divergence, suggesting both cogradient variation (microevolutionary response acting synergistically with direct environmental plastic response) and hyperplasticity (greater direct environmental response of the trait as compared to its genetic divergence). It is interesting to note that these hyperplasticity is usually expected to be associated with countergradient variation (microevolutionary response acting antagonistically with direct environmental plastic response). Under shade, the signature of *A. majus* adaptive responses to elevation differed from open light conditions, suggesting maladaptation. This finding has two implications. First, the differential impact of the open habitat and understory conditions revealed experimentally by two separate environmental treatments suggests varying selection on germination traits inside populations characterized by heterogeneous conditions of vegetation cover in

**Table 3. Results from the generalized mixed models (GLMM) testing for local adaptation on germination-related traits in both subspecies of *Antirrhinum majus*. Random factor in all models is the population effect. Marginal $R^2$ is the part of variance explained by fixed effects. Conditional $R^2$ is the part of variance explained by both fixed and random effects.**

| | *A. majus striatum* | | | | *A. majus pseudomajus* | | | |
|---|---|---|---|---|---|---|---|---|
| a) Germination rate (binomial) | Marginal $R^2$ = 0.12 | | Conditional $R^2$ = 0.13 | | Marginal $R^2$ = 0.13 | | Conditional $R^2$ = 0.14 | |
| **Fixed effects** | Estimates | CI 95% lower | CI 95% upper | p-value | Estimates | CI 95% lower | CI 95% upper | p-value |
| Intercept | -0.52 | -0.87 | -0.18 | **0.001** | -0.85 | -1.16 | -0.55 | **0** |
| Site elevation | -1.59 | -2.00 | -1.20 | **0** | -1.67 | -2.14 | -1.23 | **0** |
| Elevation of origin | -0.44 | -0.97 | 0.10 | 0.086 | -0.02 | -0.46 | 0.41 | 0.908 |
| Shade treatment | -0.69 | -1.03 | -0.36 | **0** | -0.14 | -0.46 | 0.19 | 0.408 |
| Elevation of origin x Site elevation | 0.07 | -0.60 | 0.72 | 0.833 | 0.17 | -0.46 | 0.80 | 0.593 |
| Site elevation x Shade | 1.29 | 0.75 | 1.84 | **0** | 1.47 | 0.92 | 2.05 | **0** |
| Elevation of origin x Shade | 1.23 | 0.75 | 1.73 | **0** | 0.63 | 0.18 | 1.08 | **0.006** |
| Elevation of origin x Site elevation x Shade | -1.05 | -1.91 | -0.19 | **0.016** | -1.19 | -1.99 | -0.40 | **0.003** |
| | | | | | | | | |
| b) Time to germination (poisson) | Marginal $R^2$ = 0.54 | | Conditional $R^2$ = 0.61 | | Marginal $R^2$ = 0.56 | | Conditional $R^2$ = 0.58 | |
| **Fixed effects** | Estimates | CI 95% lower | CI 95% upper | p-value | Estimates | CI 95% lower | CI 95% upper | p-value |
| Intercept | 3.53 | 3.43 | 3.63 | **0** | 3.32 | 3.26 | 3.38 | **0** |
| Site elevation | -0.66 | -0.74 | -0.58 | **0** | -0.63 | -0.73 | -0.52 | **0** |
| Elevation of origin | -0.15 | -0.30 | -0.00 | **0.023** | 0.17 | 0.08 | 0.25 | **0** |
| Shade treatment | -0.30 | -0.35 | -0.25 | **0** | -0.13 | -0.18 | -0.07 | **0** |
| Elevation of origin x Site elevation | -0.08 | -0.23 | 0.07 | 0.308 | -0.21 | -0.35 | -0.06 | **0.005** |
| Site elevation x Shade | 0.44 | 0.33 | 0.55 | **0** | 0.35 | 0.22 | 0.47 | **0** |
| Elevation of origin x Shade | 0.14 | 0.07 | 0.22 | **0** | -0.20 | -0.28 | -0.13 | **0** |
| Elevation of origin x Site elevation x Shade | 0.18 | -0.00 | 0.36 | 0.052 | 0.25 | 0.08 | 0.42 | **0.005** |

the wild. Our ability to understand adaptation in *A. majus* therefore presents a number of complications resulting from the multidimensional complexity of it environment. The multiple environmental variables that are combined to form its heterogeneous habitat can indeed affect the signature of microevolutionary and phenotypic plastic adaptive or maladaptive responses. Our findings corroborate the recent emergence of similar results in the scientific literature (Morel-Journel *et al.*, 2020). This issue has the potential to affect studies that do not test for the effect of environmental treatments on the signature of local adaptation. These complications also challenge our ability to understand adaptive responses to contrasted climate conditions.

**Local adaptation to elevation detected in a classical open habitat experimental setting.** We found divergence in the genetic variation underlying the germination rate and the time to germination which is likely to be adaptive for *A. m. striatum*. For the germination rate, partial evidence of local adaptation to elevation was found in the high-elevation site, but not in the low-elevation site. In the high-elevation site, populations of *A. m. striatum* from high-elevation habitats had higher germination rates compared to populations from low elevation habitats, whereas in the low-elevation garden we found no differences in germination rates between populations from high- and low-elevation habitats. This pattern does not on its own provide convincing evidence for the local adaptation of populations to elevation. However, it still

does suggest an imprint of natural selection imposed by the local conditions in high-elevation habitats. Climatic conditions in low elevation gardens were particularly hot and dry in southern France on that year. It is also possible that the signature of local adaptation was masked by experimental artefact (Kawecki & Ebert, 2004) with an homogenization of seed responses caused by harsh summer conditions at low-elevation with all populations suffering lower germination rates than in higher-elevation gardens. This pattern invites follow-up studies to replicate the detection of the signature of adaptation on germination.

Our results for the time to germination were consistent with our expectations. In low-elevation site, *A. m. striatum* populations from low-elevation germinated earlier than populations from high-elevation. This result is coherent with selection toward earlier germination at low elevation that is acknowledged to increase fecundity and/or to decrease summer drought mortality (Leger *et al.*, 2009). In the high-elevation site, *A. m. striatum* populations from high-elevation showed delayed germination compared to populations from low-elevation. The delayed germination of seeds where conditions turn favourable later in the season than at low elevation, or under presently favourable conditions at high elevation can be interpreted as a way to reduce the risk of mortality if the conditions were to turn unfavourable under springtime (Donohue *et al.*, 2010; Hoyle *et al.*, 2015). However, the opposite hypothesis, i.e., the benefits of earlier germination at high elevation also exists in the

literature (Schütz, 2002), although it is more suitable for annual plants and applies inside the seasonal window of favourable conditions at high elevation that might nevertheless occur later than at low elevation. We have no evidence that this pattern of delayed germination in high-elevation gardens will be advantageous to seedlings or to later adult life stages. Our results on the time to germination can therefore only be interpreted as partial evidence of local adaptation which is based on results in low-elevation site.

The findings described above show that *A. m. striatum* populations from high and low-elevation habitats have genetically diverged in terms of germination rate and time to germination. Considered altogether, these results offer a pattern consistent with local adaptation to elevation (Kawecki & Ebert, 2004) because the populations originating from high-elevation habitats outperformed the populations originating from low-elevation habitats in the high-elevation site via higher germination rates whereas the populations originating from low-elevation habitats outperformed the populations originating from high-elevation habitats in the low-elevation site via delayed germination. In contrast, we found no evidence for a pattern of local adaptation to elevation in *A. m. pseudomajus*. These results are consistent with a previous study on *A. majus* that used a $Q_{ST} - F_{ST}$ indirect approach (Marin *et al.*, 2020). They detected a potential signal of local adaptation to elevation on biomass-related traits in *A. m. striatum* but not in *A. m. pseudomajus*. This finding highlight contrasted patterns of local adaptation to elevation between these two genetically closely related subspecies subspecies that harbour different flower colours but share the same ecological range. Adaptive mechanisms related to elevation might be contributing to the divergence of these subspecies.

**The limits of forecasting responses to climate change on the basis of experimental approaches.** Our results provide evidence for the adaptation of *A. majus* populations to different elevations in the Pyrenees, which might imply that *A. majus* successfully evolved adaptations to climate differences. It is important to note that our experimental setting was made to compare the effect of climate environmental conditions while homogenising other potential effects (e.g., soil composition). Signature of past adaptive evolution are useful to identify potential traits playing a role in adaptation. At first sight, our results suggest that germination participated to the adaptation of *A. majus* populations and might play a positive role in its potential adaptation to climate change. The range of climate conditions in these mountains is already changing and set to change even more because of climate change. Conditions at high elevation are becoming more similar to conditions from lower elevation (Urli *et al.*, 2014). In this regard, the experimental evidence that seeds originating from high elevation performed as well in terms of germination rate at low elevation as seeds originating from low elevation is encouraging in terms of resilience to climate change. Again at first sight, one might speculate that seeds will keep germinating at a comfortable rate as hotter temperatures hit higher elevations. However, caution must be exercised with this type of predictions derived from experimental approaches that often require to simplify the complexity of the life cycle and the multiple dimensions of the environment. For example, the experimental evidence at low elevation that seeds from higher elevation germinate later than seeds originating from lower elevation is not reinsuring as one might speculate that plants will find themselves to grow in too hot and dry environmental conditions that will limit their survival and their reproductive success. In addition, the neglected complexity of environmental effects might also affect experimental results based on phenotypes and these types of forecasted adaptive scenarios. For example in our study, understory shade conditions modified the signature of the local adaptation of populations to elevation, which can alter predictions. Furthermore, they can also outline alternative climate change scenarios. For example, our results under understory shade suggest that some mechanisms such as a change in vegetation cover under climate change might impede adaptation.

**Sensitivity of elevation adaptation patterns to another local environmental condition.** Neglecting the multivariate nature of the environment may lead to incorrect assessments of how species adapt to their current habitat, and how they will respond to climate changes. For instance, Anderson & Wadgymar 2019 found evidence of local adaptation to elevation in *Boechera stricta* but this pattern was disturbed by changes in snow cover that lead to observe signs of local maladaptation. Examples of empirical studies testing the stability of plant adaptive responses to the complexity of environment are rare (Chevin & Lande, 2015; Westneat *et al.*, 2019). In *A. m. striatum*, we found that patterns reflecting the adaptation of populations to elevation under light were disturbed by shade. Under shade, our results reflected local maladaptation in germination-related traits. Contrary to expectation, we did not found similar patterns under shade with simply lower germination rates and earlier germination than under regular light conditions. The effect of elevation under shade could not be inferred from simply scaling down the observed patterns under light. This finding suggests heterogeneous selection at both the intra- and inter-population spatial scales. Shade is often associated with cooler temperature during daytime, warmer temperatures at night and higher air humidity and soil moisture

(Valladares *et al.*, 2016). Therefore, climate-related genetic differentiation in germination-related traits at both intra- and inter-population scales might be maintained by fluctuating selective pressures that are both influenced by microclimatic conditions (e.g. shade provided by vegetation cover vs regular light in open habitat) and larger-scale climatic conditions (e.g. due to elevation). This finding also revealed that our evaluation of the genetic signature of local adaptation in response to elevation was altered when we replicated our common garden experiment at a similar location but in a different environmental background (here understory shade). Our understanding of the adaptation of *A. majus* plants can therefore be affected by the choice of environmental conditions used in the experimental setting. Interactions between environmental factors create unexpected outcomes. This scenario is likely to be common in other systems where both plasticity to multiple environmental drivers and local adaptation are present (Palacio-Lopez *et al.*, 2015; Acasuso-Rivero *et al.*, 2019).

**Phenotypic plasticity in response to elevation.** Our findings revealed a strong plastic response of *A. majus* to elevation in germination-related traits. The magnitude of this plastic response was a lot stronger than trait genetic divergence between populations from different elevations in both subspecies. Our study therefore provided additional evidence for local adaptation and phenotypic plasticity shaping the same fitness related traits but with plasticity having a greater influence (de Villemereuil *et al.*, 2018). Elevation had a significant effect on germination rates, with lower germination rates in low-elevation gardens compared to high-elevation gardens, exception made for populations of both subspecies originating from high elevation under understory shade that were not affected. This plasticity in germination rates probably reflects between-site differences in environmental quality, with a lower germination in hot and dry environments. Elevation had also a significant impact on the time to germination. Seeds germinated earlier in low-elevation gardens compared to high-elevation gardens in a remarkable similar fashion in all populations and for both subspecies. In *A. majus*, plasticity in response to elevation appears to be neutral (under open light habitat) or maladaptive (under understory shade) for the germination rate, and adaptive (under open light habitat) or neutral (under understory shade) for the time to germination. Caution must be exercised when discussing whether plastic responses observed for the time to germination are neutral or adaptive. To support the adaptive plasticity hypothesis, it would be necessary to demonstrate that the plastic response induced by each environment is toward the phenotype favoured by selection in that environment (Ghalambor *et al.*, 2007). Although we might expect earlier germination to be favoured by mortality selection over the summer at low elevation, we have no evidence of a positive relationship between this phenotype and the plant fitness across its lifetime. Either adaptive or maladaptive, the large plasticity found in response to elevation for germination traits is likely to play a role in the response of *A. majus* to climate changes.

## 5. Conclusion

The replication under understory shade of our experiment investigating the signature of local adaptation to elevation in an open habitat (where herbaceous plant adaptation experiments in common gardens are usually conducted) greatly affected the signature of adaptation, both in terms of genetic divergence and phenotypic plasticity. This finding suggests that selection mechanisms vary across heterogeneous environments in *A. majus*. It also outlines that experimentally inferred adaptive signatures should take into account the variability of the environmental background. The multidimensional complexity of the genetic and environmental background determining phenotypic traits makes it extremely complex to extrapolate whether microevolutionary adaptive responses and phenotypic plasticity act synergistically or antagonistically. Caution should therefore be taken when asserting the existence of co- or countergradient variation and hyperplasticity as our measurements of plasticity in a given experimental background might in fact themselves harbour plasticity in different experimental backgrounds. Our findings also imply that forecasting the ability of plants to adapt to environmental changes based on common garden and reciprocal transplant experiments must account for the multivariate nature of the environment.

## 6. Author contributions

BP designed the research program. SM, JA, MI, GO, AlG and BP carried out the experiments; AG analyzed the data; AG and BP wrote the manuscript.

## 7. Acknowledgements

## 8. Conflict of interest disclosure

The authors of this article declare that they have no financial conflict of interest with the content of this article.

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