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# Rapid divergent evolution of an annual plant across a latitudinal gradient revealed by seed resurrection

**Running title: Rapid divergent evolution of plant traits**

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**Author contributions:** P.O.C. and E.I. conceived the study, performed the common garden experiment, and measured growth-related and reproductive traits at the common garden experiment. W.A.V-M. measured performance traits such as seed set and seed predators. E.F. and J.P. genotyped individuals. W.A.V-M. conducted data analysis. W.A.V-M. wrote the paper with discussions and contributions from all authors. P.O.C. supervised the entire project. No conflict of interest to declare.

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# **Rapid divergent evolution of an annual plant across a latitudinal gradient revealed by seed resurrection**

**Abstract** - Global change are expected to drive short-term evolution of natural populations. However, it remains unclear whether different populations are changing in unison. Here, we study contemporary evolution of growth-related and reproductive traits of three populations of *Cyanus segetum* across a latitudinal gradient in France. We resurrected stored seeds sampled up to 24 years apart from northern, central-western, and southern populations and conducted an *in situ* common-garden experiment. To disentangle neutral from selection-driven differentiation, we calculated  $F_{ST}$  and  $Q_{ST}$  between temporal samples. We found that phenotypic evolution was divergent across populations exhibiting different trends for rosette size, date of flowering, and capitula size. By measuring seed set as a proxy of fitness, we showed that samples with larger mean capitula size outperformed samples with smaller mean capitula size in the western and southern populations. Regression of traits on seed set showed that flowering date and capitula size are the primary determinants of fitness, and  $Q_{ST}-F_{ST}$  comparisons indicated that natural selection has likely contributed to the shifts in flowering phenology and rosette size. These findings outline the potential for rescue of natural populations through contemporary evolution and emphasize the complex interplay between spatial and temporal variation in species' responses to global change.

**Keywords:** climate change, adaptation, pollinator decline, resurrection study.

## **Introduction**

Across the globe, ecosystems are experiencing simultaneous changes in climate, atmospheric composition, pollutant deposition, habitat loss, and fragmentation through land-use intensification (Yu et al. 2016; Zhu et al. 2016). These anthropogenic disturbances have the potential to rapidly modify selection regimes in natural populations, triggering phenotypic change (Lopez-Gallego and O'Neil 2010; O'Brien et al. 2017). Although trait changes can occur due to phenotypic plasticity, growing evidence indicates that global change is a strong driver of genetic evolution in plant and animal communities at an ecological time-scale (Franks et al. 2007; Cheptou et al. 2008; Franks and Hoffmann 2012). Adaptation of natural populations to new conditions may buffer the impact of global change through short-term responses of growth-related traits and reproductive strategies (Lopez-Gallego and O'Neil 2010; Brys and Jacquemyn 2012). Yet, to what extent adaptation can rescue populations will depend on the interaction between the local selection regimes, the rate of

environmental deterioration, and the standing genetic variation (Franks and Hoffmann 2012; Thomann et al. 2015).

Contemporary plant evolution has been documented in response to different components of global change, including temperature increase, droughts, habitat degradation, spread of invasive species, chemical pollutants, and pollinator decline (Antonovics et al. 1971; Franks et al. 2007; Lopez-Gallego and O'Neil 2010; Sultan et al. 2013; Yu et al. 2016). Increased temperatures and severe droughts hinder plant growth and influence phenology by shifting flowering time (Franks et al. 2007; Yu et al. 2016; Zhu et al. 2016), while habitat loss and fragmentation result in the loss of genetic diversity and decreased gene flow (Lopez-Gallego and O'Neil 2010; Frei et al. 2014). Beyond detecting phenotypic change over time, a handful of studies have also addressed the role of natural selection in driving rapid evolution under global change through the calculation of selection differentials (Parachnowitsch and Kessler 2010),  $Q_{ST}$ - $F_{ST}$  comparisons (Rhoné et al. 2010; Thomann et al. 2015), and more recently, genome-wide scans (Franks et al. 2016; Frachon et al. 2017).

As part of global change, pollinator decline is a major ecological factor promoting evolution of plant reproductive traits (Thomann et al. 2013). Declines in pollinator visitation reduce plant fitness in allogamous species by inducing strong pollen limitation and hindering pollen transfer (Thomann et al. 2013; Thompson et al. 2013). Plants are predicted to respond to pollen limitation either by favouring the evolution of autonomous self-fertilization as a reproductive assurance strategy (Schemske and Lande 1985; Cheptou et al. 2008), or by evolving greater investment in pollinator attraction (Brys and Jacquemyn 2012; Thomann et al. 2015; Ramos and Schiestl 2019). Therefore, strict outcrossing species, such as self-incompatibles, are presumed to reinforce interactions with pollinators to increase visitation rates and efficiency of pollen transfer (Thomann et al. 2013). Traditionally, pollinator-driven selection has been studied for reproductive traits such as flowering phenology, number of open flowers, corolla size, anther-stigma distance or position, and floral morphology (Harder and Johnson 2009; Bodbyl Roels and Kelly 2011; Ramos and Schiestl 2019). Among these traits, traits related to corolla size and floral phenology have been shown to be under selection for strict outcrossing species. For instance, Thomann et al. (2015) used a common-garden experiment with stored seeds of the outcrossing *Cyanus segetum* and showed that in an interval of 18 years the studied population flowered earlier and produced larger capitula with longer receptivity and a larger floral display. However, this study was limited to one population whereas different locally adapted populations might show diverse mechanisms to ensure pollinators' visitation (Brys and Jacquemyn 2012).

In the context of global pollinator decline, differences in selective pressures can result from heterogeneity of populations proximal to forested or disturbed areas affected by pesticides, which translates into higher variance in pollinator abundance and diversity (Brys and Jacquemyn 2012).

Hence, although global change components show similar trends across diverse terrestrial ecosystems, pollinator-driven selection can strongly vary at fine-geographic scales (Brys and Jacquemyn 2012). Recent studies have used a resurrection approach to show rapid evolution of populations triggered by climate change and historical droughts across latitudinal gradients (Dickman et al. 2019; Lambrecht et al. 2020; Vtipil and Sheth 2020; Wooliver et al. 2020). However, most of these studies of contemporary evolution in response to global change have focused on showing the direction and tempo of the evolutionary change using only phenotypic data, (Franks et al. 2007; Thomann et al. 2015), thus neglecting the role of genetic drift and gene flow in explaining populations' evolutionary responses.

In this study, we investigated short-term evolution of growth-related and reproductive traits in three populations of the cornflower *Cyanus segetum* Hill (syn. *Centaurea cyanus* L.). We addressed if the direction of the evolutionary change is consistent across different populations throughout a latitudinal gradient in France. We used a combined approach of growing resurrected propagules and common-garden experiments, which allows detection of evolution while excluding plasticity, thus revealing the direction and tempo of the evolutionary process (Franks et al. 2008a; Thomann et al. 2015). We germinated stored seeds sampled 16, 23 and 24 years apart for South, North and Central-West populations, respectively. We conducted common-garden experiments in field conditions and compared growth-related and reproductive traits, as well as seed set between sampled years, to test whether: 1) evolution of growth-related or reproductive traits occurred; 2) if the direction of phenotypic change is the same for the three populations; 3) if natural selection has contributed to trait changes by  $Q_{ST}$ - $F_{ST}$  comparisons and selection gradients, 4) and whether the phenotypic changes between temporal samples translate into differences in population performance at the common garden experiment by taking seed set and number of capitula as a proxy of fitness.

## Material and methods

### *Studied species, sampling area, and climatic data*

The cornflower, *Cyanus segetum* (Asteraceae) is a self-incompatible annual plant that used to be common in cereal fields (Le Corre et al. 2014). Nonetheless, *C. segetum*, along with other rare weeds particularly in western Europe, have experienced severe declines since the second half of the twentieth century (Le Corre et al. 2014; Albrecht et al. 2016; Wilson 2017). *C. segetum* is insect-pollinated and produces blue capitula with peripheral sterile florets and central mono-ovulate flowers (Thomann et al. 2015).

Three populations were sampled to represent a latitudinal gradient from the northern, central-western

and southern regions of France. The northern population is located near Breilly in Picardie (North of France; 49°55'21.24''N, 2°09'25.40''E) (Figure 1A) and is surrounded by very few other cornflower populations (Thomann et al. 2015). Seeds were collected in 1992, 2010 and 2015, thus 18 and 5 years apart by the National Botanical Conservatory of Bailleul (CNBL) from the same agricultural field. Seeds and seedlings from the Western population were collected in an agricultural field situated in Le Mesnil-en-Vallée in Maine-et-Loire (Central West of France; 47°18'27.7463''N, 1°3'28.7636''W) (Figure 1A) at an interval of 24 years (1991 and 2015, CBN of Brest). This area, known as the Loire valley, has experienced a dramatic agricultural intensification in the last 50 years and is the leading horticultural producer in France (Chambres d'agriculture des Pays de la Loire 2013). Seeds from the southern population were collected 16 years apart (1999 and 2015) from the regional park "Parc Naturel Régional de Luberon" close to the Commune Beaumont-de-Pertuis in the Vaucluse department (Southeast of France; 43°46'10.056''N, 5°41'28.68''E) (Figure 1A). The cornflower seeds from this population were recollected in an agricultural field close to a vineyard (CBN Méditerranéen). To explore how climatic variables have changed over time and across sampled populations in France, we gathered data on monthly historical temperature (Tmin, Tmax; °C) and precipitation (cm) from CRU-TS 4.03 (Harris et al. 2014) downscaled with WorldClim 2.1 (Fick and Hijmans 2017) for the locations where we collected seeds. We summarized historical data in equivalent periods according to the time span between the ancestral and recent samples to avoid biases from comparing unequal numbers of years. For instance, the timespan between the Western ancestral (1991) and recent (2015) samples is 24 years, therefore, we defined the relevant period leading to the ancestral population as going back 24 years in time. Hence, for the western samples we compared climatic data within two periods: 1966-1990 (24 years) and 1991-2015 (24 years). Similarly, given that the span between southern ancestral and recent samples is 16 years, we defined the periods 1982-1998 (16 years) and 1999-2015 (16 years). The 1992 and 2010 sample from the northern population are separated by 18 years so we compared the periods from 1973-1991 (18 years) and 1992-2009 (18 years). Finally, we calculated mean climatic variables for the period comprised between northern 2010 and 2015 samples, although we did not perform comparisons in this case because of the difference in variances due to unequal sample sizes. Following Dickman et al. (2019), we also calculated deviations from the expected precipitation between 1975 and 2015 by subtracting the 30-year mean precipitation from the average annual precipitation to identify departures from climate normal, such as droughts (see supplementary material for more information on climatic variables).

*F0 Generation* - Seeds were collected from *ex situ* seedbank conservatories following general recommendations summarized in the ESCONET program (ENSCONET 2009). Briefly, seeds were collected randomly from at least 50 mother plants within each population to maximize genetic diversity. All seeds were stored *ex situ* in optimal conditions (low humidity and 5°C), and they were

used to generate the F0 generation for the common-garden experiment. On January 2016, we resurrected 300 randomly sampled seeds from each temporal sample from the northern and western 1991 populations, and 340 seeds from the southern temporal samples (Table S1). For each temporal sample, we transplanted 50 randomly chosen plants to create the F1 generation. For the western 2015 population, we collected 50 randomly sampled seedlings from the exact same locations as the stored seeds from 1991, to harvest seeds for the F1 generation. Hereafter, we refer to the groups of plants from seeds sampled from the northern, central-western, and southern localities, as northern, western, and southern populations (Figure 1A); and we referred to plants sampled in different dates as temporal samples or ‘ancestral’ and ‘recent’ samples.

### *Common-garden experiment*

The experiment was conducted in a common-garden in Montferrier-sur-Lez (South of France; 43°40'52.5468"N, 3°52'31.9188"E) in 2017. To control for maternal and storage effects and to create a family design for analysing plant traits on the F1 generation, we regenerated seeds through random crosses within samples. The 50 plants in the F0 generation were randomly crossed (within samples) using commercial bumblebees (*Bombus terrestris*, Koppert Biological System) in insect-proof cages during a 15-day period (Thomann et al. 2015). We set half-sib families using only one offspring per capitula and assuming that the proportion of full-sibs from different capitula was negligible. We studied 30 families each with 5 half-sibs resulting in circa 150 plants per each sampled year (nested within population). Seeds were sowed on January 25<sup>th</sup>, 2017 in individual pots in a greenhouse. Seedlings were transferred and planted on March 10<sup>th</sup>, 2017 for the northern and southern populations and on March 13<sup>th</sup> for the western population. In order to control for the environmental heterogeneity inherent to a field experiment, the seedlings from different sampling dates were randomized within populations in blocks consisting of 50 individuals (Figure S1-2). To facilitate experimental measures and because we were mostly interested in temporal differentiation, plants were only randomized within the population of origin (i.e., North, West and South).

### *Plant traits*

We recorded growth-related, reproductive, and phenological traits for each plant in the F1 generation to study differences between ancestral and recent samples in three populations. For each plant, we measured rosette size on the day of transplantation (to the nearest 0.5 cm), date of the first flowering, capitula diameter (capitula size), and number of capitula. At the end of the experiment, plants were harvested, and dry biomass was estimated as the total number of seed heads. We estimated number of seeds and number of unpollinated florets to estimate seed set for the first three capitula. Individual fitness was estimated by multiplying seed set by number of seed heads. Finally, to estimate pre-

dispersal impact caused by predators. we inspected the harvested capitulas and recorded the presence or absence of arthropods related to seed damage. We summarize this impact by estimating a “seed predators’ ratio” as the number of capitula infested.

### *Statistical analyses*

We evaluated changes in climatic variables between temporal periods by performing paired t-tests. To characterize differences on plant traits between temporal samples, we analysed data separately for each population with linear (LMM) and generalized (GLMM) mixed effect models using the functions “lmer” and “glmer”, implemented in the R package “lme4” (Bates et al. 2014). The models included the family effect as a random factor nested in the fixed effect of sampling date. We performed LMM tests for rosette size, plant biomass, mean capitula size, and days to flowering. We implemented GLMM analyses for number of capitula using a Poisson distribution and a binomial distribution for seed set and presence of seed predators. To evaluate significance of the fixed effects, we used the Satterthwaite's method for linear models as implemented in the *anova.lmerModLmerTest* function in the R package “lmerTest” (Kuznetsova et al. 2017); and likelihood ratio tests for generalized models. We corrected for multiple comparisons using the Benjamini and Hochberg method (Benjamini and Hochberg 1995).

Phenotypic selection was estimated using linear ( $\beta$ ) and quadratic selection ( $\gamma$ ) gradients with classical multiple regression models of standardized traits on relative fitness (Lande and Arnold 1983). The highest-ranking models were used to estimate the selection gradients. We assessed the significance of the inferred  $\beta$  and  $\gamma$  terms by performing t-test with a null hypothesis of  $\beta$  or  $\gamma = 0$ . All phenotypic values were standardized prior to analyses. We tested phenotypic selection only for three traits: rosette size as a proxy of vegetative growth, days to flowering representing phenology, and mean capitula size for traits associated to pollinator attraction (Renoult et al. 2013).

All the models were validated by visual inspection of residual structure and homoscedasticity. The statistical analyses were performed in R 3.6.0 (R Core Team 2019).

### *Neutral and quantitative differentiation analysis*

Leaf material was collected from the mother plants (F0) of the common-garden experiment. DNA was extracted using the CTAB method (Doyle and Doyle 1990) and genotyped at 10 microsatellite markers designed for the cornflower (Le Corre et al. 2014). Neutral genetic differentiation (Nei's  $F_{ST}$ )



between spatial and temporal samples was estimated using the R package “hierfstat” (Goudet 2015). The 95% confidence interval (CI) of the  $F_{ST}$  was estimated by bootstrapping 1,000 times across the loci. We inferred a neighbour-joining tree comprising all temporal samples and calculated support for the nodes using R packages “ape” (Paradis et al. 2004) and “hierfstat” (Jerome Goudet 2015). Note that support for the nodes might be low because of factors such as incomplete lineage sorting and gene flow. We calculated measures of genetic diversity such as the observed and expected heterozygosity (Nei’s genetic diversity) per temporal sample using the R package “adegenet” and performed permutation tests to estimate confidence intervals (Jombart 2008).

To test whether quantitative trait differentiation was significantly different from neutral genetic differentiation as a signature of natural selection, we compared  $Q_{ST}$  and  $F_{ST}$  values between temporal samples for each population. The  $Q_{ST}$  values for each trait between the temporal samples for each population were defined following Thomann et al. (2015) as:

$$Q_{ST} = \frac{\sigma_{sample}^2}{\sigma_{sample}^2 + 2\sigma_A^2}, \text{ with } \sigma_A^2 = \frac{\sigma_{fam}^2}{2\theta}$$

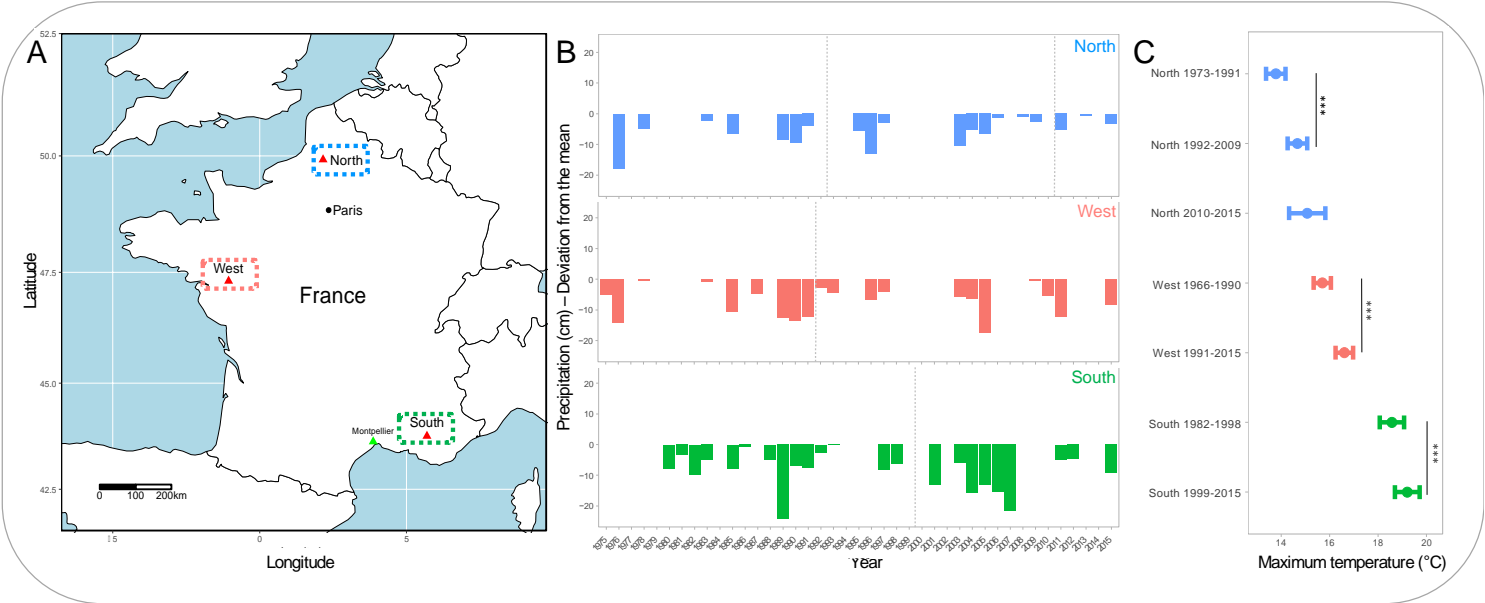
where  $\sigma_{sample}^2$  and  $2\sigma_{fam}^2$  are the between-sample and the among-family-nested within-sample variance of the quantitative traits, respectively,  $\sigma_A^2$  is the pooled additive genetic variance of the two samples, and  $\theta$  is the coefficient of coancestry. Given that families were composed of half-sibs we set  $\theta$  to 1/8. We used Linear Mixed Models (LMM) to partition the variance components for the  $Q_{ST}$  analyses with both sampling year and family as random effects. The 95% confidence interval of the  $Q_{ST}$  values were estimated by bootstrapping 1,000 times over families within each sample. The null hypothesis ( $Q_{ST} = F_{ST}$ ) was tested by comparing their 95% CI’s.  $Q_{ST}$  was considered significantly different from  $F_{ST}$  when the two 95% CI’s did not overlap or overlapped marginally with point  $Q_{ST}$  estimates outside  $F_{ST}$  bootstrapping range.

## Results

### *Climatic variation and temporal changes in traits, seed set, and seed predators*

Precipitation was lower than average for the year of collection of all recent samples (2015) across all populations (Figure 1B). The climate leading up to the year of collection for the western 1991 ancestral sample, was severely drier than average (Figure 1B). The southern population experienced the greatest variation in precipitation and a long drought from 2002 to 2007 (Figure 1B). Consistent with climate change, temperature increased between periods at the localities of the three populations (Figure 1C, Table S6).

246 Germination rates for the different accessions of the F0 and F1 plants where >70% and >91%,  
 247 respectively. Germination date in the F0 generation did not differ significantly between dates for the  
 248 northern and southern samples (Table S2, Figure S3). However, plants from the recent western sample  
 249 germinated on average one and half day later compared to the 1991 sample (Table S3, Figure S3).  
 250 Ancestral and recent samples showed significant and divergent differences across populations for  
 251 rosette size, flowering date, capitula size, and seed set (Table S2, Figure 2). Rosette size was different  
 252 between dates for the northern and southern populations, and the trend of the evolutionary change was  
 253 directional, although following opposite directions: while plants in recent northern population are  
 254 smaller, rosette size in the southern population increased over time (Table S2, Figure 2A, C). In  
 255 contrast, days to flowering was different between temporal samples only for the western populations,  
 256 whereby recent individuals flowered on average 6 days earlier (Figure 2E).

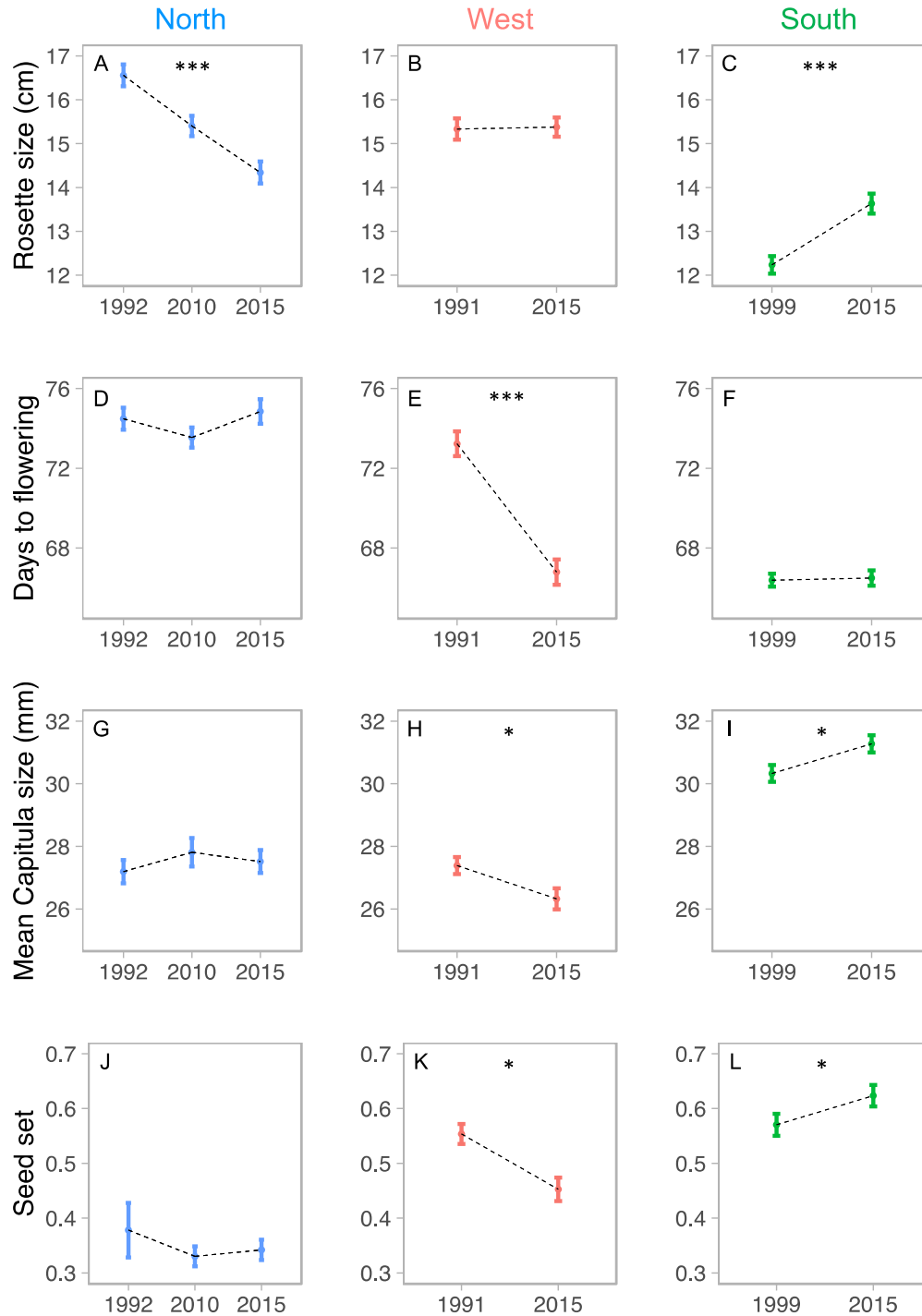


257  
 258 **Figure 1.** Sample locations and climatic variables; precipitation showed no trend with time across populations' localities,  
 259 while temperature has increased over time in all localities. **A.** Map of seed collection sites from northern, western, and  
 260 southern populations of *Cyanus segetum* in France. **B.** Precipitation (cm) at the northern, western, and southern locations  
 261 over the 30 years preceding the year of collection of the most recent temporal sample. Vertical bars correspond to  
 262 precipitation deviation (cm) from the 30-year average. **C.** Maximum temperatures (°C) at the northern, western, and southern  
 263 locations summarized over periods of time according to the time span between the ancestral and recent temporal samples.  
 264 Significance codes: p-value < 0.0001 '\*\*\*', < 0.01 '\*\*', 0.01-0.05 '\*'. Horizontal lines correspond to the standard error.

265 We found divergent evolution of capitula size in western and southern populations: the recent sample  
 266 from the South exhibited larger capitula size and outperformed the ancestral sample having a higher  
 267 seed set and lower infestation by seed predators (Table S2, Figure 2I). Conversely, the recent sample  
 268 from the West had smaller capitulas and lower fitness in terms of seed set and biomass compared to

the ancestral, yet it showed a lower proportion of attacks by seed predators (Table S2, Figure 2). Similarly to groups of seed predators found in other cornflower populations (Koprdoval et al. 2015), the most abundant seed predators were larvae of Cecidomyiidae (gall midges). We also found species of Tephritidae (fruit flies), Tortricidae (leafroller moths), Curculionidae (weevils), and parasitoid wasps.

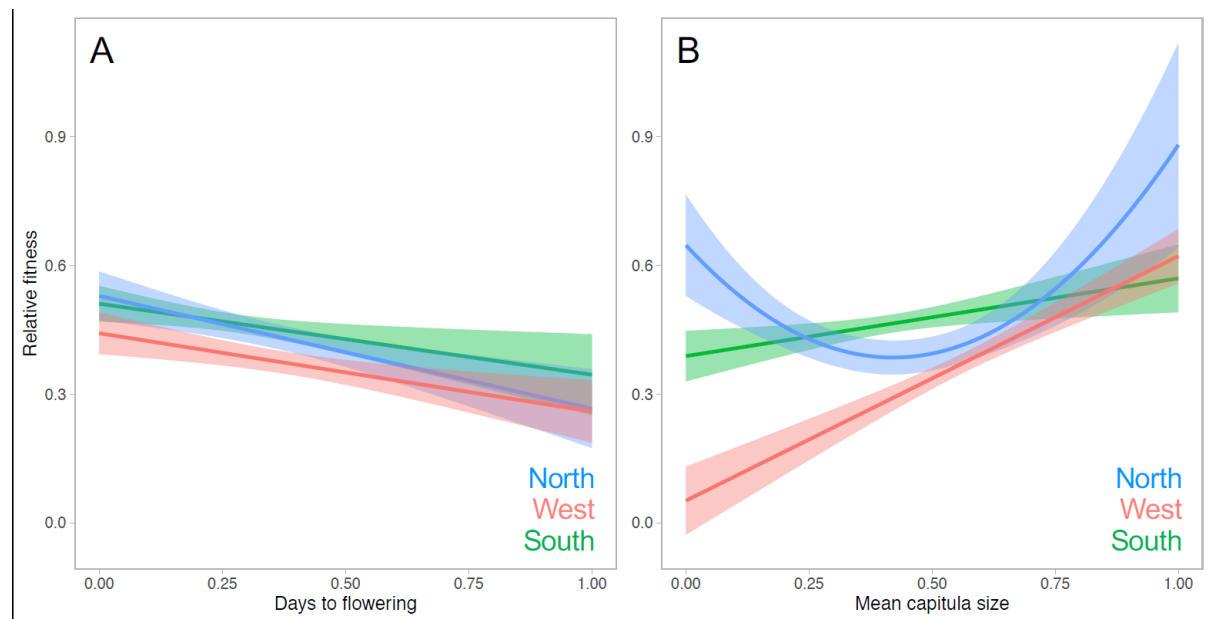
The spatial block had a pervasive effect on most measured traits (Table S2, Figure S2, S4). In particular, the block, as well as the interaction between sampling date and block, had a strong effect on condition-related estimates such as number of capitula, biomass, seed set, and seed predators ratio across all populations (Table S2, Figure S2). On average, blocks closer to the edges in the northern and southern populations presented higher seed set (Figure S2), while biomass was lower in the edges of the experimental field (Figure S2-3). Since we measured rosette size before transplantation, we were able to compare values across the three different latitudinal samples. We found that the population of origin had a significant impact on rosette size; the southern population showed smaller rosette size ( $12.89 \pm 0.15$  cm) than the northern ( $15.43 \pm 0.14$  cm) and western ( $15.35 \pm 0.16$  cm) populations ( $F = 75.4$ ,  $DF = 2$ ,  $p\text{-value} < 0.0001$ ), which did not diverge among them. Although days to flowering was affected by our block design primarily within the western population, we were able to contrast this trait throughout latitudinal populations (Table S2, Figure S2). We showed that date of flowering is different for all the populations with southern populations blooming first, followed by the western, and finally by the northern samples (Figure S2).



**Figure 2.** Phenotypic changes between ancestral and recent samples exhibited divergent trends for rosette size, days to flowering, and mean capitula size across three populations along a latitudinal gradient in France. Seeds were resurrected from a northern (North), central-western (West), and southern (South) population and traits were measured in a common garden experiment. **A-C.** Rosette size (cm). **D-F.** Mean Capitula size (mm). **G-I.** Days to flowering from germination. **J-L.** Mean seed set (a proxy for fitness). Significance codes: p-value < 0.0001 '\*\*\*', < 0.01 '\*\*', 0.01-0.05 '\*'. Vertical lines correspond to the standard error.

We found moderate correlations between most of the traits for the temporal samples within populations, including plant size, mean capitula size, biomass, and number of capitula (Table S4). The strongest phenotypic correlation was between biomass and number of capitula throughout all populations, with values ranging from 0.821 to 0.902.

Finally, all significant effects remained significant after Benjamini and Hochberg correction for multiple testing except mean capitula size in the southern population, which was marginally significant (p-value = 0.052, Table S2) suggesting moderate evidence for this shift.



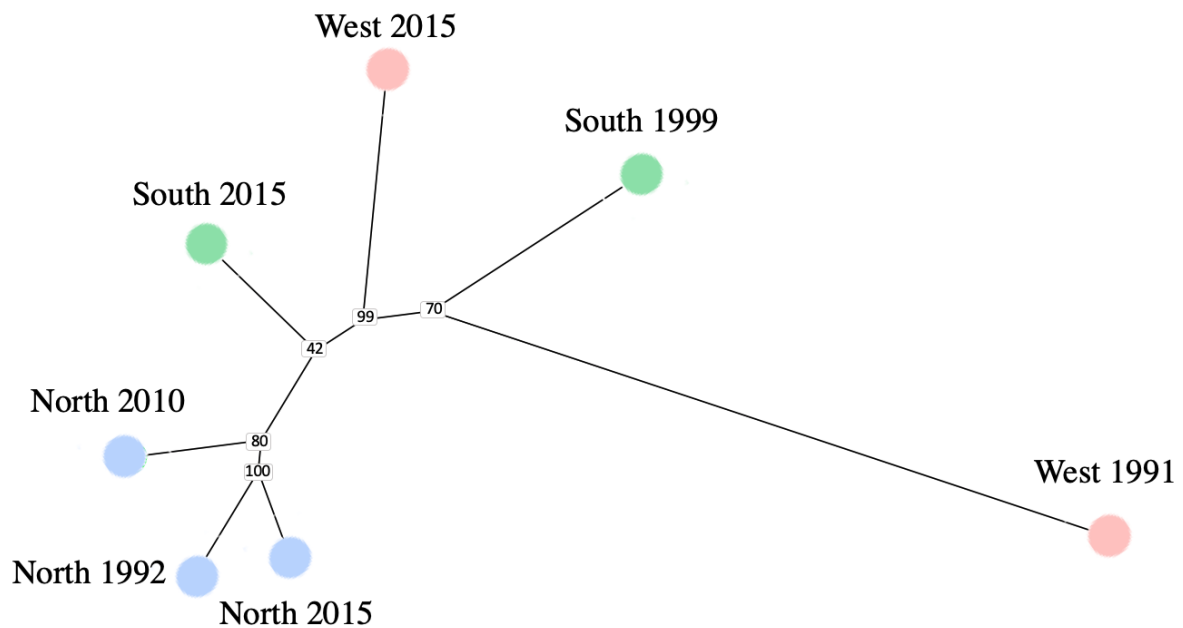
**Figure 3.** Earlier flowering and larger capitula size are correlated with higher fitness. Graphs depict the relationship between average flowering date and mean capitula size versus fitness measured in the common garden experiment in southern France. Selection gradients were inferred estimating fitness as mean seed set multiplied by number of capitulas and by fitting regression models using maximum likelihood methods. Shaded areas indicate the standard deviation.

#### *Strength of selection on phenotypic traits*

Phenotypic selection analysis at the experiment location with a natural community of pollinators indicated that reproductive traits were significantly correlated with fitness (Table 1). We found negative linear selection gradients for days to flowering across all populations indicating that earlier flowering led to higher fitness (Figure 3A). Similarly, plants with larger capitula sizes showed higher fitness, though selection is disruptive on the northern population ( $\gamma = 0.518$ ,  $p < 0.0001$ , Figure 3B). Within populations, we found directional selection for larger capitulas in the ancestral samples from

the southern 1999 sample, while for the recent 2015 sample, selection on capitula size was not significant (Table 1). In contrast, in the western population, there was strong positive selection for bigger flowers on both temporal samples with the strength of selection being higher on the recent 2015 population, which shows a lower mean capitula size compared to the ancestral 1991 (Table 1). Notably, selection for larger capitulas was stronger for the sampling dates with smaller capitulas in both western and southern populations.

In all our regression models, the inclusion of multiple predictors had only a slight influence on the  $\beta$  and  $\gamma$  terms, with no change in the estimated direction of selection, suggesting that selection is mostly acting on individual traits.

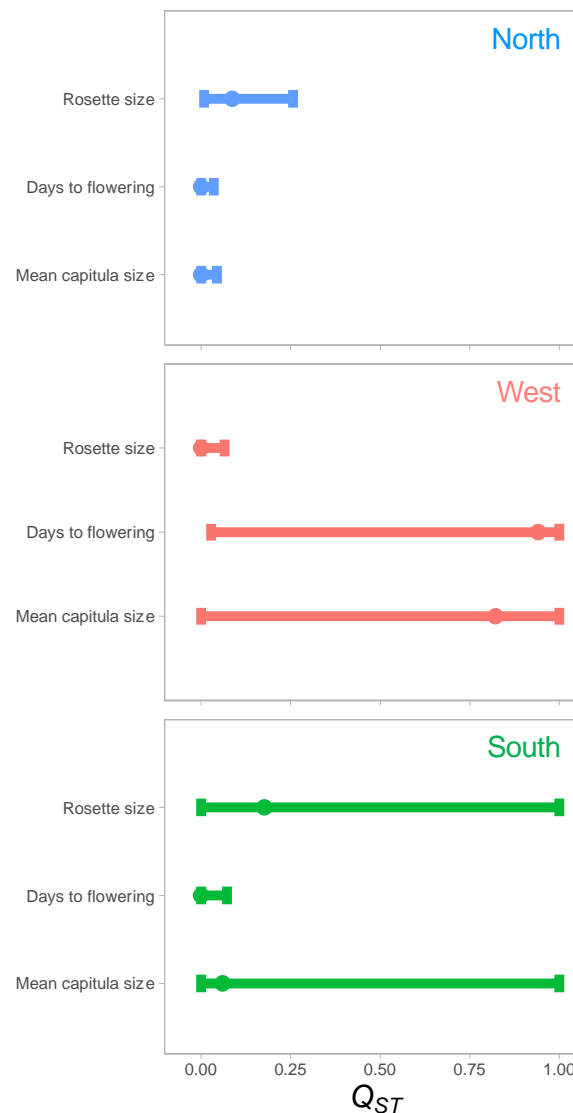


**Figure 4.** Neighbour-joining tree based on raw pairwise  $F_{ST}$  estimates from microsatellite markers among temporal samples from the different populations. Numbers at nodes denote bootstrap support. Blue, northern population; red, western population; and green southern population.

#### *Neutral genetic differentiation between temporal samples and among populations*

All populations and temporal samples exhibit high levels of genetic diversity, which did not significantly differ across temporal samples and populations (Table S1). Global  $F_{ST}$  values suggested moderate differentiation between populations (Figure 4, Table S5). Comparisons between the Northern temporal samples showed lower  $F_{ST}$  values ( $F_{ST}$  ranged from 0.015 to 0.036) compared to any other

western or southern samples ( $F_{ST}$  ranged from 0.042 to 0.1391), supporting genetic continuity of the northern samples across the three sampling dates. In contrast, ancestral and recent samples from the West and the South clustered together and showed a higher genetic affinity by temporal sample than by sampling origin (Figure 4, Table S5). Unexpectedly, the western 2015 sample showed the lowest differentiation with the southern 2015 ( $F_{ST}= 0.067$  [0.004, 0.094] 95% CI). By contrast, although the western 1991 sample showed the lowest  $F_{ST}$  with the northern 1992 ( $F_{ST}= 0.1232$  [0.083, 0.167] 95% CI), it seemed highly differentiated from all the other temporal samples. Thus, suggesting high levels of ancestral as well as ongoing asymmetrical gene-flow across populations (Figure 4, Table S5).



**Figure 5.** Natural selection played a role shaping some of the observed distributions of traits of ancestral and recent samples. Graphs depict comparisons between  $Q_{ST}$  and  $F_{ST}$  for growth-related and reproductive traits between temporal samples. Dots represent the estimated  $Q_{ST}$  between the ancestral and recent samples from northern, western, and southern populations with their 95% confidence intervals (CI). The grey shade represents the  $F_{ST}$  95% CI estimates.

Comparisons of  $F_{ST}$  and  $Q_{ST}$  estimates for the different traits indicated a significant role of selection for some traits between the ancestral and recent samples (Figure 5).  $Q_{ST}$  point estimates were higher than the  $F_{ST}$  95% CI for rosette size in the North and the South, and days to flowering and mean capitula size in the West, thus suggesting moderate evidence for divergent selection on these traits (Figure 5). Contrariwise, the  $Q_{ST}$  for rosette size in the West was significantly lower than the  $F_{ST}$ , pointing towards stabilizing selection maintaining similar values of this trait (Figure 5). Of note,  $Q_{ST}$  point estimates for days to flowering ( $Q_{ST} = 0.969$ ) and capitula size ( $Q_{ST} = 0.902$ ) for the western population were remarkably high ( $Q_{ST}$  values  $> 0.7$  are considered high, see (O'Hara 2005)) although the CI basically spanned the entire range of possible values. In the South, the empirical  $Q_{ST}$  point estimate for mean capitula size was slightly higher than the point  $F_{ST}$  estimate, yet this trait showed a wide confidence interval, similarly to other traits with higher  $Q_{ST}$  point estimates. All remaining traits showed lower  $Q_{ST}$  point values than the  $F_{ST}$  estimates (Figure 4).

**Table 2.** The linear ( $\beta$ ) selection gradients and quadratic ( $\gamma$ ) selection gradients for plant traits per temporal sample and population. (ns: not significant  $p > 0.010$ ,  $\dagger p < 0.10$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ )

Traits	North			West		South	
	1992	2010	2015	1991	2015	1999	2015
Rosette size	0.270ns	0.138ns	-0.097ns	-0.204**	0.00001ns	0.093ns	0.087ns
Days to flowering	-0.430†	-0.090ns	-0.395**	-0.286**	-0.164*	-0.069ns	-0.148ns
Mean capitula size	0.038ns	-0.002ns	-0.132ns	0.273**	0.532***	0.211*	0.008ns
$\gamma = 0.518***$							

## Discussion

We resurrected seeds from ancestral and recent samples of *Cyanus segetum* across populations at different latitudes to document plant trait evolution. The stored and recent seeds were separated by a span of about two decades, characterized by a relentless escalation of various global change components. By growing ancestral and descendant seeds in a common environment, we showed divergent evolution in plant size and capitula size for different locally adapted populations and a shift in flowering date between ancestral and recent samples from western France. Importantly, the similarity between neutral genetic diversity estimates across temporal samples suggests that genetic drift is not the sole driver of the shifts observed. We documented selection gradients for earlier flowering and bigger capitula size, which along with  $Q_{ST}$  estimates for rosette size and days to



flowering, indicate that natural selection may have played a role in triggering some of these phenotypic changes over time. Thus, our results outline the rapid pace of evolution shaping the trajectories of natural populations across local spatial gradients and the potential for adaptation of populations facing global change through contemporary evolution.

*Is the evolution of plant traits across populations consistent with global change?*

Rosette size evolved in different directions in the northern and southern populations: while it steadily decreased over time in the North, it increased in the South. We found moderate evidence that these divergent shifts were driven by natural selection with point  $Q_{ST}$  estimates higher than the  $F_{ST}$  confidence intervals. These idiosyncratic changes in face of warming are likely related to the inconsistent impact of global change across latitudes. Increases in leaf size in southern warmer habitats have frequently been demonstrated (Royer et al. 2005; Niinemets et al. 2007; Tng et al. 2013). These studies agree with predictions from simulation experiments suggesting that variations in leaf size along a climatic gradient result from greater evaporative demand of larger leaves due to enhanced thickness of the boundary layer for energy and gaseous exchange (Niinemets et al. 2007). Yet, reduced plant size and leaf size have also been observed in response to pronounced droughts (Eziz et al. 2017). An increase in plant size in the South is nonetheless consistent with a mechanism to cope with the escalation of high temperatures in the Mediterranean (Harpaz et al. 2014).

Additionally, severe droughts in southern France in the first decade of the twenty-first century may have hindered increase in plant size (Figure 1B), suggesting that the observed increase is likely the result of recent selection over the last ten years when precipitation has been, overall, higher than average (Figure 1B). Larger plant size might constitute a fast-growing strategy to reach minimum sizes for early flowering, as apparently indicated by the negative correlation between onset of flowering and plant size we found only for the southern population. Similarly to this result, Sun and Frelich (Sun and Frelich 2011) showed a negative correlation between growth rate and flowering onset across 25 herbaceous plants, hinting at a fast-growth, flower-early strategy; which might also be the case of the cornflower in the southern population. The steady decrease in rosette size in the northern population can be interpreted as the result of warming with non-limiting precipitation. In such context, selection for a fast-growing strategy is relaxed, resulting in reduced size of seedlings. This is consistent with the positive and non-significant selection gradient for rosette size in the northern population.

We observed a latitudinal gradient for flowering onset with northern populations flowering later than southern ones, closely resembling the temporal patterns of the plant communities in these latitudes (Rhoné et al. 2010). In annual plant species as the cornflower, flowering time is a major adaptive trait

because it synchronizes the initiation of reproduction with favourable environmental conditions and is expected to respond rapidly to latitudinal changes (Rhoné et al. 2010). In northern populations, where winters are colder and frost may still occur during spring, plants flowering too early are exposed to frost damage, whereas in southern populations, plants flowering exceedingly late could suffer from heat and drought stress in early summer (Rhoné et al. 2010). Besides the expected latitudinal gradient in floral phenology, we found heterogeneous flowering date shifts among temporal samples. Flowering has become earlier only for the western population whereby the recent sample flowered, on average, six days before the ancestral. Remarkably,  $Q_{ST}$  estimates moderately supported the role of divergent selection triggering this shift in the western population.

Regardless of the population and dates, selection gradients showed that earlier flowering resulted in higher fitness at the warmer southern location where the experiment was carried out. The earlier blooming of spring-flowering species is a well-characterized response to climate warming because flowering is especially sensitive to temperature changes (Abu-Asab et al. 2001; Fitter and Fitter 2002). Explicitly, advances in first flowering is directly correlated with an increase in minimum temperatures (Abu-Asab et al. 2001; Fitter and Fitter 2002; Nevo et al. 2012; Dijk and Hautekèete 2014) and minimum temperatures have significantly increased for all locations (Table S6). Nevertheless, the widespread trend of selection for earlier flowering, although consistent with global change and life history theory, can be misleading if the trait is condition-dependent, flowering duration is unaccounted for, or not all fitness elements are considered (Austen et al. 2017). Flowering date in self-incompatible species like the cornflower can also be constrained by pollinator emergence, which is expected to be a primary factor shaping the evolution of early flowering. In addition, given the low level of genetic differentiation between the western 2015 and Southern 2015 samples, and their similitude in mean flowering date, we cannot rule out a contribution of migration from populations locally adapted to southern latitudinal selection regimes into the advance in flowering date observed for the western population. Likewise, a shift towards earlier floral phenology could have contributed to greater synchronization between western 2015 and southern 2015 populations, thus facilitating gene-flow. This hypothesis is also supported by studies showing that human-transportation of seeds is a major factor reshuffling genetic variation in natural populations of *C. segetum* in France (Le Corre et al. 2014).

Capitula size exhibited shifts over time and was the main trait associated with fitness across temporal samples and populations. Selection gradients between temporal samples measured in southern France indicated that selection was stronger for ancestral samples, as well as for samples with smaller capitulas. Capitula size (flower size) plays a key role in pollinator attraction and seed set (Parachnowitsch and Kessler 2010; Gervasi and Schiestl 2017). Because seed set has been shown to positively correlate to capitula size in the cornflower (Thomann, 2014), we assume that the strong

positive relationship between larger capitulas and fitness observed in our experiment was driven by pollinators. Indeed, pollinators can drive adaptive evolution for traits that maximize their attraction and subsequent pollen delivery (Gervasi and Schiestl 2017). Particularly, pollinators select for larger flowers because they are more conspicuous and may be associated with greater rewards (Blarer et al. 2018). For instance, Parachnowitsch and Kessler (2010) manipulated the effect of pollinator on fitness through hand pollination and compared phenotypic selection in open- and hand-pollinated plants. They found that natural selection was significantly stronger in the open-pollinated plants and that hand pollination removed directional selection for larger flowers (Parachnowitsch and Kessler 2010). It is thus likely that decreases in pollinators abundance, namely pollinator decline, have driven the evolution of capitula size in the cornflower; although this remains to be tested empirically via experimental manipulation of pollinator availability. Martin et al (2019) have reported declines in pollination services in France based on reductions in pollinator-dependant crop yield. Surprisingly, in the face of pollinator decline, temporal evolution of this trait was not convergent across populations, although bigger capitulas were correlated with higher fitness. In fact, we found moderate evidence that mean capitula size has decreased in the western population while increased over time in the southern population. Conversely, in the northern population, while Thomann et al. (2015) reported a significant increase in capitula size between 1992 and 2010 (using the same seed lots but conducting an *in situ* greenhouse experiment), we report the same trend here although no statistically significant. The failure to recover the changes reported by Thomann et al. (2015) is likely associated with the higher variance in the phenotypic traits introduced by our field experiment due to an *a priori* bias in the plastic responses of the non-locally adapted populations from northern and western France (Whitlock 2008a; Rhoné et al. 2010).

In addition to the variance introduced by the field experiment, there were phenotypic differences between the spatial blocks, which suggest that pollination, competition, or resources may have differed at a fine scale in the field (Table S3, Figure S2, S4). Higher seed set closer to the edges might reflect increased accessibility to foraging pollinators. Contrariwise, since competition is expected to have played a minor role given that plants were separated by a meter, decreases in plant size towards the edges may be the result of higher wind exposure. Besides germination and rosette size, which were measured before transplantation, we were not able to make comparisons of trait values across different populations to explore for signatures of local adaptation since we only randomized within populations (Figure S1). Several studies have shown that heterogeneities among blocks mostly alter plastic responses but not the direction of the evolutionary change, which can still be detected. For instance, through a resurrection experiment, Frachon et al. (2017) grew *Arabidopsis thaliana* seeds collected across an 8-year period in six micro-habitats with different soil and competition conditions

representative of the local populations (Frachon et al. 2017). They found that evolution of the phenotypic traits towards either higher or lower values was consistent across micro-habitats, though the underlying genetic basis and the strength of the significance varied (Frachon et al. 2017). We therefore regard as unlikely that the microhabitat conditions of the terrain have affected the direction of the evolutionary change that we report but presumably only its significance. Within our findings of increased capitula size in the South, and the previous experiment using seeds from the northern locality (Thomann et al. 2015), is nested the possibility of a general trend towards larger floral display over time. A reciprocal transplant design, instead of a common garden experiment restricted to one locality, would have allowed us to test population-specific adaptation across the sample locations to assess whether fitness of the recent samples was indeed higher in their habitats. Additional refinement of the experiment to include a pollinator community under controlled conditions across all the studied localities might improve our understanding of the influence of local factors into the characterization of convergent/divergent evolution of the phenotypic traits and the adaptive value of these changes.

Self-incompatible plant species facing pollinator decline, are expected to increase flower size or other traits related to floral display to enhance pollinator visitation (Thomann et al. 2013). Gervasi et al. (2017) conducted real-time experiments showing that populations pollinated by bumblebees evolved larger plants and flowers in only six generations. Since the cornflower is a self-incompatible that entirely relies upon pollinators for fertilization, evolution towards showier capitulas might be associated with the pollinator-poor environments caused by the widespread increase in habitat destruction, pesticide use, light pollution, and other components of global change fostering declines of pollinator communities (Brys and Jacquemyn 2012; Thomann et al. 2015). Given that short-term evolution towards self-compatibility is rare among plants, the evolution of reinforced interactions with pollinators can be considered as one of the main adaptive mechanisms for outcrossing species like *C. segetum* under pollinator decline (Thomann et al. 2013). However, Ramos and Schiestl (2019) have revealed that higher degrees of self-compatibility in *Brassica rapa* can evolve by experimentally manipulating pollinator abundance, which highlights differences in evolutionary trajectories depending on species and the genetic architecture of the self-incompatible system. Moreover, floral signals used for pollinator attraction can also be perceived and used by other organism making reproductive traits particularly vulnerable to conflicting selection (Strauss and Irwin 2004; Raguso 2018). For example, antagonists such as herbivores and pre-dispersal seed predators can also exert strong selection on floral traits (Strauss and Irwin 2004). Notably, in the northern population, bigger capitulas are seemingly related to higher fitness after intermediate capitula sizes, and there seems to be a trade-off at further increases in capitula size, which might be explained in part by a higher extent of seed predators. Moreover, conflicting selection may contribute to explain the fact that the recent western population evolved towards smaller capitula size but had a lower rate of infestation compared to the ancestral one. Accordingly, we do not rule out that seed predators may have played a part

triggering this seemingly maladaptive shift towards smaller capitulas resulting in lower rates of seed predator infestation in spite of compromising pollinator visitation.

A common finding is that measures of genetic variance are increased in novel environments causing  $Q_{ST}$  estimates to be too low (O'Hara 2005a; Whitlock 2008b). Consequently, identifying traits for which  $Q_{ST}$  is significantly different from  $F_{ST}$  has proven to be extremely difficult for studies that consider less than ten populations (O'Hara 2005a; Whitlock 2008b; Leinonen et al. 2013). The impact of the number of sampled populations in the estimation of  $Q_{ST}$  seems to be particularly problematic for its use to test for natural selection in *in situ* resurrection experiments, whereby usually two temporal samples of a population are compared (Franks et al. 2008b). Furthermore, the bias in the estimation of  $Q_{ST}$  confidence intervals is appreciable when  $Q_{ST}$  true values are high ( $Q_{ST} > \sim 0.7$ ) (O'Hara 2005b). Therefore, we do not rule out that selection has played a role in the changes on rosette size, date of flowering, and capitula size across populations where the  $Q_{ST}$  true estimate was higher than the  $F_{ST}$  CI, especially for days to flowering and capitula size in the western population whereby  $Q_{ST} > 0.9$ .

#### *Can evolution contribute to population viability of species facing global change?*

On-going resurrection projects represent an exciting opportunity to test whether contemporary evolution is mitigating the impact of global change on plant demography, possibly rescuing populations from extinction (Franks et al. 2008a). For outcrossing species, shifts in flowering time and evolution towards reinforced interactions with pollinators have been predicted as the primary evolutionary changes rescuing populations facing global pollinator decline (Thomann et al. 2013). We uncovered evolutionary shifts in pollinator attractive traits such as capitula size, which was linked to higher fitness for some populations. However, we did not observe a clear signature of fitness maximization over time despite evolution across all studied populations. Migration load due to high gene flow between western and southern populations might help explain the maladaptation of the western 2015 sample. This recent western sample not only showed later germination and earlier flowering but also a smaller capitula size, lower biomass, and seed set compared to the ancestral; thus, seemingly evolving away from a fitness peak. Theoretical models and empirical evidence show that gene-flow imposes a limit on local adaptation, and may foster evolutionary change in a direction opposite to natural selection, resulting in a population sub-optimally adapted (Lenormand 2002). However, the experiment was conducted in the South of France, which undermines the unique role of gene-flow shaping this pattern.

We further hypothesize that frequency-dependent selection may have played a role hampering fitness

optimization. In particular, traits for which we report evolutionary change are presumably under frequency-dependent selection, whereby there is no optimization principle to predict the outcome of adaptive evolution (Ferriere and Legendre 2012). For instance, early flowering individuals will be rare since most conspecific plants have not yet flowered, and given that many pollinators prefer to forage in common phenotypes, these earliest bloomers can be easily skipped (Forrest and Thomson 2009). Contrastingly, the advantage of phenotypes with increased plant or flower size is higher when rare or in low-density populations (Jacquemyn et al. 2012). Nonetheless, it is hard to disentangle the effects of frequency-dependent selection from migration load and drift, and temporal data along with theoretical models will provide insights into the evolutionary dynamics of these reproductive traits in *C. segetum*.

In summary, we have shown rapid evolution of growth-related and reproductive traits. Notably, patterns of evolution are not convergent across traits and latitudes. Although compelling evidence of the adaptive nature of most of these trait shifts remains elusive, they are consistent with widespread documented responses to global change and might represent putative cases of evolutionary rescue. Nonetheless, testing for evolutionary rescue would entail linking these observed rapid changes to populations escaping the fate of extinction, which involves collecting additional demographic data through long-term monitoring of the populations in the field. Indeed, there is a wealth of possible feedback scenarios that can only be assessed by studying evolution *in situ*. Therefore, field experiments testing for contemporary evolution should be higher on the research agenda due to the importance of predicting evolutionary trajectories of natural populations in realistic environments under the pressing impacts of global change.

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