

# Rapid adaptive evolution to drought in a subset of plant traits in a large-scale climate change experiment

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## Abstract

Rapid evolution of traits and of plasticity may enable adaptation to climate change, yet solid experimental evidence under natural conditions is scarce. Here, we imposed rainfall manipulations (+30%, control, -30%) for ten years on entire natural plant communities in two Eastern Mediterranean sites. Additional sites along a natural rainfall gradient and selection analyses in a greenhouse assessed whether potential responses were adaptive. In both sites, our annual target species *Biscutella didyma* consistently evolved earlier phenology and higher reproductive allocation under drought. Multiple arguments suggest that this response was adaptive: it aligned with theory, corresponding trait shifts along the natural rainfall gradient, and selection analyses under differential watering in the greenhouse. However, another seven candidate traits did not evolve, and there was little support for evolution of plasticity. Our results provide compelling evidence for rapid adaptive evolution under climate change. Yet, several non-evolving traits may indicate potential constraints to full adaptation.

## INTRODUCTION

Rapid adaptive evolution is considered a potential pathway for species to cope with ongoing climate change. Principles of rapid evolution have been studied abundantly by artificial selection under controlled conditions with short-lived, often single-cell organisms (reviewed in Barrett & Schluter 2007; Hoffmann & Sgró 2011). However, translating these findings to real life is difficult: multiple interacting factors may substantially impede rapid adaptive evolution under natural conditions, including e.g. environmental fluctuations, various biotic interactions, low genetic variation and trait heritability, genetic drift, or trade-offs between selected traits (Hoffmann & Sgró 2011; Shaw & Etterson 2012). Thus, there is an urgent need for studying adaptive evolution within the ‘multivariate space’ of natural conditions (Hoffmann & Sgró 2011) to assess its relevance for ongoing climate change (Merilä & Henry 2014; Franks *et al.* 2014, 2018).

In plants, the limited number of tests under near-natural conditions for rapid evolution under changing climate focused on two approaches. One is the resurrection approach, where stored seeds collected years before within a plant community are revived and compared to plants from recently sampled seeds from the same location (reviewed in Franks *et al.* 2018, including eight climate-related cases). Yet, observed changes can be influenced by seed storage (Weis 2018; Franks *et al.* 2019) and attributing trait changes unambiguously to climate as the causal factor is challenging (Franks *et al.* 2018). Another approach encompasses multi-year climate manipulations imposed on natural communities in the field. When plants are subsequently screened for divergent evolution, changes can be causally attributed directly to contrasting climatic treatments - a key

asset of such experiments. Only few studies followed this highly demanding approach, reporting evolutionary change in some genetic markers (Jump *et al.* 2008, Ravenscroft *et al.* 2015) or certain phenotypic traits (Grossman & Rice 2014, Ravenscroft *et al.* 2014, Nguyen *et al.* 2016).

With either approach it has remained a great challenge though to judge whether observed changes are adaptive. This judgement is often done *a posteriori* based on ‘intuition’ or ‘common sense’ (Merilä & Hendry 2014, Franks *et al.* 2014). Yet, since species can adapt via different sets of traits to the same climatic challenge (e.g. Bilton *et al.* 2016, Bergholz *et al.* 2017, Lampei *et al.* 2017 for aridity), the traits expected to evolve can likewise differ among species. Moreover, Sandel *et al.* (2010) cautioned that initial trait responses to altered environments may differ from those for long-term adaptation. Such complexities may render common-sense interpretations misleading, i.e. we need much clearer justifications to conclude adaptive responses.

Here, we addressed this challenge using several independent lines of evidence simultaneously. Firstly, we imposed replicated *in situ* climate manipulations on entire plant communities to directly control the causal factor, and we did so in two independent sites. Consistent evolutionary responses in both sites would then strongly argue against random drift effects and in favor of adaptivity. Secondly, we combined these climate manipulations with a corresponding natural climatic gradient in a ‘space-for-time’ approach: Many species show clinal trait divergence along natural gradients when grown under common garden conditions (e.g. Kigel *et al.* 2011, Petrú *et al.* 2006, Lampei *et al.* 2017). Such clines likely reflect locally adapted ecotypes, i.e. the species-specific long-term adaptive strategy towards the corresponding climatic factor (Kawecki & Ebert 2004), and hence provide clear *a priori* predictions for directional trait evolution under climate manipulations. Thirdly, we based our selection of study traits on evolutionary theory, i.e. attested evidence for theoretical fitness advantages under differential climatic conditions. Lastly, we additionally estimated selection (Lande & Arnold 1983) on target plants grown in the greenhouse, yet under a set of contrasting abiotic conditions that mirrored our *in situ* climate manipulations. If the covariance between trait values and fitness changes with climatic condition, differential trait values should be advantageous contingent on climate. By combining these multiple approaches, we gained unprecedented strong evidence for whether potential evolutionary changes are adaptive.

Another recent debate addresses the role of phenotypic plasticity in climate change adaptation (Merilä & Henry 2014; Fox *et al.* 2019; Scheiner *et al.* 2020). High plasticity in adaptive traits, as an immediate response to altered environments, may help genotypes to better match their phenotype to these novel conditions (Crispo 2007; Lande 2009; Kelly 2019). It was therefore hypothesized that climate change can target plasticity itself for evolution and favor more plastic genotypes, even if this is a transient response that is merely ‘buying time’ until the occurrence of new genotypes with specific adaptations to the new conditions (Lande 2009; Fox *et al.* 2019; Scheiner *et al.* 2020). However, empirical tests in natural populations for evolution of increased plasticity in response to changing climate are scarce and yielded equivocal results (Franks *et al.* 2018; Arnold *et al.* 2019; Kelly 2019). This knowledge gap for evolving plasticity is unfortunate because moreover, plasticity may also interact with genetic adaptation by buffering selection and hence slowing down evolution and genetic adaption (Merilä & Hendry 2014, Kelly 2019, Fox *et al.* 2019), i.e. rapid evolution may be confined to traits with low plasticity. Yet, studies are missing that systematically compared rapidly evolving traits with their degree of plasticity.

To address these gaps, we conducted a uniquely comprehensive test for rapid evolution in ten target traits and their plasticity in a large-scale, multi-site climate change experiment (Tielbörger *et al.* 2014). Experimental rainfall manipulations (+30%, control, -30%) were imposed for ten years in two sites on entire resident plant communities in the Eastern Mediterranean, and combined with a natural rainfall gradient. Rainfall is the key abiotic factor in these ecosystems, with a projected -20% decline until 2050 (Smiatek *et al.* 2011; Samuels *et al.* 2013). Annual species dominate these communities, which allows for potentially rapid evolutionary responses (Tielbörger *et al.* 2014). Since migration of most species is limited (Siewert & Tielbörger 2010), *in situ* evolution appears crucial for climate change adaptation. We tested for evolutionary divergence in traits and plasticity in a naturally occurring annual plant species after ten years of climate manipulations, and used a comprehensive combination of lines of evidence to judge adaptivity. We hypothesized that climate

manipulation had caused adaptive evolutionary trait divergence and selection for higher plasticity, and that evolution is less probable in highly plastic traits.

## MATERIALS AND METHODS

### *Field sites and climate manipulations*

Four sites along a natural rainfall gradient from Northern to Southern Israel were chosen because they share most abiotic characteristics except for mean and variance in rainfall (detailed description: Supporting Information Fig. S1, and Tielbörger *et al.* 2014). They have Mediterranean-type climate and similar mean annual temperatures (17.7–19.1°C), yet spanned a more than eight-fold difference in mean annual rainfall (MAP). They represent mesic-Mediterranean (MM, 780 mm MAP), Mediterranean (M, 540 mm), semi-arid (SA, 300 mm) and arid conditions (A, 90 mm), respectively. The average length of the rainy season increases from the driest (Dec–March) to the wettest site (Oct–May). Rainfall variability among years decreases from the driest (40% Coefficient of Variation) to the wettest site (25% CV) (Metz *et al.* 2018). All sites are semi-natural shrublands with mostly annual species in the inter-shrub matrix, located at south exposures on limestone bedrock with similar elevation (470–620 m a.s.l.).

Three rainfall manipulation treatments (dry, control, wet) were imposed on entire resident plant communities for ten years (2002–2012) in the two intermediate sites, M and SA. Each treatment was replicated by five randomized 10m × 25m plots per site. The two extreme sites, A and MM, contained only five control plots each and extended the range of the natural rainfall gradient. For dry manipulation, rainout shelters intercepted 30% of each rainfall event; for wet manipulation, additional sprinkler irrigation after every other rain event added in total 30% of the site’s MAP; control plots received ambient rainfall (details in Tielbörger *et al.* 2014). The magnitude of the dry manipulations realistically approximated climate change scenarios for our study region, predicting 20% less rainfall until 2050 (Smiatek *et al.* 2011; Samuels *et al.* 2013), while the wet manipulations extended the general range of scenarios that were uncertain at the onset of our study.

### *Study species and seed material*

We focused on *Biscutella didyma* L., a winter annual Brassicaceae with Mediterranean and Irano-Turanian distribution (Feinbrun-Dothan & Danin 1998). This medium-sized, common and subordinate species was selected because it is one of few species occurring reliably across the entire rainfall gradient. As is typical for many annuals in our study region, *B. didyma* has short dispersal distances (Siewert & Tielbörger 2010) and low outcrossing rates (Boaz *et al.* 1990), making gene-flow among manipulated plots unlikely.

First, seeds of 100 *B. didyma* individuals (genotypes) per rainfall treatment and site were collected in spring 2012 by randomly picking twenty individuals within each 10m × 25m plot. Keeping [?]1.5m distance among genotypes and the large plot size ensured independence and capturing a representative fraction of the in-trapopulation variance. One offspring per genotype was raised with regular watering for one generation in Tübingen (Germany) to reduce potential parental effects and amplify seed material. The growth period (Nov 2012 – May 2013) approximated the natural vegetation season and photoperiod, and greenhouse temperatures mirrored day temperatures in the field sites (15–20degC in winter, gradually warming to 25–30degC in spring). Wrapping flowering plants in light, transparent fabric (organza) ensured self-pollination and facilitated seed collection separately per mother. Second, of the resulting second generation, we randomly picked 40 genotypes per rainfall treatment and site (3x40 in SA, M; 40 in A, MM; altogether 320 genotypes) for the subsequent greenhouse experiment (detailed scheme: Appendix Fig. S1).

### *Greenhouse experiment*

One season later (Nov 2013 – May 2014), we raised these 320 genotypes under conditions as above, yet under five water levels. One pot (10 x 10 x 10 cm) per genotype was included in each water level (1600 pots in total). Per pot, 15 seeds of one genotype were sown in a 1:1 mixture of nutrient-poor potting soil and sand, enriched with 0.1 g Osmocote(r) slow release fertilizer (14-14-14 NPK; Scotts GmbH, Germany). On the first and third day of the experiment, all pots were watered equally to saturation to ensure seed germination. After germination was completed, seedlings were randomly thinned to one per pot and five watering levels

were initiated (90 ml, 50 ml, 30 ml, 20 ml, and 15 ml per pot and watering event, respectively). Watering was applied manually with dispensers approx. every 2-3 days, i.e. when most plants in the lowest water level showed strong signs of drought stress. Pots were randomized across the greenhouse and re-randomized every three weeks.

### Phenotyping

We focused on ten traits across the entire plant life-cycle for which theory predicts a relationship to differential water availability.

*Germination fraction* was censused per pot after germination had ceased at the beginning of the experiment. It typically decreases towards arid populations to hedge against more frequent unfavorable years (Tielborger *et al.* 2012; ten Brink *et al.* 2020). As climate manipulations increased (dry plots) and decreased (wet plots) the occurrence of unfavorable years, reduced germination fractions should be favored in dry plots and *vice-versa* in wet plots.

*Days to flowering* (since first irrigation) were determined by inspecting plants daily for the first open flower. Accelerated phenology is expected by theory and repeatedly found in annual plants from drier sites (Cohen 1976; Kigel *et al.* 2011), i.e. earlier phenology is predicted for plants descending from dry manipulated plots and more arid sites. Moreover, the number of leaves at the day of first flowering (*leaf number at flowering*) provided an ontogenetic phenological measure and a non-destructive measure of plant size. It disentangled whether phenology changed via accelerated development (days to flowering) or shifted ontogeny (leaves at flowering) (Kigel *et al.* 2011).

*Stomata density* and *carbon isotopes* ( $\delta^{13}\text{C}$ ) assessed gas exchange and water use efficiency. Stomata density was quantified by automated high-throughput microscopy (Dittberner *et al.* 2018; see Supplementary Methods). As lower stomata density may decrease maximum transpiration (Liu *et al.* 2012) we expected lower stomata density in plants descending from drier conditions. Due to high costs, carbon isotopic ratios ( $\delta^{13}\text{C}$ , Supplementary Methods), were analyzed only for a subset (14 genotypes per site, rainfall treatment, and four water levels: 15ml – 50ml). We expected that plants from drier sites and plots exhibit higher water use efficiency, i.e. higher  $\delta^{13}\text{C}$  (Li 1999; Hartman & Danin 2010).

*Plant height* was measured on a fixed day (12<sup>th</sup> April) before the onset of senescence. Moreover, *above-ground vegetative biomass* was determined at the end of the experiment (May 15<sup>th</sup> 2014) as the dry weight (24 h, 70°C) of all stems and leaves. We expected greater height and vegetative biomass in plants from wetter conditions as adaptation to intensified aboveground competition (Westoby 1998; Schiffers & Tielbörger 2006).

*Seed number* per plant quantified fitness. Moreover, we estimated the selfing rate per plant visually as percent of flowers that developed into viable seeds; it served as covariate in some analyses because *B. didyma* populations may differ in self-compatibility (Gibson-Forty 2018).

*Reproductive allocation* quantified the biomass allocation to reproductions (i.e. weight of all diaspores and flower remains) relative to the vegetative biomass. Reproductive allocation should be higher in plants from drier conditions as they require less investment in vegetative tissue for outgrowing neighbors (Aronson *et al.* 1993).

*Diaspore weight* (maternal investment per single offspring) was measured across 30 randomly picked diaspores per plant. Diaspore weight consists of c. 50% of seed mass in *B. didyma* and both are strongly correlated ( $r^2=0.88$ ,  $p<0.001$ ; determined for 15 seeds in 32 randomly picked individuals across sites). For this complex feature of plant life-cycles (Westoby 1998), we expected increasing diaspore weight under wetter conditions (DeMalach & Kadmon 2018; DeMalach *et al.* 2019).

### Statistical analyses

Analyses of the above trait values were performed in four steps with R 3.5.2 (R core team 2018).

First, we tested for divergent trait evolution in plants descending from dry, control and wet manipulated plots in the central sites SA and M (N=240 genotypes). For each trait separately, linear mixed models were calculated with climate manipulation treatment (dry, control, wet), site (SA, M), five greenhouse watering levels, and their interactions as fixed factors, as well as genotype as random factor (accounting for five non-independent plants across water levels). Some traits were transformed prior to analyses to meet homoscedasticity (sqrt: stomata density, height, reproductive allocation, seed number; log: leaf number at flowering, vegetative biomass). Significance was assessed with Wald F-tests with Kenward-Roger approximated df in the package *car* (Fox & Weisberg 2011). Posthoc tests identified contrasting climate manipulations using the package ‘multcomp’ (Hothorn *et al.* 2008) with P-values corrected for false discovery rate (FDR) *sensu* Benjamini & Hochberg (1995). For germination fraction (binary) we used a corresponding glm with logit link-function and quasibinomial error structure.

Second, we tested for clinal trends in traits across the rainfall gradient, including only plants descending from control plots in all four sites (N=160 genotypes). We calculated linear mixed models per trait with site and greenhouse water level as fixed factors, and genotype as random factor (transformations as above). Posthoc tests with FDR-correction as above identified contrasting sites. Germination fraction was analyzed with a binomial glm as above, using only site as main factor.

Third, we estimated selection, i.e. the covariance of traits with relative fitness (Lande & Arnold 1983), under low and high irrigation in the greenhouse. This approach reveals traits that can adapt a population to drought and is independent of other environmental factors correlating with rainfall (Mitchell-Olds & Schmitt 2006). We estimated selection for all traits showing either rapid evolution (step 1) or clines with rainfall (step 2). We included all plants from sites with climate manipulation, computed the genotype trait-mean across low watering (15ml, 20ml) and high watering (50ml, 90ml), followed by standardization (zero mean, 1 SD) per population (SA and M) and watering level. Similarly, relative fitness was computed per population and watering. We fitted generalized least squares models (glms, *rms* package (Harrell 2019)), with relative fitness as the dependent variable, and trait, watering (high, low) and their interaction as predictors. A significant trait  $\times$  watering interaction indicated contrasting directional selection on that trait contingent on water availability, computed using type III sums of squares (Anova, *car* package (Fox & Weisberg 2019)) with FDR-correction.

Fourth, we tested whether field climate manipulations favored genotypes with higher plasticity. In addition to assessing the climate manipulation  $\times$  water term in step 1 above, plasticity was quantified for the above traits using the Coefficient of Variation (CV) across the five individuals (i.e. water levels) per genotype in the greenhouse. The intuitive, standardized CV allows comparing plasticity across different traits (Houle 1992) and handles well outliers and non-linear responses across several environments. Another plasticity index (PI<sub>v</sub>, see Valladares *et al.* 2006) yielded the same results. With these CV-values per genotype, we calculated two-way ANOVAs and FDR-post hoc tests separately for each trait, including the factors site (SA, M) and climate change treatment (dry, control, wet).

## RESULTS

### *Trait evolution under climate manipulations*

Three out of ten traits showed significant divergence among climate manipulations and hence rapid evolution within 10 years of field experiments. It was always the dry treatment that had diverged from control and wet treatments (Table 1, Fig. 1). Plants descending from dry manipulated plots flowered on average 3-4 days (c. 4%) earlier and with 4-5 (c. 13%) fewer leaves, and had 10-15% higher reproductive allocation than plants from control and wet manipulated plots (Table 1, Fig. 1). These evolutionary responses to drought were consistent in both sites (no site  $\times$  climate manipulation and site  $\times$  water  $\times$  climate manipulation interactions; Table 1).

The seven remaining traits showed no divergence among climate manipulation treatments (Table 1, Fig. 1).

### *Trait divergence along the rainfall gradient*

Eight traits showed significant clinal trends across the natural rainfall gradient, suggesting an adaptive role for these traits with rainfall. Towards drier sites, plants decreased in germination fraction, days to flowering, stomata density, height, and biomass, and they increased in reproductive allocation and seed production (Table 2, Fig. 1). Furthermore, flowering started at smaller plant size (fewer leaves) in drier sites, although at MM, plants invested less in lateral branches and thus possessed fewer but larger leaves along the main stem (Petrú *et al.* 2006; pers. obs.). There was a weak, non-significant tendency towards lower  $\delta^{13}\text{C}$  in arid sites ( $p=0.12$ , Fig. 1) and no clinal trend for diaspore weight despite significant differences among sites (Table 2, Fig. 1).

The clinal trends across sites in flowering time, leaf number at flowering and reproductive allocation matched the direction of their rapid evolutionary response to ten years of climate manipulation (Fig. 1). In contrast, five traits showed clines along the natural rainfall gradient but no rapid evolution under climate manipulations (germination fraction, stomata density, height, biomass, seed number).

### Selection analyses

Selection on several traits differed between high and low greenhouse watering levels (Fig. 2). In line with the field experiment, selection for early flowering was stronger under low watering in both traits (days to flowering  $\times$  watering:  $\chi^2=13.6$ ,  $df=1$ ,  $p<0.001$ ,  $b_{\text{low}}=-0.3$ ,  $b_{\text{high}}=-0.08$ ; leaf number at flowering  $\times$  watering:  $\chi^2=9.4$ ,  $df=1$ ,  $p=0.003$ ,  $b_{\text{low}}=-0.25$ ,  $b_{\text{high}}=-0.1$ ). Similarly, high reproductive allocation was stronger selected for under low than under high watering (reproductive allocation  $\times$  watering:  $\chi^2=114.6$ ,  $df=1$ ,  $p<0.001$ ,  $b_{\text{low}}=0.7$ ,  $b_{\text{high}}=0.35$ ). Among these three traits showing rapid evolution in the field, genetic correlations were relatively strong and in the direction of their joint selection (Appendix Fig. S3).

Among traits without significant rapid evolution, low watering selected stronger against high vegetative biomass (vegetative biomass  $\times$  watering:  $\chi^2=17.1$ ,  $df=1$ ,  $p<0.001$ ,  $b_{\text{low}}=-0.24$ ,  $b_{\text{high}}=0.1$ , Fig. 2). In contrast, greater plant height was overall favored ( $b=0.15$ ,  $\chi^2=11.1$ ,  $df=1$ ,  $p=0.001$ ) without difference between watering levels (height  $\times$  watering:  $\chi^2=0.21$ ,  $df=1$ ,  $p=0.65$ , Fig. 2). Since vegetative biomass and plant height showed a positive genetic correlation (Appendix Fig. S3), this suggests conflicting selection on these traits under low watering. No differential selection was found for stomata density ( $\chi^2=2.16$ ,  $df=1$ ,  $p=0.17$ , Fig. 2).

### Plasticity

The five water levels in the greenhouse significantly affected all traits (Table 1, 2; Appendix Fig. S1), indicating plasticity in response to water. Significant site  $\times$  water interactions for all traits except reproductive allocation (Table 2) indicated genetic variation in plastic responses among the four sites of the rainfall gradient.

For the two sites with climate manipulations, the magnitude of plasticity (quantified as CV) differed substantially among traits. CV was lowest in  $\delta^{13}\text{C}$  (3-4%) and days to flowering (c. 10%), intermediate for diaspore weight, height, stomata density, leaf number at flowering and total biomass, and particularly high in reproductive allocation (c. 80%) and seed number (c. 100%) (Fig. 3). The three traits showing rapid evolution thus had low (days to flowering), intermediate (leaf number at flowering) and high plasticity (reproductive allocation), respectively, signifying that plasticity and the probability of rapid evolution in the field were independent.

Climate manipulations had overall little effect on the magnitude of plasticity. Increased plasticity (CV) in plants from dry manipulated plots compared to controls was found in diaspore weight ( $p=0.01$ ) and a similar non-significant tendency in days to flowering ( $p=0.06$ ) (Table 3, Fig. 3). Plasticity in vegetative biomass increased in dry plots ( $p=0.03$ ), but only compared to wet plots (Table 3, Fig. 3). The lack of any significant climate manipulation  $\times$  site interaction showed that plasticity responded similarly to manipulations in both sites (Fig. 3, Table 3). Moreover, plasticity (CV) was significantly higher in the Mediterranean than the semi-arid site in four traits: days to flowering, height, reproductive allocation, seed number (Table 3, Fig. 3). All plasticity results were robust when using another common plasticity index,  $PI_v$  (Valladares *et al.* 2006).

## DISCUSSION

Our overall results demonstrate rapid evolution in three out of ten traits under *in situ* climate manipulations in natural plant communities after merely 10 years, i.e. at most 10 generations of our annual study species. This is a remarkably short time span, given that numerous interacting factors may hamper evolution in natural communities (Hoffmann & Sgró 2011; Shaw & Etterson 2012). The fact that this evolutionary response was consistent in two independent sites renders chance effects, e.g. genetic drift, unlikely to cause these results and underpins that the evolutionary response was directly driven by manipulated rainfall. Intriguingly, our multiple independent lines of evidence corroborate that these changes were adaptive.

After 10 years of artificial drought, phenology had evolved both in chronological (days to flowering) and ontogenetic (leaf number at flowering) time. Theory suggests accelerated life-cycles as a drought avoidance strategy that reduces the risk of mortality before reproduction (Cohen 1976; Kigel *et al.* 2011), albeit at the cost of smaller plant size and hence possibly lower competitive ability (Liancourt & Tielbörger 2009; Kigel *et al.* 2011). In line with theory, plants from dry-manipulated plots flowered earlier and with fewer leaves than plants from control and wet plots. Moreover, this rapid evolutionary response paralleled the long-term evolutionary response of *B. didyma* along the natural rainfall gradient where plants from more arid sites flowered earlier; a trend found in many other annuals along natural rainfall gradients (Kigel *et al.* 2011; Wolfe & Tonsor 2014; Kurze *et al.* 2017). Interestingly, the observed 3-4 days acceleration in phenology corresponds to an ecological distance of *c.* 100 mm lower rainfall at origin for annuals along our study gradient (Kigel *et al.* 2011; Kurze *et al.* 2017). Given the magnitude of rainfall reduction in dry plots (-90 mm in SA, -160 mm in M), this suggests that phenology could actually track a substantial part of the imposed change in rainfall. The adaptivity of accelerated phenology under drought was furthermore corroborated by our selection analyses under controlled, unconfounded (Mitchell-Olds & Schmitt 2006) watering conditions in the greenhouse. Here, earlier flowering with fewer leaves was stronger favored under low than under high water availability. These multiple lines of evidence – theory, natural rainfall gradient, selection analyses, and consistency in both sites – provide compelling evidence that the observed rapid evolution in phenology was adaptive.

Rapid evolution of earlier phenology under drought was previously reported from a Californian climate manipulation site (Nguyen *et al.* 2016) and from resurrection studies (Franks *et al.* 2007; Vigouroux *et al.* 2011; Nevo *et al.* 2012; Hamann *et al.* 2018). If phenology was reported, no evolution occurred merely in one perennial (Ravenscroft *et al.* 2014) or under elevated CO<sub>2</sub> (Grossman & Rice 2014). Therefore, phenology appears a key trait for rapid drought adaptation in annuals, congruent with similar suggestions by theory and gradient studies (Cohen 1976; Kigel *et al.* 2011; Kurze *et al.* 2017). These findings may also indicate that phenology evolves easier than other, possibly more complex traits. However, more multi-trait studies (e.g. Ravenscroft *et al.* 2014; Nguyen *et al.* 2016; Hamann *et al.* 2018) assessing comparable trait-sets are required to confirm this idea.

Here, we also observed rapid evolution in reproductive allocation. As competition is reduced in drier sites along our gradient (Schiffers & Tielbörger 2006), theory suggests reduced investment in vegetative tissue for outgrowing neighbors and increased allocation to reproduction (Aronson *et al.* 1990; 1993). In line with theory and in both sites, plants from dry manipulated plots produced 10-15% more seeds per vegetative biomass than control plants. Although reproductive allocation was rarely assessed in climate manipulation studies, a similar tendency was reported for a perennial herb (Ravenscroft *et al.* 2014). This evolutionary response was again congruent with our selection analyses in the greenhouse, and with the clinal trend in reproductive allocation along our natural rainfall gradient, and parallel clines in other species (summarized in Kurze *et al.* 2017). Thus, in all traits showing rapid evolution in the field, our independent lines of evidence demonstrate that these changes were adaptive. Intriguingly, parallel studies found that many plant community parameters were remarkably resistant to our climate manipulations (Tielbörger *et al.* 2014; Bilton *et al.* 2016). Though we have studied only a single species, our current findings suggest that rapid adaptive evolution possibly contributed to increasing population-level and community-level resistance to climate change.

Interestingly, evolutionary changes occurred solely in the dry manipulated plots, i.e. the treatment which

increased, rather than decreased stress for resident plants. Drought likely lead to direct, rapid exclusion of drought-sensitive and late-flowering genotypes, especially in dry years. In wet plots, evolution may be slower because selection was likely driven by competition for additional resources (Schiffers & Tielbörger 2006) which causes smaller fitness differences, as was shown by cross-transplants with *B. didyma* (Ariza & Tielbörger 2011).

Despite the evidence for rapid adaptive evolution, seven further traits did not evolve. This was surprising because five of them exhibited clinal shifts along the rainfall gradient, suggesting that they contribute to *B. didyma*'s long-term evolutionary response to drier climates: germination fraction, stomata density, height, vegetative biomass and seed number. In conjunction with existing theory we had expected corresponding evolution of these traits under climate manipulations (Westoby 1998; Liu *et al.* 2012; Tielbörger *et al.* 2012; ten Brink *et al.* 2020). Selection analyses supported this expectation for vegetative biomass, although not for stomata density and height; no tests were possible for germination fraction (no differential watering) and seed number (response variable in selection analyses). While empirical studies usually focused on (few) traits exhibiting rapid evolution, non-evolving traits have been reported before (e.g. Franks 2011; Ravenscroft *et al.* 2014; Nguyen *et al.* 2016). One possible explanation for the lack of evolution in some candidate traits is that selection on them was weakened by adaptation of the fast-evolving traits, i.e. evolution of further traits was unnecessary. Alternatively, the multiple potential constraints for evolution under natural conditions hindered adaptation in other traits, e.g. low genetic variation, genetic covariance and trade-offs among traits (Hoffmann & Sgró 2011; Shaw & Etterson 2012). In our case, negative genetic covariance potentially hindered evolution in vegetative biomass (Appendix Fig. S3). The observed rapid evolution in only a subset of traits may therefore indicate incomplete adaptation to new conditions, cautioning that climate change may imperil species despite rapid evolution. Importantly, most evidence for rapid adaptive evolution under natural conditions reported rather few evolving traits (e.g. Franks 2011; Nevo *et al.* 2012; Ravenscroft *et al.* 2015; Nguyen *et al.* 2016). Our findings caution that focusing on few evolving traits may overestimate the potential of rapid evolution for climate change adaptation.

High trait plasticity may further retard adaptive evolution (Shaw & Etterson 2012; Merilä & Hendry 2014; Kelly 2019), but this idea has rarely been tested in natural populations (Arnold *et al.* 2019). Our study, where the three rapidly evolving traits showed three contrasting magnitudes of plasticity (CV) indicates that plasticity and evolutionary potential are not necessarily related. However, this conclusion should be taken with caution because it is based on three partially correlated traits (Appendix, Fig. S3) which may have evolved in concert.

Our findings also provide little support for the idea that climate change leads to increased plasticity as a means to rapidly adjust the phenotype to novel conditions (Lande 2009; Arnold *et al.* 2019; Kelly 2019; Scheiner *et al.* 2020). Only a single trait, diaspore weight, had significantly increased plasticity under drought, and days to flowering showed a similar, non-significant tendency. Both responses, however, were opposite to the expected adaptive direction (e.g. later, not earlier flowering under drought; Appendix, Fig. S1), indicating non-adaptive plasticity (Acasuso-Rivero *et al.* 2019). Similarly, no clearly increased plasticity after drought was found by resurrection studies (Franks 2011; Hamann *et al.* 2018) and lower plasticity after CO<sub>2</sub> elevation (Grossman & Rice 2014; but see Sultan *et al.* 2013 for increased plasticity during plant invasion). Thus, our study joins an –albeit small– body of equivocal evidence indicating that evolution of increased plasticity is no major pathway for climate change adaptation.

Overall, our study demonstrates that rapid evolution may play an important role for climate change adaptation in natural annual plant populations. The novel setup of our study – combining *in situ* climate manipulations with a natural climatic gradient and selection analyses under controlled conditions – provided independent, compelling lines of evidence that observed evolutionary shifts were adaptive. However, with rapid evolution in merely a subset of well-justified candidate traits, our study emphasizes the importance of multi-trait studies for assessing whether rapid *in situ* evolution may safeguard species under climate change.

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## DATA ACCESSIBILITY STATEMENT

The data supporting our results are publicly available at the dryad.org data repository.

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## SUPPORTING INFORMATION

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**Table 1** Linear mixed models testing for diverging trait values in  $N=240$  genotypes originating from three Climate Change manipulations (CC) in two sites (semi-arid, Mediterranean), when grown under five water levels in the greenhouse. For CC, posthoc results are provided to identify diverging treatments (dry, control, wet).

Note that residual df are Kenward-Roger approximated and can therefore differ among models;  $^{13}\text{C}$  isotopes were measured in only 84 genotypes and 4 water levels.

	trait	CC treatment
Germination	Germination	
	germination fraction	$F_{2,234}=0.03$
Phenology	Phenology	
	days to flowering	$F_{2,234}=4.73$
	leaves at flowering	$F_{2,234}=5.05$
water & gas exchange	water & gas exchange	
	stomata density	$F_{2,229}=0.20$
	$^{13}\text{C}$ isotopes (WUE)	$F_{2,78}=2.24$
Growth	Growth	
	plant height	$F_{2,232}=2.61$
	vegetative biomass	$F_{2,231}=0.50$
Allocation	Allocation	
	reprod. Allocation <sup>a</sup>	$F_{2,231}=5.33$

	trait	CC treatment
Fitness	diaspore weight	$F_{2,231}=2.12$
	Fitness	
	seed number <sup>b</sup>	$F_{2,230}=1.81$
	*** $p<0.001$ , ** $p<0.01$ , * $p<0.05$ , ° $p<0.08$	*** $p<0.001$ , ** $p<0.01$ , * $p<0.05$
	<sup>a</sup> model included covariate '% selfing' ( $F_{1,966}=535.15$ , $p<0.0001$ )	<sup>a</sup> model included covariate '% selfing' ( $F_{1,966}=535.15$ , $p<0.0001$ )
	<sup>b</sup> model included covariate '% selfing' ( $F_{1,979}=483.15$ , $p<0.0001$ )	<sup>b</sup> model included covariate '% selfing' ( $F_{1,979}=483.15$ , $p<0.0001$ )

**Table 2** Linear mixed models testing for diverging trait values in  $N=160$  genotypes originating from four sites along a natural rainfall gradient (including control plots only), when grown under five water levels in the greenhouse. For site, posthoc results are provided to identify diverging sites (arid, semi-arid, Mediterranean, mesic-Mediterranean). Highlighted in bold are traits where trait divergence between sites followed a cline along the rainfall gradient. Note that residual df are Kenward-Roger approximated and can therefore differ among models;  $^{13}C$  isotopes were measured in only 56 genotypes.

	trait	site
Germination	Germination	
Phenology	germination fraction	$F_{3,151}=52.85$
	Phenology	
	days to flowering	$F_{3,148}=137.78$
water & gas exchange	leaves at flowering	$F_{3,148}=17.66$
	water & gas exchange	
	stomata density	$F_{3,146}=4.29$
Growth	$^{13}C$ isotopes (WUE)	$F_{3,52}=2.06$
	Growth	
	plant height	$F_{3,148}=16.42$
Allocation	vegetative biomass	$F_{3,147}=7.24$
	Allocation	
	reprod. allocation <sup>a</sup>	$F_{3,150}=26.44$
Fitness	diaspore weight	$F_{3,143}=5.07$
	Fitness	
	seed number <sup>b</sup>	$F_{3,150}=21.65$
	*** $p<0.001$ , ** $p<0.01$ , * $p<0.05$ , ° $p<0.08$	*** $p<0.001$ , ** $p<0.01$ , * $p<0.05$
	<sup>a</sup> model included covariate '%selfing' ( $F_{1,577}=303.73$ , $p<0.0001$ )	<sup>a</sup> model included covariate '%selfing' ( $F_{1,577}=303.73$ , $p<0.0001$ )
	<sup>b</sup> model included covariate '%selfing' ( $F_{1,604}=300.84$ , $p<0.0001$ )	<sup>b</sup> model included covariate '%selfing' ( $F_{1,604}=300.84$ , $p<0.0001$ )

**Table 3** Two-way ANOVAs testing for diverging plasticity (measured as Coefficient of Variation across five water levels in the greenhouse) in nine traits of  $N=240$  genotypes descending from three Climate Change manipulation treatments (CC) in two sites (semi-arid, Mediterranean). Posthoc results are provided to identify diverging CC treatments (dry, control, wet) and contrasting sites.

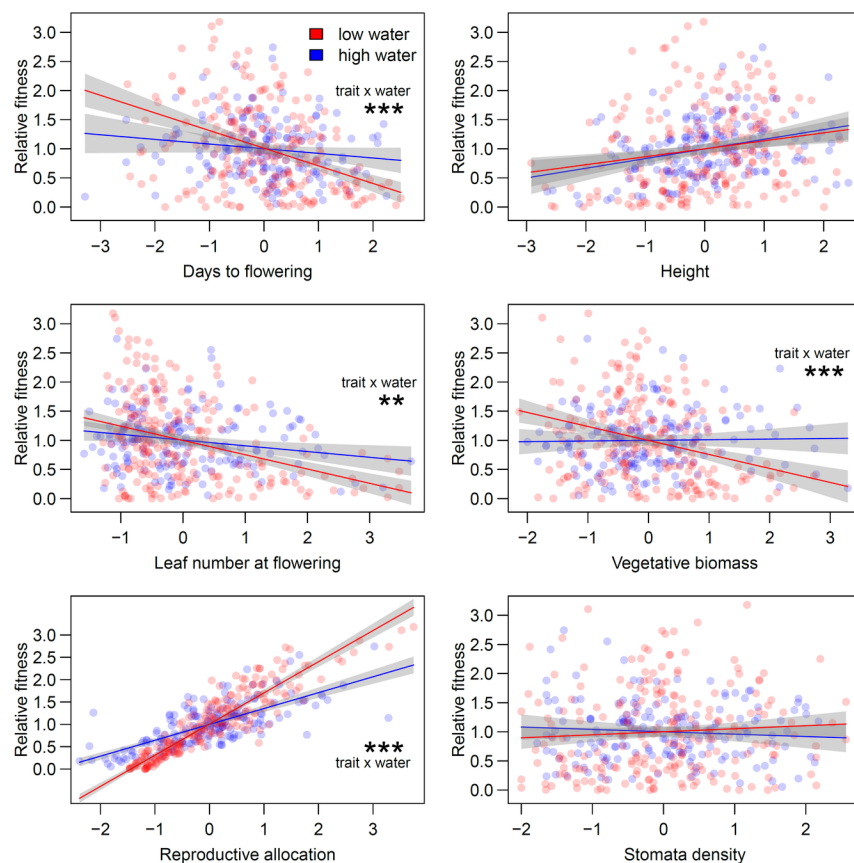
		CC	CC
phenology	trait plasticity	$F_{1,234}$	
	phenology		
	days to flowering	$F_{1,234}=2.84$	°
	leaves at flowering	1.38	

		CC	CC
water & gas exchange	water & gas exchange	water & gas exchange	
	stomata density	1.12	
	$\delta^{13}\text{C}$	0.22	
growth	growth		
	height	0.17	
	veg. biomass	3.47	* d>w
allocation	allocation		
	reprod. allocation	0.98	
	diaspore weight	4.57	* d>c,w
fitness	fitness		
	seed number	0.02	
	° p<0.08, *p<0.05, **p<0.01, ***p<0.001	° p<0.08, *p<0.05, **p<0.01, ***p<0.001	° p<0.08,

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image1.emf available at <https://authorea.com/users/297665/articles/460039-rapid-adaptive-evolution-to-drought-in-a-subset-of-plant-traits-in-a-large-scale-climate-change-experiment>

**Figure 1** Mean values ( $\pm 1\text{SE}$ ) for ten traits after ten years Climate Change manipulations (CC; dry -30% rainfall; wet +30%; control ambient rainfall) in two intermediate sites (SA semi-arid, M Mediterranean) and in two additional control sites along a natural rainfall gradient (A arid, MM mesic-Mediterranean). Significant CC effects indicate evolutionary trait divergence due to climate manipulations (full statistics in Table 1). The direction of CC responses can be compared to trait shifts along the natural rainfall gradient (effect of site; full statistics in Table 2). Points show mean trait values across 5 water levels in the greenhouse for plants from N=40 genotypes per site and CC manipulation, while statistical analyses accounted for all individual water levels. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001



**Figure 2** Directional selection under low watering (red) and high watering (blue) in the greenhouse. The analysis is based on the genotype trait-means and the relative fitness of genotypes, both computed across low water levels (15ml, 20ml) and high water levels (50ml, 90ml), excluding the intermediate water level (30 ml) and followed by standardization (zero mean, 1 SD) per population (SA and M) and watering level (high and low). Differential selection between water levels is indicated by their interaction with the tested trait (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

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**Figure 3** Mean plasticity ( $\pm 1SE$ ) in nine traits after 10 years Climate Change manipulations (CC; dry -30% rainfall; wet +30%; control ambient rainfall) in two sites (SA semi-arid, M Mediterranean). Plasticity was quantified as Coefficient of Variation (CV) across plants grown in five water levels in the greenhouse and descending from  $N=40$  genotypes per site and CC manipulation (except  $d^{13}C$  carbon isotopes:  $N=14$ ). Significant CC effects indicate evolutionary divergence in plasticity due to climate manipulations (full statistics in Table 3). Note the different y-scaling for visualization. Plasticity for germination fraction was not assessed as differential water levels were not yet applied during germination. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ )

