

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 controlled selection analyses assessed whether potential responses were adaptive. In both sites, our annual target species *Biscutella didyma* consistently evolved earlier phenology and higher reproductive allocation in dry plots. This response was adaptive, as 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, they also call attention to potential constraints for 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, a multitude of 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 for rapid evolution under changing climate under near-natural conditions 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). Yet, this approach cannot unambiguously isolate climate as the causal factor for observed changes (Franks *et al.* 2018). Another approach is multi-year climate manipulations imposed on natural communities in the field. When plants are subsequently screened for divergent evolution, as a key asset, changes can be attributed directly to contrasting climatic treatments as the causal factor.

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 remains 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.* 2018). 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 set of traits expected to evolve can likewise differ among species. Moreover, Sandel *et al.* (2010) cautioned that initial trait responses to a changing climate may differ from those for long-term adaptation. These 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 climate manipulations *in situ* on entire plant communities to control directly 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, including our study species (e.g. Kigel *et al.* 2011, Petrů *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 performed additional selection analyses 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 relative fitness changes with climatic condition, differential trait values should be advantageous contingent on climate (Lande & Arnold 1983). 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 (e.g. Merilä & Henry 2014, Kelly 2019, Fox *et al.* 2019, Acasuso-Rivero *et al.* 2019). Climate change imposes novel conditions on plants in local communities. High plasticity in adaptive traits, as an immediate response to altered environments, may help genotypes to match their phenotype to these novel conditions (Chevin *et al.* 2010; Kelly 2019). Therefore, climate change may theoretically target plasticity itself for evolution and favor more plastic genotypes within the population; 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 (Crispo 2007; Lande 2009; Merilä & Henry 2014; Fox *et al.* 2019). However, explicit tests for increased plasticity under climate change are extremely scarce, and experimental evidence from natural populations is lacking (Kelly 2019). Of 12 resurrection studies reviewed recently (Franks *et al.* 2018) and the six studies with long-term climate manipulation mentioned above, only three have tested for rapid evolution of plasticity. They found reduced (Grossman & Rice 2014) or unchanged plasticity (Franks 2011) with altered climate. This knowledge gap 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, no previous study has 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 may be 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 trait clines along the rainfall gradient and selection analyses across greenhouse irrigation levels to judge adaptivity. We hypothesized that ten years of climate manipulation had caused adaptive evolutionary trait divergence and selection for higher plasticity, and that evolution was retarded in highly plastic traits.

MATERIALS AND METHODS

Field sites and climate change manipulations

Four field sites were chosen along a natural rainfall gradient from Northern to Southern Israel which share most abiotic characteristics except for mean and variance in rainfall conditions (details in Tielbörger *et al.* 2014). The sites share a Mediterranean-type climate and similar mean annual temperature (17.7–19.1°C), yet spanned a more than eight-fold difference in mean annual rainfall. They represent mesic-Mediterranean (MM, 780 mm rainfall, 33°00'N, 35°14'E), Mediterranean (M, 540 mm, 31°42'N, 35°03'E), semi-arid (SA, 300 mm, 31°23'N, 34°54'E) and arid conditions (A, 90 mm, 30°52'N, 34°46'E), respectively. The length of the rainy season increases from the driest (Dec/Jan–March) to the wettest site (Oct/Nov–May), while rainfall variability among years decreased 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 long-term average rainfall per year; control plots received ambient rainfall (details in Tielbörger *et al.* 2014). The magnitude of the dry manipulations 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.

Study species, seed sampling and amplification

We focused on *Biscutella didyma* L., a winter annual Brassicaceae with Mediterranean and Irano-Turanian distribution (Feinbrun-Dothan & Danin 1998). It was selected because it is one of few species occurring commonly 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.

Seeds of *B. didyma* were collected in spring 2012 from twenty randomly selected individuals within each 10m × 25m plot, i.e. 100 per rainfall treatment and site. From these seeds, one offspring per individual was raised with regular watering for one generation in Tübingen (Germany) to reduce potential parental effects. The growth period (Nov 2012 – May 2013) approximated the natural vegetation season and photoperiod, and greenhouse temperatures mirrored day temperatures in the field sites (15–20 °C in winter, gradually warming to 25–30°C in spring). Wrapping flowering plants in light, transparent fabric (organza) ensured self-pollination and seed collection separately per mother. Of the resulting second-generation seed material, we randomly picked 40 mothers (hereafter 'genotypes') per rainfall treatment and site (in total 320 genotypes) for the subsequent greenhouse experiment.

Greenhouse experiment

One season later (Nov 22nd 2013 – May 15th 2014), we raised these 320 genotypes under conditions as above in the same greenhouse, yet under five water levels. One pot (10 × 10 × 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® slow release fertilizer (14-14-14 NPK; Scotts GmbH, Nordhorn, Germany).

On the first and third day of the experiment, all pots were watered equally to saturation to ensure sufficient 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 related to differential water availability across the entire plant life-cycle.

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 (Tielbörger *et al.* 2012; Lampei *et al.* 2017; ten Brink *et al.* 2020). As climate manipulations increased (dry plots) and decreased (wet plots) the occurrence of unfavorable years, we expected reduced germination fractions being 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 (reviewed in Kigel *et al.* 2011), and we expected earlier phenology in 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 ($\delta^{13}\text{C}$, see 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 (12th April) before the onset of senescence. Moreover, above-ground *vegetative biomass* was determined at the end of the experiment (May 15th 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).

Total *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). Although investment per offspring is a crucial feature of plant life cycles (Westoby 1998), predicting its evolutionary response to aridity is controversial (Kurzeet *et al.* 2017).

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. For each trait separately, linear mixed models were calculated with climate change manipulation (dry, control, wet), site (SA, M), the five greenhouse watering levels, and their interactions as fixed factors, as well as genotype as random factor. 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 Chi-square tests in the package *car* (Fox & Weisberg 2011) and 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. 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 applied *selection analyses* for trait responses to low and high irrigation in the greenhouse. They assessed adaptivity of traits without environmental factors that may correlate with water availability under natural conditions (Mitchell-Olds & Schmitt 2006; De Frenne *et al.* 2013). We ran selection analyses for all traits showing either rapid evolution (first step) or clines with rainfall (second step). We included all plants from sites with climate manipulation, computed genotype mean trait values separately across low watering (15ml, 20ml) and high watering (50ml, 90ml), followed by normalization (zero mean, 1 SD) per population (SA and M) and watering level. Similarly, relative fitness was computed per population for high and low watering. We fitted generalized least squares models (*gls*, *rms* package (Harrell 2019)), with relative fitness as the dependent variable and trait value, water availability (high, low) and their interaction as predictors. A significant trait value \times water interaction indicated contrasting directional selection on that trait contingent on water availability (Lande & Arnold 1983), computed using type III sums of squares (Anova, *car* package (Fox & Weisberg 2019)).

Fourth, we tested whether climate manipulations favored genotypes with higher plasticity. 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. CV is a standardized parameter that allows comparing plasticity across traits of different units and scales (Houle 1992; Acasuso-Rivero *et al.* 2019). 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

Rapid 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 marginally significant tendency towards lower $\delta^{13}\text{C}$ in arid sites ($p=0.08$, 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 within ten years climate manipulation. 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).

The five water levels in the greenhouse affected all traits (Table 1 & 2; Fig. S1 supp. information), indicating plasticity in response to water. Clearly significant site \times water interactions for all traits except reproductive allocation ($p=0.08$) indicated genetic variation in trait plasticity among sites (Table 2).

Selection analyses

Selection on several traits differed between low and high watering in the greenhouse (Fig. 2). As in the field experiment, days to flowering and leaf number at flowering experienced stronger directional selection under low water availability (water availability \times days to flowering: $\chi^2=13.6$, $df=1$, $p<0.001$, $b_{\text{low}}=-0.3$, $b_{\text{high}}=-0.08$; water availability \times leaf number at flowering: $\chi^2=9.4$, $df=1$, $p=0.002$, $b_{\text{low}}=-0.24$, $b_{\text{high}}=-0.1$). The third trait showing rapid evolution, reproductive allocation, was generally under strong selection due to its close correlation with relative fitness (Fig. 2); yet a significant interaction with water availability indicated that high reproductive allocation was more favored under low than under high watering ($\chi^2=114.6$, $df=1$, $p<0.001$, $b_{\text{low}}=0.7$, $b_{\text{high}}=0.35$).

Among traits without significant rapid evolution, vegetative biomass was stronger selected against under low water availability than under high water availability ($\chi^2=17.1$, $df=1$, $p<0.001$, Fig. 2). Because this negative correlation with relative fitness ($b=-0.24$) disappeared ($b=0.01$) when reproductive allocation was added to the model, this may be an indirect effect driven by the observed selection for high reproductive allocation. No differential selection was found for stomata density ($\chi^2=2.16$, $df=1$, $p=0.14$, Fig. 2) and for plant height, where tall plants were favored alike under low and high water availability (Fig. 2, $\chi^2=0.21$, $df=1$, $p=0.65$).

Plasticity under climate manipulations

The Coefficient of Variation (i.e degree of plasticity) 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). There was no apparent relation between plasticity and the probability of rapid evolution in the field as the three traits showing rapid evolution had low (days to flowering), intermediate (leaf number at flowering) and high plasticity (reproductive allocation), respectively.

Climate manipulations had overall little effect on plasticity. A tendency for increased plasticity in plants from dry manipulated plots compared to controls was found in diaspore weight ($p=0.01$) and marginally for days to flowering ($p=0.06$) (Table 3, Fig. 3). In vegetative biomass, plasticity was significantly increased in dry plots ($p=0.03$), but only in comparison to wet plots (Table 3, Fig. 3). The lack of any significant climate manipulation \times site interaction showed that plasticity responded similarly in both sites (Fig. 3, Table 3).

Plasticity 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).

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 have affected 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 in chronological time (days to flowering) and ontogenetic time (leaf number at flowering). Theory suggests accelerated life-cycles as a drought avoidance strategy that reduces the risk of mortality before reproduction (Cohen 1976; Kigel *et al.* 2011). Yet, early reproduction comes 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 (Stinchcombe *et al.* 2004; 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 a substantial part of the ‘required’ acceleration in phenology could be realized within ten years. The adaptivity of accelerated phenology under drought was furthermore corroborated by our selection analyses under controlled watering conditions in the greenhouse, which eliminated potentially confounding factors along natural environmental gradients (Mitchell-Olds & Schmitt 2006, De Frenne *et al.* 2013). 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 flowering under drought was found also in other climate change studies; it is thus far the trait most often reported to evolve under drought in natural conditions (Franks *et al.* 2007; Vigouroux *et al.* 2011; Nevo *et al.* 2012; Nguyen *et al.* 2016). Accelerated phenology therefore emerges as a key pathway for rapid evolutionary adaptation to drier climates. While this underpins the central role of phenology for drought adaptation in annuals (Cohen 1976; Kigel *et al.* 2011; Kurze *et al.* 2017), it may also signpost that phenology evolves easier than other, possibly more complex traits. However, this conclusion is still hampered by the few tests beyond our study reporting multiple traits besides phenology (Ravenscroft *et al.* 2014; Nguyen *et al.* 2016).

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 evolutionary 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 it matched 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 had reported remarkable resistance in many plant community parameters to imposed climate manipulations (Tielbörger *et al.* 2014; Bilton *et al.* 2016). The present findings highlight that rapid adaptive evolution played an important role for climate change responses in annual species, and probably contributed significantly to community resistance.

Notably, all evolutionary changes occurred solely in the dry manipulated plots, i.e. the treatment which increased, rather than decreased stress for resident plants. This appears intuitive because drought may directly lead to rapid exclusion of drought-sensitive and late-flowering genotypes, especially in dry study years. In wet plots, selection among genotypes was probably driven by competition for additional resources

(Schiffers & Tielbörger 2006), resulting in weaker, more gradual fitness differences, as was shown for *B. didyma* in a cross-transplant with and without competition (Ariza & Tielbörger 2011), and hence slower progression of exclusion and adaptive evolution.

However, seven further candidate traits did not evolve. This is surprising because five of them exhibited clinal shifts along the natural 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, and they were not possible for germination fraction (no differential watering) and seed number (response variable in selection analyses). One possible explanation for the lack of evolution in other candidate traits is that sufficient adaptation was ensured by those traits that did evolve, and hence evolution of further traits was unnecessary. Alternatively, the multiple potential constraints for evolution under natural conditions hindered adaptation in other traits (Hoffmann & Sgró 2011; Shaw & Etterson 2012). In that case, the observed rapid evolution in a subset of traits may indicate incomplete adaptation to new conditions, cautioning that climate change may imperil species despite rapid evolution. Unfortunately, almost all available evidence for rapid evolution under natural conditions was restricted to very few traits (e.g. Franks *et al.* 2018; Nguyen *et al.* 2016; Grossman *et al.* 2014; but see Frachon *et al.* 2017), i.e. there is lacking information and focus on the importance of non-evolving traits for adaptation to climate change. Our results thus highlight that studies relying on few traits might be misleading.

High trait plasticity was intensely debated as a mechanism retarding adaptive evolution to climate change, yet rarely tested in nature (Merilä & Hendry 2014; Kelly 2019; Fox *et al.* 2019). Here, each of the three rapidly evolving traits had a completely different degree of plasticity. This first assessment under field conditions suggests that traits may evolve rapidly irrespective of their degree of plasticity, and that rapid evolution appears rather restricted by other mechanisms such as the genetic architecture of traits, potential underlying trade-offs, or limited genetic variation within populations (Barrett & Schluter 2007; Hoffmann & Sgró 2011; Shaw & Etterson 2012). Our findings furthermore give little support for the idea that climate change leads to increased plasticity as a means to rapidly adjust the phenotype to novel conditions (Crispo 2007; Lande 2009; Merilä & Hendry 2014; Kelly 2019; Fox *et al.* 2019), because only a single trait (diaspore weight) showed increased plasticity under drought. Evolution of increased plasticity is therefore unlikely to be a pathway for climate change adaptation in our system, corroborating the few existing climate change studies which found plasticity either unchanged (Franks 2011) or lower (Grossman & Rice 2014).

Overall, our study demonstrates that rapid evolution plays an important role for climate change adaptation in natural annual plant communities. The novel setup of our study – combining for the first time *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 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 plants originating from three Climate Change manipulations (CC) in two sites (semi-arid, Mediterranean) and grown under five water levels. For CC, posthoc results are provided to identify diverging treatments (dry, control, wet).*

	trait	CC manipulation (df=2)
Germination	Germination	
	germination fraction	F=0.033
Phenology	Phenology	
	days to flowering	$\chi^2=9.30$
	leaves at flowering	$\chi^2=10.34$
water & gas exchange	water & gas exchange	
	stomata density	$\chi^2=0.42$
	$\delta^{13}\text{C}$	$\chi^2=4.83$
Growth	Growth	
	plant height	$\chi^2=5.36$
	vegetative biomass	$X^2=0.94$
Allocation	Allocation	
	reprod. Allocation	$\chi^2=10.98$
	diaspore weight	$\chi^2=4.36$
Fitness	Fitness	
	seed number	$\chi^2=3.73$
	^o p[?]0.08, *p<0.05, **p<0.01, ***p<0.001	^o p[?]0.08, *p<0.05, **p<0.01, ***p<0.001
	^a model included covariate '% selfing' ($\chi^2=554.18$, df=1, p<0.0001)	^a model included covariate '% selfing' ($\chi^2=554.18$, df=1, p<0.0001)
	^b model included covariate '% selfing' ($\chi^2=500.42$, df=1, p<0.0001)	^b model included covariate '% selfing' ($\chi^2=500.42$, df=1, p<0.0001)

Table 2 *Linear mixed models testing for diverging trait values in plants originating from four sites along a natural rainfall gradient (including control plots only) and grown under five water levels. 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.*

	trait	site (df=3)
Germination	Germination	
	germination fraction	F=52.85
Phenology	Phenology	
	days to flowering	$\chi^2=424.78$
	leaves at flowering	$\chi^2=54.46$
water & gas exchange	water & gas exchange	
	stomata density	$\chi^2=13.29$
	$\delta^{13}\text{C}$	$\chi^2=6.66$
Growth	Growth	
	plant height	$\chi^2=50.70$
	vegetative biomass	$\chi^2=22.37$
Allocation	Allocation	
	reprod. allocation^a	$\chi^2=81.81$
	diaspore weight	$\chi^2=15.70$
Fitness	Fitness	

trait	site (df=3)
seed number^b	$\chi^2=67.16$
^o p[?]0.08, *p<0.05, **p<0.01, ***p<0.001	^o p[?]0.08, *p<0.05, **p<0.01
^a model included covariate '%selfing' ($\chi^2=315.78$, df=1, p<0.0001)	^a model included covariate '%selfing' ($\chi^2=315.78$, df=1, p<0.0001)
^b model included covariate '%selfing' ($\chi^2=313.26$, df=1, p<0.0001)	^b model included covariate '%selfing' ($\chi^2=313.26$, df=1, p<0.0001)

Table 3 Two-way ANOVAs testing for diverging plasticity (measured per genotype as Coefficient of Variation across five water levels) in nine traits of plants 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 (df=2)	CC (df=2)
	trait plasticity	F	
phenology	phenology		
	days to flowering	2.84	^o
	leaves at flowering	1.38	
water & gas exchange	water & gas exchange		
	stomata density	1.12	
	$\delta^{13}C$	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	
	^o p<0.08, *p<0.05, **p<0.01, ***p<0.001	^o p<0.08, *p<0.05, **p<0.01, ***p<0.001	^o p<0.08,

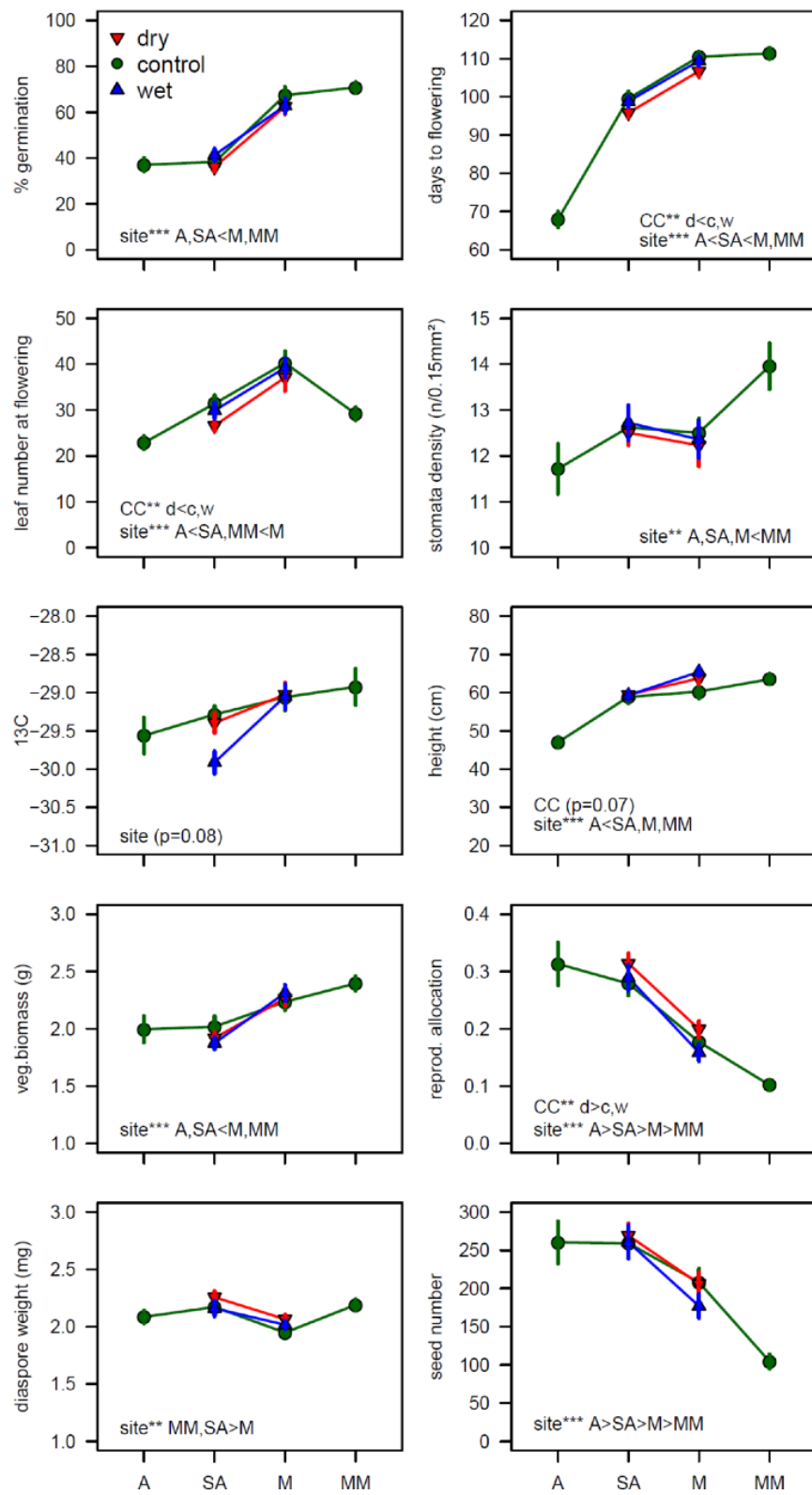


Figure 1 Mean values ($\pm 1SE$) for ten traits of genotypes originating from four sites along a natural rainfall gradient (ambient controls, in green), and from additional Climate Change manipulations in the two intermediate sites (CC; with dry/red and wet/blue). Main effects for CC (see Table 1) and site (see Table 2) are added if (marginally) significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

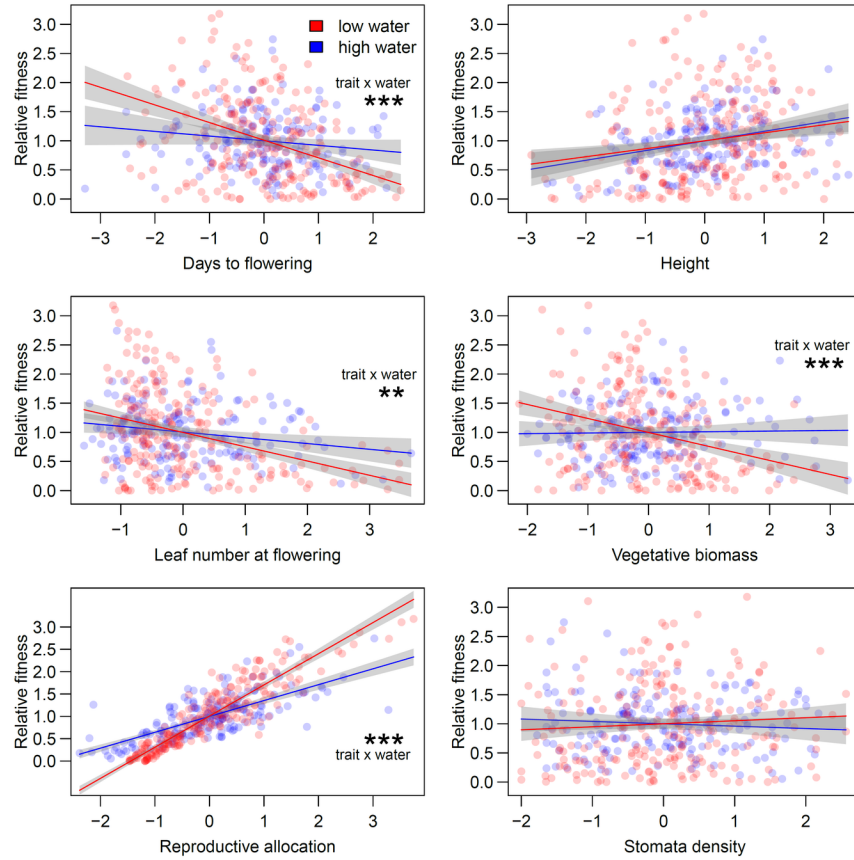


Figure 2 Directional selection in low water levels (red) and high water levels (blue) in the greenhouse. Differential selection between water levels is indicated by their interaction with the tested trait (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

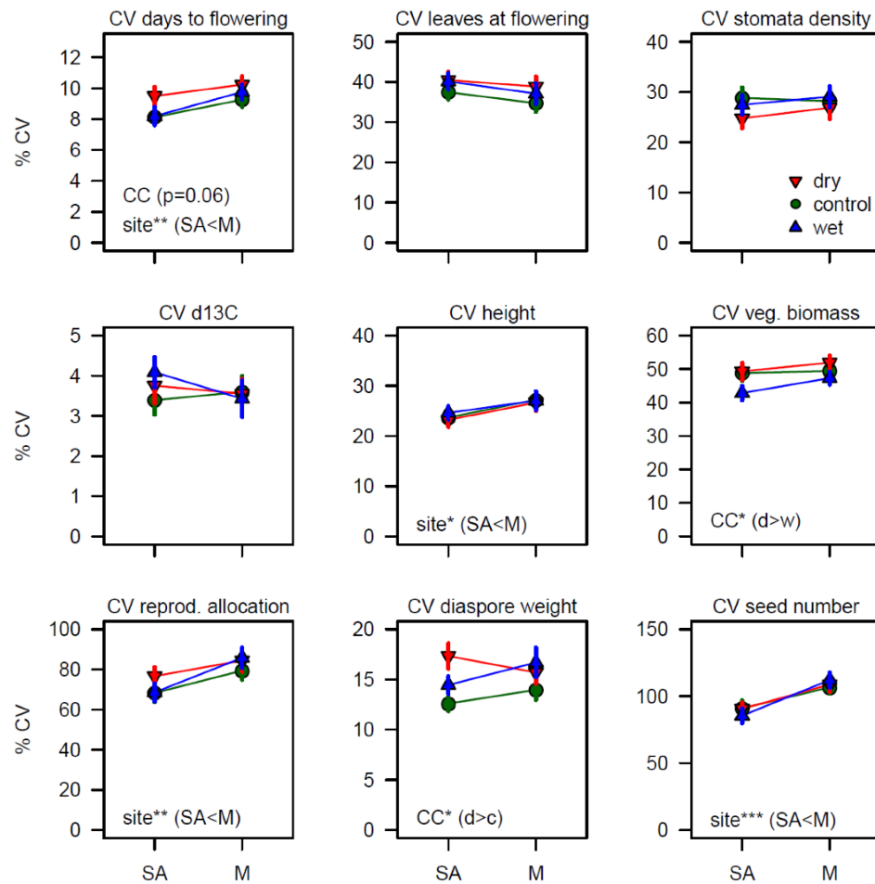


Figure 3 Mean plasticity in nine traits(measured as Coefficient of Variation across five water levels) in plants descending from three Climate Change manipulation treatments (CC) in two sites (semi-arid, Mediterranean). Error bars are $\pm 1SE$. Note the different y-scaling for visualization. Plasticity for germination fraction was not assessed as differential water levels were not yet applied during germination. Main effects for CC and site are added if (marginally) significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; see Table 3 for full results.)