Transgenerational effects of mild heat in *Arabidopsis thaliana* show strong genotype specificity that is explained by climate at origin

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Summary

- Transgenerational environmental effects can trigger strong phenotypic variation. However, it is unclear how cues from different preceding generations interact. Also, little is known about the genetic variation for these life history traits.
- Here, we present the effects of grandparental and parental mild heat, and their combination, on four traits of the third-generation phenotype of 14 *Arabidopsis thaliana* genotypes. We tested for correlations of these effects with climate and constructed a conceptual model to identify the environmental conditions that favour the parental effect on flowering time.
- We observed strong evidence for genotype-specific transgenerational effects. On average, *A. thaliana* accustomed to mild heat produced more seeds after two generations. Parental effects overruled grandparental effects in all traits except reproductive biomass. Flowering was generally accelerated by all transgenerational effects. Notably, the parental effect triggered earliest flowering in genotypes adapted to dry summers. Accordingly, this parental effect was favoured in the model when early summer heat terminated the growing season and environments were correlated across generations.
- Our results suggest that *A. thaliana* can partly accustom to mild heat over two generations and genotype-specific parental effects show non-random evolutionary divergence across populations that may support climate change adaptation in the Mediterranean.

Introduction

Plant traits often show extensive phenotypic variation between and within species, including variation in phenotypic plasticity, which is commonly observed as genotype by environment (G × E) interactions (Sultan, 2000; Brachi *et al.*, 2013). One component of phenotypic plasticity is transgenerational effects, when predecessor environments influence offspring development and responses to environmental conditions independent of genetic changes. These effects can be advantageous when parent and offspring environments are correlated (Burgess & Marshall, 2014; Leimar & McNamara, 2015; Lampei *et al.*, 2017). Transgenerational effects have been observed in many plant species (Germain & Gilbert, 2014), resulting from variable parental (Roach & Wulff, 1987; Mousseau & Fox, 1998; Galloway & Etterson, 2007; Latzel *et al.*, 2014) and grandparental environments in natural and experimental settings (Whittle *et al.*, 2009; Luna *et al.*, 2012; Rasmann *et al.*, 2012). These environments differed, for example, in the extent of herbivory (Rasmann *et al.*, 2012; Colicchio, 2017) or temperature (Whittle *et al.*, 2009; Suter & Widmer, 2013a,b). The phenotype of a plant is thus not only a reflection of the genotype and G × E interactions; it is also affected by the environment that was experienced by preceding generations and therefore may influence responses to selection (Uller, 2008; Dyer *et al.*, 2010).

However, we still know little about the evolution and adaptive value of transgenerational effects. For example, it is unclear how environmental signals experienced in different preceding generations (e.g. parental or grandparental) interact to shape the offspring phenotype. On the one hand, parents should influence their offspring more strongly than grandparents. First, taking a physiological perspective, parental effects can be transmitted by many means, including seed coat or endosperm modifications (Herman & Sultan, 2011), whereas grandparental effects lack the direct influence between generations. Therefore, some authors have argued that grandparental effects may have an epigenetic basis, which can be passed on across several generations (Whittle *et al.*, 2009; Luna *et al.*, 2012; Rasmann *et al.*, 2012). Second, theory suggests a decrease in the predictive value of environmental information in more distant generations, which implies that grandparental effects are less likely than parental effects to contribute to adaptive variation (Herman *et al.*, 2014; Leimar & McNamara, 2015). On the other hand, the environmental cues experienced over several generations may allow a more reliable prediction of the offspring environment than cues from only one generation (Herman *et al.*, 2014). Consistent with this hypothesis, empirical studies have revealed contributions of both grandparental and parental effects to offspring phenotype (Herman *et al.*, 2012; Groot *et al.*, 2016). Specifically, drought survival increased after two generations of drought exposure (Herman *et al.*, 2012), suggesting that plants can accustom to new environmental conditions over several generations.

Transgenerational effects have been found to vary among genotypes within populations (Schmitt *et al.*, 1992; Schmid & Dolt, 1994; Galloway, 2001; Holeski, 2007; Latzel *et al.*, 2014). This observation has several implications. First, it is possible to use this genetic variation to better understand how environmental signals from different preceding generations jointly influence the offspring phenotype. Disentangling the parental and grandparental effects on offspring phenotype requires experiments that consider different combinations of parental and/or grandparental treatments; performing such experiments for a range of genotypes would then allow an estimation of the genetic correlation between parental and grandparental effects (Windig, 1997). For example, a positive genetic correlation between grandparental and parental effects would indicate that genotypes show grandparental and parental effects of similar sign and comparable strength. Whereas, if no genetic correlation is found, this would indicate that these two effects trigger different phenotypes and are probably controlled via different pathways.

Second, genetic variation within populations suggests evolutionary potential (Schmitt *et al.*, 1992). Therefore, genotypes from different environments may differ in their transgenerational reaction norms, raising the question as to whether previously reported adaptive transgenerational effects observed in individual genotypes (Whittle *et al.*, 2009) are typical for a species. Genetic variation among genotypes from different sites has been observed recently for several parental environmental effects in three species (Penfield & Springthorpe, 2012; Colicchio, 2017; Lampei *et al.*, 2017). Generally, traits can diverge among genotypes from different sites for several reasons, including natural selection or mechanisms neutral to selection, such as random genetic drift (Mitchell-Olds & Schmitt, 2006). Because studies comparing the relative strength of transgenerational effects among populations are rare, little is known about how these effects diverged. However, recently, it has been shown that an adaptive parental environmental effect diverged systematically among populations, suggesting a contribution of natural selection (Lampei *et al.*, 2017). To test for systematic divergence, the reaction norm of transgenerational effects in genotypes from different sites can be correlated with environmental variables at the site of origin. This test can be enhanced by controlling for population structure (i.e. genetic relatedness among genotypes) (Price *et al.*, 2006; Kronholm *et al.*, 2012). To our knowledge, such a test of geographical association has not been conducted to date for transgenerational effects.

An ideal plant species for this study is *Arabidopsis thaliana*, which is predominantly self-fertilizing, has a short life-span, a wide native distribution range across most of Eurasia, and public sequence data exist for many ecotypes (Mitchell-Olds & Schmitt, 2006; 1001 Genomes Consortium, 2016). *Arabidopsis thaliana* predominantly exhibits a winter annual life-cycle, which is, in many regions, terminated by summer heat and drought (Wolfe & Tonsor, 2014; but see Donohue, 2009). Rising temperature in late spring, i.e. in the reproductive phase, is an environmental cue for the approaching end of the growing season. Therefore, it is not surprising that several studies have uncovered phenotypic transgenerational effects in response to mild heat (Whittle *et al.*, 2009; Suter & Widmer, 2013a,b). Heat has negative effects on all life stages in this species (Zinn *et al.*, 2010) and constitutes a potential selective agent in its natural habitat (Wolfe & Tonsor, 2014). Phenotypic differences among genotypes in *A. thaliana* are known to be partly attributable to climatic differences throughout the species range (Stinchcombe *et al.*, 2004, 2005; Fournier-Level *et al.*, 2011; Hancock *et al.*, 2011; Méndez-Vigo *et al.*, 2011; Hamilton *et al.*, 2015). Therefore, similar correlations may also be expected for the relative strength of transgenerational effects.

In this study, we systematically evaluated the transgenerational responses to mild heat stress in a set of 14 *A. thaliana* genotypes from a wide geographical range. We differentiated between three transgenerational effects, with heat exposure only in the parental, only in the grandparental or in both of these generations. The latter we named the 'two-generation effect'. Transgenerational effect sizes for each genotype were subsequently correlated among experimental groups and related to the natural ancestral environmental conditions (i.e. site of origin). In *A. thaliana*, flowering time differs strongly among genotypes (Lempe *et al.*, 2005). This complicates comparisons because heat stress effects in *Brassica* species strongly depend on the developmental stage at exposure (Gan *et al.*, 2004). Therefore, standardization of mild heat treatment at the developmental stage was essential for a valid comparison of transgenerational effects across genotypes. Because flowering time roughly matches the start of mild heat as naturally experienced in winter annuals (Wolfe & Tonsor, 2014), the treatment was started with bolting in each genotype. However, this procedure also had the potential to reduce the effects of flowering time changes on fitness estimates. Therefore, we constructed a conceptual model to test potential adaptive effects of flowering time changes. This approach had the advantage that we could apply a range of environmental scenarios, including variation in environmental correlations, across generations, a hypothesized prerequisite for the evolution of adaptive transgenerational effects (Burgess & Marshall, 2014; Ezard *et al.*, 2014; Leimar & McNamara, 2015). Hence, this approach allows for more general conclusions about environmental conditions that potentially favour the transgenerational effect on flowering time. We explicitly tested the following hypotheses: (1) transgenerational effects of mild heat are genotype specific; (2) the parental and grandparental effects show positive genetic correlations with each other and with the two-generation effect; and (3) the genotype-specific transgenerational effects of mild heat are correlated with climatic conditions at the site of origin.

Materials and Methods

Plant material

We used 14 different accessions from different environments representing a large part of the Eastern European distribution range of *A. thaliana* (L.) Heynh. (Fig. 1a; Supporting Information Table S1). Seeds were obtained from The Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/), where the accessions were propagated and maintained under uniform conditions for several generations. Because *A. thaliana* is mainly a self-pollinating species (95–99% in the native range, but see Bomblies *et al.* (2010)), and because of several previous selfing generations, these are highly homozygous lines (Mitchell-Olds & Schmitt, 2006; Hamilton *et al.*, 2015). All used accessions had been genotyped previously at high resolution (see Methods S1). In the following, we use the term 'genotype' instead of 'accession' for clarity reasons. All subsequent generations were self-fertilized.

Experimental design

For each genotype, seeds from a single founding plant were used for the start of the pedigree. To start the first generation (S1), *c.* 80 seeds were sown in a tray (18.5 × 14 × 5 cm^3) on a 1 : 1 : 2 mixture of sand, vermiculite and sieved potting soil. After stratification at 4°C for 3 d, the trays were placed for 1 wk in a climate chamber (20°C : 16°C, day : night, 16 h : 8 h, day : night and light conditions of 236 µmol m^{-2} s^{-1}) until all trays contained seedlings, and were subsequently vernalized for 3 wk (4°C day : night, 12 h : 12 h, day : night and light conditions of 101 µmol m^{-2} s^{-1}). For some genotypes (e.g. ice79, ice212, ice181 from South Tyrol), this vernalization was presumably too short as they failed to flower within the experimental period. Therefore, they were excluded from the experiment. After vernalization, 20 random seedlings per genotype (i.e. replicates) were transplanted to individual pots (diameter, 5 cm; depth, 18 cm; volume, 0.35 l) and grown on the previously described soil mixture. Pots were randomized in trays, with 13 plants per tray, to a total of 280 plants. We randomized genotypes within a tray and arranged trays in a randomized block design. All plants were placed in a climate chamber at 20° : 16°C, day : night, 16 h : 8 h, day : night and light conditions of 236 µmol m^{-2} s^{-1} (hereafter, referred to as 'control conditions'). When 90% of all plants per

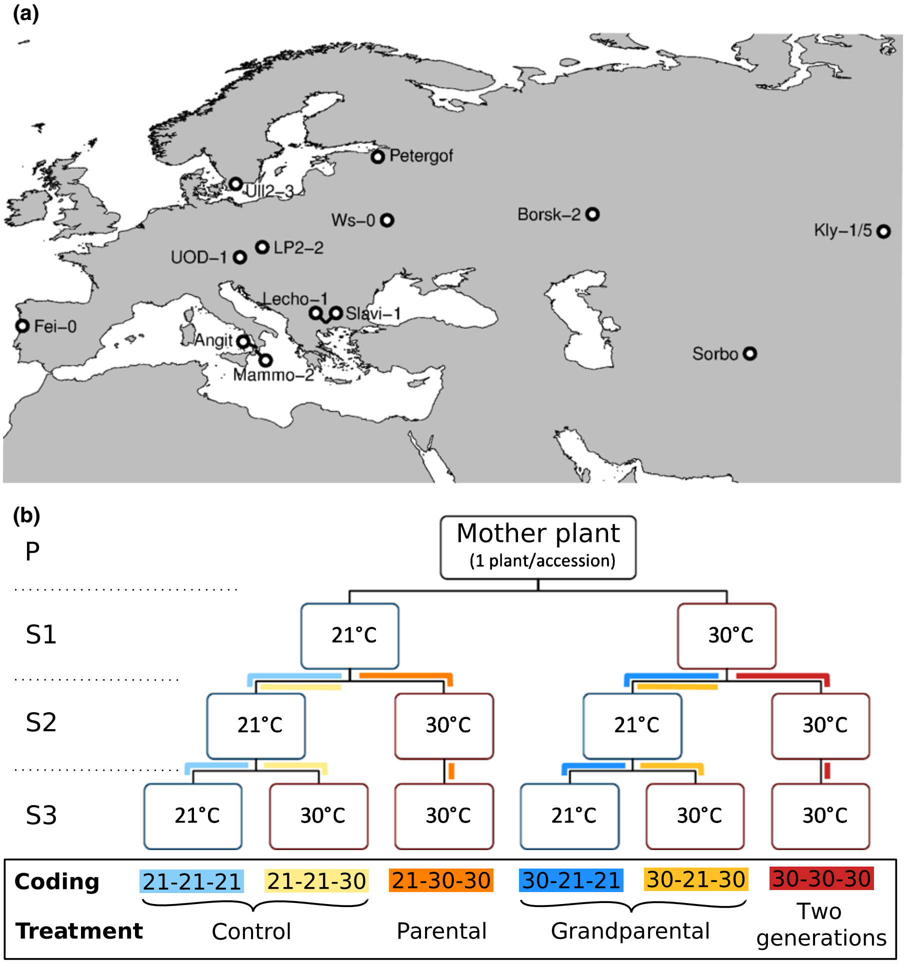

Fig. 1 (a) Collection sites of all *Arabidopsis thaliana* genotypes, with the exception of Col-0, which is from the USA. (b) Schematic of the experimental protocol across three generations (S1, S2, S3). For each *A. thaliana* accession, a single plant served as a founder for the pedigree. At each branching point, seeds of five random replicate plants per genotype were pooled and 10 offspring, raised from this pool, were allocated to each of the subsequent treatment groups. Each treatment history is colour marked, matching the colours used for treatment history identification in all later figures. Each treatment history is identified with a six-digit label consisting of the sequence of temperatures experienced and is sorted to its transgenerational treatment type. Blue colours mark S3 offspring in control conditions and yellow to red mark S3 offspring in mild heat conditions; the latter match those in Fig. 2.

genotype had started to bolt, the 20 replicates were randomly assigned to two groups (10 replicates per group). One group stayed in control conditions (21°C); the other was assigned to a mild heat treatment (30°C) in a similar climate chamber (hereafter, mild heat conditions) (Fig. 1b; S1 generation). Apart from temperature, all settings were similar to the control treatment. The plants were kept in these treatments until senescence.

On flowering, Aracons (BetaTech Bvba, Gent, Belgium) were placed over the plants to avoid cross-pollination and to prevent seed shattering. Plants were watered three times per week from the bottom with tap water. When plants had ceased flowering and started to senesce, watering was stopped and the plants were left to dry for 4–8 wk, allowing after-ripening of seeds under similar conditions. From each individual, all seeds per plant were harvested and stored in 1.5-ml reaction tubes.

For the second generation (S2), seeds from five randomly chosen plants per genotype and treatment were pooled. From these seeds, 20 seedlings were raised per parental treatment and genotype in the same manner as in the previous generation and again divided over the two treatments, control (21°C, $n = 10$) and heat (30°C, $n = 10$), when 90% of all plants per experimental treatment had started to bolt. This experimental design resulted in four experimental groups per genotype (Fig. 1b; S2 generation) with a total of 560 plants in 43 trays.

In the third generation, the procedure was repeated as in generation S2, resulting in a series of treatments across three generations (Fig. 1b), which we coded in a six-digit identifier using the ambient temperature during seed ripening in each generation (S1-S2-S3). In generation S3, because of space limitations, a full reciprocal transplant between treatments was not possible. We therefore evaluated S3 plants of all types of transgenerational effects (Fig. 1b; 21-21-30; 21-30-30; 30-21-30; 30-30-30) under mild heat treatment conditions, as we were especially interested in the offspring performance under elevated temperature. Under control conditions, we reared only plants from the control (Fig. 1b; 21-21-21) and grandparental mild heat (Fig. 1b; 30-21-21) treatment histories. This resulted in a total of six different experimental groups per genotype (Fig. 1b) with a total of 840 plants in 65 trays.

Measurements

Rosette diameter was measured 20 d after germination. Flowering time was recorded daily and calculated as the number of days from germination until opening of the first flower (all petals visible) excluding vernalization time. Total seed weight (reproductive biomass) was determined for each individual plant and used as a proxy for plant fitness. Seed size, a proxy for offspring quality, was measured by taking standardized (same distance, same object lens, same illumination) photographs of *c.* 170 seeds per S3 plant (Canon EOS 1000D camera, Canon Compact-Macro Lens EF 50 mm, 1 : 2.5; Canon, Tokyo, Japan). Customized macros in the open-source ImageJ distribution Fiji (Schindelin *et al.*, 2012) were used to identify the seeds, separate them from the background and non-seed particles (using size = 80–500 and circularity = 0.65–0.95), and measure their area in pixels.

Statistical analysis

Transgenerational effects To test for genotype specificity of transgenerational effects, we fitted a linear mixed effects model (R package NLME, v.3.1-128; Pinheiro *et al.*, 2016) for each trait of S3 plants under mild heat conditions with fixed effects of genotype and transgenerational effect (a factor consisting of 21-21-30, 21-30-30, 30-21-30 and 30-30-30) and a random effect of tray (Model 1). To infer individual genotype transgenerational effects, three predefined contrasts were extracted from Model 1 which compared S3 plants of each treatment series (21-30-30, 30-21-30 and 30-30-30) with S3 plants of the control series (21-21-30). This resulted in genotype-specific effect sizes for parental, grandparental and two-generation effects. Using the same contrasts, we also extracted average transgenerational effects. Model 1 accounted for variance heterogeneity by weighting the variance for levels of contained fixed effects, as suggested by model comparison based on the Akaike information criterion (AIC) (VARIDEN and VARCOMB functions in the R package NLME).

Pearson correlations between the genotype-specific effect sizes for parental, grandparental and two-generation effects (i.e. broad-sense genetic correlation; Windig, 1997) were calculated to investigate how transgenerational effects interact to influence the offspring phenotype. The term ‘broad-sense’ indicates that these correlations include both additive and non-additive genetic variance components (Falconer & Mackay, 1996; Windig, 1997). Further, genotypes from such a wide geographical distribution probably differ in their tolerance to mild heat. To test whether the transgenerational effects were directly related to heat tolerance, we estimated the genetic correlation between the reduction in reproductive biomass (total seed weight) caused by mild heat and the transgenerational effects. In a similar way, we approached the question as to whether the observed transgenerational effects in the traits rosette diameter, flowering time and seed size were related to transgenerational effects on fitness by estimating the corresponding genetic correlations (for details, see Methods S1).

Correlations between transgenerational effects and environmental parameters In all further analysis, we focused on transgenerational effects in flowering time, because the start of reproduction is a very important life history transition that must be well timed (Griffith & Watson, 2005; Wolfe & Tonsor, 2014) and because flowering time is one of the best-studied traits in *A. thaliana*, which allows comparisons with previous studies. Further, the high heritability of this trait (Le Corre, 2005) promises a high repeatability.

To test our hypothesis that variation in transgenerational effects among genotypes is associated with environment, we obtained geographical coordinates and bioclimatic variables from WorldClim (Hijmans *et al.*, 2005) for the collection site of each genotype (Table S1). Highly correlated variables were excluded and principal components analysis (PCA) was used to decompose climate and geographical information (Methods S1). To test whether the first PCA component (cPC1) explained the variance in the transgenerational effects on flowering time, we used linear regression. To test whether correlations with environmental variables exceeded correlations that may result purely from genetic relatedness, we extracted > 290 000 single nucleotide polymorphisms (SNPs) from public sequence data and decomposed the covariance matrix of genetic distances using principal coordinates analysis (PCoA) (Methods S1). Similar to Kronholm *et al.* (2012), the first two components of the PCoA (gPC1 and gPC2) were included in the linear regressions in the following model:

$$\text{Transgenerational effect}_{ijk} = \mu + \text{gPC1}_i + \text{gPC2}_j + \text{cPC1}_k + \varepsilon_{ijk}$$

where μ is the overall mean and ε_{ijk} is the residual. We tested the significance of regression slopes by calculating Bayesian 95% credible intervals (CrI) based on 2000 simulations with non-informative priors using the *sim* function of the R-package 'ARM' (Gelman & Su, 2014). When CrI did not include zero, the slope estimate was considered to be significant. All analyses were performed in R (v.3.3.3; R Development Core Team, 2016).

Conceptual model of parental effects on flowering time To investigate under which environmental conditions earlier flowering is an adaptive response to parental heat experience, we constructed a conceptual model to simulate the fitness consequences of transgenerational effects under different environmental conditions. The model aimed to evaluate the key aspects and was not based on our experimental data, but qualitatively informed by our results. We modelled the relation between flowering date z_i and relative fitness as a trade-off between the cost of early flowering C, which arises from the low frost resistance of flowers (Sakai & Larcher, 1987) and therefore decreases during the flowering season, and a temporally growing cost of late flowering H, which may result from increasing temperatures and heat stress over the course of the season. Based on these two cost functions, the relative fitness is then calculated as $\omega_i = (1 - C_i)(1 - H_i)$, for any individual i with flowering date z_i. In general, an individual's flowering date is determined by a combination of its genetically encoded flowering date, phenotypic plasticity and transgenerational effects. We focused on parental effects, for which we assumed that a plant transfers information about experienced conditions to its offspring, which then uses this information to adjust its own flowering time. The model reduces the genetically encoded mean flowering date by a linear function of the heat stress experienced by the mother (H_m), so that the phenotypically expressed flowering date z_i is calculated for each individual i as:

$$z_i = \alpha_i - \gamma_i H_{m,i} + \varepsilon_i \qquad \text{Eqn 1}$$

$$\varepsilon_i \sim \text{Gauss}(0, \sigma_z^2),$$

where α_i and γ_i are traits that determine the genetically encoded mean flowering date and the strength of the parental effect, respectively, and ε_i is the residual error with mean zero and variance $\sigma_z{}^2$.

We used this model to evaluate the long-term mean fitness as a function of plant traits (α, γ) and to identify optimal trait combinations for a range of environmental scenarios, focusing on simulated inter-generational variation in the timing of heat stress. Further details of the simulation model and the environmental scenarios are given in Notes S1. The data of this project is available from the Dryad Digital Repository: doi: 10.5061/dryad.km71p.

Results

Effects of offspring treatment conditions on fitness

The mild heat treatment, which started at bolting, had a strong negative effect on total reproductive biomass in all genotypes (Fig. S1a; Table S2), demonstrating the negative effect of mild heat on plant fitness. However, mild heat positively affected seed size in most genotypes (Fig. S1b; Table S2), indicating that mother plants may plastically respond to heat experience via enhanced seed provisioning. In both traits, significant genotype × S3 treatment interactions were observed, indicating that genotypes responded differently to mild heat during the reproductive phase (Table S2).

Effects of genotype and transgenerational effects

In all traits, we observed significant effects of genotype, transgenerational effects and their interaction under mild heat conditions (Table 1; Fig. 2a–d). Transgenerational effects were strong and, in flowering time, even comparable with the effects of genotype (see *F*-value in Table 1). In addition, the genotype × transgenerational effect interactions were highly significant in all traits (Table 1), indicating genetic variation in transgenerational effects of mild heat (Fig. 2a–d). The effect sizes of individual genotype included both significantly positive and negative values in all traits (Table S3).

Effect sizes for average treatment contrasts obtained from Model 1 (Table 2) showed that rosette diameter increased on average after grandparental, but decreased after parental, mild heat (Table 2). Two-generation effects on this trait were not significant. Seed size was also significantly increased by grandparental, but not by the other two transgenerational, effects (Table 2). By contrast, flowering time was significantly advanced compared with control plants by all transgenerational effects (Table 2). All of these average effects resulted from transgenerational effects in at least six genotypes (Table S2). The best fitness proxy of the four measured traits, however, is reproductive biomass. Both single-generation mild heat treatments had no effect on this trait, but the two-generation effect caused a significant increase (Table 2). This average increase in fitness under mild heat was driven mainly by three southern and one central Asian genotype (Table S3).

There was no genetic correlation between grandparental effects and parental or two-generation effects in rosette diameter, flowering time and seed size (Table 3). For example, the genotypes Angit, Kly-1/5, Lecho-1, LP2-2 and UOD-1 showed larger rosette diameters after grandparental mild heat compared with the control (21-21-30), but rosette diameter was strongly reduced

Table 1 Analysis of variance (ANOVA) table (Type I sum of squares) of the linear mixed-effects Model 1, displaying the main effects and their interaction on four traits in *Arabidopsis thaliana* under S3 mild heat conditions

Factor	Rosette diameter day 20 (mm)				Flowering time (d)				Total seed weight (mg)				Seed size (pixel)			
	numDF	denDF	*F*-value	*P*-value	numDF	denDF	*F*-value	*P*-value	numDF	denDF	*F*-value	*P*-value	numDF	denDF	*F*-value	*P*-value
Genotype	13	442	21.9	**< 0.001**	13	368	50.7	**< 0.001**	13	462	52.1	**< 0.001**	13	408	57.2	**< 0.001**
Transgenerational effect	3	442	16.0	**< 0.001**	3	368	43.7	**< 0.001**	3	462	3.8	**0.0102**	3	408	4.6	**0.0036**
Genotype × transgenerational effect	39	442	4.30	**< 0.001**	39	368	5.78	**< 0.001**	39	462	6.2	**< 0.001**	39	408	3.5	**< 0.001**

Significant *P*-values are indicated in bold.

after parental mild heat (Fig. 2). This was contrasted by significant genetic correlations in all traits among parental and two-generation effects, indicating that the parental effect dominated the phenotype when both preceding generations experienced mild heat (Table 3). Notably, an exception was observed for reproductive biomass. For this trait, all three transgenerational effects were significantly correlated, indicating that both grandparental and parental effects of mild heat contributed to two-generation effects of mild heat on fitness (Table 3).

Genotype effect sizes for transgenerational effects on all four traits were regressed on the effect sizes of fitness reduction caused by mild heat. Only parental effects on rosette diameter were significantly influenced by the negative effects of mild heat ($b = 125$, $t = 3.83$, $P = 0.002$, $R^2 = 0.55$). Specifically, genotypes that showed strong fitness reduction under mild heat exhibited reduced rosette diameter at day 20 following parental mild heat exposure. By contrast, genotypes with small fitness reduction showed enlarged rosette diameters as a parental effect. This indicates that there was a direct influence of detrimental effects of mild heat on the offspring in some genotypes, but this prevailed only in early development.

Similarly, genotype effect sizes for transgenerational effects on rosette diameter, flowering time and seed size were regressed on the effect sizes of transgenerational effects of mild heat on reproductive biomass to test their potential to contribute to a fitness gain. No association was observed for seed size. Transgenerational effects on rosette diameter showed a positive association with transgenerational effects on fitness ($b = 0.0015$, df = 1/38, $F = 10.44$, $P = 0.002$), and those on flowering time showed a negative association with transgenerational effects on fitness ($b = -0.005$, df = 1/37, $F = 6.24$, $P = 0.014$), after removal of an extreme outlier (Lecho-1, 30-21-30; see Fig. 2b; Table S3). Linear regressions for individual transgenerational effects were positive significant (rosette diameter: $b = 0.0015$, $t = 2.29$, $P = 0.041$) and negative marginally significant (flowering time: $b = -0.006$, $t = -2.12$, $P = 0.056$) only for two-generation effects. These regressions indicate that genotypes which were accustomed to mild heat over two generations were characterized by a transgenerationally increased rosette diameter and accelerated flowering.

Effects of offspring environment on grandparental effects

Two treatment series, control (21-21-21, 21-21-30) and grandparental (30-21-21, 30-21-30) mild heat, were tested in two different offspring environments, under control and mild heat S3 conditions (Fig. 1b). Because mild heat started only with bolting, the different environments were only relevant for the late-developing traits seed size and reproductive biomass. For seed size, grandparental effects did not differ among offspring treatment conditions because both the S3 treatment × grandparental effect interaction and the three-way interaction (including genotype) were not significant (Table S2 (Model 2)). For reproductive biomass, the interaction of S3 treatment × grandparental effect was not significant, indicating that the average effect of the grandparental mild heat was similar in both offspring environments. However, in this trait, the three-way interaction was

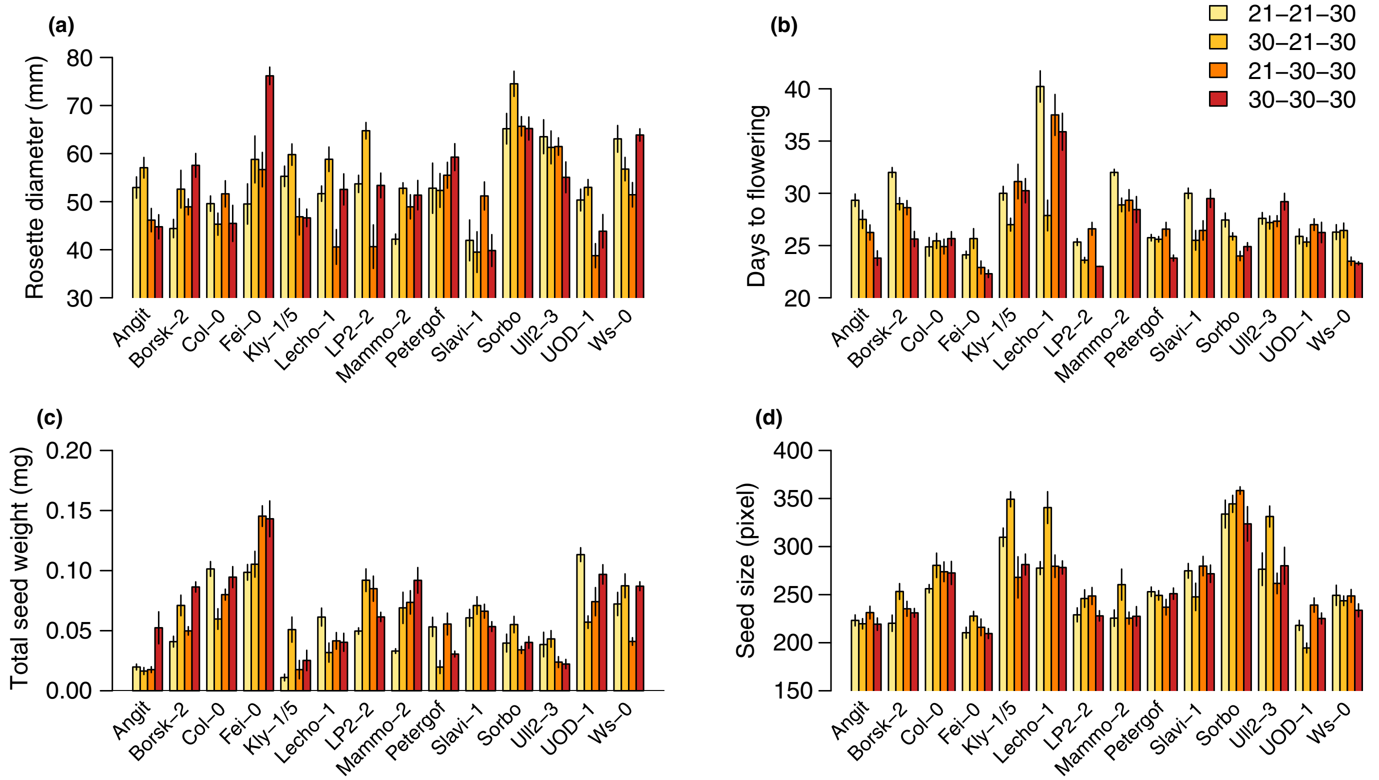

Fig. 2 Average (± SE) phenotypes of S3 *Arabidopsis thaliana* plants, illustrating the effects of different histories of mild heat exposure on the four traits, (a) rosette diameter, (b) flowering time, (c) reproductive biomass and (d) seed size, in 14 genotypes reared under mild heat conditions. The different histories of mild heat exposure are given by a colour code and three-digit identifier that match those used in Fig. 1(b). They identify the histories with 'no mild heat exposure' (21-21-30), grandparental (30-21-30), parental (21-30-30) and two-generation mild heat exposure (30-30-30).

Table 2 Effect sizes of linear mixed-effects Model 1 for treatment contrasts with the control plants averaged across *Arabidopsis thaliana* genotypes

	Rosette diameter day 20 (mm)		Flowering time (d)		Total seed weight (mg)		Seed size (pixel)	
Transgenerational effect	Effect size	*P*-value	Effect size	*P*-value	Effect size	*P*-value	Effect size	*P*-value
Grandparental effect	**4.13**	**< 0.001**	**−1.13**	**< 0.001**	0.0021	0.88	**10.4**	**0.005**
Parental effect	**−3.01**	**0.02**	**−0.79**	**0.008**	0.0004	1.00	5.56	0.22
Two-generation effect	0.004	1.00	**−2.27**	**< 0.001**	**0.0088**	**0.02**	−1.28	0.96

Significant effect sizes ($P < 0.05$) are indicated in bold.

significant (Table S2), indicating that at least some individual genotypes deviated from the average, although not strongly (Fig. S1a; Table S4). Therefore, in both traits, offspring environmental conditions had little influence on the expression of grandparental effects.

Grandparental effects on flowering time

The effect sizes (Model 1) of the grandparental effect on flowering time correlated strongly with the flowering time of control plants ($r = -0.95$, $t = -10.0$, df = 12, $P < 0.001$, Fig. 3a), but not with the flowering time of plants whose grandparents had experienced a mild heat treatment ($r = -0.39$, $t = -1.5$, df = 12, $P = 0.16$; Fig. 3b), indicating that genotype difference in the first, but not in the latter, predominantly influenced the effect sizes. In other words, grandparental mild heat exposure triggered earlier flowering only in late-flowering genotypes (Fig. 3), leading to nearly simultaneous flowering time across genotypes. The correlation with control plant flowering time remained significant and strong when the outlier genotype (Lecho-1) was excluded ($r = -0.85$, $t = -5.37$, df = 11, $P < 0.001$). None of the other transgenerational effects of mild heat induced such simultaneous flowering.

Correlations with geographical and climatic variables

In the PCA, the first two principal components captured 57% and 24% of the variance in climatic and geographical variables (cPC1 and cPC2; Fig. S2 and Table S5 for loadings of the first two axes). The highest loadings of the first axis were the mean

Table 3 Pearson correlations among effect sizes of transgenerational effects of mild heat in *Arabidopsis thaliana*

	Grandparental × parental		Grandparental × two generations		Parental × two generations	
	r	P	r	P	r	P
Rosette diameter day 20	0.034	0.91	0.43	0.13	**0.53**	**0.049**
Flowering time	0.36	0.21	0.35	0.21	**0.62**	**0.016**
Seed size	−0.33	0.26	0.06	0.85	**0.59**	**0.025**
Total seed weight	**0.67**	**0.009**	**0.63**	**0.015**	**0.66**	**0.011**

Significant terms are indicated in bold.

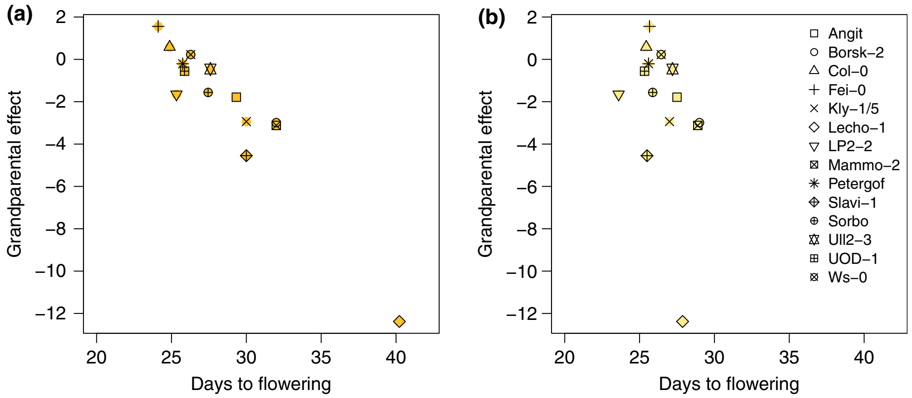

Fig. 3 Correlation between the effect size of grandparental mild heat response in *Arabidopsis thaliana* genotypes (i.e. best linear unbiased estimators, displaying the number of days 30-21-30 plants flowered earlier than control plants (21-21-30)) and (a) the average flowering time of the control plants (21-21-30) or (b) the average flowering time when the grandparents had been exposed to mild heat (30-21-30). For control plants, a strong correlation was observed ($r = -0.95$, $t = -10.0$, df = 12, $P < 0.001$), which was significant even without the outlier Lecho-1 ($r = -0.85$, $t = -5.37$, df = 11, $P < 0.001$). For second-generation offspring of grandparents that had experienced mild heat, no correlation was observed ($r = -0.39$, $t = -1.5$, df = 12, $P = 0.16$). Point colour matches the respective treatment colour in Fig. 1(b).

temperature of the driest quarter (BIO9, 0.5) and precipitation of the warmest quarter (BIO18, −0.51). The first two components of the genomic PCoA used to correct for population structure (gPC1, gPC2) together explained 60% of the total genomic variation (Fig. S3). In linear regressions including gPC1, gPC2 and cPC1, the climatic cPC1 was not significant for control flowering time ($b = 0.28$, CrI 95%: −1.63, 2.28; Fig. 4a) or flowering time effect sizes for grandparental ($b = -0.42$, CrI 95%: −2.06, 1.19; Fig. 4b) and two-generation ($b = -0.36$, CrI 95%: −1.42, 0.74; Fig. 4d) effects of mild heat. On the contrary, cPC1 was significantly associated with the parental effect ($b = -0.72$, CrI 95%: −1.40, −0.11; Fig. 4c) when accounting for population structure. With cPC1 in the model, both genomic covariates included zero in their 95% CrIs (i.e. not significant). This changed for gPC2 when cPC1 was absent ($b = 7.75$, CrI 95%: 0.258, 15.27), indicating that climate at the site of origin was a better predictor than genetic relatedness of the parental effect on flowering time. Notably, gPC1 and gPC2 clustered genotypes approximately by geographical proximity (Fig. S3). The small effect of population structure on climate regression may be partly a result of the well-distributed genotype sample, which can be seen from the star-like neighbour-joining tree with few clusters (Fig. S4).

The two climatic variables with strongest predictive power after false discovery rate (FDR) correction (Benjamini & Hochberg, 1995) across all climatic and geographical variables were the mean temperature of the driest quarter (BIO9) and precipitation of the warmest quarter (BIO18) (Table S6). The parental effect was significantly associated with BIO9 ($b = -0.012$, CrI 95%: −0.021, −0.004; Fig. 4e). As the unit of BIO9 is °C × 10, this means that the offspring of mild heat-treated plants advanced flowering on average by 1.2 d with every 10°C difference in mean temperature of the driest quarter. For BIO18, the slope was only marginally significant ($b = 0.015$, CrI 90%: 0.007, 0.029; Fig. 4f). Also, these regression models included gPC1 and gPC2.

Conceptual model of parental effects on flowering time

We used a conceptual model (Fig. 5a,b) to evaluate the theoretical environmental conditions that favour the observed parental effect of mild heat that accelerated flowering. The model predicted that this parental effect is disadvantageous when there is no temporal autocorrelation in the timing of heat stress (optimal parental effect strength $\gamma = 0$; Fig. 5c), but advantageous if parent and offspring environment are correlated (Fig. 5d). The model evaluated potential fitness advantages from transgenerational effects over a wide range of environmental scenarios, and demonstrated that these advantages become larger, not only for higher temporal autocorrelation, but also for shorter flowering seasons

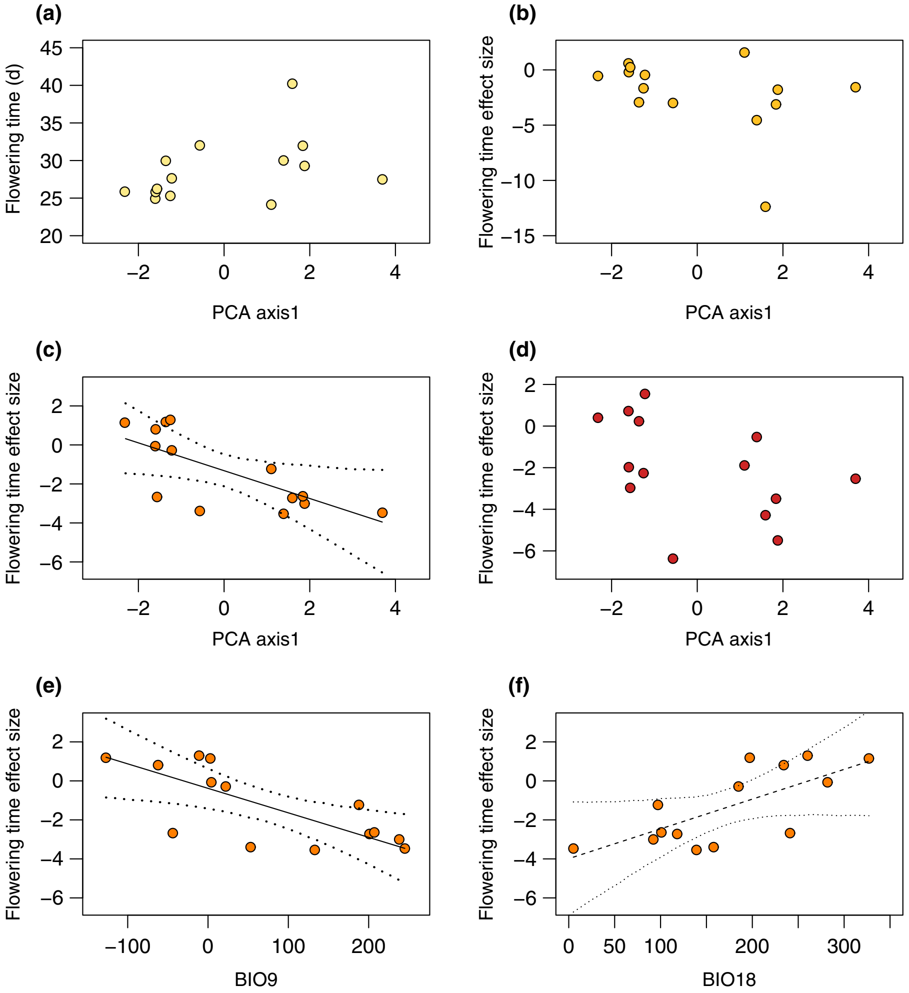

Fig. 4 Linear regression of control flowering time (a) and effect sizes of transgenerational effects (b, grandparental; c, parental; d, two-generation mild heat) on the climate PC1 in *Arabidopsis thaliana*. For the parental effect, regressions on the mean temperature in the driest quarter (e) and precipitation in the warmest quarter (f) are also displayed. Slopes are shown for significant ($P > 0.05$, solid line) and marginally significant ($P > 0.1$, dashed line) regressions, together with the Bayesian 95% credible interval from 2000 simulations (dotted line). All regressions included the first two components of the principal coordinates analysis (PCoA) of genomic differentiation to correct for population structure (see Supporting Information Fig. S3). Point colour indicates the treatment series (a) or the treatment series which is compared with the control treatment series (21-21-30, see Fig. 1b).

with, on average, an earlier onset of heat stress (Fig. 5e). In addition, the genetically encoded (i.e. fixed) fraction of the flowering phenotype increased in model results with increasing autocorrelation (Fig. S5a), indicating that the parental effect buffered the selection on early flowering.

Discussion

Transgenerational effects of mild heat are genotype specific

Transgenerational effects of mild heat were common in all genotypes and traits, illustrating that they constitute an important source of phenotypic variation (Uller, 2008; Dyer *et al.*, 2010). Beyond the principal ability of mild heat to induce transgenerational effects in *A. thaliana* (Whittle *et al.*, 2009; Suter & Widmer, 2013a,b), little was known about how these effects vary among diverse genotypes. In all traits, significant interactions between genotype and transgenerational effects were observed, indicating strong genotype specificity. Theoretically, these transgenerational effects could be a consequence of detrimental effects of mild heat on the parental phenotype. In this case, the transgenerational effect should be associated with the genotype-specific mild heat tolerance, defined as the effect of mild heat on plant fitness. Notably, only one such association was observed with parental effects on the developmental earliest trait: rosette diameter. Later traits were not affected. The genotype specificity of transgenerational effects was previously mostly observed within populations, i.e. among genotypes from the same site (Schmitt *et al.*, 1992; Schmid & Dolt, 1994; Galloway, 2001; Holeski, 2007; but see Penfield & Springthorpe, 2012; Colicchio, 2017; Lampei *et al.*, 2017). Our study focused on genotypic variation between sites, and therefore suggests evolutionary divergence of transgenerational effects among genotypes from distant geographical sites. However, as we included only one genotype per site, this is not equivalent to a test of population divergence. Nevertheless, the observation of strong genotype specificity of transgenerational effects is an important insight, not least for future studies seeking to test generality across a set of species (Germain & Gilbert, 2014) in which case, after our results, the inclusion of more than one genotype per species is recommended.

Parental effects overrule grandparental effects in most traits

The contribution of parental, grandparental and two-generation effects to phenotypic variation varied strongly among traits. This matches observations in a multi-generation experiment on

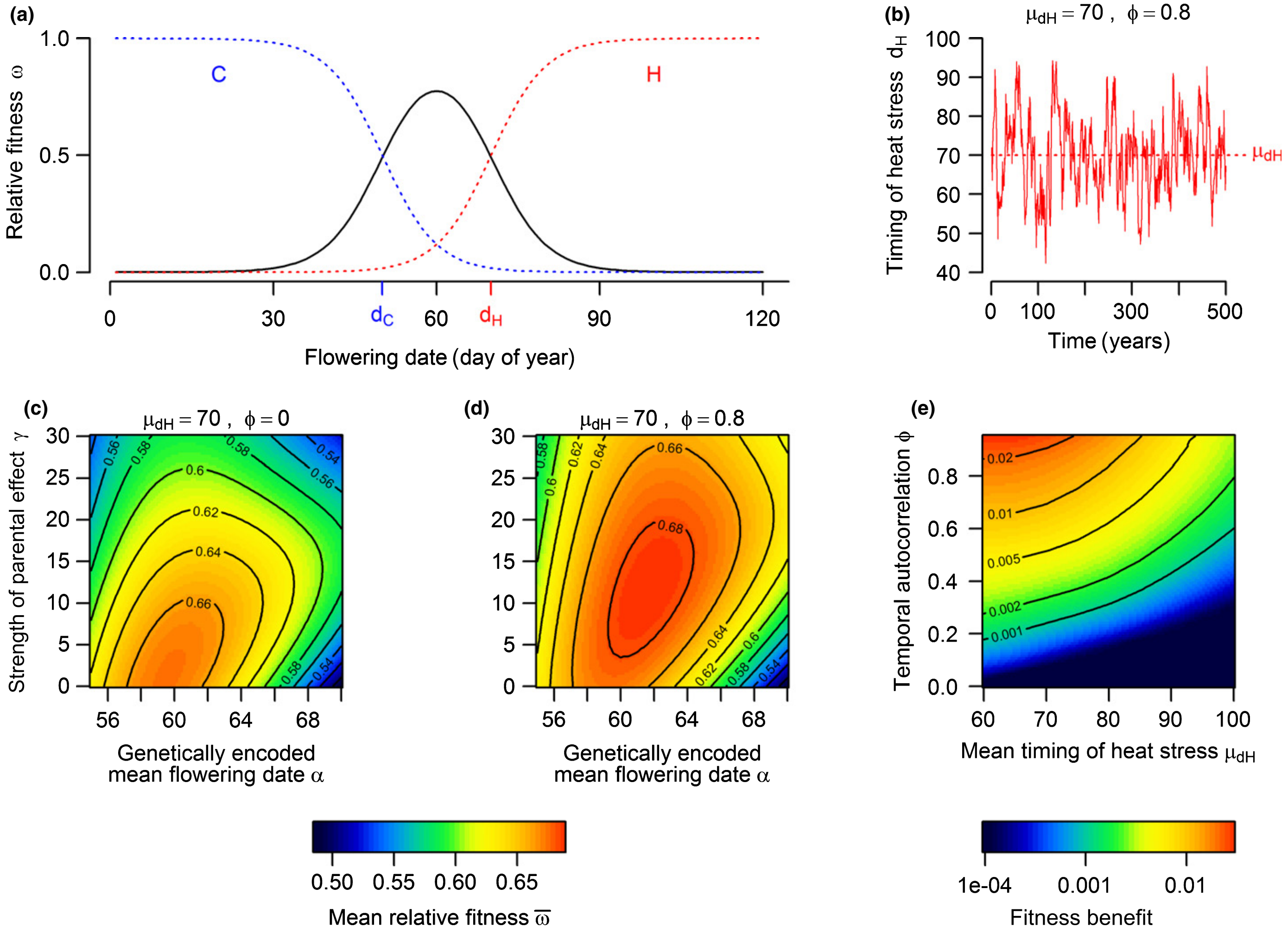

Fig. 5 Conceptual model for the investigation of the fitness consequences of parental effects in flowering schedules. (a) Relative plant fitness as a function of flowering date results from climate-dependent costs of early (C) or late (H) flowering in the season. (b) An example of an environmental scenario of inter-seasonal variation in the timing of heat stress d_H. Scenarios vary in the mean μ_{dH} and temporal autocorrelation ϕ, with constant variance $\sigma^2_{dH} = 100$. (c) Long-term mean plant fitness as a function of genetically encoded mean flowering date α and strength of parental effect γ in an environmental scenario with no temporal autocorrelation $\phi = 0$ and (d) with high temporal autocorrelation $\phi = 0.8$. (e) Potential fitness benefit from parental effects across environmental scenarios that vary in the mean timing of heat stress μ_{dH} and the degree of temporal autocorrelation ϕ. Fitness benefit from parental effects calculated as the difference between the mean fitness for optimal trait combinations and the mean fitness for optimal α in the absence of transgenerational effects ($\gamma = 0$).

transgenerational effects of salt stress (Groot *et al.*, 2016). Notably, reproductive biomass increased by two-generation effects, but not by grandparental and parental effects. When applying a published 1000-kernel weight for *A. thaliana* (Török *et al.*, 2013), the gained average reproductive biomass translates to 425 seeds, which is between 6% and 25% gain in seed number compared with the most productive and least productive genotypes with control treatment history (21-21-30). This observation in *A. thaliana* corroborates a similar effect in *Polygonum persicaria*, which was accustomed to drought over two generations (Herman *et al.*, 2012). For reproductive biomass, we observed strong genetic correlations between all transgenerational effects, indicating that both grandparental and parental effects contributed to the two-generation effect. In the other measured traits, only parental effects showed a significant genetic correlation with two-generation effects. Parental effects therefore 'overruled' grandparental effects in morphological or phenological traits. This observation corresponds to the greater possibility of parents affecting the phenotype of their offspring (Herman & Sultan, 2011). Increased rosette diameter and accelerated flowering were associated with transgenerationally increased reproductive biomass, and are therefore potentially adaptive. However, because they contributed to two-generation effects only via parental effects, we assume that grandparental effects contributed to the increased fitness after two generations of mild heat through traits which were not monitored in our experiment.

Grandparental effects are strong and frequent

Grandparental effects of mild heat were frequent in our experiment and triggered strong phenotypic responses that persisted across offspring environments. This is remarkable because grandparental effects may be less beneficial than parental effects (Herman & Sultan, 2011). Several authors have argued that grandparental effects must have an epigenetic basis as they are conserved over two generations (Whittle *et al.*, 2009; Luna *et al.*, 2012; Rasmann *et al.*, 2012). However, our results suggest that this may not always be the case. Instead, an environmental trigger in the grandparental generation may, in the form of a parental effect, condition the response of the parent to its own environment. For example, rosette diameter and seed size, the latter trait showing grandparental effects most frequently, were, on average, positively affected by grandparental effects, but little (seed size) or even negatively (rosette diameter) by parental effects. In other words, after S1 plants experienced mild heat, their offspring (S2) were prepared for the same 'stress' which, however, did not follow. As a consequence, they used the mobilized resources to 'over'-provision their offspring (S3). This is in line with a complex model of phenotypic continuity across generations (Badyaev & Uller, 2009), in which the plant's perception of its environment partly depends on the environment of the parents. These results present a challenge to unravel pure grandparental effects that are supposed to be conserved through a 'reset' generation. Instead, they suggest that the plant's perception of the 'control' environment may change after their predecessors experience a stressful environment.

However, grandparental effects are diverse. Although, in some traits, they do not seem to be conserved across generations, there is evidence that they are conserved in other traits. For example, the grandparental effect on flowering time accelerated bolting only in late-flowering genotypes. This caused genotypes to flower almost simultaneously. A similar effect on flowering time was observed as a within-generation plastic response to mild heat in *A. thaliana* (Balasubramanian *et al.*, 2006; Lutz *et al.*, 2017). This similarity between within-generation plasticity and the grandparental effect of mild heat on flowering time indeed suggests that the observed grandparental effect was conserved across an intermediate generation with lower ambient temperatures. In conclusion, an understanding of grandparental effects requires careful experimentation and possibly comparison with within-generation plasticity or the manipulation of epigenetic properties (Herman & Sultan, 2016).

Genotype-specific parental effects correlate with climate at origin

All transgenerational effects on flowering time showed significant negative average effects, indicating accelerated flowering. This corroborates earlier observations of transgenerational effects of diverse heat treatments in *A. thaliana* (Whittle *et al.*, 2009; Suter & Widmer, 2013a,b; Migicovsky *et al.*, 2014), suggesting that this effect has high repeatability. We further found that genotype-specific parental effects of mild heat were better explained by climate at origin than by genetic relatedness, indicating the contribution of past selection. This observation from *A. thaliana* corroborates the related observation of clinal population divergence in a parental effect on seed dormancy in *Biscutella didyma*, which suggests the contribution of natural selection (Lampei *et al.*, 2017). However, grandparental and two-generation effects on flowering time were not correlated with climate at origin, consistent with the lack of a genetic correlation between parental and grandparental effects. A parsimonious explanation for this discrepancy is that the parental effect constitutes an example of convergent evolution, in which different genotypes evolved a similar parental effect via mutations in different genes. Some of these paths may be conserved across a 'reset' generation and some not. Notably, convergent evolution has been suggested recently for the adaptation of *A. thaliana* to warm climates (Monroe *et al.*, 2016). This is especially interesting because the strongest correlated climate variable was 'mean temperature in the driest quarter' (BIO9), indicating that genotypes from regions with higher temperatures in the driest quarter accelerated flowering after parental heat. Although these average climatic variables are rather imprecise, they are suitable for the description of larger patterns of variation. BIO9 differentiated in our data between temperate climates, which have dry winters, and climates with dry summers (Peel *et al.*, 2007). Therefore, the parental effect on flowering time was observed in genotypes from sites in which the growing period is terminated by summer drought, which is an environmental constraint imposing strong selection on flowering time in *A. thaliana* (Wolfe & Tonsor, 2014). Temperatures during seed development provide a cue for correcting the match between parental phenology and parental environment in offspring, given that parental and offspring environments are correlated (Burgess & Marshall, 2011). It therefore seems that the divergence of genotypes in this parental effect is not a result of random processes; rather, it is favoured in sites that face a strict termination of their growing season as a result of summer drought.

In agreement with this interpretation, the conceptual model showed that a short growing period with early onset of rising temperatures favoured the parental effect on flowering time. Further, corroborating earlier, more comprehensive theoretical models for adaptive transgenerational effects (Hoyle & Ezard, 2012; Ezard *et al.*, 2014; Leimar & McNamara, 2015), our model shows that a correlation between parental and offspring environments favours parental effects. Here, correlations, even at intermediate level, were sufficient to favour the parental effect on flowering time. Notably, our model revealed that parental effects were disadvantageous when parent and offspring environment were not correlated, indicating that this scenario would select against the observed parental effect. What our model did not include was within-generation plasticity to mild heat, as we did not aim to test the relative importance of different types of plasticity with this model. In a more general theoretical model, within-generation plasticity and transgenerational effects were found to work together to shape well-adapted phenotypes (Ezard *et al.*, 2014).

Our findings are also interesting because, in a directionally changing environment, such as with climate change, the assumption of a correlation between parental and offspring environment is met. Therefore, if the trend in climate change with increasing summer temperatures and decreasing summer precipitation over the last 50 yr (Toreti *et al.*, 2009) proceeds as predicted for the Mediterranean (IPCC, 2007), genotypes from this region already possess parental effects on flowering time that can contribute to the buffering of this environmental change. Indeed, pronounced changes in plant phenology, including earlier flowering of winter annual plants, have been observed for the Iberian Peninsula (Peñuelas *et al.*, 2002). Possibly, also parental effects, such as those observed in our study, contributed to this trend. Notably, climate change predictions differ for more northern regions of our study.

Our results provide a first insight into genotype divergence of transgenerational effects caused by mild heat. As this study included only one genotype per population, we cannot draw conclusions on the evolutionary potential within populations. Therefore, more research is needed, with experiments including several genotypes per population and testing effect sizes in natural environments, also including within-generation plasticity.

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Author contributions

M.P.G., C.L., N.J.O. and K.J.S. conceived and designed the study; M.P.G. conducted the experiment with the help of P.V.; M.P.G. and C.L. performed the statistical analysis of the experimental results; A.K., C.L. and J.P. conceived the conceptual model; J.P. implemented the model, and performed the statistical analysis of the model results; M.P.G. and C.L. wrote the first version of the manuscript, with all other authors, in particular P.V., contributing to revisions.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Seed weight and seed size for control and grandparental mild heat in two offspring environments.

Fig. S2 Biplot of the principal components analysis (PCA) of climate and geographical variables.

Fig. S3 Genetic relatedness displayed as a biplot of the first two components of the principal coordinates analysis (PCoA).

Fig. S4 Neighbour-joining tree for genotypes in this experiment.

Fig. S5 Optimum plots for the genetically encoded mean flowering time (α) and the parental effect (γ).

Table S1 Geographical coordinates and bioclimatic variables

Table S2 Analysis of variance (ANOVA) table of Model 2

Table S3 Effect sizes of Model 1

Table S4 Effect sizes of Model 2

Table S5 Loadings on the first two components of the climate principal components analysis (PCA)

Table S6 Correlations between environmental variables and the parental effect on flowering time

Methods S1 Additional methods as referenced in the main text.

Notes S1 Detailed description of the conceptual model.

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