Hindawi
Journal of Botany
Volume 2018, Article ID 7836845, 10 pages
https://doi.org/10.1155/2018/7836845

Research Article

Experimental Treatment with a Hypomethylating Agent Alters Life History Traits and Fitness in *Brassica rapa*

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Received 14 June 2018; Revised 26 September 2018; Accepted 22 October 2018; Published 1 November 2018

Academic Editor: Jutta Ludwig-Müller

Epigenetic modifications to DNA can be inherited and may play a key role in evolution, with epigenetic influences on life history traits such as the timing of germination and flowering thought to be particularly important in plants. However, few studies have examined the effects of epigenetic modifications through experimental alteration of the methylome under differential growth conditions. In this study, we chemically induced global DNA hypomethylation using 5-azacytidine in three *Brassica rapa* plant populations differing in life history characteristics and under differing photoperiod treatments. We found that our 5-azacytidine treatment affected the timing of germination and that this effect differed across populations, with the treatment delaying germination in *B. rapa* Fast Plants, which have been artificially selected for rapid cycling, but accelerating germination in biennials. Rapid cycling *B. rapa* plants also were smaller and had lower reproductive fitness under the experimental demethylation treatment. There was no main effect of demethylation or photoperiod on flowering time, but the interaction was marginally significant, indicating that demethylation effects on flowering time depend on photoperiod. These results demonstrate that epigenetic modifications can influence phenotypic traits in ways that are dependent on genetic identity, life history, and light availability.

1. Introduction

Understanding the mechanisms of evolution in natural populations is a fundamental goal in evolutionary biology and is critical for understanding how species can adapt to global change [1]. There is increasing recognition that evolution can occur through mechanisms beyond base-pair changes [2]. Epigenetic modifications, such as methylation of cytosines and histones, can alter gene expression, impact phenotypes and fitness, and can also be inherited [3–7]. For plants in particular, methylation changes to DNA and histones can have wide-ranging effects [8]. However, the specific role that methylation plays in regulation of ecologically relevant phenotypes is still being explored [9–11].

The term epigenetics refers to the heritable set of molecular processes that impact an organism's phenotype without changes to the DNA sequence, including primarily DNA methylation and histone modification [8]. DNA methylation, whereby the 5′ carbon of the cytosine aromatic ring gains a methyl group through the action of DNA methyltransferase, appears to be an important factor in the regulation of gene expression [8, 12, 13]. Methylation is important in controlling transposable elements (TEs) in several species [14–16] and regulating expression of subgenomes in polyploid species [17]. In addition, widespread accessions of Arabidopsis have shown a wide range of methylation patterns, and geographic specificity, indicating that methylation may be important at the population scale [8]. Further, methylation has been linked to local adaptation in Arabidopsis across Europe [18]. However, distinguishing DNA sequence and epigenetic evolution remains an experimental challenge outside Arabidopsis, and it remains unknown how methylation changes will play a role in adaptation to global change.

One of the primary responses to global change seen in a wide variety of species is a shift in the timing of life history events such as flowering time and other phenological traits [19, 20]. The degree to which these phenotypic responses represent adaptive evolution or phenotypic plasticity remains

an active area of investigation, with evidence for both evolution and plasticity found in several studies (reviewed in [21]). Previous work has shown evidence for adaptive evolution in flowering time and other traits in *Brassica rapa* (field mustard) in response to drought [22]. A recent study of these same populations showed extensive changes in allele frequencies across the genome, with some of the evolutionary changes in genes linked to flowering time and drought response [23]. However, it remains unknown if epigenetic changes could also have contributed to this rapid evolutionary shift in flowering time, which is a trait known to be influenced by epigenetic modifications [24]. *Brassica rapa* has also evolved via artificial selection, which was used to create rapid cycling fast plant cultivars that flower in only 14 days [25]. This life history pattern contrasts with biennial *B. rapa* varieties, which take two years, including a period of vernalization, to initiate flowering. This species is prime for this research as a previous study confirmed with Methylation-Sensitive Amplified Polymorphism profiling that treatment with 5-azacytidine results in altered DNA methylation in *Brassica rapa* [26]. They found that in some *B. rapa* populations, the 5-azaC demethylation treatment altered plant size, and this change in size was heritable, but they did not examine life history changes or environmental interactions.

One approach to studying epigenetics is to map out existing methylation patterns in the genome and compare these patterns across populations and species [27]. This method is fruitful, but it is resource intensive as it requires high-resolution reference genome assemblies and bisulfite sequencing methods to generate many individual methylomes, and it is descriptive rather than experimental. Another approach to directly assess responses of organisms to epigenetic modifications is to experimentally alter the methylome.

An agent commonly used in this experimental approach is 5-azacytidine (5-azaC), which has been consistently shown to alter nucleotide methylation patterns across the genome [28–30]. 5-Azacytidine blocks the active site of DNA methyltransferase enzymes which confer methylation patterns to the novel strand during DNA replication, inhibiting the methylation transferral process [28]. Administration of 5-azaC at the pregermination stage has been shown to directly reduce 5-methylcytosine content in the genomic DNA of mature plants [31] as well as induce heritable phenotypic change [32]. However, it is important to note that when used at high concentrations, 5-azaC is incorporated into DNA and RNA causing cell death and not necessarily DNA demethylation, so due caution must be used in the interpretation of results generated from this methodological approach [33]. Despite the rather crude nature of this method, it is nevertheless advantageous because it allows researchers to quickly and easily generate populations that are genetically identical but epigenetically variant and perform manipulative experiments with these lines.

Previous studies have found that 5-azaC induced demethylation altered development rates and flowering time, two particularly important life history traits related to climate adaptation, in *Arabidopsis thaliana* [12], *Linum usitatissimum* [34], and *Thlaspi arvense* [35]. Furthermore, it has been shown that 5-azaC treated *Arabidopsis thaliana* presents not only DNA demethylation, but altered expression of FLC and FWA, two genes responsible for plant flowering [36].

In this study, we experimentally altered the methylome of *B. rapa* varieties that differed in life history characteristics. We then grew these populations under differing photoperiod conditions, as it has previously been shown that DNA demethylation via 5-azaC can impact flowering under differing light conditions [31]. We investigated the effects of this methylation treatment and photoperiod on the timing of germination and flowering, as well as on traits such as size and growth rate. We predicted that if methylation patterns are important for determining responses of plants to environmental signals that influence the timing of germination and flowering, the demethylation treatment would alter these traits and would have differential effects on populations with different life history characteristics. We further predicted that demethylation might induce flowering under conditions in which flowering normally does not occur, including under short days and in biennials before vernalization.

2. Methods

2.1. Study System. The study species is *Brassica rapa L. (Brassicaceae)*, commonly known as field mustard. The species originated in the Middle East, but has spread widely throughout Europe, North America and Asia and has become naturalized in many areas. In agriculture, *B. rapa* has been cultivated to create a number of vegetables such as turnips, bok choy, and Chinese cabbage. *B. rapa* is also used extensively in laboratory research. Fast Plant cultivars have been artificially selected to undergo a full lifecycle in 30-40 days [25].

We studied three populations of *B. rapa*. Two of the populations are annual plants that flower within one year and do not require a vernalization treatment to flower. These are a Fast Plant cultivar from the Wisconsin Fast Plants Program and a naturalized population collected from the Back Bay site in the Upper Newport Bay Nature Preserve in Newport Beach, California in 2008 (permit #19699-21901 from the UC Reserve System RAMAS). The third is an agricultural population from the Netherlands that is biennial (flowering once every two years) which was obtained from the USDA National Genetic Resources Program, via the Germplasm Resources Information Program (GRIN). This cultivar normally requires a vernalization treatment to flower, but plants were not vernalized in this study to determine if hypomethylation could replace vernalization.

2.2. Experimental Design. We used a full factorial design, with hypomethylation (demethylated or control), photoperiod (24hr or 8 hr), and population (California, Fast plant, or biennial) as factors. For each population, we randomly assigned 80 plants to one of each combination of methylation treatment and photoperiod, with 20 replicates for each.

For hypomethylation, we used the global demethylating agent 5-azacytidine (5-azaC), which is an effective inhibitor of methyltransferase, the enzyme that methylates newly synthesized DNA [29, 37]. We cold-stratified 100 seeds from each of our three *B. rapa* populations (annual Californian, annual Fast Plant, and biennial Dutch) at 1-4°C in petri dishes.

We placed half of each population on filter paper soaked with 1.4mL-distilled water, and half on filter paper with 1.4mL of a 500μmol 5-azaC solution (a concentration on the high-end of the spectrum used in previous studies using 5-azaC for demethylation; [12]). After five days of cold-stratification, we moved the plants to room temperature to germinate.

Once the seeds germinated, we transplanted seedlings into 6.5 cm^2 pots in trays positioned in temperature and light-controlled growth chambers so that seedlings were 5-10cm below the fluorescent lights, as per Fast Plant protocol (www.fastplants.org). We watered plants daily and fertilized twice per week with balanced NPK (20-20-20) fertilizer. We kept the growth chambers at 22°C.

We recorded the dates of first observed budding, flowering, and senescence. To assess plant size, we noted the number of leaves and measured the length and width of the longest leaf at budding and flowering. Plants that did not flower by the end of the experiment had leaf number and size traits recorded at 6 weeks after germination.

2.3. Statistical Analysis. We determined the effects of hypomethylation treatment and population for time to germination with an ANOVA using SYSTAT v. 13 (Systat, Chicago, IL, USA). We further assessed differences between the treated plants and controls within each population using planned orthogonal contrasts in R version 3.2.2 (R Core Team 2014). We did not assess photoperiod for germination because all seeds were germinated under identical conditions of 24 hours/day of light. We transformed data as needed to meet model assumptions. We assessed the effects of the hypomethylation treatment, photoperiod, and variety on plant size characters using ANOVA. As the Fast Plant variety was the only population to flower with a sufficient sample size, and the biennial size data was collected at a different time than the Fast Plants, we also performed separate ANOVAs for all measured traits within these groups. Results were considered significant for $p < 0.05$ and marginally significant for $0.05 < p < 0.10$. We confirmed results for all traits related to timing (time to germination, flowering time) with survival time analysis using a Cox proportional hazards model.

3. Results

3.1. Germination. Both the Fast Plants and biennials germinated at relatively high rates (~80% each), while germination rate was low for the California population (15%). Fast Plants were the earliest to germinate, followed by the biennials and then the California population (Figure 1).

Populations differed significantly in germination time ($p < 0.001$), but there was no significant main effect of 5-azaC on germination time ($p = 0.968$) (Table 1). However, we found a significant interaction between population and 5-azaC treatment ($p = 0.002$). Planned orthogonal contrasts showed that demethylation significantly increased time to germination in Fast Plants ($p < 0.05$), with demethylated plants germinating about a day later than control plants, and marginally significantly decreased time to germination in for Biennials ($p = 0.0557$), with control plants flowering less than a day later than treated plants (Table 1; Figure 1). There was no significant effect of the 5-azaC treatment on germination in the California population ($p = 0.487$).

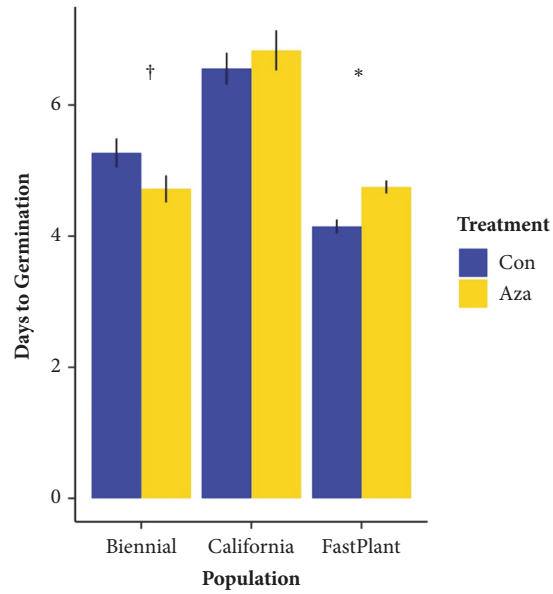

Figure 1: Mean number of days from methylation treatment to germination in three varieties of *Brassica rapa*. Blue bars (left) indicate control individuals ("Con") while yellow bars (right) denote individuals under 5-azacytidine hypomethylation treatment ("Aza"). Varieties have differing source locations and flowering regimes. Biennial: Netherlands, biennial; California: California, annual; FastPlant: Lab-selected, rapid annual. Error bars represent 1 SE. The following symbols denote significance, quantified by planned orthogonal contrast between "Aza" and "Con": $^{\dagger}p < 0.1$, $*p < 0.05$.

3.2. Growth and Flowering Time. The plant populations differed significantly in number of leaves and the width and length of the longest leaf (Table 2). All three of these size traits were also significantly affected by photoperiod (Table 2). There was not a significant effect of 5-azaC demethylation on number of leaves or longest leaf length, but there was a marginally significant ($p < 0.1$) effect of 5-azaC on longest leaf width (Table 2). There was a significant interaction between population and photoperiod for number of leaves and leaf length (Table 2).

In the Fast Plants (the only variety with a large proportion of individuals flowering during the study), photoperiod had a significant effect on number of leaves, length and width of the longest leaf, and flowering time, but not on number of flowers (Table 3).

Methylation treatment alone did not have a statistically significant effect on flowering time; however the interaction between methylation treatment and photoperiod treatment on flowering time was marginally significant (Table 3). Specifically, while there was little effect of photoperiod on flowering time in the control treatment, the short photoperiod delayed flowering time by about 2.5 days in the hypomethylation treatment (Figure 2). This suggests that altering the epigenome affected plant response to differing

Table 1: Cross-population germination analyses.

(a) ANOVA

factor	df, residual df	mean squares	f-ratio	p-value
Population	2, 172	31.698	27.948	< 0.001 ∗ ∗ ∗
5-AzaC	1, 173	0.002	0.002	0.968
Population ∗ 5-AzaC	2, 172	6.69	6.226	0.002 ∗∗

(b) Planned orthogonal contrast

comparison	estimate	std. error	t-value	p-value
Biennials	-0.06428	0.03343	-1.923	0.0557 †
Fast Plants	0.07184	0.03334	2.155	0.0322 ∗

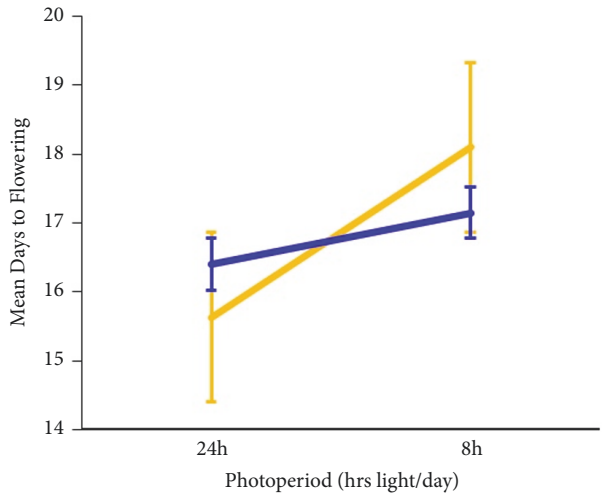

Figure 2: Interaction effect between photoperiod and demethylation on flowering time in *B. rapa* Fast Plants. Yellow indicates individuals under 5-azacytidine hypomethylation treatment ("Aza"), while blue indicates control individuals ("Con"). Error bars represent 1 SE above and below the mean.

light availability, in this case with hypomethylation increasing plasticity. (Note for use of ANOVA: homogeneity of variance was violated for flowering time. However, the difference is just under the threshold for significance, and ANOVA is generally robust to minor violations of the homoscedasticity assumption [38].) Methylation did not affect number of leaves or leaf length but significantly reduced leaf width and number of flowers (Table 2; Figure 3). The hypomethylation treatment reduced leaf width by about 22% and reduced fitness, based on number of flowers, by approximately 30% (Figure 3).

4. Discussion

In this study, we show that treatment of seeds with 5-azacytidine affects several plant traits at different life history stages in different populations of *Brassica rapa*, and that these effects can differ among populations and depending on environmental conditions such as photoperiod. 5-Azacytidine is a well-established demethylation agent [29, 30, 39] with a known mechanism of activity [28] that has previously been used in a number of studies to cause global demethylation in seeds [12, 26, 31, 32, 34–36, 39]. It has been shown to demethylate DNA globally at a higher level than the comparable Zebularine, including transposable element regions that would normally be silenced [39]. Still, studies conducted in mammalian systems have brought to light important evidence that 5-azacytidine and similar demethylating agents can have additional unintended chemical effects. It has been theorized that cytotoxic effects observed in 5-azaC treated mice lines are due to covalent binding of DNA methyltransferase to 5-azaC rather than hypomethylation [33] and that this binding can cause an increased risk of mutations [37]. Additionally, 5-azacytidine can be incorporated into RNA and this may incur additional adverse effects due to transcriptomic changes occurring haphazardly in different parts of the genome [40]. 5-Azacytidine has also been shown to elicit changes to root system development [41] potentially due to one of these alternate mechanisms. However, the different effects we observed under different environmental conditions suggest that general cytotoxicity is unlikely to be the sole cause of our observed results. Because of this, we proceed with the assumption that DNA demethylation was involved in the effects we observed. But we do acknowledge that other side-effects of 5-azacytidine administration cannot be ruled out as potential contributing factors.

Previous studies using 5-azaC have found that demethylation influences a variety of traits, including shade-avoidance [29], premature ripening [42], and somatic embryogenesis [30]. Since it is thought that one of the main effects of methylation is the suppression of transposable elements (TEs), and TEs influence a variety of phenotypes in different ways, it is not surprising that the effects of 5-azaC are widespread and unpredictable [8].

In our experiment, we found that treatment with 5-azaC influenced the timing of germination, and that the effects differed for populations differing in life history characteristics. Recent studies of germination in Arabidopsis have shown that the major DNA methylation changes occur during the germination process [8, 43] and that changes in germination rate are impacted by histone demethylation [44]. Germination is a critical life history trait, given that germinating at the wrong time under the wrong conditions can be fatal. Although the effect of demethylation on germination was relatively small, causing differences of only about a day,

Table 2: ANOVA of plant size traits, all lines.

		Log Number of Leaves			Longest Leaf Length [mm]			Longest Leaf Width [mm]		
factor	**df, residual df**	**ms**	**f-ratio**	**p-value**	**ms**	**f-ratio**	**p-value**	**ms**	**f-ratio**	**p-value**
5-AzaC	1, 156	0.2623	2.6827	0.1035	1310	1.4664	0.2279	775	3.3100	0.07091 †
Population	2, 155	1.7729	18.1291	>0.001∗ ∗ ∗	389646	436.0645	>0.001∗ ∗ ∗	64771	276.5248	>0.001∗ ∗ ∗
Photoperiod	1, 156	3.5565	36.3671	>0.001∗ ∗ ∗	8965	10.0330	0.0019∗∗	987	4.2145	0.0419∗
5-AzaC ∗ Population	2, 155	0.0119	0.1216	0.8856	93	0.1045	0.9009	56	0.2388	0.7879
5-AzaC ∗ Photoperiod	1, 156	0.0989	1.0111	0.3163	258	0.2884	0.5921	7	0.0297	0.86344
Population ∗ Photoperiod	2, 155	0.9035	9.2392	>0.001∗ ∗ ∗	20312	22.7312	>0.001∗ ∗ ∗	510	2.1766	0.11709
Line ∗ 5-AzaC ∗ Photoperiod	2, 155	0.1090	1.1150	0.3307	41	0.0460	0.9551	57	0.2419	0.78548

Table 3: ANOVA of flowering and plant size traits in Fast Plants.

		Flowering Time			Log Leaves		
factor	**df, residual df**	**ms**	**f-ratio**	**p-value**	**ms**	**f-ratio**	**p-value**
5-AzaC	1, 75	0.296	0.065	0.799	0.244	1.194	0.278
Photoperiod	1, 75	50.511	12.95	0.001∗∗	3.857	24.503	<0.001∗ ∗ ∗
5-AzaC ∗ Photopd	1, 75	14.573	3.827	0.054 †	0.234	1.504	0.224
		Longest Leaf Width [mm]			**Log Number of Flowers**		
Factor	**df, residual df**	**ms**	**f-ratio**	**p-value**	**ms**	**f-ratio**	**p-value**
5-AzaC	1, 75	1008.6	8.304	0.005∗∗	5.507	10.38	0.002∗∗
Photoperiod	1, 75	1806.53	16.26	<0.001∗ ∗ ∗	1.369	2.337	0.131
5-AzaC ∗ Photopd	1, 75	1.536	0.015	0.902	0.002	0.005	0.946

†: $p < 0.06$, ∗: $p<0.05$, ∗∗: $p < 0.01$

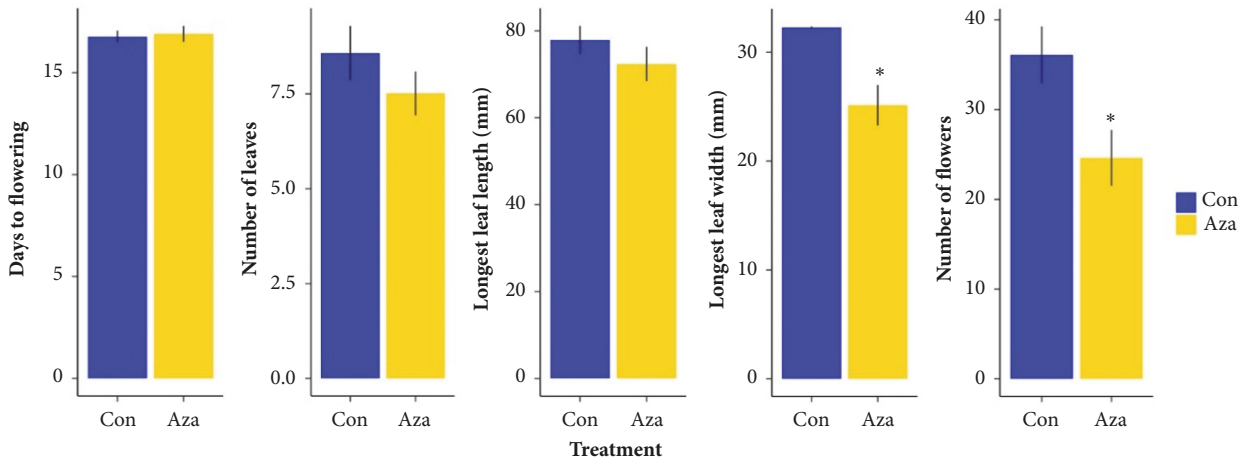

Figure 3: Differences in phenotypic traits between 5-AzaC treated and untreated *B. rapa* Fast Plants. Blue bars indicate control individuals ("Con"), while yellow bars denote individuals under 5-azacytidine hypomethylation treatment ("Aza"). Leaf size measurements are in mm. Error bars represent 1 SE above and below the mean. Significance quantified by planned orthogonal contrast: $**(.001<p<.01)$.

this can still be biologically significant, since seedlings with even a one day head start could potentially overtop and outcompete neighbors. In any case, our results support the idea that epigenetic effects can influence germination. The fact that the different populations differed in the effects of hypomethylation on germination suggests that there may be epigenetic differences underlying the different populations.

As in the study by Bossdorf et al. [12] in *Arabidopsis thaliana*, our studied populations demonstrated variation in the response to the 5-azaC treatment. We found that 5-azaC influenced the expression of some adult plant size and flowering traits, but that this effect was only present in the Fast Plant population and varied depending on photoperiod. In the Fast Plant population, we found that 5-azaC reduced leaf width and number of flowers by a substantial amount. This result is consistent with the overall trend of reduced plant growth traits and fitness in 5-AzaC treated individuals in Bossdorf et al. [12]. The administration of demethylating agents has also been shown to reduce plant growth in *Taraxacum officinale* [45], and DNA methylation has been linked to variation in other leaf traits [46]. Although it is possible that off-target effects of 5-AzaC such as mutagenesis could have played a role, it appears that the results support the idea that methylation patterns are important for plant growth, and that disrupting these patterns results in a loss of fitness, which has important consequences for the evolution of plant traits.

The 5-azaC treatment did not eliminate the vernalization requirement in the biennial population, as was seen in previous studies with the plants *Thlaspi arvense* [35] and *Linum usitatissimum* (Fields 1994). It is possible that the lack of an effect was due to the 5-azaC concentration we used in our experiment. Fieldes' 1994 study administered various concentrations of 5-azaC to vernalization-dependent populations of *L. usitatissimum* and found that some concentrations produced the same flowering effect as vernalization, but that concentrations higher than 250μM failed to produce flowering the way that lower concentrations did [34]. A previous study generating hypomethylation in *B. rapa* using 5-azaC did not use a vernalization-dependent population [26], so whether a different concentration of 5-azaC would remove the vernalization requirement in biennial *B. rapa* is not known and could be the subject of future investigations.

One intriguing outcome of our experiment was the results for flowering time in the Fast Plant variety. We saw no significant effect of 5-azaC on flowering time, a significant effect of photoperiod on the trait, and a marginally significant interaction between the two (Table 3). The fact that there was no main effect of hypomethylation on flowering time is somewhat surprising given the fact epigenetics plays a key role in the regulation of flowering time [47], and that 5-azaC hypomethylation has previously been shown to alter this trait in *Arabidopsis thaliana* [12]. While this may appear to present conflicting evidence, a closer look at the populations assessed in the respective studies reveals a more nuanced picture. Of the 22 genotypes used in Bossdorf et al. [12] study, all but two were derived from natural populations, and only one of the two artificial populations made it into the final analysis. Artificial selection may have a role mediating epigenetic impacts. The *B. rapa* Fast Plant cultivar we used has been bred for short generation time in order to increase their utility as a lab study organism. It is possible that the strong artificial selection for early flowering time in Fast Plants reduced the role of epigenetic regulation of flowering time. Studies of *A. thaliana* found that epigenetic regulation of flowering time is particularly important through the vernalization pathway [48, 49], and vernalization is not necessary for flowering in the Fast Plant variety of *B. rapa*. In addition, we saw that 100% of Fast Plants that survived transplantation grew to flower during the course of our study, regardless of methylation treatment. This contrasts sharply with the results of Amoah et al. [26], who found a much lower rate of mature individuals flower in the 5-azaC demethylated population of *B. rapa* which they generated from a 5 mM concentration. This provides further evidence that flowering time in artificially

selected *B. rapa* populations is less influenced by epigenetic and other environmental factors than this trait is in plant populations that have not been subjected to artificial selection for early flowering.

We found a marginally significant ($p = 0.054$) interaction effect between methylation and photoperiod treatments for flowering time in Fast Plants. No interaction effects were observed for other traits. As shown in Figure 2, chemically demethylated individuals exhibit a greater difference in mean days to flowering time between photoperiod treatments, with later flowering in treated versus control under short day-length conditions. This result supports the hypothesis that methylation may influence responses to harsher environmental conditions. Several prior studies have explored how epigenetic variation is correlated with and in some cases induces plasticity in response to variation in factors such as nutrient availability and drought [45, 50, 51]. Bossdorf et al. [12] found that 5-azaC increased phenotypic plasticity in traits such as flowering time to environmental variation in nutrients. The groundbreaking work of Herrera et al. [46] demonstrated quantitatively that methylation-controlled plasticity in resource use became more critical as environmental conditions became harsher. Here we also show that methylation may influence plasticity, in this case plastic response of flowering time to environmental variation in photoperiod. The potential for artificial selection to limit the possible contribution of variable DNA methylation on flowering makes this interaction effect even more intriguing. Even in this highly artificially selected population, the 5-azaC treated plants respond to differential light cues and demonstrate greater variation than control plants. Thus epigenetic modifications may be important in influencing plant traits as well as plasticity, showing that epigenetic patterns can influence responses of plant populations to environmental variation both within and across generations.

5. Conclusions

In this study we demonstrated that treatment with 5-azacytidine, a demethylating agent, had effects on plant traits at different life history stages in plant populations with differing life-histories. We also showed that this treatment increased phenotypic plasticity in response to light conditions, even in a population that has been bred for rapid flowering. These results contribute to the body of literature investigating how epigenetic modifications such as DNA methylation can affect plasticity and evolution. Continued work in this field can further elucidate the relationship between genetic variation, epigenetic variation, life history traits, and response to more environmental conditions such as temperature, humidity, and soil type. This can be done in both lab-generated demethylated populations, such as those used in this study, and naturally epigenetically variant populations or individuals of the same population grown under differential conditions. Although we are still far from fully understanding the role of epigenetic variation in an organism's response to this environment, this work is crucial to understanding how natural populations will adapt as our world undergoes rapid changes in climate.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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